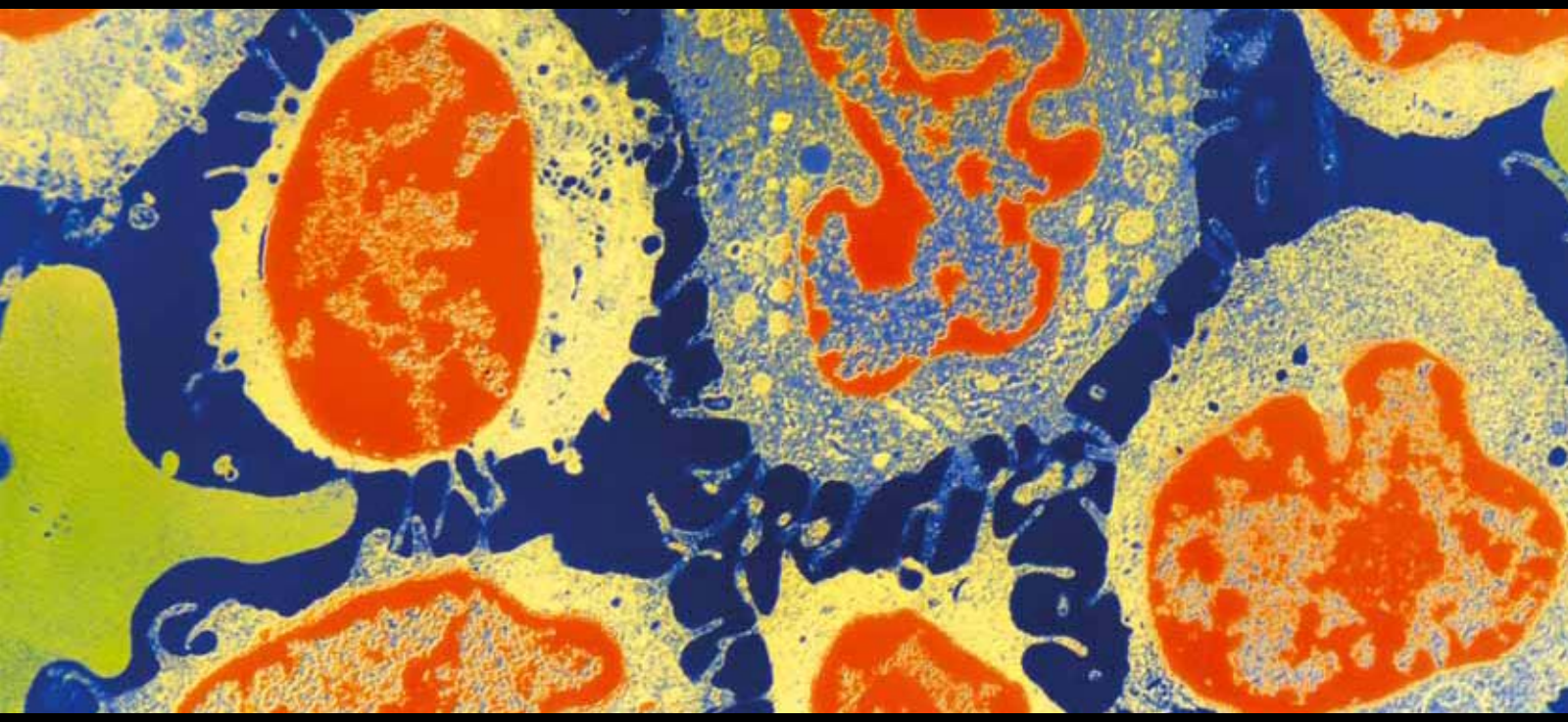
Journal of Oncology

Epithelial Ovarian Cancer: Focus on Targeted Therapy

Guest Editors: M. Markman, Jalid Sehouli, Charles F. Levenback, and Dennis S. Chi



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Editorial Board

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Editorial

Epithelial Ovarian Cancer: Focus on Targeted Therapy

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Over the past five decades ovarian cancer has been of considerable interest to clinical cancer investigators due to the fact that it is among the most chemosensitive of all solid tumors [1]. Unfortunately, despite the advances in the chemotherapeutic management of this malignancy, the large majority of patients ultimately recur, progress, and ultimately die as a direct result of complications of the disease process. Thus, there is a critical need to find novel agents that may favorably impact the natural history of ovarian cancer.

In recent years there has been a particular focus in the cancer research community to discover and subsequently develop clinically active drugs that are capable of specifically *targeting* biological pathways relevant in a particular tumor type (e.g., ovarian cancer) and even within a specific patient with that type of cancer (so-called, "personalized medicine"). Research in this arena in ovarian cancer remains in its early stages although a number of quite exciting developments have recently been reported in the peer-reviewed medical literature that suggest the realistic potential that this novel general class of drugs will soon become important components of "standard-of-care" in the management of this difficult malignancy.

In this special issue, investigators from around the world have contributed to this literature by summarizing a number of important developments. In the papers of this special issue, an initial discussion of the role of surgical cytoreduction in the malignancy is followed by an overview of the management of recurrent ovarian cancer and the relevance of molecular abnormalities in specific ovarian cancer subtypes.

This is followed by several excellent and comprehensive overviews of the possible roles of targeted therapy in ovarian cancer, the potential impact of antiangiogenic drugs, epidermal growth factor and PARP inhibitors, disruption of insulin and glucose pathways, and novel treatments affecting histone deacetylase and metastatic colonization, as well as an innovative approach to immunotherapy in the malignancy.

The peer-reviewed papers in this special issue provide important insight into both the current and future management of epithelial ovarian cancer.

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Review Article

When Should Surgical Cytoreduction in Advanced Ovarian Cancer Take Place?

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Initial surgical management is commonly accepted to date as paramount in the treatment of women presenting with epithelial ovarian cancer and permits the assessment of the disease (staging), the histological confirmation of disease type and grade, and the practice of maximal debulking preceding platinum-based chemotherapy. Many studies have shown that the volume of residual disease after initial surgical cytoreduction inversely correlates with survival. Thus, women with optimal debulking performed by a trained specialist have improved median survival. In this review, we will focus on the answers gleaned from clinical trials on primary and interval surgery, which prompts the question on the timing of surgery in respect to chemotherapy. Interval debulking surgery (IDS) is secondary cytoreduction following primary debulking and is carried out in between the courses of chemotherapy. The major clinical trials and the latest systematic reviews seem unable to give any definitive guidance or recommendation for clinical practice. The choice of aggressive primary cytoreduction or upfront chemotherapy followed by second line surgical cytoreduction seems among others to have to be individualized according to tumour load, prediction of its resectability, and response to chemotherapy. The role of tumour biology must also be kept in mind. Finally, concrete answers are awaited on the timing of surgery from the ongoing prospective randomized control trials (CHORUS and EORTC 55971) though preliminary data from the latter have already been presented at major meetings (IGCS 2008; SGO 2009) and ignited strong debate.

1. Introduction

Ovarian cancer represents the sixth most commonly diagnosed cancer among women in the world and causes more deaths per year than any other cancer of the female reproductive system [1]. In advanced disease which constitutes about 75% of women at presentation, the accepted management is a combination of surgery and platinum based chemotherapy. This has been the approach for some decades, though the 5-year survival remains poor at about 40%. Epithelial ovarian cancer constitutes the majority of disease types, and this review will focus on reports relating to advanced epithelial ovarian carcinoma.

2. Materials and Methods

A Medline database search (January 1966 to April 2009) was undertaken using key words: epithelial ovarian cancer, debulking surgery, and interval debulking surgery resulting in 80 articles with 14 relevant papers. The articles in full were obtained for each of the papers and reviewed by the authors. Results in terms of overall survival (OS) and progression free survival (PFS) were evaluated in each study.

3. Results

The 80 resulting articles were screened and 14 relevant papers were retained: 3 meta-analysis [2–4], 3 randomized control trials (RTC) [5–7] (Table 1) 2 Cochrane Reviews (CRs) [8, 9], and 6 case/control (CC) reports enrolling more than 50 patients [10–15] (Table 2).

4. Discussion

4.1. Primary Debulking Surgery. The initial studies supporting the concept of debulking surgery were published in the

Table 1: RCTs investigating the role of IDS.

Name of Study & year	Rose PG et al. [6] (GOG)	Van der Burg et al. [5] (EORTC)	Redman et al. 1994[1] [7]
N	550 with 448 randomized 226 IDS versus 222 no IDS	425 with 319 randomized 278 evaluated: 138 IDS versus 140 no IDS	86 randomized with 7 excluded* 37 IDS42 no IDS
FIGO stage	II-IV	IIB-IV	II-IV
Trial characteristics	RD > 1 cm after primary surgery and responding/stable after 3 cycles of Cisplatin/pacitaxelStage IV only pleural effusion	RD > 1 cm & maximum primary debulking not attempted in all cases with high proportion of RD >5 cm Randomization after 3 cycles of CP	Primary surgery and RD > 2 cm 1–4 CP Or 3 PAB followed by 5* escalating CP
PFS for IDS versus No IDS	12.5 versus 12.7	18 versus 13	
OS for IDS versus No IDS	36.2 versus 35.7	26 versus 20	15 versus 12 months

CP: cisplatin/cyclophosphamide; overall survival in months; PAB: cyclophosphamide/doxorubicine/bleomycin; PSF: progression free survival in months; RD: residual disease.

*Surgery was non suboptimum.

[1] Randomization begins at the start of the trial.

1970s by Griffiths et al. [16]. The premise for considering the potential impact of reducing intra-abdominal tumour burden was based on the findings of work by Magrath et al. [17], which reported enhanced survival outcome by reducing intra-abdominal disease, in patients with Hodgkin's disease. Griffiths undertook a retrospective analysis of just over 100 women and noted that those with residual disease masses <1.6 cms in largest diameter had an improved survival outcome compared with patients left with a greater disease volume. A subsequent small prospective study [18] on a heterogeneous population of patients, who underwent aggressive radical surgery, also revealed the better survival pattern associated with less tumour burden. Thus, the concept of debulking surgery in ovarian cancer became the normal approach to this disease. The use of adjuvant chemotherapy, which is platinum based, is also the accepted norm in care. The question as to whether the surgical ability of the operator or the inherent tumour biology of the disease is the main factor impacting on survival remains a debate. Indeed, the benefit of radical debulking has already come under criticism [19] while some have advocated that tumour biology rather than the surgical effort might determine prognosis [20]. In a study of 213 patients with Stage IIIC epithelial ovarian cancer who underwent complete cytoreduction before initiation of systemic platinum-based combination chemotherapy, Eisenkop and Spirtos [21] came to the conclusion that the need to remove a large number of peritoneal implants correlates with biological aggressiveness and diminished survival, but not significantly enough to preclude long-term survival or justify abbreviation of the operative effort.

Regarding primary surgery, there is a plethora of published papers, all of which support the findings of Griffiths, though none are randomized controlled trials, and hence, all with similar inherent biases. It is also important to note that various definitions of optimal cytoreduction have been proposed [22–24]. The Gynaecologic Oncology Group (GOG) currently defines optimal cytoreduction as leaving residual disease less than 1 cm in maximum tumour diameter. Some may argue that optimum should only mean no macroscopic residual disease.

There are 3 systematic reviews on residual disease and outcome, which have conflicting conclusions. In an analysis of 81 cohorts of patients (over 6000 women) with advanced-stage ovarian carcinoma treated with platinum-based chemotherapy Bristow et al. [4] found a 5.5-percent increase in median survival for every 10-percent increase in the proportion of patients achieving maximal cytoreduction. Contrary to these findings was the meta-analysis by Hunter et al. [2], (again over 6000 women) whereby the administration of platinum was deemed more important in influencing survival rather than the achievement of optimum debulking surgery. The main difference between these papers is that in Bristow's study, all patients were exposed to adjuvant platinum therapy, which was not the case in Hunters study. The third and smaller study also concluded that optimum debulking was associated with improved survival patterns, though further prospective trials were necessary [3].

4.2. Secondary Surgical Cytoreduction. At the beginning of the eighties, Berek et al. [25] noticed that secondary cytoreduction could also improve survival. Subsequently, the role of interval debulking surgery (IDS) has been investigated in three prospective Randomized Controlled Trials (RCTs) [5–7] where conclusions are different. Interval debulking surgery is defined as a second operation performed after 3 or 4 cycles of platinum chemotherapy in woman who had

Table 2: Nonrandomized case control studies evaluating delayed primary debulking surgery.

Name of Study	Colombo et al. [10]	Oksefjell et al. [11]	Hegazy et al. [12]	Le T et al. [13]	Rafii et al. [14]	Vergote [15]
N	203	789(217 IDS 572 non IDS)	59 all submitted to prior surgical exploration	61	109	285
FIGO stage	IIc-IV	All stages treated for 1st relapse	II-IV	IV without bowel obstruction	IV	III-IV
Important study data	Gr 1 conventional OS = 38 m Gr 2 with NACT OS = 26 m	Platinum single or combination/taxol single or combinationor other	*N = 27 (*OS = *25 m) unresectable NACT with 18 for IDSN = 32 primary cytoreduction (*OS = *28)*	NACT platinum-taxol OS = 41.7 m	NACT platinum-taxol + IDSOS = 45.5 m (under 20% of patients in study)	*Choice of treatment:* upfront surgery or NACT according to disease extent and patient PPS
Main conclusions	*Upfront surgery* for advanced operable disease	Benefit of IDS versus chemotherapy alone when tumour is localised.	*NACT* for unresectable tumours leads to a group of sensitive patients for successful IDS	Response rate to NACT comparable to that of upfront surgery stated in literature	Benefit of IDS in patient responding to NACT	OS was higher for patients with high tumour load treated with NACT than with upfront surgery
	NACT for non operable or poor performance status with IDS ideally after 3 cycles	Best OS (48 m) with radical primary cytoreduction, TFI >24 m & ≤ 39 years		Importance of maximal secondary cytoreduction in IDS	NACT can select patients for surgery	

IDS: interval debulking surgery; m = months; NACT: neoadjuvant chemotherapy; OS: overall survival; PFS: progression free survival; PPS: patient performance status; TFI: treatment free interval.

suboptimal debulking primary surgery. Table 1 summarizes the main features of these trials.

The trials by Redman et al. [7] and the GOG by Rose et al. [6] failed to show any advantage of IDS. The study by Redman was closed prematurely, as no survival benefit was noted at interim analysis, and of note, optimum debulking was defined as <2 cms residium compared with <1 cms in the other studies. In the GOG study, 550 women with suboptimally debulked stage III/IV ovarian cancer received three cycles of paclitaxel/cisplatin and then were randomly assigned to interval cytoreduction or no surgery. Chemotherapy was continued up to a maximum of 6 cycles. A secondary attempt at cytoreduction was not associated with an improvement in progression free survival (PFS) (12.5 versus 12.7 months) or overall survival (OS) (36.2 versus 35.7 months). This was not the case with the EORTC trial carried out by Van de Burg et al. [5], which showed that the IDS group had a significantly increased median survival of 6 months compared to those who had not undergone this procedure. Indeed this is still the only prospective RCT showing a survival benefit with "debulking" surgery. Nevertheless, it is important to point out some differences between these trials. At the time of the EORTC trial, chemotherapy consisted of cisplatin/cyclophosphamide as Paclitaxel was not available, unlike the GOG trial. Another major difference was that in the EORTC trial, primary surgery was not necessarily performed by a trained gynaecological oncologist, resulting in different extents of debulking. The number of patients with less than 5 cm of residual tumour following primary cytoreduction in the EORTC trial was less than a third, compared to 55 percent in the GOG trial. Surgery performed by a trained gynaecological oncologist has been shown to increase survival [26], and the GOG study therefore concludes that with appropriate persons undertaking primary surgery, IDS is not required.

4.3. Neoadjuvant Chemotherapy (NACT) and Debulking Surgery. The term IDS should be confined to patients who have had primary surgical debulking, but it has been used in situations whereby a primary surgical attempt is delayed until during chemotherapy. Six large case-control studies [10–15] relating to "delayed" primary surgery were identified, and are summarized in Table 2.

One of the studies [10, Colombo et al.] divided patients into 2 groups to evaluate the place of surgery in the therapeutic sequence of care: group 1 receiving upfront surgery and group 2 where first debulking was undertaken after chemotherapy. In group 1 the OS was 38 months and 3 factors significantly predicted suboptimal upfront surgery: poor performance status, extensive mesenteric involvemen, and stage IV disease. The second group showed OS of 26 months, and despite a response to NACT in 90% of cases, there was no long-term survivors in the patients whose interval cytoreduction was suboptimal. Generally, OS was stated to be influenced by three main factors: the extent of the disease at the time of diagnosis, the biology of the

tumour, and its chemosensitivity, and the authors concluded that optimal surgery with limited morbidity (14% in their case) can be achieved in many cases at primary surgery setting. Hegazy et al. [12] found, in a population of patients with advanced ovarian carcinoma where resectability was not possible, that neoadjuvant chemotherapy *helped to select patients for feasible and relatively less aggressive* IDS, thus preventing initial surgical failure, in terms of optimal debulking. However, Morris et al. [27] in 1989 demonstrated that patients resistant to chemotherapy during primary treatment had little benefit from IDS. This was also concluded by Rafii et al. [14] as well as the selection effect of NACT for the second intention surgery.

In another recent study [13], the complete response rates after three cycles of platinum/taxane chemotherapy was 36.1%. After IDS, 80% of all patients were left with optimal residuals (<2 cms). The response rate to chemotherapy given in a neoadjuvant setting was comparable to those published in literature in patients who were treated with conventional upfront tumour reduction surgery followed by adjuvant chemotherapy. They also found that residual decease after IDS is the only significant predictive factor associated with prolonged PFS ($P = .003$). To date, there is very little good quality evidence to either support or refute the use of neoadjuvant chemotherapy in the treatment of ovarian cancer [9].

A retrospective study between 1980 and 1997 from Vergote et al. [15] included 285 patients with stages III and IV ovarian cancer. In the period from 1980 to 1988, optimal primary cytoreduction (0.5 cm residual disease) was achieved in 82% of cases, but patients with stage IV disease or a metastatic tumour load of >1 kg prior to this procedure had poorer survival with high postoperative mortality (6%). Between 1989 and 1997 patients received either upfront surgery or chemotherapy depending on the extent of the disease and the performance status. This subsequent management improved overall survival, despite a reduction of 25% in the rate of primary debulking.

4.4. Surgery at Relapsed Disease. A large Norwegian retrospective study ($n = 789$) [11] carried out at the Radium Hospital looked at treatment model for 1st relapse of ovarian cancer of any stage. They found that treatment free interval (TFI) following primary therapy is a significant prognostic factor for OS in multivariate analysis. They also report age as prognostic factor for OS at the time of secondary cytoreductive surgery. Survival benefit was clear for patients with optimum secondary cytoreductive surgery followed by chemotherapy compared with chemotherapy alone at the time of recurrence. Complete secondary cytoreductive surgery was found possible in a significant percentage of patients properly selected for this secondary surgery. Localised tumour was found to be a significant factor to predict this optimum surgery. This selection of patients for secondary cytoreductive surgery is thus crucial. Guidelines at relapse [11] for local and disseminated disease have been set up, where secondary cytoreductive surgery is recommended as independent of TFI for localized tumours and should be considered for TFI > 24 months in case of disseminated disease (Table 3).

Table 3: Radium hospital guidelines for IDS based on TFI and number of recurrence sites, taken from Oksefjell et al. 2009 [11].

TFI, months	Local disease	Disseminated disease
0–5	Consider SCR	No SCR
6–11	Offer SCR	No SCR
12–23	Offer SCR	No SCR
>24	Offer SCR	Consider SCR

SCR: secondary cytoreduction; TFI: treatment-free interval.

Selecting the right patients for the right treatment sequence is challenging. Predicting the possibility to perform successful surgery has been studied [28, 29] with one model having an 85% specificity or ability to identify patients undergoing optimal surgery [30]. In certain situations laparoscopy is recommended as the most valuable tool for evaluating the operability in upfront or second line debulking surgery [31].

5. Conclusions

This paper has reviewed only RCTs and large series, which do reflect the findings of many other reports on the specific debates surrounding the role and timing of surgery in ovarian carcinoma. There is agreement that one of the most important prognostic factors for survival in the treatment of ovarian cancer is the amount of residual tumour after cytoreduction [4, 16]. It is welcome to note that in more recent times surgical approaches have undergone scrutiny in RCTs. Indeed there is evidence of a shift from debulking for all to debulking for a select group, or put another way increased individualisation of therapy. Unlike in previous decades the use of neoadjuvant chemotherapy seems to have gained some popularity, though the real impact requires the formal publication of the randomized trials EORTC 55971 and CHORUS. The EORTC study has been presented at the IGCS in Bangkok and generated a lot of debate, as to the role of neoadjuvant chemotherapy. The finalised peer-reviewed publication is awaited with interest.

Another factor which cannot be ignored in the debate is the inherent tumour biology where the question, raised by some [32] and still requiring an answer, is to know if it is the surgeon's skills or tumour biology which determines survival outcome. In this respect, opinions vary regarding its impact on the ability to surgically debulk [21]. On the other hand, others have put forward the strong expression of the p53 tumour suppressor gene correlating with reduced likelihood of achieving complete cytoreduction [33]. The progress and accessibility to novel technologies applied to biology will make possible in the future the assessment of new prognostic profilesbased on genetic and/or proteomic tumour characteristics. The future also relies on the identification of predictive factors of response to treatment [34].

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Review Article

Treatment for Recurrent Ovarian Cancer—At First Relapse

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Recurrent ovarian cancer is a lethal disease, and few patients can be cured. Although most patients receive standardized surgery and chemotherapy, the status of recurrent disease is heterogeneous. The site of recurrence and the survival intervals after recurrence are also widely distributed. Among a number of factors, many clinical trials identified time to recurrence was the factor most related to chemosensitivity at first relapse. The current recommendation for platinum sensitive ovarian cancer is a carboplatin containing combination chemotherapy. Generally, a single agent is chosen for platinum resistant ovarian cancer. Patients with single site recurrence and a long disease free interval are candidates for secondary cytoreduction, which may provide longer survival. There are several treatment choices at first relapse, and disease status, chemotherapy-free interval, and the patient's condition play a major role in the decision making process.

1. Introduction

Ovarian cancer has the second highest incidence of any gynecologic malignancy in western countries. In Asian countries, ovarian cancer has the third highest incidence, but it is rapidly increasing. In spite of recent progress in treatment strategy, it is still the leading cause of death among cases of gynecologic cancer. After recurrence, generally 70% of advanced stage ovarian cancer relapses, and even in stage I or II patients, the relapse rate is 20%–25%. The survival curve after recurrence never plateaus, which means that the goal of treatment for recurrent ovarian cancer is controlling the disease and disease-related symptoms, limiting treatment-related toxicity, and maintaining or improving quality of life [1]. Nevertheless, the period up to first relapse varies widely, from a few months to more than 5 years. Several prognostic factors have been reported, and clinical trials have provided us with some treatment options. In this paper, patterns and treatment for ovarian cancer at first relapse are discussed.

2. Pattern and Classification of Recurrence

The median interval to first recurrence is 18 to 24 months in ovarian cancer. To clarify the prognostic factors and to determine the treatment procedure, grouping of recurrent patients was applied. They were distinguished by pretreatment or initial treatment profiles, such as FIGO stage, histologic type, and size of residuals. Furthermore, the status at recurrence, such as time to recurrence, site of recurrence, number of recurrent sites, and treatment procedure was also tested as prognostic factors. Time to recurrence was divided to three groups as follows: more than 12 months, less than 6 months, and 6 to 12 months. Sites of first recurrence were divided to two groups, primary site (pelvis and abdominal cavity), and other areas. The number of recurrent sites was divided into two groups: single and multiple. Treatment procedure was divided into surgery and surgery plus chemotherapy.

Half of the recurrences occur at more than 12 months from the end of the first-line therapy, and one quarter of all recurrences occur at less than 6 months. Regarding recurrent sites, Table 1 shows the distribution of first relapse sites from our data on 112 recurrent cases. Fifty-five percent of first relapse was found at the primary site (pelvis or abdomen); the rest was found at distant lesions similar to previous reports [16]. There was a wide variety of recurrent sites, such as, retroperitoneal nodes, liver or spleen, brain, and bone. There was no difference in first recurrent sites between early and advanced stage cancers [17].

Table 1: Site distribution at first relapse of ovarian cancer (n = 112).

Abdominal cavity	33 (29.4%)*
Pelvic cavity	29 (25.9%)*
Vaginal stump	17 (15.2%)
Retroperitoneal lymph node	8 (7.1%)
Superficial lymph node	7 (6.3%)
Liver, spleen	7 (6.3%)
Bladder	3 (2.7%)
Bone	3 (2.7%)
Brain	2 (1.8%)
Lung	2 (1.8%)
Adrenal	1 (0.9%)

Select one main site in case of multilocated.
*Recurrence at the primary site.
Kurume University 1990–2005.

An Italian study showed statistical significance between survival from recurrence and initial clinical stage (I, IIA versus IIB–IV), residual disease after initial surgery (≤1 cm versus >1 cm), time to recurrence (≤6 months versus 6–12 months, >12 months), and treatment at recurrence (surgery plus chemotherapy versus others) by univariate analysis. In multivariate analysis, residual disease and time to recurrence were the only two independent prognostic factors after recurrence. Conversely, histological type (serous versus non serous), tumor grade (G1 versus G2, G3), number of recurrence sites (single versus multiple), and symptoms at recurrence (symptomatic versus asymptomatic) had no prognostic relevance [16]. Hawkins identified predictive factors of survival after first relapse by time to progression (TTP) (>593 days), original tumor grade ≤2, and performance status ≤2 using tree model analysis. In this analysis, the good response group showed longer survival than the intermediate or poor response groups [18]. Data from our 110 recurrence cases showed that time to recurrence (≤6 months versus >6 months), the number of recurrence sites (single versus multiple), and treatment at recurrence (chemotherapy plus surgery and/or radiotherapy versus chemotherapy only) had statistical significance in survival after recurrence. On the other hand, histologic type (serous or endometrioid versus mucinous or clear cell) and initial clinical stage (I, II versus III, IV) did not have any correlation with the survival interval after recurrence (Ushijima, unpublished data).

3. Treatment Option for Recurrence

3.1. Chemotherapy

3.1.1. Patient Selection. Regarding the secondary response of platinum-based chemotherapy, Markman clarified patients with more than 24-month platinum free interval as showing superior response compared to patients with between 5- and 12-month interval (59% and 27%, resp.) [22]. Gore reported that patients with at least an 18-month progression free interval (PFI) showed a remarkably higher response rate compared to patients with less than 18 months of PFI (53% versus 17%) [23]. This theory was supported by the following literature. It is generally accepted that response to second-line chemotherapy correlates well with PFI, from the end of initial treatment [24]. For the selection of a chemotherapeutic regimen at first relapse, patients are categorized according to their estimated chemosensitivity depending on PFI. Many studies have employed the 6-month interval as the borderline when defining the criteria [25]. Figure 1 explains the criteria of platinum sensitivity. Patients whose disease showed initial response to platinum and recurred having >6 months PFI are defined as platinum sensitive. Patients whose disease showed response to or stable disease prior to platinum treatment and who recurred within 6 months of final treatment are defined as platinum resistant. For a more strict separation of the platinum sensitive disease, patients with a more than 12-month interval are defined as highly sensitive disease and patients with 6 to 12 months are considered intermediate sensitive disease [26]. Patients, whose disease progressed during platinum treatment and have less than 3-month platinum free interval, are defined as refractory disease, which means that these patients have very little chance to respond to a platinum-based therapy [27].

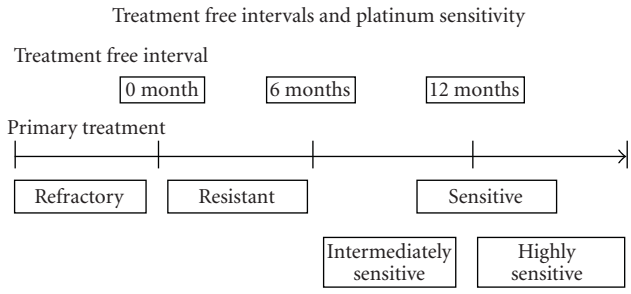

Figure 1: Treatment free intervals and platinum sensitivity.

3.1.2. Chemotherapy for Patients with Platinum Sensitive Ovarian Cancer. The single use of carboplatin has been a standard regimen for the patients with platinum sensitive disease. A phase II trial on paclitaxel and carboplatin combination for sensitive (≥6months) patients showed a considerably high response rate (90%) and 9 months PFI among 20 measurable and assessable patients [2] (Table 2). The ICON4/AGO-Ovarian Cancer 2.2 trial which was a large international phase III study showed that paclitaxel and platinum combination had a statistically higher response rate and improved progression free survival (PFS) than conventional platinum combination in 802 patients with platinum sensitive disease [6]. Nevertheless, this trial had only 34% of the patients who were treated with paclitaxel in the front-line chemotherapy and included several combinations in the treatment regimens as conventional platinum-based chemotherapy.

A Spanish trial was conducted by more stringent design. Eighty-one patients with platinum sensitive disease were randomized to receive carboplatin (AUC: 5) alone as a standard arm, or paclitaxel (175 mg/m^2) and carboplatin

Table 2: Phase II study results for platinum sensitive recurrent ovarian cancer.

	PTX/CBDCA	GEM/CBDCA	PLD/CBDCA	DTX/CBDCA
Author/group	Rose et al. [2]	du Bois et al. [3] AGO	Power et al. [4] *	Ushijima et al. [5] WJGOG
Dose (mg/m^2)	135/AUC 5-6	1000/AUC 4	30/AUC 5	70/AUC 5
Number of patients	20	25(16)**	54	29
Response rate (%)	91	63	46	59
PFS (months)	9	10	10	11
OS (months)	10	18	19.1	NA

PTX: paclitaxel; CBOCA: carboplatin; GEM: gemcitabine.
PLD: pegylated liposomal doxorubicin.
PFS: progression free interval; PS: overall survival.
*Patients who recur within 6 to 12 months.
**Patients with measurable disease.
NA: not applicable.

(AUC: 5) as an experimental arm. More than 80% of patients received paclitaxel previously. Those who were treated with paclitaxel and carboplatin had a significantly higher response rate (75.6% versus 50.0%) and a PFS that was 4 months longer than those treated with carboplatin only [7]. Furthermore, the platinum and paclitaxel combination did not increase toxicity except moderate neurotoxicity. These results suggested the benefits of a paclitaxel and platinum combination and it became the standard treatment of choice for the patients with platinum sensitive disease (Table 3).

A phase I, II study of gemcitabine and carboplatin combination showed 62.5% of objective response in patients with platinum sensitive ovarian cancer [3] (Table 2). According to this result, a large randomized study was conducted by AGO, NCIC CTG, EORTC intergroup trial to compare gemcitabine (1.000 mg/m^2) plus carboplatin (AUC: 4) with carboplatin (AUC: 5) alone for platinum sensitive ovarian cancer. In total, 356 patients were recruited and higher response rates were shown in the gemcitabine plus carboplatin combination including a higher CR rate (14.6% versus 6.2%) and a significantly longer PFS (8.6 months versus 5.8 months). There was no difference in nonhematologic toxicities, but grades 3 and 4 hematologic toxicities were greater with the combination [8] (Table 2).

Pegylated liposomal doxorubicin (PLD) is one of the alternatives for platinum resistant ovarian cancer. A phase II study of PLD (30 mg/m^2) plus carboplatin (AUC: 5) q/4weeks combination chemotherapy showed a 46% objective response rate for the patients who recur within six to twelve months [4]. A randomized phase III study of PLD (30 mg/m^2) plus carboplatin (AUC: 5) q/4w versus carboplatin (AUC: 5) q/4w alone for platinum sensitive ovarian cancer was done by SWOG. The PLD containing regimen showed a 4-month longer PFS (12 months versus 8 months). The lack of incidence of carboplatin-related allergic reaction in the PLD-treated patients may be an additional benefit of this combination [9]. Gynecologic Cancer Intergroup (GCIG) conducted a large phase III study for patients with taxane pretreated sensitive relapse disease, which consisted of PLD (30 mg/m^2) plus carboplatin (AUC: 5) q/4weeks (CD) versus retreatment by paclitaxel (175 mg/m^2) plus carboplatin (AUC: 5) q/3weeks (CP) (CALYPSO study), and the result was presented at ASCO in 2009. The number of patients was 466 in CD and 508 in CP. When comparing median PFS, CD showed statistically longer PFS (11.3 months versus 9.4 months). These two regimens had different toxicity profiles. CD had more incidences of thrombocytopenia and palmar-plantar erythrodysesthesia (PPE). Nevertheless, CD had extremely less incidence of Grade 4 neutropenia, Grade 2 alopecia, Grade 2–4 neurotoxicity, and carboplatin hypersensitivity reaction, which resulted in significantly less incidence of discontinuation of treatment by toxicity than CP. This combination may be a good option for platinum or taxane sensitive relapse [10].

Docetaxel showed similar response to paclitaxel and a different toxicity profile in first line chemotherapy [28]. The Japanese group conducted a phase II trial with docetaxel (70 mg/m^2) and carboplatin (AUC: 5) combination for platinum sensitive patients. The objective response rate was 59% (17/29, including 5 CR) in 29 evaluable patients (Table 2). They showed 46% sensory neurotoxicity and only 7% motor neurotoxicity, without any grade 3 or 4 neurotoxicity, even though most patients were previously treated by paclitaxel [5]. According to this result, a new trial which consisted of biweekly docetaxel (35 mg/m^2) with bolus carboplatin (AUC: 5) repeated every 4 weeks is now ongoing. We can expect a similar response to bolus DC treatment with less hematologic toxicity.

In summary, a carboplatin-based combination is strongly recommended for patients with platinum sensitive disease rather than carboplatin monotherapy. Paclitaxel with carboplatin is the most frequently used combination and showed favorable result for these patients. Nevertheless, alternative combinations of gemcitabine or PLD with carboplatin have responses and prolonged survival rates similar to paclitaxel and carboplatin with different toxicity profiles. Gemcitabine combination showed similar bone marrow toxicity but less neuropathy or alopecia. PLD combination showed less neurotoxicity or bone marrow toxicity, but more PPE.

Table 3: Phase III study results for platinum sensitive recurrent ovarian cancer.

	PTX/CBOCA (COOP) versus Pt combination	PTX/CBOCA versus CBDCA	GEM/CBOCA versus CBDCA	PLO/CBOCA versus CBDCA	PLO/CBOCA versus PTX/CBOCA
Dose (mg/m^2)	175–185/ AUC 5 (50–75) versus AUC 5 (50–75)	175/AUC 5 versus AUC 5	1000/AUC 4 versus AUC 5	30/AUC 5 versus AUC 5	30/AUC 5 versus 175/AUC 5
Author	ICON4/ [6]	González-Martín et al. GEICO [7]	Pfisterer et al. AGO OVAR, [8]	Alberts et al. SWOG [9]	Pujiade-Lauraine et al. GClG [10]
Study group	AGO-OVAR2.2		NCIC CTG, EORTC GCG		
Number of patients	392 versus 410	41 versus 40	178 versus 178	31 versus 30	466 versus 508
Response rate (%)	66 versus 54	75.6 versus 50.0*	47.2 versus 30.9*	52 versus 29	NA
PFS (months)	12 versus 9*	12.2 versus 8.4*	8.6 versus 5.8*	12 versus 8*	11.3 versus 9.4*
OS (months)	29 versus 24*	NA	18 versus 17.3	26 versus 18*	NA

PTX: paclitaxel; PT: cisplatin or carboplatin.
CBDCA: carboplatin; GEM: gemcitabine; PLD: pegylated Iiposomal doxorubicin.
PFS: progression free interval; PS: overall survival.
NA: not applicable.
*Statistically significant.

Table 4: Study result for platinum resistant recurrent ovarian cancer (single agent).

	PLD	GEM	Topotecan	PLD versus Topotecan	PLO versus GEM
Dose	50 mg/m^2/4w	1 g/m^2 d1.8.15/4w	1.5 mg/m^2 d1–5/3w (1 mg/m^2/3w)	50 mg/m^2/4w 1.5 mg/m^2 for 5 d/3w	50 mg/m^2/4w 19/d1.8/3w
Author	Gordon et al. [31]	Markman [11]	Bookman et al. [15] (Rodriguez et al. [36])	O'Malley et al. [13]	Mutch et al. [14]
Number of patients	82	51	112 (37)	130 versus 124	96 versus 99
Response rate					
CR + PR (%)	18.3	16	12.4 (22)	12.3 versus 6.5	8.3 versus 6.1
+SD (%)	66.1	NA	NA(44)	40 versus 49.2	46.9 versus 60.6
Most frequent Adverse effect	PPE	neutropenia	neutropenia	PPE/neutropenia	fatigue/fatigue
PFS (weeks)	17	16	12.1 (18)	9.1 versus 13.6	12.4 versus 14.4
OS (weeks)		15 (months)	47 (NA)	35.6 versus 41.3	50.8 versus 54

PPE: palmar-plantar erythrodysesthesia.

3.1.3. Chemotherapy for Patients with Platinum Resistant Ovarian Cancer. Many phase II trials of single agents for patients with platinum resistant disease showed at most only a 5%–20% response rate (Table 4). Therefore, duration of disease control and low incidence of toxicity should be an important factor in choosing the proper drugs [29]. PLD demonstrated a response in the treatment of recurrent ovarian cancer in some phase II studies [30, 31]. Although the recommended dose of PLD is 50 mg/m^2 q/4weeks, a reduced dose (40 mg/m^2 q/4weeks) showed a lower incidence of PPE which is schedule limiting toxicity. So, a modified dose 40 mg/m^2 q/4weeks may be used for patients with platinum resistant ovarian cancer to minimize adverse effects [32]. PLD is recognized as the first choice nonplatinum agent for patients with relapse, who have failed first-line therapy, or who cannot tolerate platinum retreatment due to toxicity [33].

Gemcitabine has less toxicity except for manageable neutropenia. Single use gemcitabine (1000 mg/m^2 on day 1, 8, and 15 q/4w) is well tolerated and showed 16% of partial response in 51 platinum-paclitaxel refractory ovarian cancer (Table 4) [11]. Dose limiting toxicity is bone marrow suppression; so starting at reduced doses (800 mg/m^2) for heavily pretreated patients is reasonable [11]. A randomized phase III study comparing gemcitabine (1000 mg/m^2 on day 1, 8 q/3w) with PLD (50 mg/m^2 q/4w) in platinum resistant patients showed similar response and PFS. Fatigue (grade2) is frequently the worst toxicity, nausea (grade3) and neutropenia (grade3 and 4) are also statistically frequent in gemcitabine, and PPE is more frequent in PLD [14]

Table 5: Study result for platinum resistant recurrent ovarian cancer (combination).

	GEM/weekly PTX	GEM/PLD	PLD /Topotecan
Dose (mg/m^2)	1000/80 day 1.8. 15/4w	1000/30 day 1.8/day l/3w	30/1 day l/day l–5/3w
Author	Garcia et al. [12]	Ferrandina et al. [19]	Verhaar-Langereis et al. [20]
Number of patients	35	66	27
Response rate			
CR + PR (%)	40	21.6	28
+ SD (%)	77	53.9	72
PFS (months)	5.7	20 weeks	30 weeks
OS (months)	13.1	50 weeks	41 weeks

(Table 4). The different mechanisms and noncross-resistance of gemcitabine can be expected to overcome drug resistance in combination with other nonplatinum drugs [34]. A combination of gemcitabine (1000 mg/m^2) with weekly paclitaxel (80 mg/m^2) on days 1, 8 and 15 q/4w showed a 40% response rate and the median PFS was 5.7 months for 35 patients with platinum resistant disease [12]. A large multicenter phase II study of combination of PLD (30 mg/m^2) day 1 and gemcitabine (1000 mg/m^2) days 1 and 8 every 3 weeks showed a 22% overall response and a 32% stable disease for patients with platinum resistant disease. In that study, the lower PLD dose might contribute to the very low incidence of PPE (Table 5) [19].

Topoisomerase inhibitors, topotecan, irinotecan, and oral etoposide can also be used for platinum resistant disease. The standard treatment of topotecan is 1.5 mg/m^2 for five consecutive days, every 3 weeks. The response rate for platinum resistant ovarian cancer was 12%–14% [15, 35]. High bone marrow toxicity was seen (82% of patients with Grade 4 neutropenia) in heavily pretreated patients [19]. Recently, a lower dose (1.0 mg/m^2) 5-day q/3w protocol [36], weekly schedule (2.5 mg/m^2 days 1, 8, 15 q/4w) [13], or 3-day schedule (1.5 mg/m^2 days 1–3 q/3w) [37] improved treatment tolerability for heavily pretreated patients (Table 4). The combination of lower dose of PLD (30 mg/m^2) and topotecan (1 mg/m^2 for days 1–5) was tested for platinum resistant ovarian cancer. Although relatively higher response rate (28%) was seen in a phase II study, severe bone marrow toxicity (grade 3/4 thrombocytopenia in 41% of cases) limited further clinical use (Table 5) [20].

A randomized phase III study for topotecan and PLD for refractory or recurrent ovarian cancer was conducted. The PFS rates were similar between two arms. In patients with platinum sensitive disease, PLD was demonstrated to be significantly superior to topotecan in overall survival. On the other hand, in the platinum-refractory subgroup there was no statistically significant survival trend in favor of either liposomal doxorubicin or topotecan (Table 4) [38].

Table 6: Recommendation for secondary cytoreduction based on disease free interval and number of recurrence site.

Disease free interval	Single site	Multiple site no carcinomatosis	Carcinomatosis
6–12 Mo	offer SC	consider SC	No SC
12–30 Mo	offer SC	offer SC	consider SC
>30 Mo	offer SC	offer SC	offer SC

DFI: disease free interval. SC: secondary cytoreduction. Chi et al. [21].

4. Surgery

Complete response by chemotherapy for recurrent ovarian cancer is rare, and shrinkage of the tumor does not always ensure prolongation of survival. A surgical approach may bring clinical benefit to some patients. Surgery for clinical recurrence is defined as secondary cytoreductive surgery, similar to surgery for persistent disease at the completion of chemotherapy. In multivariate analysis, disease free interval, the number of sites of recurrence, and residual disease after secondary cytoreduction were factors found to influence prognosis. The patients with the longer disease free interval (>30 months) and a single site of recurrence were most likely to reap the benefits of secondary cytoreduction (Table 6) [21]. A longer period of PFS and complete resection at secondary cytoreductive surgery are common favorable prognostic factors [39–41]. Nevertheless, the surgical result is dependent on the number of sites and the skill of the surgeon. Onda et al. proposed four prognostic factors as follows: >12 months PFS, no liver metastasis, solitary tumor, and <6 cm tumor size. Patients with three or all four of these factors who received complete surgical resection at secondary cytoreduction showed a favorable prognosis [42].

At secondary reduction, bowel or other organ resections are often also performed. More than 30% of surgeries included bowel resection [21], and some of them accompanied considerable morbidity, such as colostomy or pelvic exenteration [40]. On the other hand, patients with longer PFS are also expected high response to second-line chemotherapy. Therefore, careful consideration must be made when deciding which strategy, surgery, or chemotherapy to use to most benefit each patient.

5. Conclusion

Recurrence of ovarian cancer is a lethal and chronic disease. Nevertheless, patients with recurrent platinum-sensitive ovarian cancer may have increased response rates and longer PFS when treated with combination platinum-based chemotherapy compared to carboplatin alone. Most recurrent patients with platinum resistant disease have little chance for a long PFS, but less toxic treatment may

contribute to extending their survival interval. Complete secondary cytoreduction combined with further adjuvant therapy at the time of relapse may improve clinical outcome in selected patients. There are several treatment choices from first relapse to terminal state; however these choices cannot be made uniformly. They should be decided on an individual basis depending directly on the patients' condition.

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Review Article

Molecular Abnormalities in Ovarian Cancer Subtypes Other than High-Grade Serous Carcinoma

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Ovarian cancer is the fifth leading cause of cancer death in women in North America, and approximately two-thirds of cases of ovarian cancer are of high-grade serous type. The remaining cases are comprised of a mix of different tumor types (e.g., endometrioid, clear cell, mucinous, etc.), with no single tumor type accounting for more than 10% of ovarian cancer cases. These tumor types can be reproducibly diagnosed, and each features distinct underlying molecular events during oncogenesis, with a characteristic natural history and response rate to conventional cytotoxic chemotherapy. In this review the molecular abnormalities present in the more common non-high-grade serous subtypes of ovarian cancer will be presented. Development of targeted therapies for these tumor types will require understanding of the genetic basis of each tumor type, and may lead to subtype-specific therapy.

1. Introduction

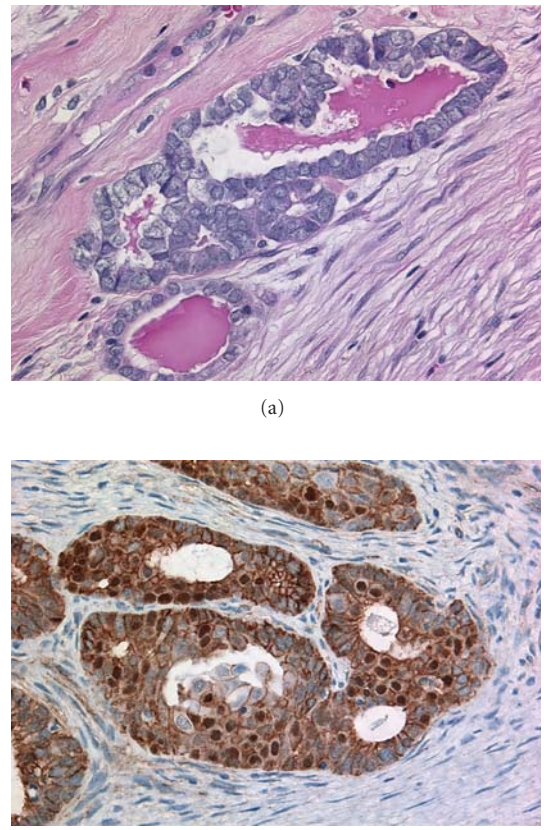
Ovarian cancer is not a single disease but is comprised of more than 15 distinct tumor types, each characterized by subtype-specific risk factors (environmental and genetic), precursor lesions, histopathological features, molecular events during oncogenesis, response to chemotherapy, and patient outcome [1, 2] (N.B. the terms "tumor type" and "subtype" are used interchangeably in this paper to refer to the morphologically defined variants of ovarian cancer, as diagnosed in routine surgical pathology practice). More than 90% of ovarian malignancies are carcinomas, commonly referred to as surface epithelial carcinomas, even though there are now significant doubts about the cell of origin of these tumors, and an increasing belief that many, if not most, do not arise from ovarian surface epithelium. Of the group of surface epithelial carcinomas (referred to hereafter simply as carcinomas), approximately 70% are of high-grade serous type [3].

High-grade serous carcinomas are chromosomally unstable tumors, and usually have mutations in the TP53 tumor suppressor gene [4]. In most cases they also have germline or somatic mutations in BRCA1 or BRCA2, or promoter methylation of BRCA1 with loss of expression [5]. This underlying loss of BRCA function and inability to repair double-strand repair breaks, leading to chromosomal instability, are an attractive therapeutic target for drugs that target DNA repair (e.g., PARP inhibitors) [6–8].

There is an unfortunate tendency to use the terms "ovarian cancer" and "high-grade serous carcinoma" interchangeably. While this is understandable, given high-grade serous carcinomas account for most cases of ovarian cancer, at least in North America and Europe, and most of the deaths due to ovarian cancer, this has resulted in failure to significantly advance treatment for other ovarian cancer subtypes, particularly the carcinoma subtypes. Although it is current practice to treat all subtypes of carcinoma with the same platinum/taxane chemotherapy, some subtypes do not respond well to this approach and subtype-specific trials of chemotherapy have been recommended for clear cell and mucinous carcinoma in particular [9]. The large randomized clinical trials leading to refinement of the current chemotherapy for ovarian carcinoma have been based on case series that, by current diagnostic criteria, would be composed almost exclusively of high-grade serous carcinomas, and none of these trials permit

any conclusions to be drawn about appropriate treatment of other ovarian cancer subtypes. This minority of non-high-grade serous ovarian cancers, consisting of a patchwork of carcinoma subtypes and malignant tumors other than those of surface epithelial type, will be a challenge to study as there are relatively few cases of any given subtype, and large mixed-case series are not appropriate to explore targeted or subtype-specific therapies for these subtypes.

Targeted therapy for ovarian carcinoma, if defined as a therapy directed specifically at molecular abnormalities in individual tumors, will probably require consideration of the tumor subtype, as the molecular abnormalities underlying each of these subtypes are different. The aim of this paper is to present the most common subtypes of ovarian cancer apart from high-grade serous type, discussing first the clinical significance and then presenting an overview of the molecular abnormalities for each subtype. This paper does not cover histopathology, but an important point is that, with recent advances in diagnostic criteria and development of sensitive and specific immunomarkers, all can be reproducibly diagnosed [2, 10]. This reproducibility is recent and historical case series, or more recent retrospective case series without contemporary slide review, are not useful in understanding these uncommon tumor subtypes as a significant number of cases will have been misclassified [10]. The frequency estimates for each subtype are from our center (British Columbia Cancer Agency, which serves a population of 4.1 million) [3], unless otherwise indicated.



(a)

(b)

Figure 1: Ovarian carcinoma of endometrioid type (a). Immunostaining for beta-catenin shows both nuclear and membranous localization within the tumor cells (b).

2. Endometrioid Carcinoma

Endometrioid carcinomas account for approximately 10% of ovarian carcinomas, with most diagnosed at stage I or II. Historical data on endometrioid carcinomas is not reliable as many tumors that were diagnosed as endometrioid in the past are now known to be high-grade serous carcinomas, based on their immunoprofile [11]. Most endometrioid carcinomas are grade 1 or 2 and there is a strong association with endometriosis. Although one of the most common non-serous subtypes, because they are predominantly low stage and low grade at presentation, the burden of morbidity and mortality associated with this subtype is relatively low. While there is a need for adjuvant chemotherapy for advanced-stage endometrioid carcinomas, there is no data currently available specifically on this subtype, and such data will be hard to aquire given that advanced-stage or recurrent tumors are rare.

The most common genetic abnormalities in endometrioid carcinoma are somatic mutations in the beta-catenin (CTNNB1) and PTEN genes [12–14]. CTNNB1 mutations are present in 38% to 50% of cases; mutations in codons 32, 33, 37, and 41, of exon 3, involve the phosphorylation sequence for glycogen synthase kinase 3-beta and are thought to lead to decreased APC-mediated downregulation, with accumulation of beta-catenin protein in the nucleus. Nuclear accumulation of beta-catenin protein can be demonstrated in 80% of cases (Figure 1); this contrasts with the exclusively membranous localization seen in other carcinoma subtypes. PTEN is mutated in approximately 20% of cases. BRCA abnormalities and loss of function are not seen in endometrioid carcinomas. Endometrioid carcinomas of the ovary are associated with hereditary nonpolyposis colon cancer syndrome, in patients with germline mutations in a gene encoding a DNA mismatch repair enzyme. This results in microsatellite instability in the tumor cells, which can also occur in sporadic cases as a result of MLH1 promoter methylation. There is coexistence of endometrioid carcinoma of ovary and endometrium relatively frequently (up to 20% of cases of endometrioid carcinoma of the ovary are associated with synchronous atypical hyperplasia or endometrioid adenocarcinoma of the endometrium) [15, 16]. The favorable outcome of such cases suggests that these are independent primaries and also suggests a role of hormonal environment in the genesis of endometrioid carcinoma of the ovary, given the well-characterized role of unopposed estrogenic stimulation as a risk factor for endometrial adenocarcinoma of endometrioid type. Virtually all endometrioid carcinomas of the ovary express estrogen receptor protein [10].

3. Clear Cell Carcinoma

Clear cell carcinomas occur at a similar frequency as endometrioid carcinomas, and account for approximately 10% of ovarian carcinomas in North America. They are more common in Japan, at least relatively, although this may reflect only a proportional increase, with fewer high-grade serous carcinomas. Clear cell carcinomas also usually present with low-stage disease. All clear cell carcinomas are considered high-grade [1], and they would all be treated with adjuvant chemotherapy in most centers, because of a significant likelihood of relapse, but the available evidence suggests that responses to adjuvant platinum/taxane chemotherapy are uncommon [17–22]. The range of reported response rates is wide (15%–45%), and it is likely that this reflects differences in diagnostic accuracy historically, rather than biological differences in cases series, although there is no proof of this. Because of this poor response rate, and the relatively aggressive nature of clear cell carcinoma, there is an acute need for more effective treatments. Clear cell carcinomas were a subtype specifically mentioned at a recent National Cancer Institute State of the Science meeting on ovarian cancer as being a priority for subtype-specific trials of novel therapeutic agents, in an attempt to identify more effective treatment [9].

The molecular origins of clear cell carcinomas remain obscure. They are not associated with germline or somatic BRCA mutations and typically do not show the complex karyotypes associated with chromosomal instability [5]; most clear cell carcinomas are diploid or tetraploid (B. Risberg and C. B. Gilks, unpublished data). Clear cell carcinomas show relatively low-mitotic rates [5, 23], and it is therefore not surprising that responses to agents targeting dividing cells are less successful than those in high-grade serous carcinoma. Clear cell carcinomas, like endometrioid carcinomas, are strongly associated with the presence of endometriosis and are not uncommonly seen arising in endometriotic cysts. Unlike endometrioid carcinomas, however, they lack expression of hormone receptors (estrogen receptor or progesterone receptor) [24], suggesting that the hormonal influence during oncogenesis is different; clear cell carcinomas may be analgous to the nonhormonally dependent Type 2 endometrial carcinomas while endometrioid carcinomas share many features (both morphological and molecular) with Type 1 carcinomas of the endometrium [25]. Clear cell carcinomas of the ovary show striking similarities to renal clear cell carcinomas, based on gene expression profiling [26], raising the possibility that responses to treatment could be similar in clear cell carcinomas arising at different sites. To investigate this possibility, we treated mice carrying xenografts of an ovarian clear cell carcinoma with sunitinib, a kinase inhibitor that targets VEGF action that is approved for use in patients with renal clear cell carcinoma, and demonstrated a response to sunitinib in the clear cell carcinoma xenograft but not in xenografts derived from three different high-grade serous carcinomas (Y. Z. Wang and C. B. Gilks, unpublished data). Clear cell carcinomas have not been specifically studied for sensitivity to agents targetting angiogenesis/VEGF in human patients, but this may prove to be a fruitful avenue of study. Interestingly, there is evidence that ovarian clear cell carcinomas are sensitive to radiotherapy [27]; given that clear cell carcinomas are not rapidly proliferating tumors, this may reflect targetting of intratumoral neovascularization by the radiotherapy.

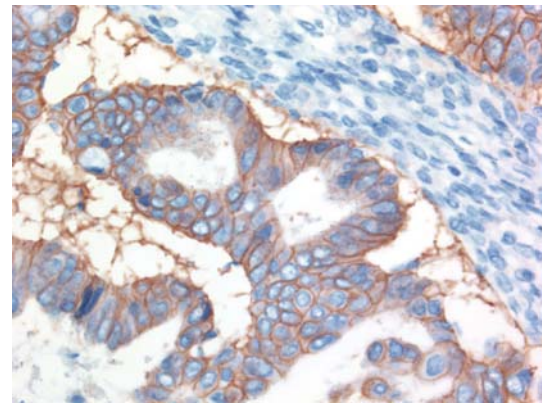


Figure 2: HER2 immunostaining of a mucinous carcinoma shows diffuse membranous positivity. This was associated with high-level HER2 amplification on FISH analysis.

4. Mucinous Carcinoma

Mucinous carcinomas are much less common than was previously thought, as historically many case series included cases of metastic carcinoma with mucinous differentiation, that were primary in gastrointestinal or biliary tract. Only 3%-4% of ovarian carcinoma are of mucinous type and most are confined to the ovary at presentation. Nonetheless, some will recur and when they do, there are no effective treatments. Mucinous carcinomas, like clear cell carcinomas, were singled out as being a priority for subtype-specific clinical trials, given the ineffectiveness of current therapy [9].

Mutations in KRAS, involving codons 12 and 13, are the most common mutations described in mucinous carcinomas [28]. Mutations can be seen in benign-appearing areas of mucinous tumors, adjacent to frank mucinous carcinoma, suggesting that they are an early event during oncogenesis. HER2 amplification, with overexpression of the protein on the membrane of the tumor cells, is present in 15%–20% of mucinous carcinomas of the ovary (J. N. McAlpine et al. BMC Cancer, in press.) (Figure 2). This is a higher frequency of HER2 amplification than is seen in breast cancer, and it is similar to the frequency encountered in adenocarcinoma of the gastroesophageal junction. Trastuzumab (Herceptin) therapy is an obvious treatment choice for these cases but there is no data yet on response of mucinous carcinomas of the ovary with HER2 amplification/overexpression to such treatment. Although there are large clinical trials of trastuzumab therapy in ovarian cancer, with discouraging results, almost all tumors in these studies were of high-grade serous type [29]. High-grade serous carcinomas only rarely show high-level amplification of the HER2 gene or overexpression of HER2 protein on the cytoplasmic

membrane of tumor cells [30], and these studies are not informative about efficacy of this therapeutic option in mucinous carcinoma.

5. Low-Grade Serous Carcinoma

The separation of serous carcinomas into low-grade and high-grade types is a recent development. Comparison of low-grade and high-grade serous carcinomas shows that the low-grade serous carcinomas often arise from a serous borderline tumor while the precursor lesion of high-grade serous carcinoma is tubal intraepithelial carcinoma, in most cases. Low-grade serous carcinomas can be reproducibly distinguished from high-grade serous carcinomas, based primarily on their very uniform nuclei, using low-mitotic rate as a secondary diagnostic criterion [31, 32]. Low-grade serous carcinomas are much less common than high-grade serous carcinomas and account for only 2% of ovarian carcinomas. As many present with high-stage disease, however, there is a need for effective chemotherapy. An unusual feature of the natural history of low-grade serous carcinomas is that they may follow a relatively indolent course; this in turn allows for multiple opportunities to treat [33]. The response rate to platinum/taxane chemotherapy within this group is difficult to gauge, as there are no studies of large series of well-characterized cases. In the case of serous borderline tumors that have progressed to low-grade serous carcinomas, however, response rates are relatively low, with most patients showing no response [34]. As is the case for clear cell carcinomas, low-grade serous carcinomas have a low-mitotic rate, and poor response to platinum-based chemotherapy is not unexpected.

KRAS or BRAF mutations, which target the same molecular pathway, are present in most low-grade serous carcinomas [35–37]. These tumors are also almost invariably positive for hormone receptor expression (estrogen and/or progesterone receptors). Low-grade serous carcinomas are not chromosomally unstable; they are usually diploid or near diploid and do not show the complex genetic abnormalities seen in high-grade serous carcinomas [38]. Low-grade serous carcinomas are not associated with either germline or somatic abnormalities in BRCA1/2 and typically do not have TP53 mutations. Only rarely do low-grade serous carcinomas progress to higher-grade tumors [39].

6. Granulosa Cell Tumor

Granulosa cell tumors are the most common malignant tumors within that group of tumors arising from ovarian sex cord or stromal cells, and account for a large majority of the malignant tumors within this category [1]. They are still relatively uncommon, and have been reported to account for approximately 1%-2% of ovarian tumors (benign or malignant), although in our experience this is an overestimate. They may account for 2% of ovarian cancers, however. They are the most common primary ovarian malignancies, apart from carcinomas. As with some of the other subtypes discussed above, they have not been reliably diagnosed in the past, so that older data regarding their natural history or molecular abnormalities is not reliable. There are two distinct granulosa cell variants and the discussion that follows relates only to the adult-type granulosa cell tumors, which account for 95% of granulosa cell tumors. These tumors are usually confined to the ovary at presentation, and recurrences can be many years after presentation [1]. The only effective therapeutic option at present is surgery.

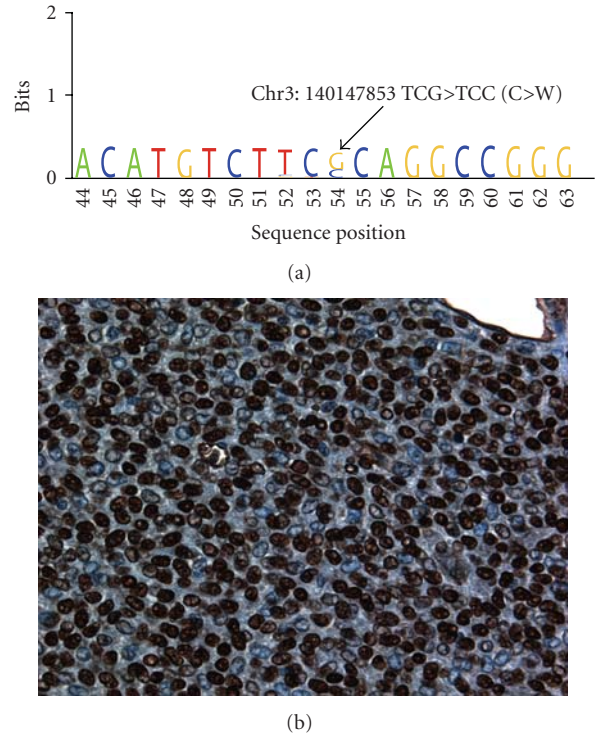


Figure 3: Results of sequencing of the transcriptome of a granulosa cell tumor, showing sequence from the FOXL2 gene (a). At nucleotide 402, both G and C were identified, indicating that this tumor was hemizygous for the 402C->G mutation charateristic of adult-type granulosa tumor. Granulosa tumor cell nuclei show high-level expression of the FOXL2 protein by immunostaining (b), in association with this mutation.

Granulosa cell tumors are genomically stable and diploid. They show few abnormalities by cytogenetic analysis. It is likely that some tumors considered to be aneuploid granulosa cell tumors in the past were undifferentiated carcinomas, based on their natural history (early recurrence and poor prognosis). Recently 4 granulosa cell tumors were subjected to transcriptome sequencing, revealing a missense G > C mutation at nucleotide 402 of the FOXL2 gene in every case (Figure 3) [40]. Extension of this study by examination of additional cases revealed that these identical 402G->C FOXL2 mutations were present in more than 95% of cases diagnosed as adult-type granulosa cell tumors, as well as occasional thecomas, and a single juvenile granulosa cell tumor (of ten tested) [40]. It is likely that at least two of the three cases of purported adult-type granulosa cell tumor lacking an FOXL2 mutation were misdiagnosed

and were not granulosa cell tumors, based on a review of the tumor's immunophenotypes, while the thecomas showing the FOXL2 mutation did have minor granulosa cell components, on retrospective review of the cases.

The FOXL2 gene is a member of the forkhead/winged-helix family of transcription factors, and this point mutation results in a cysteine to tryptophan change at position 134 in the amino acid sequence of the protein, a highly nonconservative change, which is predicted to affect protein-protein interactions. FOXL2 is a crucially important transcription factor in granulosa cell development; an autosomal recessive disorder, blepharophimosis-ptosis-epicanthus inversus syndrome, occurring as a result of two mutant alleles of FOXL2, is associated with ovarian failure [41–43]. In granulosa cell tumors, the FOXL2 mutations are somatic; in all cases tested, the germline sequence has been normal. The near universal presence of this FOXL2 mutation in adult-type granulosa cell tumors, the fact that most tumors are hemizygous for the mutation, and the presence of abundant FOXL2 protein in tumor cell nuclei (Figure 3) suggest that this mutation is a critical genetic abnormality in the genesis of adult-type granulosa cell tumors and that it is an activating mutation. FOXL2 interacts with SMAD and AP1 proteins, and it is possible that this interaction is disrupted, leading to uncontroled growth. The presence of a single mutation suggests the possibility of targeted therapy, similar to what has been developed for other cancers where specific recurrent genetic abnormalities are present (e.g., chronic myelogenous leukemia, gastrointestinal stromal tumor, and dermatofibrosarcoma protuberans).

7. Dysgerminoma

Dysgerminomas are within the group of primitive germ cell tumors, which are defined as malignant, nonteratomatous germ cell tumors [1]. Dysgerminomas are morphologically indistinguishable from their much more common counterpart in the male, testicular seminoma. Although dysgerminomas are the most common of the primitive germ cell tumors of the ovary, they are rare and account for less than 1% of ovarian cancers. These tumors are chemosensitive, and most patients, even with advanced-stage disease at presentation, can be cured.

The genetic abnomormalities in dysgerminoma are identical to those of seminoma. Cytogenetically, abnormalities of chromosome 12, particularly i(12p), are commonly present [44]. Activating mutations in KIT are present in a significant minority of patients with dysgerminoma and are associated with high-level expression of KIT protein in the tumor cells [45–47]. KIT protein can also be present in dysgerminomas without an identifiable KIT mutation; the mechanism underlying KIT overexpression in these cases is not known.

8. Summary

Although there has been progress in elucidating the molecular basis of the less common subtypes of ovarian cancer, there remains much work to be done if targeted therapy is to become a routine option clinically. There are compounds available that can target some of the molecular abnormalities identified (HER2 amplification in mucinous carcinoma, neovascularization and VEGF signaling in clear cell carcinoma, hormone receptor signaling in low-grade serous carcinoma, and KIT mutations in dysgerminoma); future studies should focus on both identifications of additional targets; rational preclinical studies and subtype-specific clinical trials of targeted therapies aimed at promising molecular targets.

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Review Article

Targeted Therapies in Epithelial Ovarian Cancer

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Epithelial ovarian cancer remains a major women's health problem due to its high lethality. Despite great efforts to develop effective prevention and early detection strategies, most patients are still diagnosed at advanced stages of disease. This pattern of late presentation has resulted in significant challenges in terms of designing effective therapies to achieve long-term cure. One potential promising strategy is the application of targeted therapeutics that exploit a myriad of critical pathways involved in tumorigenesis and metastasis. This review examines three of the most provocative targeted therapies with current or future applicability in epithelial ovarian cancer.

1. Introduction

Ovarian cancer represents the sixth most common malignancy as well as the seventh leading cause of cancer-related death in women worldwide [1, 2]. In the USA, this neoplasm ranks second among gynecologic cancers, yet it is by far the most lethal one, accounting for more than 15,000 deaths annually [3]. One of the major reasons underlying this dismal prognosis is the fact that nearly 75% of cases are diagnosed at an advanced stage (i.e., tumor already spread beyond the ovary) [4, 5], despite great efforts to develop reliable screening and prevention strategies.

To date, advanced ovarian cancer management has predominantly consisted of surgery followed by chemotherapy consisting of a combination of platinums and taxanes. More recently, neoadjuvant chemotherapy, a therapeutic alternative traditionally reserved for those patients considered poor candidates for upfront surgery, has emerged as a potential first-line option [6]. Even though up to 80% of these patients will respond to initial treatment, most of them will subsequently recur [7]. Chemotherapy success rates after relapse range from 10% to 50%, depending on whether the tumor is platinum sensitive or resistant (i.e., a progression-free interval (PFI) following platinum-based first-line therapy of more or less than 6 months, resp.). Unfortunately, almost all responses are invariably transient. Thus, the 5-year overall survival (OS) for late-stage disease is approximately 45% [2].

Since "nonspecific" therapies, namely, surgery, radiation, and conventional chemotherapy, have largely failed to achieve cure in the majority of patients affected by epithelial ovarian cancer, investigators have focused on developing novel treatment approaches. Many of these new strategies are based upon an understanding of the critical molecules and pathways specifically involved in tumorigenesis and metastasis. This has led to the development of "targeted" oncologic therapies that might be ultimately more effective and less toxic.

Although significant overlap occurs, targeted therapies can be broadly divided into two categories:

(i) those focused on cellular mechanisms that are disregulated in carcinogenesis,

(ii) those directed against the neoplasm's microenvironment, a tumor component lately recognized as highly relevant in both cancer growth and dissemination.

The present article addresses targeted therapies currently being employed or tested in epithelial ovarian cancer (EOC). Since their number has become as numerous as the

myriad of critical pathways involved in ovarian neoplastic transformation, this review will focus on three of the most promising and/or well-studied targeted weapons in ovarian cancer therapeutics to date, namely,

(i) antiangiogenesis compounds,

(ii) epidermal growth factor receptor (EGFR) antagonists,

(iii) poly (ADP) ribose polymerase (PARP) inhibitors.

2. Materials and Methods

A comprehensive literature search was conducted using the following key terms: "ovarian cancer", "targeted therapies", "antiangiogenesis", "epidermal growth factor receptor (EGFR) inhibitors", and "poly (ADP) ribose polymerase (PARP) inhibitors". For this purpose, primary sources used were PubMed and Cochrane Databases. Articles' selection was limited to those written in English, without restriction to year of publication. The main analysis was focused on those studies providing clinical evidence, although preclinical data were included either when background information was required or when clinical assays were absent. Highly valuable references cited by primarily collected studies as well as pivotal abstracts presented at prominent oncologic meetings, such as the Society of Gynecologic Oncologists (SGO), the American Society of Clinical Oncology (ASCO), the European Society of Gynaecological Oncology (ESGO), and the International Gynecologic Cancer Society (IGCS), were also assessed and their data incorporated whenever pertinent.

3. Antiangiogenesis

Angiogenesis (i.e., the formation of new blood vessels) plays a critical role in cancer expansion and propagation. While many tumors start as avascular nodules, early data demonstrated that growth is impaired beyond 2 mm^3 unless effective neovascularization is established [8]. Hence, this phenomenon appears to be a rate-limiting step in tumor progression. Antiangiogenic therapies have been shown to inhibit new blood vessels development, induce endothelial cells apoptosis, and normalize vasculature [5].

Many components interact in this process, such as proangiogenic factors, metalloproteinases, and endothelial precursor cells. Among angiogenesis-promoting molecules, the vascular endothelial growth factor (VEGF) is the most sensitive and potent one, as well as the best characterized [9]. It is overexpressed in many human tumors, including ovarian cancer. In ovarian malignancies, high levels of VEGF have been associated with poor prognostic features, such as advanced stage, carcinomatosis, distant metastasis, as well as a decreased survival [10]. Thus, the VEGF pathway has become one of the most attractive research areas in EOC therapeutics. Preclinical data from animal models showed that VEGF blockade was associated with inhibition of ascites formation and tumor growth [11].

Bevacizumab, a recombinant humanized monoclonal antibody directed against VEGF-A, was the first of these agents to be evaluated in EOC. Case reports and small series constituted the initial clinical evidence supporting its therapeutic value, mainly in recurrent, heavily pretreated patients [12, 13]. Based on these findings, two phase II trials using single-agent bevacizumab in recurrent ovarian cancer, predominantly platinum-resistant disease, were subsequently conducted (Table 1) [14, 15]. Their results demonstrated the following.

(a) An overall response rate (RR) was of 15%–21%. Unfortunately, less than 5% were complete responders.

(b) One study showed that additionally 50% of patients had stable disease.

(c) A 6-month progression-free survival (PFS) ranged from 30% to 40%.

(d) Hypertension was the most common side effect documented, being usually well controlled with standard antihypertensive medication. However, two major complications emerged, gastrointestinal perforation and thromboembolic disease, both venous and arterial, ranging from 0% to 11% and 3% to 7%, respectively. Indeed, one of the studies, Cannistra et al. [15], carried out in heavily pretreated patients, was prematurely closed due to the high incidence of bowel perforation observed.

(e) Bevacizumab-related deaths were estimated in up to 7% of treated patients.

This drug has been and continues to be tested in combination with chemotherapy, as a part of the first line treatment in newly diagnosed EOC and recurrent disease. Table 1 both summarizes the most relevant past and ongoing trials conducted in this setting.

Other anti-VEGF as well as non-VEGF mediated Antiangiogenic drugs are currently in clinical development. Table 2 illustrates some of these initiatives.

In conclusion, to date antiangiogenesis appears as one of the most promising targeted strategies explored in EOC. Given the encouraging initial results, bevacizumab has entered phase III trial evaluation. Meanwhile, it is considered a viable option in the recurrent setting. Appropiate bevacizumab dose (7.5 versus 15 mg/kg) and the ability to combine with other biologics require further study as well. Safety issues must be considered when using this compound. Adequate patient selection may potentially reduce the incidence of serious adverse events by excluding those at a highest risk for gastrointestinal perforation or a thromboembolic event. Major risk factors for these two complications have been described (Table 3), yet it should be noted that they still require further validation.

4. Epidermal Growth Factor (EGF) Receptors Antagonists

The family of EGF receptors (EGFRs) is composed of 4 structurally similar receptors which exert a tyrosine kinase function: ErbB1 (commonly referred to as epidermal growth

Table 1: Clinical trials testing Bevacizumab in EOC.

	Type	Study's Scope and Population	Intervention	Outcomes or Planned End Points
Published				
Burger (2007) [14]	Phase II	62 patients with persistent or recurrent Ov or PP cancers 66% had received two prior chemotherapy regimens 42% were platinum-resistant	Single-agent Bevacizumab	CR: 3% PR: 18% SD: 52% MPFS: 4.7 6-mon PFS: 40% MOS: 17 GIP: 0% TED: 0%
Cannistra (*) (2007) [15]	Phase II	44 patients with recurrent Ov or PP cancers 48% had received three prior chemotherapy regimens 84% were platinum-resistant	Single-agent Bevacizumab	CR: 0% PR: 16% SD: Not reported MPFS: 4.4 MOS: 10.7 GIP: 11% TED: 7%
Micha (2007) [16]	Phase II	Adjuvant treatment in front-line 20 patients stage III Ov, PP, or FT cancers 85% optimally cytoreduced	Carboplatin + Paclitaxel + Bevacizumab	CR:30% PR:50% SD: 5% TED: 10% (**) GIP: 0%
Garcia (2008) [17]	Phase II	70 patients with recurrent Ov or PP cancers Median n° of prior chemotherapy regimens: 2 40% were platinum-resistant	Metronomic Cyclophosphamide + Bevacizumab	CR: 0% PR: 24% SD: 63% MOS: 17 MPFS: 7 TED: 4% GIP: 4%
Ongoing				
TEACO	Phase II	Adjuvant treatment in front-line Stage IB-IV Ov, PP, or FT cancers Either optimally or suboptimally cytoreduced	Oxaliplatin + Docetaxel + Bevacizumab (both first line and maintenance)	1-year PFS Safety RR PFS OS
GOG 218	Phase III	Adjuvant treatment in front-line Stage III-IV Ov or PP cancers Either optimally or suboptimally cytoreduced	Carboplatin + Paclitaxel with or without Bevacizumab, either short-term or extended (maintenance)	PFS (primary) OS RR Toxicity QoL Translational objectives
ICON 7	Phase III	Adjuvant treatment in front-line High-risk early stage (I-IIA, clear cell or grade 3) or advanced stage (IIB or greater), either optimally or suboptimally cytoreduced Ov, PP, or FT cancers	Carboplatin + Paclitaxel with or without extended Bevacizumab	PFS (primary) QoL Cost effectiveness

Table 1: Continued.

	Type	Study's Scope and Population	Intervention	Outcomes or Planned End Points
GOG 213	Phase III	Platinum-sensitive recurrent Ov, PP, or FT cancers	Carboplatin + Taxane with or without Bevacizumab with or without Secondary cytoreduction	OS (primary) PFS Toxicity
OCEANS (AVF4095g)	Phase III	Platinum-sensitive recurrent Ov, PP, or FT cancers	Carboplatin + Gemcitabine with or without both short-and long-term (manitenance) Bevacizumab	PFS OS RR Safety profile of the combination

Ov: Ovarian; PP: Primary peritoneal; FT: Fallopian Tube
RR: Response rate; CR: Complete response; PR: Partial response; SD: Stable disease
TED: Thromboembolic disease (either arterial or venous); GIP: Gastrointestinal perforation
MPFS: Median progression-free survival (months); MOS: Median overall survival (months);
QoL: Quality of life
(*) Study stopped prematurely due to the high rate of severe complications (i.e., GIP)
(**) TED cases were not directly attributed to bevacizumab.

factor receptor), ErbB2 (Her2/neu), ErbB3 (Her3), and ErbB4 (Her4). Their activation triggers a cascade of events ultimately resulting in cell proliferation and survival. Like VEGF, EGFRs are frequently overexpressed and/or dysregulated in solid tumors. Ovarian cancer is not an exception, with up to 70% of cases exhibiting this aberrant phenotype, which has been linked to poor oncologic features and outcomes [5, 7, 29].

These observations have suggested that EGFRs might represent a viable target for novel therapies in EOC. While blockade of these receptors can be achieved by several mechanisms, two of these have been most extensively explored: (a) small molecules capable of inhibiting the tyrosine kinase domain and (b) monoclonal antibodies directed against the extracellular region.

Preliminary preclinical data demonstrated antitumoral activity and a reversion of the chemoresistant phenotype secondary to EGFR inhibition [30]. Nonetheless, and in contrast with the promising results obtained with anti-VEGF therapies, to date clinical trials with EGFR inhibitors alone have produced disappointing results.

4.1. Tyrosine Kinase Inhibitors (TKIs). Gefitinib and erlotinib are two of the main compounds in this category. Both are orally administrated and relatively well tolerated [31], which would represent a significant advantage in terms of patients' quality of life. Core findings from the most relevant trials conducted on these agents can be summarized as follows [32–38].

(a) An RR in recurrent ovarian cancer is of less than 10% along with stable disease in up to 44% of patients when used as single agents.

(b) These results were improved either when gefitinib was combined with standard chemotherapy or when erlotinib was combined with bevacizumab. The combination gefitinib-tamoxifen did not appear to add any clinical benefit.

(c) As a part of the first-line treatment in conjunction with a platinum and a taxane, either upfront or as consolidation therapy, TKIs have yet to confirm a demonstrable survival advantage. The EORTC has just finalized the recruitment of a phase III trial exploring erlotinib as maintenance therapy in both high-risk early-stage and advanced diseases.

(d) In terms of side effects, the most frequently observed were diarrhea (up to 30%, being the dose-limiting toxicity), nauseas and vomiting (nearly 10%), and an acne-like cutaneous rash (5%–15%), which interestingly correlated positively with tumor response. As expected, increased toxicity was seen when a cytotoxic agent was coadministrated.

As noted above, the initial experience with these agents has not revealed a definitive role in the treatment of unselected EOC population, either in the first-line or the relapsed setting. However, a subgroup of patients showing an increased likelihood to respond to these compounds has been identified. In Schilder's trial, published in 2005, clinical outcomes correlated with EGFR status, with a significantly longer progression-free survival (PFS) as well as a trend in improved overall survival (OS) among those who were EGFR (+). Specifically, an enhanced response to gefitinib was linked to the presence of an infrequent mutation affecting the catalytic domain of this receptor [32]. This relationship closely resembles what previously has been described in lung cancer [39, 40]. Thus, it has been suggested that prescreening patients for specific active EGFR mutations could define the population most likely to benefit from this therapy. Further investigation to validate this finding in EOC is warranted.

Novel EGFR inhibitors in development for EOC include lapatinib, canertinib, PKI-166, and EKB-569. Until better evidence supporting a relevant therapeutic value becomes available, the role of TKIs in this neoplasm remains predominately confined to clinical trials.

Table 2: Examples of other promising Antiangiogenic agents in EOC.

	Mechanism of action	Current evidence
VEGF-mediated		
Aflibercept (VEGF-Trap)	Soluble receptor which binds VEGF-A and-B as well as placenta-derived growth factor (PlGF) 1 and 2	Preliminary results reported by a Phase II trial conducted in recurrent setting showed similar results than bevacizumab, with a remarkable less incidence of bowel perforation (1%) [18] A phase III trial is ongoing
Cediranib	Small molecule that inhibits the tyrosine kinase domain of the VEFG receptor (VEGFR) Other members of this family are sorafenib and sunitinib	Two phase II trials in relapsing EOC demonstrated a response rate of nearly 20%, increasing up to 30% if disease stabilization is considered [19, 20] ICON 6, a phase III trial in recurrent platinum-sensitive patients, is now testing this agent in combination with carboplatin and paclitaxel
Sorafenib	Multitargeted TKI that inhibits raf kinase, VEGFR-2, VEGFR-3, Flt-3, c-kit, and platelet-derived growth factor receptor (PDGFR)	Phase I trial reported that 50% of patients showed stable disease [21]. Early data from a subsequent phase II study testing the combination of sorafenib with gemcitabine in recurrent EOC confirmed encouraging activity, with an overall response rate of 33% [22] Several other phase II trials employing sorafenib either in front-line, maintenance phase, or recurrent settings, alone or in combination with standard chemotherapy or biologics (e.g., bevacizumab) are underway A randomized phase III trial is currently evaluating Sorafenib as a maintenance therapy after first-line treatment in EOC
Pazopanib	Oral tyrosine kinase inhibitor that targets VEGFR, PDGFR, and c-kit	Preliminary results of a phase II trial conducted in recurrent EOC defined by CA-125 elevation showed a biochemical response of 47%, with stable disease observed in other 27% [23] A phase III trial is currently evaluating pazopanib as a maintenance therapy after first-line treatment in EOC
Non VEGF-mediated		
Vascular disrupting agents (VDAs)	Represent a new approach to deprive tumor from its blood supply, by causing the collapse of the established tumor vasculature. Their main targets are the endothelial cells Examples include tubulin destabilizers and flavanoids, among others	Preclinical data indicate that these drugs can improve tumor response to chemotherapy [24], radiation, and other Antiangiogenic therapies Zweifel and coworkers presented recently the final results of a phase II trial employing Fosbretabulin (a tubulin binder) along with carboplatin and paclitaxel in platinum-resistant EOC, revealing a response rate of 32% [25]

Table 3: Major risks factors potentially associated with bevacizumab-induced arterial thrombo-embolism and gastrointestinal perforation.

1- Arterial Thromboembolic Events (ATEs) [26]
- –Age ≥65 years
- –Prior history of ATE

2- Gastrointestinal Perforation [27, 28]
- –Multiple prior chemotherapy regimens (heavily pretreated patients)
- –Large intraabdominal tumor burden
- –Neoplastic bowel involvement
- –Clinical evidence of partial obstruction

4.2. Monoclonal Antibodies. Various humanized antibodies against the extracellular region of EGFR have been thought to be potentially effective in EOC. Nonetheless, similar to what has occurred with TKIs, the theory has not been confirmed clinically. Probably the most emblematic example illustrating this unfulfilled potential has been trastuzumab. Multiple initial studies confirmed that Her-2/neu overexpression was associated with an adverse prognosis of patients with epithelial ovarian cancer [41, 42]. Trastuzumab, a selective Her-2/neu inhibitor approved for the treatment of ErbB 2 (+) metastatic breast cancer, was proposed to have antitumoral activity comensurate with that observed in breast cancer. Further clinical evidence in a large GOG trial, however, demonstrated a response rate of only 7%, with disease

Table 4: Other Anti-EGFR antibodies explored in EOC.

Antibody	Target	Clinical data available
Cetuximab	ErbB1	Three phase II trials (one as a component of the first-line treatment and two performed in recurrence), alone or in combination with conventional cytotoxic therapy, have evidenced null or only modest impact of cetuximab in the management of EOC [44–46]
Matuzumab	ErbB1	One phase II study conducted in platinum-resistant, EGFR (+) population, concluded that matuzumab was well-tolerated, but lacked significant clinical activity [47]
Pertuzumab	Her2/neu	One phase II trial involving advanced, refractory EOC patients has been conducted using this agent. Like matuzumab, pertuzumab was associated with a poor response rate (approximately 4%) [48]

stabilization in other 39% of ErbB 2(+) recurrent ovarian cancer patients [43].

Results obtained with other monoclonal antibodies, alone or in combination with standard chemotherapy, are outlined in Table 4.

5. PARP Inhibitors

Approximately 10% of ovarian cancers are considered hereditary. Germline mutations affecting two genes, BRCA1 and BRCA2, account for the vast majority of these cases. The lifetime risk of developing an epithelial ovarian carcinoma among women who carry these genetic defects has been estimated to be up to 60% [49]. The proteins encoded by these tumor suppressor genes participate in multiple cellular processes, including transcription, cell cycle regulation, and repair of DNA double-strand breaks [50]. When inactived, chromosome instability occurs, an event potentially facilitating carcinogenesis.

Many other DNA-repair mechanisms are generally available within the normal cell. The base-excision repair (BER) complex constitutes one of them. The enzyme poly (ADP) ribose polymerase (PARP) is a key component of this pathway. Its scope is restricted to single-strand defects. Accordingly, its malfunction theoretically should not affect double-strand repair; however, a persistent single-strand defect may ultimately result in DNA replication interruption or a double-strand break [51]. When this occurs in a cell that is already unable to repair DNA damage , as the case for BRCA-defective cells, cell cycle arrest or death occurs. This observation, known as synthetic lethality [52], supports the contention that PARP blockade would be therapeutically effective in hereditary EOC. This premise was initially confirmed in preclinical studies demonstrating a highly increased sensitivity to PARP inhibition among BRCA-deficient cells, with a subsequent decreased cell survival, compared to those heterozygous or BRCA wild-type cells [53, 54].

Clinical studies exploiting this approach have been recently conducted in multiple human solid tumors. Initial trials used these agents primarily as chemosensitizers, mainly in association with methylating compounds [55]. However, with the demonstration of BRCA specific sensitivity, single-agent inhibitors were assessed. Recently, final results of the first phase I trial evaluating Olaparib, an orally administrated PARP inhibitor, in BRCA-defective malignancies, including ovarian cancer, showed a low toxicity along with a response or disease stabilization rate of 63% [56]. Multiple PARP inhibitors are currently being examined in phase II trials. Of interest, Audeh et al. lately reported the interim analysis of a phase II study employing Olaparib in BRCA-deficient advanced ovarian cancer [57]. Overall 57% of patients demonstrated response to PARP inhibition, using either RECIST or CA-125 criteria. Potential use of PARP inhibitors as chemoprophylactics in BRCA mutation carriers [58] and for treating sporadic ovarian cancers [49] has been proposed, as well.

A potential barrier to PARP inhibitors use has been the recently described emergence of resistance by reversal of the BRCA-deficient phenotype [59]. The clinical implications of this phenomenon require further clarification.

6. Conclusions and Future Overview

Women with epithelial ovarian cancer are living for longer periods of time than ever before. Development of novel chemotherapeutics has in part contributed to this improved outcome. However, a significant proportion of affected patients still succumbs to this difficult disease. Thus, progress is still needed. To this end, targeted therapies appear to be a promising platform for clinical development.

Many cellular pathways have been implicated in ovarian carcinogenesis, and exploitation of these perturbations critical in forming or maintaining the malignant phenotype has yielded a number of promising compounds. However, to date only Antiangiogenic agents have reached clinical relevance in EOC management. New therapeutic tools showing promising results, such as PARP inhibitors that exploit the abnormality responsible for the initial neoplastic transformation, have demonstrated encouraging clinical potential.

Some relevant lessons learned in targeted therapy development thus far include [7] the following.

(i) The mere presence of a particular molecule or pathway dysregulated in a particular tumor does not guarantee that its inactivation will have therapeutic benefit.

(ii) Response does not always translate into prolonged survival, symptom relief, or other valuable clinical endpoints. Conversely, there may be significant improvements in time-to-event endpoints such as time-to-progression or PFS, and yet objective responses

may be rare. Thus different clinical parameters may be neccesary for efficacy assessment of targeted agents.

(iii) Given the multiplicity and redundancy of aberrant pathways involved in ovarian cancer, it is unlikely that inhibition of a single cascade will be highly effective. Thus agents that act upon multiple levels or interconnected pathways simultaneously appear potentially more promising.

The future of cancer therapeutics will likely include tailored, individualized treatments, designed on the basis of an even deeper understanding of the critical alterations in ovarian carcinogenesis. Gene expression profiles have established that this neoplasia is far from being a uniform disease [60]. Thus, genotype-directed and pharmacogenomic therapies emerge as the next frontier for fruitful exploration and novel drug development.

Conflict of Interest Statement

Nicanor I. Barrena Medel and Jason D. Wright both have no conflicts of interest to declare. Thomas J. Herzog received Honoraria from Educational Programs: GSK, J&J, Lilly, Merck, and Genentech.

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Review Article

Targeted Therapy in Ovarian Cancer

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Ovarian cancer is the most common cause of mortality of tumors from gynecologic origin and is often diagnosed after patients have already progressed to advanced disease stage. The current standard of care for treatment of ovarian cancer includes cytoreductive surgery followed by adjuvant chemotherapy. Unfortunately, many patients will recur and ultimately die from their disease. Targeted therapies have been evaluated in ovarian cancer as a method to overcome resistant disease. Angiogenesis inhibitors have shown success in many tumor types and have also demonstrated promise in trials involving patients with ovarian cancer. PARP inhibitors may be potentially active agents in patients with BRCA-associated ovarian cancer. Trials that have evaluated combinations of targeted agents have often revealed untoward toxicities, thus tempering enthusiasm for this approach.

1. Introduction

Ovarian cancer is the most common cause of mortality from gynecologic cancer and will be responsible for 14 600 cancer related deaths this year. Secondary to vague presenting symptoms and the lack of effective screening, most patients will present with advanced disease. The current standard of care for ovarian cancer therapy is surgery followed by adjuvant carboplatin and taxane-based chemotherapy. Unfortunately, these protocols often do not allow for cure at initial diagnosis, and many patients will often recur and eventually die from their disease. Chemoresistance is an important hurdle in the treatment of recurrent cancer. Targeted therapy has subsequently come to the forefront of research and clinical trials in an effort to overcome resistant disease and achieve improvement in patient outcomes.

2. Epidemiology

Ovarian cancer is the second most common gynecologic malignancy, but is the most common cause of mortality from gynecologic cancer. It accounts for about 3 percent of all cancers among women and is the fifth most common cause of cancer-related death in women [1]. Approximately 21 550 cases will be diagnosed and 14 600 deaths will occur this year [2].

Surveillance Epidemiology and End Results (SEER) database shows that the incidence of ovarian cancer has decreased over the past 30 years [2]. Age-based incidence increases from 0.26/100 000 at age 5–9 to a peak of 58.3/100 000 at age 80–84. Following this, there is a downward trend in incidence rate. The lifetime risk of ovarian cancer in the general population is 1.7 percent. Most women who are diagnosed with epithelial ovarian cancer (EOC) are between the ages of 40 and 65.

3. Diagnosis and Initial Treatment

Unfortunately, the initial signs and symptoms of ovarian cancer are vague. These can include nonspecific complaints of bloating, gastrointestinal symptoms, and pain [3]. The subtle nature of symptoms can often delay patient presentation. It is important for a provider to retain a high index of suspicion if a patient presents with abdominal or pelvic symptoms, particularly if these symptoms occur daily, are more severe than expected, or present as a constellation of complaints. Secondary to lack of screening tools and the indolent nature of presenting symptoms, ovarian cancer often presents when patients have already progressed to disseminated disease. A prior analysis by the International Federation of Gynecology and Obstetrics showed that distribution by stage is I (23 to 33 percent), II (9 to 13 percent), III

(46 to 47 percent), and IV (12 to 16 percent) [4]. Those who present with advanced stage are often incurable.

Cytoreduction is the goal in initial surgical therapy for patients with ovarian cancer. Decreasing the remaining tumor burden has been shown to improve response to post-operative systemic chemotherapy. This finding is biologically plausible, in that small tumors are better perfused and more mitotically active, thereby allowing chemotherapeutic drugs to have better efficacy. A meta-analysis of over 53 studies with advanced stage ovarian carcinoma treated with platinum-based chemotherapy found a 5.5 percent increase in median survival for every 10 percent increase in the proportion of patients achieving maximal cytoreduction, which was defined as less than or equal to 3 cm in the analysis [5].

The current standard of care for initial adjuvant chemotherapy in EOC is a platinum drug, usually carboplatin, and a taxane. The Gynecologic Oncology Group (GOG) evaluated the efficacy of cisplatin versus carboplatin in a noninferiority trial. The authors concluded that a chemotherapeutic regimen consisting of carboplatin plus paclitaxel results in less toxicity, is easier to administer, and is not inferior, when compared with cisplatin plus paclitaxel [6].

4. Second Line and Targeted Therapy

Unfortunately, despite optimal cytoreduction and adequate adjuvant therapy, many patients with EOC will experience disease recurrence. Over 70–80 percent of patients will relapse and ultimately die of their disease [7]. Therapy for recurrent disease is varied and depends upon time to recurrence.

Patients are categorized into groups based on their disease-free period, including platinum-sensitive (those patients who recur greater than 12 months after therapy), partially platinum-sensitive (those who recur between 6–12 months after therapy), platinum-resistant (those who recur before 6 months after therapy), and platinum-refractory (those who never achieve disease free status). Traditionally, patients who recur more than 6 months after initial therapy are given a second course of platinum-taxane-based chemotherapy. Platinum-sensitive disease has a greater than 50 percent response rate to single agent carboplatin, while resistant disease has a 10–20 percent response rate and refractory disease response is even lower [8]. The latter groups are therefore typically treated with other FDA approved chemotherapy regimens including pegylated liposomal doxorubicin, gemcitabine, topotecan, and etoposide [9].

The bane of ovarian cancer therapy is the failure of currently established treatment protocols to allow for cure of the disease at diagnosis, even in patients with initially chemosensitive tumors. Despite efforts of clinical trials to identify more efficacious regimens to overcome the chemoresistance encountered after front-line platinum-taxane treatment, clinical response to second-line therapy continues to be short lived and results in only marginal improvements in progression free and overall survival [8]. In response to this challenge, the idea of overcoming resistant disease with targeted therapy has come to the forefront of investigation in ovarian cancer therapy.

5. Angiogenesis Targeted Therapy

Angiogenesis is the development of new blood vessels in areas of new tissue growth. This is a normal phenomenon associated with routine processes including wound healing and embryogenesis. It is also an important process that occurs almost universally in solid tumors as a response to the expansion of the cancer mass and its subsequent growth away from existing blood supply. This causes the oxygen tension to decrease beneath physiologic levels needed for oxidative metabolism [10].

An important interplay of proangiogenic signaling occurs in response to the hypoxic state. A protein called hypoxia-inducible factor (HIF) 1 alpha is stabilized in these conditions and enters the nucleus where it forms a complex with another protein (HIF 1 beta) [11]. This complex is then able to act as a transcription factor allowing upregulation of growth factors including vascular endothelial growth factor (VEGF) [12, 13]. The VEGF family includes six closely related molecules, but the most important angiogenic agent is VEGF-A.

Molecular markers of angiogenesis have been studied in ovarian cancer. Prior studies have shown associations between VEGF-A levels and microvessel density in primary tumors and disease extent as well as progression-free and overall survival following initial antiangiogenic therapy [14]. Preclinical models have also shown the importance of the VEGF pathway in ascites formation [15, 16].

Bevacizumab is a monoclonal antibody directed against VEGF-A. Studies evaluating this agent have shown improved survival in colorectal [17], breast [18], and lung cancers [19]. A GOG phase II study of bevacizumab in persistent or recurrent EOC or primary peritoneal carcinoma was performed by Burger et al. [20]. This study revealed a 21% clinical response rate. Of the 62 patients on trial, 25 experienced at least 6-months progression free survival (PFS), with a median PFS of 4.7 months and median overall survival of 17 months. This study was unique in that none of the patients experienced gastrointestinal perforation, a known complication of bevacizumab in other clinical trials. Cannistra et al. performed a phase II trial of single agent bevacizumab in patients with platinum-resistant disease [21]. As opposed to the GOG trial, this study was closed early secondary to the proportion of patients that experienced GI perforations (5/44), but the study did show a 16% response rate and a median durable response of 12 weeks. Toxic events that were similar between these two trials include hypertension and vascular thrombosis. Garcia and colleagues performed a phase II trial of bevacizumab that evaluated the use of bevacizumab and low-dose metronomic oral cyclophosphamide in recurrent ovarian cancer [22]. The authors found a 28% response rate with 6 month PFS of 28%; see Table 1.

Based on the activity of bevacizumab as documented in these phase II trials, there are currently two trials that are ongoing to evaluate the activity of bevacizumab in the setting

Table 1: Results of three pivotal trials evaluating bevacizumab in ovarian cancer. Burger et al. and Cannistra et al. evaluated bevacizumab as a single agent whereas Garcia et al. evaluated bevacizumab with low-dose metronomic oral cyclophosphamide. All studies were performed in patients with recurrent disease.

Author	Progression free survival	Overall survival	Bowel perforation
Burger et al.	3.4 months	7.29 months	0%
Cannistra et al.	4.4 months	10.7 months	11.4%
Garcia et al.	7.2 months	16.9 months	5.7%

Table 2: Review of studies of antiangiogenic agents in recurrent or persistent ovarian cancer. RR: response rate, HTN: hypertension, RF: renal failure, P/S: platinum-sensitive, P/R: platinum resistant. *2 confirmed and one unconfirmed partial response. **1 unconfirmed partial response.

Agent	Authors	RR	Toxicities
VEGF trap	Tew et al.	5/45 partial	HTN, proteinuria, encephalopathy, RF
Cediranib	Hirte et al.	P/S: 3/17 partial* P/R: 1/24 partial**	Diarrhea, HTN, fatigue, anorexia

of front line adjuvant therapy. The first is GOG 218, a study that evaluates stages III and IV EOC patients who have undergone surgery and are subsequently randomized to one of three arms; arm 1 utilizes the traditional chemotherapy regimen of carboplatin (AUC 6) and paclitaxel (175 mg/m2) and placebo, arm 2 includes the active drugs of arm 1 and adds bevacizumab (15 mg/kg every 21 days for 6 cycles, starting with cycle 2), while arm 3 includes the drugs of arm 2 and adds maintenance bevacizumab given every 21 days to complete 22 cycles. A second trial is run by the Gynecologic Cancer InterGroup in Europe (ICON7) and is an open label trial. The ICON7 study population includes both high risk early stage disease (stage I-IIa with grade 3 or clear cell histology) and advanced disease IIb-IV EOC or primary peritoneal cancer. Patients are randomized to one of two arms: carboplatin and paclitaxel or carboplatin, paclitaxel, and bevacizumab. The bevacizumab arm also includes a maintenance schedule continuing the drug every three weeks for 12 cycles. The study aims to evaluate PFS as a primary endpoint and overall survival, duration of response, and response rate as secondary endpoints [23].

Bevacizumab has also been studied in conjunction with other targeted agents. A phase I study of bevacizumab and a vascular disrupting agent (VDA) combretastatin 4A phosphate (CA4P) in patients with advanced solid tumors demonstrated no additive toxicity and the evidence for efficacy was encouraging [24]. This is of interest because preclinical evidence exists for synergy between VDA, which causes a surge in VEGF-stimulated circulating endothelial progenitor cells, and bevacizumab, which suppresses this induced effect [25].

VEGF Trap is a fusion protein consisting of the extracellular domains of human VEGF-1 and -2. This protein binds to VEGF-A and placental growth factor. In mouse models VEGF Trap treatment resulted in decreased ovarian cancer growth and ascites [26]. Tew and colleagues reported on a phase II study evaluating patients with recurrent, platinum-resistant EOC. The participants received VEGF trap (2 or 4 mg/kg) administered intravenously every two weeks. This study yielded an 11% partial response, with grade 3/4 toxicities including hypertension, proteinuria, encephalopathy, and renal failure [27]. A phase II trial involving VEGF trap combined with docetaxel in patients with recurrent EOC, primary peritoneal cancer or fallopian tube cancer with measurable disease is currently ongoing [28]. Patients in this study will receive VEGF trap at the maximum tolerated dose (as determined in Phase I of the trial which has closed to accrual) over 1 hour on day 1 of course 1, followed by VEGF trap IV over 1 hour and docetaxel over 1 hour on day 1 in all subsequent courses. The courses repeat every 21 days in absence of disease progression or unacceptable toxicity.

C-kit is a growth factor receptor of the tyrosine kinase subclass III family, the ligand of which is Stem Cell Factor, and is normally expressed in many cell lines, including gametocytes [29]. C-kit signaling promotes cell proliferation, differentiation, migration, adhesion, and survival [30]. The platelet derived growth factor receptor beta (PDGRF-B) gene encodes a cell surface tyrosine kinase receptor for members of the platelet derived growth factor family. This receptor is essential for cell migration and development of microvasculature.

Cediranib is an oral VEGFR-1, -2, and -3, PDGFR-B, and c-kit inhibitor. Hirte et al. performed a phase II trial of cediranib in patients with recurrent or persistent EOC, primary peritoneal or fallopian tube cancers [31]. The trial design initially included daily oral dosing of 45 mg, which was decreased to 30 mg continuously secondary to toxicity. Of the patients with platinum sensitive disease, 41% responded to therapy, while those with platinum-resistant disease demonstrated a 29% response rate. Significant side effects included diarrhea, hypertension, fatigue, and anorexia. Median time to progression was 4.1 months, while median overall survival was 11.9 months. A phase III study of cediranib in patients with platinum sensitive recurrent EOC is currently ongoing in Europe; see Table 2.

6. Epidermal Growth Factor Receptor

Epidermal growth factor receptor (EGFR) is overexpressed in 70% of cancers and is associated with chemoresistance, poor prognosis, and advanced disease at presentation [32, 33]. The mechanism of growth factor receptors is via activation of the intracellular tyrosine kinase domain, which triggers downstream targets and subsequently cell proliferation and survival [34]. Preclinical studies suggested that inhibiting this target might reverse chemoresistance and demonstrate antitumor activity [35–37]. Unfortunately, clinical trials evaluating drugs affecting these pathways, such as studies of EGFR tyrosine kinase inhibitors (gefitinib and erlotinib) and monoclonal antibodies directed against EGFR (cetuximab, panitumumab, and matuzumab), have not been met with significant success, showing only modest efficacy [8].

Gefitinib is a small molecule tyrosine kinase inhibitor that binds to the ATP-binding site of the EGF receptor and thereby prevents its activation. A GOG phase II study of gefitinib in patients with relapsed or persistent ovarian or primary peritoneal carcinoma assessed the activity and tolerability of a daily oral dose of 500 mg. The trial showed that only four of 27 eligible and evaluable patients exhibited progression-free survival greater than 6 months. One objective response was seen, and interestingly this patient was found to have the rare presence of an EGFR mutation. EGFR expression was associated with longer PFS ($P = .008$) and possibly longer survival ($P = .082$). Gefitinib was well tolerated, with dermatologic (15%) and diarrhea (30%) the most common grade 3 toxicities [38]. A phase II trial performed by the AGO Ovarian Cancer Study group evaluated gefitinib (500 mg/day) in combination with tamoxifen (40 mg/day) given until progression or unacceptable toxicity in patients with platinum-resistant EOC [39]. While this study demonstrated no tumor responses, 16 of 56 patients had stable disease. Notably, there was an 11% discontinuation rate secondary to side effects including diarrhea and skin rash.

Erlotinib is an oral epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor. Gordon and colleagues performed a phase II study in patients with refractory, recurrent, HER1/EGFR positive EOC [34]. Patients received 150 mg erlotinib orally once a day for up to 48 weeks or until disease progression or dose-limiting toxicity. This study found little clinical activity, with an objective response rate of 6% (2/34), both of which were partial responses. Stable disease was seen in 15/34 patients. Rash (68%) and diarrhea (38%) were the most frequent adverse events. Erlotinib was recently investigated as a single agent medication in maintenance therapy after first-line chemotherapy in a large study performed by the European Organization for Research and Treatment of Cancer (EORTC), with results forthcoming.

Disappointment was also encountered in clinical trials examining erlotinib in combination with other agents, where toxicity led to premature termination [40]. A phase II trial by Nimeiri and colleagues of bevacizumab (15 mg/kg) administered intravenously every 21 days and erlotinib (150 mg) given orally every day was performed in 13 patients with recurrent ovarian, primary peritoneal and fallopian tube cancer. This study showed a 15% response rate and seven patients had a best response of stable disease. Two patients had fatal gastrointestinal perforations, which lead to the early termination of the trial.

Overexpression of ERBB2 is also found in patients with ovarian cancer. Trastuzumab, or Herceptin, is a monoclonal antibody directed against ERBB2, and has been studied in a phase II trial by the GOG. This study evaluated the drug in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2 [41]. Patients initially received trastuzumab at a dose of 4 mg/kg, then weekly at 2 mg/kg. Patients without progressive or excessive toxicity could continue indefinitely, and those with stable or responding disease at 8 weeks were offered treatment at a higher dose (4 mg/kg) at time of progression. The authors reported that only 7% of the patients responded to treatment and a median time to progression of 2 months was seen.

Pertuzumab is a monoclonal antibody that inhibits dimerization of ERBB2 with EGFR, ERBB3, and ERBB4. A phase II trial of single agent pertuzumab administered as an intravenous loading dose of 840 mg followed by 420 mg every three weeks (in cohort 1) and as 1050 mg every three weeks (in cohort 2) was performed in advanced, refractory ovarian cancer. The authors reported a 4.3% partial response rate and 6.8% of patients with stable disease lasting at least 6 months. Median PFS was 6.6 weeks [42]. Patients who were phosphoHER2 positive had a trend toward higher median PFS (20.9 weeks) versus those who were negative (5.8 weeks, $P = .14$). Two trials have evaluated the efficacy of pertuzumab when combined with chemotherapy, one phase II study in combination with carboplatin and another phase II trial in combination with gemcitabine [43, 44]. The gemcitabine and pertuzumab trial was performed in patients with platinum resistant EOC, and there was the suggestion of some benefit of pertuzumab in patients with low levels of ERBB3 mRNA expression and platinum-resistant disease; see Table 3.

7. Multikinase Inhibitors

Sorafenib is an oral multikinase inhibitor that targets the mitogen-activated protein kinase (MAPK) pathway or Raf/MEK/ERK pathway [45]. This drug also inhibits VEGFR-1, -2, and -3 and platelet-derived growth factor receptor (PDGFR) beta tyrosine kinase activity. Sorafenib is currently FDA-approved for treatment of advanced renal cell cancer, and the biologic rationale for attempting its use in other solid tumors is the fact that MAPK pathway is well conserved evolutionarily and may serve as a central and common target [23].

A phase II trial of single agent sorafenib in persistent or recurrent EOC or primary peritoneal cancer was performed by the GOG [46]. Patients received sorafenib 400 mg orally twice daily until disease progression or prohibitive toxicity. Of the 59 patients with measurable disease, there were 2 partial responders and 20 patients with stable disease,

Table 3: Review of drugs in the epidermal growth factor receptor family that have been evaluated in ovarian cancer. RR: response rate, SD: stable disease, HTN: hypertension.

Agent(s)	Authors	RR	SD	Toxicities
Gefitinib	Schilder et al.	1/27		Dermatologic, diarrhea
Gefitinib + tamoxifen	Wagner et al.	0/56	16/56	Diarrhea, skin rash
Erlotinib	Gordon et al.	2/34 partial	15/34	Rash, diarrhea
Erlotinib + bevacizumab	Nimeiri et al.	1/13 complete 1/13 partial	7/13	Anemia, nausea, vomiting, HTN, diarrhea, 2 fatal GI perforations
Transtuzumab	Bookman et al.	1/41 complete 2/41 partial		
Pertuzumab	Gordon et al.	5/107 partial	8/107	Diarrhea

30 patients had progressive disease reported, and 7 were unable to have their tumor assessed. Grade 3 and 4 toxicities included rash, gastrointestinal, cardiovascular, metabolic, and pulmonary.

Sorafenib has also been studied in conjunction with other medications. A Phase I dose escalation study of sorafenib (200 mg orally twice daily) and bevacizumab (5 mg/kg or 10 mg/kg intravenously every two weeks) showed six Response Evaluation Criteria in Solid Tumors (RECIST) partial responses in 13 ovarian cancer patients, with duration of response from 4 to over 22 months [47]. Unfortunately, this combination yielded significant toxicity, with grade 3 hypertension, diarrhea, hand-foot syndrome, thrombocytopenia, proteinuria, and two episodes of fistula formation at sites of disease response. A phase II trial evaluated sorafenib in combination with gemcitabine [48]. Patients were given gemcitabine 1000 mg/m^2 intravenously weekly for 7 out of 8 weeks of the first cycle, then weekly for the first 3 weeks of a 4-week cycle and sorafenib 400 mg orally twice daily continuously. Using RECIST criteria, the authors reported 1 out of 18 evaluable patients had a partial response and 5 had a confirmed partial response by CA125 criteria. An additional 10 patients exhibited stable disease. Median time to progression was 5.4 months and overall survival was 13.3 months. The most frequent grade 3 and 4 toxicities were hematologic (lymphopenia, neutropenia, and thrombocytopenia), fatigue,

hypokalemia, and hand-foot syndrome.

Imatinib mesylate inhibits abl, c-kit, and PDGFR tyrosine kinases, thereby inhibiting tumor growth. It is FDA approved for some forms of adult and child chronic myelogenous leukemia as well as gastrointestinal stromal tumors (GISTs). Activating mutations of kit have not been found in ovarian cancers, but abnormal kit expression has been described [49]. The activity of single agent imatinib in patients with recurrent EOC has been poor. A phase II trial of imatinib administered orally at 600 mg daily for six weeks and repeated in absence of measurable progressions was performed in patients with platinum and taxane-resistant ovarian and primary peritoneal cancer. This trial showed no complete or partial responders during a median followup of 6.6 months [50]. A phase II trial of imatinib mesylate (400 mg orally) in recurrent ovarian cancer with positive c-kit or PDGFR found no objective responders and a median PFS of only 2 months [51]. The GOG also conducted a phase II trial of single agent imatinib (400 mg orally twice daily) in recurrent or persistent EOC or primary peritoneal cancer [49]. Eligibility for this trial included expression of at least one target (c-kit, PDGFR-alpha, PDGFR-beta) in the tumor. Only 9/56 patients were progression free for at least 6 months, with a median PFS of 2 months and median overall survival of 16 months. The most common grade 3 and 4 toxicities included GI, pain, electrolyte disturbances, dermatologic, and neutropenia; see Table 4 .

8. PARP Inhibition

Poly(ADP-ribose) polymerase (PARP) is an enzyme involved in repair of DNA single-strand breaks using the base excision repair pathway [52, 53]. A recent review by Yap et al. detailed the mechanism by which PARP inhibition can lead to cancer cell death. Inhibition of PARP leads to the accumulation of DNA single-strand breaks, which may subsequently lead to DNA double-strand breaks at replication forks [54]. In normal cells, double-strand breaks would be repaired in part by error-free homologous recombination DNA repair mechanisms [8]. Two proteins involved in this process are functional BRCA1 and BRCA2, which have a role in homologous recombination repair and maintenance of genomic stability [55]. If somatic mutations or epigenetic silencing leads to the absence of either BRCA1 or BRCA2, alternative DNA repair pathways such as nonhomologous end joining are employed; this subsequently results in chromosomal instability and cell death [54]. The use of PARP inhibitors in BRCA mutation carriers exploits the concept of synthetic lethality via combination of base excision repair inhibition with a defective homologous DNA repair pathway which results in the generation of unrepaired DNA single-strand breaks, an accumulation of double-strand breaks, collapsed replication forks, and eventual cell death [56–58]; see Figure 1 .

Olaparib is an oral small-molecular PARP inhibitor. Preclinical studies confirmed that BRCA-deficient cells were up to 1000-fold more sensitive than wild-type cells to PARP inhibition [57]. Cells that are heterozygous for BRCA mutations, with an intact homologous recombination function, had a lack of sensitivity to PARP inhibitors similar to wild-type cells. This finding suggests that a therapeutic index

Table 4: Review of multikinase inhibitors that have been studied in ovarian cancer. RR: response rate, SD: stable disease, HTN: hypertension, HFS: hand-foot syndrome.

Agent(s)	Authors	RR	SD	Toxicities
Sorafenib	Matei et al.	2/59 partial	20/59	Rash, GI, cardiovascular, metabolic, pulmonary
Sorafenib + bevacizumab	Azad et al.	6/13 partial		HTN, diarrhea, HFS, thrombocytopenia, proteinuria, fistula
Sorafenib + gemcitabine	Welch et al.	6/18 partial	10/18	Lymphopenia, thrombocytopenia, HTN, HFS, pain, neutropenia, hypokalemia
Imatinib	Coleman et al.	0/12	4/12	Fatigue, nausea/vomiting, rash, neutropenia
Imatinib	Alberts et al.	0/19		Hematologic, metabolic
Imatinib	Schilder et al.	1/56 complete		Neutropenia, GI, dermatologic, pain, electrolyte disturbances

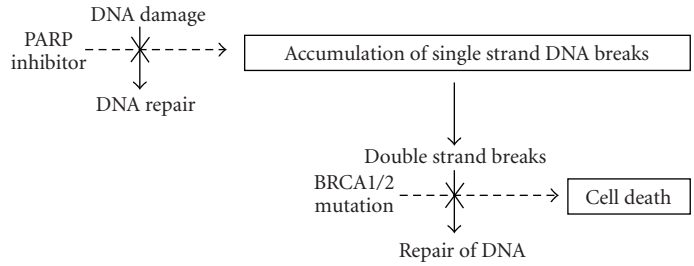

Figure 1: Poly(ADP-ribose) polymerase is involved in base excision repair of DNA single-strand breaks. If PARP is inhibited, these breaks can accumulate, potentially leading to double-strand breaks. These double-strand breaks are normally repaired by error-free homologous recombination, of which BRCA1 and BRCA2 proteins are involved. If these proteins are affected by somatic mutation or epigenetic silencing, eventual chromosomal instability and cell death can be seen.

for antitumor therapy may be present in BRCA-associated ovarian cancer [54, 57].

In phase I trials, olaparib was well tolerated, and there were no obvious differences in the pattern of toxicities between BRCA and non-BRCA patients [59–61]. A phase I trial in BRCA deficient ovarian cancer included 41 BRCA1 mutation carriers, 8 BRCA2 mutation carriers, and one patient with compelling family history for BRCA mutation. Of the 46 patients evaluable for RECIST or Gynecologic Cancer Intergroup CA125 response, 41% responded. An additional 11% of patients had meaningful stabilization of disease by RECIST criteria. Median response duration was 30 weeks. Responses were more frequent in the platinum-sensitive group, but were also seen in platinum-refractory and platinum-resistant populations. A phase I trial included 60 patients, 22 of which were carriers of a BRCA1 or BRCA2 mutation and one had a strong family history of BRCA-associated cancer but declined mutational testing [62]. The olaparib dose and schedule were increased from 10 mg daily for 2 of every 3 weeks to 600 mg twice daily continuously. Dose limiting toxicities including mood alteration, fatigue, thrombocytopenia, and somnolence seen at 400–600 mg twice daily led to the recruitment of a second cohort of patients consisting of only BRCA1 or 2 mutation carriers, who received olaparib at 200 mg twice daily. Objective antitumor activity was reported only in mutation carriers.

A randomized phase II trial comparing olaparib (200 or 400 mg orally twice daily) with pegylated liposomal doxorubicin (50 mg/m2 monthly intravenous) in patients with BRCA-mutated ovarian cancer with a platinum-free interval of 0–12 months is currently underway (NCT00628251). Another ongoing trial is a randomized placebo-controlled study of olaparib (400 mg orally twice daily) as maintenance therapy in patients with serous/sporadic ovarian cancer at high risk of early recurrence (NCT00753545).

9. Summary

Newer targeted therapies are undergoing evaluation in ovarian cancer. The most promising at this time are those directed towards inhibition of angiogenesis. Combining targeted therapeutics has resulted in significant toxicities, tempering enthusiasm for this approach. The finding of PARP inhibitors as potentially active agents in BRCA-associated ovarian cancer further supports the importance of screening patients for potential BRCA-associated disease and offering mutational testing when appropriate. Finally, given that the patient population who has typically entered trials evaluating targeted therapeutics includes those with recurrent or resistant disease, perhaps the finding of stable disease has some merit in the context of treatment effectiveness. Deeper understanding of biological pathways in ovarian cancer will be needed to select patients who enter these trials.

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Review Article

A Current Review of Targeted Therapeutics for Ovarian Cancer

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Difficult to detect, ovarian cancer typically presents at an advanced stage. Significant progress has been achieved in the treatment of ovarian cancer with therapeutics focused on DNA replication or cell division. However, despite sensitivity to induction chemotherapy the majority of patients will develop recurrent disease. Conventional agents for recurrent disease offer little in terms of long-term responses. Various targeted therapeutics have been explored in the management of ovarian cancer. These include monoclonal antibodies to epidermal growth factor receptors, small molecule tyrosine kinase inhibitors, monoclonal antibodies directed at the vascular endothelial growth factor (bevacizumab), and the small tyrosine kinase inhibitors that target the vascular endothelial growth factor receptor. Recently, several other agents have come forth as potential therapeutic agents in the management of ovarian cancer. These include monoclonal antibodies to the folate receptor, triple angiokinase inhibitors, PARP inhibitors, aurora kinase inhibitors, inhibitors of the Hedgehog pathway, folate receptor antagonists, and MTOR inhibitors.

1. Introduction

Various targeted therapeutics have been explored in the management of ovarian cancer. These include monoclonal antibodies to Her 2 neu [1, 2] and other epidermal growth factor receptors [3] (i.e., Trastuzumab [1], Pertuzumab [2], and EMD 7200 [3]), small molecule tyrosine kinase inhibitors that targeted the various EGFR receptors (gefitinib [4], erlotinib [5], CI-1033 [6]), monoclonal antibodies directed at the vascular endothelial growth factor [7–19] (bevacizumab), and the small tyrosine kinase inhibitors that target the vascular endothelial growth factor receptor [20–25]. Recently, several other agents have come forth as potential therapeutic agents in the management of ovarian cancer. These include monoclonal antibodies to the folate receptor, triple angiokinase inhibitors, PARP inhibitors, aurora kinase inhibitors, inhibitors of the Hedgehog pathway, folate receptor antagonists, and MTOR inhibitors.

This paper will explore the current data on the various targeted approaches in ovarian cancer. Attention will be directed at understanding the molecular mechanisms of these agents balanced with their application to clinical practice.

2. Angiogenesis

Enthusiasm for cytotoxic agents in the management of ovarian cancer has been tempered by the emergence of resistance. As such, a focus on alternative innovative therapeutics has emerged. One such direction is the inhibition of angiogenesis. Angiogenesis is one of the cardinal processes leading to invasion and metastasis of solid tumors. The angiogenic-signaling pathway may be triggered by the release of angiogenic ligands such as the vascular endothelial growth factor from tumor cells. Tumor angiogenesis is well established as essential for the growth and metastasis of solid tumors, [26–28] This process involves the recruitment of mature vasculature and circulating endothelial cells [29, 30] and proangiogenic soluble mediators one of which includes the vascular endothelial growth factor (VEGF) [31]. This factor has several known activities [31], such as mitogenesis, angiogenesis, endothelial survival, enhancement of vascular permeability, and effects on hemodynamic status. In ovarian cancer increased levels of VEGF are associated with poor prognosis and have been confirmed in multivariate analysis as an independent prognostic indicator of survival [28, 32–38]. Given the poor long-term responses appreciated with

conventional cytotoxic agents that target VEGF have taken center stage.

Agents targeting angiogenesis include monoclonal antibodies to the VEGF ligand [7–19], small tyrosine kinase inhibitors that target the vascular endothelial growth factor receptor [20–25], and soluble decoy VEGF receptors [39, 40]. The most studied agent to date has been bevacizumab, a recombinant humanized monoclonal antibody to the VEGF ligand.

To date several investigators [7–19] (Table 1) have explored bevacizumab as a single agent or in combination with chemotherapy in the management of advanced ovarian cancer.

Several studies in both the upfront and in the recurrent setting are underway. GOG 218 is a randomized placebo controlled three-arm study examining the role of bevacizumab in combination with carboplatin and paclitaxel and also as a maintenance therapy. ICON-7 is a two arm trial comparing carboplatin and paclitaxel (six cycles) versus carboplatin, paclitaxel, and bevacizumab (7.5 mg/kg) for six cycles followed by 12 cycles of maintenance bevacizumab. Campos et al. [20] is conducting a phase II trial of carboplatin/paclitaxel/bevacizumab in optimally and suboptimally debulked patients. Patients achieving a clinical complete response, partial response, or stable disease are subsequently randomized to either bevacizumab for 12 months or the combination of bevacizumab and erlotinib. Preliminary safety results have noted an increase in hypertension but to date no evidence of gastrointestinal perforations.

Given the recent data that has emerged on the role on intraperitoneal chemotherapy [41–43] investigators are exploring the role of IP chemotherapy with IV bevacizumab. Several abstracts were highlighted at the recent American Society of Clinical Oncology meeting. Konner et al. [44] and McKeekin et al. [45] in independent studies reported the feasibility of utilizing bevacizumab (IV) in conjunction with intraperitoneal therapy. One bowel perforation [44] was noted the Konner study while McKeekin et al. [45] colleagues noted one deep venous thrombosis and one fistula.

In the recurrent setting several trials are being conducted. The OCEANS trial is a randomized study of carboplatin/gemcitabine and bevacizumab (NCT 00434642) versus carboplatin/gemcitabine. GOG 213 (Figure 1) is randomized trial in recurrent ovarian cancer patients. Patients are stratified as to whether or not they are surgical candidates. If the patients are deemed to be surgical candidates they are randomized to surgery or no surgery followed by randomization to chemotherapy. If patients are randomized to no surgery they are subsequently randomized to carboplatin and paclitaxel or carboplatin/paclitaxel and bevacizumab.

The combination of carboplatin/DOXIL and bevacizumab is also being studied. The later trial may prove to be intriguing given the recently reported results of the CALYPSO trial [46]. In the CALYPSO trial the combination of Carboplatin-Doxil demonstrated a superior therapeutic index (benefit/risk ratio) versus current standard, carboplatin-paclitaxel.

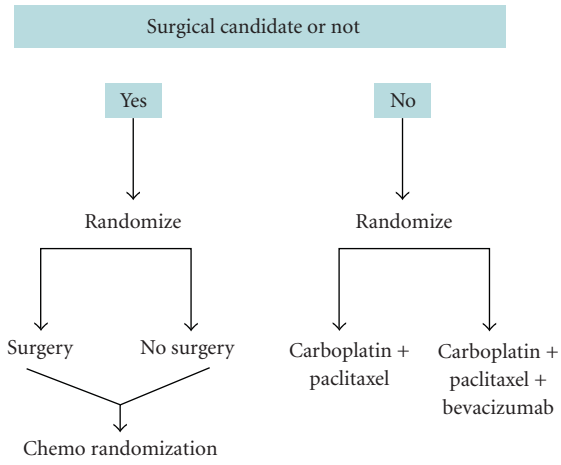

Figure 1: GOG 213.

3. Small Molecules that Target the VEGFR Receptor

Small molecule tyrosine kinase inhibitors that target the vascular endothelial growth factor receptor are currently being investigated in numerous clinical trials. AZD2171 (Cediranib) is a novel oral tyrosine kinase inhibitor of VEGFR2, VEGFR1, and c-kit. Matulonis et al. [21] reported the initial results of this agent in the management of patients with recurrent ovarian cancer. Five patients had confirmed partial responses with an overall response rate of 18.5%. Three patients had stable disease lasting 30, 27+, and 24 weeks. Hirte et al. [22] reported a response rate of 40.5% in platinum sensitive patients and a response rate of 29% in platinum resistance disease with AZD 2171 (Cediranib). Prevalent side effects included fatigue and hypertension. Currently, ICON-6 is conducting a study of AZD2171 (Cediranib) in platinum sensitive relapsed ovarian cancer in a three arm randomized placebo-controlled phase III trial in combination with paclitaxel and carboplatin. (Figure 2).

Pazopanib is tyrosine kinase inhibitor of vascular endothelial growth factor receptor (VEGFR) -1, -2, and -3, platelet-derived growth factor receptor (PDGFR) $-\alpha$ and $-\beta$, and c-Kit. Friedlander et al. [24] have reported activity with pazopanib in women with advanced epithelial ovarian cancer. Eleven of 36 subjects (31%) experienced a cancer antigen-125 (CA-125) response to pazopanib. Overall response rate based on modified Gynecologic Cancer Intergroup (GCIG) criteria (incorporating CA-125, Response Evaluation Criteria in Solid Tumors (RECIST), and clinical assessment) was 18% in subjects with measurable disease at baseline and was 21% in subjects without measurable disease at baseline. Median PFS was 84 days.

Sunitinib, an inhibitor that targets the VEGFR 1, 2, 3, and platelet–derived growth factor receptors, has also been studied in the management of patients with recurrent ovarian cancer. Biagi et al. [25] investigated the role of sunitinib in the management of patients with recurrent ovarian cancer. Sunitinib was administered at 50 mg every

Table 1: Current trials in ovarian/fallopian/peritoneal cancer.

Author	N	Prior lines	Platinum S/R*/first line	Regimen**	RR:CR + PR	TTP/PFS median
Burger R et al. [7]	62	1-2	+/+	Single	21 %	PFS 4.7mo
Cannistra et al. [8]	44	2-3	+/+	Single	15.9%	PFS 4.4 mo
Garcia et al. [9]	70	1–3	+/+	Combo	24%	TTP 7.2 mo
Wright et al. [10]	23	2–15	−/+	Combo	35 %	TTP 5.6 mo
Chura et al. [11]	15	5–15	+/+	Combo	43 %	PFS 3.9 mo
Nimeiri et al. [12]	13	1–3	+/+	Combo	15 %	PFS 4.1 mo
Monk et al. [13]	32	2–10	−/+	Single	16 %	PFS 5.5 mo
Simpkins et al. [14]	25	2–12	−/+	Combo	28 %	TTP 9.0 mo
McGonigle et al. [15]	18	0–2	−/+	Combo	22%	PFS 3.8 mo
Azad et al. [17]	13	NR	NR	Combo	46%	NR
Micha et al. [16]	20	0	First line	Combo	80%	NR
Campos/Penson et al. [18, 19]	58	0	First line	Combo	75%	PFS:11mo

*Enrolled patients: platinum sensitive/resistant/first line.
**Single bevacizumab or combination therapy with cytotoxic or other biological agents.
NR: not reported.

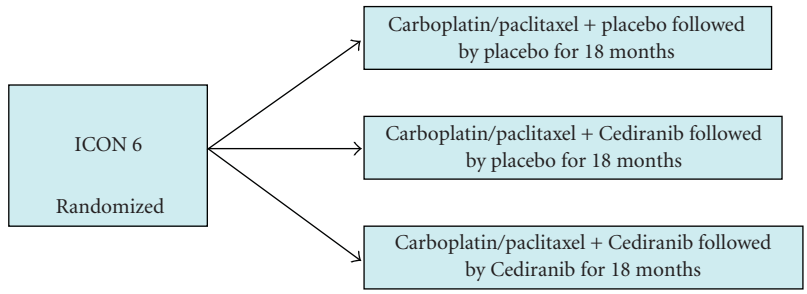

Figure 2: ICON-6.

day on a 4-week on 2-week off schedule. Noted in this study was the development of pleural effusions during the 2-week rest period. Of the seventeen patients that were studied 12% of patients had a partial response, and 59% of patients had disease stabilization. Currently the Harvard Cancer Center Gynecological Group (NCT00768144) is conducting a phase II trial using sunitinib in refractory ovarian caner patients. The dose of sunitinib is held constant at 37.5 mg every day.

AMG 706 is an investigational inhibitor of vascular endothelial growth factor receptors 1, 2, and 3, platelet-derived growth factor receptor, and stem-cell factor receptor. A Phase II Evaluation of AMG706 (NCT00574951) in the Treatment of Persistent or Recurrent Epithelial Ovarian Fallopian Tube or Primary Peritoneal Cancer is currently active.

Matei et al. [26] reported on the activity of sorafenib in patient with recurrent ovarian cancer. Sorafenib is a tyrosine kinase inhibitor targeting raf and other receptor kinases (VEGF-R, PDGF-R, Flt3, c-KIT). Patients received sorafenib at 400 mg QD. Patients in this study have a 3% partial response, and 20% of patients had stable disease for > than 6 months. Toxicities included rash, metabolic abnormalities, gastrointestinal, cardiovascular, and pulmonary toxicity.

4. VEGF Trap (Aflibercept)

VEGF trap (Aflibercept) is fusion protein containing the VEGF binding regions of both VEGFR-1 and 2 linked through the Fc region of a human IgG1. Aflibercept binds VEGF-A and neutralizes all VEGF-A isoforms plus placental growth factor. This agent is currently being explored in platinum resistant ovarian cancer. Columbo et al. [39] reported the results of VEGF Trap in patients with symptomatic malignant ascites. Aflibercept, 4 mg/kg, i.v. was administered every 2 weeks, in patients with advanced ovarian cancer and symptomatic ascites requiring frequent paracentesis. Primary endpoint was repeat paracentesis response rate (RPRR) defined as at least a doubling of time to the first paracentesis compared to a baseline average. Patients received 1–13 cycles of aflibercept. The authors reported that the time to the first paracentesis was 12–205 days. Eight out of ten evaluable patients achieved a RPRR response as per protocol. Adverse events included bowel obstruction, nausea, vomiting, anorexia, edema, and 1 case of bowel perforation. Tew et al. [40] reported the preliminary results of a randomized phase II study in patients with recurrent platinum-resistant epithelial ovarian cancer. VEGF Trap was (2 or 4 mg/kg) administered intravenously every 2 weeks in patients with recurrent

ovarian cancer was conducted. Five partial responses in a sample size of 45 patients (11%) were reported.

5. Epidermal Growth Factor Inhibitors

In addition to the VEGF inhibitors, the epidermal growth factor receptor (EGFR) has emerged as an attractive target [47–49]. The activation of EGFR signaling pathways is known to increase proliferation, angiogenesis, and decrease apoptosis. Several strategies that target the EGFR in gynecologic cancers have included monoclonal antibodies [1–3], (trastuzumab, pertuzumab, EMD7200) and tyrosine kinases inhibitors [4–6] (gefitinib, erlotinib, lapatinib and CI-1033). Bookman and colleagues [1, 50] reported a response rate of 7% in a phase II trial of ovarian cancer patients treated with trastuzumab. Kaye et al. [51], Amler et al. [52], and Makhija et al. [53] in independent studies examined pertuzumab, a humanized recombinant monoclonal antibody that inhibits the dimerization of HER2 with EGFR, HER 3, and HER4, in patients with ovarian cancer. As a single agent there were only modest responses. Gordan et al. [54] recently published the clinical activity of pertuzumab in advanced ovarian cancer. There were five partial responses (response rate 4.3%), eight patients (6.8%) with stable disease lasting at least 6 months, and 10 patients with CA-125 reduction of at least 50%. Median progression-free survival (PFS) was 6.6 weeks. Twenty eight percent of the tumor biopsies were pHER2+ by ELISA. Of note the progression free survival for pHER2+ patients was 20.9 weeks ($n = 8$) versus 5.8 weeks for pHER2−.

Several studies are ongoing. The EORTC have recently completed a trial investigating erlotinib as maintenance therapy following first-line chemotherapy in patients with ovarian cancer (NCT00263822). A phase II open label trial of erlotinib and bevacizumab is being conducted by Alberts et al. in patients with advanced ovarian cancer (NCT00696670).

Unlike other disciplines there is lack of data in the gynecological literature on who, if any, will benefit from EGFR inhibitors. Schilder et al. [55] reported that in a sample size of 55 ovarian cancer patients 3.6% had mutations in the EGFR tyrosine kinase domain and that the mutation correlated with a response to gefitinib. Exploratory analyses in the pertuzumab studies [51–53] suggested that patients with platinum resistant disease and low levels of HER3 mRNA might benefit from pertuzumab. An additional study by Tanner et al. [56] demonstrated an influence of HER 3 expression on the survival of patients with ovarian cancer.

Selection of ovarian cancer patients with EGFR amplifications, increased pHER2, and low expression of HER 3 ratios may represent the selected few that may respond to EGFR inhibitors.

6. Combination Therapy with EGFR and VEGF Inhibitors

EGFR activation has been reported to promote VEGF [57] secretion. Several clinical studies are exploring the combination of EGFR inhibitors and VEGF inhibitors. Nimeiri et al. [12] investigated the clinical activity and safety of bevacizumab and erlotinib patients with recurrent ovarian, primary peritoneal, and fallopian tube cancer. In this study patients were heavily pretreated. Two patients had a fatal bowel perforation.

Currently investigators at the Harvard Cancer Center are conducting a randomized phase II trial of Bevacizumab or Bevacizumab and Erlotinib as First Line Consolidation Chemotherapy after Carboplatin, Paclitaxel, and Bevacizumab (CTA) Induction Therapy for Newly Diagnosed Advanced Ovarian, Fallopian Tube and Primary Peritoneal Cancer & Papillary Serous Mullerian Tumors (NCT00520013) [20].

7. Platelet Derived Growth Factor Inhibitors

Platelet-derived growth factor (PDGF) a prototype for understanding the function of growth factors and receptor tyrosine kinases (TK) [58] induces cell growth and survival, transformation, migration, vascular permeability, and wound healing [59]. PDGF receptor (PDGFR) activation in cancer occurs as a consequence of gene amplification, chromosomal rearrangements, or activating mutations [60–62]. PDGFR activation is critical to tumor initiation in addition to functioning as a mediator of connective tissue stroma [63].

PDGFR has been shown in 50–80% of ovarian tumors [63]. Several agents that target the PDGFR have been studied. These include imatinib mesylate [63–66], sorafenib, [17, 26], sunitinib [25], dasatinib [67], 3G3 [68], and CDP 860 [69]. Imatinib mesylate is a selective Abl, c-Kit, and PDGFR inhibitor. Three phase II clinical trials [64, 70, 71] in patients with ovarian cancer failed to demonstrate clinical benefit.

The GOG (170 M) is currently studying dasatinib in a Phase II Evaluation of Dasatinib in the Treatment of Persistent or Recurrent Epithelial Ovarian, Fallopian Tube, or Primary Peritoneal Carcinoma

BIBF1120 [72] is a novel agent. It is a triple angiokinase inhibitor that targets the VEGFR, PDGRF, and the fibroblast growth factor receptor (FGFR). Sustained pathway inhibition is a distinct feature of this agent. Ledermann et al. [73] recently conducted a randomized phase II placebo-controlled trial using maintenance therapy to evaluate the vascular targeting agent BIBF 1120 following treatment of relapsed ovarian cancer. The 36-week PFS rate for BIBF 1120 was 15.6% and 2.9% for placebo. The authors concluded that maintenance BIBF 1120 could delay disease progression in ovarian cancer patients who had previously responded to chemotherapy.

8. Folate Receptor Inhibitors

Folic acid is an essential vitamin and of importance for one-carbon transfer processes medicated by enzyme systems involved in DNA synthesis [74]. Increased expression of α-FR has been described in various tumor tissues, including ovarian, endometrial, and breast cancer [75]. While the

Table 2: PDGF-targeted therapies in ovarian cancer.

clinical trial.gov ID	Therapeutic regimen	Study PI
NCT00913835	Doxil ± IMC 3G3 in platinum refractory or resistant EOC	W. McGuire
NCT00768144	Sunitinib in refractory/recurrent ovarian, fallopian tube, or peritoneal cancer	S. Campos
NCT00437372	Sunitinib and radiation therapy	A . Dicker
NCT00792545	Dasatinib + bevacizumab in surgically metastatic, or unresectable solid tumors	E. Kohn
NCT00672295	Dasatinib + paclitaxel + carboplatin in ovarian, fallopian tube, and peritoneal cancer	A. Secord
NCT00436215	Sorafenib + bevacizumab in recurrent/refractory ovarian, fallopian tube, or peritoneal cancer	E. Kohn
NCT00526799	Sorafenib + topotecan in platinum resistant EOC	D. Matei
NCT00390611	Paclitaxel + carboplatin ± sorafenib for first-line therapy for EOC	J. Hainsworth
NCT00096200,	Sorafenib + paclitaxel + carboplatin in recurrent platinum-sensitive ovarian, fallopian tube, or peritoneal cancer	V. von Gruenigan
NCT00510653	Gleevac study for patients with ovarian cancer	D. Gershenson
NCT00840450	Gleevac and paclitaxel with recurrent mullerian cancers	F. Muggia

function of α-FR in cancers is not fully understood, folates are critical metabolites for nucleotide synthesis and methylation reactions. Its overexpression might confer a tumor growth advantage by increasing folate availability to cancer cells [75]. Over 90% of nonmucinous ovarian cancers overexpress α-FR [76].

Several strategies have been employed to target the folate receptor. Some of these include the use of anti-α-FR antibodies or folic acid conjugates. There has also been recent research to show that α-FR may have a potential as a target for immunotherapeutic approaches in ovarian cancer. α-FR is a tumor-associated antigen that induces detectable immune responses in 70% of patients with breast and ovarian cancer [77]. The presence of endogenous immune reactivity raises the possibility that the immune response could be further enhanced by vaccines targeting the α-FR. Hernando et al. [78] presented a case of a women with recurrent epithelial ovarian cancer treated with a vaccination regimen created with autologous dendritic cells engineered with mRNA-encoded α-FR [78]. An initial contrast-enhanced CT of the abdomen before vaccination had shown para-aortic lymph node metastasis at the level of the left renal hilus and lower abdominal aorta. Follow-up CT 16 months after last vaccination depicted a more than 50% regression of lymph node metastasis and a dramatic decrease in CA125 concentrations 4 weeks after the first vaccination [78].

Farletuzumab (MORAb-003) is a monoclonal antibody to α-FR that activates antibody-dependent cell-mediated cytotoxicity and complement-mediated toxicity [79]. In a recent Phase II trial of 54 patients [80] with platinum-sensitive relapsed disease patients who received combination therapy exhibited a prolongation of their remission when compared to their previous remission. Ongoing clinical trials looking at Farletuzumab include a Phase III trial comparing the efficacy and safety of intravenous carboplatin and taxanes with and without farletuzumab in subjects with first platinum-sensitive relapse, a Phase II trial examining intravenous paclitaxel with and without farletuzumab in patients with first platinum-resistant or refractory relapse.

EC145 is a drug that is specifically designed to enter cancer cells via the folate vitamin receptor (FR). Early clinical evidence in a small number of phase I patients suggests that EC145 may have antitumor effect in women with advanced ovarian cancer. Current independent studies include a study of EC145 in patients with advanced ovarian and endometrial cancers (NCT00507741) and a study in patients with platinum resistant ovarian cancer with a combination of Doxil and EC145 Combination Therapy (NCT00722592).

9. Poly-ADP-Ribose Polymerase (PARP) Inhibitors

Between 5 and 10% of all ovarian cancer cases are associated with inheriting a mutation in the BRCA1 or BRCA2 gene [81]. The lifetime risk of ovarian cancer for BRCA1 and BRCA2 mutation carriers is estimated at 40–50% and 10–20%, respectively. BRCA1 and BRCA2 are essential for the repair of double strand DNA breaks (DSBs) and maintenance of genomic stability [82].

Poly (ADP-ribose) polymerase (PARP) is a key nuclear enzyme involved in the repair of DNA single-strand breaks (SSBs) using the base excision repair pathway [83]. PARP-1 and PARP-2 are the only members of the PARP family known to be activated by DNA damage, and PARP-1 has been best characterized. PARP inhibition results in the accumulation of DNA SSBs, which may lead to DSBs. Thus, the use of PARP inhibitors in BRCA mutation carriers uses the concept of synthetic lethality and hence can be described a therapeutic exploitation.

In the first human phase I clinical trial using Olaparib (AZD2281, KU-0059436; AstraZeneca) an oral small-molecule PARP-1 inhibitor, toxicities included nausea, vomiting, anorexia, and fatigue. Efficacy has been reported. Olaparib has shown antitumor activity in BRCA-associated ovarian cancer [84, 85]. Fifty patients were treated at various doses, of which 41 were BRCA1 mutation carriers, eight were

Table 3: α-folate receptor inhibitors and ovarian cancer.

Clinical trial.gov ID	Therapeutic regimen	Study PI
NCT00722592	Doxil and EC145in platinum resistant EOC	R. Messmann
NCT00738699	MORAb-003 in first platinum resistant or refractory relapsed EOC	D. Chakraborty
NCT00849667	MORAb-003 in platinum sensitive, first relapse EOC	D. Chakraborty

BRCA2 mutation carriers and one had a compelling family history for BRCA mutation. Of the 46 patients with evaluable disease, 41% reached either a complete or partial response. Eleven percent had meaningful stabilization of disease for 4 months, giving a total clinical benefit rate of 52%. The median response duration was 30 weeks.

Recently reported were the results of a phase II trial of the oral PARP inhibitor Olaparib (AZD 2281) in BRCA-deficient advanced ovarian cancer [86]. An international, phase II study examined two cohorts of patients that received oral olaparib in 28-day cycles, initially at the MTD, 400 mg bid (33 pts), and subsequently at 100 mg bid (24 pts). The confirmed overall response rate was 33% at 400 mg bid dose and 12.5% at 100 mg bid dose. Clinical benefit rate (ORR and/or confirmed ≥50% decline in CA125) was 57.6% at 400 mg bid and 16.7% at 100 mg bd. Toxicity was mild.

Olaparib is currently being evaluated in randomized Phase II trials in platinum-sensitive recurrent ovarian cancer, and in known BRCA or high grade recurrent ovarian cancer. It is also being compared with pegylated liposomal doxorubicin in patients with BRCA mutated ovarian cancer with a 0-12 month platinum-free interval (NCT00628251).

Other PARP inhibitors are also being evaluated in BRCA mutation carriers with cancer, including AG0146999 (Pfizer) ABT888 (Abbott), BSI-201 (Bipar), INO-1001 (Inotek/Genentech), and MK4827 (Merck).

The use of PARP inhibitors might be extended to sporadic ovarian cancers with homologous recombination defects. These sporadic tumors seem to phenocopy BRCA1/2 deficient tumors even though they do not possess the germline mutations in either gene. This phenomenon is called "BRCAness." This can occur due to loss of heterozygosity, hypermethlyation, and haploinsufficiency (inactivation of one BRCA allele), thereby, genetically silencing the BRCA gene without an actual germline mutation. A recent study suggests that over 50% of high-grade serous ovarian cancer had loss of BRCA function, either by genetic or epigenetic events [87]. A randomized placebo-controlled trial of olaparib as a maintenance therapy in patients with serous (sporadic) ovarian cancer at high risk for recurrence is now underway.

10. Aurora Kinase Inhibitors

Aurora kinases are protein kinases that are important mitotic regulators [88, 89]. They are central to many cellular functions notably mitosis, centromere separation, as well as mitotic spindle formation. Three aurora kinases (A, B, C) exist. The activity of aurora kinase is cell cycle dependent and active during the G2M phase of the cell cycle. Several investigators [88, 90, 91] have described the oncogenic potential of these proteins. Aurora-A also phosphorylates the tumor suppressor protein p53, resulting cell cycle progression [92].

Aurora A is overexpressed in 83% of human epithelial ovarian carcinomas [93]. In addition, amplification of human chromosome 20q13.2, which contains Aurora-A, frequently occurs in ovarian cancer [94]. Aurora kinase A has been significantly associated with tumor grade, FIGO stage, and survival [93, 95].

Lin et al. [96] studied the role of MK-0457, a small molecule pan-aurora kinase inhibitor in ovarian cancer cell models. Two chemosensitive human ovarian cancer cell lines, HeyA8 and SKOV3ip1, were used to study the effects of aurora kinase inhibition. Additionally two chemoresistant cell lines (Hey A8-MDR and A2780-CP-20) were also studied. Both cell lines showed that aurora kinase inhibition alone significantly reduced tumor burden. Combination treatment with docetaxel resulted in significantly improved reduction in tumor growth beyond that afforded by docetaxel alone ($P <$ or $= .03$). Scharer et al. [97] also reported that aurora kinase inhibitors synergize with paclitaxel to induce apoptosis in ovarian cancer cells.

Manfredl et al. [98] reported the antitumor activity of MLN8054, an orally active small molecule inhibitor of aurora kinase. Growth of human tumor xenografts in nude mice was dramatically inhibited after oral administration of MLN8054 in human tumor xenografts. MLN8054 induced mitotic accumulation and apoptosis. Given these findings MLN8054 is currently being explored in the management of patients with platinum-refractory or resistant epithelial, fallopian, or primary peritoneal carcinoma (NCT00853307).

11. Hedgehog Pathway Inhibitors

Hedgehog signaling plays a role in many processes such as cell differentiation, growth, and proliferation. This pathway is active during embryonic development and remains active in the adult where it is involved in the maintenance of stem cell populations.

The Hedgehog family [99] has several proteins which function as signaling molecules. These include Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). There are two receptors that are involved in the Hedgehog pathway. PATCHED1 is a hedgehog receptor. In the absence of a ligand PATCHED1 inhibits SMOOTHENED, a transmembrane G-coupled protein. However, when the ligand binds PATCHED1, SMOOTHENED suppression is relieved resulting in transcription of the Hedgehog genes. PATCHED1 or

Table 4: PARP inhibitors and ovarian cancer.

clinical trial.gov ID	Therapeutic regimen	Study PI
NCT00753545	AZD2281 in platinum sensitive EOC	J. Lederman
NCT00679783	AZD2281 in known BRCA or recurrent EOC	K. Gelman
NCT00749502	MK4827 in BRCA mutant ovarian cancer	
NCT00664781	AG014699 in BRCA mutant ovarian cancer	R. Plummer
NCT00647062	AZD2281 and carboplatin in BRCA mutant EOC	E. Kohn

SMOOTHENED receptor mutations or overexpression of the Hedgehog ligand leads to uncontrolled cell proliferation.

Bhattacharya et al. [99] studied the role of Hedgehog signaling in ovarian cancer. They utilized a hedgehog pathway blocker and studied the proliferation of ovarian tumors. They noted that PATCHED1 is downregulated in ovarian cancer and that this low level expression of the PATCHED1 contributed to the proliferation of ovarian cancer cells. Chen et al. [100] also examined the expression and the functional role of the hedgehog signal molecules in ovarian cancer. They reported that the hedgehog molecules (Shh, Dhh, Ptch, Smo, and Gli 1 proteins) were increased in malignant disease. Decreased cell proliferation in ovarian carcinoma cell lines was observed with Hedgehog pathway inhibitor-cyclopamine.

Recently reported was the effect of IPI-926 (Infinity Pharmaceuticals, Inc., Cambridge, Mass) a novel inhibitor of the Hedgehog signaling pathway in ovarian cancer grafts. Data revealed that treatment with cyclopamine, the natural product of IPI-926 in animals with primary ovarian cancer grafts, resulted in tumor growth inhibition. This agent is currently being explored as a Phase I study in patients with solid tumors (NCT00761696).

Currently recruiting is a study of GDC-0449 (Genentech, Inc), a Hedgehog pathway inhibitor, as maintenance therapy in patients with ovarian cancer in a second or third complete remission. GDC-0449 will be evaluated in approximately 100 patients with ovarian cancer in second or third complete remission in a randomized, placebo-controlled, double-blind, multicenter Phase II trial. Patients are randomized in a 1 : 1 ratio to receive either GDC-0449 or a placebo comparator and are stratified based on whether their cancer is in a second or third complete remission. The primary endpoint of the trial is progression-free survival. Secondary outcome measures include overall survival, measurement of Hedgehog ligand expression in archival tissue, and number and attribution of adverse events.

12. MTOR Inhibitors

Numerous investigators have reported alterations in PTEN in gynecological malignancies [101]. PTEN is a lipid phosphatase that is associated with cell cycle G1-phase arrest and apoptosis through the PI3K/AKT/mTOR pathway [102]. The mTOR pathway is a central regulator of cell growth, proliferation, and apoptosis. The loss of functional PTEN either through deletion, mutation, or inactivation leads to the constitutive activation of PI3K effectors in the absence of exogenous stimuli. Potential therapies targeting the mTOR pathway include mTOR inhibitors Temsirolimus (CCI-779), everolimus (RAD001), and deforolimus (AP23573).

In ovarian cancer, AKT activity is frequently elevated and is closely associated with the upregulation of mTOR signaling [103]. High levels of AKT activity in vitro result in hypersensitivity to mTOR inhibitors [103]. An in vivo study [104] using xenografts of SKOV-3 cells revealed that RAD001 inhibited tumor growth, angiogenesis, and production of ascites suggesting the potential of mTOR inhibitors in the treatment of women with ovarian cancer.

GOG trial 170I has recently closed a Phase II Evaluation of Temsirolimus (CCI-779, mTOR inhibitor) in the Treatment of Persistent or Recurrent Epithelial Ovarian, Fallopian Tube or Primary Peritoneal Carcinoma. Currently recruiting studies include (NCT00926107), a study of the mTOR inhibitor Temsirolimus (CCI-779) to treat ovarian cancer with Ca125 relapse only, a Phase I study of DOXIL and Temsirolimus in Resistant Solid Malignancies NCT00703170, and a Phase I study of Docetaxel and Temsirolimus in resistant solid malignancies (NCT00703625).

13. Conclusion

Multiple attractive targets for the design of targeted therapeutics in ovarian cancer are currently under investigation. Recent studies employing monoclonal antibodies have revealed improvements in time to progression. Studies with tyrosine kinases inhibitors remain in their infancy of development but have provided the basis for continued research.

Despite these advances there are multiple goals for the future. These include a better understanding of the redundant pathways that exist in cell signaling, creative targeting of horizontal and vertical signaling pathways, identification of other predictive markers to better identify a targeted subpopulation of patients that will respond, and an underlying of the mechanisms of resistance. Achieving these goals will be of paramount importance in the study of targeted therapy in ovarian cancer.

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Review Article

Antivascular Therapy for Epithelial Ovarian Cancer

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Ovarian cancer is the fifth largest cancer killer in women. Improved understanding of the molecular pathways implicated in the pathogenesis of ovarian cancer has led to the investigation of novel targeted therapies. Ovarian cancer is characterized by an imbalance between pro- and antiangiogenic factors in favor of angiogenesis activation. Various antivascular strategies are currently under investigation in ovarian cancer. They can schematically be divided into antiangiogenic and vascular-disrupting therapies. This paper provides a comprehensive review of these new treatments targeting the tumor vasculature in this disease. Promising activities have been detected in phase II trials, and results of phase III clinical trials are awaited eagerly.

1. Introduction

Ovarian cancer is the fifth largest cancer killer in women. Primary surgical cytoreduction followed by platinum-based chemotherapy is the standard treatment for patients with advanced epithelial ovarian cancer. However, despite this aggressive approach, all stages combined, the 5-year survival rate remains only around 45% [1]. Novel approaches to improve disease outcome are thus urgently needed.

There is a strong rationale to use antivascular therapies in epithelial ovarian cancer. Ovarian cancer is characterized by an imbalance between pro- and antiangiogenic factors in favor of angiogenesis activation, with an increase in the tumor levels of proangiogenic factors (i.e., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factors (PDGFs), tumor necrosis factor (TNF)-alpha, angiopoietins, interleukin (IL-6 and IL-8, etc.) and a decrease in anti-angiogenic factors (i.e., angiostatins, endostatins, etc.) [2]. Angiogenesis is necessary for tumors to grow beyond a few millimeters and is triggered by tumor hypoxia that induces the release of pro-angiogenic factors [3]. Angiogenesis has also an important role in the formation of ascites, a frequent clinical feature of advanced ovarian cancer. The accumulation of ascites results mainly from the increased permeability of the peritoneal capillaries. VEGF, also known as the "vascular permeability factor," plays a key role in this process [4] (see Figures 1 and 2).

Various antivascular strategies have been investigated in ovarian cancer. They can schematically be divided into antiangiogenic therapies and vascular-disrupting therapies. Given the important role of vascular biology in ovarian cancer, it is not surprising that these new treatment approaches have shown promising activity in this disease, even when administered as a single agent.

2. Antiangiogenic Therapies

2.1. VEGF. The most studied antiangiogenic strategies target the VEGF/VEGF receptor (VEGFR) pathway through inhibition of its ligands and/or receptors. The VEGF family includes 6 glycoproteins (VEGF-A to E and placental growth factor) and 3 tyrosine kinase receptors (VEGFR1 to 3). VEGF-A promotes angiogenesis through enhancement of permeability, activation, survival, migration, invasion, and proliferation of endothelial cells [5]. VEGFR1 and VEGFR2 mediate the effects of VEGF-A [6]. Recent studies suggest a direct effect of VEGF-A on tumor cell proliferation the VEGFR2 via a mechanism thought to involve the

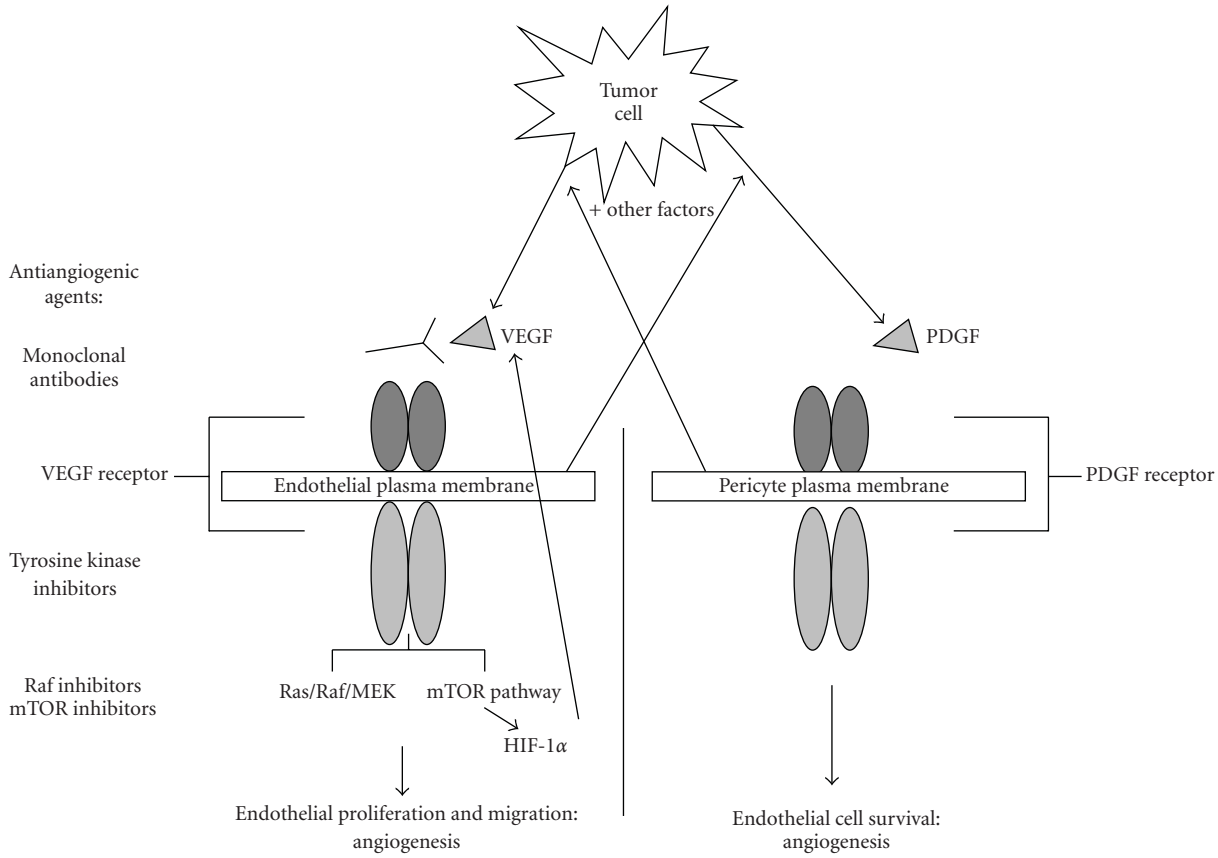

Figure 1: Major pathways promoting angiogenesis in epithelial ovarian cancer. VEGF: vascular endothelial growth factor, PDGF: platelet-derived growth factor, mTOR: mammalian target of rapamycin.

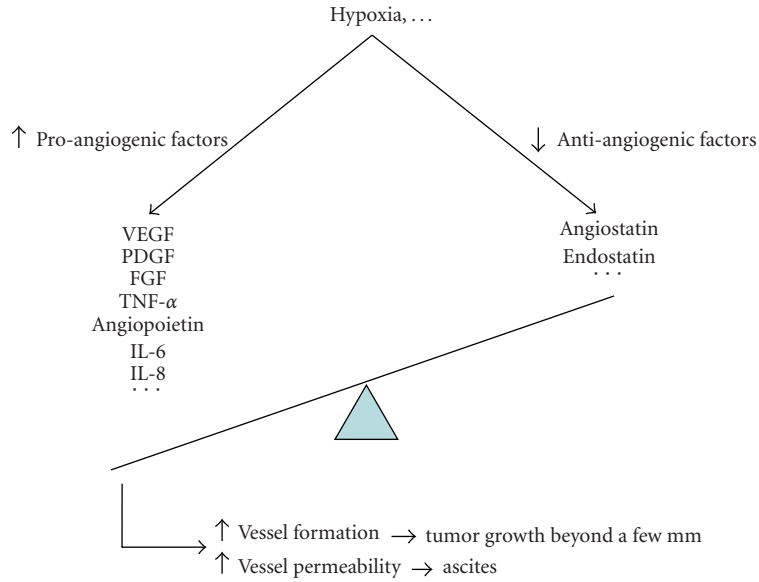

Figure 2: Molecular events leading to increased angiogenesis in epithelial ovarian cancer. VEGF: vascular endothelial growth factor, PDGF: platelet-derived growth factor, FGF: fibroblast growth factor, TNF = tumor necrosis factor, IL: interleukin.

AKT/mTOR pathway [7]. VEGF-A also regulates the invasiveness of cancer cells by altering the expression of matrix metalloproteinase-2 [8].

2.1.1. Agents Directed Against VEGF Ligand(S). (1) The most widely investigated anti-VEGF ligand agent is *bevacizumab (BEV)*. BEV is a recombinant humanized monoclonal antibody that binds and neutralizes all biologically active isoforms of VEGF. Published studies are presented in this section, while ongoing trials are summarized in Table 1.

(a) Single-Agent Activity. In 2005, Monk et al. reported an objective response lasting more than 5 months in a patient treated with BEV monotherapy after failing eleven lines of chemotherapy and radiation therapies [9]. Later, the same group found a 16% objective response rate (ORR) in a retrospective analysis of 32 patients with refractory epithelial ovarian cancer treated with BEV alone or in combination with chemotherapy (after failing 2 to 10 prior cytotoxic regimens) [10].

In the phase II GOG 170-D trial, Burger et al. reported a partial response (PR) rate of 18% (11 out of 62) and a complete response (CR) rate of 3% (2 out of 62) in patients with persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer having received 1 or 2 prior cytotoxic regimens and treated with BEV monotherapy. Median progression-free survival (PFS) was 4.7 months [11]. These results were confirmed by Cannistra et al. who observed PR in 15.9% (7 out of 44) with a median PFS of 4.4 months with single-agent BEV in women with refractory or resistant ovarian cancer or peritoneal serous cancer [12].

BEV maintenance therapy after complete response to cisplatin-based chemotherapy is an interesting concept and showed promising results in xenograft models of ovarian cancer by prolonging survival [13]. This approach is currently explored in scheduled and ongoing trials (see Table 1).

(b) BEV and Chemotherapy. The vessels formed during tumor angiogenesis are structurally and functionally abnormal. This leads to an impaired tumor blood supply that may interfere with the delivery of therapeutics. Hypoxia also renders tumor cells more resistant to both radiation and cytotoxic drugs [14]. It has been proposed that the "normalization" of the tumor vasculature by BEV could allow a better delivery of chemotherapy and decrease hypoxia, making tumors more chemosensitive [15].

In a retrospective analysis of 23 patients with recurrent platinum-refractory epithelial ovarian cancer progressing after 2 to 15 prior cytotoxic regimens, Wright et al. observed PR in 35% with a combination of BEV associated with various chemotherapy regimens (cyclophosphamide, 5-fluorouracil, docetaxel, or gemcitabine/liposomal doxorubicin). Median PFS was 5.6 months in the patients who achieved a PR [16]. Richardson et al. reported an ORR of 78% and a median PFS of 12 months in a retrospective analysis of 35 patients with recurrent ovarian cancer treated with a combination of gemcitabine, cisplatin or carboplatin and BEV. The higher ORR observed in this last study could be explained by the inclusion of a vast majority of platinum-sensitive patients [17]. Cohn et al. retrospectively identified 10 patients with advanced, recurrent and refractory ovarian cancer who were treated with a combination of BEV and weekly taxane (paclitaxel or docetaxel) after failure of 1 to 4 prior chemotherapy regimens. The 5 symptomatic patients in this study experienced a rapid subjective palliation of pain, nausea, and ascites [18].

Metronomic administration of chemotherapy, defined as the frequent administration of doses substantially lower than the maximum tolerated dose, can suppress tumor growth, probably through stimulation of the release of thrombospondin 1, a potent and endothelial-specific inhibitor of angiogenesis [19]. Shortening the time between cycles provides more sustained apoptosis of endothelial cells within the tumor vascular bed [20]. Metronomic chemotherapy regimens deliver lower doses of cytotoxic agents, thereby decreasing potential side effects and improving patient tolerance [21]. In a retrospective analysis, 15 heavily pretreated patients with recurrent ovarian cancer (5–15 prior chemotherapy regimens) received a combination of BEV and metronomic oral cyclophosphamide with encouraging results: CR 13.3% and PR 40% [22]. However, in a prospective phase II trial that included 70 less heavily pretreated patients with recurrent ovarian cancer, combination of BEV with metronomic cyclophosphamide showed PR in only 24% with a median PFS of 7.2 months [23].

Various combinations of BEV and chemotherapy are currently being *tested*; these studies are briefly described in Table 1.

(c) BEV and Other Targeted Therapies. Combination therapy in this context can be divided into horizontal and vertical molecular pathway blockade. The horizontal approach involves the association of targeted agents to inhibit two or more different pathways simultaneously, while the vertical approach involves the inhibition of various molecular steps of the same pathway, thus counteracting negative feedback loops. By inhibiting the activation of alternate molecular pathways, these combinations could theoretically decrease treatment resistance [24].

Epithelial growth factor receptor (EGFR) is overexpressed in up to 70% of advanced epithelial ovarian cancers [25] and an increased level of EGFR expression has been correlated with poorer overall survival [26]. The VEGF and the EGFR pathways are interconnected: VEGF signaling is upregulated by EGFR expression and VEGF upregulation independent of EGFR signaling seems to contribute to resistance to EGFR inhibition [27]. Since EGFR inhibitors alone have shown limited activity in epithelial ovarian cancer [28, 29], it was postulated that combining an EGFR tyrosine kinase inhibitor like erlotinib with BEV might improve response rates. Unfortunately, in a phase II trial conducted in 13 patients with recurrent ovarian cancer treated with a combination of BEV and erlotinib after failure of 1 to 3 prior chemotherapy regimens, ORR was relatively low (15%) and median PFS (4.1 months) did not seem to be improved over BEV alone [30]. Other trials investigating this combination are planned (see Table 1).

Table 1: Ongoing studies with bevacizumab (BEV) in ovarian cancer.

Stage of the disease	Phase	Intervention	Trial number
Monotherapy			
Recurrence after prior therapy with maintenance BEV	II	BEV monotherapy	NCT00866723
Combination with chemotherapy			
Newly diagnosed	III	Carboplatin and paclitaxel with versus without BEV	ICON7 NCT00483782
Previously untreated stage III or IV	III	Carboplatin and paclitaxel versus carboplatin, paclitaxel, and concurrent BEV with versus without extended BEV	GOG218 NCT00262847
Adjuvant	II	Carboplatin, paclitaxel and BEV (BEV omitted in first cycle)	OVCA NCT00129727
Newly diagnosed stage III/IV	II	Carboplatin, paclitaxel and BEV	AV53206s NCT00127920
Newly diagnosed stage IB-IV	II	Oxaliplatin and docetaxel with BEV	TEACO NCT00296816
Newly diagnosed stage II-III	II	IV paclitaxel, IP cisplatin and IV BEV followed by BEV consolidation	AVF3953 NCT00511992
Initial treatment of optimal stage II or III (adjuvant)	II	IV and IP paclitaxel, IP cisplatin, and IV BEV	06-064 NCT00588237
Platinum-sensitive recurrent	III	Carboplatin and paclitaxel with versus without BEV followed by secondary cytoreduction surgery	GOG213 NCT00565851
Platinum-sensitive recurrent	III	Carboplatin and gemcitabine with versus without BEV	AVF4095g NCT00434642
Platinum-sensitive recurrent	II	Gemcitabine, carboplatin and BEV	2005CO073 NCT00267696
Platinum-sensitive recurrent	II	Carboplatin and liposomal doxorubicin plus BEV	CR015094 NCT00698451
Platinum-sensitive recurrent	II	Oxaliplatin, gemcitabine, and BEV	DF 04-356 NCT00418093
Recurrent having failed platinum- and taxane-based regimens	II	Pemetrexed and BEV	08-0508 NCT00868192
Platinum-resistant recurrent	II	Weekly topotecan with BEV	AVF3648s NCT00343044
Platinum-resistant recurrent	II	BEV and docetaxel	MCC-14920 NCT00504257
Platinum-resistant recurrent	II	BEV and carboplatin	2008-000878-20 NCT00744718
Platinum-resistant recurrent	II	BEV and liposomal doxorubicin	AVF3910s NCT00846612
Platinum-resistant recurrent	II	Sequential BEV and metronomic cyclophosphamide	08-148 NCT00856180

Table 1: Continued.

Stage of the disease	Phase	Intervention	Trial number
Monotherapy			
Recurrence after prior therapy with maintenance BEV	II	BEV monotherapy	NCT00866723
Combination with chemotherapy			
Platinum-resistant recurrent	II	BEV and albumin-bound paclitaxel	ALSSOPR0501 NCT00407563
2nd or later complete remission, or untreated or refractory to platinum treatment or no response to salvage treatment	II	Stem-cell transplant trial evaluating treatment with BEV plus gemcitabine, docetaxel, melphalan, and carboplatin	2007-0368 NCT00583622
Advanced peritoneal carcinomatosis	I	IP oxaliplatin and paclitaxel plus IV paclitaxel and BEV	2006-1068 NCT00491855
Combination with other targeted therapies			
Newly diagnosed	II	BEV and erlotinib as 1st line consolidation chemo after carboplatin, paclitaxel, and BEV induction therapy	07-039 NCT00520013
Relapsed or refractory	II	BEV and erlotinib	UARIZ-05-0178-01 NCT00696670
Recurrent or metastatic	II	BEV and erlotinib	NCI-6759 NCT00126542
Refractory or recurrent	II	BEV and sorafenib	NCI-07-C-0058 NCT00436215
Persistent or recurrent	II	BEV with or without everolimus	GOG-0186G NCT00886691

Studies were accessed from http://www.clinicaltrials.gov/ on May 17, 2009
IV = intravenous, IP = intraperitoneal

Sorafenib inhibits, among others, the VEGFR2 and Raf kinases. In a phase I dose-escalation study with a combination of BEV and sorafenib, 6 of 13 (46%) patients with ovarian cancer had a PR [31]. A phase II study with this combination is ongoing (see Table 1).

Other combinations of BEV with targeted therapies have been tested in preclinical models. Since the mammalian target of rapamycin (mTOR) pathway regulates VEGF expression in cancer cells [32], researchers combined BEV and rapamycin in an ovarian cancer xenograft model and found a 94% reduction in tumor growth as well as a prolonged survival [33]. Everolimus, another mTOR inhibitor, is now under investigation in a randomized phase II trial (see Table 1).

(d) Toxicities of BEV. Angiogenesis inhibitors are not easy drugs to manipulate with some specific toxicities. Common *complications* following treatment with BEV in colorectal cancer, where this drug is widely used, include hypertension (25% grade 1-2, 5% grade 3-4), proteinuria (9% grade 1-2, 1% grade 3-4), bleeding (28% grade 1-2, 3% grade 3-4), wound-healing complications (3% grade 1-2, 1% grade 3-4), arterial thrombo-embolic events (1.5%, mostly grade 3-4), and gastrointestinal (GI) perforations (2%, mostly grade 3-4, with only 0.4% grade 5) [34]. The complication rate in ovarian cancer is quite similar, but there are some noteworthy specificities. In the published phase II ovarian studies, the rate of GI perforations varied from 0% [11] to 11.4% [12], leading to the early closure of the latter study. It was hypothesized that the increased rate of bowel perforation in the latter study was due to the fact that these patients were more heavily pretreated, but this finding could not be confirmed in other studies. Intestinal obstruction and bowel wall involvement by the tumor were other potential risk factors, but they were not statistically significant. In a retrospective review of 62 patients treated with BEV after a median of 5 prior chemotherapy regimens, researchers found grade 3–5 toxicities in 24% of patients, including grade 3-4 hypertension in 7%, GI perforations in 7%, and chylous ascites (probably due to lymphatic disruption by targeting VEGF-C) in 5%. Development of GI perforations and chylous ascites appeared to correlate with tumor response [35].

There is a trend towards increased toxicity when BEV is combined with a cytotoxic agent [35]. GI perforation seems to be more frequent in ovarian cancer than in other solid tumors and could be favored by peritoneal carcinomatosis.

In a retrospective cohort of patients without clinical symptoms of bowel obstruction and without evidence of bowel involvement, there were no cases of GI perforation or other grade 3/4 toxicities [36]. Careful patient selection might reduce the risk of GI perforations but all toxicities will not be avoided. Researchers recently reported two cases of GI perforations in a retrospective analysis of 35 patients treated with gemcitabine, platinum, and BEV. These patients had none of the abovementioned risk factors and were not heavily pretreated [17]. It seems in any case preferable to withhold therapy for at least 30 days before surgery [37].

Rare complications reported specifically in ovarian cancer patients treated with BEV include spontaneous nasal septal perforation [38] and erosive osteoarthritis [39].

(2) Aflibercept (VEGF-trap) is a VEGF-ligand-binding antiangiogenic agent that binds and inactivates VEGF-B and placental growth factor in addition to VEGF-A. In preclinical models of ovarian cancer, it significantly reduced both tumor burden and ascites [40, 41]. A phase I trial of VEGF-trap in patients with advanced solid tumors included one patient with ovarian cancer. This patient experienced a PR. Fatigue (9 out of 10 patients), pain (4 out of 10 patients), and constipation (4 out of 10 patients) were the most common side effects of this new drug [42]. In a phase II trial of VEGF-trap in patients with platinum-resistant and topotecan and/or liposomal doxorubicin-resistant advanced ovarian cancer [43] an interim analysis after accrual of 162 patients showed that 11% of the patients receiving the study drug experienced a PR [44]. A phase II trial of VEGF-trap in advanced ovarian cancer patients with recurrent symptomatic malignant ascites [45] has been completed but not yet reported. A phase II trial combining VEGF-trap with docetaxel in patients with persistent or recurrent ovarian epithelial cancer is currently ongoing [46].

(3) *HuMV833* is another monoclonal antibody directed against VEGF. In a phase I study conducted in patients with advanced cancer, one patient with ovarian cancer experienced a PR that lasted 9 months [47].

2.1.2. VEGFR Tyrosine Kinase Inhibitors. *Cediranib* (AZD 2171, CED) is a highly selective and potent oral tyrosine kinase inhibitor (TKI) of VEGFR1, VEGFR2, VEGFR3, and c-Kit. In a phase II study conducted in recurrent epithelial ovarian cancer, researchers found CED to have an ORR of 18.5%. Grade 3 toxicities included hypertension (13 out of 27 patients), fatigue (5 out of 27 patients), diarrhea (3 out of 27 patients), vomiting (2 out of 27 patients), hyponatremia (2 out of 27 patients), oral cavity pain (2 out of 27 patients), and nausea, constipation, abdominal pain, headache, and hypothyroidism (1 out of 27 patients). Grade 4 toxicities included central nervous system hemorrhage (1 out of 27 patients), lipase elevation (1 out of 27 patients), and hypertriglyceridemia (1 out of 27 patients) [48]. Two phase II trials are currently studying CED in recurrent ovarian cancer [49, 50], while a phase III randomized study is comparing chemotherapy with carboplatin and paclitaxel (PBC) versus concurrent CED and PBC versus concurrent CED and PBC followed by maintenance CED in women with platinum-sensitive relapsing ovarian epithelial carcinoma [51].

Ramucirumab (IMC-1121B) is a fully human antibody that blocks the interaction between VEGF and VEGFR2, resulting in potent inhibition of an array of biological activities of VEGF, including activation of the receptor and its signaling pathway, intracellular calcium mobilization, and migration and proliferation of endothelial cells [52]. It is currently under study in a phase II trial of persistent or recurrent epithelial ovarian carcinoma [53].

Semaxinib (SU5416) is a tyrosine kinase inhibitor with activity against VEGFR2. It reduced microvessel density and tumor growth in a preclinical tumor model with high VEGF expression [54].

Despite these promising data, some combination trials resulted in very disappointing results. In a recent preclinical study of metronomic paclitaxel with the VEGFR2 inhibitor SU5416, researchers found that the combination therapy showed an additive effect in tumors with low VEGF expression, while they observed an antagonism in tumors with high VEGF expression. They postulated that the lack of additive effect between these 2 drugs in tumors with high VEGF expression might be due to the fact that these two agents acted through the same pathways, and that their concomitant use could not produce more effects than each drug used in monotherapy [54]. These experiments outline that a better knowledge of the various molecular pathways implicated will help us to investigate the optimal combination partners and schedules.

2.2. PDGF. Platelet-derived growth factor (PDGF) is a potent mitogen and chemotactic factor for a variety of mesenchymal cells, such as fibroblasts and vascular smooth muscle cells. They exert their effects on target cells by activating two structurally related protein tyrosine-kinase receptors, α and β located on pericytes [55]. High expression of PDGF receptors is a common characteristic of solid tumors [56].

PDGF is expressed in 73% of ovarian carcinomas, while 36% express PDGF-receptor alpha (PDGFRA). In addition, overexpression of PDGFRA is an independent poor prognostic factor in ovarian carcinoma [57]. *Imatinib* mesylate is a small molecule that inhibits the tyrosine kinases abl, c-kit, PDGFRA, and PDGFRB. It inhibits the growth of ovarian cancer cells through PDGFRA inactivation [58], and decreases the secretion of VEGF by epithelial ovarian cancer cells [59]. However, in the clinical setting, imatinib has failed to show relevant clinical activity as a *single* agent. There was no complete or partial response with imatinib monotherapy in a phase II trial that enrolled 16 patients with platinum/taxane-resistant disease overexpressing at least one imatinib molecular target [60]. In another phase II trial with imatinib in a less pretreated ovarian cancer population, median PFS was also disappointingly low: 2 months [61]. There are various reasons for the ineffectiveness of imatinib monotherapy in ovarian cancer: downregulation of c-kit and PDGFR may lead to induction of VEGF, inhibition of a single tyrosine kinase might be insufficient to impact downstream signaling cascades, and the molecular targets of imatinib might not be relevant in the occurrence of ovarian

cancer in comparison with gastrointestinal stromal tumor or chronic myeloid leukemia where a single specific mutation or a translocation, respectively, can be responsible for the genesis of these two cancers [62]. Despite these results, a phase II study of imatinib monotherapy in patients with recurrent platinum and taxane-resistant epithelial ovarian cancer whose tumor expresses either c-kit, PDGFR, or ABL is currently accruing patients [63].

By dysregulating proangiogenic signaling, there was some hope that the use of imatinib in a *combination* approach might be more effective. This was supported by a preclinical model of human ovarian carcinoma in which combination treatment with imatinib and paclitaxel induced increased apoptosis of tumor-associated endothelial cells, which resulted in a reduced tumor burden [64]. However, combination therapy with imatinib and docetaxel in 23 heavily pretreated patients with advanced, platinum-resistant ovarian cancer, and primary peritoneal carcinomatosis resulted in a disappointing ORR of 21.7% (1 CR and 4 PR) and a median PFS of 1.8 months [65]. A phase II study is currently studying the combination of paclitaxel with imatinib in taxane-pretreated ovarian cancer [66].

2.3. Multitargeted Tyrosine Kinase Inhibitors. Targeting the PDGF/PDGFR axis alone or in combination with classical chemotherapy is not very effective in the clinical setting. Endothelium homeostasis is regulated to a large extent by the PDGF/PDGFR system expressed by pericytes. Pericytes are perivascular cells that provide local survival signals for endothelial cells. Combination approaches targeting the VEGF/VEGFR and the PDGF/PDGFR axes are thus very appealing [67].

Sunitinib (SUN) is an orally bioavailable small molecule that inhibits multiple tyrosine kinases including all the PDGF receptors and VEGF receptors, as well as c-kit, RET, CSF-1R, and flt-3. A patient with recurrent clear cell ovarian carcinoma briefly responded to SUN as fifth-line therapy [68]. At least three phase II trials of SUN in recurrent and refractory ovarian carcinoma are currently ongoing (see Table 2). Typical side effects of SUN in other diseases are fatigue (28% grade 2-3), diarrhea (20% grade 2-3), dyspepsia (16% grade 2-3), hypertension (16% grade 2-3), hand-foot syndrome (15% grade 2-3), nausea (13% grade 2), stomatitis (13% grade 2-3), anorexia (12% grade 2-3), neutropenia (40% grade 2-3, 2% grade 4), thrombocytopenia (21% grade 2-3), lipase elevations (25% grade 2-3, 3% grade 4) [69], and hypothyroidism (53–85%) [70].

Sorafenib (SOR) is an oral small molecule that predominantly inhibits the serine/threonine raf-1 kinase. The molecule also inhibits other tyrosine kinase receptors including VEGFR1, VEGFR2, VEGFR3, PDGFRB, flt-3, and c-kit. The Ras/raf/MEK kinase pathway plays a key role in cellular proliferation. In addition, the Raf kinase is a downstream modulator of the VEGF signaling pathway [71]. Oncogenic b-raf mutations have been found with high frequency in ovarian cancer [72, 73]. After encouraging phase I results, where about 50% of patients with epithelial ovarian cancer had evidence of stable disease [74], SOR is now being tested in various combinations (see Table 2).

Vatalanib is a multitargeted tyrosine kinase inhibitor targeting angiogenesis that inhibits PDGFRB, VEGFR1, VEGFR2, c-Kit, and c-Fms. In a preclinical model of VEGF-dependent human ovarian carcinomas, vatalanib inhibited the formation of malignant ascites and the tumor growth [75, 76]. It is currently under investigation in advanced solid tumors.

BIBF 1200 is a combined inhibitor of PDGFR, VEGFR, and FGFR [77]. It was tested as maintenance therapy in a phase II randomized double-blind trial in ovarian cancer patients who responded to their last (at least second line) chemotherapy. Median time to RECIST progression was 4.8 months for BIBF 1120, and 2.8 months for placebo. Grade 3 and 4 adverse events were seen in 54 and 7% (BIBF 1120) and 25 and 3% (placebo) of patients. The rate of gastrointestinal toxicities was slightly higher in the BIBF 1120 arm (16 versus 10%, all grade 3; no grade 4 events). Elevation of liver enzymes occurred in 43% (BIBF 1120) versus 6.3% (placebo) [78]. *Other multitargeted tyrosine kinase inhibitors currently under investigation are summarized in Table 2.*

2.4. Endothelin. The *endothelin* axis comprises 3 small peptides (ET-1 to -3) that mediate various physiological processes by binding to endothelin A (ET_A) and endothelin B (ET_B) surface receptors. Activation of the ET_A receptor (ET_AR) by ET-1 increases tumor cell proliferation, survival, angiogenesis, migration, invasion, and metastasis in ovarian cancer [79]. Endothelins also modulate angiogenesis indirectly, as VEGF and ET-1 have reciprocal stimulatory interactions in vivo [80]. More than 90% of primary ovarian cancers express ET-1, and ET-1 expression in tumors is significantly elevated compared to normal ovarian tissue. Moreover, the vast majority of ovarian carcinomas express the ET_AR [81], which is emerging as an attractive target for anti-angiogenesis therapy.

Atrasentan is a selective ET_AR antagonist. In ovarian carcinoma xenografts, atrasentan significantly reduced microvessel density, expression of VEGF, matrix metalloproteinase-2, and increased the percentage of apoptotic tumor cells. Combined treatment with atrasentan and paclitaxel produced additive antitumor, apoptotic, and antiangiogenic effects [82].

In humans, the most common side effects of atrasentan include fatigue, edema, and rhinitis [83].

In a preclinical model, ZD4054, another selective ET_AR antagonist, significantly reduced tumor growth and angiogenesis [84]. The reduction in new vessel formation was even more pronounced when ZD4054 was combined with gefitinib [85]. As is the case with atrasentan, the combination of ZD4054 with paclitaxel also produced additive antitumor effects [86].

2.5. mTOR Inhibitors. Inhibition of mTOR reduces secretion of VEGF by the tumor through inhibition of HIF-1α. In addition, mTOR inhibitors can also decrease cancer cell proliferation and survival [87]. RAD001 (everolimus) diminished the expression of VEGF and inhibited angiogenesis in a transgenic mouse model of ovarian cancer [88]. RAD001 significantly enhanced cisplatin-induced apoptosis in vitro

Table 2: Ongoing trials with multitargeted tyrosine kinase inhibitors in ovarian cancer.

Agent	Targets	Phase	Intervention	Stage of the disease	Trial number
Sunitinib	VEGFR PDGFR c-kit RET CSF-1R flt-3	II	Sunitinib monotherapy	Platinum-resistant recurrent	AGO-OVAR 2.11 NCT00543049 (Germany)
		II	Sunitinib monotherapy	Recurrent or refractory	DF08-056 NCT00768144 (United States)
		II	Sunitinib monotherapy	Advanced and/or metastatic	CAN-NCIC-IND185 NCT00388037 (Canada)
Sorafenib	Raf-1 VEGFR PDGFRB flt-3 c-kit	II	Sorafenib maintenance versus placebo	CR after standard platinum therapy	NCT00791778
		II	Paclitaxel and carboplatin +/− sorafenib	1st line	SCRI GYN 19 NCT00390611
		II	Paclitaxel and carboplatin +/− sorafenib	Platinum-sensitive recurrent	CASE-CWRU-2804 NCT00096200
		II	Topotecan + sorafenib	Platinum-resistant recurrent	GYN06-111 NCT00526799
Pazopanib	VEGFR PDGFR c-kit	III	Pazopanib maintenance versus placebo	After 1st line chemo	AGO-OVAR16 NCT00866697
		II	Pazopanib monotherapy	Recurrent	VEG104450 NCT00281632
		I	Metronomic topotecan + pazopanib	Persistent or recurrent	NCT00800345
XL999	VEGFR PDGFR FGFR flt-3 Src	II	XL999 monotherapy	Recurrent	NCT00277290
Motesanib	VEGFR PDGFR c-kit	II	Motesanib monotherapy	Persistent or recurrent	NCT00574951
Vandetanib	VEGFR EGFR	II	Docetaxel +/−vandetanib	Persistent or recurrent	SWOG-S0904 NCT00872989
		I/II	Pegliposomal doxorubicin +/− vandetanib	Platinum-refractory recurrent	NCT00862836

Studies were accessed from http://www.clinicaltrials.gov/ on May 17, 2009
VEGFR = vascular endothelial growth factor receptor, PDGFR = platelet-derived growth factor receptor,

[89]. A randomized phase II study of BEV with or without everolimus in patients with recurrent or persistent ovarian epithelial cancer is ongoing [90].

2.6. Src Inhibition. Src plays a critical role in tumor angiogenesis, probably through the regulation of IL-8, an important angiogenic cytokine [91–93]. It is also essential for the hypoxia-mediated induction of VEGF [94]. Src inhibition through a novel small-molecule inhibitor, AP23994, alone or in combination with cytotoxic chemotherapy, significantly reduced tumor growth in ovarian cancer models [95]. Src is thus emerging as a new target for antiangiogenic treatment of ovarian cancer. A phase I trial of a Src kinase inhibitor, dasatinib, in combination with paclitaxel and carboplatin in patients with advanced or recurrent ovarian cancer is currently ongoing [96]. Src inhibition is also being evaluated

in a phase I study combining dasatinib and BEV in patients with metastatic or unresectable solid tumors [97].

AZD0530 is a dual inhibitor of Src and abl. It is currently in phase II study in combination with carboplatin plus paclitaxel in platinum-sensitive ovarian cancer patients [98].

EphA2 is a protein overexpressed by many tumor cells. Use of an agonistic antibody of EphA2 (EA5) in combination with paclitaxel substantially reduced tumor growth in an ovarian cancer model, including a paclitaxel-resistant model. EA5 led to dissociation of Src from EphA2, resulting in decreased phosphorylation of Src and thus VEGF expression [99].

2.7. Integrin $\alpha5\beta1$ Targeting. Endostatin is a COOH-terminal fragment of collagen XVIII and is a potent angiogenesis inhibitor. Integrin $\alpha5\beta1$ is the major target for endostatin-mediated inhibition of endothelial cell proliferation and migration. Endostatin was shown to block peritoneal attachment and vessel cooption by ovarian cancer cells [100]. It is currently being investigated in phase I studies in advanced refractory solid tumors [101, 102]. Volociximab is a chimeric monoclonal antibody that blocks $\alpha5\beta1$ binding to fibronectin and induces apoptosis in proliferating endothelial cells. It was tested in a phase I/II study in combination with pegylated doxorubicin in patients with recurrent platinum-resistant ovarian cancer. Since a preliminary analysis of PFS suggested that there was a low probability of detecting a statistically significant difference in favor of the combination regimen, the study was closed to enrollment [103].

2.8. Thalidomide (THAL). Multiple mechanisms of action have been proposed for THAL. It could, at least in part, act through an antiangiogenic effect, by inhibiting tumor-necrosis alpha, VEGF and/or fibroblast growth factor 2 [104]. In a phase I study involving 17 heavily pretreated patients with recurrent epithelial ovarian cancer, 18% experienced a PR and 35% a stable disease after 6 months. Median time to progression was 10 months. Common grade 1 or 2 side effects included constipation (76%), neuropathy (71%) and fatigue (65%). Among the 5 grade 3/4 toxicities, 2 patients (12%) had a venous thrombosis [105]. A single-institution prospective cohort study conducted in patients with recurrent ovarian or primary peritoneal cancer who had received a minimum of 2 prior therapeutic regimens compared any standard intravenous chemotherapy to THAL or treatment holiday. There was a trend towards comparable responses in the chemotherapy and THAL arms. There was a high rate of grade 3 dyspnea, with 8 out of 18 (44%) patients who presented subjective shortness of breath at rest in the THAL arm. At least one of these patients had pulmonary embolus, a dreaded complication of THAL [106]. In a randomized phase 2 trial comparing topotecan to topotecan plus THAL in 75 women with recurrent epithelial ovarian cancer, the addition of THAL to topotecan appeared to improve response rates: ORR was 47% in the THAL arm versus 21% in the topotecan alone arm. Median PFS was 6 months in the THAL arm compared to 4 months in the control arm [107]. A randomized phase II study is currently comparing carboplatin and THAL with carboplatin alone in patients with stage Ic-IV ovarian cancer [108].

2.9. Prostaglandin E2 (PGE2). PGE2 enhances angiogenesis through the induction of VEGF [109]. Clofibric acid is a peroxysome proliferator-activated receptor α (PPARα) ligand that reduces PGE2 levels, leading to repression of VEGF expression, inhibition of angiogenesis and tumor cell apoptosis in a preclinical ovarian cancer model [110]. In a preclinical ovarian cancer model, celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, and ciglitazone, a PPARγ ligand, reduced tumor growth by decreasing angiogenesis through inhibited VEGF production in relation to PGE2 reduction [111]. Ongoing trials are investigating celecoxib in advanced ovarian cancer; one phase II study is combining paclitaxel with celecoxib [112] and another randomized phase II study is comparing cyclophosphamide with or without celecoxib [113].

2.10. Antiangiogenic Gene Therapy. (i) Phosphatase and tensin homologue on chromosome 10 (PTEN) is a cancer suppressor gene. Overexpression of the PTEN gene by transfection in ovarian cancer cell lines without PTEN mutations leads to decreased VEGF concentrations and a reduced number of new blood vessels. PTEN gene therapy in murine models of human ovarian cancer suppresses intraperitoneal dissemination and extends survival [114].

(ii) Increased IL-8 expression is associated with poor clinical outcome in human ovarian carcinoma, and IL-8 gene silencing with small interfering RNAs (siRNAs) can decrease tumor growth through antiangiogenic mechanisms in preclinical models [115].

(iii) Ribozymes are catalytic RNA molecules that can cleave other RNA molecules in a target-specific manner, thereby downregulating the expression of any pathogenic gene product. Angiozyme inhibits angiogenesis by selectively downregulating VEGFR1 through targeted cleavage of VEGFR1 mRNA [116]. After encouraging phase I testing, it has now completed the phase II setting in renal cancer [117]. There is a strong rationale to try this approach in ovarian cancer.

(iv) Shiga-like toxin 1 mutants $Stx1^{W203F}$ and $Stx1^{R170H}$ have been shown in preclinical models to have antiproliferative and antiangiogenic effects in murine xenograft models of ovarian cancer. They are good candidates for gene therapy [118].

2.11. Other Antiangiogenic Targets. (i) Squalamine is an aminosterol that inhibits mitogen-induced proliferation and migration of endothelial cells in vitro and causes significant in vivo inhibition of angiogenesis [119]. It is currently in phase II testing in combination with carboplatin in patients with recurrent or refractory stage III or stage IV ovarian cancer [120].

(ii) CAI is a synthetic carboxyamidotriazole that inhibits proliferation, invasion and metastasis, and neovascularization both in vitro and in vivo. In a phase II study of 38 heavily pretreated patients with recurrent epithelial ovarian cancer, median PFS was 3.6 months [121].

(iii) Angiopoietins are emerging as crucial regulators of the angiogenic switch in tumors [122]. AMG 386 is a peptibody that binds to and inhibits angiopoietin 1 and 2. It is being investigated in a phase 1b study in combination with either pegylated liposomal doxorubicin or topotecan in subjects with advanced recurrent epithelial ovarian cancer [123].

3. Vascular-Disrupting Agents

Tumor vessels have different characteristics than normal vessels. They have been found to be more tortuous, less organized, and more leaky [124]. Vascular-disrupting agents (VDAs) are a new class of agents that cause a pronounced shutdown in blood flow to solid tumors, resulting in extensive tumor-cell necrosis due to lack of oxygen and nutrients supply, while they leave the blood flow in normal tissues relatively intact [125]. Small molecules VDAs are the major class of VDAs. They can be divided into 2 groups: the tubulin-binding agents and the flavonoids [126].

Combretastatin A-4 (*CA-4*), its prodrug ZD6126 and AVE8062 (a water-soluble analog of CA-4) are tubulin-binding agents that are structurally related to the colchicines and possess potent antivascular properties [126]. CA-4 was shown to exert its antivascular effects through selective disruption of the tubulin cytoskeleton of endothelial cells [127]. In a murine model of ovarian carcinoma, AVE8062 effectively inhibited tumor growth and was even more effective in combination with docetaxel [128]. VDAs are currently in clinical development, alone or in combination. 5, 6-dimethyl-xanthenone-4 acetic acid (*DMXAA*) is a flavonoid causing DNA damage to endothelial cells that induces apoptosis in preclinical models [126]. When given 1–4 hours after cisplatin chemotherapy, DMXAA or CA-4 induced a markedly increased tumor response in a xenograft model of ovarian carcinoma [129].

The differences between normal and tumor vessels can also be exploited to selectively deliver chemotherapeutic drugs to the tumor vasculature. Peptides containing the asparagines-glycine-arginine (*NGR*) motif, which binds to a specific isoform of CD13 exclusively found in angiogenic vessels, have been used to deliver various antitumor compounds to the tumor vasculature [130]. Targeted liposomal doxorubicin (TVT-DOX) is a form of ligand-targeted nanomedicine that contains the NGR motif on its surface. In a murine xenograft of doxorubicin-resistant ovarian cancer, it was able to more effectively kill angiogenic tumor blood vessels and indirectly the tumor cells that these vessels support than an untargeted formulation of doxorubicin [131].

4. Biomarkers

4.1. Classical Markers. Plasmatic CA125 concentration is routinely used in clinical practice as a surrogate marker for clinical response of ovarian cancer treatment [132]. However, CA125 has not been validated in the context of targeted therapies. The mechanism regulating the production and/or secretion of mucin MUC16, which is recognized by the OC125 antibody, is as yet unknown, and it could potentially be altered by biochemical modulation of the tumor [133]. Moreover, in a phase II study of patients receiving BEV and SOR, the authors found a poor concordance between CA125 changes and objective imaging (67% concordance) [134], raising the question whether CA125 monitoring can be used to monitor tumor response to antiangiogenic therapy.

Response Evaluation Criteria in Solid Tumors (*RECIST*) are routinely used to assess tumor response [135]. They can however not be considered entirely reliable in the context of agents that reduce tumor blood flow because changes in blood flow may precede changes in tumor size [136].

4.2. Markers of Angiogenesis. There is a clear correlation in ovarian cancer between markers of angiogenesis and poor *prognosis*. Increased angiogenesis can be identified in various ways.

Microvessel density evaluated by the specific endothelial cell marker CD34 is correlated with poor prognosis in ovarian cancer [137, 138]. The Chalkley count with CD34 immunostaining is the most validated method of microvessel density determination [139].

In small retrospective analyses of ovarian tumor samples after surgery and prior to standard chemotherapy, *overexpression of VEGF* as detected by immunohistochemistry on tumor tissue was present in up to 48% of samples and was shown to be independently predictive of poor prognosis [140–142]. However, in recent series of 339 primary ovarian cancers, only 7% showed a high expression of VEGF. The use of different antibodies, scoring systems, and cutoff points might explain the discrepancies between studies. In any case, these latest data suggest that the benefit of anti-VEGF therapy might be limited to a small subset of patients [143].

Other markers of angiogenesis are currently under study. Serum VEGF levels are independent prognostic markers in ovarian cancer patients [144]. *Genetic testing* also showed promising results, as the simultaneous carriage of 3 single nucleotide polymorphisms associated with increased VEGF production was shown to lead to a significantly impaired overall survival [145], while a 34-gene-profile of angiogenesis-related genes was able to predict the overall survival of ovarian cancer patients [146]. Finally, high expression of new tumor vascular markers, like *STC2, EGFL6, and FZD10*, which are specifically expressed by tumors harboring tumor endothelial cells, have been shown to be associated with a significant decrease in disease-free interval [147].

Other biomarkers could be used in the future to *predict* the outcome after targeted therapy. IL-8 plays a significant role in mediating human ovarian carcinoma-derived angiogenesis and tumorigenesis [148], probably independently of VEGF [149]. It was recently shown that the *IL-8 A-251T polymorphism* might be a molecular predictor of response to BEV-based chemotherapy in ovarian cancer patients [150]. *pAKT* may serve as a predictor of resistance to imatinib treatment in ovarian cancer cells [151].

4.3. Imaging of Angiogenesis. New noninvasive imaging techniques are currently under study. In a retrospective study of 49 women with primary ovarian cancer or metastatic tumors to the ovary, three-dimensional power Doppler *ultrasound*

(3D-PDU), which allows tumor vascularization assessment, showed that vascularization was higher in advanced stage and metastatic ovarian cancers than in early stage ovarian cancer [152]. In a retrospective study of 41 women with epithelial ovarian cancer, researchers found that dynamic contrast-enhanced *magnetic resonance* imaging (DCE-MRI) could help distinguish among benign, borderline, and invasive tumors and was correlated with tumoral angiogenic status, specifically the pericyte coverage index and VEGF expression [153].

Tracers focusing on VEGF and VEGFR2 have been developed to visualize angiogenesis-related events with noninvasive *positron emission tomography* (PET) imaging [154, 155]. In preclinical murine models of ovarian carcinoma treated with vascular-disrupting agents, [^{18}F]FDG PET imaging could predict tumor response as early as 2 hours after therapy [128].

5. Conclusion

Antiangiogenic therapy in ovarian cancer is very promising so far, at least in phase II trials. This is probably due to the highly angiogenesis-dependent pathophysiology of this disease. We should however keep in mind that angiogenesis might not be the driving force behind all cases of epithelial ovarian cancer and that we are still missing large placebo-controlled phase III trials that show a benefit in term of PFS or overall survival. Tools to detect the patients that are likely to benefit from antiangiogenic treatment have yet to be validated in the clinic. This would allow us to restrict the use of these very potent but also onerous new drugs to those who are most likely to benefit. A better selection of patients would also help to reduce the high complication rate seen with these agents, in particular GI perforations. The optimal duration of maintenance treatment with BEV will also have to be evaluated, and pharmaco-economic considerations will have to be addressed. Finally, combined targeting of tumor cells, endothelial cells, and pericytes (which play an important role in the stabilization of endothelial cells) is a very interesting approach that warrants further studies.

In conclusion, antivascular treatment for epithelial ovarian cancer is a very promising approach that still needs to be validated in the phase III setting. As many patients as possible should be encouraged to take part in well-designed clinical trials.

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Review Article

Targeting the EGF Receptor for Ovarian Cancer Therapy

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Ovarian carcinoma is the leading cause of death from gynecologic malignancy in the US. Factors such as the molecular heterogeneity of ovarian tumors and frequent diagnosis at advanced stages hamper effective disease treatment. There is growing emphasis on the identification and development of targeted therapies to disrupt molecular pathways in cancer. The epidermal growth factor (EGF) receptor is one such protein target with potential utility in the management of ovarian cancer. This paper will discuss contributions of EGF receptor activation to ovarian cancer pathogenesis and the status of EGF receptor inhibitors and EGF receptor targeted therapies in ovarian cancer treatment.

1. Introduction

Ovarian carcinoma is the leading cause of death from gynecologic malignancy, with an estimated 15 520 deaths in the USA in 2008 [1]. Ovarian cancer is a highly metastatic disease that is rarely detected when disease is confined to the ovary (stage I) and 5-year survival is >90%. The great majority of ovarian cancer patients are initially diagnosed with disseminated intra-abdominal disease (stages III–IV) and have a 5-year survival of <20% [2]. Clinically, ovarian tumors often involve the ovary and omentum, with diffuse, multifocal intraperitoneal metastases and malignant ascites [2, 3]. The combined factors of late diagnosis and the cellular and molecular heterogeneity of ovarian cancers hamper efforts to effectively treat this disease.

For many cancers, including those of the ovary, there is growing emphasis on the identification and development of targeted therapies to disrupt specific molecular pathways contributing to disease progression [4]. The epidermal growth factor (EGF) receptor is one such molecular target. The EGF receptor impinges on multiple key hallmarks of cancer defined by Hanahan and Weinberg [5] and the EGF receptor is associated with a gene expression pattern unique to invasive tumor cells [6]. Aberrant expression and activity of the EGF receptor is generally recognized to have a deleterious impact on the clinical outcome of cancer patients which has fueled development of targeted therapeutics (reviewed in [7–12]). This paper will discuss potential contributions of EGF receptor activation to ovarian cancer pathogenesis and the status of EGF receptor inhibitors and EGF receptor targeted therapies in ovarian cancer treatment.

2. The EGF Receptor in Ovarian Cancer

The EGF receptor is a member of the receptor tyrosine kinase (RTK) family of growth factor receptors and the founding member of the ErbB subfamily that includes four proteins: ErbB1 (EGF receptor), ErbB2 (HER-2), ErbB3 (HER-3), and ErbB4 (HER-4). The ErbB receptors are single membrane spanning proteins possessing intrinsic tyrosine kinase catalytic activity. Ligand binding promotes EGF receptor homo- and heterodimerization with ErbB family members, activation of the intracellular tyrosine kinase domain, and stimulation of numerous downstream signaling cascades associated with cell growth and survival, increased angiogenesis, and tumor metastasis (reviewed in [7–10], [13–17]).

The most common form of ovarian cancer arises from the ovarian surface epithelium (OSE). The OSE expresses EGF receptors in vivo and EGF receptor activity is implicated in gonad development, growth and differentiation of the ovarian follicle, and postovulatory repair [18–20].

It has been proposed that EGF stimulation of the OSE contributes to its rapid post-ovulatory proliferation and to epithelial-mesenchymal transition (EMT) of OSE cells within the ruptured follicle. Malfunctions in post-ovulatory repair are believed to contribute to formation of epithelial inclusion cysts, which are the preferential sites of malignant transformation [15, 21, 22]. The normal OSE responds to EGF receptor generated signals by displaying a phenotypic plasticity characterized by transition between epithelial and fibroblastic phenotypes, a characteristic usually limited to immature, regenerating, or neoplastic epithelia [23]. These attributes of the adult OSE suggest that this tissue is "primed" to respond to the EGF receptor during tumor development and progression.

In addition to its role in normal ovarian epithelium, there is abundant evidence of aberrant EGF receptor and/or ligand expression in ovarian cancer. A recent review [15] provides an excellent and comprehensive summary of immunohistochemical studies evaluating ErbB receptor and ErbB ligand expression in malignant ovarian tumors. Briefly, published reports estimate EGF receptor expression in 10–70 percent of human epithelial ovarian cancer cases (reviewed in [15]). A smaller subset of studies has examined amplification of the EGF receptor gene in ovarian cancer. An advantage of this approach is the relative stability of DNA in archived samples, but because EGF receptor overexpression can occur in the absence of gene amplification, these studies may underestimate the frequency of elevated EGF receptor protein in tumors. Despite this caveat, EGF receptor gene amplification is detected in ~10–20 percent of ovarian cancer cases [24–26], with low-level gains detected more frequently in 43 percent of tumors [24]. Thus, based on detection of protein or gene amplification, there is strong evidence for elevated EGF receptor expression in a significant fraction of ovarian cancer cases.

Overall, elevated EGF receptor is associated with less favorable disease outcomes in a number of human tumors [17, 27–29]. Despite evidence for EGF receptor expression in ovarian tumors [15], studies on the relationships between receptor and patient outcomes do not provide a uniform picture on the clinical consequences of elevated EGF receptor levels. Based on studies with normal tissue reference controls, elevated EGF receptor levels significantly correlated with aggressive disease characteristics [24] and high tumor EGF receptor expression was proposed as the most significant prognostic factor for disease-free and overall survival [30]. An overall conclusion that aberrant EGF receptor status is a factor in ovarian cancer outcome is supported by a meta-analysis study revealing a relationship between EGF receptor and decreased survival [31], and the abundant evidence linking EGF receptor to poor patient outcome in other cancers of epithelial origin.

3. Consequences of EGF Receptor Activation in Ovarian Cancer

A limited number of studies examine activated (tyrosine phosphorylated) EGF receptor in ovarian tumors and overall, little attention has been given to receptor activation status and disease parameters. In one study, 11.8 percent of ovarian tumors were positive for phosphorylated EGF receptor (pEGFR) but no clinicopathological parameter or survival differences were noted [32]. In another study, twenty-four heavily pretreated patients with epithelial ovarian cancer all had detectable EGF receptor and p-EGFR (Y1148), suggesting that EGF receptor activation might be more evident in advanced disease [33]. We conducted a tumor tissue array analysis and found evidence for pEGFR in approximately 1/3 of ovarian tumor samples [34]. EGF receptor activation was statistically positively correlated with matrix metalloproteinase (MMP)-9 expression, a protein associated with tumor invasion and metastasis. Together, these in vivo data indicate that activated EGF receptor is present in ovarian tumor specimens, likely driving aspects of tumor behavior.

The mitogenic effects of EGF receptor activation in ovarian tumor cells are well documented. EGF increases the growth potential of primary ovarian surface epithelial (OSE) cells in culture [35] and gene expression profiling of normal rat ovarian surface epithelium following EGF treatment demonstrates EGF-dependent activation of genes involved in cell cycle and proliferation, apoptosis, and protein turnover [36]. In addition, malignant transformation of rat OSE cells results in alteration of downstream effectors of the EGF receptor pathway [36]. Regarding ovarian tumor cells, numerous studies demonstrate that autocrine and paracrine stimulation of the EGF receptor promotes ovarian tumor cell growth (reviewed in [37, 38]). Furthermore, blockade of EGF receptor activation or signaling inhibits ovarian tumor cell growth in vitro and in vivo (reviewed in [37]).

In addition to fostering cell growth, activation of the EGF receptor is associated with stimulation of metastasis-associated cellular responses. Many aspects of tumor metastasis resemble features of epithelial-mesenchymal transition (EMT) [39–43]. Notably, EGF receptor activation is capable of driving EMT-associated events in epithelial ovarian carcinoma cells in culture including migration and invasion, disruption of E-cadherin-mediated intercellular junctions, and production of matrix degrading proteinases (reviewed in [37, 38, 44, 45]). In contrast to the well-defined events that characterize EMT in development, tumor-associated EMT is currently viewed as a continuum of phenotypic plasticity and gain of mesenchymal characteristics. Tumor phenotype likely reflects the particular complement of EMT regulatory factors expressed in cells or within the tumor microenvironment [42–45]. The functional consequences of this phenotypic plasticity are not fully understood, but may play a role in modulation of cell survival in suspension (ascites), chemoresistance, and intraperitoneal anchoring of metastatic lesions (reviewed in [42, 44, 46]).

Based on the evidence that (1) ovarian tumors share certain characteristics (EGF receptor overexpression and activation) with tumors approved for treatment with EGF receptor inhibitors, (2) receptor activation drives tumor-relevant responses in ovarian tumor cells, and (3) ovarian tumor growth is reduced by EGF receptor directed therapeutics in preclinical models, the EGF receptor inhibitors have

Table 1: FDA approved EGF receptor inhibitors.

Generic, brand name	Type	Mechanism	Clinical Dose Range (route)	Approved Tumors	Company
Gefitinib, Iressa ZD1839	Small molecule TKI	Inhibits intracellular EGFR tyrosine kinase phosphorylation	250 mg daily (oral)	Platinum and taxane resistant nonsmall cell lung cancer	Astra-Zeneca
Erlotinib, Tarceva OS-774 CP-358774	Small molecule TKI	Inhibits intracellular EGFR tyrosine kinase phosphorylation	100 mg–150 mg daily (oral)	Nonsmall cell lung cancer, pancreatic cancer	OSI Pharmaceuticals/Genentech
Lapatinib, TYKERB GW 572016	Small molecule dual TKI, EGFR-1 and EGFR-2,	Inhibits heterodimerization and her1/her2 phosphorylation	1250 mg daily days 1–21 (oral)	Her2+breast cancer refractory to herceptin and chemo	Glaxo-Smith Kline
Cetuximab, Erbitux IMC-C225	Human/mouse chimeric MAb	Extracellular domain binding and ligand blockade	400 mg/m^2 load then 250 mg/m^2 weekly (IV)	Metastatic colorectal cancer, head, and neck	ImClone
Panitumamab, Vectibix ABX-EGF	Humanized MAb	Extracellular domain binding and ligand blockade	6 mg/kg every 14 days (IV)	Metastatic refractory colorectal cancer	Amgen/Abgenix

moved forward into clinical trials for ovarian cancer and are discussed in the following section.

4. Clinical Status of EGF Receptor Inhibition in Ovarian Cancer

With the advent of better understanding of the molecular mechanisms contributing to ovarian cancer, novel receptor targeted therapeutics or "biologic therapeutics" either administered alone or in combination with conventional chemotherapy have become a rapidly developing strategy in clinical trials design. Based on expression of the EGF receptor in ovarian cancer and the known consequences of receptor activation, this pathway could be a prime target for therapeutic blockade [4]. Numerous anti-EGF receptor agents are under active development and each compound has subtle differences in target binding, downstream signaling, ease of administration and toxicity profiles. Yet despite favorable preclinical studies using EGF receptor antagonists, clinical trial outcomes in ovarian cancer have been overall disappointing. Investigations are underway to understand the mechanism of escape from EGF receptor blockade as well as to identify clinical predictors of antagonist response. The following sections will summarize the success and shortcomings of these agents in ovarian cancer trials.

The majority of EGF receptor inhibitor agents in clinical trial development fall into two categories: small molecule tyrosine kinase inhibitors (TKIs) that compete with ATP for its binding site in the tyrosine kinase domain or monoclonal antibodies (MAbs) against the extracellular domain that interfere with ligand binding and/or receptor dimerization. Additional EGF receptor directed therapeutic strategies include development of EGF vaccines, receptor downregulation by antisense oligonucleotides [47]. EGF receptor dependent targeting of imaging agents, chemotherapeutic agents, and toxins will be discussed later in this paper.

A significant clinical difference between the small molecule TKIs and MAbs is that the TKIs are orally administered and require daily dosing (especially the reversible inhibitors) to maintain target blockade whereas the MAbs are given intraveneously usually weekly or every 2 weeks. The TKIs and MAbs share a toxicity profile which includes fatigue, diarrhea, and a robust acneiform rash. The cutaneous rash has been described as a clinical indicator of EGF receptor blockade due to abrogation of receptor signaling in nontumor tissues such as the skin and gut mucosa [47]. In addition, hypersensitivity reactions are a concern with MAbs, especially the nonhumanized or chimeric agents. Several TKIs and MAbs are FDA approved for treatment of specific solid tumors, yet none have performed well enough in ovarian cancer trials to warrant such approval (Table 1). Additional compounds are under clinical development in ovarian cancer and other solid tumors (Table 2).

4.1. EGF Receptor Specific Inhibitors. In clinical trials EGF receptor inhibitors have been administered as single agents and in combination with chemotherapy. Generally the trials are conducted in patients with recurrent ovarian cancer, and often patients have been heavily pretreated before receiving the targeted therapeutics. The common dosing schedules

Table 2: Non-FDA approved EGFR inhibitors. Data derived from the NCI Drug Dictionary and Clinical Trials Search http://www.nci.nih.gov/Templates/drugdictionary and [4, 47].

Generic or research name	Type	Mechanism	Clinical trial-ovarian cancer, other	Clinical dose range (route)	Company
CI-1033 PD 183805 Canertinib	Small molecule TKI	Irreversible binding to ATP-binding site EGFR 1, 2, 3, 4	Phase II	50 mg–200 mg daily day 1–21 (oral)	Pfizer
EKB-569 Pelitinib	Small molecule TKI	Irreversible binding to TK domain of EGFR 1, 2, 4	None, Phase I in solid tumors	25 mg daily (oral)	Wyeth-Ayerst
PKI-166	Small molecule TKI	Reversible binding to TKI domain EGFR 1, 2	None, Phase I in solid tumors	600 mg–700 mg 2 weeks on/off	Novartis
AV-412	Second generation dual TKI	Reversible binding to TKI domain EGFR 1,2	None, active Phase I trial in solid tumors	Dose escalation daily, dose escalation three times/wk	AVEO Pharmaceuticals
BIBW-2992 Tovok	Second generation dual TKI	Irreversible binding to TKI domain EGFR 1, 2	None, Phase I in solid tumors and Phase II in lung, breast, cancer	50 mg daily (oral), 70 mg daily 2weeks on/off	Boehringer Ingelheim's
CUDC-101	Small molecule TKI	Multi-targeted HDAC/EGFR 1, 2	None, Phase I solid tumors	Dose escalation, unknown starting dose	Curis, Inc.
BMS-690154	Small molecule TKI	Binds tyrosine kinase domains of EFGR1, 2 and VEGFR-2	None, Phase I in combo with paclitaxel and carboplatin	Dose escalation, unknown starting dose	Bristol-Myers Squibb
Matuzumab, EMD 72000	Humanized MAb	Extracellular domain binding and ligand blockade	Phase II EGFR+, other head+neck, lung, gastric	800 mg weekly (IV)	EMD Serono/Merk KGaA
Pertuzumab	Humanized MAb	Extracellular her2 ligand blockade, prevents dimers with EGFR-1	Phase II, lung, breast, prostate	840 mg load followed by 420 mg every 3 weeks (IV)	Merck Serono
RO5083945	Glycoengineered MAb	Binds to EGFR extracellular domain, inhibits dimers	None, Phase I EGFR+ solid tumors	Dose escalation start at 50 mg (IV)	Roche Pharmaceuticals

from multiple Phase I trials for the oral TKIs are shown in Table 1. Gefitinib alone (500 mg) performed poorly in Phase II trials with minimal clinical response for ovarian cancer patients. The only responder had an activating mutation in the EGF receptor catalytic domain similar to the mutations evident in responsive lung cancer patients [48]. Erlotinib alone (150 mg) performed slightly better with 6% of the patients responding based on tumor regression and 44% of patients had stable disease [4]. Gefitinib has been combined with cytotoxic chemotherapy such as carboplatin, paclitaxel, topotecan, oxaliplatin, vinorelbine, and the aromatase inhibitor anastrazole in multiple Phases I and II trials with some patients responding to treatment [4, 47]. Eroltinib has been combined with carboplatin, docetaxel, paclitaxel, and the VEGFR inhibitor bevacizumab [4]. Several of these trials were performed as front line treatment after cytoreductive surgery demonstrating good clinical and some pathologic complete response rates, but the response rates do not appear dramatically different when compared to historic controls for conventional therapy alone. The pipeline of EGF receptor

tyrosine kinase inhibitors continues to expand (Table 2). A randomized Phase II trial of the irreversible EGF receptor inhibitor CI-1033 was performed in a heavily pretreated population of women with recurrent ovarian cancer. Two different oral dose regimens were given (50 mg versus 200 mg daily) for 21 days. Unfortunately there were no responders to single agent treatment and no association between baseline ErbB expression and disease stability [49]. Future studies will likely see these new agents in combination with cytotoxic and other biologic agents.

There are many possible reasons to account for the modest responses to EGF receptor inhibitors. The oral tyrosine kinase inhibitors can be difficult to use in this patient population, as advanced disease causes loss of bowel function and potential unreliable absorption of drug. Another significant concern is the lack of validated biomarkers for response to these TKIs. To date, activating mutations in the EGF receptor kinase domain are the only known predictors of response, but these mutations have not been fully explored in ovarian tumors.

The monoclonal antibodies against the EGF receptor ligand binding domain have some pharmacologic advantages and may perhaps lead to better clinical outcomes compared to the TKIs. Cetuximab is the prototype MAb and has been administered alone or in combination with carboplatin +/− paclitaxel. A Gynecologic Oncology Group (GOG) Phase II trial of cetuximab and carboplatin in platinum sensitive recurrent ovarian cancer showed a 35% response rate (partial and complete responses) in patients with tumors displaying EGF receptor overexpression documented by immunohistochemistry (IHC). Of note, 93% of patients had overexpression of EGF receptor in the primary archived tumor as determined by immunohistochemistry [50]. Although it is tempting to conclude that EGF receptor immunohistochemical analysis of formalin fixed, paraffin embedded tissue is of predictive value for response rate, this has been neither quantified nor validated. A Phase II trial of EMD 72000 (matuzumab) given at 800 mg IV weekly enrolled 37 women with heavily pretreated platinum resistant recurrent ovarian cancer. EGF receptor status was not evaluated for entry criteria or for correlation to clinical response and there were no objective responses in this group when matuzumab was used as monotherapy [51]. The authors concluded that matuzumab monotherapy was not effective for this heavily pretreated group of women. Panitumumab is a fully humanized EGFR MAb under active investigation, particularly in lung and colorectal cancer. It is expected to elicit fewer hypersensitivity reactions than the chimeric human/mouse cetuximab, but to date, there is little direct clinical trial emphasis in ovarian cancer.

4.2. Dual Receptor Inhibition. Dual inhibition of ErbB receptor family members is an interesting approach for targeted therapy as much of the signaling is generated by heterodimers, particularly heterodimers of EGF receptor and ErbB2. Lapatinib is an oral small molecule tyrosine kinase inhibitor that reversibly inhibits both ErbB1 and ErbB2. It is well tolerated alone and in combination with chemotherapy as determined by Phase I trials [4, 47]. Our group recently completed a Phase I/II trial of weekly metronomic carboplatin and paclitaxel in combination with lapatinib (1250 mg daily) in 25 evaluable patients with recurrent ovarian cancer. Interval evaluation showed a 50% response rate (complete and partial response) with the expected gastrointestinal and hematologic toxicities [52]. The final analysis and publication of this study is pending. Canertinib (CI-1033) is a newer oral dual TKI which inhibits autophosphorylation of all ErbB receptors including a highly tumorigenic, constitutively active mutant form of the EGF receptor (EGFRvIII) [47]. This agent showed no significant activity as a single agent in a Phase II study in patients with recurrent ovarian cancer.

Monoclonal antibody dimerization inhibitors have shown the most promise in preclinical studies. Pertuzumab is the prototype of this inhibitor class and prevents ErbB2/HER2 dimerization with the EGF receptor, ErbB3/HER3, and ErbB4/HER4 leading to inhibition of MAP kinase and PI3 kinase signaling. A Phase II trial was conducted by Gordon et al. that included 123 patients with recurrent ovarian cancer (the majority platinum resistant). Two different dosing strategies of pertuzumab as a single agent demonstrated an overall response rate of 4.3% and a mean response duration of 18.6 weeks [53]. Only 28 patients had biopsy material accessible for evaluation of phosphorylated HER2 (pHER2) status by ELISA. Of this group only 8 patients had pHER2+ tissues with one patient in this group experiencing a partial response. The 20 other tumors did not show pHER2 expression and there were no treatment responses in this group [53]. This suggests that pHER2 rather than HER2 overexpression may be a viable biomarker for response although validation studies are desperately needed. Two ongoing randomized Phase II trials in relapsed ovarian cancer are evaluating pertuzumab versus placebo in combination with gemcitabine or carboplatin [54, 55]. In these trials treatments were tolerated, but clinical response endpoints have not yet been reached. In an early analysis of the data, low ErbB3/HER3 mRNA levels as measured in 122 of the 130 patient archival tumor tissues appeared to predict clinical benefit in the cohort receiving gemcitabine + pertuzumab versus the gemcitabine + placebo group [54]. Final analyses of both pertuzamab trials are pending. Additional monoclonal antibodies developed to inhibit EGF receptor family members are listed in Table 2 and studies to test the toxicity and efficacy of these agents in ovarian cancer are needed.

5. EGF Receptor as a Targeting Molecule for Imaging Agents and Therapeutics

In addition to therapies directed against the EGF receptor as discussed previously, this receptor has been used to deliver imaging agents or therapeutics to tumors. To target the EGF receptor on tumor cells, EGF receptor ligands or anti-EGF receptor MAbs are incorporated into complexes containing a therapeutic or imaging agent. EGF receptor ligands such as mouse EGF can be conjugated through its N-terminus without affecting receptor binding ability. In contrast, human EGF has two additional amino groups

due to internal lysines, and their conjugation can interfere with receptor binding [56]. For that reason mouse EGF rather than human EGF is usually employed for EGF receptor targeting. Novel peptides that specifically bind to EGF receptor provide alternative targeting moieties. Such peptides have been identified either through screening of a virtual peptide library [57], or through screening phage display libraries [58] for peptides that specifically bind to the EGF receptor, including lysine-deficient EGF variants [56]. EGF receptor-targeting moieties are conjugated with imaging or therapeutic agents such as radionuclides, cancer chemotherapeutic agents, toxins, RNase, or photosenstizers. In addition, delivery of oligonucleotides or expression vectors to either suppress or express certain genes in EGF receptor-positive cells through the use of viral or nonviral delivery systems has been reported. Recently more complex systems have been designed that employ various nanocarriers as targeted delivery systems.

The simplest form of an EGF receptor-targeting complex is radiolabelled-EGF, TKI inhibitor, anti-EGF receptor MAb, or engineered anti-EGF receptor fragments, which can be used for in vivo imaging or for therapeutic purposes [59, 60]. The targeted radionuclide delivery serves as a cytotoxic agent by itself and has been employed in boron neutron capture therapy [61, 62], although optimal therapeutic effects may not be achieved with stand alone boron therapy [63]. Radionuclides as imaging agents can be used to evaluate whether tumors are EGF receptor positive and thus likely to respond to EGF receptor-targeted therapies, or monitor response to therapy. Imaging techniques used to detect EGF receptor-expressing tumors in small animals include positron emission tomography (PET), magnetic resonance (MR), and single photon emission computed tomography (SPECT) [59, 60, 64]. These techniques involve positron emitting radionuclides (such as ^{11}C, ^{18}F, among others), beta emitters (such as Technetium (^{99m}Tc) and Lutetium (^{177}Lu)), gamma emitters (such as iodide (^{125}I) and Indium (^{111}In)), and alpha emitters (such as astatine (^{211}At) and bismuth (^{212}Bi, ^{213}Bi)) [59, 60, 64, 65]. Numerous preclinical studies indicate that tumor targeting can be achieved through the EGF receptor; however, most of these studies did not include ovarian tumor models.

In addition to radionuclides, cancer chemotherapeutic agents such as cisplatin [66], doxorubicin [67, 68], carminomycin [69], and tyrosine kinase inhibitors [70, 71] have been delivered to EGF receptor-positive cells through conjugation to EGF or to anti-EGF receptor mAb either directly or through a polymer linker. Numerous toxin conjugates that inhibit specific molecular targets within the cell have been delivered to EGF receptor-positive cells including pseudomonas exotoxin (PE) [72], amanitin [73], gelonin [74], and ricin chain A [75–78]. Furthermore, RNases targeted to the EGF receptor were cytotoxic to cancer cells [79–83] and photosensitizers used for photodynamic therapy have been successfully targeted to EGF receptor-positive cells [84–87]. Phase I clinical trials for TP-38 which is a fusion of a mutated PE and the EGF receptor ligand transforming growth alpha demonstrate that it is well tolerated with promising clinical response in patients with recurring malignant brain tumors [88]. The main challenges to expanding use of these toxin conjugates in clinical trials include reducing their immunogenicity by shielding the toxin portion of the complex, and the need to improve delivery to solid tumors [72].

EGF receptor targeted approaches have been used for viral and nonviral gene delivery to cells. As an example of viral systems, avidin-adenovirus (ADV) that expresses GFP was functionalized with EGF, and GFP expression was enhanced in EGF receptor-overexpressing cells compared to cells that moderately express EGF receptor or relative to naked or PEG-ADV [89]. DNA/polycation complexes have been employed for efficient gene delivery as nonviral systems. EGF or anti-EGF receptor MAb was conjugated to cationic polymers such as poly-L-lysine (PLL) [90–95] or polyethyleneimine (PEI) [96–102] that are positively charged and thus interact with negatively charged oligonucleotides or expression vectors. These systems efficiently transfected tumor cells in a receptor-dependent fashion. A number of strategies to improve EGF receptor-specific gene transfer or specificity include PEG or poly–L-glutamic acid (PLG). Other modifications that enhance EGF receptor gene transfer include incorporation of melittin, a membrane active peptide [103], or incorporation of PEG to reduce albumin-caused aggregation [104] and protect the complexes from serum proteins [105].

New generations of nanocarriers are under intense investigation as they offer advantages over administering a drug alone or in a simple conjugated targeting moiety. Nanocarriers have numerous benefits including their ability to deliver hydrophobic drugs, increased drug loading, the potential to load multiple drugs or imaging agents, and the ability to functionalize nanocarriers with multiple molecules. Moreover, because of their size these nanocarriers can passively target tumors through the enhanced retention effect caused by large gaps between vascular endothelial cells tissue and defective lymphatic drainage in tumor tissue [106]. In addition to passive targeting, active targeting of cancer tissue can be achieved using nanocarriers functionalized with a targeting moiety such as an EGF receptor ligand or an anti-EGF receptor MAb. Several nanocarriers have been employed as delivery vehicles for drugs or imaging agents to target EGF receptor-positive cancers including liposomes [107–112], gelatin nanoparticles [113, 114], gold [115], dendrimers [116], and carbon nanotubes [117]. These nanocarriers specifically bound to and were internalized by EGF receptor-expressing cancer cells in vitro [109, 115, 116, 118], or preferentially accumulated at tumor sites in vivo [107, 109, 113].

We successfully targeted carbon nanotubes functionalized with EGF and a PEG-fluorescein conjugate to ovarian tumor cells [118]. Specific EGF receptor targeting and cellular uptake was achieved by coating the nanotubes with PL-PEG2000. Furthermore, we find that these vehicles were trafficked to lysosomes, consistent with the fate of ligand-activated EGF receptor (Zeineldin, unpublished data). Lysosomes provide an acidic environment that is conducive to release of drugs attached to the delivery vehicle through acid-labile linkers. This property may allow for the design of

Table 3: Clinical trials combining the EGF receptor antagonists with other signaling pathway inhibitors.

American Society of Clinical Oncology (ASCO) 2009 Annual Meeting Proceedings
Phase I trial of bevacizumab + everolimus + panitumumab in refractory solid tumors [117]
Phase I trial of cetuximab and erlotinib in solid tumors [119]
Phase I trial of dasatinib + cetuximab in advanced solid tumors [120]

therapeutics that will release drugs intracellularly following EGF receptor targeted internalization. In addition, nanocarriers are being developed as efficient drug delivery systems to improve the cellular uptake of certain therapeutic agents such as inhibitory RNA or to enhance the therapeutic efficacy of drugs [106]. A pioneering example of a targeted nanocarrier that just completed phase I trials is CALAA-01. CALAA-01 is a stabilized cyclodextrin-containing polymer that delivers inhibitory RNA through transferrin targeting (Calando Pharmaceuticals: http://www.insertt.com). It is expected that nanotechnology will lead to innovative platforms for targeted drug delivery in future therapeutics.

6. Summary and Future Perspectives

There is abundant evidence that EGF receptor activation drives cellular processes linked to ovarian tumor development, tumor cell survival, and metastasis. However, the overall clinical impact of targeting the EGF receptor and its dimers in ovarian cancer, either by monoclonal antibodies or inhibition of the tyrosine kinase domain, has been modest in unselected women with advanced or recurrent ovarian cancer. Although the EGF receptor is a genetically validated target for non-small-cell lung cancer, therapeutic EGF receptor inhibition results in significant tumor regression in only 10–20% of patients [121]. One key goal in applying these agents to ovarian and other cancers will be to identify patients most likely to benefit from targeted therapies and to validate biomarkers of response [2, 4]. This type of preselection is standard in breast cancer, for example, where the estrogen receptor status of a tumor plays a major role in therapeutic decision-making strategy.

Clearly, a better understanding of in vivo efficacy, improved predictive biomarkers of response, and an understanding of the molecular "escape" pathways for EGF receptor antagonists is needed in ovarian cancer. Given concurrent activation of signaling pathways and pathway crosstalk in tumor cells, inhibition of multiple pathways has been proposed as a strategy to improve the impact of targeted therapeutics [2]. Accordingly, the latest approach in clinical trials is to combine the EGF receptor antagonists with inhibitors of other related or downstream signaling pathways. Phase I clinical trials in solid tumors have been presented recently at the 2009 American Society of Clinical Oncology (ASCO) meeting demonstrating this strategy (Table 3). Agents such as the mTOR inhibitor everolimus and vascular endothelial growth factor receptor inhibitor bevicizumab have been combined with panitumumab, and cetuximab has been combined with the BCR/ABL and src tyrosine kinase inhibitor. Dose limiting toxicities are similar as seen in other combined trials. The impact on biologic endpoints in vivo will be critical to assess the mechanisms of action of these combined therapies.

Ongoing research continues to identify new and more effective inhibitors of EGF receptor activity, and novel approaches to target antitumor therapies via the EGF receptor. Exploiting the EGF receptor to target and deliver drugs or imaging agents to tumor cells shows promise in preclinical models and an EGF receptor targeted toxin is in clinical trials for glioblastoma [88]. There is resurgence of interest in this strategy based on new generations of nanocarriers with improved drug delivery characteristics and the potential to deliver multiple drugs to tumor cells. Although application of EGF receptor antagonists and EGF receptor targeted therapies to ovarian cancer treatment lags behind that of certain other tumors such as lung and colorectal cancers, lessons learned in using these agents in other diseases are likely to benefit ovarian cancer patients in the future.

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Review Article

Targeting the Epidermal Growth Factor Receptor in Epithelial Ovarian Cancer: Current Knowledge and Future Challenges

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The epidermal growth factor receptor is overexpressed in up to 60% of ovarian epithelial malignancies. EGFR regulates complex cellular events due to the large number of ligands, dimerization partners, and diverse signaling pathways engaged. In ovarian cancer, EGFR activation is associated with increased malignant tumor phenotype and poorer patient outcome. However, unlike some other EGFR-positive solid tumors, treatment of ovarian tumors with anti-EGFR agents has induced minimal response. While the amount of information regarding EGFR-mediated signaling is considerable, current data provides little insight for the lack of efficacy of anti-EGFR agents in ovarian cancer. More comprehensive, systematic, and well-defined approaches are needed to dissect the roles that EGFR plays in the complex signaling processes in ovarian cancer as well as to identify biomarkers that can accurately predict sensitivity toward EGFR-targeted therapeutic agents. This new knowledge could facilitate the development of rational combinatorial therapies to sensitize tumor cells toward EGFR-targeted therapies.

1. Introduction

Epithelial ovarian cancer, defined as cancers arising either from the mesothelial lining of the ovaries (either from the epithelial surface lining or cortical ovarian cysts formed by invaginations of the surface epithelium) or from the fallopian tube epithelium [1], accounts for 90% of ovarian malignancies [2]. Epithelial ovarian cancers are further divided into 5 histologic subtypes: serous, endometrioid, mucinous, clear cell, and undifferentiated. Aberrant epidermal growth factor receptor (EGFR) expression is detected in up to 60% of ovarian cancers and occurs in all histologic subtypes [3, 4]. Further, aberrant EGFR expression is associated with poor outcome of ovarian cancer patients [5, 6]. In this article, we review the EGFR family, the role of EGFR in ovarian cancer, and the methods used to determine this role. We also summarize the results of anti-EGFR therapies in ovarian cancer clinical trials and discuss challenges and future work in effective treatments utilizing anti-EGFR therapies in ovarian cancer, focusing on epithelial ovarian cancer whenever possible.

1.1. The Epidermal Growth Factor Receptor Family. The EGFR family (also known as the HER or ERBB family) consists of 4 members: EGFR, HER2, HER3, and HER4 (alternately known as ERBB1–4). Structurally, the EGFR family consists of an extracellular ligand binding domain, a single transmembrane-spanning region, and an intracellular region containing the kinase domain (Figure 1; reviewed in [7–10]). In humans, more than 30 ligands have been identified that bind to the EGFR family, including EGF and EGF-like ligands, transforming growth factor (TGF)-α, and heregulins (HRGs, also known as neuregulins) [11].

EGFR is activated upon ligand binding, which results in a conformational change in the extracellular domain, leading to homo- or heterodimerization with another EGFR family member. The EGFR binding partner appears to depend on several properties, including the proportion of EGFR

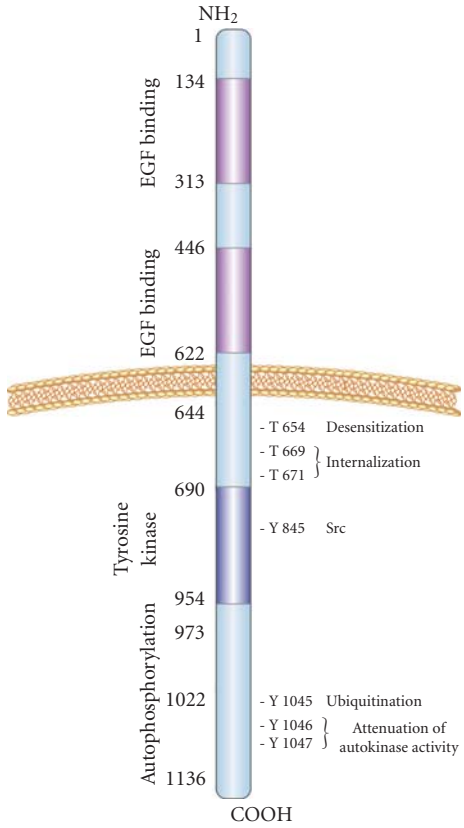

Figure 1: Structure of EGFR. EGFR consists of extracellular, transmembrane, and intracellular domains. The extracellular domain is the least conserved domain among the EGFR family members and consists of 4 subdomains—two ligand-binding domains and two receptor dimerization domains, which are cysteine-rich (reviewed in [12]). The transmembrane domain, which spans the cell membrane, is hydrophobic. The cytoplasmic tail of the EGFR family is highly conserved and contains the tyrosine kinase domain. Activation of EGFR family members leads to autophosphorylation of the tyrosine residues in the cytoplasmic tail. The phosphorylated tyrosine residues become docking sites for proteins with SRC homology 2 and phosphotyrosine binding domains, which transduce the signals downstream. EGFR phosphorylation at selected residues and their functional outcomes are indicted in the diagram. T: threonine; Y: tyrosine.

family members in the membrane, type and proportion of ligand (reviewed in [10, 13]), and cell lineage likely reflected in the expression of additional members of the signaling complex (see below). Strikingly, HER2 is the preferred binding partner for all EGFR family members [14], while HER3 is an obligatory partner [15], being inactive on its own or as a homodimer as it lacks intrinsic kinase activity due to mutation of critical amino acids in the kinase domain [16, 17]. This combination has lead to the suggestion by Yarden and colleagues that HER2 and HER3 are "deaf and dumb" members of the EGFR family, functioning in normal physiology as part of signaling complexes with other EGFR family members [18].

Activation of the EGFR family members results in transduction of EGFR signals, via intracellular cascades, such as mitogen-activated protein kinases (MAPKs), and AKT (also known as protein kinase B), resulting in perturbation of multiple cellular responses including proliferation, differentiation, cell motility, and survival (reviewed in [9, 19]). A summary of selected EGFR family pathways is shown in Figure 2.

The EGFR family members can also be activated by other signaling proteins independent of addition of exogenous EGFR ligands. These include other receptor tyrosine kinases (RTKs) such as insulin-like growth factor-1 receptor (IGF-1R) (reviewed in [20, 21]) and tyrosine kinase receptor B (TRKB, [22]) as well as other types of receptors such as G protein-coupled receptors (GPCRs) (reviewed in [23]), the leptin receptor [24], and adhesion proteins such as E-cadherin (reviewed in [25]) and integrins (reviewed in [26]). While the details of EGFR transactivation upon crosstalk are not yet fully elucidated, transactivation has been shown to occur by a variety of mechanisms. For example, there is evidence that EGFR can be transactivated by IGF-1R by direct binding [27]. Additionally, EGFR transactivation by GPCR has been shown to occur intracellularly, such as by activation of SRC upon GPCR stimulation (e.g., [28]), as well as extracellularly, such as by GPCR activation by gastrin releasing peptide [29]. This induces the formation of a GPCR complex containing SRC, Phosphatidylinositol 3′-kinase (PI3K), PDK1, and TNF-α converting enzyme (TACE), resulting in activation and translocation of TACE to the membrane where it releases the EGFR ligand amphiregulin, resulting in subsequent EGFR activation [29]. Lysophosphatidic acid (LPA)-GPCR-induced ectodomain shedding of pro Heparin Binding-EGF also activates EGFR [30]. LPA-mediated signaling is of particular importance in ovarian cancer as abnormalities in LPA metabolism and function likely contribute to initiation and progression of ovarian cancer [31–33]. Additionally, TRKB may also play a role in ovarian cancer as its activation has been shown to enhance migration and proliferation and suppress anoikis in human ovarian cancer cells [22, 34].

1.2. EGFR in Ovarian Cancer. The *EGFR* gene, located on chromosome 7p12, is amplified in ovarian cancer in approximately 4%–22% of cases [3, 6, 35, 36], including about 13% in epithelial ovarian cancers [35]. Activating *EGFR* mutations, as determined by sequence analyses of potential activating mutation sites in the catalytic domain, is rare in ovarian cancer, with a frequency of 4% or less [6, 35, 37]. The constitutively active mutant *EGFRvIII*, while reported earlier to be detected in 73% (24/32) of ovarian cancers [38], was not detected in subsequent and more extensive studies examining serous [6] or various types of ovarian cancers [39]. Overexpression of the EGFR protein has been detected in 9%–62% of human ovarian cancers [6, 36, 40, 41]; the differences in frequencies from these studies likely reflect utilization of different antibodies and cutoffs for overexpression. *EGFR* gene amplification or protein overexpression occurs across all epithelial ovarian cancer histotypes [3, 4]. Increased EGFR expression has been associated with high tumor grade [3, 5, 6], high cell proliferation index [6], aberrant P53 expression [6], and poor patient outcome [5, 6].

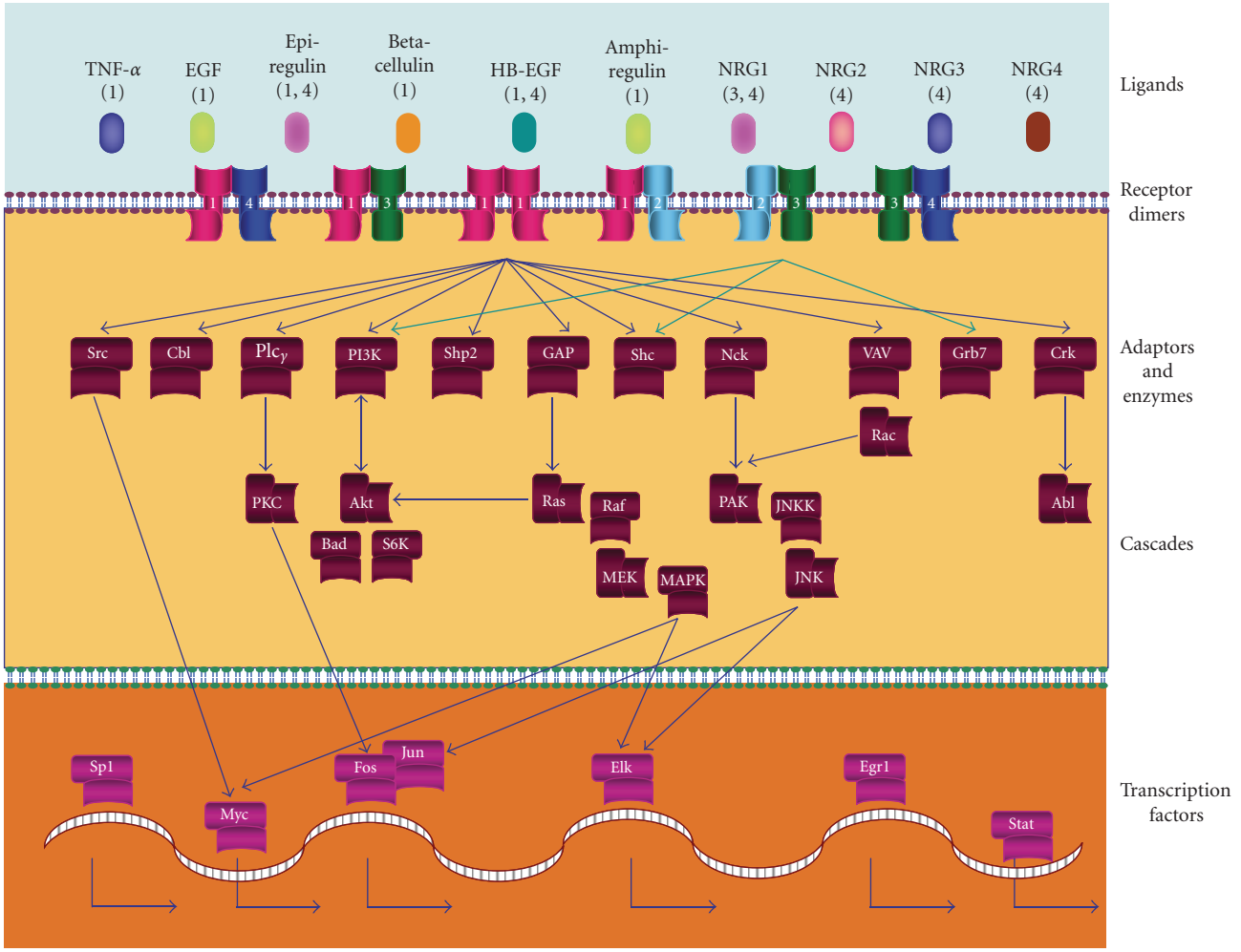

Figure 2: Selected representation of canonical EGFR family signaling pathways. The EGFR family consists of 4 members: EGFR, HER2, HER3, and HER4 (indicated by numbers 1–4 in the diagram). EGFR family ligands include EGF-and EGF-like ligands, transforming growth factor (TGF)-α and heregulins (HRGs, also known as neuregulins, NRGs). As indicated by the numbers in parentheses beneath the ligands, each ligand binds preferentially to a particular EGFR family member. HER2, while lacking any known ligand, is the preferred binding partner of for all EGFR family members. HER3 lacks intrinsic kinase activity due to mutation of critical amino acids in the kinase domain; therefore, it is inactive on its own or as a homodimer. Transduction of EGFR signals occurs through intracellular adaptor proteins, which transmit signals through cascades such as the RAS/RAF/MEK/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3′-kinase (PI3K)/AKT cascades. The downstream proteins in these signaling cascades can shuttle from the cytoplasm to the nucleus, where they signal to transcription factors and their complexes such as MYC, ELK, and FOS/JUN. Signal transduction through the EGFR family to downstream pathways and cascades controls diverse cellular responses such as proliferation, differentiation, cell motility, and survival as well as tumorigenesis. Figure adapted from [13]. Abbreviations: PLCγ: Phospholipase Cγ; SHP2: SRC homology phosphatase 2; GAP: GTPase activating protein; SHC: SRC homology 2 domain and collagen-containing protein; PKC: Protein kinase C; MEK: MAPK/ERK kinase; PAK: P21-activated kinase; JNKK: JNK kinase; JNK: JUN N-terminal kinase; EGR1: Early growth response protein 1; STAT: Signal transducer and activator of transcription.

One of the first studies implicating the EGFR pathway in ovarian cancer was the detection of TGF-α in human ovarian cancer effusions as determined by radioimmunoassay [42]. TGF-α was also shown to increase proliferation as measured by [^{3}H]thymidine incorporation [43] as well as increase levels of the tumor markers cancer antigen-125 and tissue polypeptide antigen [44] in EGFR-positive primary human serous ovarian cancer cells. In the human ovarian adenocarcinoma cell line OMC-3, TGF-α induced migration and invasion as well as gelatinolytic, caseinolytic, and plasmin activity in a dose-dependent manner [45].

While initial studies suggested that EGF, due to the inability to detect transcripts in Northern blotting, might not play a significant role in ovarian cancer [43], subsequent studies indicated that exogenous EGF can also induce effects associated with transformation. Like TGF-α, treatment of OMC-3 cells with EGF induced cell migration and invasion and degradation of extracellular matrix components [45].

Additionally, human ovarian cancer cell lines treated with EGF showed significant increases in expression of proteins associated with invasion (urokinase plasminogen activator and its receptor, and plasminogen activator inhibitor-1 [46]). EGF can also affect pathways associated with angiogenesis, as EGF stimulation of the human ovarian adenocarcinoma cell line OVCAR-3 leads to increased H_2O_2 levels, which in turn activates the AKT-P70S6K pathway and increases vascular endothelial growth factor transcription through hypoxia-inducible factor-1α expression [47].

While earlier studies focused on EGFR ligands in ovarian cancer, emerging studies examined the mechanism of EGFR activation itself. For example, Campiglio et al. detailed the activation characteristics of the EGFR family members upon addition of EGF or HRG in human ovarian cancer cell lines containing different levels of EGFR family proteins [48]. In this report, they show that the pattern of EGFR family activation in human ovarian cancer cells appears to be distinct from that of human breast cancer cell lines; while EGFR and HER2 were consistently activated upon EGF treatment, HER3 and HER4 activation depended upon the relative abundance of each receptor in ovarian cancer cells. Additionally, HER3 activation could occur independently of HER2 [48]. This complex pattern of EGFR family activation could in part explain the poor rate of response to EGFR inhibition in ovarian cancer.

Further elucidation of the effects of EGFR signaling in ovarian cancer comes from inhibition of EGFR in cultured human ovarian cancer cells. For example, treatment of the human ovarian serous epithelial cancer cell line OVCA420 with the anti-EGFR murine monoclonal antibody (mAb) C225 resulted in decreased levels of cell cycle progression-associated proteins Cyclin-dependent kinase (CDK) 2, CDK4, and CDK6 and increased expression of the cell cycle-inhibiting protein $P27^{Kip1}$, along with increased association of $P27^{Kip1}$ with the CDKs [49]. Additionally, modulation of other cell cycle proteins was observed, including decreased expression and phosphorylation of the CDK substrates RB and P130 and decreased protein levels of cyclin A. Modulation of these proteins upon C225 treatment was associated with an increase in the proportion of cells in the G1 phase of the cell cycle. The effects observed upon EGFR inhibition were enhanced upon combined treatment of human ovarian cancer cells with the anti-HER2 murine mAb 4D5 [49].

As transactivation pathways in various cell systems have been delineated, so have the pathways associated with EGFR family activation in ovarian cancer. For example, Vacca et al. have provided evidence that the GPCR ligand, endothelin (ET)-1, can activate EGFR in the human ovarian cancer cell line OVCA 433 [50]. ET-1 has been observed to play a role in mitogenic autocrine loops in various cultured cell types including human ovarian cancer [51, 52] and is proposed to contribute to tumor growth in vivo [53]. ET-1 treatment increased phosphorylation of EGFR and its downstream proteins SRC homology 2 domain and collagen-containing protein (SHC) and ERK2 as well as increased SHC-GRB2 association [50]. These effects were reversed upon pretreatment of OVCA 433 cells with the EGFR inhibitor tyrphostin AG1478 as well as the ET_A-specific antagonist BQ-123 [50].

More recent studies have found additional signaling molecules or pathways that contribute to EGFR-mediated malignant phenotype in human ovarian cancer cell lines, including EGFR-interleukin-6 crosstalk through Janus kinase 2/Signal transducer and activator of transcription 3 signaling to mediate epithelial-mesenchymal transition [54], coactivation of Src/EGFR and axin/glycogen synthase kinase (GSK)-3β pathways and induction of invasion by β-arrestin activation of the ET-A receptor [55], and Src/EGFR transactivation, cyclooxygenase-2 expression, and cell migration upon LPA2 stimulation in CAOV-3 cells [56].

2. Disease Models, Knockouts, and Assays for EGFR in Ovarian Cancer

In addition to the studies alluded to above in determining the effects of molecular modulations of EGFR and its biochemical and biological effects, several other approaches for studying EGFR have been used; these are summarized in Table 1. As EGFR is an extracellular signaling protein, the assays most commonly used in examining EGFR in human ovarian cancer cell lines or tissues involve methods that directly or indirectly measure EGFR activity. Assays include methods for detecting increased levels of the *EGFR* gene (e.g., fluorescence in situ hybridization) or protein (e.g., immunohistochemistry, Western blotting) as well as expression of activating *EGFR* mutations (e.g., polymerase chain reaction + sequencing) or measurement of EGFR protein activity (e.g., Western blotting of EGFR phosphorylation sites, in vitro kinase assays).

To determine the effects of EGFR activation or inhibition in tumor formation, human ovarian tumor cells are most frequently implanted heterotopically (subcutaneously) in immunocompromised mice (Table 1). No reports of "true orthotopic" implantation such as in the ovarian bursa of mice have been found in EGFR studies in ovarian cancer, presumably due to the complex and labor-intensive nature of these procedures, while a few reports of "semiorthotopic" implantations via intraperitoneal (IP) injection were identified. While IP tumor implantation offers a model potentially more reflective of advanced ovarian cancer in the patient than subcutaneous injection [57], the difficulty in measuring tumor volume in intact mice has precluded its widespread use in anti-EGFR drug studies.

In addition to implantation of human tissues or cells via xenografts, animal models utilizing other methods of tumor formation have been used to study ovarian cancer. (For comprehensive reviews on animal tumor models, see [58–61].) Most of these animal models utilize mice, and the methods used to induce tumor formation include (1) exposure to radiation (e.g., [62]) or chemicals (carcinogens or hormones) introduced at or near the ovary (e.g., [63]), (2) syngeneic models in which spontaneously transformed murine ovarian epithelial cells are transplanted into immunocompetent mice (e.g., [64]), and (3) knockout or transgenic models in which selected genes are removed

Table 1: *Summary of assays used in detecting EGFR in vitro and in vivo*. Aside from high-throughput methods (such as cDNA arrays, comparative genomic hybridization, and reverse phase protein arrays) and xenograft tumor assays, more broadly encompassing biological methods such as assays for invasion, migration, or gene knockouts have been excluded. cDNA: complementary DNA; PCR: polymerase chain reaction.

EGFR assay method	Assay output	Performed in ovarian cancer?	Platform for ovarian cancer	References for ovarian cancer
cDNA Array	Detection of mRNA levels of various genes	Yes*	Patient tissue, Human cell lines	[172]
Comparative Genomic Hybridization	Detection of copy number changes in chromosomes	Yes*	Patient tissue, Human cell lines	[173]
Chromatin Immunoprecipitation	Detection of stable protein-DNA associations	No		
Coimmunoprecipitation + Western blotting	Detection of stable protein-protein associations	No		
Crystallography	Determination of entire structure or portions of molecule; interacting molecules	No		
Enzyme-linked Immunosorbent Assay	Determination of amount of protein in sample	Yes	Patient tissue	[174]
Fluorescence/ Chromogenic in situ Hybridization	Determination of gene copy number	Yes	Patient tissue	[3, 6, 35, 36]
Flow Cytometry/ Fluorescence-Activated Cell Sorting	Determination of protein levels at cell surface	Yes	Patient tissue, Human cell lines	[175–179]
Immuno-histochemistry/ Immunocyto-chemistry/ Immunofluorescence (includes Tissue Microarrays)	Determination of presence, location, or amount of protein in tissue/cell	Yes	Patient tissue, Patient effusions, Human cell lines	[4, 5, 35–37, 40, 41, 43, 46, 97, 117, 123, 178, 180–195]
In vitro Kinase Assay	Measurement of intrinsic kinase activity	No		
Mass Spectrometry after Protein Enrichment /Purification (e.g., Immunoprecipitation, Chromatographic Separation, Baculovirus Expression)	Detection of protein modification sites (e.g., phosphorylation, glycosylation); changes in protein levels or proteomic profiles, protein-protein complexes	No		
Microscopic Techniques (e.g., Confocal)	Determination of presence, location, or amount of protein in cell	No		
Mulitplex Antibody Arrays (Solid Phase or Bead Based)	Detection of multiple molecules (usually proteins) of interest	Yes*	Patient serum, Human cell lines	[196, 197]
Northern Blotting	Determination of steady-state RNA levels	Yes	Patient tissue, Human cell lines	[43, 186, 193, 198, 199]
PCR + DNA analysis (e.g., Sequencing, Restriction Fragment Length Polymorphisms, Denaturing Gradient Gel Electrophoresis)	Detection of known mutations/ polymorphisms	Yes	Patient tissue, Human cell lines	[6, 35–37, 117, 130, 187, 200]

Table 1: Continued.

EGFR assay method	Assay output	Performed in ovarian cancer?	Platform for ovarian cancer	References for ovarian cancer
Quantitative PCR	Measurement of RNA levels of interest	Yes	Human cell lines	[39, 174, 201]
Radioligand Binding/ Radioimmunoassay	Estimation of number of receptors; determination of ligand or agonist/ antagonist binding kinetics	Yes	Patient tissue, Patient effusions, Human cell lines	[42–45, 199]
Reverse Phase Protein Array	Determination of levels of several proteins and protein modifications of interest	Yes	Patient tissue, Patient effusions	[202, 203]
Reverse Transcription-PCR + Southern Blotting	Determination of mRNA levels	Yes	Human cell lines, Rat cell lines	[198, 204]
Southern Blotting	Detection of gene of interest	Yes	Rat cell lines	[198]
Tryptic Digests + Peptide Resolution (e.g., Reverse Phase High Performance Liquid Chromatography)	Determination of phosphorylation sites of protein	No		
Western Blotting	Determination of protein abundance, protein-associated modifications (e.g., phosphorylation, cleavage, ubiquitination)	Yes	Patient tissue, Human cell lines	[38, 39, 46, 48–50, 56, 147, 175, 177, 178, 181, 186, 196, 200, 201, 204–212]
Xenograft Tumors	Determination of effect of gene/cell perturbation on tumor growth	Yes	Human and mouse cell lines	[47, 49, 147, 178, 213–219]

*EGFR was detected and reported, but samples were not necessarily preselected for alteration of *EGFR* sequence, expression, or activity.

or activated within the mouse. While none of these methods have directly examined the role of EGFR aberrations in ovarian cancer, some of these methods have been applied to other tumor models (e.g., glioma [65], lung adenocarcinoma [66]) in which EGFR perturbations (activating mutations) have been studied, indicating that EGFR-mediated tumor development can be successfully developed in transgenic mice.

In one study where signaling proteins downstream of EGFR induced ovarian cancer, transgenic mice harboring exogenously controllable ("floxed") expression of phosphatase and tensin homolog (*PTEN*) and mutated K-*RAS* genes were induced to gain oncogenic K-*RAS* and lose tumor suppressing *PTEN* expression in the ovaries via injection of an adenovirus-Cre recombinase vector into the infundibulum [67]. All animals developed endometrioid adenocarcinoma of the ovary and, unlike previous ovarian tumor models, were well differentiated, reflecting similar histomorphology to human epithelial ovarian cancers. Thus, this model allows for detailed study of the endometrioid subtype of epithelial ovarian cancer at various stages of tumor development and with some manipulations could be used to study the effects of EGFR aberrations in ovarian tumor development. Mouse models for other subtypes of epithelial ovarian cancers (serous, mucinous, clear cell, transitional) await further development.

3. Targeting EGFR in Ovarian Cancer

While several strategies have been attempted to block EGFR activity, two types of inhibitors are currently used in the clinic: (1) monoclonal antibodies (mAbs), and (2) small molecule tyrosine kinase inhibitors (see [68, 69] for reviews). A summary of these inhibitors and their uses in clinical trials is shown in Table 2. While the various natural functions of antibodies may contribute to their utility as anticancer agents, including their role as modulators or effectors of the immune response, molecular carriers, and pharmacologic agents that directly interfere with activation of the receptor and its downstream pathways (reviewed in [70]), the focus of this paper will be on mAbs as pharmacologic agents. As indicated above by the in vitro studies in human ovarian cancer cells, EGFR and its downstream effectors may be activated directly or indirectly by numerous other signaling molecules. Since determination of which molecules are key to EGFR signaling in ovarian cancers is

not completely understood, the focus will be on inhibition of EGFR and its family members.

3.1. Anti-EGFR Monoclonal Antibodies. Anti-EGFR mAbs that are used in the clinic typically bind to the extracellular domain of EGFR (e.g., [71, 72]). While there are potentially many different mechanisms of inhibition, in many of the known cases, the antibodies prevent ligand binding (in the case of wild-type EGFR), promote antibody-receptor complex internalization [73–75], induce transient decrease of EGFR expression [76], inhibit EGFR heterodimerization [72, 77, 78], and increase ubiquitin-mediated degradation [79]. The downstream effects of inhibition in EGFR-dependent cancer cells include decreased TGF-α secretion, angiogenesis, cell migration, invasion (reviewed in [80]), and induction of apoptosis [81]. Additionally, certain engineered IgG subclass antibodies in which the F_c region is maintained can induce antibody-dependent cell-mediated cytotoxicity or complement activation (see [82, 83] for comprehensive reviews). To reduce the likelihood of patient immune response against the therapeutic antibody, mouse mAbs have been humanized (reviewed in [84]); these are reflected by their antibody names. For example, human-mouse chimeric antibodies of 30% mouse composition are designated as "-ximab" (e.g., cetuximab); humanized antibodies with 10% mouse composition are given the "-zumab" designation (e.g., trastuzumab, matuzumab), while fully humanized antibodies are designated as "-mumab" (e.g., panitumumab).

Cetuximab (Erbitux) was the first anti-EGFR mAb tested in the clinic. Cetuximab inhibits growth of a variety of cultured cancer cells including breast, prostate, lung, colon, kidney, head and neck (reviewed in [85]), pancreas [86], and bladder [87] and can induce regression (either alone or as a combined therapy) of a number of human tumor xenografts such as epidermoid carcinoma [88], renal cell carcinoma [89], pancreatic cancer [86, 90], non-small cell lung cancer (NSCLC) [91], thyroid carcinoma [92], and glioblastoma multiforme [93]. Cetuximab demonstrates activity in patients with colorectal, head and neck, and lung cancers [94, 95].

Reports for cetuximab in ovarian cancers have appeared recently (Table 2), including its use as a single agent in a phase II trial [96] and in two other phase II trials in combination with carboplatin with or without paclitaxel (Taxol) [97, 98]. In all studies, EGFR positivity was determined by immunohistochemistry (IHC) and in two cases was used among the criteria for inclusion [96, 98]. Cetuximab therapy alone showed 4% (1/25 patients) partial response (PR) [96], while the cetuximab + carboplatin trial showed 12% (3/26 patients) complete response (CR) and 23% (6/26 patients) PR [97]. While no response rate was reported in the cetuximab + carboplatin + paclitaxel trial, progression-free survival (PFS) at 18 months was 39%, which did not meet the authors' criteria for meaningful response [98] and did not proceed to the next phase of accrual. There was no evidence of correlation between EGFR levels and patient response in any of the reports. The implications of these and subsequent results will be discussed in the "Next frontiers" section.

Among other anti-EGFR antibodies, a single multi-institution open-label phase II trial was reported in patients with ovarian cancer using matuzumab (EMD 72000) [99]. While screening for this phase II trial included EGFR positivity in the ovarian tumor as determined by IHC, no responses to therapy were observed. To date there are no approved anti-EGFR antibodies for ovarian cancer, and while there was one clinical trial involving panitumumab (Vectibix) in combination with AMG 706 and gemcitabine-cisplatin in patients with advanced cancers (including ovarian), this trial was terminated. Currently, there are no full reports of clinical trials for ovarian cancer with other anti-EGFR antibodies such as zalutumumab (HuMax-EGFr) and nimotuzumab ($BIOMAb^{EGFR}$). Among patented mAbs directed towards EGFR that are not yet in clinical use, one has been proposed for use in ovarian cancer (patent number WO2005010151); however, as it is directed against deletion mutants of EGFR (particularly EGFRvIII), its use in ovarian cancer is likely to be limited.

Due to potential EGFR transactivation by other EGFR family members, mAbs targeting other EGFR family members have also been tested or used clinically against various cancer types such as breast and urothelial malignancies (reviewed in [100]). This includes clinical trials targeting HER2 such as a phase II multi-institutional trial in ovarian cancer in which trastuzumab (Herceptin) was used as a single agent in patients determined HER2 positive by IHC [101]. An overall response rate of 7.3% (1 CR, 2 PR) was reported. However, the relatively low frequency of *HER2* amplification in unselected ovarian cancers (e.g., 10%–23%; [35, 102]) has precluded more extensive studies. Pertuzumab (Omnitarg), a HER2 dimerization inhibitor, was administered with gemcitabine (Gemzar) in platinum-resistant ovarian cancer patients in a phase II safety study [103]; efficacy awaits further reports.

Among antibodies targeted toward other signaling molecules known to activate EGFR are monoclonals for IGF-1R, including 19D12 and EM164. These antibodies have been demonstrated to inhibit proliferation of human ovarian cancer cells [104] as well as tumor growth in mouse xenograft studies [105]. However, whether EGFR aberrations affect response to anti-IGF-1R treatment or whether inhibition can be enhanced by anti-EGFR treatment is unknown.

3.2. Small Molecule EGFR Inhibitors. Small molecule inhibitors, based on modeling by structure-based drug design [106] or by screening (e.g., erlotinib, [107]), appear to act intracellularly by competing with ATP binding in the catalytic region of the kinase domain, thereby abrogating enzymatic activity of the kinase and its subsequent downstream signaling effects (reviewed in [108]). Small molecule inhibitors directed against EGFR generally prevent homo- and heterodimerization between it and other EGFR family members; however, in some cases the inhibitor allows heterodimerization but prevents activation of these dimers [109]. While most mAbs are designed to target full length EGFR, many small molecule inhibitors can target mutant RTKs such as EGFRvIII that lack a critical extracellular regulatory region targeted by some of the antibodies. Small

Table 2: *Summary of clinical trials using EGFR inhibitors in ovarian cancers* References are in parentheses next to the first author of the study. CT: clinical trial; IHC: immunohistochemistry; RPPA: reverse phase protein array; CR: complete response; PR: partial response; SD: stable disease; pt: patient; PFS: progression free survival; GOG: Gynecologic Oncology Group; VEGFR: vascular endothelial growth factor receptor.

(a) Monoclonal Antibodies

Study and Year	CT no.	Phase	# Pts	Therapy	Selection criteria	Outcome	Comments
Secord et al. 2008 [97]	NCT 00086892	II	28	Cetuximab + Carboplatin	Recurrent, platinum-sensitive disease	CR: 3 pts PR: 6 pts SD: 8 pts	Response rate criteria not met for next stage of accrual. 26 pts were EGFR positive by IHC.
Konner et al. 2008 [98]	NCT 00063401	II	40	Cetuximab + Paclitaxel + Carboplatin	Grade III-IV debulked tumor, EGFR positive by IHC	Median PFS: 14.4 months PFS at 18 months: 39%	Combination was adequately tolerated. No increase in PFS when compared to historical data.
Schilder et al. 2009 [96]		II	25	Cetuximab	Persistent or recurrent ovarian or primary peritoneal disease, EGFR positive tumors by IHC	PR: 1 pt SD: 9 pts	12 serologic markers examined before and during treatment. No correlation between PFS and marker changes, but high baseline of markers associated with earlier disease progression.
Seiden et al. 2007 [99]	NCT 00073541	II	37	Matuzumab	Recurrent platinum-refractory disease, EGFR positivity by IHC	No objective response SD: 16%–22%	Primary objective was pharmacodynamic; signal transduction evaluation. 75 pts were screened for EGFR status.
Bookman et al. 2003 [101]	GOG-160	II	41	Trastuzumab	Persistent and/or refractory disease with 2-3+ HER2 by IHC	CR: 1 pt PR: 2 pts	Serum HER2 levels not associated with clinical outcome.

(b) Small Molecule Inhibitors

Study and Year	CT no.	Phase	# Pts	Therapy	Selection criteria	Outcome	Comments
Posadas et al. 2007 [203]	NCT 00049556	II	24	Gefitinib	Platinum-refractory disease	No objective response SD: 37% for >2 months	Protein correlates done with RPPA. No significant correlation between EGFR phosphorylation and tumor response
Schilder et al. 2005 [112]	NCT 00023699	II	27	Gefitinib	Persistent or recurrent disease	PR: 1 pt	Analyses suggest trend towards responsiveness in EGFR positive (by IHC) pts. Activating mutations documented in the PR pt.
Wagner et al. 2007 [115]	NCT 00189358	II	56	Gefitinib + Tamoxifen	Disease refractory or resistant to platinum-taxane-based therapy	No objective response SD: 16 pts	EGFR positivity not a prerequisite; EGFR status not determined
Gordon et al. 2005 [116]		II	34	Erlotinib	Relapsed or progressive disease, EGFR positivity by IHC	PR: 2 pts SD: 15 pts	Primary goal was to estimate the objective tumor response rate to erlotinib as a single agent.
Vasey et al. 2008 [118]		Ib	45	Erlotinib + Docetaxel + Carboplatin	Chemonaïve pts	CR: 5 pts PR: 7 pts (23 evaluable)	Phase Ib dose finding study. Addition of erlotinib to other agents did not increase response rate.

(b) Continued.

Study and Year	CT no.	Phase	# Pts	Therapy	Selection criteria	Outcome	Comments
Nimeiri et al. 2008 [117]	NCT 00126542	II	13	Erlotinib + Bevacizumab	Recurrent or refractory disease, ≤2 prior cytotoxic chemotherapies; no previous anti-EGFR or VEGFR therapies	CR: 1 pt PR: 1 pt	No indication of improvement over bevacizumab treatment only. No *EGFR* mutations detected; one EGFR 2+ IHC staining detected.
Kimball et al. 2008 [122]	NCT 00317434	I	11	Lapatinib + Carboplatin	Recurrent, platinum-sensitive disease	PR: 3 pts SD: 3 pts	No screening or measurement of EGFR or HER2 performed.
Campos et al. 2005 [123]		II	105	CI-1033	Relapsed or refractory disease	No objective response SD: 26–34%	Baseline HER1-2 levels determined by IHC. No association between HER levels and SD.

molecule inhibitors can bind reversibly (e.g., gefitinib or erlotinib) or irreversibly (e.g., CI-1033) to EGFR. The clinical significance of these different mechanisms of inhibition is not yet known.

Gefitinib (Iressa or ZD1839), which inhibits a variety of cancer cell lines and xenograft tumors (reviewed in [110]), including ovarian [111], was tested as a single agent in two trials [112, 113]. In both trials, EGFR aberrations were not included as selection criteria but were assayed via IHC for EGFR protein expression [112] or via reverse phase protein array (RPPA) for total and phospho-EGFR levels [113] as well as for *EGFR* mutations in exons 18–21 via polymerase chain reaction (PCR) amplification and nucleotide sequencing [112]. In both studies, there was no CR; 0%–4% had PR, and 4%–37% had stable disease (SD) [112, 113]. While decreased EGFR phosphorylation and expression, as determined by RPPA, was observed in >50% of gefitinib-treated patients, this was not associated with clinical benefit or response [113]. However, EGFR positivity via IHC was associated with longer PFS [112]. Additionally, a mutation in exon 19 was detected in the one partially responding patient [112], a location that was shown to be responsive to gefitinib treatment in NSCLC patients [114].

Gefitinib was also used in combination with tamoxifen in a phase II study in Germany involving patients refractory or resistant to platinum-taxane-based treatment but not prescreened for estrogen receptor or EGFR expression [115]. While this combination therapy was well tolerated, it was reported to be ineffective against platinum refractory/resistant ovarian cancer as there were no tumor responses.

Another small molecule inhibitor, erlotinib (Tarceva), demonstrated limited activity for ovarian cancer patients in a multicenter phase II trial, with only 2 chemorefractory patients in 34 demonstrating a partial response to treatment [116]. While EGFR expression was determined by IHC, low expression was not used as a criterion for exclusion. Erlotinib has also been tested in combination with other chemotherapeutic agents, including the antivascular endothelial growth factor (VEGF) antibody bevacizumab (Avastin) in a phase II trial [117], and docetaxel (Taxotere) with carboplatin in a phase Ib trial [118]. EGFR aberration or positivity was not an inclusion criterion in either study, and EGFR status was reported in only one study [117], which examined EGFR positivity via IHC and activating mutations in exons 19 and 21 via PCR amplification and sequencing. The objective response rates were 15% (2/13 patients) for the erlotinib + bevacizumab therapy [117] and 52% (12/23 patients) for erlotinib + docetaxel + carboplatin [118]. No *EGFR* mutations were detected, and one patient demonstrated EGFR positivity, but this patient was unresponsive to erlotinib + bevacizumab therapy [117]. Due to lack of improvement over bevacizumab therapy alone and two incidents of fatal gastric perforations, the erlotinib + bevacizumab study was discontinued [117]. Whether these are due to the combinatorial effects of the drugs or due to bevacizumab alone, which has been reported to induce gastric perforation [119], remains undetermined. The response rate of the erlotinib + docetaxel + carboplatin therapy was slightly lower than that of a docetaxel + carboplatin therapy previously conducted by the same group (52% versus 59%, [118, 120]), but due to good patient tolerance of the 3-drug combination, it was recommended for further studies, particularly as maintenance therapy.

Lapatinib (Tykerb, Tyverb), a dual EGFR-HER2 inhibitor [121], was tested in a multicenter phase I trial in combination with carboplatin in patients with platinum-sensitive recurrent ovarian cancer [122]. Patients were not prescreened or measured for EGFR in this study. Three of 11 patients (27%) had PR, and 3 patients (27%) had SD [122]. This treatment regimen was not recommended, as it had a low response rate and significant treatment toxicities, including grade 3–4 neutropenia and grade 4 thrombocytopenia. In addition, 2 other patients had treatment delays due to development of nondose limiting grade 3 neutropenia using the initial combination therapy regimen [122].

The irreversible pan-EGFR family inhibitor CI-1033 (Canertinib) was administered in a multicenter open-label phase II trial for ovarian cancer patients who had failed prior platinum-based therapy [123]. While baseline EGFR family levels were determined via IHC from archival patient tumor specimens, it was not used as a selection criterion.

No objective response was observed, although SD was confirmed in 26%–34% of the patients (depending on the dosage). There was no association between EGFR family levels by IHC and stable disease.

Due to the relatively unremarkable results of anti-EGFR small molecules in earlier clinical trials, more recent trials have focused on small molecules that bind irreversibly or have a broader target range. For instance, BIBW2992 (Tovok) binds irreversibly to EGFR and HER2 and can inhibit both wild type EGFR and activated mutants of EGFR and HER2 [124]. BIBW2992 was shown to inhibit growth of human NSCLC cells implanted in nude mice more effectively than erlotinib [124]. Several phase I and II trials are underway with BIBW2992 as a single agent or in combination with various agents such as paclitaxel, cisplatin, or temozolomide (Temodar, Temodal) in patient groups consisting of various solid tumors including glioma, NSCLC, prostate, breast, and colorectal cancer (http://www.clinicaltrials.gov/). A few trials will screen patients for EGFR or HER2 status, whether by detection of gene amplification or by activating *EGFR* mutations. An example of a small molecule with an even broader target range is AEE788, which inhibits EGFR, HER2, and vascular endothelial growth factor receptor (VEGFR) [125]. While the current focus of AEE788 is on glioblastoma, there is also a study that assesses the safety and clinical activity of AEE788 in various solid tumors. There is currently no complete report indicating which tumor types were included, patient response, and follow up. Other small molecule EGFR family inhibitors undergoing clinical trials against solid tumors of various types (specific types not yet reported) include HKI-272 and EKB-569.

In lung cancers, sensitivity to EGFR inhibition by small molecules such as gefitinib and erlotinib is associated with *EGFR* mutation [126–129]. Therefore, Lacroix et al. analyzed *EGFR* sequences from exons 18–24 in 18 advanced epithelial ovarian carcinoma specimens from patients that displayed objective response or disease stabilization to carboplatin-paclitaxel-gefitinib treatment, along with NSCLC [130]. While 2 of 20 NSCLC samples displayed an activating deletion in exon 19 (consistent with previous reports), no *EGFR* mutations were detected in the ovarian carcinomas. However, the potential role of mutations, insertions, or deletions elsewhere in *EGFR* or other EGFR family members was not explored.

4. Next Frontiers in Anti-EGFR Drug Discovery

4.1. Improving Response to EGFR Inhibitors in Ovarian Cancer. As detailed by the list of clinical trials, the use of EGFR inhibitors as single agents or in early combination studies in ovarian cancer has met with limited success. The regimens have included EGFR-selective or less selective inhibitors and administration as single agents or in combination with other non-EGFR antineoplastic agents. One not yet widely explored possibility is whether using a combination of an externally targeting EGFR drug (i.e., mAb) with an internally targeting drug (i.e., small molecule kinase inhibitor) would produce better results. So far, there is one complete report of a phase I study that has determined optimal doses of combined cetuximab and gefitinib therapy in patients with advanced or metastatic NSCLC previously treated with platinum therapy [131]. These patients had no detectable *EGFR* amplifications or K-*RAS* mutations. The regimen, with the exception of the development of hypomagnesemia, was well tolerated. There was no objective response; however, 4 of 13 had SD. Based on these results, the group has recommended an optimum tolerated dose to use in a phase II trial.

While later studies selected patients based on EGFR positivity or overexpression via IHC, many of these trials still demonstrated low efficacy, suggesting that other methods of EGFR detection might be better suited for pre-drug screening. Quantitative approaches to assess protein level, RNA levels, gene amplification, and mutations might prove less subjective and more robust than IHC and could be included as one of the predictors of patient response. In lung cancer, gene copy number assessed by fluorescence in situ hybridization (FISH) has been reported to indicate sensitivity to EGFR inhibition (reviewed in [132]). Whether *EGFR* amplification as determined by FISH is a reliable indicator of EGFR inhibitor sensitivity for other types of cancers has not yet been conclusively assessed. Additionally, it is possible that gene increase is associated with mutational activation of EGFR, serving as a surrogate marker for mutation, and would suggest that screening by FISH might be limited to cancers in which *EGFR* is frequently mutated. At any rate, clinical trials in which better-defined measurements of EGFR status are taken into consideration have been emerging, such as screening of *EGFR* mutations in NSCLC patients prior to administration of erlotinib.

An understanding of the mechanisms leading to resistance of EGFR inhibitors could help enrich for patients likely to respond to therapy and more importantly identify rational combinatorial therapy. Resistance of tumors to anti-EGFR therapies has been discussed in a number of reviews (e.g., [133]). Furthermore, various mechanisms of chemoresistance in tumor treatment have been described (e.g., see [134, 135]). Resistance can be apparent from the onset of treatment ("intrinsic") or develop over time ("acquired"). While resistance at the physiologic level has been attributed to mechanisms such as suboptimal immune system activity or rapid metabolism or poor absorption of the drug, resistance at the molecular level has been attributed to expression or activation of molecules or signaling pathways that can directly or indirectly override the effects of the drug (reviewed in [136]). This activation may occur via intracellular or intercellular mechanisms, and the activating intercellular source could either be another tumor cell or be the surrounding stroma (reviewed in [137]).

Anti-EGFR therapy resistance mechanisms include production of EGFR-activating ligands, receptor mutations, constitutive activation of downstream pathways, and activation of alternative signaling pathways (reviewed in [138, 139]). Another mechanism recently suggested is increased resistance to autophagic cell death upon increased EGFR expression via stabilization of the facilitated glucose transporter sodium/glucose cotransporter 1 (SGLT1) [140]. SGLT1 can transport glucose "upstream" of a glucose

gradient, enabling cells to accumulate higher glucose concentrations than their environment, as in the case of cancer cells, and providing more "food" for the cell [141]. Increased SGLT1 stability is dependent on EGFR expression and not its activity [140]. Thus, agents that target EGFR activity but not its expression are likely ineffective.

Another potential mechanism of EGFR inhibitor resistance is inflammation, such as by release of the inflammatory cytokine prostaglandin E2, which in lung cancer cells induced phosphorylation of MAPK, indicating a bypass of EGFR activation (reviewed in [142]). One other consideration regarding chemoresistance is the sequence or timing of multidrug administration. Proliferation of an esophageal squamous epithelial cancer cell line possessing autocrine EGFR activity was either inhibited or enhanced depending on whether a cytotoxic drug (platinum derivative or taxane) was administered before or after an EGFR inhibitor [143]. While many of these mechanisms have been studied in other cancer types, the data for ovarian cancer is currently sparse.

Experimental results have also indicated the need to better understand the interaction of EGFR with other family members, signaling events, and the tumor environment in ovarian as well as in other cancers. As noted earlier, relative differences in levels of EGFR family members induced different dimerization partners upon stimulation by a given ligand in ovarian cancer cell lines [48]. Further, there is evidence that HER3, a family member also present in ovarian cancers and associated with increased tumor aggressiveness [144] and poor prognosis [145], plays a critical role in EGFR- and HER2-driven tumors (reviewed in [146]). Therefore, only targeting EGFR will likely be insufficient due to functional overlap by other EGFR family members. Also, in mouse studies using SU11925, a small molecule that targeted both EGFR and HER2, a higher concentration of SU11925 was required to inhibit HER2 phosphorylation in xenograft tumors than in cultured human or murine cells when relative HER2 levels in the cell were higher than EGFR [147]. These results point to a potential shortcoming of small molecule inhibitors in vivo.

As evident here and in numerous other reports on EGFR inhibitors in various cancer cell types, other signaling molecules affected by or effecting EGFR family members will have to be concomitantly examined in solid tumors. First, signaling of the EGFR family occurs primarily in *trans* with HER2 being the preferred binding partner [14]. Also, in human breast cancer cells, there is evidence that cells can escape gefitinib treatment due to increased HER3 expression induced by AKT-mediated negative feedback signaling [148]. Additionally, examining signaling proteins further downstream indicates that constitutive activation of these pathways must also be taken into consideration. For example, EGFR-overexpressing human cell lines treated with gefitinib were resistant when PTEN, the negative regulator of the PI3K/AKT pathway, was not functional [149, 150]. In NSCLC, 0 of 8 patients with both *EGFR* amplification and K-*RAS* mutation responded to erlotinib treatment compared to 4 of 5 responders with *EGFR* amplification alone [151]. Further, tumors with *RAS* mutations in several cell lineages such as NSCLC, colon, and bronchioalveolar carcinoma are resistant to anti-EGFR receptor agents and may have a worsened outcome with therapy [151–153]. This is leading to widespread testing of *RAS* mutations in patients (such as the recent study in ovarian cancer [154]) and, indeed, is approved by the European Medicines Agency as an exclusion criterion for anti-EGFR therapy in colorectal cancer in Europe. Optimal efficacy of anti-EGFR therapy is likely to require concurrent targeting of the PI3K/AKT or RAS/MAPK pathways in patients with mutational activation of these downstream components. To this end, trials that target both EGFR and the PI3K/AKT pathway have been performed or are underway, including cancers for glial cells and head and neck. While new agents that target the PI3K/AKT pathway, including XL765 or XL147, are being tested against various solid tumors in combination with erlotinib, no known combination trials exist in ovarian cancer. Also, while trials utilizing the farnesyl transferase inhibitor lonafarnib (Sarasar), which targets RAS [155], are underway, none are currently examining the combination of EGFR and RAS inhibition in any tumor type.

In addition to signaling across EGFR family members and proteins downstream, consideration of other transmembrane signaling molecules must be taken into account. Considerable data in various cell types including hepatoma [156], prostate [21], and breast [157] has shown that EGFR inhibition can be overridden by IGFR stimulation. Moreover, there is in vitro evidence in human NSCLC and head and neck squamous cell cancer cells to support therapies combining EGFR and GPCR inhibitors, such as antagonists for bradykinin (CU201) or gastrin (PD176252) (e.g., [158, 159]). Recently, amplification of the RTK gene *MET* has been shown to bypass EGFR receptor inhibition in human lung cancer cells and was present in 4 of 18 lung cancer specimens that developed resistance to gefitinib or erlotinib, supporting the idea that MET should also be targeted in EGFR-dependent cancers [160]. On the other hand, treatment of solid tumors with the dual EGFR-VEGFR inhibitor vandatanib (ZD6474 or Zactima) was ineffective [161]. Based on these reports and the emergence of numerous potential EGFR-mediated signaling proteins of interest in ovarian cancers, determination of which proteins play crucial roles in ovarian tumors might prove to be a challenging process. High-throughput methods such as gene expression arrays and RPPA should help in determining which genes and proteins are modulated upon single and combination treatment of ovarian cancer cell lines and tissues. For example, Skvortsov et al. have used 2-dimensional gel electrophoresis and mass spectrometry to identify proteins associated with sensitivity or resistance to C225 in two colon cancer cell lines [162]. Additionally, development of robust algorithms to predict effective drug combinations (e.g., [163]) should aid in streamlining high-throughput studies and increase the likelihood of finding successful combinations.

Despite these challenges, reports utilizing adherent human epithelial cancer cell lines and tumor types suggest that mechanisms of resistance and methods to overcome resistance could be determined and incorporated into

ovarian cancer therapies. For instance, MAPK phosphorylation was not inhibited in an EGFR-positive, gefitinib-resistant human bladder cancer cell line upon gefitinib treatment, while MAPK phosphorylation decreased in an EGFR-positive, gefitinib-sensitive cell line [164]. Moreover, in the gefitinib-sensitive cell line, increased GSK-3β activity and decreased cyclin D1 levels were observed upon gefitinib treatment and correlated with responsiveness. Additionally, platelet-derived growth factor receptor-β (PDGFR-β) was observed to short circuit the EGFR/MAPK pathway in the gefitinib-resistant cells [164]. These results suggest that, in bladder cancer, MAPK kinase phosphorylation could be a marker for resistance while GSK-3β activation or cyclin D1 levels could be a marker for sensitivity of EGFR drug treatment, and that inhibition of both EGFR and PDGFR-β would be more effective in treatment of EGFR-positive bladder cancers than EGFR alone.

4.2. Improving Understanding of EGFR Processes in Ovarian Cancer. With the emergence of high-throughput technologies and their accompanying development and refinement of data analyses, reports contributing to further understanding of ovarian cancers have emerged. Among the first reports utilizing gene arrays was that of Wang et al., who identified genetic differences between human ovarian tumor specimens (comprising 5 different histopathologic types) and normal ovarian tissue [165]. Later studies expanded the number and refined the analyses of histopathologic types of samples (serous papillary, clear cell, endometrioid, undifferentiated, and adenocarcinomas) included in the analyses (e.g., [166]), as well as compared drug (primarily platinum) sensitive and resistant samples [167]. While the number of samples analyzed in depth is increasing, this number is still relatively small; whether the profile of EGFR-positive ovarian cancers is different from that of other prominent molecular markers is unknown. Moreover, the most comprehensive profiles characterized thus far have focused on gene alterations, via comparative genomic hybridization or gene microarrays (reviewed in [168, 169]), which provide an incomplete profile of ovarian cancer cells, particularly in the case of protein signaling-dependent alterations such as EGFR activation. Thus, more information derived from proteomic studies is needed.

Based on the current outcomes of EGFR targeted therapy in ovarian cancers, it is evident that patients should be screened for EGFR status including amplification and mutation; additionally, screening for other EGFR family members and key downstream effector proteins such as RAS and PTEN would be preferable. Also, while *EGFR* in ovarian cancers has been screened for potential activating events via presence of *EGFRvIII* [38, 39] or activating mutations in the kinase domain [6, 35–37], it is possible that ovarian cancers might have a yet unidentified *EGFR* activating "hot spot." Screening and analysis of full-length *EGFR* will be required to determine if this is the case.

Determination of other molecular markers for likely responders or nonresponders toward anti-EGFR therapies should also be performed; identification of such markers could be facilitated by high-throughput methods that can be correlated with patient response. High-throughput methods could also be used to aid in developing predictive models of drug combination in patients, such as by testing well-defined chemotherapeutic drugs in a large number of cancer cell lines and performing cell "population studies," to better correlate drug response with precisely defined oncogene status (e.g., specific mutations, gene amplification), such as with EGFR [170]. Further studies of other proteins affecting or affected by EGFR activity, some of which have been discussed above, should also be performed to clarify their roles in ovarian cancer, both independently and in context with EGFR activation. Further, the role of EGFR in different ovarian cancer histotypes should be examined. Additionally, preclinical combination therapy reports such as by Morelli et al. [143] suggest that more studies should be performed on determining proper scheduling of multiple therapies as well as examination of previously untested drug combinations. Also of great benefit is designing more streamlined and rational methods for performing drug combination studies, such as by development of search algorithms to determine optimal doses of combined drugs [171].

5. Conclusion

EGFR and its family members play a variety of roles in oncogenesis and tumor progression in different cancer and cell types. To date, clinical studies using EGFR antagonists in ovarian cancer have shown limited efficacy. As we learn more about the complexities of specific signaling changes associated with EGFR mutation and overexpression, future studies using EGFR antagonists in ovarian cancer should focus on determining reliable predictors for patient responsiveness to anti-EGFR therapy such as by obtaining good biomarker profiles and utilizing assays most appropriate to determine EGFR status as well as developing rational combination therapies with EGFR inhibitors. These determinations should be facilitated by the use of high-throughput methods, as well as development of robust algorithms to help design experiments and analyze results. Continuing these studies in ovarian and other types of cancers will increase our likelihood of achieving success in targeting EGFR-dependent tumors.

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Research Article

Downregulation of Epidermal Growth Factor Receptor Expression Contributes to α-TEA's Proapoptotic Effects in Human Ovarian Cancer Cell Lines

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RRR-α-tocopherol derivative α-TEA (RRR-α-tocopherol ether-linked acetic acid analog) has been shown to be a potent antitumor agent both in vivo and in vitro. In this study, we investigated the effects of α-TEA on the expression of epidermal growth factor receptor (EGFR) family members, ErbB1, 2 and 3, and the role of ErbB 2 and 3 in α-TEA-induced apoptosis and suppression of Akt, FLIP and survivin in the cisplatin-sensitive (A2780S) and -resistant (A2780/CP70R) human ovarian cancer cell lines. Data show that α-TEA's ability to induced apoptosis was associated with reduced expression of ErbB1 (cisplatin-resistant cells), 2 and 3 (both cell types) and reduced levels of the phosphorylated (active) form of Akt; as well as, reduced levels of FLIP and survivin proteins in both cell types. Ectopic overexpression and siRNA knockdown studies showed that ErbB2, ErbB3, Akt, FLIP and survivin are involved in α-TEA-induce apoptosis and that α-TEA downregulates FLIP and survivin via suppression of pAkt, which is mediated by ErbB2 and ErB3. Thus, α-TEA is a potent pro-apoptotic agent for both cisplatin-sensitive and -resistant ovarian cancer cell lines in cell culture and it produces cell death, at least in part, by downregulation of members of the EGFR family.

1. Introduction

Ovarian cancer ranks eighth among all cancers in women in terms of estimated new cases and fifth in estimated deaths [1]. A majority of patients with ovarian cancer require treatment with cytotoxic chemotherapy. Platinum agents, cisplatin or carboplatin, are the most effective first-line treatments; however, despite initial promising responses, a high percentage of cases develop chemoresistance which significantly hinders successful treatment outcomes [2, 3]. Thus, there is a great need to develop agents for treatment of drug resistant ovarian tumors.

The epidermal growth factor receptor (EGFR) family (ErbB family) of type I receptor tyrosine kinases (RTKs) has four members: EGFR/ErbB1, ErbB2, ErbB3, and ErbB4 (also referred to as HER1, HER2, HER3 and HER4). All ErbB family members share common features including an extracellular ligand-binding domain (except ErbB2), a transmembrane domain, and an intracellular protein tyrosine kinase domain (except ErbB3). The receptors have notable differences in their sequences, which account for differential ligand-binding and diverse affinities for down-stream signaling molecules [4, 5]. Data suggest that the homo- and heterodimerization between members of the ErbB family as well as the ability of their ligands to bind and activate more than one receptor help produce the complex signaling pathways of these membrane-bound proteins [6]. ErbB receptors not only play key roles in normal developmental processes but also are implicated in malignant transformation [6]. Activated ErbB receptors stimulate many intracellular signaling pathways, especially the phosphatidylinositol 3-kinase (PI3K)-AKT pathway [7].

Akt/protein kinase B is a family of serine-threonine protein kinases that promote cell survival and proliferation [8, 9]. Abnormal activation of Akt signaling has been reported in several human cancers [8, 10, 11] including approximately 30–40% of ovarian cancers [9, 12]. Akt promotes cell survival by mediating inactivating phosphorylation of proapoptotic proteins like Bad and caspase-9 and mediating activating phosphorylation of NF-kappaB, which controls expression of prosurvival proteins such as survivin, Bcl-2, and the caspase-8 inhibitor FLIP [13, 14]. Additionally, active Akt has been shown to confer resistance to chemotherapy in human cancers and is considered a therapeutic target [8, 11, 12].

Impairment of apoptotic processes can also be mediated by factors such as FLIP and survivin. FLIP and survivin are cytoplasmic proteins that function as inhibitors of caspase-8 and caspase-9/3, respectively, [15, 16]. FLIP has a similar structure to caspase-8 without the catalytic domain and thus competitively inhibits caspase-8 binding to the tumor necrosis factor family of cell surface death receptors thus blocking their apoptotic signaling [15]. Survivin is a structurally unique member in the IAP (inhibitor of apoptosis protein) family [16] and suppresses the processing and catalytic activity of execution caspases, such as casapse-9 and 3 [17].

Our lab has developed a vitamin E analog, 2,5,7,8-tetramethyl-2R-(4R, 8R, 12-trimethyl-tridecyl chroman-6-yloxy) acetic acid, referred to as alpha-tocopherol ether acetic acid analog (α-TEA), which differs in structure and function from natural vitamin E (RRR-α-tocopherol). α-TEA has an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head of RRR-α-tocopherol by an ether linkage yielding a stable, nonhydrolyzable entity [18, 19]. Studies have demonstrated that α-TEA can reduce tumor burden and inhibit lung metastases when delivered by aerosol or in the diet in preclinical syngeneic transplantable mouse mammary cancer studies [18–21]; as well as in xenograft models using immune compromised mice transplanted with human ovarian, breast or prostate cancer cells [22–24]. Immunohistochemical analyses of tumor tissue from α-TEA treated animals indicated that α-TEA reduction of tumor burden was associated with increased apoptosis and reduced cell proliferation in tumor tissue [18–20, 22–24]. Cell culture studies have shown that α-TEA induces human ovarian, prostate and breast cancer cells to undergo DNA synthesis arrest and apoptosis, and that α-TEA-induced apoptosis involves activation of Fas/Fas Ligand and c-Jun NH2-terminal kinase (JNK) proapoptotic pathways; as well as, suppression of Akt, FLIP, and survivin antiapoptotic/prosurvival factors [24–27]. In this study, we investigated the effect of α-TEA on the expression of EGFR family proteins and studied the roles of ErbB2 and ErbB3 in α-TEA-induced apoptosis and suppression of Akt, FLIP and survivin antiapoptotic/prosurvival factors.

2. Materials and Methods

2.1. Chemicals. α-TEA (F.W. = 488.8) was prepared in house as described previously [18]. LY294002, a specific inhibitor of the p110 catalytic subunit of PI3K [28], and Wortmannin [29], a cell-permeable, irreversible inhibitor of PI3K, were purchased from Calbiochem (San Diego, CA).

2.2. Cell Lines and Treatments. The human ovarian A2780 cisplatin-sensitive parental cancer cell line (designated A2780S), provided by Dr. J. Rebecca Liu (University of Michigan Medical School, Ann Arbor, MI), was originally established from an untreated ovarian cancer patient [30]. The cisplatin-resistant A2780/CP70 variant (designated A2780/CP70R), provided by Dr. Michael J. Birrer (Department of Cell and Cancer Biology, National Cancer Institute, Rockville, MD), was created through intermittent exposure of A2780 cells to increasing concentrations of cisplatin (up to 70 μM) in vitro [31]. A2780S and A2780/CP70R cells were grown as monolayers on plastic (Corning Plastic Ware, Corning, NY) and maintained at 37°C in RPMI 1640 (Invitrogen-Life Technologies, Inc., Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (Sigma Chemical Co., St. Louis, MO). Cultures were maintained and routinely examined to verify the absence of *Mycoplasma* contamination as described previously [32].

For experiments, the percentage of FBS was reduced to 2%. Exponentially growing A2780S and A2780/CP70R cells were plated at a density of 3×10^6 in T75 flasks (10 mL) for Western analyses, or at a density of 1.5×10^5/well in 12-well plates (1 mL) for apoptosis analyses. Cells were allowed to attach overnight before treatment initiation. Treatments were conducted at various concentrations of α-TEA in a final concentration of 0.1–0.25% ethanol. Equal volume of ethanol was used as vehicle control.

2.3. Cell Proliferation Assay. To study the effects of α-TEA on proliferation, A2780S or A2780/CP70R cells were trypsinized, and seeded in triplicate into 96-well plates at a density of 5000 cells/well which yields a 20–30% cell density. α-TEA at 1.25, 2.5 or 5 μM, or 2.5, 5, or 10 μM were then added to A2780S or A2780/CP70R, respectively, and cells were grown in media containing 2% FBS for 1 to 3 days. Viable cell numbers were determined using Promega's CellTiter 96 Aqueous One Solution Cell Proliferation MTS assay (Promega, Madison, WI) according to the manufacturer's instructions.

2.4. Apoptosis Assay. Assessment of apoptosis was performed based on nuclear morphology of DAPI-stained cells as described previously [33]. Cells in which the nucleus contained clearly condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic. Apoptotic data are reported as percentage of apoptotic cells in a given cell population sample. For each sample, a minimum of 3 counts involving a minimum of 100–200 cells/count were scored. Apoptotic data are presented as the mean ± SD for three independently performed experiments. Reagents for morphological analyses of apoptosis were purchased from Boehringer Mannheim Corp. (Indianapolis, IN).

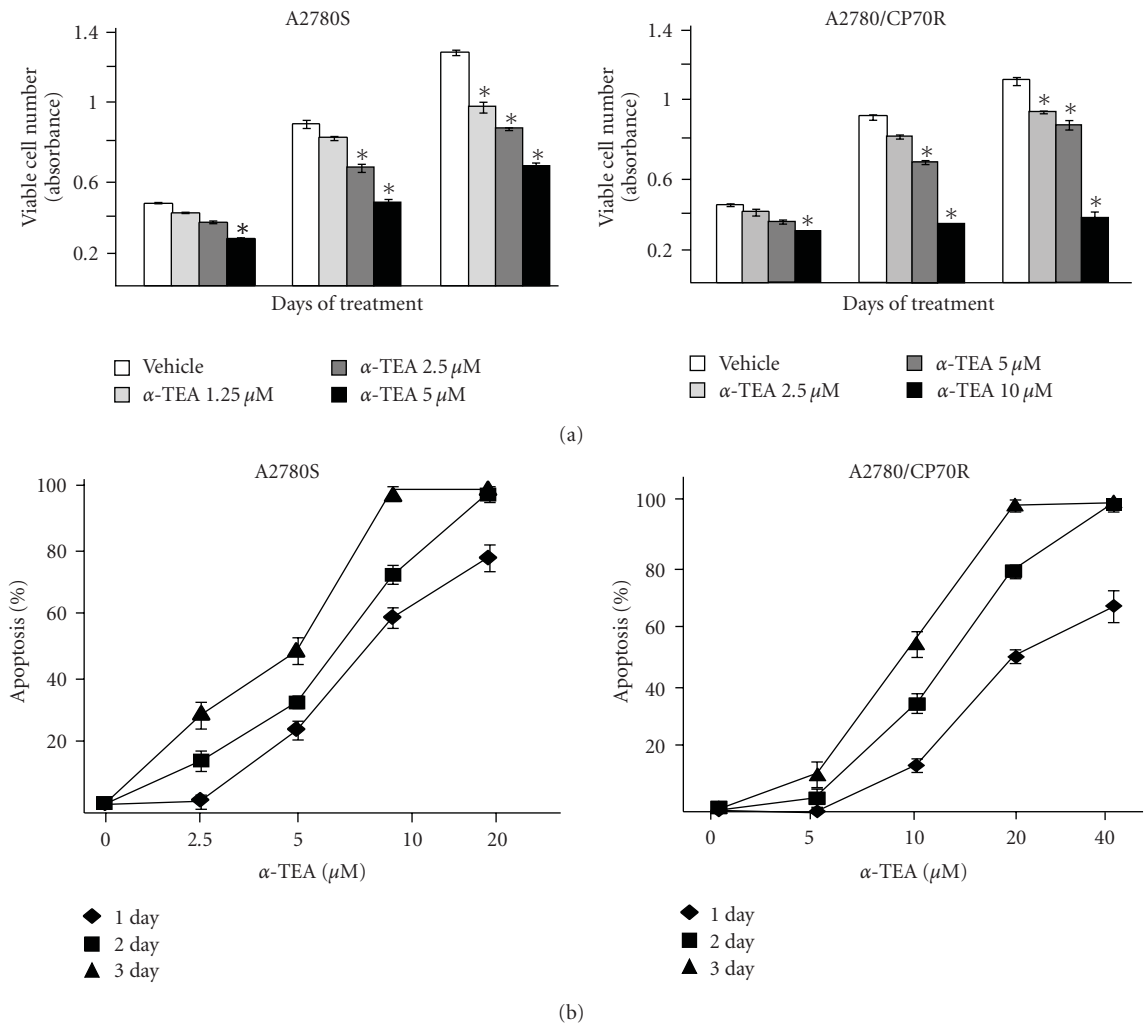

Figure 1: Effects of α-TEA on cell growth and apoptosis in A2780S and A2780/CP70R cells. (a) A2780S and A2780/CP70R cells were plated, cultured and treated as described in Section 2. Assessment of viable cell numbers (absorbance) was determined by the MTS assay following treatment with different doses of α-TEA for 1, 2 and 3 days. (b) Following treatment of cells with various concentrations of α-TEA or vehicle control for 1, 2, or 3 days, floating and adherent cells were harvested, washed, and stained with DAPI. Apoptosis was determined by counting the number of cells exhibiting condensed and fragmented nuclear morphology, and reported as percent apoptosis. Data in (a) and (b) are depicted as the mean ± SD for three independent experiments ($^* = P < .05$ in comparison to vehicle control).

2.5. Western Immunoblot Analyses. Antibodies used to detect pro- and cleaved caspase 3 (sc-7148), pro- and cleaved caspase 9 (sc-8355), HA-probe (sc-805), survivin (sc-17799), ErbB-2 (sc-284), ErbB-3 (sc-285), ErbB-4 (sc-283), and PARP (Poly(ADP-Ribose) Polymerase) (sc-7150), were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Antibodies used to detect pro- and cleaved caspase 8 (#9746), phospho-AKT (Ser 473) (#9271), AKT (#9272), phospho-GSK-3α/β (Ser 21/9) (#9331), GSK-3β (#9332), and ErbB1 (#2232) were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal antibody to human FLIP was purchased from Alexis Biochemical (ALX-804-428-C050); and GAPDH was made in-house. GAPDH was used for monitoring lane loads as described previously [33]. Whole cell lysates were prepared and 50 μg protein was loaded per lane. Proteins were separated by using 10–15% SDS-PAGE under reducing conditions and were electroblotted onto a nitrocellulose membrane. Immunoblotting was performed using primary rabbit or mouse antibodies and peroxidase-conjugated goat antirabbit or antimouse, respectively, as the secondary antibodies (Jackson Immunoresearch Laboratory, West Grove, PA) at a 1 : 2000 dilution, followed by detection with ECL (Pierce, Rockford, IL). Quantification of band intensity was performed using Scion Image Software (Scion Corporation, Frederick, MD).

2.6. RNA Interference. ErbB2 and ErbB3 siRNA duplexes were synthesized by Qiagen (Valencia, CA, USA) [34]. The targeted sequences (sense strand were as follows: ErbB3: AAGAGCGACTAGACATCAAGC; ErbB2: AAGTACACG-ATGCGGAGACTG. Nonsilencing control siRNA (sc-37007) was from Santa Cruz Biotechnology, (Santa Cruz, CA): Cells

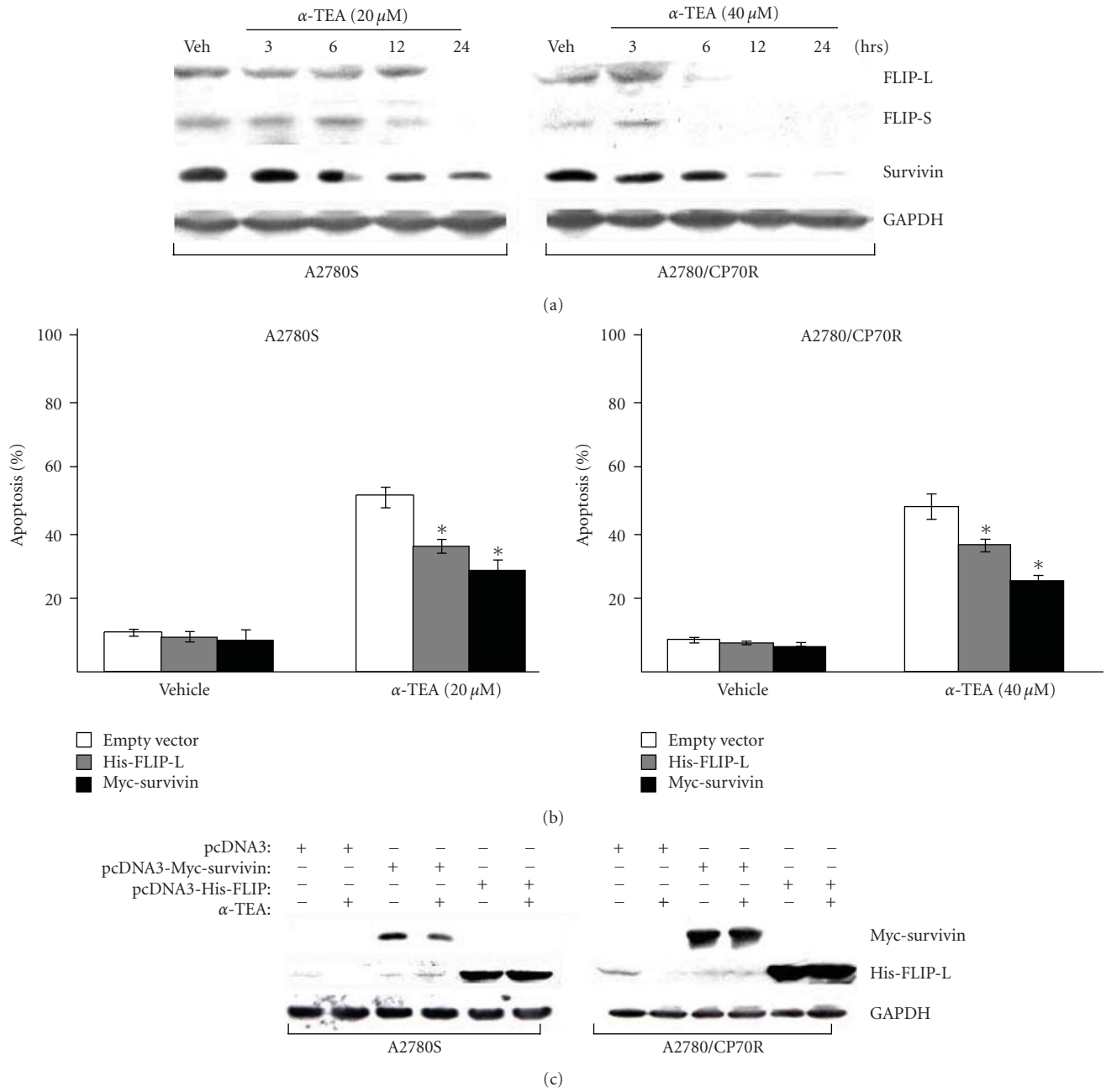

Figure 2: Two antiapoptotic molecules, FLIP and survivin, are downregulated by α-TEA in both cell types, and overexpression of either FLIP or survivin blocks α-TEA-induced apoptosis. (a) Cells were treated with α-TEA for various time periods and whole cell lysates were analyzed by western immunoblotting. ((b) and (c)) Cells were transiently transfected with empty vector control (pcDNA3), survivin (myc-survivin)- or FLIP (his-FLIP-L)-expression plasmids. After 24 hours of transfection, cells were treated with or without α-TEA (20 or 40 μM for A2780S and A2780/CP70R, resp.) for 1 day prior to DAPI analysis for apoptosis (b), and for 12 hours prior to immunoblot analysis (c). Data in (a) and (c) are representative of a minimum of two independent experiments. Data in (b) are depicted as the mean ± SD for three independent experiments (* = $P < .001$ in comparison to vector control).

were permitted to attach overnight and then were transiently transfected with ErbB2, ErbB3 or non-silencing control siRNAs at a final concentration of 30 nM in Lipofectamine 2000 Reagent from Invitrogen Corporation (Carlsbad, CA) following manufacturer's instructions. After one day transfection, the cells were recultured in 100 mm dish at 2×10^6 cells/dish for western blot and 1.5×10^5/12 well plate for apoptosis.

2.7. Ectopic Expression of ErbB-2 and ErbB-3. The hemagglutinin epitope-tagged constitutively active (Myr)-AKT2 construct, HA-Myr-AKT2, and wildtype survivin expression

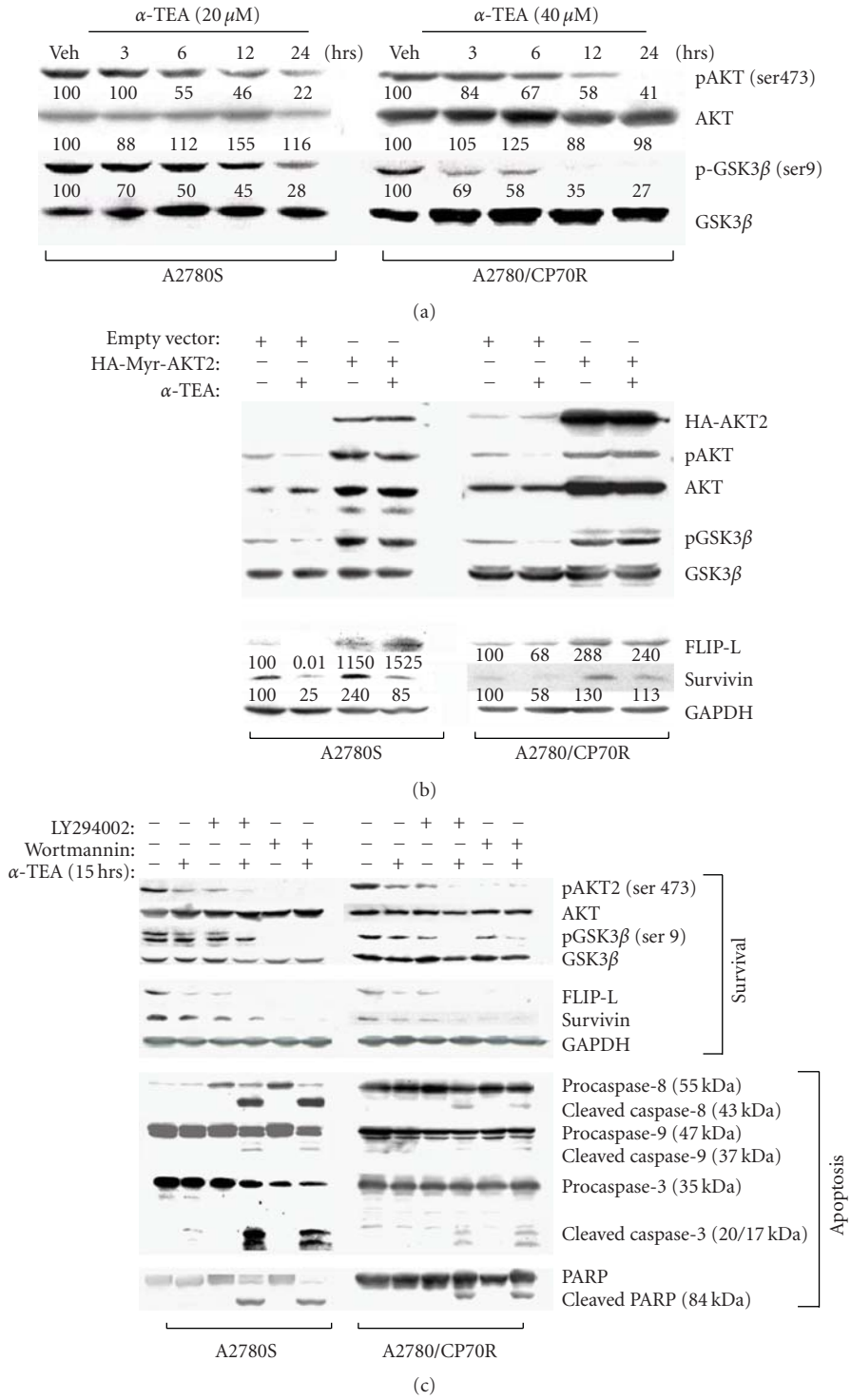

Figure 3: α-TEA decreased levels of the phosphorylated (active) form of Akt and its downstream substrate p-GSK3β. Overexpression of constitutively active Akt blocks α-TEA's ability to downregulate FLIP and survivin, while combinations of α-TEA and chemical inhibitors of PI3K are better at inducing apoptosis and downregulating FLIP and survivin than single treatments. (a) Cells were treated with 20 μM (A2780S) or 40 μM (A2780/CP70R) of α-TEA for 3, 6, 12, and 24 hours or vehicle control for 24 hours. Cell lysates from each treatment were analyzed by immunoblotting for pAkt, total Akt, pGSK3β (Ser-9), and GSK3β. Numbers cited under lanes represent densitometric analyses of α-TEA treated samples compared to vehicle control treated samples normalized for any lane load differences. (b) Immunoblotting analyses were conducted on whole cell lysates from A2780S and A2780/CP70R cells transiently transfected with HA-Myr-AKT2 plasmid, following treatment with α-TEA at 20 or 40 μM, respectively, for 12 hours. (c) Cells were treated with two PI3K inhibitors, LY294002 (10 μM) or wortmannin (1 μM), and cultured with or without α-TEA (10 or 20 μM for A2780S and A2780/CP70R cells, resp.) for 15 hours. At the end of the treatment period, cells were collected and total proteins were extracted for Western blot. All data are representative of a minimum of two independent experiments.

plasmids were kindly provided by Dr. Jin Q. Cheng (Department of Pathology, Molecular Oncology, and Drug Discovery Programs, University of South Florida College of Medicine, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL) [35]. The constitutively active AKT is tagged at the carboxy-terminus with a hemagglutinin epitope tag and is modified at its aminoterminus with the c-Src-derived myristylation signal (MGSSKSKPK) [36]. The wildtype survivin expression plasmid was created by subcloning a PCR product of survivin into Myc-tagged pcDNA3.1 and confirmed by DNA sequencing analysis [37]. The wildtype His-tagged FLIP expression construct, pcDNA3.1-His-cFLIP-L, was kindly provided by Dr. John C. Reed (The Burnham Inst. La Jolla, CA) [38]. The pEGFP-C1 vector (Clontech, Mountain View, CA) was used to express enhanced green fluorescent protein (EGFP) in cells as a measure of transfection efficiency. The pcDNA3.1 expression construct containing ErbB3 was provided by Dr. Xiaofeng Li (MD Anderson Cancer Center) [39], and the expression construct of ErbB2 was kindly provided by Dr. Atanasio Pandiella (Instituto de Microbiologia Bioquimica and Centro de Investigacion del Cancer, CSIC, Universidad de Salamanca, Salamanca, Spain) [40]. A2780S and A2780/CP70R cells were plated at 1.5×10^6 cells/100 mm^2 cell culture dishes for Western immunoblot analyses and at 1.5×10^5 cells/well in 12-well plates for apoptosis analyses. Cells were permitted to attach overnight and then were transiently transfected with mammalian expression vectors or appropriate vector control. Briefly, cells were washed two times with serum-free media (RPMI) and were incubated with 0.5 mL of serum-free media (OPTI-MEM I, Gibco, Grand Island, NY) containing 100 μL of DNA/LipofectAMINE/Plus (Invitrogen, Carlsbad, CA) complex for apoptosis studies, and 4 mL of serum free medium (MEM-Option) containing 800 μL of DNA/LipofectAMINE/Plus complex for Western immunoblot studies. DNA/LipofectAMINE/Plus reagent complex was made by first mixing 0.7 μg of DNA/50 μL of serum-free medium with 5 μL of Plus reagent followed by 15-minute incubation, and then mixing the DNA/Plus reagent with 2 μL of LipofectAMINE reagent/50 μL of serum-free media followed by 14-minute incubation. After overnight transfection, cells were treated with α-TEA for 2 days before analyses for apoptosis or for 12 hours before Western immunoblot analyses.

2.8. Statistical Analysis. All experiments were performed two or more times and experimental results were analyzed for statistical significance using 2-tail t test. The significance level was set at $P < .05$.

3. Results

3.1. α-TEA Inhibits Cell Growth and Induces Apoptosis in a Dose- and Time-Dependent Manner. Cells in monolayer cultures were treated with α-TEA (0, 1.25, 2.5, or 5 μM for A2780S or 0, 2.5, 5, or 10 μM for A2780/CP70R cells) for 1, 2, or 3 days, and viable cell numbers were determined by MTS assay. As illustrated in Figure 1(a), α-TEA decreased viable A2780S and A2780/CP70R cell numbers in a dose- and time-dependent manner. To confirm that the reduction in cell viability following α-TEA treatment was due to the induction of apoptosis, cells were treated with different levels of α-TEA for 1, 2, and 3 days, and apoptosis was measured by morphological analyses of cells stained with the DNA dye DAPI (Figure 1(b)). A2780S cells treated with 2.5, 5, 10, or 20 μM α-TEA for two days exhibited dose dependent apoptosis of 16, 34, 72, and 98 % apoptotic cells; whereas, A2780/CP70R cells treated with two-fold higher levels of α-TEA (namely, 5, 10, 20, or 40 μM) for two days exhibited dose dependent apoptosis of 5, 34, 75, and 97 % apoptotic cells (Figure 1(b)). EC50 values for 2-day α-TEA treatments of A2780S and A2780/CP70R cells were 5.6 and 13 μM, respectively. Vehicle control treated cells of either type exhibited a low background level of apoptosis of approximately 4%. Since treatment of A2780 and A2780/CP70R with 20 and 40 μM α-TEA, respectively, induced optimal amounts of apoptosis, these two different concentrations were used for all the following mechanistic studies.

3.2. α-TEA Downregulates Two Prosurvival (Antiapoptotic) Factors, FLIP and Survivin in Both Cell Types and Overexpression of Either FLIP or Survivin Partially Rescues A2780S and A2780/CP70R Cells from α-TEA-Induced Apoptosis. Treatment of both cell lines with α-TEA decreased protein levels of FLIP-L, FLIP-S and survivin in a time-dependent manner (Figure 2(a)). To assess the importance of FLIP and survivin to α-TEA-induced apoptosis, cells were transiently transfected with wild-type, his-tagged FLIP-L, myc-tagged survivin or empty vector (pcDNA3), and treated with either vehicle control or α-TEA for 24 hours. Percentage of apoptotic cells were determined (Figure 2(b)) and level of ectopically expressed proteins were measured (Figure 2(c)). Overexpression of either wild-type FLIP-L or wild-type survivin in both cell lines significantly suppressed α-TEA-induced apoptosis, compared to empty vector control ($P < .001$; Figure 2(b)), demonstrating that ectopic expression of FLIP-L or survivin rescues cells from α-TEA-induced apoptosis. Western blot analyses confirmed that transfections yielded high levels of the ectopically expressed proteins (Figure 2(c)). Please note, the Western blot depicted in Figure 2(c) is from a short exposure time that is inadequate for visualizing endogenous wildtype survivin expression. These results demonstrate that α-TEA downregulation of FLIP-L and survivin is required, at least in part, for maximum induction of apoptosis.

3.3. α-TEA Decreased Levels of Phosphorylated (Active) Akt. Phosphorylation of Akt at Ser 473 is required for its full activation [41]. α-TEA decreased the levels of phospho-Akt (Ser 473) while having no major effect on levels of total Akt protein expression in both ovarian cancer cell types in a time-dependent manner (Figure 3(a)). To verify that the decrease in phosphorylation status of Akt is correlated with decreased kinase activity, the phosphorylation status of GSK3β, a substrate of Akt, was assessed following α-TEA

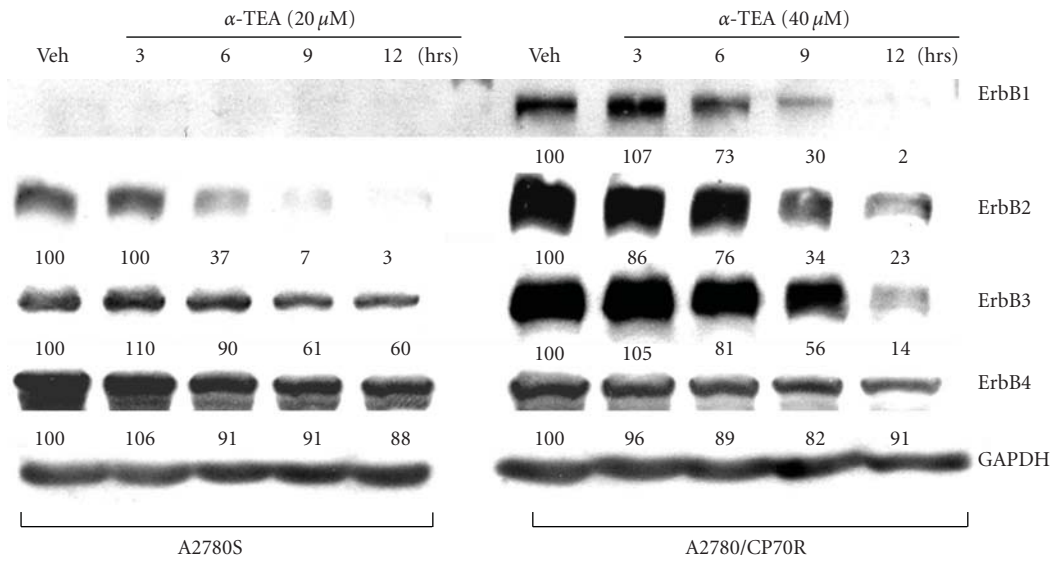

Figure 4: α-TEA downregulates ErbB1, -2, or -3 receptors in A2780S and A2780/CP70R cells. A2780S and A2780/CP70R cells were treated with 20 μM and 40 μM of α-TEA, respectively, for 3, 6, 9, and 12 hours or vehicle control for 12 hours. Cell lysates from treatment groups were analyzed by immunoblotting for ErbB1, ErbB2, ErbB3 and ErbB4 protein levels. GAPDH levels were determined to verify lane loads for densitometric analyses with vehicle control given a value of 100. Data are representative of two or more experiments.

treatments. Reduced phosphorylation status of pGSK3β (Ser 9) was detected with little to no corresponding decreases in protein levels, indicating that α-TEA treatments inhibited Akt activity (Figure 3(a)).

3.4. Overexpression of Constitutively Active Akt Blocks α-TEA's Ability to Reduce FLIP and Survivin Levels. Studies were conducted to determine if α-TEA downregulation of FLIP and survivin is mediated by Akt. A constitutively active form of Akt2 (HA-Myr-AKT2) was overexpressed in both cell types. Cells transfected with the pcDNA3 vector alone served as control. Data show that cells transfected with the constitutively active form of Akt exhibited inhibition of α-TEA's ability to reduce FLIP and survivin expression (Figure 3(b)). These data suggest that Akt is an upstream mediator for both FLIP and survivin and α-TEA downregulation of FLIP and survivin is mediated via suppression of Akt.

3.5. Inhibition of Akt Using PI3K Inhibitors Enhances α-TEA's Ability to Induce Apoptosis and Reduce FLIP and Survivin Levels. To further investigate the function of active Akt in the regulation of FLIP and survivin, we used PI3K inhibitors LY294002 [28] and wortmannin [29] to determine whether reduced Akt activity effects α-TEA-induced apoptosis and FLIP and survivin protein expression. Data show that both LY294002 and wortmannin effectively inhibited the levels of phosphorylated Akt and GSK3β in both cell lines without markedly changing total protein levels (Figure 3(c), first to fourth panels). Likewise, FLIP and survivin expression levels were decreased by treatment with either PI3K inhibitor or α-TEA singly, and further decreased by co-treatments (Figure 3(c), fifth and sixth panels). Cleavage of caspases-8, -9, and -3 were enhanced by combination treatments. Likewise, PARP proteolytic cleavage as a measure of apoptosis showed that LY294002 and wortmannin augmented the apoptotic response induced by α-TEA (Figure 3(c), last 4 panels).

3.6. α-TEA Downregulates ErbB Protein Levels in Both Cell Lines. ErbB family members play important roles in regulating epithelial cell proliferation and survival via their downstream mediators, including PI3K/Akt [7]. Therefore, we investigated whether α-TEA suppresses Akt/FLIP and survivin pathways via targeting ErbB family members in A2780S and A2780/CP70R cells. Western immunoblot data (Figure 4) show that A2780S cells expressed undetectable ErbB1 and lower levels of ErbB2 and ErbB3 in comparison with A2780/CP70R cells. α-TEA at 20 or 40 μM for 3, 6, 9, or 12 hours in A2780S and A2780/CP70R, respectively, decreased ErbB1 (A2780/CP70R), and ErbB2 and ErbB3 in both cell lines (Figure 4). GAPDH levels were used to normalize densitometric values for any variation in lane loads.

3.7. Investigation of Causal Roles of ErbB2 and ErbB3 in Akt Signaling in α-TEA-Induced Apoptosis. In this study, we tested if α-TEA down regulation of ErbB2 or ErbB3 contributes to α-TEA-induced suppression of pAkt and α-TEA-mediated apoptosis. First, we examined the effects of functional knockdown of ErbB2 or ErbB3 using siRNA. Data show that ErbB2 and ErbB3 targeted siRNAs produced reductions in levels of pAkt in comparison to nonsilencing siRNA treated controls (Figure 5(a)). As predicted, knockdown of ErbB2 and ErbB3 enhanced α-TEA suppression of pAkt and increased α-TEA-mediated apoptosis as measured by PARP-cleavage (Figure 5(a)).

As shown in Figure 5(b), ErbB2 and ErbB3 were successfully over expressed and α-TEA's ability to reduce pAkt levels and induce apoptosis were diminished but not totally

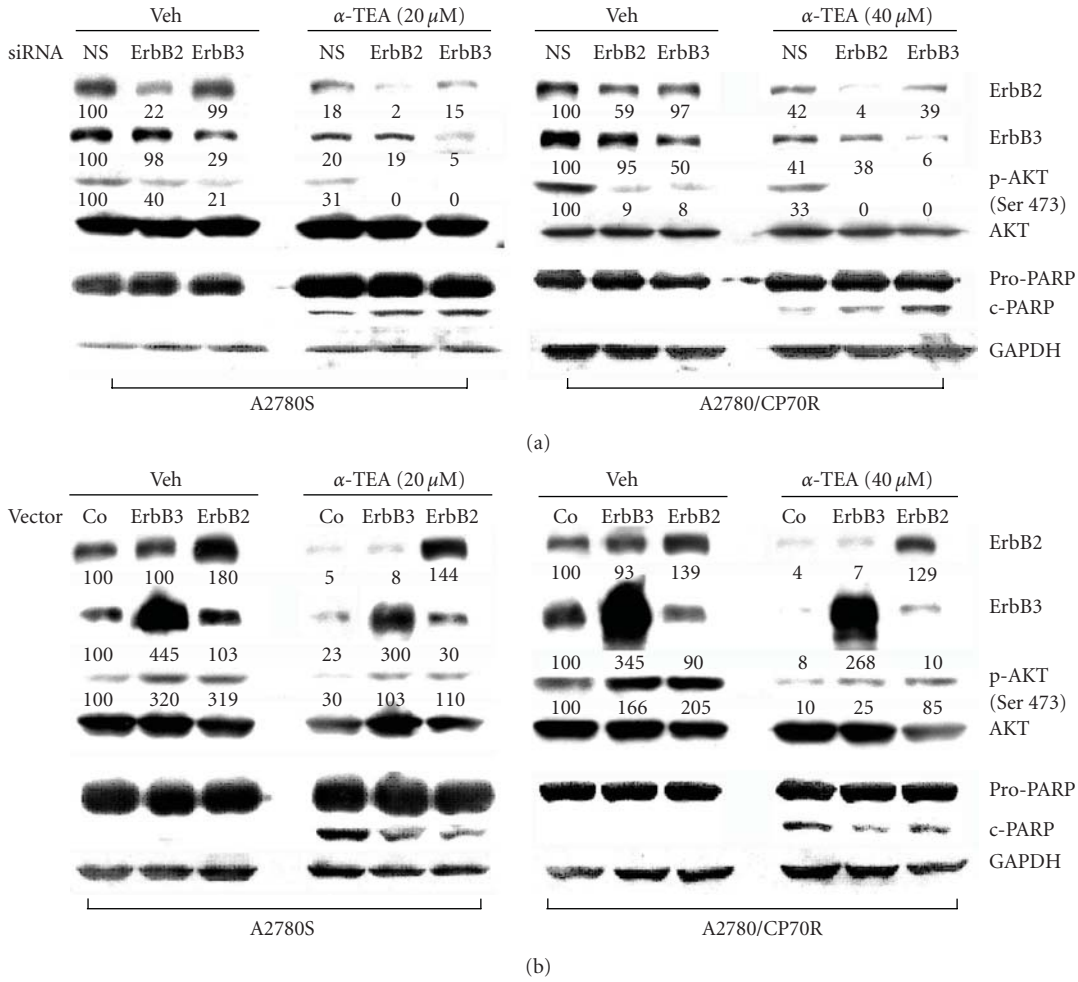

Figure 5: Downregulation of ErbB2 or -3 using siRNAs sensitize A2780S and A2780/CP70R cells to α-TEA-induced apoptosis and inhibit Akt phosphorylation; whereas overexpression of ErbB2 or -3 blocks α-TEA-induced apoptosis and enhances phosphorylation of Akt. (a) Cells were transfected with 20 μM of sequence-specific synthetic siRNAs against ErbB2 or -3. Nonsilencing (NC) siRNA-transfected cells were used as control. Cells were treated with either vehicle or α-TEA for 9 hours and then lysed. 50 μg of total lysate protein was used for detection of ErbB2, ErbB3, pAkt (Ser 473), total Akt, and PARP by Western blot. Data are depicted as the mean ± SD for three independent experiments. (b) Cells were transiently transfected with empty vector control (pcDNA3), ErbB2 or ErbB3 plasmids. After 24 hours of transfection, cells were treated with or without α-TEA for 9 hours, and whole cell extracts examined by immunoblotting. Numbers cited under lanes represent densitometric analyses for comparative purposes. Data are representative of three independent experiments.

eliminated, suggesting that α-TEA might still be an effective anticancer agent even in cases of highly elevated ErbB mediated survival.

Collectively, these results show that ErbB2 and ErbB3 control basal, constitutively active levels of pAkt and down regulation of ErbB2 and ErbB3 contributes to α-TEA-induced suppression of pAkt and induction of apoptosis in both A2780S and A2780/CP70R human ovarian cancer cells. Conversely, ectopic over expression of ErbB2 or ErbB3 limited α-TEA's ability to induce apoptosis.

4. Discussion

Data in this paper showed the following: (i) α-TEA is an effective stand alone anticancer agent for human ovarian cancer cell lines in that it inhibits both cisplatin-sensitive and -resistant ovarian cancer cells growth in culture by both decreasing cell proliferation and inducing apoptosis. (ii) The downregulation of ErbB2 and ErbB3/Akt/FLIP and survivin signaling events is necessary for α-TEA-induced apoptosis, and (iii) ErbB1 is highly expressed in the A2780/CP70R cells and below levels of detection in the A2780S cells, suggesting that ErbB1 may play a role in cisplatin resistance. Taken together with previous data that showed that α-TEA induces apoptosis via Fas Fas(CD95)/FasL mitochondrial dependent signaling events [27], we have summarized our current understanding of α-TEA induced apoptosis in human ovarian cancer cells in Figure 6. α-TEA is an effective anticancer agent not only because it triggers apoptosis via activation of membrane death receptor Fas (CD95)-mediated proapoptotic signaling but also because it downregulates ErbB family members and their downstream antiapoptotic effectors.

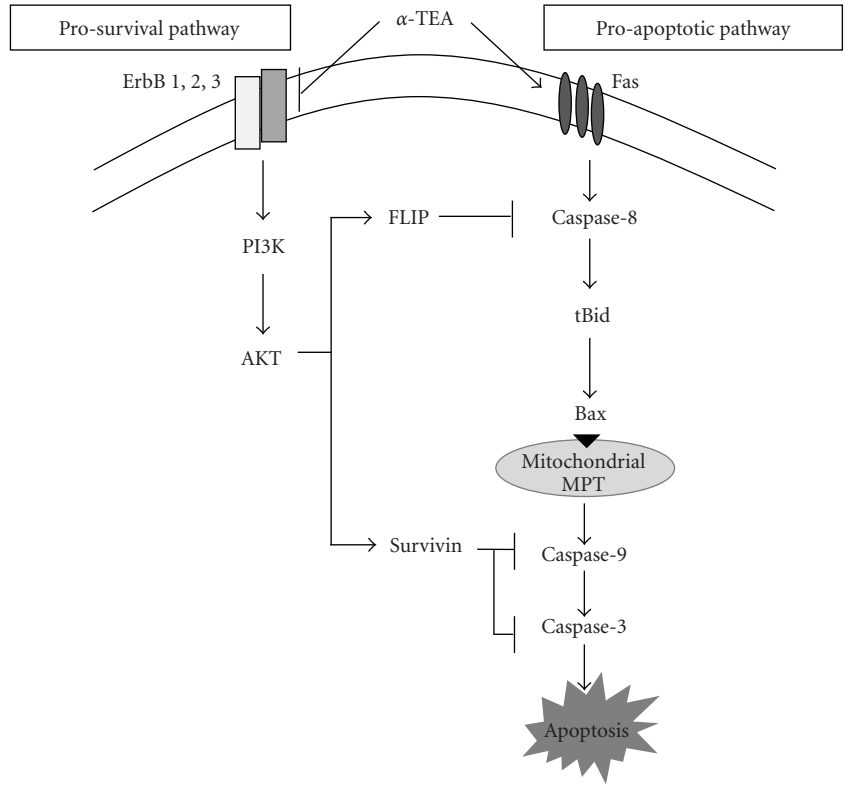

Figure 6: Schematic of signaling pathways involved in α-TEA-induced apoptosis in human ovarian cancer cell lines. Based on previously reported data [27] and data reported here, we propose both blockage of prosurvival and activation of proapoptotic signaling pathways are involved in α-TEA-induced apoptosis of human ovarian cancer cell lines. α-TEA triggers activation of the proapoptotic Fas (CD95) pathway, leading to a caspase-8- and mitochondria/caspase-9-dependent proapoptotic cascade [27]. Additionally, α-TEA downregulates ErbB1 (in the A2780/CP70R cells) and ErbB2 and ErbB3 in both A2780 and A2780/CP70R cell lines, leading to suppression of PI3K/Akt signaling and expression of the downstream antiapoptotic factors FLIP and survivin, which potentiates the α-TEA-induced proapoptotic cascade via enhancing the activation of caspase-8 and caspase-9/3, respectively, [15–17].

Vitamin E analogs have not been extensively studied in ovarian cancer. Previously, we reported that α-TEA in combination with cisplatin significantly reduced A2780/CP70R ovarian cancer tumor burden and lung metastasis in comparison to single treatments [22], and that the apoptotic properties of α-TEA in human ovarian cancer cells was mediated, at least in part, via Fas (CD95) mitochondrial-dependent apoptotic signaling pathway [27]. Studies by Shanker et al. [42] showed that the succinate analog of RRR-α-tocopherol, vitamin E succinate, in cell culture inhibited the growth of MDAH2774 human ovarian tumor cells via extrinsic and intrinsic apoptotic pathways. In a study that compared the efficacy of α-TEA versus vitamin E succinate, we showed α-TEA was a more effective anticancer agent because esterase activity in ovarian cancer cells clipped off the succinic moiety of vitamin E succinate, yielding RRR-α-tocopherol which does induce apoptosis [26]. The ability of α-TEA to induce apoptosis in human ovarian cancer cell lines is not restricted to A2780 and A2780/CP70R cells but has been shown in a number of human ovarian cancer cell lines including 2008, 2008-C13, Hey, OVCA-429, OVCA-433, OVCA-432 and SK-OV-3 [26].

The ability of α-TEA to induce apoptosis in both A2780S and A2780/CP70 ovarian cancer cells requires both downregulation of ErbB-mediated prosurvival factors (ErbB/Akt/FLIP and survivin) and activation of Fas-mediated mitochondrial dependent apoptosis, two complementary and necessary events.

The membrane associated epidermal growth factor receptor family (ErbB) members possess protein tyrosine kinase activity, are involved in cell survival and proliferation and are amplified in many cancers [43]. Amplification of ErbB2 protein is found in approximately one third of ovarian cancers and is an indicator of poor prognosis in advanced disease [44]. ErbB2 initiates several signaling networks involved in a variety of cellular processes, including PI3K/Akt [44]. Akt is constitutively active in ovarian cancers, and contributes to tumor cell survival by promoting the expression of survivin [37, 45].

Although direct measurements of Akt activity were not performed, decreased phosphorylation status of a downstream target of Akt, namely GSK3β was observed following α-TEA treatment, indicating that α-TEA-is downregulating Akt activity. Akt was shown to play a role in α-TEA induced apoptosis since expression of constitutively active Akt2

partially prevented α-TEA-induced apoptosis, and chemical inhibition of the PI3K/Akt pathway augmented α-TEA-induced apoptosis as measured by caspase-8 and casapse-9 activation, as well as PARP cleavage. Furthermore, the ability of α-TEA, via downregulation of Akt, to reduce FLIP and survivin provide further evidence that ErbB/Akt/FLIP/survivin signaling events help maintain ovarian cancers.

Based on studies reported here that suppression of Akt, FLIP or survivin in the absence of α-TEA did not induce apoptosis, suggests that these ovarian cancer cells are not "addicted" to PI3K/Akt/cFLIP/survivin for survival. Rather data show that suppression of ErbB/Akt/FLIP/survivin anti-apoptotic pathway cooperates with α-TEA-induced death receptor-mediated mitochondrial-dependent apoptotic cascade to sensitize the cells to cell death signals. As depicted in Figure 6, inhibition of FLIP and survivin are predicted to impact the death signaling pathway at both initiation (caspase 8) and execution (caspases 9 and 3) phases [15, 16]. Both FLIP and survivin have been implicated in contributing to cisplatin resistance in ovarian cancer [27, 46]. Therefore, downregulation of ErbB/Akt/FLIP and survivin prosurvival pathway by α-TEA not only enhances α-TEA-induced apoptosis but may also sensitize ovarian cancer cells to other proapoptotic agents.

5. Conclusion

Aberrant activation of ErbB receptors and downstream PI3K/Akt signaling contributes to the development of many cancers, including ovarian cancer. This report demonstrates that α-TEA is a potent inducer of apoptosis in both cisplatin-sensitive and -resistant human ovarian cancer cell lines in culture. α-TEA's ability to initiate apoptosis is enhanced by its ability to downregulate ErbBs and subsequent downstream prosurvival mediators, Akt, and Akt mediated FLIP and survivin, yielding a dual-acting agent. A more complete understanding of α-TEA's multiple actions, not only adds to our basic understanding of dysregulated signaling in cancer pathophysiology but hopefully will aid in selecting the proper application of α-TEA in the clinic.

Acknowledgments

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Review Article

The Emerging Role of PARP Inhibitors in the Treatment of Epithelial Ovarian Cancer

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Poly(ADP-ribose) polymerase-1 (PARP-1) is an important novel target in cancer therapy. This enzyme is essential in the repair of single-stranded breaks in DNA via the base excision repair pathway. Drugs which inhibit PARP are emerging as a promising new class of anticancer agents particularly effective against tumors which have lost homologous recombination (HR) through loss of functional BRCA1 and BRCA2. PARP inhibitors potentially represent a major breakthrough for patients with hereditary BRCA-associated cancers. Furthermore their role in sporadic epithelial ovarian cancer is emerging with identification of additional subpopulations of women who may benefit a priority. This paper will summarize the mechanism of action of PARP inhibition and its role in the treatment of BRCA1- and 2-associated cancers. We will then expand on the broader relevance and future directions for PARP inhibition in the clinical setting.

1. Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of death in women in North America [1]. Despite the efficacy of platinum-based chemotherapy, over 75% of women with stage III/IV EOC ultimately relapse and die from their disease. Median survival for women whose disease does not respond or in whom duration of response is short is less than 12 months [2]. Traditional cytotoxics topotecan and liposomal doxorubicin demonstrate only modest efficacy in women with platinum resistant EOC and are associated with significant toxicity [3]. New therapeutic approaches, and the ability to identify patients groups who will derive benefit from them, are urgently required.

Over recent years the investigation of DNA repair in cancer cells has been a very active area of translational research. All cells have a number of overlapping pathways to protect the genome from DNA damage which occurs as a result of normal cell cycling, environmental insults, or cytotoxic chemotherapy. It is well recognized that when mutations occur within these DNA repair pathways there is an increased risk of malignant transformation and chemotherapy resistance [4]. Much research has focused on protecting cells from DNA damage and/or restoring DNA repair function. However, emerging data suggest that the concept of "synthetic lethality," that is, exploiting the vulnerability of cancer cells which have lost one mechanism of DNA repair by targeting a second pathway, may be a particularly attractive therapeutic approach. Poly(ADP-ribose) polymerase (PARP) is an enzyme which plays an important role in the recognition and repair of single-strand DNA breaks via the base excision repair (BER) pathway [5]. Over the last few years it has become apparent that in cells which have lost BRCA1 or BRCA2, components of a second DNA repair pathway, homologous recombination (HR), are particularly sensitive to PARP inhibition. These data suggest that PARP inhibitors may be particularly useful for the treatment of women with hereditary BRCA1/2-associated EOC [6, 7]. Targeted therapy using PARP inhibitors has become an important novel strategy for treating those with hereditary ovarian cancer. Furthermore the identification of other subpopulations of women with EOC who may benefit from this approach is an active area of research.

This paper will outline the mechanism of PARP inhibition and discuss this in relation to loss of BRCA function. We will summarize the preclinical and clinical evidence from the most recent studies and discuss future directions for PARP inhibition in EOC.

2. BRCA1 and BRCA2

BRCA1 or BRCA2 mutations occur in 0.1–0.8% of the general population and are inherited in an autosomal dominant manner [8]. They are well recognized to have a higher incidence in certain ethnic groups, such as women of Ashkenazi Jewish descent [9]. Women carrying a mutation in BRCA1 have a lifetime risk of developing ovarian cancer of between 40 and 50%, while those carrying a BRCA2 mutation have a slightly lower risk of 10–20% [10]. Over the past ten years, the focus of management for those identified as BRCA1/2 mutation carriers has been on cancer prevention and early cancer detection. However, despite prophylactic measures to reduce risk of EOC, many BRCA1/2 carriers will already have cancer at the time their mutation is diagnosed.

The BRCA1 gene is located on chromosome 17q21, while BRCA2 is located on chromosome 13q12 [11, 12]. BRCA1 and BRCA2 play major roles in the repair of DNA double-strand breaks (DSBs) by homologous recombination (HR). HR repairs DSBs that occur in late S and G2 phase of the cell cycle and also has a key role in repairing DSBs that result from unrepaired single-strand break (SSB) [13]. BRCA1 signals the presence of DSBs, while BRCA2 is directly involved in the mechanism of HR. In the absence of BRCA1 or BRCA2, alternative DNA repair pathways are used, which result in chromosomal instability and cell death. Normal cells of carriers are usually heterozygote with loss of the second allele occurring during tumorigenesis in the tumor cells of these women [14].

Currently, the treatment of patients with BRCA-associated EOC is identical to those with sporadic EOC. However, even prior to the emergence of the PARP inhibitors, data suggested that cancers associated with BRCA mutations responded differently to chemotherapy [15]. Tan et al. compared 22 BRCA-positive patients with EOC to 44 nonhereditary EOC controls in a matched case-control study. They found that BRCA-positive patients have higher response rates to first line platinum-based treatment (81.8% versus 43.2%, $P = .004$), subsequent lines of platinum-based treatments (second line, 91.7% versus 40.9%, $P = .004$), longer tumor-free intervals between relapses, and improved overall survival (8.4 versus 2.9 years, $P < .002$) [16]. This data implies that different strategies may be required in this group of women.

3. Poly(ADP-Ribose) Polymerase Inhibitors

There are currently 17 members of the PARP superfamily identified [17]. PARP-1 is the most studied enzyme, which is involved in the repair of SSBs of DNA by the base excision repair (BER) pathway [5]. Targeting the nuclear enzyme PARP-1 represents a new and novel approach to the treatment of EOC and appears to be particularly promising for those carrying mutations in the BRCA1 and 2 genes [14].

Cells utilize several overlapping DNA repair mechanisms to maintain the integrity of the genome. PARP-1 activation occurs in response to metabolic, chemical, or radiation-induced DNA SSBs and forms part of the BER pathway [18, 19]. PARP-1 detects and signals the presence of an SSB by binding to DNA adjacent to the damage. Once bound, PARP-1 catalyzes the cleavage of the coenzyme nicotinamide adenine dinucleotide (NAD+) into nicotinamide and ADP-ribose to produce highly negatively charged branched chains of poly(ADP-ribose) (PAR). A multiprotein repair complex is then formed including repair enzymes DNA ligase III, the DNA polymerase pol B, and scaffolding proteins such as XRCC1 (X-ray repair cross-complementing 1). Following ADP-ribosylation, PARP-1 has reduced affinity for DNA and is released. After repair, the PAR polymers are degraded via poly(ADP-ribose) glycohydrolase (PARG) [14] (Figure 1). The role of PARP-1 may not be limited to just SSB repair; roles in the DSB repair response have also been proposed [20].

The new generation of PARP inhibitors inhibits PARP by competitive inhibition of NAD+. In the preclinical setting, PARP-1 inhibitors enhance the cytotoxic effects of ionizing radiation and cytotoxic chemotherapy [21]. Additionally, in the preclinical setting, the use of PARP-1 inhibitors as single agents did not cause any measurable toxicity, but the combination of PARP-1 inhibitor with temozolomide in the tumor-bearing mice caused significant toxicity [22]. There did not seem to be a correlation, however, between the antitumor activity and the toxicity of the PARP inhibitor-temozolomide combinations, suggesting that toxicity and chemosensitization were by different mechanisms. While promising in combination with other agents, PARP inhibitors appear to be particularly potent in patients who have defects in DNA repair.

In a normal cell, PARP-1 inhibition leads to failure of SSB repair, resulting in the formation of a DSB in the DNA when a replication fork encounters the SSB. Thus the DSB can be repaired by HR and the fidelity of the genome maintained. However, in cells carrying defects in BRCA1/2, HR is defective, resulting in an attempted repair of the DSB by the more error prone nonhomologous end joining (NHEJ) pathway [18]. As a result, the cell acquires lethal levels of damage and cellular viability is lost, a prime example of "synthetic lethality" with the malignant cell able to function with the loss of one DNA repair mechanism (HR) but ceasing to be viable with the loss of a second (BER) [23, 24]. As most BRCA1/2 carriers have one normal allele, the hope was that inhibition of PARP would be selective for tumor cells.

In 2005, two preclinical papers demonstrated the sensitivity of BRCA1- and BRCA2-deficient cell lines to PARP inhibition [6, 7]. The first paper by Bryant et al. demonstrated reduced survival of BRCA2-deficient cell lines with four PARP inhibitors. They concluded that BRCA2-deficient cells were sensitive to PARP inhibition, and that monotherapy with one of these agents could selectively kill cancer cells [6]. In the same year, Farmer et al.

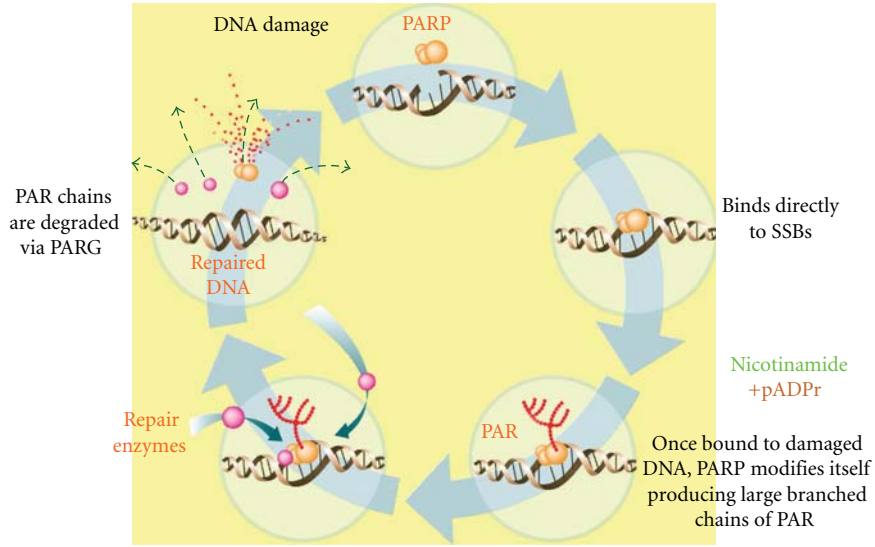

Figure 1: The role of PARP in the repair of single-strand DNA breaks via the base excision repair pathway.

demonstrated how both BRCA1- and BRCA2-deficient cells lines were sensitive to inhibition of PARP-1, and that BRCA2 deficient cells were more than 1000 times more sensitive to nanomolar concentrations of PARP inhibitor [7]. Both of these papers demonstrated how homozygotes (tumor cells) are sensitive to the mechanism of PARP inhibition; whereas heterozygotes (the rest of the patient's cells) are insensitive to this mechanism and should not exhibit toxicity. These findings from two independent groups using different chemical classes of PARP inhibitors on different BRCA-deficient cell lines were the first to suggest the potent effect of PARP inhibition.

4. Clinical Evidence in Phase I and II Trials

A number of PARP inhibitors have entered the clinic in both intravenous and oral formulations. The four which are furthest along in terms of development are AGO14699 (Pfizer), AZD2281 (AstraZeneca), ABT-888 (Abbott), and BSI-201 (BI Par), and all four of these compounds demonstrate profound inhibition of PARP-1.

Olaparib (AZD2281, KU- 0059436, AstraZeneca) is an oral small-molecule PARP inhibitor. The first clinical evidence demonstrating the sensitivity of BRCA-mutated cancers to PARP inhibitor monotherapy was presented in a study by Yap et al. in 2007 [25]. This phase I trial included 44 patients, of which 11 patients had a BRCA mutation associated cancer. Dose escalation was guided by toxicity, pharmacokinetic and pharmacodynamic data. Based on the encouraging antitumor activity, many in whom had BRCA1/2 mutations, the trial was subsequently expanded to concentrate on cancers in patients with BRCA mutations and was presented in 2008 by Fong et al. [26], followed by publication of the manuscript in 2009 [27]. The drug was well tolerated in both BRCA mutated and normal populations. Most toxicities were grade 1-2 (≥95%), consisting of fatigue (28%), nausea (28%), vomiting (18%), loss of taste (13%), and anorexia (12%). Grade 3-4 toxicities were rare, consisting of myelosuppression (≤5%), nausea and vomiting (2-3%), and dizziness or mood changes (2-3%) [27]. Of the 60 patients that were enrolled and treated, 19 of 23 BRCA-positive carrriers were evaluable. 12 of the 19 (63%) had a clinical benefit from olaparib, with radiologic or tumor marker responses, or stable disease for 4 months or more [27]. Patient response was seen in those receiving a minimum of 100 mg twice daily up to 400 mg twice daily. Response was the greatest in patients with platinum-sensitive disease, although duration of response was the same regardless of the platinum-free interval [26].

Recently data was presented from a phase II study of olaparib in women with advanced EOC with known mutations in BRCA1/2 [28]. Two patient cohorts received continuous oral olaparib in 28-day cycles; 33 patients received 400 mg orally twice daily, while 24 patients received 100 mg twice daily. The choice of dosing and schedule was based on the phase I trial above [25]. The objective response rate measured by RECIST criteria was 33% at the 400 mg dose, and 12.5% at the 100 mg dose, suggesting that there may be a dose response effect. The toxicity profile was mainly mild, consisting of grade 1 or 2 nausea (44%) and fatigue (35%), with few grade 3 or 4 toxicities. Interestingly, although numbers were low, in this study there appeared to be a higher response rate in platinum resistant patients (38% versus 14%), which was opposite to that observed in the earlier phase I study (Table 1), where response was the greatest in platinum-sensitive patients. Laboratory studies have previously suggested that platinum resistant patients may reacquire BRCA function [29] thus potentially making them resistant to the effects of PARP inhibition. Taken together, the clinical data suggest that we still have a lot to learn with regard to target populations and the role of PARP inhibition. Furthermore, data from

Table 1: Responses rates of women with epithelial ovarian cancer to olaparib (AZD2281) by platinum sensitivity in Phase I (Fong et al.) [26] and Phase II trials (Audeh et al.) [28].

	No. evaluable		Responders by RECIST (%)		Responders by RECIST or GCIG (%)	
	Phase I [26]	Phase II [28]	Phase I [26]	Phase II [28]	Phase I [26]	Phase II [28]
Total	46	33	13 (28%)	11 (33%)	21 (46%)	20 (61%)
Platinum sensitive (>6 months)	10	7	5 (50%)	1 (14%)	8 (80%)	—
Platinum resistant (≤6 months)	25	26	8 (32%)	10 (38%)	11 (44%)	—
Platinum refractory	11	—	0 (0%)	—	2 (18%)	—

the phase II study appears to give an early indication that response (both RECIST and CA125) may be greater in those patients with BRCA2 mutations. This would be in line with the known mechanism of action of the two BRCA proteins as BRCA2 plays a key role in the repair pathway; whereas BRCA1 functions as a signaling molecule [30]. This phase II study concluded that oral olaparib is well tolerated and highly active in advanced, chemotherapy-refractory BRCA-deficient EOC, with greater activity seen at a higher dose of 400 mg twice daily. The optimal patient group with respect to platinum sensitivity has not been defined.

Reassuringly in the clinical studies there does not appear to be an increase in toxicity between BRCA mutation carriers compared to noncarriers, supporting the theory that PARP inhibitors should not result in increased toxicity to heterozygote cells [6, 7].

These recent phase I and phase II trials are particularly promising for patients with BRCA-associated EOC. Further phase II trials are currently underway which will help further elucidate the role and potential for this new targeted therapy.

5. PARP Inhibitors in Sporadic Ovarian Cancers

BRCA-associated EOC is associated with only 10% of all ovarian cancers. However, loss of BRCA1/2 function is not exclusive to inheriting a mutation in the BRCA1/2 genes [31]. The results seen in known BRCA1 and 2 mutation carriers may also be relevant to the sporadic EOC patient population.

Epigenetic gene inactivation is a well-recognized phenomenon with 31% of EOC exhibiting aberrant methylation of the BRCA1 promoter [32]. Furthermore, genetic or epigenetic events occurring in other components of the HR pathway can be found in sporadic EOC [15, 33]. These tumors seem to be similar to BRCA1- or BRCA2-mutated tumors, even though they do not have mutations to either of these genes, a concept called "BRCAness." [15, 33]. One molecular characterization study suggested that over 50% of patients with high-grade EOC had loss of BRCA function, either by genetic or epigenetic events [34]. Studies have shown that the loss of functional proteins in the HR pathway may lead these cells to be sensitive to PARP inhibition [35]. Identification of "BRCA-like" EOC populations who may benefit from this new therapy through the identification and validation of biomarkers is an active area of ongoing research.

6. Future Directions

At least 6 PARP inhibitors, including AG0146999 (Pfizer) and MK4827 (Merck), are under investigation either as single agents and/or in combination with other agents or treatment modalities. Phase II studies in women with advanced EOC in both BRCA1/2 mutation carriers and high-grade EOC of unknown BRCA status are ongoing, many incorporating translational research questions which are vital to our understanding of the biology of PARP inhibition. Currently, olaparib is being evaluated in a randomized phase II trial comparing this agent with pegylated liposomal doxorubicin in patients with BRCA-mutated EOC with a platinum-free interval of 0–12 months [36].

Early data combining PARP inhibitors with cytotoxics suggested that the combinations may be toxic and that substantial dose reductions of the cytotoxic agents may be required [37]. Intriguingly, a randomized study in women with triple negative breast cancer presented at this year's American Society of Clinical Oncology (ASCO) suggests that this may not always be the case. Patients were randomized to receive either gemcitabine 1000 mg/m^2 and carboplatin AUC 2 on days 1 and 8 with or without the PARP inhibitor BSI-201 [38]. In this study there was no difference in the rates of toxicity or dose adjustments between the two arms. Response rates were significantly higher ($P = .002$) for women receiving the PARP inhibitor. Currently many combination studies are underway; the results are awaited with interest. Combination studies in women with both hereditary and sporadic EOC are expected in the future.

Further defining the role of PARP inhibitors in the clinic is ongoing. Olaparib is being evaluated in a randomized placebo-controlled trial as a maintenance therapy in patients with sporadic EOC at high risk of early recurrence [39]. Furthermore, some suggest that PARP inhibitors could be used to prevent cancers in patients who are BRCA mutation carriers [40]. This approach, however, requires careful consideration and some caution with the potential for the development of drug resistance in long-term use of PARP inhibitors.

Investigation of the PARP inhibitors in the nonhereditary EOC population is very active with both the impact of treatment on patients without BRCA defects and the search for populations of women who have lost functional proteins in the HR pathway. Investigation of PARP inhibitor resistance and ways to overcome this resistance are emerging fields.

7. Conclusions

We are living in exciting times as our knowledge of tumor cell biology expands and new agents become available. As we move into the era of personalized medicine, the emerging data regarding the use of PARP inhibitors in patients with BRCA-associated EOC are encouraging and inspiring. Expansion and identification of further patient groups who will benefit from this approach are a priority. Over the next few years we expect to see an explosion in the publication of studies exploring the use and role of PARP inhibitors in the clinic. Careful clinical trial design, and the development and validation of biomarkers are essential if we are to make the optimal use of these exciting agents and improve outcome for women with EOC.

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Review Article

BRCA1 as a Therapeutic Target in Sporadic Epithelial Ovarian Cancer

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In sporadic epithelial ovarian cancer (EOC), the inactivation of BRCA1 through various mechanisms is a relatively common event. BRCA1 protein dysfunction results in the breakdown of various critical pathways in the cell, notably, the DNA damage response and repair pathway. Tumors from patients with *BRCA1* germline mutations have an increased sensitivity to DNA damaging chemotherapeutic agents, such as cisplatin, due to defective DNA repair. Thus, inhibiting BRCA1 in sporadic EOC using novel targeted therapies is an attractive strategy for the treatment of advanced or recurrent EOC. Several classes of small molecule inhibitors that affect BRCA1 have now been tested in preclinical and clinical studies suggesting that this is a rational therapeutic approach. The aim of this paper is to provide an understanding of how BRCA1 has evolved into a promising target for the treatment of sporadic disease and to outline the main potential small molecule inhibitors of BRCA1 in EOC.

1. Introduction

Up to 10% of epithelial ovarian cancers (EOCs) are caused by germline mutations in the tumor suppressor genes, *Breast Cancer 1* (*BRCA1)* and *BRCA2* [1, 2]. Carriers of these mutations have a risk of developing ovarian cancer of 18%–54% by age 70; rates significantly are higher than those of the general population [3]. The majority of sporadic EOCs display BRCA1 dysfunction or reduced expression, due to factors such as somatic mutations or promoter hypermethylation [4–6]. The *BRCA1* tumor suppressor gene codes for a 220 kD nuclear phosphoprotein which has been shown to be involved in many cellular processes such as cell cycle checkpoint control, DNA damage recognition and repair, apoptosis, the ubiquitin-proteasome pathway, and transcriptional regulation [7–10]. BRCA1 is located downstream in the cascade of the DNA damage sensors ATM and ATR and is phosphorylated by these kinases upon their activation in response to genotoxic stressors such as radiation and chemotherapeutic agents. Once in its phosphorylated state, BRCA1 becomes part of a number of different complexes which relocate to areas of damaged DNA and coordinate cell cycle checkpoints in order to execute DNA repair.

Ovarian cancer patients with tumors known to harbor a germline mutation in *BRCA1* are believed to display a better response to platinum-based therapies and improved survival compared to patients without *BRCA1*-associated disease [11]. BRCA1 deficiency is believed to result in deregulation of the carefully coordinated DNA repair cascade and thereby renders tumor cells more vulnerable to DNA damaging agents and genomic instability. While this may appear to be a distinct disadvantage for these cells in terms of tumorigenesis, this situation can be advantageous and potentially exploitable in the context of enhancing the response to DNA damaging chemotherapeutic drugs. The majority of patients demonstrate an initial response to debulking surgery along with the first-line therapy regimen of platinum and taxane-based agents. However, the majority will recur and develop platinum-resistance. To overcome platinum resistance, there is a significant need to develop novel therapeutic options that will either enhance the effectiveness of standard chemotherapeutics or target a subset of patient tumors based on molecular markers. This

paper focuses on novel therapeutic drugs in sporadic EOC that directly or indirectly target BRCA1 and its interrelated pathways. A review of *BRCA1* gene therapy is provided as well as an overview of the preclinical and clinical studies on the most relevant small molecular inhibitors, poly(ADP-ribose) polymerase-1 (PARP), histone deacetylases (HDAC), checkpoint kinases (CHKs), and proteasome inhibitors in the context of how these agents alter the BRCA1 pathway to enhance sensitivity to platinum-based chemotherapy. Finally, the potential for clinical use of BRCA1 as a biomarker in EOC is reviewed.

2. Gene Therapy

The first efforts to target BRCA1 in EOC involved restoring BRCA1 function via gene therapy [12]. In its normal state, BRCA1 functions as a tumor suppressor gene, inhibiting the aberrant proliferation of tumor cells. However, BRCA1 rarely displays normal expression and function in EOC [5]. Thus, a logical therapeutic option is to restore the tumor suppressor function of BRCA1 in cancer cells in order to suppress cell proliferation. In a cell culture model, a normal splice variant of BRCA1 was overexpressed by a retroviral vector resulting in decreased cell proliferation. The cell line was then implanted into a mouse xenograft model and tumor growth suppression was observed [12]. Preclinical findings indicated that restoration of normal function of BRCA1, in a disease where its loss has been shown to contribute to both its development and progression, could have the therapeutic potential to inhibit tumor growth. In the Phase I trial, twelve patients with recurrent metastatic ovarian cancer, who had been treated with standard surgery and chemotherapy, received one to three cycles of intraperitoneal injections of BRCA1 in a retroviral vector. Two-thirds of patients demonstrated stable disease for 4–16 weeks and one third showed reduction of tumor burden. Given the absence of significant toxicity, a Phase II trial in patients with less advanced disease was performed [13]. This trial demonstrated little to no vector stability as well as a rapid development of a neutralizing antibody response, which was not observed in the previous trial. Furthermore, there was no evidence of clinical response. The authors postulated that this stark difference in results was likely due to differences in immunocompetence between the patient groups in each trial, attributable to differences in factors such as tumor burden, number of chemotherapy treatments, and nutritional status. The same group went on to design a second-generation retroviral vector containing *BRCA1*. In preclinical efficacy studies in mouse xenograft models, this new vector was found to be minimally immunogenic and increased survival compared to both the control vector and the first generation vector used in the Phase II trial [14]. No clinical trials with this vector have been reported to date.

Vector reconstitution, in an attempt to regain normal function of BRCA1, has never proceeded to Phase III study. Thus, attention has recently focused on taking advantage of the inherent weakness of BRCA1-deficient tumor cells, namely, the inability to effectively repair DNA damage.

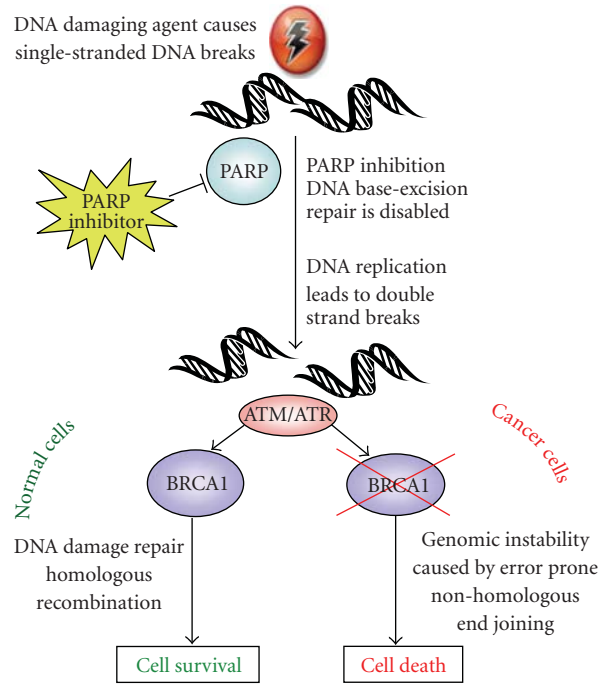

Figure 1: Targeting BRCA1 as a therapeutic strategy in the treatment of EOC. BRCA1 is central to the DNA damage response which is initiated following insults such as platinum-based chemotherapeutic agents. Various small molecule inhibitors may target BRCA1 directly or indirectly, ultimately leading to failure to repair damaged DNA and apoptosis.

3. PARP Inhibitors

A novel therapeutic option which has been the closest to widespread clinical use in the treatment of EOC is PARP inhibitors (PARPi) [15]. The PARP family of enzymes catalyzes the polymerization of poly ADP-ribose from a nicotinamide adenine dinucleotide (NAD^+) substrate. They play a critical role in the repair of single-stranded DNA breaks (SSBs) via the base excision repair (BER) pathway and inhibition of PARP results in failure of SSB repair. Unrepaired SSBs which encounter a DNA replication fork result in double-strand breaks (DSBs). Normal cells are able to repair DSBs via the homologous recombination (HR) DNA repair pathway. However, cells with defective BRCA1 are unable to repair DSBs due to defective HR repair and are forced to use the error-prone nonhomologous end-joining (NHEJ) pathway. The ensuing genomic instability ultimately results in cell death (Figure 1). This ability to preferentially target BRCA1-defective cells and spare those with normal function made PARPi an attractive option for the treatment of ovarian and breast cancer patients with BRCA1 germline mutations. It is noteworthy that preclinical studies examining the effect of PARP inhibitors on BRCA-deficient cancers have focused on breast cancer models.

The first preclinical work to demonstrate susceptibility to PARP inhibitor-induced cytotoxicity in *BRCA*-null cells was published simultaneously by two different groups in 2005 [16, 17]. Bryant et al. showed that *BRCA2*-null V-C8

cells were extremely sensitive to PARP1 inhibitors of varying potency and that this was likely due to their inability to execute effective HR repair. Farmer et al. also demonstrated similar findings in *BRCA2*−/− mouse embryonic stem (ES) cells, as well as *BRCA1*−/− ES cells. Treatment of V-C8 and *BRCA*-null ES cells with PARP inhibitors resulted in an increase in chromosomal instability, cell cycle arrest, and apoptosis. The results of the in vitro studies were validated in vivo by creating xenograft mouse models using the same V-C8 and *BRCA2*-null ES cell lines. It was consistently found that treatment of the mice with PARP inhibitors blocked the growth of *BRCA*-null tumors but had no significant effect on reconstituted *BRCA* control tumors.

PARPi have also been shown to enhance the cytotoxicity of platinum-based agents in vitro and in vivo, irrespective of *BRCA1* status. One study looked at a PARP-1 inhibitor, 3-aminobenzamide, and found increased cisplatin cytotoxicity in CH1cisR cisplatin-resistant ovarian tumor cells [18]. A novel 3-aminomethyl carbazole imide PARP-1 and PARP-2 inhibitor, CEP-6800, was combined with cisplatin to treat Calu-6-NSCLC cells [19]. Combination treatment displayed more DNA damage than cisplatin alone. Furthermore, when Calu-6-NSCLC tumor cells were implanted into a nude mouse model, there was a 35% reduction in tumor growth with CEP-6800/cisplatin combination treatment compared to single-agent cisplatin.

There are preclinical data evaluating the combination of PARPi and platinum-based agents in both *BRCA*-mutant and BRCA-deficient breast cancer models. Donawho et al. used a *BRCA1*-deleted and a *BRCA2*-mutated MX-1 breast carcinoma xenograft mouse model to perform the in vivo evaluation of the Abbott Cancer Research PARPi, ABT-888. [20]. ABT-888 was shown to potentiate cisplatin and carboplatin cytotoxicity by inducing a greater regression of established tumors compared to modest tumor inhibition by platinum chemotherapy alone. Two studies analyzed a PARP-1 inhibitor by AstraZeneca (AZD2281) in BRCA-deficient models [21, 22]. In the study by Evers et al., AZD2281 displayed strong growth inhibition of BRCA2-deficient mouse mammary tumor cell lines compared to a BRCA2-proficient control tumor cell line [21]. Synergistic cytotoxicity in combination with cisplatin in the same model system was shown. Rottenberg and colleagues used the genetically engineered BRCA1-deficient breast cancer mouse model to establish AZD2281's efficacy alone and in combination with platinum-based agents [22]. When mice were treated with AZD2281 alone versus vehicle-treated controls, inhibited tumor growth and increased survival was observed. Subsequently, AZD2281 was combined with either cisplatin or carboplatin. This combination significantly prolonged the recurrence-free and overall survival compared to either platinum drug alone.

As a result of these promising findings, there are currently several PARPi in various stages of clinical development for use in patients with *BRCA1/2*-mutant breast and ovarian cancers (Table 1). The AstraZeneca/KuDOS compound KU-0059436 (AZD2281) was evaluated in *BRCA1/2* germline mutation positive breast, ovarian, and prostate cancers in a phase I trial [23]. This study showed that AZD2281 had few adverse effects, inhibited PARP and had antitumor activity in *BRCA1/2* mutation positive patients.

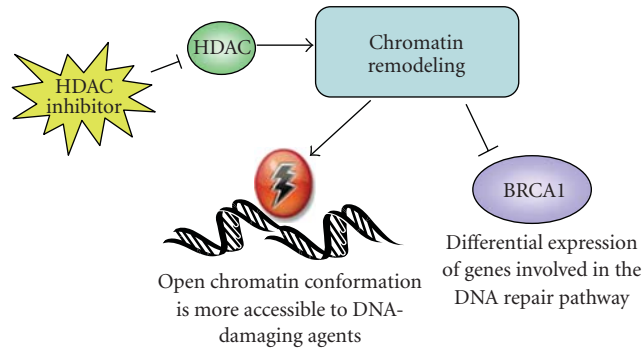

Figure 2: HDAC inhibition induces DNA damage and disrupts DNA repair. Inhibiting HDAC causes hyperacetylation of DNA and chromatin remodeling leading to more relaxed and open DNA. This conformational change renders DNA more accessible to cytotoxic DNA-damaging agents causing both upregulation and downregulation of genes in the DNA damage and repair cascade.

A large proportion of sporadic EOCs display BRCA1 dysfunction and may behave similarly to *BRCA1* germline mutation-related disease in terms of overall survival, sensitivity to platinum drugs and defects in DNA damage repair. Given these findings, it is rational to extrapolate the use of PARPi for the treatment of patients with sporadic EOC. A number of clinical trials are currently underway examining the effect of PARPi alone or in combination with platinum agents in ovarian cancers irrespective of *BRCA1/2* mutation status (Table 2).

4. HDAC Inhibitors

Histone Deacetylase Inhibitors (HDACi) have recently generated interest as a potential therapeutic option in the treatment of cancer, having demonstrated their ability to inhibit the proliferation of cancer cell lines in vitro and in vivo [31]. Histone deacetylases are enzymes involved in the posttranslational regulation of chromatin structure [32]. Their role is to catalyze the removal of acetyl groups from lysine residues in the core histones of chromatin, resulting in a more compact and transcriptionally repressed chromatin structure. The mechanism by which HDACi suppress the growth of cancer cells might be due to their inhibition of acetyl group removal, resulting in the hyperacetylation of chromatin structure. This causes chromatin to become more relaxed and open, making it more accessible to DNA-damaging agents and changing the expression of genes in the DNA damage recognition and repair cascade (Figure 2). There are several classes of HDACi including hydroxamic acid-derived compounds (e.g., Trichostatin A and SAHA), short-chain fatty acids (e.g., Sodium butyrate and valproic acid), benzamides, cyclic peptides, and thiolates.

HDACi may have a significant effect on the sensitivity of EOC tumors to DNA-damaging agents. Zhang et al. performed a gene expression profile on squamous carcinoma cells and observed that most genes involved in cell cycle

Table 1: Completed clinical trials evaluating potential drug targets of BRCA1.

Drug class	Compound	Phase	Study population
PARP inhibitors	KU-0059436 (AZD2281)	Phase I	BRCA1/2 germline mutation in advanced breast and EOC [23]
HDAC inhibitors	SAHA (vorinostat)	Phase II	Recurrent EOC [24]
CHK inhibitors	UCN-01 (staurosporine derivative) in combination with topotecan	Phase I	Advanced solid tumors, including EOC [25]
	UCN-01 in combination with topotecan	Phase II	Recurrent EOC [26]
Proteasome inhibitors	PS-341 (bortezomib) in combination with carboplatin	Phase I	Recurrent EOC [27]
	PS-341 in combination with carboplatin	Phase I	Platinum/taxane resistant EOC [28]
	PS-341	Phase II	Recurrent, platinum-sensitive EOC [29]
	PS-341 in combination with paclitaxel	Phase I	Advanced solid tumours, including EOC [30]

Table 2: Ongoing clinical trials evaluating potential drug targets of BRCA1.

Drug class	Compound	Phase	Clinical trial number	Study population
PARP inhibitors	MK4827	Phase I	NCT00749502	EOC
	AG014699	Phase II	NCT00664781	Advanced EOC
	KU-0059436 (AZD2281)	Phase I	NCT00647062	EOC with or without BRCA1 mutation
	KU-0059436 (AZD2281) in combination with doxorubicin	Phase II	NCT00628251	BRCA1/2 mutation positive EOC
	ABT-888 in combination with bevacizumab, carboplatin, paclitaxel	Phase I	NCT00989651	EOC
	ABT-888 in combination with temozolomide	Phase I	NCT00526617	EOC
	KU-0059436 (AZD2281)	Phase II	NCT00679783	EOC with or without BRCA1 mutation
	KU-0059436 (AZD2281)	Phase II	NCT00753545	Platinum sensitive serous EOC
	BSI-201	Phase II	NCT00677079	Advanced EOC
	ABT-888 in combination with topotecan	Phase I/II	NCT01012817	EOC
HDAC inhibitors	SAHA (vorinostat) in combination with paclitaxel, carboplatin	Phase I/II	NCT00772798	EOC
	SAHA (vorinostat) in combination with carboplatin, gemcitabine	Phase I/II	NCT00910000	EOC
	Hydralazine and magnesium valproate	Phase III	NCT00533299	Advanced EOC
CHK inhibitors	UCN-01 in combination with irinotecan	Phase I	NCT00031681	Metastatic EOC
Proteasome inhibitors	PS-341(bortezomib) in combination vandetanib	Phase I/II	NCT00923247	EOC
	PS-341	Phase II	NCT00023712	Platinum-sensitive EOC

control, DNA replication, and DNA damage repair were downregulated when treated with Trichostatin A (TSA) [33]. Our group has shown that the treatment of A2780s/cp ovarian cancer cells with the TSA analogue, M344, causes the downregulation of BRCA1 mRNA and protein levels [34]. We have also shown that M344 was able to increase the sensitivity of A2780s/cp ovarian cancer cell lines to cisplatin and carboplatin, but not to taxol. Strait and colleagues showed that TSA alone induced apoptosis in cisplatin resistant ovarian cancer cell lines OVCA-3 and SKOV-3 [35]. Another study looked at several different HDACi and found that they all enhanced the cytotoxicity of cisplatin, but not to metabolic antagonists or microtubule-damaging agents, in six human ovarian cancer cell lines of varying cisplatin sensitivity [36]. R306465 and PXD101, hydroxamate-based HDACi, have shown efficacy in A2780 xenograft mouse models [37, 38]. Oral administration of R306465 in immunodeficient mice was well tolerated and antitumor activity of 76%–87% was observed compared to vehicle controls. PXD101 showed single-agent antitumor effect in xenograft mice that was enhanced by the combination with carboplatin treatment.

SAHA (vorinostat) has been approved for the treatment of cutaneous T-cell lymphoma and subsequently, a number of clinical trials are currently underway to evaluate the toxicity and dose of HDACi in solid tumors, including ovarian cancer (Tables 1 and 2). A phase II study of SAHA in recurrent ovarian cancer found that the treatment was well tolerated but had minimal activity as a single agent [24]. There are phase I/II trials underway examining the combination of taxol, carboplatin, and SAHA as well as carboplatin, gemcitabine, and SAHA. A phase III trial is recruiting advanced ovarian cancer patients for treatment with magnesium valproate, an HDACi, in combination with hydralazine, an antihypertension agent. Since current trials have focused on all ovarian cancer patients with advanced/recurrent disease, there may also be a future role for targeting a specific subset of the patient population based on tumor biomarkers.

5. CHK1/2 Inhibitors

Following DNA damage, BRCA1 is involved in the control of cell cycle checkpoints, which represents another potential mechanism to target BRCA1 therapeutically. Two genes involved in this aspect of the DNA damage cascade are Checkpoint Kinase 1 and 2 (CHK1 and CHK2). CHK1 is activated by ATR in response to stressors such as replication stress, chemotherapeutic agents, and SSBs; whereas CHK2 is activated by ATM in response to ionizing radiation, chemotherapeutics, or DSBs [39]. Activation of CHK1/2 leads to arrest of the cell cycle at different phases depending on the specific kinase activated, allowing for DNA repair to occur. The functional BRCA1 protein has been shown to be phosphorylated and activated by CHK2, resulting in the activation of CHK1. The presence of phosphorylated BRCA1 affects the expression and localization of Cdc25C, a downstream target of CHK1 [40]. Inhibitors of CHK1/2 abrogate normal cell cycle arrest induced by their activation, thereby preventing the repair of DNA damage (Figure 3). Altering the function of the checkpoint kinases may directly or indirectly impact BRCA1 function and thus may be a suitable target for therapy in EOC.

Husain et al. found that the CHK inhibitor UCN-01, a staurosporine derivative, potentiated the cytotoxicity of cisplatin in a panel of ovarian cancer cells, with a notable increase in apoptosis [41]. Furthermore, the cytotoxic effect was more pronounced in *p53*-wildtype cells. A phase I clinical trial of UCN-01 in combination with topotecan was performed in patients with advanced solid tumors, including a significant proportion of EOC [25]. This treatment combination demonstrated some efficacy and overall was well tolerated. However in the Phase II trial examining the same treatment in patients with advanced recurrent ovarian cancer, no significant antitumor effect was seen [26]. The efficacy of CHK inhibitors in the context of BRCA1 expression levels in EOC has not been examined, but warrants investigation due to the interaction between CHK1/2 and BRCA1 in the DNA damage cascade.

6. Proteasome Inhibitors

BRCA1 is known to have a role in the ubiquitin-proteasome proteolysis pathway, whereby damaged and misfolded proteins are tagged with a polyubiquitin chain and targeted for ATP-dependent degradation by the 26S proteasome [42]. *BRCA1* contains a zinc ring finger domain in its amino-terminal region which has E3 ubiquitin ligase activity and aids in the transfer of ubiquitin to the target substrate. Mutations in the RING finger domain of *BRCA1* are thought to predispose to the development of cancer because they abrogate ubiquitin ligase activity [43]. It has been suggested that this particular function of BRCA1 may be critical to the DNA recognition and repair process. As such, inhibitors of the ubiquitin-proteasome pathway may offer an alternative therapeutic option in EOC, as inhibition of this pathway may result in defective repair of DNA damage (Figure 3).

Proteasome inhibitors include compounds such as peptide aldehydes, boronates, and epoxyketones as well as β-actones, which prevent the degradation of ubiquitinated proteins. Several groups have demonstrated that the treatment of ovarian cancer cell lines with a proteasome inhibitor in combination with cisplatin treatment increased the cytotoxicity of platinum drugs, increased DNA damage and inhibited repair. Mimnaugh et al. pretreated ovarian cancer cells with either ALLnL or lactacystin proteosome inhibitors prior to cisplatin treatment and observed an abrogation in the expected increase in excision repair cross-complementation group 1 (ERCC1) expression with cisplatin and more efficient apoptosis [44]. The same group also evaluated the combination treatment of the proteasome inhibitor lactacystin with cisplatin in cisplatin-resistant ovarian cancer cells [45]. They observed the suppression of ERCC1 expression and inhibition of DNA repair with resultant enhanced cisplatin cytotoxicity. In addition, the proteasome inhibitor ALLnL was used in combination with cisplatin treatment in A2780s and A2780cp ovarian cancer cells, a cisplatin sensitive/resistant pair, and OVCAR3 cells [46].

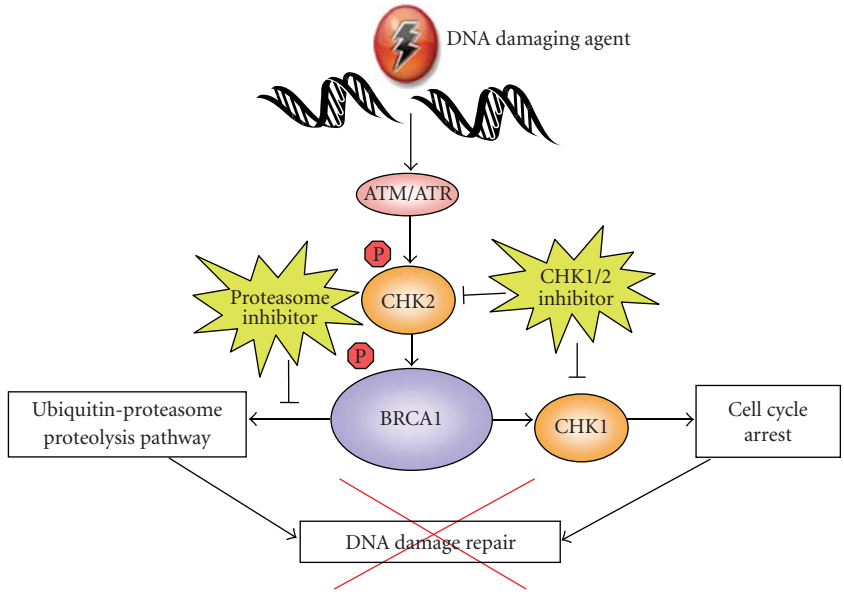

Figure 3: CHK1/2 and proteasome inhibition cause defects in the DNA damage repair pathway. CHK1 and CHK2 function to arrest the cell cycle when DNA has been damaged, thus allowing time to repair the DNA lesions. CHK inhibition leads to continued cell cycling in the presence of damaged DNA and is thought to alter the function of the DNA repair pathway.

They again found that cisplatin sensitivity was increased in all of the cell lines, along with an increase in DNA damage and defect in repair. They also noted a significant increase in the accumulation of cisplatin in the cells and a reduction in cisplatin efflux. Recent work by Jandial et al. has shown that the reduction of cisplatin efflux in ovarian cancer cotreated with the proteasome inhibitor, PS-341, is due to the prevention of cisplatin-induced downregulation of the copper transporter 1 (CTR1), a major transporter of platinum drugs [47].

Based on the preclinical results, proteasome inhibitors, namely, PS-341, have entered into clinical trials, including studies in EOC. In a Phase I trial examining a combination of carboplatin and PS-341 in recurrent EOC, an overall response rate of 47% was observed, including two patients demonstrating a complete clinical response, one of whom had platinum resistant disease [27]. Another recent Phase I trial evaluated PS-341 in combination with carboplatin in ovarian cancer patients with recurrent and platinum/taxane-resistant disease [28]. The drug combination was well tolerated, with just under half of the patients demonstrating stable disease. There are currently Phase II clinical trials assessing PS-341 either as a single agent or in combination with other therapies in ovarian cancer presently underway (Tables 1 and 2) [29, 30]. The combination of proteasome inhibitors to platinum chemotherapy taking account BRCA1 mutation status or expression levels is a potential area of future study in EOC.

7. BRCA1 as a Biomarker in EOC

A range of preclinical studies using both in vitro and in vivo models have supported the association of low BRCA1 mRNA and protein expression with an enhanced sensitivity to platinum agents [48, 49]. An ovarian cancer cell line SKOV3, which expresses high levels of BRCA1, was the model used to assess the role of BRCA1 in cisplatin sensitivity. Husain et al. depleted BRCA1 levels by an antisense inhibition approach to sensitize SKOV3 cells to cipslatin [48]. Zhou et al. used a retrovirus-mediated siRNA interference approach to show similar results [50]. Another group used both BG-1 and OVCAR5 ovarian cancer cell lines that were either stably transfected with a BRCA1 antisense construct or transiently transfected with a siRNA against BRCA1 [49]. The cells that displayed reduced BRCA1 expression were more sensitive to platinum agents than their empty vector and scrambled oligonucleotide controls. Using two different models to target the inactivation of Brca1 in mouse ovarian surface epithelial cells, an increase in chemosensitivity and an enhanced apoptotic response to cisplatin in the absence of Brca1 in this tissue was reported, suggesting that this phenomenon is also present in normal cells [51, 52]. Furthermore, in the ID8 mouse EOC cell line, Brca1 expression has also been shown to mediate sensitivity to platinum agents [53].

EOC patients with germline mutations in the *BRCA1* tumor suppressor gene have an improved initial response to treatment with platinum-based chemotherapy regimens and have an improved overall survival [54]. Several studies have also shown a reduction in BRCA1 expression in sporadic EOCs compared to normal ovarian tissue, as assessed by methods such as immunohistochemistry (IHC), loss of heterozygosity, mRNA levels, and hypermethylation of the *BRCA1* promoter [55]. Recent data also indicates that BRCA1 levels may be predictive of response to treatment and overall survival in sporadic EOC. In the largest study

of 230 patients with sporadic EOC, Thrall et al. analyzed BRCA1 protein expression by IHC and found that decreased BRCA1 expression was protective for survival [56]. IHC was performed on formalin-fixed-paraffin-embedded (FFPE) samples with the mouse monoclonal antibody specific to the amino-terminal of the BRCA1 protein. The study was scored based on previously published methods used in breast cancer [57], with a score of 0–4, based on the number of cells stained in a field of view. Several other groups have also assessed BRCA1 protein levels via IHC in sporadic EOC in smaller sample sizes, with reduced expression found in between 34% and 90% of tumors [58–60]. The studies by Wang et al. and Zheng et al. followed the same experimental conditions as Thrall's group in terms of using FFPE samples and the same BRCA1 antibody epitope. Russell's study performed IHC on flash frozen sections and they used six different antibodies ranging in epitope from the amino to the carboxy terminus. With this rigorous approach, they were able to find reduced or absent BRCA1 protein expression in 90% of their cases. However, it must be considered that such a wide range of results may be attributed to variations in inclusion criteria as well as the relatively subjective nature of IHC scoring.

The group of Quinn et al. was the first to report that decreased BRCA1 mRNA expression by quantitative RT-PCR in tumors from patients with sporadic EOC who received platinum-based chemotherapy was predictive of an improved overall survival [49]. Our recent study substantiates these results by showing that lower BRCA1 expression predicts for longer overall survival, especially in patients who were optimally debulked <2 cm at the time of staging laparotomy [34]. While RNA analysis is a more quantitative approach, it not only requires the availability of frozen tissue, but RNA extraction is a more time-consuming approach than IHC on samples which are processed on a tissue microarray. Furthermore, unless the mRNA analysis has been done from microdissected samples, the tissue sample itself may be a mixture of heterogeneous tissue including normal, nonmalignant tissue.

A consistent finding in the few studies on human EOC tumors is that BRCA1 mRNA and protein are frequently expressed at low levels within cell nuclei relative to the commonly used positive tissue control MCF7, a breast cancer cell line. This may represent a potential challenge in utilizing BRCA1 as a clinically useful predictive marker. Distinguishing "high" expressors from "low" expressors can be particularly difficult, especially at the protein level via IHC, where the scoring method is usually qualitative. Quantitative methods such as analysis of mRNA levels may provide more accurate results and could facilitate differentiating between true high and low expressors in a population where baseline levels are low. Considerable success using this method has been achieved in the use of BRCA1 as a predictive marker in nonsmall cell lung cancer (NSCLC) [61].

8. Conclusion

The array of cellular processes in which BRCA1 plays an integral role offers several mechanisms by which its function could be targeted for the treatment of EOC. All of these options take advantage of the weakness that is central in a BRCA1-deficient cell, the inability to effectively repair damaged DNA. As a result, the therapies outlined in this review offer promise not just in *BRCA1* mutation-associated EOC, but to the large proportion of patients with sporadic disease with tumors that display BRCA1 deficiency due to epigenetic changes. Furthermore, as BRCA1 shows promise as a prognostic and predictive marker in sporadic EOC, patients identified as being high expressors could be treated with agents that downregulate BRCA1, thus sensitizing them to standard therapies. Further work, both in vitro and in clinical trials, is needed to assess the correlation between BRCA1 expression levels and response to these potential targeted therapies.

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Review Article

Targeting Insulin and Insulin-Like Growth Factor Pathways in Epithelial Ovarian Cancer

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Ovarian cancer is the most lethal of all gynecological malignancies, due in part to the diagnosis at an advanced stage caused by the lack of specific signs and symptoms and the absence of reliable tests for screening and early detection. Most patients will respond initially to treatment but about 70% of them will suffer a recurrence. Therefore, new therapeutic modalities are urgently needed to overcome chemoresistance observed in ovarian cancer patients. Evidence accumulates suggesting that the insulin/insulin growth factor (IGF) pathways could act as a good therapeutic target in several cancers, including ovarian cancer. In this paper, we will focus on the role of insulin/IGF in ovarian cancer tumorigenesis and treatment.

1. Introduction

Ovarian cancer is the leading cause of death among all gynecological cancers in western countries. When compared to other gynecological cancers, the fatality rate of ovarian cancer surpasses that of cervical and endometrial cancers put together [1]. This high death rate is due to the diagnosis at an advanced stage in most patients caused by the relative lack of specific signs and symptoms of the disease and the lack of reliable tests for early detection. It is estimated that this year in North America, 24 150 women will be newly diagnosed with ovarian cancer and that 17 220 women will die of the disease [2]. Epithelial ovarian cancer (EOC) constitutes 90% of ovarian malignancies and is classified into distinct histologic categories including serous, mucinous, endometrioid, clear cell, transitional, mixed, and undifferentiated subtypes [3]. Nowadays, data suggest that the cell of origin for an important proportion of high-grade pelvic serous carcinomas, including the ovary, is derived from the distal fallopian tube [2].

Although most patients with EOC experience a reasonable initial clinical response to debulking surgery and chemotherapy, the majority of these patients will not be cured. Approximately 70% will experience a recurrence and this chemoresistance is responsible for the majority of ovarian cancer-related deaths [4]. Presently, there are no available treatments capable of curing recurrent ovarian carcinomas due to their rapid evolution into a chemoresistant disease. It has therefore become essential to introduce new therapeutic modalities that will change response to treatment into cure and salvage these patients. Over the last decade, accumulating data suggest that the insulin/IGF pathway might be one such good therapeutic target in cancers, including ovarian cancer. In this paper, we intend to review the role of insulin/IGF pathway in ovarian cancer and the various strategies to target it.

2. Physiological Roles of Insulin and Insulin-Like Growth Factor

Insulin and Insulin-like growth factor (IGF) signaling regulates cellular growth, proliferation, metabolism, and survival. Insulin was discovered in 1922 and is a crucial regulator of metabolic pathways. It is under the tight control of blood glucose levels and is excreted by the pancreas solely in periods of rising blood glucose levels [5]. When released by the beta-cells of the pancreas, insulin binds to receptors

on the surface of most cells. Hepatocytes, adipocytes, and muscle cells are classic insulin responsive cells and express high levels of insulin receptors. Insulin is primarily involved in regulating metabolism but was also shown to have a mitogenic effect [6]. On the other hand, IGF signaling plays a fundamental role in regulating embryonic growth and regulates specific differentiation in most adult tissues [7]. IGF is a major downstream target of growth hormone (GH) and is essential for regulating growth and body size both in the prenatal and postnatal stage [8]. The insulin and IGF-I receptors, though separate gene products, are structurally very similar. In addition, insulin and IGF-I are closely related peptides. Amino acid similarities range between 40 and 85% in different domains with the highest degree of homology being found in the tyrosine kinase domain [9].

Interestingly, the expression, signaling mechanisms, and roles of members of the insulin/IGF family such as ligands, receptors, binding proteins, and binding protein proteases and their inhibitors have been elucidated in ovarian follicle function in humans and other species. In vitro studies and genetic approaches using mouse knockout models for IGF family members have revealed that IGFs are key intraovarian regulators of follicular growth, selection, atresia, cellular differentiation, steroidogenesis, oocyte maturation, and cumulus expansion [10]. Some of these actions are synergistic with gonadotropins, although most are not sustainable with IGFs alone and require gonadotropin actions. In fact, IGFs are designated as copartners of gonadotropins. Moreover, recent studies demonstrate that endocrine-disrupting chemicals can compromise IGF activity and signaling in the ovarian follicle, affecting follicular development, steroidogenesis, and oocyte quality. The successful development of a healthy oocyte and appropriate granulosa and theca cell steroidogenesis on a cyclic basis are contingent on multiple factors, including a properly functioning of intraovarian IGF system [11]. Disruption of even one component of this system can lead to abnormal follicular development and function. Interaction of the IGF system with other growth factor systems and ovarian peptides during follicular development is still in early investigative stages.

3. Insulin and IGFs Structure and Signaling

3.1. Insulin and IGF Ligands. Insulin/IGF signaling system is comprised of three ligands, IGF-I, IGF-II, and insulin itself. These ligands interact with at least four receptors: the type I IGF receptor (IGF-IR), the type II IGF receptor (IGF-IIR), the insulin receptor (IR), hybrid receptors of IGF, and insulin [12]. The circulating and biologically active form of insulin ligands is a monomer consisting of two chains, an A chain of 21 amino acids and a B chain of 30 amino acids linked by two disulfide bridges [13]. On the other hand, IGFs are small, single-chain polypeptide ligands (7-8 kD) that are derived from prepropeptides in a similar way to insulin, but contain the C-peptide bridge between B and A chains that is normally cleaved in insulin [14]. The mature IGF-I and IGF-II peptides consist of B and A domains that are homologous to B and A chain of insulin.

3.2. Insulin and IGFs Receptors and Signaling. Insulin action is mediated through its receptor. The IR is a heterotetrameric protein consisting of two extracellular α-subunits and two transmembrane β-subunits. The binding of ligand to the α-subunits of IR stimulates the intrinsic tyrosine kinase activity of the β-subunits of the receptor [15]. The ability of the receptor to autophosphorylate and phosphorylate intracellular substrates is essential for the mediation of the complex cellular responses to insulin. The activated IR tyrosine kinase phosphorylates several immediate substrates including insulin receptor substrate proteins (IRS1-4), DOK4, DOK5, SHC, Gab1, Cbl, APS, and signal regulatory protein family. These adaptor proteins provide an interface between the activated receptors and the downstream-located effector molecules. Insulin activates the mitogenic (via MAP kinases and Erk1/2) and metabolic branches of insulin signaling, the latter involving PI3 kinase, PKB/Akt, mTORC1, p70S6 kinase, as well as PLCγ [16–18]. There are two isoforms of IR that are involved in different cellular functions. These two isoforms of IR are generated by alternative splicing of exon 11, giving rise to the B-isoform (IR-B) and A-isoform (IR-A) [19]. They are expressed in a developmentally specific manner, with high expression of IR-A in fetal tissues and IR-B in adult tissues. Moreover, IGF-II binds IR-A with high affinity whereas IGF-I does not [20, 21].

The IGF-I and IGF-II ligands interact with an array of cell receptors that may be present singly or in various combinations on target cells. IGF-I has a twofold higher affinity for the IGF-IR than for the IR, most of the effects of IGF-I result from activation of the IGF-IR. IGF-I and IGF-II interact with the IGF-IR, a transmembrane tyrosine kinase that is structurally and functionally related to the IR [21, 22]. Homology between IR and IGF-IR ranges 45–65% and 60–85% for the ligand binding, tyrosine kinase, and substrate recruitment domains, respectively [23]. Ligand binding of IGF-I or IGF-II to IGF-IR results in a conformational change leading to transphosphorylation of one β-subunit by the other. Activated IGF-IR recruits and phosphorylates adaptor proteins belonging to the insulin receptor substrate (IRS) family or SHC. The phosphorylated adaptor proteins then serve as docking sites for other signaling molecules, resulting in the activation of the downstream pathways. The IGF-1R plays a central role in integrating signals of nutrition and stress into energy shifts from energy expensive anabolic processes such as growth and reproduction [12, 24].

IGF-IIR is a multifunctional receptor that lacks an intracellular signaling domain. It is known as the cation-independent mannose-6-phosphatase receptor that binds to a diverse group of mannose-6-phosphatase tagged proteins for endosomal trafficking and degradation by the lysosome. The IGF-IIR or the cation-independent mannose-6-phosphate receptor binds IGF-II and causes internalization and subsequent clearance by the lysosome. IGF-IIR is involved in the regulation of the extracellular concentration of IGF-II [25].

Furthermore, many cells and tissues have hybrid receptors assembled with one chain of the IGF-IR and one of the IR. IGF-IR/IR-B hybrids have higher affinity for IGF-I whereas IGF-IR/IR-A hybrids have equal affinity for IGF-II

and insulin. Insulin binding to hybrid receptors initiates similar cellular responses as when binding to IR or IGF-IR. In both cases, ligand binding to their receptors will stimulate the activity of their intrinsic tyrosine kinase [26, 27]. However, the exact role of hybrid receptors in signaling needs further investigation.

3.3. Insulin-Like Growth Factor Binding Proteins (IGFBPs). The IGFs action is under the control of six binding proteins. IGFBPs are a family of secreted proteins that bind IGFs with equal of greater affinity than to IGF-IR. Six designated IGFBPs (1–6) have been isolated and characterized so far in human and in a variety of vertebrate species. These IGFBPs, with apparent molecular mass of 24–45 kDa, share a common domain organization. All of them have a highly conserved N-terminal domain, a conserved C-terminal domain, and a variable central linker domain. Most IGFBPs function as carrier proteins for circulating IGFs and regulate IGF turnover, transport, and tissue distribution, thus determining the physiological concentration of IGFs. Another important role of IGFBPs may be to help in the storage of IGFs in the extracellular matrices of certain tissues [28].

IGFBPs are produced by a variety of biological tissues and are thus found in various biological fluids. Although all six known IGFBPs belong to the same gene family, several features distinguish IGFBPs from each other. IGFBP-1, IGFBP-2, IGFBP-4, and IGFBP-6 inhibit IGFs actions by preventing their binding to IGF receptors. In the circulation, IGF-I and IGF-II are mainly bound to IGFBP-3, which is the most abundant IGFBP in serum. Moreover, IGFBP-3 was found not only to regulate the mitogenic actions of IGFs but also to inhibit their antiapoptotic effect. Intriguingly, IGFBP-3 has been localized in the nucleus, implying a more direct transcriptional regulatory role, but the way extracellular IGFBP-3 enters the cell remains largely unknown. IGFBPs bind to IGF-I and IGF-II with the same affinity as the latter do with IGF-IR [29, 30]. Under different physiological conditions, the IGFBPs can either increase or decrease IGF signaling, probably related to the fact that IGFBPs can prolong the half-lives of IGFs but also can compete with receptors for free IGF-I and IGF-II. However, IGFBP-1, IGFBP-3, and IGFBP-5 can also mediate their effects on the target cells by an IGF independent pathway [31]. Table 1 summarizes the physiological roles of each insulin/IGF family members.

An additional important variable is the presence of specific IGFBP proteases. IGFBPs have been reported to be proteolytically degraded by a variety of serine and matrix metalloproteases. Proteolytic activity has been described for IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5. Since the IGFBP fragments that are generated bind IGF-I weakly or not at all, proteolysis is believed to play an important role in controlling the bioavailability of IGF-I to receptors at the cellular level. Although fragments that are generated usually have reduced affinity for the IGFs, the cleavage of IGFBP-3 generates a 30-kDa fragment with relatively intact affinity for IGF-II [32]. This raises the possibility that these proteases may function to release IGFs, making them available to bind to receptors. Overall, the bioavailability and biological activity of IGFs are modulated by these IGFBPs and their proteases.

4. Insulin/IGFs in Human Cancers

IGF ligands, receptors, and IGFBPs have been shown to play a critical role in the development and progression of human cancers. Elevated plasma concentrations of IGF-I or IGFBP-3 have been linked to a high risk for several types of cancers including breast, prostate, and lung cancer [33–35]. In addition, the expression levels of the IGF-IR and IR are predictive of breast cancer outcome. Several studies have also reported that inhibition of IGF-IR reduces metastasis of various cancer cells emphasizing the importance of IGF signaling in cancer progression. IGF/IGF-IR have been studied extensively in metastatic colon, pancreatic, prostate, and breast cancer [21, 36]. In many human cancers, there is a strong association with dysregulated insulin/IGF signaling pathway that has been extensively reviewed. However, the role of insulin/IGF in ovarian cancer warrants further description.

5. Components of the IGF Axis Expression in Human Ovarian Cancer Risk

The first study showing the expression of IGF-I mRNA in ovarian cancer cells and tissues was published back in 1991 by Yee et al. [37]. They also reported several IGFBPs and the IGF-IR expression by ovarian cancer cells. This study suggested that all necessary components for an IGF-I-mediated autocrine loop are present in ovarian cancer cells, an observation that was also confirmed in one of our early studies using the OVCAR-3 cell line [38]. Two other groups described the expression of the IGF and insulin receptors in ovarian tumors [39, 40]. During the same period, it was reported that IGF-I levels were higher in cyst fluid from invasive malignant neoplasms compared to benign tumors [41]. Later, another group confirmed the presence of the IGF-IR expression by immunohistochemistry (IHC) in 100% of the ovarian carcinomas samples tested [42]. These initial studies opened the door to a widespread area of research in ovarian cancer, indicating an involvement of the insulin/IGF system in ovarian tumorigenesis.

5.1. Tissue Expression of the Insulin/IGF System in Ovarian Cancer. A strong support for a role of IGF-I in ovarian cancer progression came from a recent study by Brokaw et al., who showed that high free IGF-I protein expression in ovarian tumor tissue was independently associated with the progression of ovarian cancer [43]. Moreover, IGF-I mRNA expression was also associated with disease progression, implying that both endocrine and paracrine/autocrine regulations of IGF-I activity are involved in ovarian cancer [43]. Similarly, microarray expression profiles from 64 EOC patients demonstrated that individual genes including IGF-I, IGF-IR, and several genes downstream of the receptor were overexpressed in tumors associated with an unfavorable prognosis [44].

Table 1: Normal physiological role of insulin/IGF family members.

	Origin	Gene regulation	Serum levels	Affinity for insulin/IGF family members	Function
Insulin	Beta cells of the pancreas	Glucose uptake, protein synthesis	11–14 μU/mL	IR >IGF-IR> IGF-IIR	–Regulates carbohydrate, fat, and protein metabolism. –Mitogenic effect.
IGF-I	Liver, bone, and several other tissues.	Hormones, growth factors, cytokines, nutrition, smoking, exercise.	100–200 ng/mL	IGF-IR> IGF-IIR > IR	–Regulates embryonic growth and specific differentiation in adult tissues. –Involved in cell proliferation, transformation, and antiapoptotic activity.
IGF-II	Liver, kidney, bone, and several other tissues.	Tumor suppressor proteins WT1 and p53, HIF-1, genomic imprinting.	400–700 ng/mL	IGF-IIR> IGF-IR>IR	–Functional during embryonic and fetal growth.
IGFBPs	IGFBP-1: liver, decidua. IGFBP-2: CNS. IGFBP-3: several tissues.	BP-1: insulin, steroids. BP-2: insulin, metabolic process. BP-3: GH, PTH, cytokines, p53, estradiol, steroids.	BP-1, BP-2: vary during the day and meals.	BP-1, BP-3, BP-5: IGF-I> IGF-II	–Regulate transport and half-life of IGFs between different body compartments.
	IGFBP-4: bone, CNS, prostate. IGFBP-5: kidney, bone, mammary gland. IGFBP-6: ovary, prostate.	BP-4: vitamin D, parathyroid hormone. BP-5: GH, prolactin, vitamin D. BP-6: GH, FSH.	BP-3: vary in relation to age and sex 1500–5580 ng/mL.	BP-2, BP-6: IGF-II>IGF-I	–Regulate IGF independent effect on cell proliferation and apoptosis.

Another member of the IGF family that seems to be involved in ovarian cancer is the IGF-II. It has been reported that IGF-II gene expression is increased more than 300-fold in cancer tissues compared to normal ovarian surface epithelium (NOSE) samples [45]. Interestingly, two studies showed that IGF-II is associated with disease progression, and proposed that it can be a predictor of poor survivals for patients with EOC [45, 46]. Recently, the protein expression of IGF-II mRNA-binding protein 3 (IGF2BP3, also known as IMP3) was reported to be an independent marker for reduced disease-specific survival in the rarely studied clear cell carcinoma subtype of ovarian cancer [47].

Finally, it was demonstrated that IGFBP-2 relative mRNA expression was 38-fold higher in ovarian cancer than in NOSE [48]. A concomitant elevation in serum IGFBP-2 was also observed in cancerous specimens, conveying the notion that IGFBP-2 might represent a novel biomarker for detection and/or monitoring of EOC [48]. In opposition to the above described studies, serum IGFBP-3 levels are decreased in patients with ovarian cancer [49] and low IGFBP-3 levels are associated with a higher risk for disease progression [50] and poor survival [51]. The studies mentioned above are detailed in Table 2.

5.2. Circulating Levels of the Insulin/IGF System in Ovarian Cancer. In the same order of idea, a lot of efforts were made to verify the use of certain components of the IGF system expression as predictive markers for ovarian cancer. Thus, IGFBP-2 levels were determined in the serum of EOC patients and found to positively correlate with cancer antigen 125 (CA125) [49], a widely used marker for ovarian cancer follow-up. Overall, in retrospective studies, lower IGF-I levels were found in serum of disease patients versus controls [41, 49, 52–55].

On the other hand, two recent prospective studies reported a higher ovarian cancer risk among women aged 55 or less at time of diagnosis when comparing the top and bottom tertile of IGF-I levels [56, 57]. However, in a recent nested case-control study using data from three prospective cohorts, namely, the Nurses' Health Study (NHS), NHSII, and the Women's Health Study (WHS), no significant positive association between IGF related proteins (IGFBP-2, IGFBP-3, and IGF-I) and ovarian cancer risk was found [58].

In general, studies aimed at determining an association between ovarian cancer risks and circulating IGF concentrations have been few and inconsistent [59] (Table 3). Clearly more investigative efforts are needed to confirm the role of this hormone in ovarian cancer although biological evidence suggests a mitogenic role of insulin and IGF-I in the development of this disease.

6. Role of IGF Family in Ovarian Carcinogenesis: Proliferation, Angiogenesis, Invasion, and Metastasis

A primary study using ovarian cancer cell lines implicated IGF-II in cell adhesion and invasion through the stimulation of the extracellular matrix glycoprotein tenascin-C [60]. Later, accumulating evidence depicted a role for IGF-I in cellular proliferation, invasion, and angiogenesis. Firstly, Shen et al. demonstrated an induction of KCl Cotransport (KCC) in response to IGF-I in OVCAR-3 cells. This KCC was necessary for IGF-I-induced cancer cell invasiveness and proliferation [61].

Next, the induction of cell invasion and proliferation by IGF-I occurred through phorpshorylation of AKT and ERK1/2 in human ovarian cancer cells HRA [62]. IGF-I also induced cyclooxygenase-2 (COX-2), a crucial player in tumor angiogenesis, partly by enhancing vascular endothelial growth factor (VEGF) production [63]. This elevation of COX-2 expression was followed by an augmentation of prostaglandins E_2 (PGE_2) biosynthesis and was associated with the activation of PI3K, MAPK, and PKC pathways. Finally, IGF-I and insulin stimulated the migration of SKOV-3 cells by favoring the urokinase-type plasminogen activator (uPA) over the plasminogen activator inhibitor-1 (PAI-1) through the PI3K/AKT pathway [64]. An induction of uPA is linked to a poor prognosis and correlates to a more aggressive phenotype of ovarian cancer [65–68]. As stated earlier, IGFBP-2 is overexpressed in ovarian malignant tissues and in the serum and cystic fluid of ovarian cancer patients [41, 48, 49, 69], indicating a role in the biology of ovarian cancer. Indeed, it was reported that IGFBP-2 stimulated the invasion of SKOV-3 cells using the Matrigel invasion assay, an effect reversible by an attenuation of its expression by small interference RNA (siRNA) [70].

On the contrary, two IGFBPs seem to have a suppressing effect on invasion, metastasis, and angiogenesis. Interestingly, it was recently shown that IGFBP-3 inhibited cell migration, invasion, and metastasis in the human ovarian endometrioid carcinoma cell line OVRW59-P4 [51], an observation that correlates with the low levels of IGFBP-3 expression in high tumor grade, advanced stage, and poor survival in endometrioid carcinoma and EOC patients [50, 51]. IGFBP-5 function in angiogenesis was also studied in a xenograft model of ovarian cancer. IGFBP-5 expression prevented tumor growth and tumor vascularity, indicating a tumor suppressor role in ovarian cancer [71].

7. Development of Inhibitors of the Insulin/IGF-I Pathways

The strategies to target IGF in cancer consist of (1) reducing circulating ligand levels or bioactivity, (2) blocking receptor function using receptor-specific antibodies or small-molecule tyrosine kinase inhibitors, and (3) activating AMP-activated protein kinase (AMPK) (see Figure 1).

7.1. Ligand-Targeted Approach. The first-generation strategies that included the use of somatostatin analogues to diminish circulating IGF-I levels were unsuccessful [7]. It was reported in one of the largest clinical trials that the suppression of ligand levels was not achieved using this approach [72], suggesting a failure of this particular strategy rather than an evidence of a wrong targeting [7, 73]. This targeting strategy has never been tested in ovarian cancer.

Table 2: Tissue expression modulations of the insulin/IGF system in ovarian cancer.

Insulin/IGF components	No. of patients	Modulation	Reference
Free IGF-I mRNA and protein	215 EOC	↑	[43]
IGF-I, IGF-IR mRNA, and several genes downstream of the receptor	64 EOC	↑	[44]
IGF-II mRNA	109 EOC	↑	[45]
IGF-II mRNA	215 EOC	↑	[46]
IGFBP3 protein	128 clear cell carcinoma	↑	[47]
IGFBP-2 mRNA	113 EOC	↑	[48]
IGFBP-3 protein	147 EOC	↓	[50]
IGFBP-3 protein	35 endometrioid carcinoma	↓	[51]

EOC: epithelial ovarian cancer.

Table 3: Circulating protein levels of the insulin/IGF system in ovarian cancer.

Insulin/IGF components	No. of patients	Modulation	Reference
IGFBP-2	20 EOC	↑	[49]
IGF-I	58 EOC	↓	[53]
IGF-I	24 EOC	↓	[52]
IGF-I	59 EOC	↓	[54]
IGF-I	9 EOC	↓	[55]
IGF-I	132 EOC (<55 yrs.)	↑	[56]
IGF-I	214 EOC (< 55 yrs.)	↑	[57]
IGF-I, IGFBP-2, IGFBP-3	222 EOC	↔	[58]

EOC: epithelial ovarian cancer.

7.2. Receptor-Specific Antibodies. These agents have been designed to be highly specific for the IGF-IR; that is, they do not bind to the insulin receptor. As described earlier, there exist hybrid receptors whose expression depends on the relative expression of the genes encoding the IGF-I and insulin receptors [73]. Based on this theory of "half receptors," the novel antibody drug candidates have been designed to act against IGF-IR and hybrid receptors. Many have been studied in preclinical models and about a dozen are being evaluated in clinical trials simultaneously [7, 73, 74].

The first study targeting IGF-IR in ovarian cancer was published in 2003 by Hongo et al., in which they used a soluble form dominant negative of the type I IGF-IR designated 486/STOP in CaOV-3 cells [75]. This soluble IGF-IR is a truncated receptor at the 486th amino acid, located within the extramembranous α-subunit. They showed that the 486/STOP expression could reverse transformed phenotype of the CaOV-3 in vitro and inhibit tumorigenicity in vivo. Likewise, the administration of the 486/STOP recombinant protein retarded the tumor growth of CaOV-3 cells in vivo.

Simultaneously, another group tested an antagonistic monoclonal antibody designated EM164, specific to the IGF-IR, in various cancer cell lines, including ovarian cancer [76]. They demonstrated a reduction of IGF-I-stimulated proliferation and survival of the human ovarian cancer OVCAR-5 cells.

7.3. Receptor Kinase Inhibitors. Small molecule inhibitors block IGF-IR activation by binding to the ATP-binding pocket of the receptor [77]. Most of the developed tyrosine kinase inhibitors have the side effect of attenuating insulin receptor signaling as well. However, despite this lack of specificity, they were found to be active in preclinical models and some are being evaluated in clinical trials [24, 74, 78]. There is a possibility that these agents might be more potent anticancer drugs since insulin receptor present on malignant cells may have an important role as well in carcinogenesis [7].

In the last couple of years, studies targeting IGF or insulin pathways in ovarian cancer mostly used small molecule IGF-IR kinase inhibitors. Indeed, our group reported an inhibition of cell survival in response to NVP-AEW541 in two human epithelial ovarian cancer cell lines, namely, OVCAR-3 and OVCAR-4 [38]. Interestingly, this effect was not reversible by the addition of recombinant IGF-I. We further demonstrated that this inhibitor sensitized cells to the effect of cisplatin, an effect described in other types of cancer cells as well [77]. This observation is relevant to the clinical application of the drug. Finally, NVP-AEW541 induced apoptosis and decreased AKT activation. We also performed a preliminary in vivo study using this small-molecule inhibitor in a human ovarian cancer xenograft model that gave promising results [79]. We confirmed our in vitro results using another IGF-IR kinase inhibitor produced by Bristol-Myers-Squibb, BMS-536924. BMS-554417 is a derivative of BMS-536924 and shares the same properties. Using the OV202 cells, Haluska et al. showed an antiproliferative effect of BMS-554417 at an IC50 of 7,5 μM [80]. Moreover, the drug inhibited the phosphorylation of the IGF-IR, insulin receptor, AKT, and ERK1/2 and also induced apoptosis. In addition, treatment of OV202 with BMS-554417 stimulated

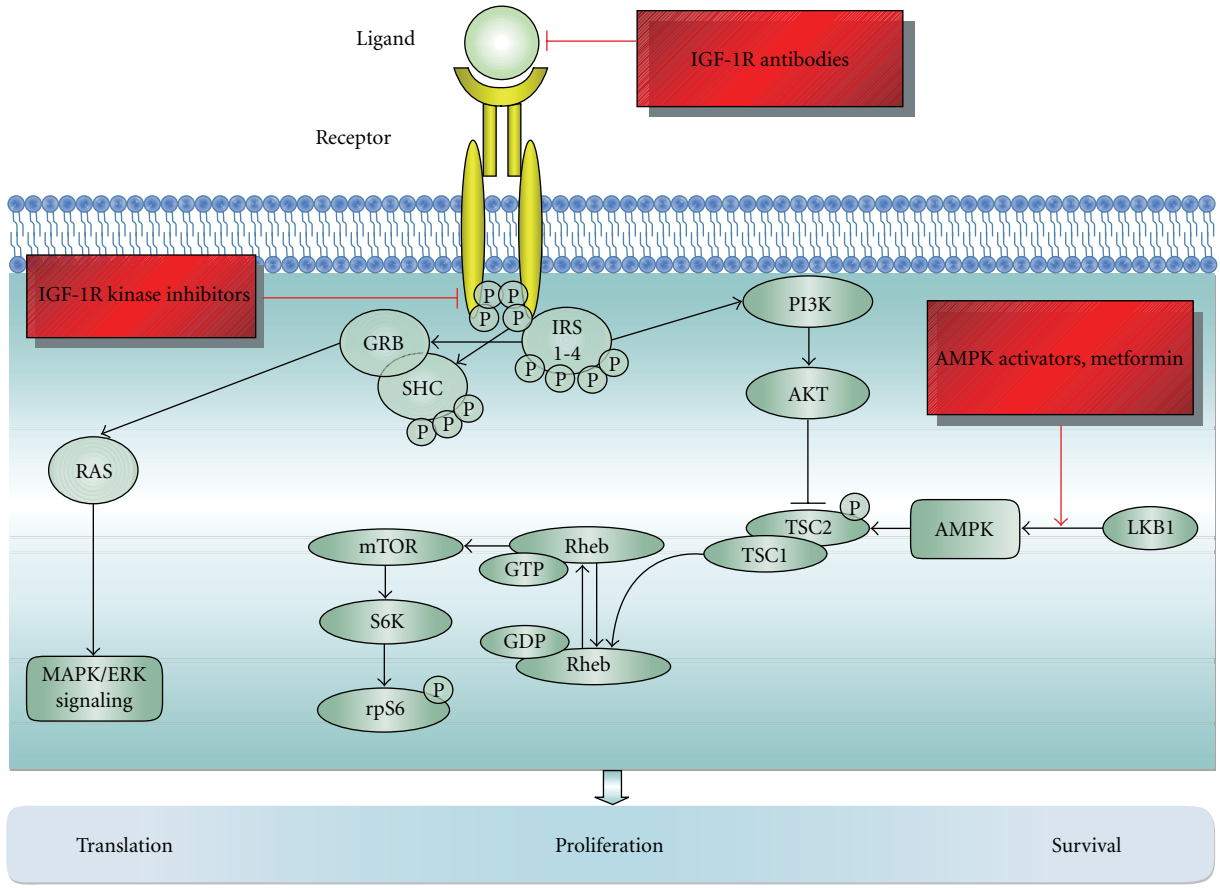

Figure 1: IR and IGF family signaling pathway. Upon the binding of the ligand, the activated receptor will undergo autophosphorylation and in turn will phosphorylate IRS and SHC. Activated IRS will recruit GRB to the phosphorylated form of SHC adaptor protein. The SHC-GRB complex will induce RAS and turn on the MAPK/ERK pathway, inducing cell proliferation and survival. Phospho-IRS will also stimulate the PI3 kinase to phosphorylate AKT thus initiating its downstream effectors such as mTOR, promoting translation, proliferation, and cell survival. Generally, activated AKT will have an inhibitory effect on TSC2, allowing Rheb-GDP to be converted to its GTP-bound state, thereby activating mTOR and its downstream signaling molecules to promote cellular translation. Three different potential targeted therapies are underway of investigation in ovarian cancer, including IGF-IR antibodies, IGF-IR kinase inhibitors, and AMPK activators such as metformin.

the phosphorylation of HER-2. Inversely, treatment with the pan-HER inhibitor increased the phosphorylation of IGF-IR, suggesting a reciprocal cross-talk mechanism [81]. Therefore, the combination of BMS-536924 and a pan-HER inhibitor resulted in a synergistic antiproliferative effect in various ovarian cancer cell lines. A concomitant reduction of AKT and ERK phosphorylation and apoptosis induction were also demonstrated. Furthermore, HER receptor expression could confer resistance to IGF-IR-targeted therapy using breast cancer cells expressing HER-1 or HER-2. This suggests that combining targeted therapies to the HER and IGF-I family of receptors might be an effective strategy to overcome potential clinical resistance to IGF-IR inhibitors.

Concurrently, we showed a dose and time-dependent growth inhibition of human epithelial ovarian cancer cell lines, the OVCAR-3 and OVCAR-4 in response to BMS-536924 [82]. This effect was partly mediated by AKT and the ribosomal protein S6. BMS-536924 provoked cell apoptosis as shown by the activation of PARP cleavage. We finally showed that this IGF-IR kinase inhibitor could sensitize cells to PARP inhibitors, possibly via the induction of DNA damage as indicated by the increased phosphorylation of histone H2AX. This study reinforced the concept that IGF-IR is a good therapeutic target in ovarian cancer. In addition, it proposes that combination therapy using BMS-536924 with a PARP inhibitor might be an effective strategy to circumvent resistance to treatment in clinical settings.

7.4. Metformin. Another potential drug targeting agent related to the insulin and/or IGF pathway is metformin. Metformin is an oral biguanide widely used since the 1950s for the treatment of type 2 diabetes, that lowers both circulating glucose and insulin levels. Two population studies provided preliminary evidence that metformin may reduce cancer risk and improve prognosis in patients with type 2 diabetes [83, 84]. Importantly, recent data demonstrated that the key mechanism of action of metformin is by activating the AMPK-LKB1 pathway [85, 86]. Other AMPK activators have been demonstrated to have growth inhibitory effects in various cancer cell types [87–89]. Therefore, metformin

might have two potential antineoplastic effects: reducing circulating insulin levels and directly inhibiting growth through the AMPK-LKB1 pathway.

We published the original study evaluating the antineoplastic effect of metformin in human epithelial ovarian cancer cell lines [90]. We demonstrated that metformin decreased in a dose and time-dependent manner ovarian cancer cells survival, an effect partly mediated by AMPK. Moreover, metformin potentiated the effect of cisplatin. The activation of AMPK by metformin was associated with an inhibition of downstream targets of AKT, such as phospho-p70S6 and phospho-S6. These findings led us to evaluate the potential applicability of metformin in the treatment of ovarian cancer by testing it in preclinical animal models. These experiments are currently underway of investigation in our laboratory.

Only two other recent studies showed a cytotoxic effect of other AMPK activators. The first one is C93, a synthetic fatty acid synthase inhibitor that increased AMP/ATP ratio in SKOV3 human ovarian cancer cells, thereby provoking AMPK activation and leading to cell toxicity [91]. Using compound C, a specific inhibitor of AMPK, the authors clearly implicated AMPK in the cytotoxic action of C93. Interestingly, these findings were confirmed in vivo in an SKOV3 xenograft mice model [91]. The second study provided evidence that curcumin caused CaOV3 ovarian cancer cell death through AMPK, suggesting that the latter is a new molecular target of curcumin [92].

7.5. Clinical Trials. To the best of our knowledge, only two clinical trials using targeted therapy against IGF-IR are currently ongoing in ovarian cancer patients (clinicaltrials.gov identifier: NCT00719212 and NCT00718523). Both studies are testing the same human anti-IGF-IR human monoclonal antibody, namely, the AMG-479 [74, 93] that was previously tested clinically in other types of cancer [94, 95]. The objective of the first study is to verify whether the addition of AMG-479 to paclitaxel and carboplatin in first line chemotherapy could improve the progression-free survival in patients with optimally debulked FIGO stage III and IV ovarian epithelial carcinoma. The second study aims to obtain an estimate of the objective response rate (ORR) of AMG-479 in patients with recurrent platinum-sensitive ovarian epithelial carcinoma failing frontline chemotherapy. The completion dates of both studies are estimated in 2015 and 2012, respectively.

8. Conclusion

All members of the IGF family are expressed in malignant ovarian epithelial cells. On the other hand, circulating levels of IGF have not been undoubtedly associated with ovarian cancer risk or disease progression. However, a role of some of the components of the IGF family, such as IGF-I and IGF-IR, has been clearly involved in ovarian tumorigenesis. In the past few years, various inhibitors of IGF-IR have been developed, including AMPK activators. These were tested in ovarian cancer in vitro and in vivo models, obtaining promising results for the potential of this targeted strategy in ovarian carcinoma, supported by the currently ongoing clinical trials.

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Review Article

The Role of Dysregulated Glucose Metabolism in Epithelial Ovarian Cancer

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Epithelial ovarian cancer (EOC) is the most lethal gynecologic cancer and also one of the most poorly understood. Other health issues that are affecting women with increasing frequency are obesity and diabetes, which are associated with dysglycemia and increased blood glucose. The Warburg Effect describes the ability of fast-growing cancer cells to preferentially metabolize glucose via anaerobic glycolysis rather than oxidative phosphorylation. Recent epidemiological studies have suggested a role for hyperglycemia in the pathogenesis of a number of cancers. If hyperglycemia contributes to tumour growth and progression, then it is intuitive that antihyperglycemic drugs may also have an important antitumour role. Preliminary reports suggest that these drugs not only reduce available plasma glucose, but also have direct effects on cancer cell viability through modification of molecular energy-sensing pathways. This review investigates the effect that hyperglycemia may have on EOC and the potential of antihyperglycemic drugs as therapeutic adjuncts.

1. Introduction

The poor survival statistics of epithelial ovarian cancer (EOC) are mentioned by way of introduction in almost all review literature pertaining to the disease. Unfortunately, in the past forty years there have been only small improvements in overall ovarian cancer survival rates. Specific challenges to the treatment of EOC include the problems of late detection, metastasis within the peritoneal cavity, drug resistance, and cancer recurrence even after initial response to treatment. Up to 90% of EOCs do not have an identified genetic component, and the development of specific and sensitive screening tools has proven elusive [1]. A metabolic approach to the targeted treatment of EOC has the potential to address many of the issues that make this the most deadly gynecologic cancer.

In recent years, it has been noticed that the influence of lifestyle, in particular the high-fat Western diet, is associated with the multisite development of cancers. The state of chronic positive energy balance is linked to a cluster of conditions including impaired glucose regulation and insulin resistance, collectively called the metabolic syndrome [2]. Hyperglycemia is a distinguishing feature of over-nutrition and it is believed to be an independent risk factor for cancer development. To provide an idea of the clinical importance of hyperglycemia, it is estimated that the incidence of type two diabetes mellitus (T2DM), a common consequence of the syndrome, will double in many regions in the next fifteen years. However, the burden of T2DM, where as many as one third of individuals are undiagnosed [3], almost certainly underestimates the true incidence of abnormal glucose homeostasis in the population. Given the emerging association between hyperglycemia and cancer, it is conceivable that there will be an increase in the incidence of EOC in the near future.

We hypothesize that hyperglycemia provides a nutrient-rich, growth signal-rich environment for epithelial ovarian cancer cells, where tumour formation and growth

is encouraged by free radical-induced DNA damage. We address possible cellular mechanisms by which a hyperglycemic environment may increase the rate of development of ovarian tumours, and discuss the implications for metabolically targeted EOC treatments.

2. Hyperglycemia and EOC: Epidemiological Evidence

While significant associations have been reported between elevated glucose [4, 5], glycemic load [6], T2DM [2, 7], and a number of cancers, there is little information to support the influence of preexisting hyperglycemia on EOC [8]. However, much of the literature relating cancer and glucose abnormalities comes from clinical or epidemiological studies that were not originally designed to evaluate the effects of hyperglycemia on cancer development [9]. This is a particular limitation when looking at EOC because of its relatively low population incidence. In addition, many of the studies used diabetic status or a single glucose measurement as a proxy for classifying glucose abnormalities, likely underestimating the true hyperglycemic population. The changing profile of insulin status over the course of T2DM [10] probably further obscured any associations and there was poor consideration of confounding variables such as insulin, obesity, medication, and time since diagnosis.

The design of these population studies presumed that hyperglycemia was a direct and sufficient cause of ovarian cancer, when it may in fact be more important in the growth promotion of previously transformed cells. In this way, endpoint analyses such as case-control or retrospective cohort studies would not be expected to show any association. A more useful consideration may be that of time to tumour development in patients with hyperglycemia. For example, in women already diagnosed with ovarian cancer, high glucose appears to be a poor prognostic factor [11]. A further complication of these studies is that both hyperglycemia and EOC are notoriously quiet diseases in their early stages. This makes it very difficult from a population health standpoint to infer an association, or suggest causality, as the underlying pathologies of both diseases begin and may interact well before diagnosis.

Although population-based studies have not been supportive for a role of preexisting hyperglycemia in the development of ovarian cancer, recent basic science still suggests that EOC may be subject to the influence of high blood sugar. The rate of glucose uptake, which increases with increasing extracellular glucose [12], has been linked with tumour aggressiveness [13]. EOC cells are also sensitive to complete glucose deprivation than nontransformed ovarian epithelial cells [14]; thus, they may also be very responsive to hyperglycemia.

3. Hyperinsulinemia versus Hyperglycemia

The impact of hyperinsulinemia on cancer has received much more research attention than the impact of hyperglycemia, although the two conditions are very closely related. It is well established that insulin promotes tumour growth. Insulin is mitogenic via its signaling through the insulin receptor and the insulin-like growth factor (IGF) pathways and direct anabolic signaling which is mediated by changes in the insulin receptor (IR) population. Expression of the IR is elevated in EOC, suggesting a tumour-promoting role in this cancer [15].

However, we contend that the specific impact of hyperglycemia on EOC is also an important area of research as abnormalities in glucose metabolism typically underlie hyperinsulinemia. Elevated insulin levels arise as a result of persistent hyperglycemia and peripheral insulin resistance. Thus, although insulin has direct, isolated actions on tumour growth, changes in glucose metabolism predispose changes in insulin signaling. In addition, it is becoming clear that there are insulin-independent mechanisms of glucose action on cancer risk, particularly through energy-sensing pathways and glucotoxic damage.

4. Hyperglycemia

4.1. Historical Perspective on Hyperglycemia and Cancer. Almost 80 years ago, Dr. Warburg observed that, compared to normal cells, cancer cells show a preference for glycolysis and lactate production over oxidative phosphorylation [16]. Because glycolysis is 18 times less efficient at producing ATP, this glycolytic switch suggests that cancer cells have an inherently high need for glucose. Furthermore, tumours are very active metabolically and require copious amounts of cellular fuel to meet growth demands. Aerobic glycolysis has been successfully exploited in EOC diagnostics in which tumour visualization occurs through the detection of the differential uptake of glucose in cancer cells compared to normal cells [17]. The use of FdG-PET (18-fluoro-2-deoxyglucose positron emission tomography) demonstrates the association between tumour growth and energy availability.

Glucose metabolism of tumours was studied extensively starting in the 1950s. Warburg's initial observation was bolstered by evidence that tumours could induce host hypoglycemia in a tumour mass-dependent fashion [18, 19]. In many tumour-bearing animals, there appeared to be host compensation for hypoglycemia at the level of the liver, with increased gluconeogenesis and glycogen mobilization [18]. Local hypoglycemia in the area around the tumour was particularly pronounced [18, 20]. It was found that while tumours had the capacity to take up larger volumes of glucose in mildly hyperglycemic environments they were poor at compensating for low blood glucose by increasing glucose uptake [18, 20]. An important role for the vasculature was identified in hyperglycemic conditions, as tumours were able to increase glucose uptake by increasing glucose transfer across the capillary walls [20].

Following these metabolic observations, a number of groups looked at the growth characteristics of tumours in hyperglycemic environments. It was reported widely that profound hypoinsulinemia usually caused by chemical destruction of pancreatic β-cells consistently caused a decrease in tumour growth [19, 21, 22]. The

hypoinsulinemia was generally associated with significant hyperglycemia. However, in diabetic animals, combined treatment of both antitumour and antihyperglycemia therapies gave the best tumour-reductive outcome [19].

Although they demonstrated a negative effect of hyperglycemia on tumour development, these early studies have a number of limitations. The large transplantable tumours used were sustainable in vivo only for several weeks. The alloxan used to induce diabetes was toxic and administered systemically, and so may have had effects outside the target endocrine cells within the pancreas. Also, the studies that showed a decrease in tumour mass in the diabetic animals did not report the changes with respect to total animal mass, which is generally smaller in the diabetic animals.

The studies also seem to make the assumption that all glucose taken up is immediately metabolized. However, it was noted independently by several groups that glucose uptake was too high to be fully explained by the amount of tumour growth [20, 23]. These results suggest the possibility that cancer cells may be able to store fuel in times of high abundance. Nigam et al. concluded that low glycogen was due to defective glycogen synthesis and reported low activities of key glyconeogenic enzymes phosphoglucomutase and glycogen synthetase as compared to normal tissues [24]. The low tumour glycogen was also linked to abnormally high rates of glycogen breakdown by phosphorylase. A recent article looking at glycogen levels in human colorectal cancer, however, reported that tumour cells actually had higher glycogen content than normal tissue [25]. The authors noted that there was less glycogen in poorly differentiated tumours compared to well-differentiated tumours, suggesting that low glycogen may be an indicator of a poor prognosis. They also found a very clear negative correlation between glycogen level and proliferation index [25]. The little research in this area has been carried out in normoglycemic conditions. It seems likely that, given the high rate of fuel usage in a tumour, at normoglycemic levels, there would be little need for storage as most would be used immediately. This brings up an intriguing question: could hyperfueled conditions favour a storage phenotype in cancer cells? This might explain the low growth rates of tumours in type one diabetic conditions.

Glycogen synthase kinase 3β (GSK3) phosphorylates and inactivates glycogen synthase, preventing the formation of glycogen. High levels of GSK3 have been implicated in the progression of a number of cancers, including ovarian cancer [26]. GSK3 affects tumour growth through many different mechanisms, including NF-κB and Wnt signaling activation [26]. Although it was not discussed in the literature reviewed here, GSK overexpression may be linked with glycogen storage and proliferation index. In summary, despite a number of investigations, carbohydrate metabolism by tumours is still poorly understood.

4.2. Hyperglycemia in EOC. We consider the possible effects of glucose on EOC development to be either "permissive" or "contributing". Permissive effects are those that alter the energy status of cells, allowing tumour cells greater access to fuel. Contributing effects are those that directly damage protein or DNA in some cancer-promoting way.

Persistent elevations in blood sugar occur once hypersecretion of insulin is no longer able to compensate for combined insulin resistance and high glucose levels. The failure of insulin to facilitate glucose entry into cells is evaluated on a continuum, meaning that patients may have significant pathological changes while being in a "prediabetic" state. In fact, by time of diagnosis of T2DM, hyperglycemia has already caused vascular complications in at least 20% of patients [3, 27]. However, poor glycemic control is not solely due to impaired insulin signaling, as glucose has the ability to regulate its own clearance by mass action [12]. Glucose self-regulation is impaired in people with hyperglycemia, leading to a state of glucose resistance [12]. Chronic hyperglycemia downregulates enzymes responsible for glucose metabolism, including those of the energy-sensing AMP-activated protein kinase (AMPK) pathway [28]. This results in fewer glucose transporters translocating to the cell surface, further impeding the cell's ability to take up fuel. Gluconeogenesis also appears to be increased in patients with already elevated blood sugar [29]. Thus, the effects of glucose join insulin resistance in maintaining and exacerbating hyperglycemia.

4.3. Permissive Effects of High Glucose: Energy Excess. It is postulated that where there is energy available tumour cells will have a suitable soil to grow. The biological plausibility of this excess energy hypothesis has been supported by a number of in vitro studies: Yamamoto et al. found that increasing glucose concentration in the culture media of MCF-7 breast cancer cells increased proliferation [30], mediated by an upregulation of cdk2 and cyclin D1 [31]. In a line of choriocarcinoma cells, sustained hyperglycemia was found to stimulate the cell's glucose transport system, increasing glucose uptake rates [32]. In contrast, most nontransformed cells downregulate glucose transport in the presence of hyperglycemia. Studies in human breast cancer xenografts also suggest that the amount of glucose metabolism is not determined by metabolic demand, but rather by substrate availability [33]. Conversely, energy restriction is protective in several cancer models [34]. Together, these findings support the idea that the fuel availability in hyperglycemia may be permissive for cancer growth.

In hyperglycemia-induced insulin resistance, the ability of normal cells to access fuel is impaired. The correlation between cancer risk and T2DM suggests that where normal cells fail metabolically cancer cells excel. Mechanistically, this may involve the overexpression of components of the AMPK pathway [35]. It is possible that in hyperglycemia cancer cells are inherently better at responding to the effects of insulin compared to insulin-resistant "normal" cells. In their 2004 paper, Gatenby and Gillies argue that mutations affecting substrate use cannot be early events in carcinogenesis because they would offer no advantage when there are no constraints on fuel availability, which typically arise in a larger tumour mass [13]. While this is true in a normal cellular environment, in hyperglycemia there is a limit on substrate availability because of insulin

resistance. Better access to the abundance of extracellular glucose, therefore, confers a selective growth advantage and could be an early marker of tumourigenic potential.

If conditions such as dysglycemia and diabetes prove to be involved in EOC initiation as well as promotion, then we propose that the selective pressures of the energy status may be an early event in the formation of EOC tumours. Cells that are best able to survive high glycemic conditions necessarily have a key characteristic of cancer cells, essentially obtaining self-sufficiency in growth signals [36]. Thus, cancers that arise in a hyperglycemic environment may represent an unregulated adaptive survival response. Although there is currently no directly supportive data for this hypothesis, possible mechanisms for this relationship are described in the following sections.

4.4. Contributing Effects of High Glucose: Cellular and Genetic Damage. The consequences of chronic exposure to high glucose tend to be detrimental to cellular function and affect the physiology of the normal ovary [37]. In fact, most long-term diabetic complications (retinopathy, neuropathy, and nephropathy) are consequences of hyperglycemia and cannot be reversed despite glucose normalization [38]. However, this damage might also provide a mutational advantage to some cells by altering cellular proteins or DNA. Cancer development is often thought of in terms of a series of "hits". The conditions of the tumour microenvironment, many of them determined by an altered metabolic profile, have been shown to contribute to the genetic instability of cancer cells [39], providing the necessary "hits" for a more aggressive tumour. Acidity, hypoxia, and formation of reactive oxygen species may all be enhanced in tumours in a hyperglycemic environment.

4.4.1. Acidic Environment. In tumour cells, high glucose flux through the glycolytic pathway produces large quantities of lactate, resulting in tumour tissue with pH 0.5 units lower than normal tissue [40]. Cancerous cells adapt to this acidification, exhibiting maximal growth at the relatively low pH of about 6.8 [41]. Tumours also have a capacity, similar to working skeletal muscle, to share lactate between hypoxic and nonhypoxic cells, so it is not extruded as a waste product [42]. Despite these survival adaptations, tumour acidity has been shown to impair DNA repair mechanisms [39] and to upregulate angiogenic molecules such as vascular endothelial growth factor (VEGF) and IL-8 in order to enhance lactate clearance [43, 44]. Experimental evidence demonstrates that the acidic environment is supportive of tumourigenesis, increasing resistance to chemotherapy [45], mutation rate [46], and invasion capability [47]. The acid-mediated tumour invasion hypothesis postulates that H^+ ions from the tumour microenvironment diffuse down their concentration gradient into the surrounding normal tissue [48]. Because the normal cells cannot survive the increase in acidity, the border of malignant tissue is progressively pushed forward. In fact, mathematical modeling has shown that tumour acid production alone can explain patterns of tumour growth [40]. The effects of acidity are particularly important in a hyperglycemic environment because increased glucose flux through tumour cells has been shown to create a large increase in lactate production [33, 49].

4.4.2. Transient Hypoxia. The characteristic microvascular damage caused by hyperglycemia [50] may lead to periods of hypoxia, possibly through a nitric-oxide-mediated mechanism. The bioavailability of the vasodilator is decreased in diabetes [51] as it is scavenged by superoxide radicals to form the highly reactive $ONOO^{\cdot}$ molecule [52]. Transient hypoxia is thought to be one of the strongest pressures for cells to undergo transformation and is a central hypothesis explaining the glycolytic switch [13, 53]. Hypoxic conditions also increase the activity of hypoxia-inducible factor (HIF-1α) and VEGF, which are strongly associated with both tumour angiogenesis and EOC tumour aggressiveness [54, 55].

4.4.3. Oxidative Stress. Levels of oxidative stress reflect the ability to balance production and elimination of highly reactive free radicals, which include the family of reactive oxygen species (ROS). Oxidative stress is known to be higher in diabetic patients than in healthy individuals [56], and it is often cited as a unifying theory to explain tissue damage by hyperglycemia [57]. Because ROS can also create DNA damage through a number of mechanisms [58], it has similarly been proposed that carcinogenesis in general is caused by oxidative stress [59]. This stress in ovarian epithelial cells specifically is thought to be a potential initiator of tumourigenesis [60]. Hyperglycemia also causes increased flux of glucose through the aldose-reductase (polyol) pathway, which has been postulated to increase sensitivity to oxidative stress by reducing regeneration of the antioxidant glutathione [50]. While epidemiological studies evaluating antioxidant use in diabetes [52, 61] and ovarian cancer [62] have not been conclusive, preliminary results suggest that this therapeutic avenue is worth further exploration. A recent study of flavonoids with antioxidant effects found that they inhibited cell growth and VEGF expression in ovarian cancer cells [63].

4.4.4. Glycation. Much of the tissue damage and cellular dysfunction associated with hyperglycemia has been attributed to advanced glycation end products (AGEs) created by the nonenzymatic glycation of proteins [64]. While AGE accumulation is a normal part of aging, it occurs at an accelerated rate in diabetes where progressive modifications can lead to irreversible cross-linking, impairing the actions of other molecules [64, 65]. Receptors for AGE (RAGE) mediate many more severe actions and potentiate the cellular response [66]. RAGEs are upregulated by presence of AGE ligands, and AGE-RAGE binding protects the ligands, allowing them to persist in the environment [66]. AGE-RAGE interaction has been shown to stimulate tumour cell growth or invasiveness in pancreatic cancer [67], melanoma [68], and glioma [69], while blocking the RAGE inhibits tumour formation and metastasis [68, 69]. The ovarian surface epithelium may be particularly susceptible to the effects of glycation damage because not only the tissue is well vascularized, but it is also in constant contact with peritoneal

fluid, whose glucose content is reflective of blood glucose levels [70].

Mechanistically, AGE-RAGE signaling has been linked to induction of an inflammatory response in the vasculature [71], as well as an increase in matrix metalloproteinases (MMPs)-2 and -9 [66], and may, therefore, play a role in determining tumour invasiveness. Because AGE-RAGE signaling seems to be part of the chronic rather than acute response [66], its contributions to the development of tumour formation are quite plausible.

Glucose reactivity in hyperglycemia can also lead to glucose autoxidation, generating hydroxide radicals, and contributing to the burden of oxidative stress [72]. Also, apart from RAGE signaling, glucose moieties on proteins can donate electrons to form hydrogen peroxide, directly activating NF-κB [73, 74] and contributing to an inflammatory response. There is evidence that changes to local tissue can enhance the possibility of tumour spread [75], possibly implicating glucose-induced damage to the peritoneal cavity as a permissive factor for ovarian tumour metastasis [76].

4.5. The Role of Glucose Transporters. Glucose is a large, hydrophilic molecule that cannot diffuse through the lipid bilayer of cells on its own, and thus requires specific transporter proteins. Glucose enters cells by facilitated diffusion mainly through glucose transporters (GLUTs), and the activation of GLUT genes is one of the earliest events in oncogenesis [77]. Because GLUTs have a role in glucose sensing and respond to extracellular glucose concentrations, these transporters may be very important in a hyperglycemic environment. GLUT1 in particular is highly expressed in ovarian cancer [78], where tumour status (benign, borderline, or malignant) is correlated with the level of GLUT1 expression [79]. Almost all invasive epithelial carcinomas are positive for GLUT1, independent of stage, grade, or histological subtype [79, 80]. Antibodies to GLUT1 decrease proliferation, induce apoptosis in nonsmall cell lung cancer and breast cancer cell lines, and appear to synergize with a number of chemotherapeutics to enhance their apoptotic effects [81].

Very recently, another class of transporters, sodium/glucose cotransporters (SGLTs), was shown to be associated with the epidermal growth factor receptor (EGFR) in cancer cells [96]. The authors of the study proposed that SGLTs may enhance tumourigenesis by making cells independent of the glucose concentration gradient, allowing them to take up fuel in any situation. This hypothesis is in line with the proposal made here that permissive effects of glucose are cancer causing: removing restrictions on fuel availability seems to enhance tumourigenesis. The EGFR is particularly important in ovarian cancer; it is normally expressed on ovarian surface epithelium and is often overexpressed in EOC. The expression of key glucose transporters in ovarian cancer is summarized in Table 1.

5. Inflammation and EOC

In both rats and humans, hyperglycemia has been shown to be a major cause of the systemic inflammatory response [99, 100]. Both oxidative stress [101] and AGE-RAGE [66] signaling are also implicated in promoting systemic inflammation in hyperglycemic environments.

Inflammation is thought to be associated with cancer development mechanistically because of rapid cell division, DNA excision and repair, oxidative stress, and high concentrations of cytokines and prostaglandins; all of which are promoters of mutagenesis [102]. Moreover, inflammation has been proposed as a unifying hypothesis for the development of EOC [103]. The high concentrations of circulating growth-promoting and inflammatory cytokines as a result of hyperglycemia may mean that factors, which normally in an autocrine or paracrine fashion [104] are instead coming from the systemic environment and exerting an endocrine effect, potentiate tumour growth. In support of this, animal knockout studies have shown that MMP production by the host may be more important in carcinogenesis than MMP production by tumour cells themselves [105].

Cytokines can affect EOC tumour growth by acting as growth factors, increasing angiogenesis, or an immunomodulatory pathway whereby they prevent cellular recognition and destruction of the tumour. A number of cytokines that are increased as part of systemic inflammation in diabetes also have tumour promoting effects in ovarian cancer [106]. IL-1 and TNF-α are thought to increase production of IL-6, which promotes cell attachment and migration [107] and also blocks apoptosis induced by cytotoxic agents [106]. IL-8 and TGF-β promote tumour angiogenesis [106]. In addition, although TGF-β normally inhibits epithelial cell proliferation [108], repeated exposure to high levels may attenuate the response of cancerous epithelial cells [106].

The inflammatory hypothesis lends itself to testing with a variety of antiinflammatory drugs and indeed early studies show promise. A study evaluating human ovarian tumours in nude mice concluded that cyclooxygenase inhibitors limited tumour growth, in part through an antiangiogenic mechanism [109]. Epidemiologically, patients with chronic aspirin, NSAID, or acetaminophen use have been shown to have a reduced risk of EOC [110]. However, as with antioxidant trials, these observational studies are still preliminary [103].

5.1. The Incessant Ovulation Hypothesis. Recently, the inflammation associated with postovulatory follicle repair has received attention as a possible contributor to EOC promotion [103]. The incessant ovulation hypothesis purports that the repeated damage and repair cycles associated with ovulation enhance the possibility for mutagenesis. Incessant ovulation also increases the likelihood that inclusion cysts will form, trapping epithelial cells in the hormone-rich environment of the ovarian stroma [1, 111]. If these trapped cells are inappropriately maintained, they are more likely to transform [111–113]. Wound healing in hyperglycemia is characteristically slow and almost certainly influenced by the effects of inflammation and damage from glycation. Lowered nitric oxide bioavailability in combination with the tissue damage caused by hyperglycemia may be partly responsible [114]. In one study AGE-RAGE blockade decreased expression of inflammatory cytokines and MMPs resulting

Table 1: Glucose transporter expression in ovarian and other cancers.

Facilitative Transporters: Class 1 GLUTs				
	Major site of expression	Expression in EOC [77–80]	Localization in EOC [77–80]	Expression in other cancers
GLUT-1	Fetal tissue, erythrocytes; widely distributed	Overexpressed in almost all invasive carcinomas; expression increases from benign to invasive tumours	Cell membrane, cytoplasm; more in membrane in more invasive; some studies say stronger closer to periphery; some say farther from tumour-stromal interface	Breast [82, 83], head, and neck [84], colorectal [85], prostate [86], pancreatic [87], cervical [88]
GLUT-2	Liver, pancreas	Negative	Unknown	Islet cell tumours [89], sarcoma [90]
GLUT-3	Brain	Conflicting: reported to be high in >90% of EOC tumours; also weak, homogenous expression in all ovarian tissue; also in ovarian tumours but not normal tissue	Cytoplasm and cell membrane	Lymphoma [91], head and neck [92], lung [93]
GLUT-4	Insulin-responsive tissues (skeletal muscle, heart, adipose tissue)	Conflicting: no expression in normal or malignant; also present in up to 84% in ovarian tumour cells	Unknown	Lung [94], breast [95]
Active Transporters: SGLTs.				
	Major site of expression	Expression in EOC	Localization in EOC	Expression in other cancers
SGLT1	Kidney and small intestine	Not investigated	Unknown	Breast [96], prostate [96], head and neck [97], pancreatic [98]
SGLT2	Kidney and small intestine	Not investigated	Unknown	No reports
SGLT3	Skeletal muscle and small intestine	Not investigated	Unknown	No reports

in normalization of wound closure in a genetic mouse model of diabetes [115]. Taken together, the mutagenic risk and the risk of entrapment in inclusion cysts from repeated ovulations, combined with impaired wound healing, might mean a greater risk for ovarian cancer development in a hyperglycemic environment. This idea provides a possible mechanism by which hyperglycemia may initiate cancer, in addition to playing a role in promotion of EOC from an unrelated transforming event.

6. Glucose, Angiogenesis, and Tumour Formation

As hypothesized by Dr. Folkman [116], solid tumours must recruit new blood vessels in order to grow beyond 1-2 mm in size. Most of the tumour vascularization occurs through angiogenesis, which is the development of new blood vessels from preexisting vasculature. The angiogenic process is regulated by a balance between pro- and anti-angiogenic factors and in ovarian cancer there is a concomitant overexpression of proangiogenic factors and an inhibition of anti-angiogenic molecules [117]. There are numerous reports concluding that elevated glucose levels contribute to increased angiogenic processes. Granulosa cell tumours of the ovary have been shown to have increased expression of members of both the glycolytic and angiogenic pathways [118]. Glucose directly increases expression of the potent proangiogenic factor VEGF, which is thought to be the mechanism involved in the vascular complications associated with diabetes (reviewed in [119]). In a similar fashion to tumour cells, endothelial cells that comprise the

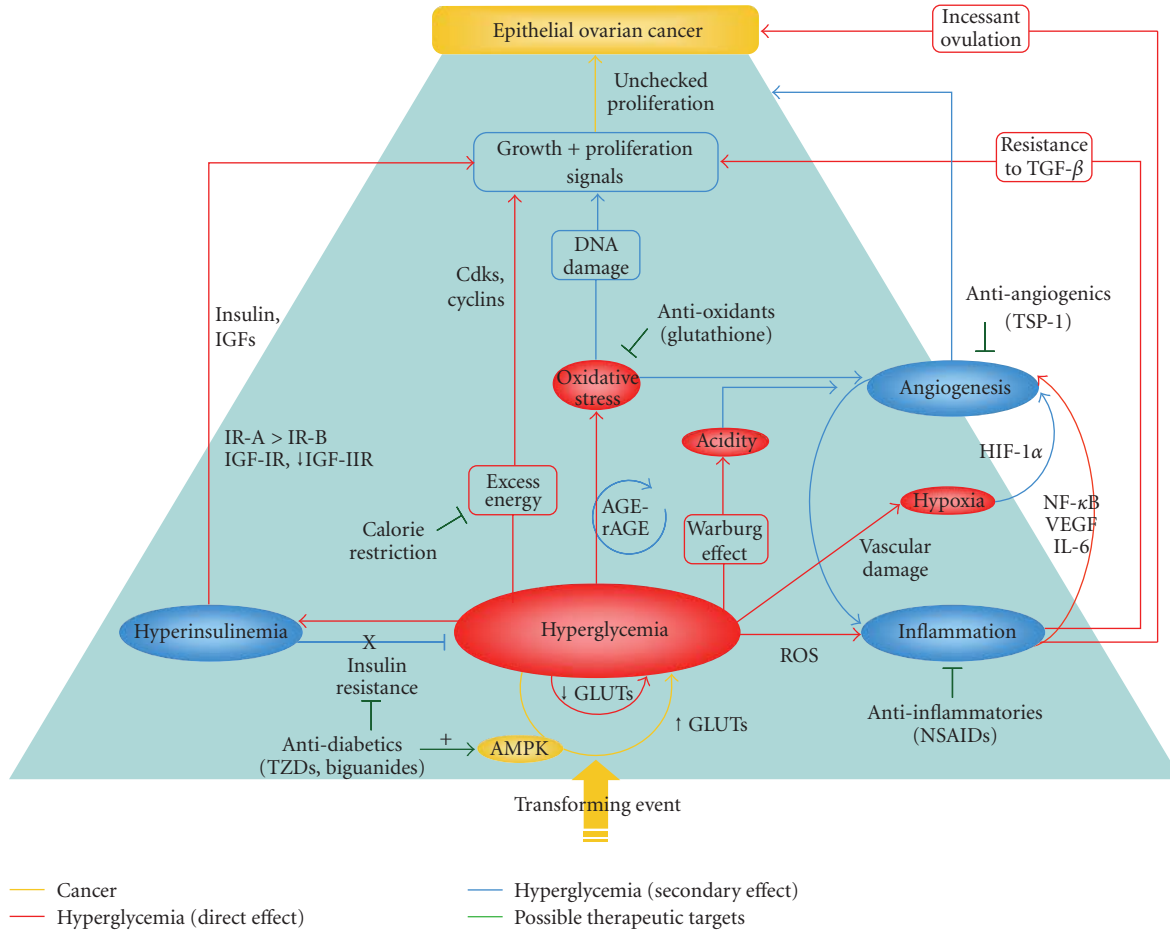

Figure 1: Summary diagram of factors hypothesized to link hyperglycemia to the development of epithelial ovarian cancer. Hyperglycemia, leading to hyperinsulinemia and inflammation, underlies the development of parallel pathologies affecting growth and death signaling, formation of reactive species, and angiogenesis. Together, these aberrant signals converge on a hyperproliferative phenotype that may promote or initiate the development of cancer. Possible therapeutic approaches, including the novel application of antidiabetic drugs, are shown in green. *Abbreviations:* TZDs, thiazolidinedoines; GLUTs, facilitative glucose transporters; ROS, reactive oxygen species; NSAIDs, nonsteroidal antiinflammatory drugs; AGE-RAGE, advanced glycation end product receptor complex; IR-A and IR-B, insulin receptor isoforms A and B; IGF(R), insulin-like growth factor (receptor); cdk, cyclin-dependant kinase; TSP-1, thrombospondin-1; HIF-1α, hypoxia-inducible factor alpha; NF-κB, nuclear factor kappa B; VEGF, vascular endothelial growth factor.

tumour vasculature also increase their utilization of glucose. Glucose transporter expression is increased in the hypoxic environment associated with most solid tumours [120], and glucose increases survival of both tumour epithelial and endothelial cells [96]. Because increased tumour vascularity is correlated with increased metastatic potential and tumour progression [121, 122], the proangiogenic inflammatory environment of hyperglycemia may also promote carcinogenesis. Unfortunately, inflammation may be self-promoting as increased tumour perfusion can act to further exacerbate the immune response [121].

In addition to the direct effects of glucose, the effects of inflammation are likely mediated by VEGF. Inflammatory mediators upregulate VEGF and VEGF receptors, which are correlated with the clinical outcomes of ovarian cancer patients [123]. For example, NF-κB can promote angiogenesis by activating VEGF and IL-8 [124] and may be central to inflammation-induced tumour growth and progression [125]. MMPs can also stimulate proliferation and release of VEGF [126].

The possible impact of hyperglycemia-related inflammation on cancer suggests that anti-angiogenic molecules such as thrombospondin-1 may be of great benefit in treating diabetic tumours [127]. The relationship between angiogenesis, inflammation, and carcinogenesis is illustrated by the fact that a number of anti-angiogenic drugs that are promising in the treatment of cancer are also effective against chronic inflammatory diseases [128].

7. Antidiabetic Drugs as Targeted EOC Therapy

Because of the multitude of protumour effects of glucose, it is intuitive that glucose deprivation may be a potent antitumour treatment approach. From the literature, it is apparent that glucose is an important energy substrate,

survival factor, and proangiogenic molecule. There are a number of antihyperglycemic treatments currently available for reducing serum blood glucose and these drugs may effectively inhibit glucose availability to the tumour. Although the effects of antihyperglycemic drugs are well documented in diabetes, their effects in cancer are relatively unknown. Preliminary reports show that these drugs may have multi-modal effects in slowing tumour growth. In an approach similar to that using anti-angiogenic drugs, the class of antihyperglycemic drugs such as metformin and rosiglitazone may reduce glucose availability to the tumour and essentially starve the tumour of nutrients. These drugs have also been shown to have direct effects on metabolic and signaling pathways that may be independent of glucose.

Metformin is in the biguanide class of antidiabetic drugs and decreases circulating glucose levels by suppressing hepatic production of glucose [129]. Metformin, by reducing insulin and glucose levels, reduced the size and increased latency of mammary adenocarcinomas in HER-2/neu transgenic mice, demonstrating a potent antitumour effect [130]. In vitro, metformin significantly inhibits the growth of epithelial ovarian cancer cells and may potentiate the effects of the common chemotherapy drug cisplatin [131]. Metformin may preferentially increase peripheral glucose uptake in skeletal muscle, as administration increases AMPK activity in skeletal muscle [132] and stimulates translocation of muscle GLUT-4 [133]. This favoured packaging of glucose into skeletal muscle cells would decrease serum glucose levels and availability to the tumour cells resulting in nutrient depletion. Stimulation of AMPK by metformin also contributes to the reduced hepatocyte production of glucose [134]. In fact, AMPK activation is associated with an inhibition of tumourigenesis through apoptosis induction, decreased cell proliferation and may be a communal molecule utilized by metformin as well as a number of anti-tumour drugs that have been shown to have effects in EOC. C93 [135], resveratrol [13, 136], 2-deoxy-D-glucose [137], and AICAR [138] are targeted therapies that are effective in the treatment of ovarian cancer. Interestingly, these molecules also cause the stimulation of AMPK, indicating a common pathway intersection with metformin. Although not yet investigated, there is a possibility that metformin may have a synergistic interaction with these molecules, in addition to its glucose deprivation effects.

Rosiglitazone is another antidiabetic agent in the thiazolidinedione class of drugs designed to reduce the hyperglycemia associated with this disease. Rosiglitazone activates the peroxisome proliferator activated receptors (PPAR) in target tissues, increasing insulin sensitivity and decreasing serum levels of glucose. As with metformin, rosiglitazone also stimulates increased expression of GLUT-4 [139] causing glucose uptake in skeletal muscle [140]. One of the mechanisms by which rosiglitazone may have a significant antitumour effect is through the inhibition of angiogenesis. Rosiglitazone has been shown to inhibit VEGF-induced angiogenesis [141] and is suggested as a treatment option for vascular disorders associated with diabetes such as diabetic retinopathy, macular degeneration, and so forth. As VEGF expression is significantly elevated in EOC [142] and is responsible for some of the ovarian tumour vascularization (reviewed in [143]), rosiglitazone may have a bimodal anti-tumour effect by decreasing glucose availability and also by reducing tumour angiogenesis. Simply by decreasing tumour vascularity, rosiglitazone will decrease glucose delivery to the tumour by decreasing tumour tissue perfusion.

8. Summary and Conclusions

An emerging view of cancer relies on an initiation-promotion paradigm that suggests a fundamental role of the tumour environment on cancer development. New data suggests that hyperglycemia may be a contributing factor to the onset and progression of EOC through a number of complex mechanisms (summarized in Figure 1). We propose that hyperglycemia has important effects on both the progression and somatic evolution of epithelial ovarian cancer. Altered glucose homeostasis is common in cancer patients, so antihyperglycemic therapies are applicable to even those who have normal blood sugar. Although there are a number of cellular mechanisms through which hyperglycemia may effect the promotion or initiation of ovarian cancer, there is almost no in vivo experimental data exploring the link between hyperglycemia and EOC. Further research in this area not only has applications in the development of cancer therapeutics, but also will provide new insights into EOC pathogenesis, early detection, and possible prevention.

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Review Article

Histone Deacetylase Inhibitor Therapy in Epithelial Ovarian Cancer

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Since epigenetic alterations are believed to be involved in the repression of tumor suppressor genes and promotion of tumorigenesis in ovarian cancers, novel compounds endowed with a histone deacetylase (HDAC) inhibitory activity are an attractive therapeutic approach. In this review, we discuss the biologic and therapeutic effects of HDAC inhibitors (HDACIs) in treating ovarian cancer. HDACIs were able to mediate inhibition of cell growth, cell cycle arrest, apoptosis, and expression of genes related to the malignant phenotype in a variety of ovarian cancer cell lines. Furthermore, HDACIs were able to induce the accumulation of acetylated histones in the chromatin of the $p21^{WAF1}$ gene in human ovarian carcinoma cells. In xenograft models, some of HDACIs have demonstrated antitumor activity with only few side effects. Some clinical trials demonstrate that HDACI drugs provide an important class of new mechanism-based therapeutics for ovarian cancer. In this review, we discuss the biologic and therapeutic effects of HDACIs in treating ovarian cancer, especially focusing on preclinical studies and clinical trials.

1. Introduction

Ovarian cancer is the most lethal gynecologic malignancy [1]. Early-stages of ovarian cancer are frequently asymptomatic and difficult to detect and thus diagnosis usually occurs after the disease advanced. The search for agents effective in the treatment of either advanced or recurrent ovarian cancer has been disappointing. To date, platinum and paclitaxel demonstrate the greatest efficacy [1]. However, although reported response rates have been as high as 70%, the duration of response remains brief. In patients with stage III and IV disease, the median duration of response (as measured by progression free survival) following first line therapy is approximately 18 months (reviewed in [2]). Therefore, innovative approaches are needed for the treatment of ovarian cancer.

1.1. Histone Modification. One of the most important mechanisms in chromatin remodeling is the posttranslational modification of the N-terminal tails of histones by acetylation, which contributes to a "histone code" determining the activity of target genes [3]. Transcriptionally silent chromatin is composed of nucleosomes in which the histones have low levels of acetylation on the lysine residues of their amino-terminal tails. Acetylation of histone proteins neutralizes the positive charge on lysine residues and disrupts nucleosome structure, allowing unfolding of the associated DNA with subsequent access by transcription factors, resulting in changes in gene expression. Acetylation of core nucleosomal histones is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDACs catalyze the removal of acetyl groups on the amino-terminal lysine residues of core nucleosomal histones, and this activity is generally associated with transcriptional repression. Aberrant recruitment of HDAC activity has been associated with the development of certain human cancers [4]. HDAC inhibitors (HDACIs) can inhibit cancer cell growth in vitro and in vivo, revert oncogene-transformed cell morphology, induce apoptosis, and enhance cell differentiation [5].

1.2. Mechanisim of Action of HDACI. HDACs catalyze the removal of acetyl groups from the chromatin core histones.

HDACs induce neutralization of the charge on the histones which allows the phosphate backbone of the DNA to open up and therefore facilitate the transcription of many genes, including tumor suppressor genes silenced in cancer. Moreover, acetylation of histones facilitates destabilization of DNA-nucleosome interaction and renders DNA more accessible to transcription factors [6]. In parallel to effects on gene expression and differentiation, HDACIs have also been shown to be efficient inducers of apoptosis in several cellular systems [7]. The precise mechanisms of this effect are under investigation, with suggestions ranging from effects on cellular networks to oxidative stress induction and to DNA damage induction [8].

1.3. Different Classes of Drug. Several classes of HDACIs have been identified, including (a) organic hydroxamic acids (e.g., Trichostatin A (TSA) and suberoyl anilide bishydroxamine (SAHA)), (b) short-chain fatty acids (e.g., butyrates and valproic acid (VPA)), (c) benzamides (e.g., MS-275), (d) cyclic tetrapeptides (e.g., trapoxin), and (e) sulfonamide anilides [9] (see Table 1).

1.4. Postulated Downstream Effects of Inhibition. HDACIs markedly upregulated the level of $p21^{WAF1}$ and $p27^{KIP1}$ proteins, which were expressed at negligible levels in the untreated ovarian cancer cell lines. Conversely, HDACIs decreased the levels of cyclin D1 and cyclin D2. HDACIs decreased bcl-2 levels. E-cadherin binds to β-catenin and can act as a tumor suppressor gene; its promoter has CpG islands which are frequently methylated in selected cancers. Although some investigators believed that the expression of E-cadherin can promote carcinogenesis from normal ovarian surface epithelial cells unlike the other carcinomas [10], HDACIs markedly increased the expression level of E-cadherin in endometrial and ovarian cancer cells and exhibit antiproliferative activity in these cells [11] (Figure 1).

2. Preclinical In Vitro Studies

SAHA (vorinostat) is one of the most promising HDACIs in treatment of epithelial ovarian cancer. To date, three studies have evaluated vorinostat in ovarian cancer. Takai et al. elucidated for the first time that vorinostat caused cell cycle arrest and markedly induced apoptosis in nine ovarian cancer cell lines [11]. Second, Sonneman et al. found that vorinostat had cytotoxic activities and caspase-3 activities in three ovarian cancer cell lines as well as in primary cancer cells that were isolated from malignant ascites collected from five patients with stage III ovarian carcinomas. They also found that paclitaxel-resistant ovarian cancer cell line (2780AD) cells were responsive to varinostat [12]. Third, Cooper et al. reported that in an ovarian cancer cell line, vorinostat decreased viability and increased apoptosis similarly to paclitaxel, but the combination was not statistically significantly different from the single agents [13].

The anticonvulsant VPA has HDAC inhibitory activity [14]. VPA has an extensive safety history and well-established pharmacokinetics. In cell culture models, exposure to VPA results in dose-dependent cell cycle arrest as well as apoptosis in nine ovarian cancer cell lines [11]. Furthermore, Lin et al. suggested that VPA synergizes with cytotoxic anticancer agents [15].

HDACIs that demonstrated antiovarian cancer activity in single agent are TSA [11], vorinostat [11], CBHA [16], scriptaid [17], sodium butyrate [11], VPA [11], MS-275 [18], M344 [19], apicidin [20], and PDX101 [21].

There are some combination studies in ovarian cancer cells looking at HDACIs in combination with multiple different agents; these include traditional cytotoxic agents (paclitaxel [12, 13, 21, 22], docetaxel [21], cisplatin [15], carboplatin [21]), biologic agents (bortezomib [23]), and aspirin [19]. All of these combination studies in ovarian cancer seek to capitalize on the multiple different mechanisms of action of HDACIs in order to create a synergistic effect with the other modalities and to increase the tumoricidal impact.

3. Preclinical In Vivo Studies

We previously tested the ability of VPA to inhibit the growth of human SK-OV-3 ovarian cancer tumors growing in immunodeficient mice during 5 weeks of therapy [11]. Administration of VPA remarkably suppressed the growth of the tumors. During the study, all the mice were weighed once per week. No significant differences in the mean weights, histology of internal organs, mean blood chemistries including liver parameters and hematopoietic values were found between diluent-treated mice and those that received 5 weeks of therapy. It meant that there was no side effect during VPA treatment. Histological analysis of these tumors from untreated mice revealed moderately differentiated carcinomas with small foci of necrosis and fibrosis. Approximately 50%–60% of each of the tumor sections from mice treated with VPA revealed necrosis and histologic changes of apoptosis including formation of apoptotic bodies. These tumors were sampled for expression of $p21^{WAF1}$ using immunohistochemistry on formalin-fixed paraffin-embedded sections. SK-OV-3 ovarian cancer cells treated with VPA showed strong nuclear staining. Control cancer cells from untreated mice had negative or focal weak staining for $p21^{WAF1}$. $p21^{WAF1}$ is cyclin dependent kinase inhibitors (CDKIs) that bind to cyclin-dependent kinase complexes and decrease kinase activity and may act as key regulators of the G0/G1 accumulation (reviewed in [24]).

Qian et al. demonstrated that PXD101 displayed single-agent antitumor activity on human A2780 ovarian cancer xenografts which was enhanced when combined with carboplatin [21]. Cooper et al. reported that a nude mouse ovarian cancer model found limited single agent efficacy with vorinostat; however, paclitaxel followed by vorinostat and paclitaxel alone increased survival compared to either vorinostat alone or vorinostat followed by paclitaxel [13]. These studies raised several questions regarding the optimal sequencing of future combination therapy with HDACIs and chemotherapy.

Table 1: Overview of frequently used histone deacetylase inhibitors being available for clinical and research purposes.

Substance groups	Derivatives	Isotype	Study phase
Hydroxamates	Trichostatin A (TSA)	I, II	
	Suberoylanilide hydroxamic acid (SAHA, vorinostat)	I, II, IV	III
	LBH589 (panobinostat)	I, II, IV	II
	PCI24781 (CRA-024781)	I, IIb	I
	LAQ824	I, II	I
	PXD101 (belinostat)	I, II, IV	II
	ITF2357	I, II	II
	SB939	Unknown	I
	JNJ-16241199 (R306465)	I	I
	m-carboxycinnamic acid bishydroxamide (CBHA)		
	Scriptaid		
	Oxamflatin		
	Pyroxamide		
	Cyclic hydroxamic acid containing peptides (CHAPs)		
Short chain fatty acids	Butyrate	I, IIa	II
	Valproate	I, IIa	II
	AN-9		II
	OSU-HDAC42		
Benzamides	MS-275 (entinostat)	1, 2, 3, 9	II
	MGCD0103	1, 2, 3, 11	II
	Pimelic diphenylamide	1, 2, 3	
	M344		
	N-acetyldinaline (CI-994)		II
Cyclic tetrapeptides	Apicidine	I, II	
	Trapoxins		
	HC-toxin		
	Chlamydocin		
	Depsipeptide (FR901228 or FK228) (romidepsin)	1, 2, 4, 6	II
Sulfonamide anilides	N-2-aminophenyl-3-[4-(4-methylbenzenesulfonylamino)-phenyl]-2-propenamide		
Others	Depudecin		
	NDH-51		
	KD5150	Pan-HDACI	

Class I: HDAC 1, 2, 3, 8; class IIa: HDAC 4, 5, 7, 9; class IIb: HDAC 1, 2, 3, 8; class III: HDAC 6, 10; class IV: HDAC 11.

4. Clinical Trials

HDACIs require a significant period of exposure (≥24 hours) to achieve maximum tumor cell killing in culture, presumably because of their action as cell cycle agents. Sequestration and elimination may also be problems in vivo. Thus continuous administration may be required to achieve efficacy in the clinic [9]. Some HDACIs (e.g., TSA and trapoxin) are of limited therapeutic use because of poor bioavailability in vivo as well as toxic side effects at high doses. Sodium butyrate and phenylbutyrate are degraded rapidly after IV administration (short half life) and therefore require high doses exceeding 400 mg/kg/day [25]. Furthermore, these compounds are not specific for HDACs because they also inhibit phosphorylation and methylation of proteins as well as DNA methylation [26].

There is only one phase I data including ovarian cancer patients treated with HDACI. Camacho et al. conducted phase I dose escalation clinical trial of phenylbutyrate sodium administered twice daily to patients with advanced solid tumors at Memorial Sloan-Kettering Cancer Center. Administration of phenylbutyrate sodium in a twice-daily infusion schedule is safe. The maximum tolerated dose is 300 mg/kg/day [27].

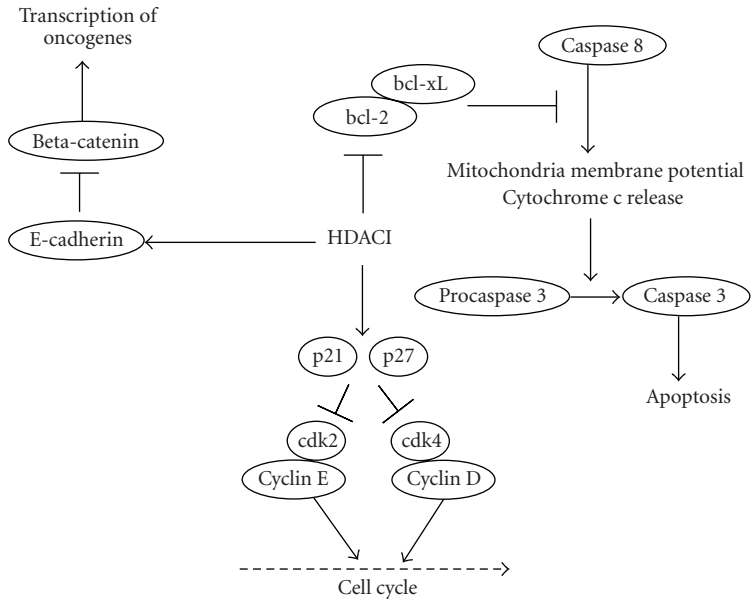

Figure 1: The mechanism of action of HDACIs against ovarian cancer [9].

The multi-institutional phase II trial assessed the activity and toxicity of a new histone deacetylase inhibitor, vorinostat in patients with recurrent or persistent epithelial ovarian, or primary peritoneal carcinoma [28]. The initial dose of vorinostat was 400 mg orally daily and a cycle was defined as a period of 3 weeks (21 days) and was given at a fixed daily dose until progressive disease or adverse effects prohibited further therapy with this agent. The primary endpoints were progression-free survival (PFS) at 6 months and toxicity. Two women of twenty-seven enrolled patients survived progression-free over 6 months, with one having a partial response. The estimated probability of PFS for at least 6 months was 7.4% (90% C.I. was 1.3%–21.5%). Major grade 4 toxicities were leucopenia and neutropenia (7%). While there has not been clear evidence of QTc prolongation due to vorinostat in either preclinical or clinical studies to date, isolated clinical events of QTc prolongation have been reported for other HDAC inhibitors [29]. This phase II GOG study of vorinostat in recurrent ovarian cancer patients demonstrated that, in this platinum-resistant or refractory patient population, there is limited efficacy for this drug as a single agent. Authors discussed that it could be classified as a biologic response modifier rather than a traditional cytotoxic agent. In ovarian cancer, the potential role for this drug may be in overcoming chemotherapy resistance in recurrent disease or in combination with paclitaxel and platinum agents in the upfront treatment. Due to the nature of vorinostat, it may be more effective in low-volume disease for stabilization or prevention of recurrence. Future preclinical and clinical trials will need to focus on potential synergistic effects of vorinostat with other agents, particularly paclitaxel and platinum agents.

Phase II study, single-arm study of hydralazine and magnesium valproate added to the same schedule of chemotherapy on which patients were progressing, has been conducted [30]. Patients received hydralazine at 182 mg for rapid, or 83 mg for slow, acetylators, and magnesium valproate at 40 mg/kg, beginning a week before chemotherapy. Response and toxicity were evaluated. Seventeen patients were evaluable for toxicity and 15 for response. A clinical benefit was observed in 12 (80%) patients: four PR, and eight SD. The most significant toxicity was hematologic.

There were two clinical presentations from ASCO 2008 with PDX101 (belinostat) both alone and in combination with chemotherapy in ovarian cancer [31, 32]. Mackay et al. demonstrated a phase II trial of belinostat in patients with platinum resistant epithelial ovarian cancer (EOC) and borderline ovarian tumors. Belinostat 1,000 mg/m^2/day was administered IV on days 1–5 of a 21-day cycle. Tumor response was assessed by RECIST and CA125 criteria every 2 cycles. Of 18 patients with EOC, 9 patients have SD, 6 progressive disease (PD), 3 are nonevaluable (NE), and 2 remain on study. Of 12 patients with borderline tumors, 1 patient had a partial response (PR), 9 SD, and 2 are NE. 1 further patient had a CA125 response. 5 patients remain on study. The most frequent grade 3 adverse events (both patient groups) were bowel obstruction, thrombosis, dyspnea, fatigue, lymphopenia, elevated ALP, and nausea. Belinostat shows promising activity in borderline ovarian tumors. Finkler et al. conducted phase II multicenter trial of belinostat, carboplatin, and paclitaxel in patients with relapsed epithelial ovarian cancer. BelCaP (Bel 1,000 mg/m^2 $\times$ 5 days; carboplatin AUC 5 $\times$ 1 day 3; paclitaxel 175 mg/m^2 $\times$ 1 day 3) was given in 3-week cycles. The primary endpoint was overall response rate (OR). OR was 31%, including 1 complete response and 10 PR. In addition, 16 patients (46%) had SD.

5. Conclusions

In this review we summarize recent studies on the use of HDACIs especially in human ovarian cancer cells. Many questions are currently still unanswered with respect to HDACI specificities for definite tumor subtypes and the molecular mechanisms underlying HDACI-induced differentiation, cell cycle arrest and apoptosis, and the regulation mechanisms of the specific gene expression and recruitment of HDAC complex to the specific promoter sites remain still to be determined. Also, it is still unclear to what extent different HDACs exhibit different and potentially overlapping functions, and it is important to distinguish the HDAC specificity of HDACIs for the development of selective therapy on the molecular level. Certainly, further work will be required to improve the understanding on why transformed cells are more susceptible to the effect of HDACIs than normal cells. Also, combinations of HDACIs with differentiation-inducing agents, with cytotoxic agents, and even with gene therapy may represent novel therapeutic strategies and new hope on the horizon in the treatment of ovarian cancer.

Acknowledgments

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Review Article

Disrupting Ovarian Cancer Metastatic Colonization: Insights from Metastasis Suppressor Studies

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Ovarian cancer affects approximately 25,000 women in the United States each year and remains one of the most lethal female malignancies. A standard approach to therapy is surgical cytoreduction, after which the remaining microscopic residual disease is treated with chemotherapy. The vast majority of patients have disease recurrence, underscoring the crucial need for approaches to control the regrowth, or colonization, of tissues after local treatment. Improved therapies require mechanistic information about the process of metastatic colonization, the final step in metastasis, in which cancer cells undergo progressive growth at secondary sites. Studies of metastasis suppressors are providing insights into events controlling metastatic colonization. This paper reviews our laboratory's approach to the identification, characterization, and functional testing of the JNKK1/MKK4 metastasis suppressor in ovarian cancer metastatic colonization. Specifically, we demonstrate that interaction of ovarian caner cells with the omental microenvironment activates JNKK1/MKK4 resulting in decreased proliferation without affecting apoptosis. The potential role of the omental microenvironment, specifically milky spot structures, is also described. It is our goal to provide this work as a usable paradigm that will enable others to study metastasis suppressors in clinical and experimental ovarian cancer metastases.

1. Introduction

Management of metastatic ovarian cancer continues to be a critical clinical problem. Ovarian cancer affects close to 25,000 women yearly [1] and most patients have extensive metastatic disease at the time of diagnosis. Ovarian cancer metastasis is thought to result from exfoliation of tumor cells from the ovary and/or direct extension onto the peritoneal surfaces, the omentum, and the surface of organs such as the liver and bowel. A standard approach to therapy is to surgically remove surgically as much of the tumor(s) as possible, a process known as surgical cytoreduction. This technique, which leaves only microscopic residual disease, is used in conjunction with chemotherapy. Unfortunately, more than 80% of patients have cancer regrowth. These dismal statistics show the need for improved understanding of the process of *metastatic colonization*, the final step in metastasis, in which cancer cells undergo progressive growth at secondary sites [2, 3] (see Figure 1). While invasion and adhesion have been well studied, mechanisms regulating metastatic colonization are largely unknown. Studies of metastasis suppressors are providing insights into events controlling metastatic colonization [4].

Remarkably, in 2000 when our laboratory began working on metastasis suppressors in ovarian cancer, there were only a handful of papers that specifically addressed aspects of ovarian cancer metastasis. Not surprisingly, research in the molecular underpinnings of ovarian cancer metastasis continues to lag behind other cancer types. In addition to fundamental aspects of metastasis, there are promising developments in the area of therapeutic application of metastasis suppressors. Work from the laboratories of Dr. Patricia Steeg (National Cancer institute) and Dr. Dan Theodorescu (University of Virginia) demonstrates the feasibility of taking

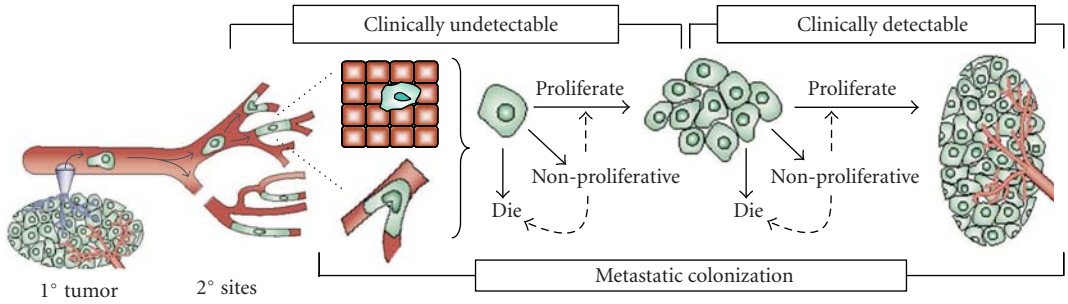


Figure 1: Metastatic colonization is the final step in the development of metastases. After lodging at 2° sites, cells can either remain intravascular or extravasate. To form detectable metastases, disseminated cancer cells must activate signaling cascades, enabling them to survive, enter the cell cycle, and divide. Progressive growth requires the fraction of proliferating cells to exceed the fraction of cells that are nondividing or apoptotic,(*Adapted from* [4]).

metastasis suppressors into the clinic (reviewed in [5]). The following sections describe our approach to using the JNKK1/MKK4 metastasis suppressor to dissect molecular events governing omental metastatic colonization in the SKOV3ip.1 model. It is our goal to encourage others to examine metastasis suppressors in clinical and experimental ovarian cancer metastases.

2. Metastasis Suppressors Can Be Used to Query the Metastatic Process and Regulate Metastatic Growth

Clinically and experimentally, tumor formation and metastasis are distinct processes. Locally growing tumors can progress without the development of metastases. This observation prompted the hypothesis that molecular processes regulating tumorigenicity and metastasis are distinguishable and could be targeted therapeutically [4]. To identify events specifically involved in metastasis regulation, our laboratory and others hypothesized that genes and their encoded proteins that specifically regulate metastasis formation could be functionally identified [4–7]. *Metastasis suppressors* are operationally defined as genes which, when ectopically expressed in metastatic cells, can inhibit the development of spontaneous overt metastases without significantly affecting primary tumor growth [4]. This definition has been extended to include *genes and their encoded proteins which specifically inhibit metastatic colonization* (i.e., experimental metastasis formation using intravenous or intraperitoneal injection) [4]. Identification of metastasis suppressors requires in vivo testing since in vitro assays generally do not model the process of metastasis.

When efforts to find metastasis suppressors were initiated, it was expected that their utility would be in predicting disease outcome; however, robust in vivo studies have showed that metastasis suppressors can control the growth of cancer cells *at metastatic sites* [4, 8]. As a result there now is evidence that metastasis suppressors can influence the interaction of disseminated cells with the microenvironment of distant organs and impair metastatic colonization. Interestingly, other investigators, working on completely different questions, also identified metastatic colonization as a rate-limiting step in metastasis formation [8, 9]. To date our laboratory and others have identified 23 *bona fide* metastasis suppressors, many of which would not have been predicted *a priori* based on their previously known function(s) [4, 5]. Determining how metastasis suppressors modulate cancer cell-microenvironmental interactions will shed light on their function in metastatic colonization, a clinically tractable therapeutic target [2, 10].

3. The JNKK1/MKK4 Stress-Activated Kinase Has a Novel Metastasis Suppressor Function

Our laboratory identified c-Jun NH2-terminal kinase (JNK) kinase 1/mitogen-activated protein kinase (MAPK) kinase 4 (JNKK1/MKK4) as a prostate cancer metastasis suppressor in 1999 [11] and subsequently as an ovarian cancer metastasis suppressor in 2002 [12]. JNKK1/MKK4 is a MAP kinase within the SAPK signaling cascade. MAP kinases occupy a central position in cell growth, differentiation, and transformation. To date, three MAP kinase modules have been well characterized: extracellular signal-regulated protein kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38 [13]. Each consists of a MAP3K, a MAP2K, and a MAPK. The JNK and p38 pathways are generally activated by stress stimuli. The JNK signaling cascade consists of two MAP2Ks, JNKK1, and MKK7, while the p38 signaling cascade MAP2Ks includes JNKK1, MKK3, and MKK6. JNKK1/MKK4 is a dual-specificity kinase which, in response to extracellular stimuli, can become activated and in turn can phosphorylate and activate the JNK and p38 MAPKs (Figure 2 [2–4]). In contrast, the MKK7 MAP2K can only phosphorylate JNK, while the MKK3 and MKK6 MAP2Ks can only phosphorylate p38.

Downstream targets of MAPK signaling include components of the AP-1 transcription factor complex [14]. The biological outcome of MAPK activation can depend, in part, on the transcriptional regulation of target genes. Specificity depends on factors such as cell type, cell environment, signal strength and duration, and the particular composition of the transcription factor, such as AP-1. While conventional

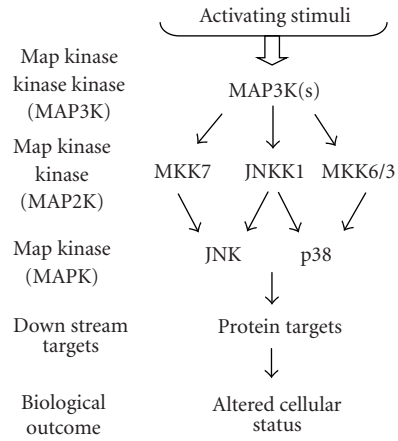


Figure 2: Overview of interactions in JNKK1/MKK4 signaling.

wisdom stipulates that the JNK and p38 pathways mediate viability to stresses, increasing evidence from several model systems indicates a role for both of these MAPKs in cell cycle and consequent proliferation. For instance, reports demonstrate important functions for JNK in the G1/S transition, G2/M progression, and/or cytokinesis [15]. Similarly, p38 can activate the G2/M and spindle assembly checkpoints in mammalian cells and delay entry into mitosis or may prevent anaphase entry when the mitotic spindle is damaged [16, 17]. In sum, the biological and biochemical functions of JNKK1 were consistent with its putative role in metastasis suppression; however, there were no published studies testing its function in complex and dynamic pathological processes such as metastasis. Comprehensive in vivo studies were needed to test its role in metastasis regulation.

4. Testing the Ability of JNKK1/MKK4 to Suppress Ovarian Cancer Metastatic Colonization

Various studies support a role for JNKK1/MKK4 dysregulation in clinical disease [2]. In ovarian cancer, the relationship between its expression and metastasis has been particularly informative. JNKK1/MKK4 protein levels were significantly decreased in metastases as compared to normal ovarian surface epithelium [12]. Profiling studies identified high JNKK1/MKK4 expression as a significant predictor of improved response to surgical cytoreduction [18]. In vivo functional studies used SKOV3ip.1 human ovarian cancer cells, which form metastatic deposits of a serious papillary histology and produce highly reproducible numbers of metastases on the omentum, liver, and bowel [12]. After intraperitoneal injection of 1×10^6 parental SKOV3ip.1 or SKOV3ip.1-vector control cells into female immunodeficient mice, the cells adhere to target organs and by 30 days post injection (dpi) animals have ~30 metastases. SKOV3ip.1 cells have low endogenous levels of JNKK1/MKK4 but retain physiologic levels of other components of its signaling cascade [12].

Ectopic JNKK1/MKK4 decreased the number of SKOV3ip.1 metastases by 88% ($P < .0001$) and increased the animal lifespan by 70% (Wilcoxon, $P = .0045$) [12]. Its metastasis suppressor function is kinase-dependent and studies showed that selective activation of p38 by ectopic MKK6 reduced SKOV3ip.1 metastasis formation by 70% ($P = .0082$), while selective activation of JNK by ectopic MKK7 had no effect ($P = .43$) (Figure 3, 3(a) [19]). These data further defined JNKK1/MKK4's metastasis suppressor activity and prompted the question—What is the biological mechanism of JNKK1/MKK4-mediated metastasis suppression?

5. Determining the Biological Mechanism of JNKK1/MKK4-Mediated Metastasis Suppression

JNKK1/MKK4-mediated metastasis suppression could be due to decreased adhesion of cells, increased apoptosis of cells, or inhibition of cell proliferation. Quantitative real time PCR showed that there was not a significant difference between the numbers of vector-only and JNKK1/MKK4-expressing cells present on the omentum at 3 dpi ($P = .06$; [20]). The TUNEL reaction was used to evaluate apoptosis in SKOV3ip.1-vector or SKOV3ip.1-JNKK1/MKK4 microscopic foci. This showed rare apoptotic cells (<1%) in both groups ($P = .43$, Figure 4(a)). These data were confirmed by morphological assessment as well as immunohistochemistry (IHC) for cleaved caspase 3, which is an early marker of apoptosis [20]. To determine if SKOV3ip.1-JNKK1/MKK4 cells were deficient in proliferation, incorporation of BrdU (a marker of S-phase cells) and endogenous levels of phosphohistone H3 ((pH3), a marker of M-phase cells) were evaluated in microscopic metastases [20]. These studies showed that BrdU incorporation was decreased in SKOV3ip.1-JNKK1/MKK4 cells (Figure 4; 6% versus 19% positive cells, $P < .0001$). Similarly, pH3 staining showed decreased numbers of mitotic SKOV3ip.1-JNKK1/MKK4 cells (average of 0.7% versus 2.5% positive cells in the SKOV3ip.1-vector cells, $P = .004$) [20].

The decrease in BrdU incorporation and pH3-staining in SKOV3ip.1-HA-JNKK1/MKK4 microscopic lesions suggested that fewer cells were traversing S- and subsequently M-phase compared to controls. This prompted the examination of cell cycle inhibitory proteins, including p21 and p27, using IHC [20]. This showed a nearly 10-fold increase in p21 in SKOV3ip.1-JNKK1/MKK4 microscopic lesions in vivo as compared to controls (average 9% versus 1%, $P < .0001$, Figure 4(c)). Since only a portion of the total population of SKOV3ip.1 cells is in cell cycle at any point in time (with 19% entering S-phase in a 4-hour window), the observed increase in p21 (9% of the population) is biologically relevant [20]. The observation that JNKK1/MKK4 activation inhibits disseminated cell growth prompted us to examine the extent and duration of this suppression.

Despite the reduction in the number of SKOV3ip.1-JNKK1/MKK4 metastases at 30 dpi and extension of survival, ultimately animals succumb to metastatic disease [20].

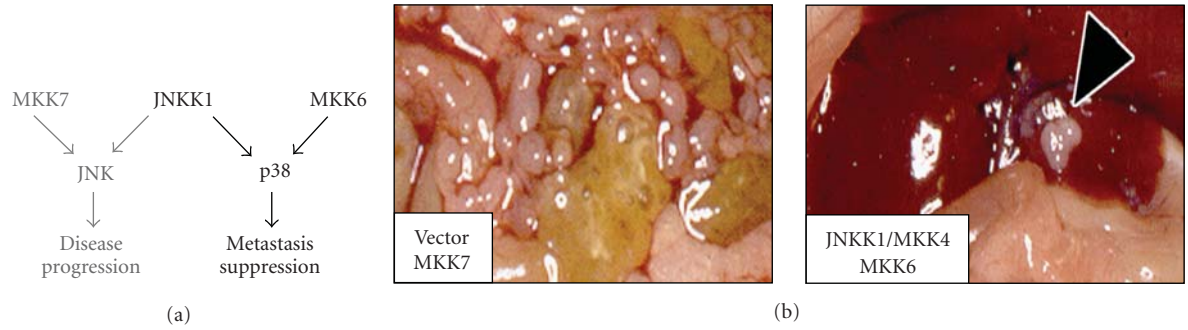


Figure 3: Summary of the effect of MKK7, JNKK1/MKK4, and MKK6 on SKOV3ip.1 metastasis formation. (a) Schematic of JNKK1/MKK4's signaling cascade. In vivo studies show that in SKOV3ip.1 cells, activation of p38 by ectopic expression of JNKK1/MKK4 or MKK6 causes metastasis suppression. (b) Images depicting the effect of specific proteins on metastasis formation, (*Complete primary data can be found in* [12, 19]).

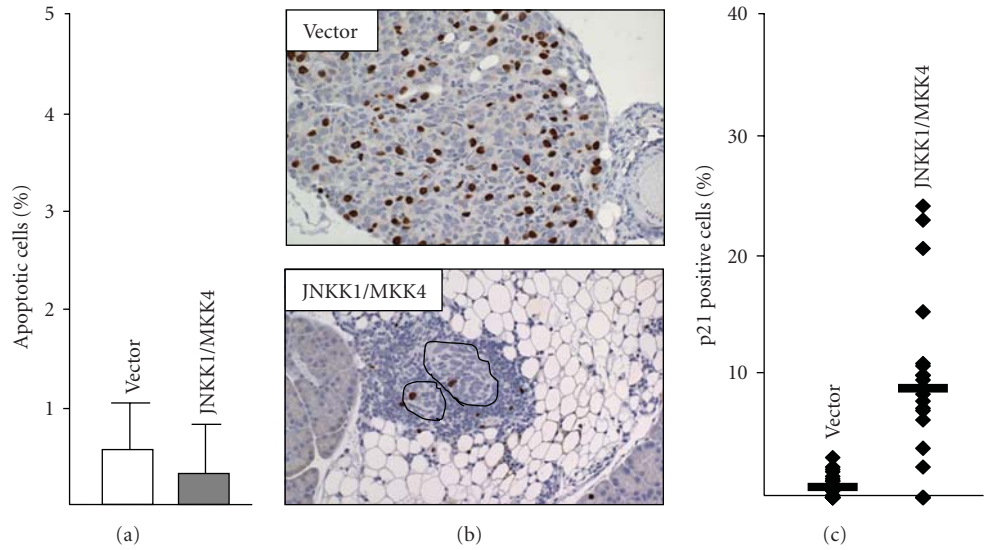
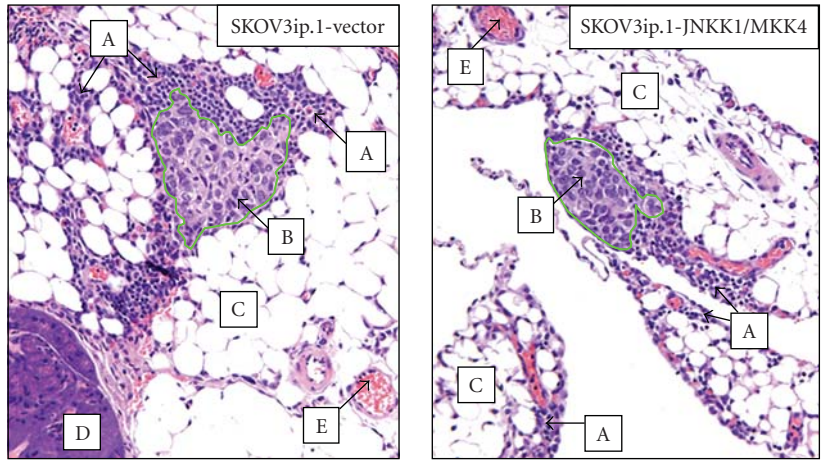


Figure 4: SKOV3ip.1-JNKK1/MKK4 microscopic metastases show decreased proliferation. (a) TUNEL reaction for apoptotic cells was quantitated and showed only rare positive cells. (b) Immunolabeling for BrdU in SKOV3ip1-vector and SKOV3ip.1-HA-JNKK1 microscopic metastases (outlined in black) at 14 dpi (100 × magnification). Both size and BrdU incorporation were significantly decreased in SKOV3ip.1-JNKK1/MKK4 metastases compared to SKOV3ip.1-vector metastases. (c) p21 nuclear staining was significantly decreased in SKOV3ip.1-JNKK1/MKK4 metastases compared to SKOV3ip.1-vector metastases, (*Data adapted from* [20]).

A mathematical analysis of the rates of overt metastasis formation suggested that suppression and outgrowth of JNKK1/MKK4 cells are due to the behavior of *the population* and not selection of a subset of cells, as would occur with increased apoptosis or differential adhesion to the omentum [20, 21]. Molecular analyses showed that overt metastases still express functional JNKK1/MKK4, supporting the notion that metastasis formation was not due to selection for cells that have permanently altered their JNKK1/MKK4 signaling status [20]. Our accumulated data support a model in which binding of cells to the omentum results in the activation of JNKK1/MKK4 and induction of a cell cycle arrest [20]. In order to determine what cellular and molecular signals activate JNKK1/MKK4 and how overt metastases ultimately form, we must consider the microenvironment in which suppression is taking place. In essence we are ahead of ourselves and need to step back and consider what is known about the structure, function, and morphology of the omentum and integrate this knowledge into our current understanding of JNKK1/MKK4-mediated suppression of metastatic colonization.

6. Examining the Structure and Function of the Omentum and of the Omental Microenvironment

The omentum, the primary site for ovarian cancer metastases, is a fatty peritoneal fold that covers most of the abdominal organs and serves as a storage site for lipids, as a regulator of fluid exchange, and as a reservoir for immune cells [22]. Despite its importance, prevailing views



(a) (b)

Figure 5: SKOV3ip.1-vector and SKOV3ip.1-JNKK1/MKK4 cells are found in association with immune cells early in the process of metastatic colonization. Histology of omental tissues harvested at 3 dpi from mice. A: immune cells; B: cancer cells (demarcated by added green border); C: adipose; D: pancreatic tissue; E: vessels (*Data adapted from* [20]).

of ovarian cancer metastasis formation do not consider the potentially dynamic and specialized functions that the omentum may contribute to this process. Historically, the omentum is viewed as being somewhat of an inert, black box—malignant cells attach and cancer proliferates. The implication is that ovarian cancer metastasis formation is the result of uncontrolled growth of cancer cells and not a regulated process which is in part controlled by the omental microenvironment. A review of the literature challenges the view that the omentum plays a passive role in ovarian cancer metastasis formation.

The human and murine omenta are structurally similar, being composed of both adipose-rich and translucent membranous tissues covered by a mesothelial layer [22]. Mesothelial cells share characteristics of both epithelial and mesenchymal cell types and range from flattened to cuboidal in shape, depending on the body site or state of activation [23, 24]. It is well established that omenta from a wide variety of animals, including immunodeficinet rodents, contain aggregates of immune cells known as milky spots. These were first described by von Recklinghausen in 1863 [25] and termed "milky spots" by Ranvier in 1874 [26]. In the omentum, these structures are specialized to enable mobilization of immune cells for migration into the peritoneal cavity. They may also facilitate reentry of immune cells from the peritoneum into the connective tissue (and therefore bloodstream) [18, 22–30]. Remarkably, physiologic functions of milky spots, or even their existence, have not been integrated into generally accepted models of ovarian cancer metastasis. This is a crucial oversight, as it does not consider the possibility that ovarian cancer cells may exploit a highly regulated physiologic system in order to adhere, survive, and grow into metastases.

There is a limited amount of published data that suggests that cancer cells can specifically interact with milky spot structures [31, 32]. Interestingly, in our studies, Lotan et al. found the association of SKOV3ip.1-vector and SKOV3ip.1-JNKK1/MKK4 cells with immune aggregates which we now suspect that they are milky spot structures (Figure 5 [20]). Our laboratory is currently investigating the potential role for milky spot interactions in JNKK1/MKK4-mediated suppression of metastatic colonization. We hypothesize that disseminated SKOV3ip.1 cells interact with milky spots in the omentum, and these interactions contribute to the microenvironmental context-dependent activation of JNKK1/MKK4, resulting in impaired metastatic colonization. Evidence for specific interactions of ovarian cancer cells with milky spot structures immediately identifies a target for mechanism-based studies of ovarian metastatic colonization.

7. Controlling Metastatic Growth by Targeting Ovarian Cancer Metastatic Colonization

There is considerable interest in controlling the growth of cancer cells at metastatic sites. Therapeutic leads may be discerned by determining why disseminated cancer cells, which have molecular modifications that should enable their growth at distant sites, often lodge at target organs and persist as undetectable, or dormant disease. Our data to date support the hypothesis that activated JNKK1/MKK4 impairs proliferation of cells early in the course of metastatic colonization. It is remarkable that few, if any, studies have been conducted that specifically examine growth control of cells during metastatic colonization. From the standpoint of translational science, the crucial yet underexplored question is how disseminated cells ultimately bypass suppression and form progressively growing metastases.

Historically, the fundamental tenets of metastasis biology dictate that acquisition of metastatic ability is the result of the "drive" of malignant cells towards growth [21]. Thus it

was predicted that bypass of suppression is simply the result of mutation-selection cycles which permanently inactivate JNKK1 or members of its signaling cascade. Findings of Lotan et al. and Hickson et al. challenge this paradigm and suggest that JNKK1-mediated suppression may be due to a reversible cell cycle arrest concomitant with changes in JNKK1 activation status [20, 21]. These findings demonstrate a crucial need to reexamine important but scattered literature on population-dependent behaviors of metastatic cells, which have heretofore been refractory to mechanistic study [33–36]. This also presents an opportunity to examine the interaction of ovarian cancer cells with their microenvironment of the omentum during metastatic colonization. Given the rich literature on the bidirectional communication between cancer cells and their microenvironments, it is important that we consider microenvironmental functions and adaptations as we examine the population-dependent behaviors of cancer cells. Ultimately such studies can lay the foundation for the development of adjuvant therapies that can be used in conjunction with local therapy to delay the onset of disease recurrence, extend survival, and improve quality of life for patients with ovarian cancer.

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Research Article

Repeated Intraperitoneal α-Radioimmunotherapy of Ovarian Cancer in Mice

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The aim of this study was to investigate the therapeutic efficacy of α-radioimmunotherapy of ovarian cancer in mice using different fractionated treatment regimens. The study was performed using the monoclonal antibody MX35 F(ab′)$_2$ labeled with the α-particle emitter ^{211}At. *Methods*. Nude mice were intraperitoneally inoculated with $\sim 1 \times 10^7$ cells of the cell line NIH:OVCAR-3. Four weeks later 6 groups of animals were given 400 kBq ^{211}At-MX35 F(ab′)$_2$ as a single or as a repeated treatment of up to 6 times ($n = 18$ in each group). The fractionated treatments were given every seventh day. Control animals were treated with unlabeled MX35 F(ab′)$_2$ ($n = 12$). Eight weeks posttreatment the animals were sacrificed and the presence of macro- and microscopic tumors and ascites was determined. *Results*. The tumor-free fractions (TFFs) of the animals, defined as the fraction of animals with no macro- and microtumors and no ascites, were 0.17, 0.11, 0.39, 0.44, 0.44, and 0.67 when treated with 400 kBq ^{211}At-MX35 F(ab′)$_2$ once or 2, 3, 4, 5, or 6 times, respectively. Repeated treatment 3 times or more resulted in a significantly higher ($P < .05$) TFF than compared to treatment once or twice. The presence of ascites decreased from 15 out of 18 animals in the group given only one treatment to zero for the 2 groups given 5 or 6 fractions. Treatment with unlabeled MX35 F(ab′)$_2$ resulted in a TFF of zero. *Conclusion*. Weekly repeated intraperitoneal injections of tolerable amounts of activity of ^{211}At-MX35 F(ab′)$_2$ of up to 6 times produced increased therapeutic efficacy without observed toxicity, indicating a potential increase of the therapeutic index.

1. Introduction

Ovarian cancer frequently recurs on the peritoneal surface from remaining micrometastatic growth in spite of debulking surgery and systemic chemotherapy. External abdominal radiotherapy has proven unsuccessful due to absorbed dose limitations of normal tissues. Therefore, adjuvant locoregional treatment with intraperitoneal targeted ligands could be decisive in the treatment of remaining micrometastatic disease. Several studies have been performed on radioimmunotherapy (RIT) of ovarian cancer, mostly mAbs labeled with ^{90}Y and ^{131}I, in animals [1–6] and humans [7–12]. The β-emitting radionuclides however have too long a range for effectively treating microscopic tumors. Thus we believe it is important to continue our investigations of the efficacy of mAbs labeled with α-particle emitters when treating microscopic disease on the peritoneum [13]. In this study, as in a series of earlier studies [14–19], we used the α-particle emitter ^{211}At, with a half-life of 7.21 hours, a mean range in tissue of $\sim 62\,\mu$m, and a mean linear energy transfer (LET) of ~ 111 keV/μm. The half-life of this radionuclide makes it ideal for local treatment as the target cells are easily reached while the transfer of the radioimmunocomplex to the systemic circulation is delayed. The short range ensures a significant absorbed dose in microscopic tumors or even single cells. The high LET, together with the high relative biological effectiveness (RBE) of the α-particles necessitating only a few hits to devitalize the cell, indicates that only a small number of ^{211}At-atoms have to be targeted to each cell [20, 21].

In this study we used the monoclonal antibody (mAb) MX35 F(ab′)$_2$, which recognizes the sodium dependant phosphate transport protein 2b (NaPi2b) of ~ 90 kDa on ovarian cancer cells. We used an animal model mimicking

the clinical situation with intraperitoneal RIT. The intraperitoneal approach allows a high absorbed dose to nonvascularized peritoneal tumor cells with low myelotoxicity as the clearance rate from the peritoneal cavity to the systemic circulation delays systemic exposure.

Fractionated external radiotherapy widens the therapeutic index compared to using a single fraction and higher absorbed doses can be delivered with acceptable toxicity. We hypothesize that this could be true for internal α-RIT.

2. Materials and Methods

2.1. Radionuclide. ^{211}At was produced by the ^{209}Bi(α, 2n)^{211}At reaction in a cyclotron (Scanditronix MC32 at the PET and Cyclotron Unit, Rigshospitalet, Copenhagen, Denmark) by irradiating a ^{209}Bi target with 28-MeV α-particles. The ^{211}At was isolated using a dry-distillation procedure [22].

2.2. Monoclonal Antibodies. MX35 is a murine IgG1-class mAb, developed and characterized at the Memorial Sloan-Kettering Cancer Center (MSKCC), Ny, USA. MX35 is directed towards the sodium dependant phosphate transport protein 2b (NaPi2b) of ~90 kDa on OVCAR-3 cells [23] and is expressed strongly and homogeneously on ~90% of human epithelial ovarian cancers [24]. A batch of MX35 F(ab′)$_2$, produced by Strategic BioSolutions (Newark, USA) for clinical use, was provided by MSKCC.

2.3. Antibody Labeling. MAbs were labeled with ^{211}At using the intermediate labeling reagent m-MeATE (*N*-succinimidyl 3-(trimethylstannyl)benzoate) [25]. Briefly, to a dry residue of ^{211}At (50–100 MBq) was added a mixture of m-MeATE and N-iodosuccinimide in methanol: 1% acetic acid. This solution was then incubated for 20 minutes at room temperature and the labeling reaction was stopped by adding sodium ascorbate. The mAb MX35 F(ab′)$_2$ was then added to the labeling mixture and conjugation was allowed to proceed for 20 minutes. Finally, the mAb fraction was isolated using a NAP-5 column (Amersham Biosciences, Uppsala, Sweden), resulting in a specific activity of 120 kBq/μg, that is, 1 labeled mAb out of ~1200 mAbs.

2.4. Cell Line. The cell line OVCAR-3 (NIH:OVCAR-3, ATCC, USA) was used [26]. The cell line was obtained from the American Type Culture Collection, Rockville, MD, USA. The cells were cultured in T-75 culture flasks at 37°C in a humidified atmosphere of 95% O_2/5% CO_2 with RPMI-1640 cell culture medium supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin.

2.5. Immunoreactivity of Antibodies. After conjugation, the immunoreactivity of the mAbs was analyzed in vitro by determination of the immunoreactive fraction, representing conditions of infinite antigen excess, which was derived from a plot of the total applied radioactivity divided by cell-bound radioactivity as a function of the inverse of the cell concentration [27].

2.6. Animals. We used 120 female, nude Balb/c nu/nu mice (Charles River Laboratories International Inc., Wilmington, MA, USA) in this study. The animals were housed at 22°C and 50%–60% humidity with a light/dark cycle of 12 hours. They were given autoclaved standard pellets and water ad libitum. All the experiments were approved by the Ethics Committee of the University of Gothenburg.

2.7. In Vivo Procedures and Study Groups. At the age of 5 weeks all mice were intraperitoneally inoculated with ~1×10^7 OVCAR-3 cells suspended in 0.2 mL saline. Four weeks after cell inoculation the animals were divided into 7 groups. The animals in groups 1–6 were intraperitoneally injected with 400 kBq ^{211}At-MX35 F(ab′)$_2$ in 1 mL saline as a single or as a weekly treatment of 2, 3, 4, 5, or 6 times, respectively (n = 18 in each group). As controls (group 7), animals were treated once with unlabeled MX35 F(ab′)$_2$ (n = 12). All the animals were thereafter weighed weekly. Eight weeks after the last treatment occasion for each group the animals were sacrificed by cervical dislocation and dissected. The abdominal cavity was opened and the presence of ascites and macroscopic lesions was judged as "yes" or "no". Peritoneal biopsies were taken from the upper left quadrant since tumor propagation is most frequently seen in this area. Suspected lesions were also biopsied. All biopsies were processed for light microscopy and judged as "yes" or "no." Animals dissected and judged were blinded from knowledge of exposure conditions. Differences in TFF and weight between the different study groups were tested using a 2-sample test for equality of proportions.

3. Results

The radiochemical yields were 30%–40% and the radiochemical purity was over 95% as determined by methanol precipitation and gel-permeability chromatography. The immunoreactivity measurements of the ^{211}At-MX35 F(ab′)$_2$ and OVCAR-3 cells gave an immunoreactive fraction of 0.95.

The TFFs of the study groups, defined as the fraction of animals with no macro- and microtumors and no ascites, were 0.17, 0.11, 0.39, 0.44, 0.44, and 0.67 when treated with 400 kBq ^{211}At-MX35 F(ab′)$_2$ as a single or as a repeated treatment regimen of 2, 3, 4, 5, or 6 times, respectively (Table 1). Repeated treatment of 3, 4, 5, or 6 times resulted in a significantly higher ($P < .05$) TFF than that compared to 1 or 2 treatment. The presence of ascites decreased from 15 out of 18 animals in the group given only one treatment to zero for the 2 groups given 5 or 6 repeated treatments. The presence of tumors did not decrease as drastically as the presence of ascites when the number of treatments increased. Treatment with unlabeled MX35 F(ab′)$_2$ resulted in a TFF of zero.

The findings on the peritoneal biopsies at the time of dissection revealed both larger tumor cell clusters of several millimetres in diameter as well as clusters consisting of only a few tumor cells. The tumor cells were sometimes only loosely adhered to the peritoneum but had sometimes penetrated under the mesothelial cell layer.

Table 1: Study groups and number of mice with macroscopic and microscopic tumors and ascites.

Group	n	Treatment	Number of treatments	Macroscopic tumors	Microscopic tumors	Ascites	TFF*
1	18	400† kBq ^{211}At-MX35 F(ab′)$_2$ in PBS‡	1	11/18	15/18	15/18	0.17
2	18	400† kBq ^{211}At-MX35 F(ab′)$_2$ in PBS‡	2	16/18	16/18	8/18	0.11
3	18	400† kBq ^{211}At-MX35 F(ab′)$_2$ in PBS‡	3	11/18	11/18	5/18	0.39
4	18	400† kBq ^{211}At-MX35 F(ab′)$_2$ in PBS‡	4	10/18	10/18	1/18	0.44
5	18	400† kBq ^{211}At-MX35 F(ab′)$_2$ in PBS‡	5	10/18	10/18	0/18	0.44
6	18	400† kBq ^{211}At-MX35 F(ab′)$_2$ in PBS‡	6	6/18	6/18	0/18	0.67
7	12	MX35 F(ab′)$_2$ in PBS	1	12/12	12/12	10/12	0

*TFF : tumor-free fraction (i.e., fraction of animals with no macro- and microscopic tumors and no ascites). Injected activities were †400 ± 14 kBq (mean ± SEM). ‡PBS : phosphate-buffered saline. The presence of macroscopic tumors and ascites was assesed by careful ocular inspection during dissection 2 mo after the last administration of the radioimmunocomplex. Microscopic tumor growth was assessed by conventional histopathology. Judgements were blinded from treatment information.

The general condition of the animals seemed to be unaffected by the different treatment regimens, although the weights of the control animals were significantly higher ($P < .05$) than those of the animals given different regimens of ^{211}At-MX35 F(ab′)$_2$ (groups 1–6), due to the ascites production. No mutually significant difference ($P > 0.5$) in weight between the groups given different regimens of ^{211}At-MX35 F(ab′)$_2$ could be detected and no deaths occurred during followup.

4. Discussion

Fractionated radiotherapy in humans results in an increased therapeutic efficacy as compared to single doses and allows for increased total absorbed dose delivered to the target area. Mimicking such fractionation using RIT presents challenges with respect to physical half-lives and biodistribution. Reasons why fractionated RIT would appear promising are the possibility of reducing the systemic toxicity and hence increasing the maximum tolerated activity, achieving a more uniform absorbed dose distribution in the tumor, and increasing the therapeutic index. We have in a previous animal study found significantly less myelotoxicity dividing the injected activity into 3 fractions, with only a minor decrease in therapeutic efficacy [28]. In that study we also discussed the potential risk of treatment interruption in the human situation due to human antimouse antibody (HAMA) response. However, in our recently published phase I study in which we used a fragmented IgG1 mAb (MX35 F(ab′)$_2$) we could not detect any signs of any HAMA response, indicating a low probability for an HAMA responses in potential future fractionated clinical RIT treatments [29]. In the present study we chose an activity well tolerated as a single injection (400 kBq), with a white blood cell recovery approximately within a week, to be repeated weekly for up to 6 times, that is, a total activity of up to 2400 kBq, not tolerated as a single injection [20]. An interval of 7 days was chosen from the bone marrow recovery data [28] as well as from logistics, that is, a weekly delivery of ^{211}At. The rationale for choosing the fragmented mAb instead of the whole IgG in this study is due to 4 facts. (i) The fragmented mAb was the only clinical grade version of the mAb available at the time of the study; (ii) we have received an approval by the Swedish Medical Products Agency for carry through a phase I study with this fragmented mAb; (iii) we believe that the diffusion into tumors using the fragmented mAb is higher than compared to whole IgG; (iv) We believe that the immunogenicity of the fragmented mAb is lower than the whole IgG, reducing the risk for HAMA response, especially if repeated treatments are considered in the future.

In the series of experiments in this paper the efficacy expressed as the tumor-free fraction (TFF) was less than in previous studies from our group [14–19], but a significant total activity and TFF relation were shown without any signs of toxicity. The difference in the efficacy between the studies probably reflects varying proliferation of the injected cells resulting in different sizes of the tumor deposits at the time of treatment, that is, 4 weeks postinjection. Since the α-particle track length is limited to 60–70 μm the size of the tumor cell clusters is crucial. In an earlier study [19] tumor dimensions were measured and the largest clusters at 4 weeks postinoculation were ~95 μm, actually exceeding the α-particle path length. A significant peeling of the outermost cell layers of the tumor cell clusters and/or a uniform absorbed dose distribution does not seem probable since ~1/3 of the animals were not free of tumors in spite of up to 6 treatment fractions. In the interval of 400–1200 kBq in our earlier preclinical studies the TFF was not correlated with the administered activity. This could be explained by the saturation of the antigenic sites, which—according to the dynamic compartmental model introduced in one of those studies [17]—occurs within a few hours after the injection, resulting in a similar absorbed dose for those activity levels.

In our recently published phase I study on women in clinical complete remission after ovarian cancer occurrence we disclosed no marrow toxicity after an intraperitoneal injection of ~200 MBq ^{211}At-MX35 F(ab′)$_2$ in 1 L, which is in accordance with a low absorbed dose to the bone marrow derived from biokinetic data [29]. This, together with a low probability for HAMA response discussed above, could indicate a possibility of using a fractionated regimen in a phase II study now under planning.

In conclusion, weekly repeated intraperitoneal injections of tolerable amounts of activity of ^{211}At-MX35 F(ab′)$_2$ of up to 6 times produced increased efficacy without observed

toxicity, indicating a potential increase of the therapeutic index.

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