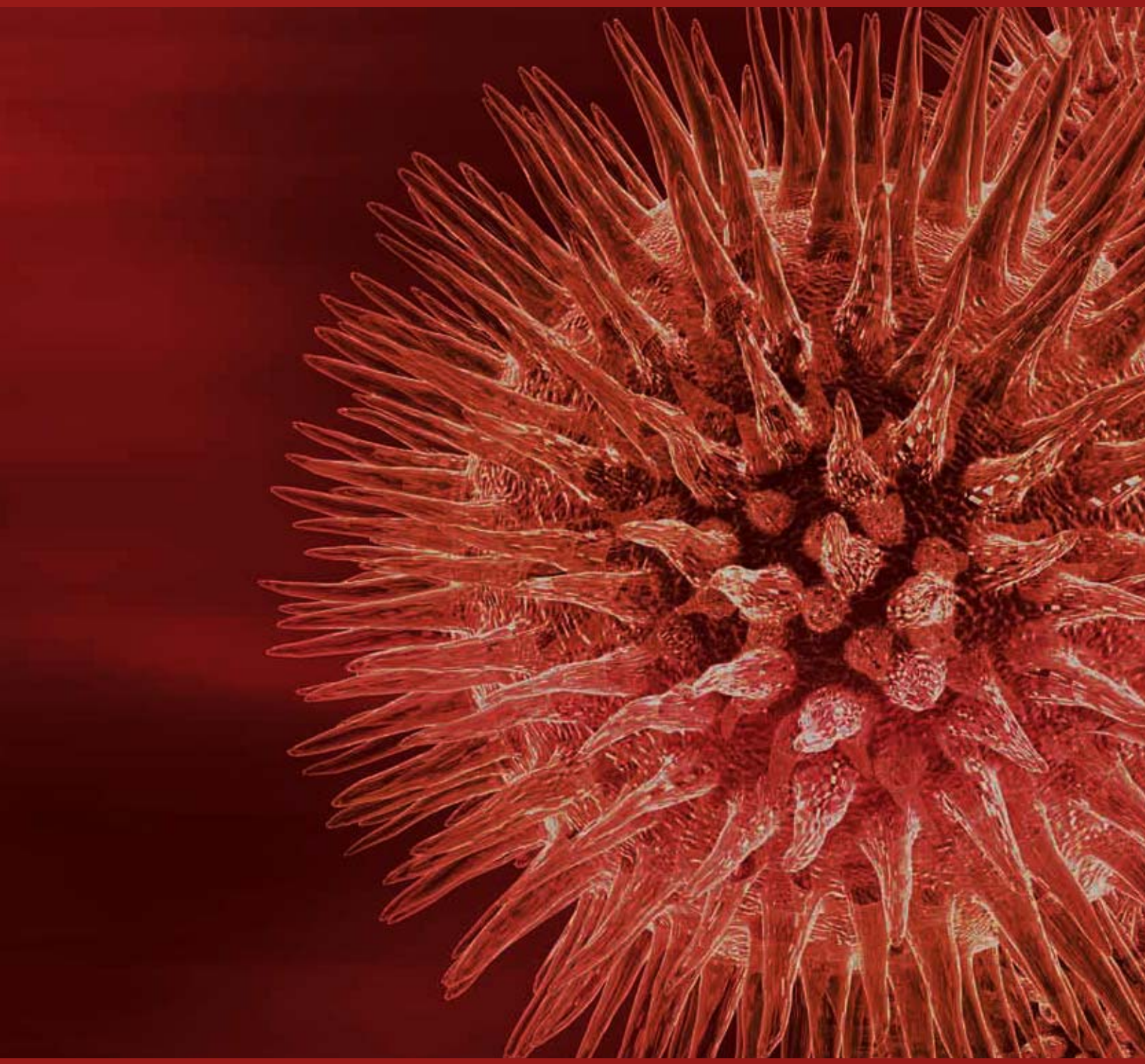
Journal of Biomedicine and Biotechnology

Cancer Chemoprevention

Guest Editors: Amr Amin, Metka Filipič, Su S. Chen,
and Regine Schneider-Stock



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Editorial
Cancer Chemoprevention

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Cancers are characterized by the dysregulation of cell signaling pathways at multiple steps. Most current anticancer therapies however involve the modulation of a single target. The lack of safety and high cost of monotargeted therapies have encouraged alternative approaches. Both natural compounds, extracted from plants or animals, and synthetic compounds, derived from natural prototype structures, are now being used as cancer therapeutics and as chemopreventive compounds.

Given the limited treatments available, preventive control approaches have been considered among the best strategies to protect against cancer. Chemoprevention takes advantage of the long latency of the disease that presents various opportunities for intervention. Chemopreventive intervention can cater to a defined population that is relatively at a higher risk for developing the disease with an objective to end, turn around, or slow down the progression of the disease. Exogenous phytochemicals are usually utilized to enhance endogenous mechanisms against various stages of cancer development.

Natural drugs have found direct medical application as drug entities, but they also serve as chemical models or templates for the design, synthesis, and semisynthesis of novel substances, such as paclitaxel (Taxol), vincristine (Oncovin), and camptothecin, in the treatment of human cancer. Although there are some new approaches to drug discovery, such as a combination of chemistry and computer-based molecular modeling design, none of them can replace the important role of natural products in drug discovery and development.

In this special issue, M. Cazzaniga and B. Bonanni review the current clinical research in both ER-positive and ER-negative breast cancer chemoprevention, explaining the biologic effect of the various agents (such as: Tamoxifen, Raloxifene, Retinoids) on carcinogenesis and precancerous lesions, and finally present an excursus about new molecular targets under investigations in breast cancer settings. In one of the papers, C. Stolfi et al. highlight the available experimental data supporting the ability of mesalazine and its derivatives to interfere with intracellular signals involved in colorectal cancer cell growth.

Natural products encompass three main categories of compounds, phenylpropanoids, isoprenoids, and alkaloids, which are widely distributed in plant foods and medicinal herbs. This large array of molecules is crucial to human nutrition and health. Plant-derived foodstuffs and beverages also constitute the so-called functional foods and beverages, which include mainly fruits, vegetables, herbs, and spices. Curcumin is among the widely known spices that have been used in traditional medicine for many years and by different cultures. K. Sintara and his group in another paper here clearly show that Curcumin treatment for 20 weeks also decreased 8-hydroxy-2′-deoxyguanosine (8-OHdG) expression in benign tumor-bearing rats compared with N-methyl-N-nitrosourea and saturated sodium chloride. Curcumin can attenuate cancer via a reduction of phospho-IκBα and 8-OHdG expressions, which may play a promising role in gastric carcinogenesis. In another paper, A. Amin and his colleagues have provided clear evidence that oral *Ginkgo biloba* (GB) administrations to cisplatin-treated rats effectively alleviated cisplatin-induced toxicity in reproductive system. The present results provide further insights into the mechanisms of protection against cisplatin-induced reproductive toxicity and confirm the essential

antioxidant potential of a GB extract. Finally, A. Sapone et al. show that the idea that the physiological roles of specific catalysts may be easily manipulated by regular long-term administration of isolated nutrients and other chemicals derived from food plants is not viable. In contrast, it is the consumption of wholesome healthy diets that is most likely to reduce mutagenesis and cancer risk. They suggest that both research endeavors and dietary recommendations be redirected away from single molecules to dietary patterns as a main strategy for public health policy.

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Review Article

On Enzyme-Based Anticancer Molecular Dietary Manipulations

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Evidence from both epidemiological and experimental observations has fuelled the belief that the high consumption of fruits and vegetables rich in nutrients and phytochemicals may help prevent cancer and heart disease in humans. This concept has been drastically simplified from the dietary approaches to the use of single bioactive components both as a single supplement or in functional foods to manipulate xenobiotic metabolism. These procedures, which aim to induce mutagen/carcinogen detoxification or inhibit their bioactivation, fail to take into account the multiple and paradoxical biological outcomes of enzyme modulators that make their effects unpredictable. Here, we show that the idea that the physiological roles of specific catalysts may be easily manipulated by regular long-term administration of isolated nutrients and other chemicals derived from food plants is not viable. In contrast, we claim that the consumption of healthy diets is most likely to reduce mutagenesis and cancer risk, and that both research endeavours and dietary recommendations should be redirected away from single molecules to dietary patterns as a main strategy for public health policy.

1. Introduction

Strategies for cancer prevention necessarily focus on eliminating unhealthy lifestyle habits such as alcoholism or cigarette smoking or improving both diet and exercise patterns which are believed to contribute to about one-third of annual cancer deaths worldwide [1–4]. Over the last decades, accumulating epidemiological evidence and animal investigations have suggested that consumption of a diet rich in food plants significantly reduces the risk of several types of cancers and recent recommendations point to plant-based diets [5–7]. This raises the theoretical possibility that such protective effects could be attributed to specific micronutrient or phytochemical constituents of food plants and that such components might have beneficial effects in the field of cancer chemoprevention either as naturally occurring dietary constituents/pharmaceuticals or in functional foods [8–10].

It has been speculated that they could manipulate the activity of metabolic enzymes that break down chemical mutagens and carcinogens to reduce lifetime cancer risk. It is indeed widely believed that the postoxidative enzymes (also, i.e., phase II enzymes), such as glutathione *S*-transferase, UDP-glucuronosyl transferase, sulphotransferase, and acetyl transferase, are able to promote health by detoxifying xenobiotics. On the contrary, the oxidative enzymes (e.g., phase I), represented mainly by the superfamily of cytochrome P450 (CYP) and FAD-containing monooxygenases, raise cancer risk by the bioactivation of ubiquitous mutagenic compounds [11–17]. This rather simplistic dichotomy has in turn suggested that food plant-derived nutrients or phytochemicals might be employed to reduce the risk of cancer through two enzyme-based strategies such as boosting the "good" detoxifying phase II enzymes (using, for example, representative phytochemical-containing fruits and vegetables such as grapes, cauliflower, kale, and broccoli), or inhibiting the "bad" activating phase I enzymes (using those contained in garlic, tea, and onion).

We must remember here that these strategies were extrapolated from epidemiological observations on populations consuming diets varying in both quantity and type of food plant containing thousands of chemical agents which

are able to modulate the specific activity of the metabolizing enzyme battery in a very complex way. They have been popularized by the media and exploited by marketers of supplements of phytochemicals and desiccated vegetables labelled as containing suitable amounts of detoxifying enzyme modulators.

However, this approach totally fails to address the complexity of the multiple interactions between dietary components and xenobiotic metabolism simultaneously generating health benefits or harmful outcomes, depending on circumstances that cannot yet be predicted. Consequently, the potential effects of whole-food plant-derived single constituents on xenobiotic metabolism and cancer risk are also uncertain.

2. The Metabolic Manipulation Approach

This modulation strategy foresees large-scale induction of postoxidative phase II enzymes that "detoxify" xenobiotics by means of single green constituents, thereby accelerating the clearance of mutagens and protecting cells against cancer. The potential benefits of this strategy have stimulated active *in vitro* and in *ex vivo* studies on the molecular mechanism and specificity of such chemical compounds [18–23]. Particular attention has been devoted to cruciferous vegetables of the Brassica genus, such as kale, cabbage, broccoli, Brussels sprouts, and cauliflower. These vegetables contain considerable amounts of glucosinolates which are the precursors (via the enzymatic conversion by the enzyme myrosinase) of isothiocyanates [24–26], which are phase II enzyme inducers [27–30]. Some researchers have actually created hybrid plants specifically to produce higher amounts of single phytochemical inducers [31]. Resveratrol, a phytoalexin found in grapes and other food products, is also able to boost postoxidative-linked activities [23], but many other compounds contained in plants could be cited.

An alternative anticancer approach is to inhibit the oxidative "bioactivating" phase I enzymes [12, 13]. This hypothesis is emphasized by both the scientific literature and the media, as exemplified by numerous reports urging regular consumption of green or black tea containing catechins as well as onion and garlic rich in diallyl sulfide [32–34].

Finally, both proposed strategies also must be considered in the context of genetic metabolic polymorphisms, which may differentially, *per sè*, modulate the effects of any one dietary factors on individuals.

3. The Limitations of Such Strategies

We would like to point out that the main difficulty with these strategies is that they totally ignore the complexity of metabolizing enzymatic machinery. Indeed, if on one hand the consumption of food plants, which contain thousands and thousands of phytochemicals (an apple, e.g., seems to contain more than 700 chemical compounds, and a simple fruit salad?) is linked to a reduced cancer risk, on the other, the induction of xenobiotic metabolism by one specific food component may also stimulate the unwanted formation of highly reactive mutagens [35, 36]. The use of single naturally occurring dietary constituents such as isothiocyanates or individual drugs such as disulfiram, oltipraz, or food additives such as BHA [2(3)-tert-butyl-4-hydroxyanisole], for example, also elicits unhealthy effects [37–40].

It should be pointed out that in addition to the increase in xenobiotic clearance, each postoxidative (phase II) enzyme is also involved in electrophilic species generation and, therefore, must be considered as a "bioactivating system" for specific chemical classes such as halogenated hydrocarbons by glutathione *S*-transferases, for example, or polycyclic aromatic hydrocarbons (PAHs) by sulphotransferases [41–64]. So, the activation or inactivation of a compound depends on the chemical nature of the compound itself and not on the metabolic enzyme involved. More in general, the manipulation of the activity of one or more phase II enzyme can either increase or reduce the bioactivation of specific compounds. Whereas induction increases the detoxification of some promutagens, thereby favoring chemoprevention, it also increases the bioactivation of countless other foreign chemicals to which humans are simultaneously exposed. As the population is exposed to a myriad of potentially harmful molecules, any modification of the activity of these enzymes could actually lead to unexpected dangerous effects [40]. For example, cruciferous isothiocyanates such as the sulforaphane, widely considered as a beneficial phase II inducer, turn out to be genotoxic or a strong promoter of urinary bladder and liver carcinogenesis, also inducing cell cycle arrest and apoptosis [65–67]. Similarly, engineered *Salmonella typhimurium* TA1535 transfected with the plasmid vector pKK233-2 containing rat glutathione S-transferase 5-5 cDNA has been shown to activate many genotoxicants, whereas the nontransfected counterpart does not [68]; in addition, heterologous expression of mammalian theta class glutathione transferases in *S. typhimurium* and *Escherichia coli* systems has been used to demonstrate the role of glutathione conjugation in the genotoxicity of dihalomethanes [61, 69]. Paradoxically, liver metabolic S9 fractions isolated from rodents treated with the monofunctional postoxidative inducer BHA have been proposed as a "complementary" S9 metabolizing system to bioactivate pro-mutagens in typical short-term mutagenicity bioassays [70].

Similar considerations should be made for the inhibitory strategy, a hypothesis that has stimulated recommendations to increase, for example, consumption of green and black teas, as they contain phytochemicals such as catechins able to inhibit the oxidative (phase I) enzymes thus reducing the production of mutagens and carcinogens such as N-nitroso compounds [13, 71]. The inhibition of dimethylhydrazine-induced colon cancer by diallyl sulfide, a flavour component of garlic (*Allium sativum*), has encouraged garlic consumption increase [72, 73]. Moreover, the flavonoid naringin, present in grapefruit and related citrus fruits, has been found to inhibit aflatoxin B1 activation by CYP3A4 in cells and animal models supporting the general idea that green-based metabolism inhibition may reduce carcinogenesis risk [74].

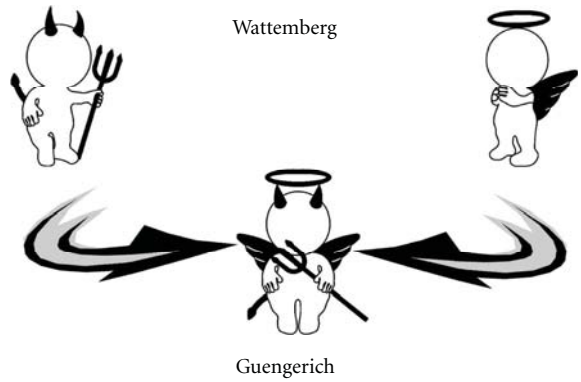

Figure 1: Allegoric representation of main difference between classic theory and data emerging from scientific literature. Since 1985 Wattenberg proposed chemoprevention strategies, including the ones that foresee the above mentioned manipulation of metabolic (according to the belief that they are classified as "bad-phase I" and "good-phase II") enzyme activities, and Guengerich published a comprehensive review that suggested the "dual bioactivating and detoxifying nature" of each metabolic enzyme regardless of whether belongs to the phase I or II battery. The last theory (Guengerich) seems the correct ones, since the data emerging from scientific literature.

The concept of metabolic manipulation, however, ignores the simple fact that the "institutional" role of CYP superfamily toward xenobiotics is to promote their detoxification from the cells and body as well [75]: it is our misfortune that some of them are bioactivated. In other words, due to the dual beneficial and detrimental nature of any of the P450 enzymes, the reduced activation of certain toxins occurs simultaneously with the reduced detoxification of other environmental toxicants to which humans are exposed daily, a phenomenon clearly unpredictable. Once again, it should be noted that the bioactivation of chemicals by phase I apparatus, as occurs for phase II ones, depends on the nature of the substance itself and not on the involved enzyme. Of course, as it is impossible to select safe, personalized human exposure levels to environmental toxins in such a way as to systematically avoid harmful compounds, the regular inhibition of oxidative enzymes might actually lead to an increase toxicological risk. In addition, it should take into account that a selective inhibitor of one CYP enzyme (and due to the existence of many CYP isoforms this strategy should foresee the use a cocktail of multiple inhibitors) may be an inducer of other CYPs; for example, phenethyl isothiocyanate derived from Brassica and diallyl sulfide from garlic are able to inhibit CYP2E1 but also induce CYP2B1 and CYP1A2 [76].

Paradoxically, in 1985 when Wattenberg proposed chemoprevention strategies [12], including the ones that foresee the above mentioned manipulation of metabolic (according to the belief that they are classified as "bad-phase I" and "good-phase II") enzyme activities, Guengerich published a comprehensive review on the "dual bioactivating and detoxifying nature" of each metabolic enzyme regardless of whether belongs to the phase I or II battery (Figure 1) [41].

Not least, the use of enzyme activity modulators can lead to other serious unhealthy consequences stemming from the alteration of endogenous metabolism where these catalysts are involved (e.g., arachidonic acid derivatives, nitric oxide, aldosterone, cholesterol, or vitamins) as well as alteration of fundamental physiological functions (growth, differentiation, apoptosis, homeostasis, and neuroendocrine functions) [77]; the effects on the pharmacokinetics of coadministrated drugs should not be overlooked as well.

4. The Role of Metabolic Polymorphisms

The illogical effects of single daily consumed dietary constituents on xenobiotic metabolism are further complicated by genetic (metabolic) polymorphisms that lead to the occurrence of high- or low-metabolizer phenotypes in the population, each at increased toxicological risk from exposure to specific chemicals [78, 79]. The multiple polymorphisms (e.g., occurrence of high or low (or intermediate in some cases) metabolizers for any oxidative or postoxidative isoforms) characterizing the so called "individual metabolic fingerprint" further complicate the issue. This phenomenon can indeed be interpreted as a sort of a "constitutive up- or down-regulation" of any phase I or II dependent enzyme. In other words, the infinite number of possible combinations of human genetic metabolic polymorphisms constitutes another set of variables in the xenobiotic metabolism [80]. Thus, it appears even more clear that the possibility of manipulating enzyme activity, which in its "constitutive" diversity already may determine genetic disorders as well as perturbations on the chemical biotransformation (including drugs), raises further questions about the effectiveness of the chemical-based enzymatic modulation of cancer risk [81, 82]. In our opinion, these considerations suggest the need for considerable caution before allowing for any form of enzyme-activity manipulation for a generalized prevention, particularly in healthy individuals.

5. On the Clinical Significance

What is the clinical significance of the perpetual manipulation of such enzymatic systems by single nutrient or phytochemicals? Summarizing the various aspects depicted above, the scenario that arises shows how both oxidative and postoxidative enzymes are highly multifunctional and can be induced or inhibited or both by a great number of dietary components. Noteworthy, is the often ignored existence of the dual activating and detoxicating nature of these enzymatic systems. So, the impressive number of chemical compounds that can modulate them, the presence in greens of chemicals that induce both activation and inhibition of mutagenesis, the genetically determined interindividual variability that may moderate (increasing and/or inhibiting) the effects of specific dietary factors on any metabolic enzyme, and the complexity of the interactions among food constituents and enzyme systems have fed the ongoing debates as to whether phytochemicals can alone explain the anticancer ability of plants [9, 83].

It is very difficult to imagine how a single phytochemical, today selected as representative of this or that green, such as lycopene in tomato, resveratrol in grapes, sulforaphane in broccoli, and beta-carotene in carrots, used as a food supplement would offer an advantage, since a variety of fruit and vegetables seems necessary to provide the mixture of vitamins and minerals that appear to favour protection against neoplasia [84]. How can we imagine that the beneficial effects of consuming entire fruits and vegetables, in which enzyme modulating components appear in varying amounts and proportions, and in which unpredictable synergistic and antagonistic (or both depending on the enzyme involved) interactions occur among thousands of different chemicals in their natural matrix, could be just reproduced by supplements of single representative phytochemicals? [85] The beneficial or harmful outcomes of a single compound (*portio facit venenum*, Paracelso) can be quite different from those elicited by the same compound within complex mixtures (*portio and interactiones faciunt venenum*) [86].

The fact that a great number of clinical investigations using single "natural" components failed to reproduce the beneficial effects of the plants from which they were derived should not be underestimated. For example, we can cite the "unexpected" results of cancer chemoprevention trials of antioxidant provitamins and vitamins which we believe can constitute an exemplary warning about the vulnerability of single-nutrient strategies [87–90]. Beta-carotene administered alone or in combination with Vitamins A, E, or C for the prevention of lung cancer and other cancers in heavy smokers or asbestos workers failed to reduce cancer risk and, in some cases, actually increased the risk, raising the suspicion that single chemical supplements may have harmful and beneficial effects as well [91–93]. It has been documented that the deleterious effect of beta carotene can be linked to its ability to stimulate the metabolizing machinery, such as activators of polycyclic aromatic hydrocarbons, and to generate an oxidative stress [94]. In addition, supplementation with commercial doses of vitamin C for 6 weeks is enough to induce DNA damage in human lymphocytes [95], probably by means of its ability to generate oxidative stress connected to phase I upregulation [96, 97].

6. Concluding Remarks

In the field of cancer prevention, the idea of producing the so called "magic-bullet," as conceived by Paul Ehelich for antibacterials, too easily evokes the long-life elixir on a molecular level capturing the imagination of both the public and researchers. From the standpoint of cancer research policy, the possible role of single dietary constituents is of pivotal interest in cancer research but basic information about the role of metabolizing apparatus, however, makes it clear that the role of any single anticarcinogenic phytochemical cannot be understood except in the context of broader dietary patterns. The ongoing scientific controversy surrounding the effects of single molecules on cancer risk seems to provide a salutary warning for health policymakers. Considering that unhealthy lifestyle factors are also taken into account, educational campaigns encouraging the consumption of fruit, fibres, and greens should be encouraged.

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Review Article

Breast Cancer Chemoprevention: Old and New Approaches

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In 1976, Sporn has defined chemoprevention as "the use of pharmacologic or natural agents that inhibit the development of invasive breast cancer either by blocking the DNA damage that initiates carcinogenesis, or by arresting or reversing the progression of premalignant cells in which such damage has already occurred." Although the precise mechanism or mechanisms that promote a breast cancer are not completely established, the success of several recent clinical trials in preventive settings in selected high-risk populations suggests that chemoprevention is a rational and an appealing strategy. Breast cancer chemoprevention has focused heavily on endocrine intervention using selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs). Achieving much success in this particular setting and new approaches as low-dose administration are actually under investigations in several topics. Unfortunately, these drugs are active in prevention of endocrine responsive lesions only and have no effect in reducing the risk of estrogen-negative breast cancer. Thus, recently new pathways, biomarkers, and agents likely are to be effective in this subgroup of cancers and were put under investigation. Moreover, the identification of new potential molecular targets and the development of agents aimed at these targets within cancer have already had a significant impact on advanced cancer therapy and provide a wealth of opportunities for chemoprevention. This paper will highlight current clinical research in both ER-positive and ER-negative breast cancer chemoprevention, explaining the biologic effect of the various agents on carcinogenesis and precancerous lesions, and finally presenting an excursus on the state-of-the-art about new molecular targets under investigations in breast cancer settings.

1. Introduction

While decreases in both breast cancer incidence and mortality have been apparent in recent years, the societal and economic impact of this malignancy continues to be enormous [1]. Breast cancer remains the most commonly diagnosed malignancy among females [2]. The idea of preventing breast cancer dates back to history (Figure 1). Positive associations between environmental and individual factors and increased risk of breast cancer development have been alleged for at least a century. Several progresses were made in understanding the underlying mechanisms of cancer development and some drugs were recently approved for the preventive approach of this disease. Thus, the current thinking is that prevention is a highly feasible approach to breast cancer control. Despite several factors which increase the woman' risk (gender, age, and family history) are not changeable, other modified risk factors such as alcohol intake, dietary fat, obesity in postmenopausal age, and hormonal stimulations have been identified and for these reasons interest in strategies to prevent breast cancer remains strong and intriguing [3]. Cancer chemoprevention is defined as the use of natural, synthetic, or biochemical agents to reverse, suppress or prevent carcinogenic process to neoplastic disease [4]. The epithelial carcinogenesis is a multistep, multipath, and multiyear disease of progressive genetic and associated tissue damage (Figure 2) [5]. In detail, the carcinogenetic process starts with unspecified accumulations of genetics events which lead to a progressive dysplastic cellular appearance with genotypic and phenotypic alterations, deregulated cell growth, and finally cancer [6]. This process is similar in every epithelial cancer, and the ability to arrest one or the several of these steps may impede or delay the development of cancer.

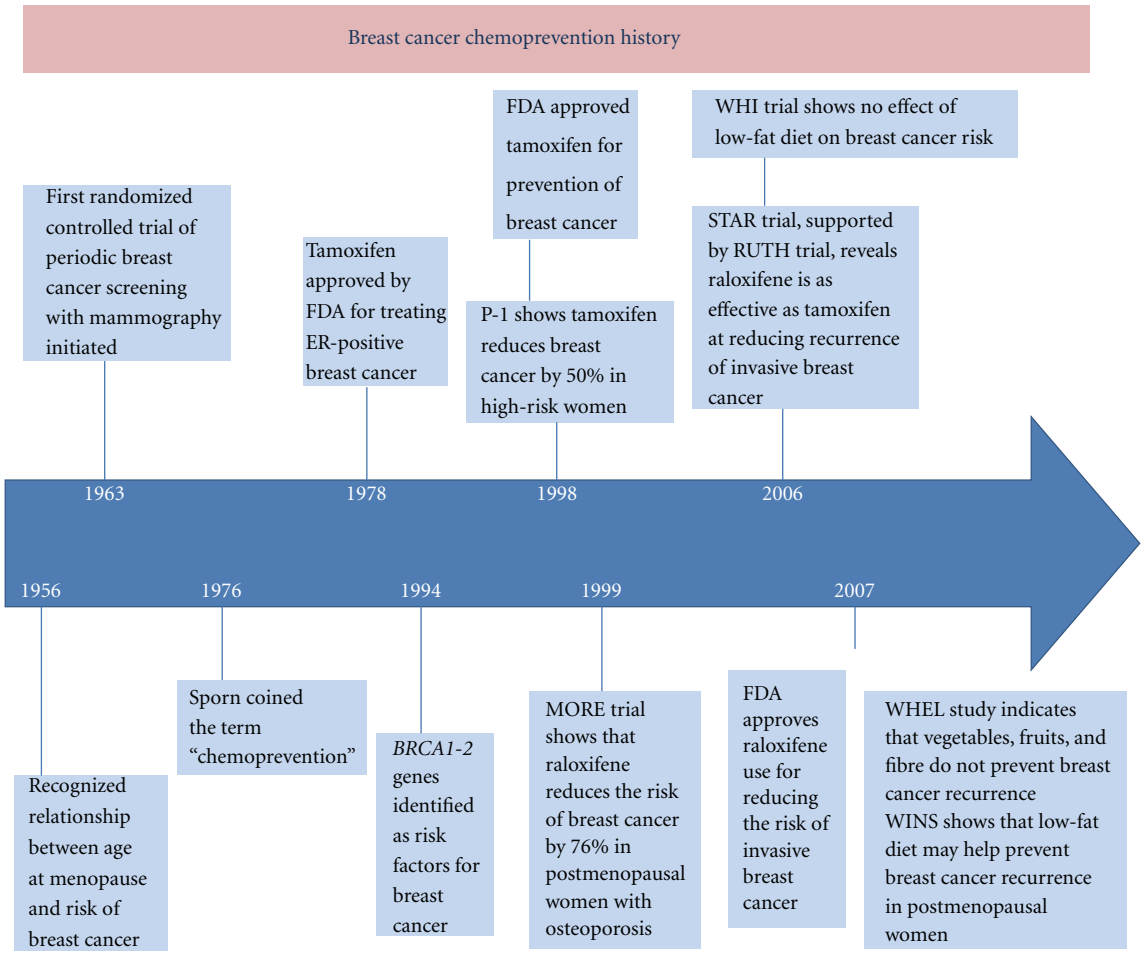

Figure 1: Breast cancer chemoprevention history.

2. ER-Positive Breast Cancer Prevention

Although the precise mechanism that causes breast cancer is not fully established itis recognized that hormones play a significant role in almost 70% of cases [7] and current chemopreventive strategies have targeted hormonally responsive breast cancers.

Estrogen is well established as a promoter of cell division in the breast, where it causes proliferation of both normal and malignant cells [8]. The two major classes of antiestrogenic drugs, the selective estrogen receptor modulators (SERMs) and the aromatase inhibitors (AIs), have been recently used for their activity in breast cancer prevention.

3. SERMs

3.1. Tamoxifen. This class of drugs includes in particular Tamoxifen (TAM) and Raloxifene, acting as both estrogen agonist and antagonists. Tamoxifen citrate is the first generation of SERMs that competes with circulating estrogen for binding the estrogen receptor (ER) [9]. Like tamoxifen, also raloxifene, a second generation of SERMs, has both estrogen agonist and antagonist properties. It differs from tamoxifen principally by its lack of stimulation of endometrium [10].

TAM has been in clinical use for breast cancer treatment for more than 30 years to reduce the risk of both recurrence and contralateral neoplasia, 42% and 47%, respectively [11]. These data lead to choose TAM as a potential chemopreventive agent, and several studies were conducted in last decades in this particular setting.

The BCPT NSABP-1 [12] was a placebo-controlled trial of TAM in more than 13000 women at high risk of breast cancer. Women were randomized to receive tamoxifen 20 mg/die or placebo for 5 years. This trial was closed early after the interim analysis showed a 49% reduction in incidence of invasive breast cancer in the treatment arm. Moreover, the highest level of benefits was observed in patients with precancerous lesions as LCIS (relative risk = 0.44) and atypical ductal hyperplasia (relative risk = 0.14). Tamoxifen appeared able to reduce breast cancer incidence also in healthy BRCA2 carriers by 62% but not in BRCA1 [13]. The study showed also an increased risk of endometrial cancer and thrombotic events, and these conclusions suggested that despite its extraordinary preventive efficacy the utilization of TAM in this particular setting should be extremely individualized.

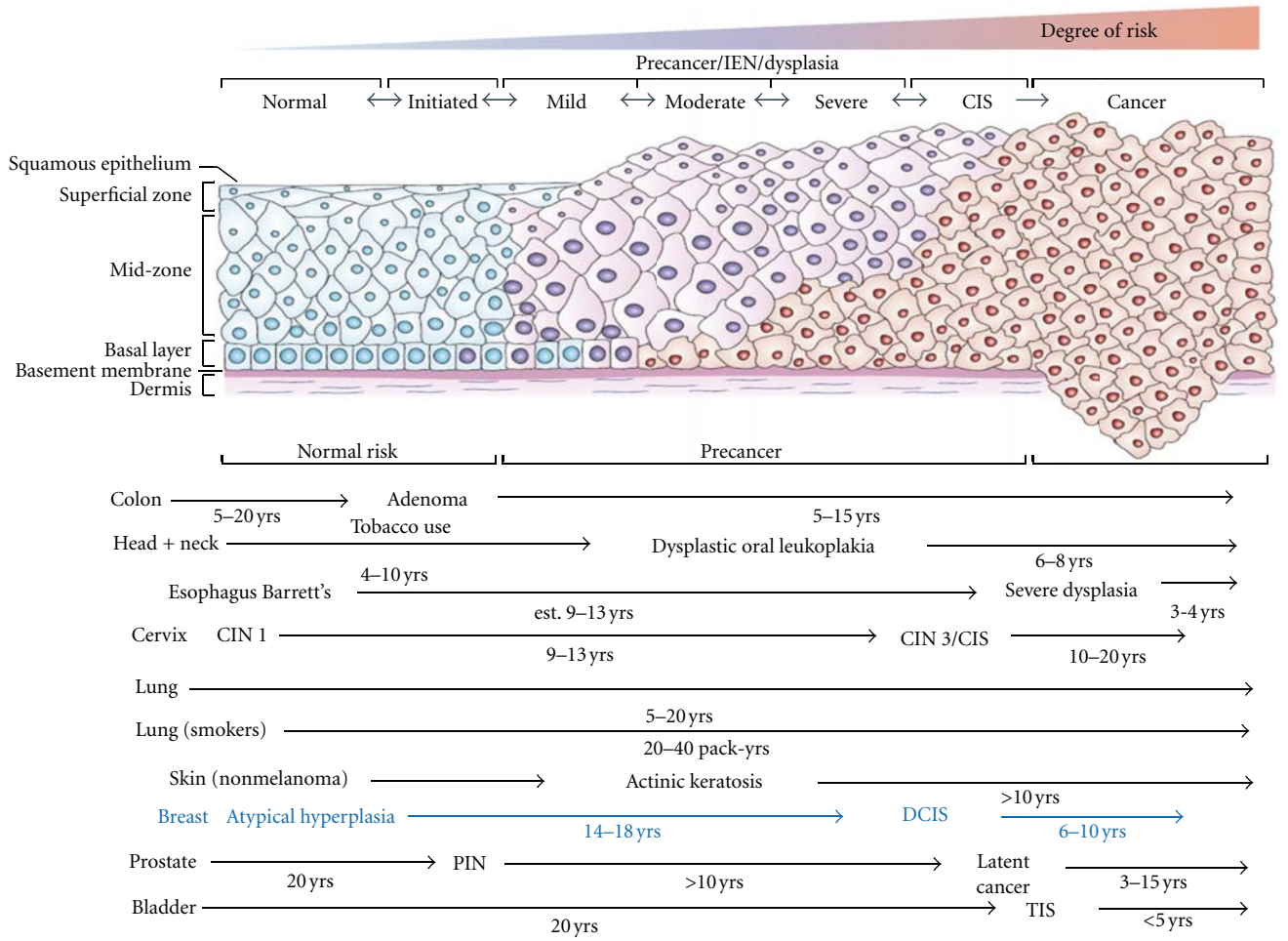

Figure 2: Model of human carcinogenesis.

The results of this study were the first to show the benefit of TAM in breast cancer prevention.

In addition, 3 European tamoxifen prevention trials have been completed and have reported long-term follow-up data of the effect of this agent in BC incidence: The Italian Tamoxifen Prevention Study, The Royal Marsden Hospital tamoxifen randomized chemoprevention trial, and the International Breast Cancer Intervention Study (IBIS)-1 [14]. Although they differ in many details in study design and other, these trials were similar enough to be evaluated together in an overview of their main outcomes [15]. Their combined data indicate an overall 30 to 40% reduction in breast cancer ER-positive incidence following 5 years of TAM versus placebo (Figures 3 and 4), and these effects remains also after more than ten years of followup. The serious adverse events that occurred with TAM were as anticipated from previous adjuvant trials, an increase of endometrial cancers and venous thromboembolic events (VTEs). Other expected TAM-associated toxicities were observed as cataracts, hot flushes, and vaginal discharge.

The data from the NSABP P-1 trial, which showed a reduction in both invasive and noninvasive breast cancers, led to the 1998 US Food and Drug Administration (FDA) approval of TAM for reduction of breast cancer incidence

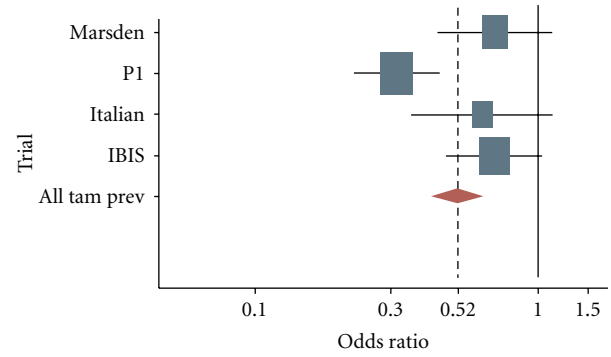

Figure 3: ER+: odds ratios for developing an estrogen receptor-positive invasive breast cancer among women involved in tamoxifen prevention trials.

in high-risk women. However, the adverse effects of this drug have hampered its uptake by women at increased risk. When TAM's benefits are balanced against its major toxicities, younger women at very high risk and possibly hysterectomized postmenopausal women appear to be the best candidates for preventive TAM.

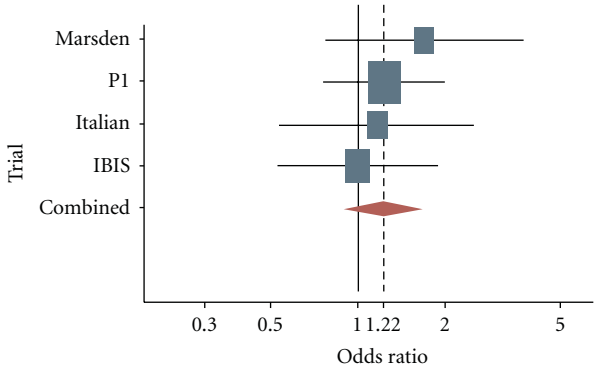

Figure 4: ER−: odds ratios for developing an estrogen receptor-negative invasive breast cancer among women involved in tamoxifen prevention trials.

3.2. Tamoxifen at Lower Dose. A simple and economic approach to retain tamoxifen efficacy while reducing the risks may be a dose reduction. In a study conducted by our group, standard dose of tamoxifen (20 mg/die) and two differ lower doses (10 mg/die and 10 mg on alternate days) were administered for 2 months in a cohort of more than 120 healthy women [16], and changes in serum biomarkers regulated by the ER were evaluated. No evidence for a concentration-response relationship was observed for most of these biomarkers. The concept of dose reduction was further supported by the observation of tamoxifen as very high tissue distribution (5–60 times its blood concentrations) [17] and a prolonged half-life [18]. Moreover, the low-dose concept has been confirmed in a preoperative trial in which 120 breast cancer women were treated with either 20, 5, or 1 mg/die of TAM for 4 weeks before surgery [19]. The effects of these different doses of Tam on proliferation were analyzed using the Ki67 expression as the main surrogate endpoint marker. Interestingly, the change in Ki67 expression induced by lower doses of tamoxifen was comparable to that achieved with the standard dose, implying that low-dose TAM retains its antiproliferative activity. Moreover, several blood biomarkers of TAM estrogenicity associated with the risk of breast cancer, cardiovascular disease and bone fracture showed a dose-response relationship, and suggesting that this approach may be associated with reduced, positive and negative oestrogenic effects of TAM.

These fundamental data provide a strong rationale for the formal assessment of low-dose TAM in preventive setting, and for these reasons we started two phase III randomized placebo trials (actually ongoing in our institute) in order to assess the efficacy of 5 mg/die of TAM in high-risk women as current HRT (HOT study) and with breast intraepithelial neoplasia (IEN).

3.3. Raloxifene. Raloxifene, a second generation of SERMs has reduced the incidence of breast cancer in preclinical models and several clinical trials evaluated it for the prevention of osteoporosis and heart disease [20–22]. Raloxifene, which has a well-established and favourable effect on bone metabolism, was in fact initially approved (by FDA) for the prevention and treatment of osteoporosis in postmenopausal women. In the Multiple Outcomes of Raloxifene Evaluation (MORE) trial [23], raloxifene (60 mg or 120 mg compared to placebo) shows a 30% reduction in the risk of vertebral fracture [24] in postmenopausal women with osteoporosis. One of the secondary endpoints of the study was the incidence of breast cancer in this target of population, and in raloxifene-treated group the risk of invasive breast cancer was significantly reduced by 72% (RR = 28; 95% CI 0.17–0.46) that becomes 62% after 4 years of followup in the Continuing Outcomes Relevant to Evista (CORE) trial [25]. As was noted in the tamoxifen trials, the benefits appeared to be specific only to receptor-positive invasive breast cancer. The adverse events were different in tamoxifen. An increasing risk in thromboembolic events included DVT (deep venous thrombosis) and pulmonary embolism as observed in a raloxifene study (RR = 3.1; 95% CI 1.5–6.2), but unlike what occurs in tamoxifen there was no difference in the incidence of endometrial carcinoma compared with placebo arm [23, 24].

Results of MORE and CORE trials led researchers to conduct a comparative, randomized phase III study of raloxifene versus tamoxifen in more than 19000 postmenopausal women at increased risk for breast cancer [26]. The Study of Tamoxifen and Raloxifene (STAR) trial or NSABP-P2 compared 20 mg of tamoxifen daily to 60 mg of raloxifene daily for 5 years with the incidence of breast cancer as a primary endpoint. The secondary end points included noninvasive breast cancer, uterine malignancies, thromboembolic events, fractures, cataracts, quality of life, and death from any cause. Interestingly, although no untreated control group was included, there was no difference in the incidence of the disease between the two groups (RR: 1.02; 95% CI: 0.82–81.28). Furthermore, while there was a difference between the two treatment groups for the rate of in situ(ductal and lobular) breast cancer, this was not shown to be statistically significant (RR: 1.40; 95% CI: 0.98–92.00). The numbers of invasive breast cancers in both groups of women were statistically equivalent. Conclusive results based on the risk reduction seen in the BCPT for tamoxifen show that both drugs reduced the risk of developing invasive breast cancer by about 50%. While tamoxifen reduced the incidence of LCIS and DCIS, raloxifene did not have an effect on these diagnoses.

A mechanism to explain the difference in noninvasive breast cancer incidence is unknown, but long-term follow-up results for the STAR trial may result in additional information regarding this issue. Regarding the side effects, more uterine malignancies occurred in the Tamoxifen arm and no statistically significant differences were noted between the 2 groups relative to the incidence of any cardiovascular events.

More recently, results from the Raloxifene Use for the Heart (RUTH) study affirmed the benefit of raloxifene with regard to reduced risk of breast cancer. This trial, designed to focus on heart disease, randomized more than 10,000 postmenopausal women with coronary health disease or multiple coronary health disease risk factors to receive either raloxifene 60 mg per day or placebo [27].

Data from the STAR trial and the other raloxifene/placebo trial resulted in the approval of raloxifene by the US Food and Drug Administration for a reduction in the risk of invasive breast cancer in postmenopausal women with osteoporosis and reduction in the risk of invasive breast cancer in postmenopausal women at high risk of invasive breast cancer. No data are currently available on the use of raloxifene in patients with BRCA1 or BRCA2 mutations, nor was raloxifene approved for women with a previous invasive breast cancer or for the treatment of invasive breast cancer. However, the approval of raloxifene gives an important new option to postmenopausal women beyond that of tamoxifen, one that avoids an excess of endometrial cancers and reduces the risk of thromboembolic events.

4. Aromatase Inhibitors (AIs)

High circulatory estrogen levels, as well as high aromatase levels in breast tissue, have been known to increase breast cancer risk. Thus, inhibition of aromatase would be expected to decrease estrogen production and ultimately estrogen-related breast carcinogenesis.

In adjuvant setting, third generation of AIs (anastrozole, letrozole, and exemestane) has been found to superior to tamoxifen and be able to reduce the incidence of contralateral breast cancers by 37 to 55% [28–33]. These agents have resulted in improved disease-free survival and are associated with fewer life-threatening side effects than SERMs [34]. The principle toxicity of AIs is accelerated bone resorption. AIs are generally welltolerated with the primary side effects being musculoskeletal and joint discomfort. Thus, the third-generation aromatase inhibitors (AIs) have been introduced into the treatment of breast cancer, and their greater efficacy compared to tamoxifen, along with a more favorable side-effect profile, makes them attractive agents for use in breast cancer prevention [35, 36].

The International Breast Cancer Intervention (IBIS)-II prevention trial [37], direct consequence of ATAC trial, is actually ongoing and is comparing anastrazole (ANA) to placebo in 6000 postmenopausal women at increased risk to breast cancer. A second complimentary study (IBIS-II) will look at the role of ANA in affected postmenopausal women who underwent a locally excised (or mastectomy) hormone receptor-positive intraductal neoplasia with clear margins. In this second group, ANA is compared to tamoxifen. Both arms address the ability of ANA to reduce the incidence of first primary invasive breast cancers.

Another prevention trial with AIs (MAP3) is actually underway with exemestane (EXE). Authors are comparing placebo or EXE, or EXE plus celecoxib for 5 years in more than 5000 high-risk postmenopausal women. In September 2004 the disclosure of an excess of adverse cardiovascular events in the COX-2 inhibitor arm has recommended authors to revised the study design. They modified it in two different arms (exemestane vs placebo) and a new simple size of 4.560. Despite this, the MAP3 study was reopened to accrual in March 2005 with a revised sample size of 4,560 and two arms, EXE 25 mg/d alone and placebo. The primary endpoint is the incidence of breast cancer specifically to determine if EXE is able to reduce invasive breast cancer by 65% compared to placebo. Secondary endpoints regard also safety and incidence of noninvasive breast cancer.

These data obtained by adjuvant trial provide a rational for exploring AIs in prevention setting. They are superior to tamoxifen, and we hypothesized that the major of ER-positive breast cancer (but not for ER negative) can be prevented by these drugs. Moreover, they are also well tolerated than tamoxifen without uterine and thrombotic effects, but they do lead to bone mineral loss. These effects should be contrasted by the use of bisphosphonates.

5. ER-Negative Breast Cancer Prevention

Although a number of antiestrogenic agents are being extensively tested in clinical trials, all these agents affect the endocrine pathway and suppress only the development, of estrogen receptor (ER)-positive breast cancer. They have no effect in reducing the risk of ER-negative breast cancer, which accounts for 20–30% of breast cancers and has a poor prognosis [38].

Thus, it is worth identifying new pathways, biomarkers, and agents that are effective in the treatment and prevention of these subtypes. With the accumulating knowledge in understanding the biology of cancer development several classes of a new generation of chemopreventive agents modulating the nonendocrine biochemical pathways have been developed and many of these are still currently under investigation.

These agents include retinoids, epidermal growth factor receptor (EGFR), tyrosine kinase inhibitors (TKIs), cyclooxygenase-2 (COX-2) inhibitors, bisphosphonates, vitamin D receptor (VDR), statins, peroxisome proliferator-activated receptor (PPAR), and others. A complete summary of involved agents, with their specific pathways, is shown in Table 1 and a brief state of the art of the more compounds involved are analyzed below.

5.1. Retinoids. Retinoids are natural and synthetic derivative of Vitamin A (Retinol) that have profound effects on development, metabolism, differentiation, and cell growth.

The retinoid, the most widely studied in chemoprevention clinical trials, is the synthetic amide of retinoic acid *N*-(4-hydroxyphenyl) retinamide (4-HPR), or fenretinide. Fenretinide has been found to exert significant chemopreventive activity in various in vitro and in vivo studies [39–42]. A phase III clinical trial, using 4-HPR to reduce the incidence of secondary breast cancer in almost 3000 patients, was published in 1999 and showed no difference in contralateral and ipsilateral breast cancers; however, a posthoc analysis suggested a significant treatment interaction with menopausal status. In particular, it showed a 35% reduction in premenopausal women and an opposite trend in postmenopausal women [43]. Moreover, the 15-year follow-up of this trial substantially confirmed these results, and the risk reduction is of the order of 50% in women aged

Table 1: Class, specific pathways, and agents actually involved in the treatment and prevention of ER-breast cancer.

Class	Targets	Drugs or agents
Nuclear receptors	Retinoid acid receptor RXR	Fenretinide (4-HPR) 9 cis-retinoic acid (Targretin)
	VDR	VIT D3 analogues
	$PPAR_{\gamma}$	Troglitazone, rosiglitazone, pioglitazone
Membrane receptors and signal transduction	HMG-CoA	Statins
	Tyrosine kinase	Gefitinib (Iressa)
	HER-1, HER-2	Trastuzumab (Herceptin), lapatinib, gefitinib, erlotinib
	IGF-R, IGF-1, IGFBP3	Metformin
Anti-inflammatory and antioxidant	COX-2	celecoxib, rofecoxib, NSAIDs
Angiogenesis	VEGF	Bevacizumab
DNA modulation	BRCA1-BRCA2	PARP inhibitors

4-HPR: N-(4-hydroxyphenyl) retinamide; COX: cyclooxygenase; ER: oestrogen receptor; HMGCoA: 3 hydroxy-3 methylglutaryl coenzyme A; NSAIDs: nonsteroidal anti-inflammatory drugs; PARP: poly (ADP-ribose) polymerases; PPAR: peroxisome proliferator-activated receptor; RXr: retinoid X receptors, VDR: vitamin D receptor.

40 years or younger and persists for 10 years after retinoid cessation [44]. Moreover, 4-HPR was observed to reduce secondary tumours in premenopausal women irrespective of the hormone receptor status of the primary cancer, suggesting that retinoids have a potential chemopreventive effect on ER-negative and ER-positive breast cancers.

Recently, also a new RXR-selective retinoid, commonly named as rexinoids, has been studied as cancer preventative agent. Preclinical studies in fact have demonstrated that this compound is able to maintain the chemopreventive efficacy of the retinoids, also in ER-negative setting, but with substantial minor toxicity [45, 46]. For this reason this agent is actually considered particularly attractive in prevention setting.

5.2. EGFR-Tyrosine Kinase Inhibitors. The EGFR is one of a family of four closely related receptors (EGFR or erbB-1, HER-2/neu or erbB-2, HER-3 or erbB-3, and HER-4 or erbB-4) that uses tyrosine kinase activity and contributes to a large number of processes involved in tumour survival and growth, including cell proliferation and inhibition of apoptosis, angiogenesis, and metastasis [47], thus making it an attractive target for cancer prevention and treatment, because agents that are able to block the erbB-signaling pathways are promising in the treatment and prevention of breast cancer.

In particular, the researchers focused their attention to EGFR-HER-1 and HER-2 pathways, because the mechanism of resistance to antioestrogen therapy is usually associated with an increased expression of HER-1 and HER-2 receptors. Inhibition of tyrosine kinase activity, with TKIs, involved in the EGFR signaling cascade could be the right pathway for the treatment and prevention of ER-independent breast.

There are two different and concomitant strategies able to inhibit erbB activity. One involves blockade of this activity with monoclonal antibodies (trastuzumab), whereas the second involves the TKIs. The two strategies differ in several pharmacological properties [48].

Amplification of the HER2 gene and overexpression of it's related protein have been found in almost 30% of human breast cancer and it is generally correlated with poorer outcomes compared with tumors HER2 negative [49, 50]. Moreover, there is substantial evidence of an inverse correlation between HER2 expression and hormone receptor [51]. In an effort to improve the prognosis of these HER2+ cancers, research has focused therapies directly against this pathway and in particular included the monoclonal antibodies *trastuzumab (Herceptin)*.

Trastuzumab has largely showed its benefit in adjuvant therapy; in particular, it is able to increase the clinical benefit of first-line chemotherapy in metastatic HER-2 breast cancer [52], and this benefit seems to be irrespective of the ER status [53]. Important results were also obtained in early breast cancer setting [54–56]. The drug is generally well tolerated, but its possible cardiotoxicity and its route of administration (intravenously) make it difficult to propose it to healthy women as chemoprevention.

Apart from the monoclonal antibodies directed against the extracellular receptor domain of HER-2, there is another way to contest erbB activity. As previously explained, the use of small molecules inhibit intracellular tyrosine kinase activity, named TKIs. TKIs have several advantages over monoclonal antibodies such as oral bioavailability, potentially less toxicity, and ability to inhibit truncated forms of EGF receptors [57].

Lapatinib (Tykerb) is a reversible small-molecule TKI that targets both HER-2 and EGFR tyrosine kinase. It is able to interrupt signal transduction from both EGFR and HER-2 receptors, and because of its dual-receptor activity it has been evaluated in several phase II and III trials in various forms of breast cancer [58–60]. Moreover, in the prevention setting, it showed a significant delay in the ER-negative mammary tumors development [61]. This preventive action was seen in premalignant mammary lesions, and this suggests a drug efficacy also in initiation and progression of breast carcinogenesis.

Gefitinib (Iressa), another EGFR tyrosine kinase inhibitor that suppressed ER-negative mammary tumor formation in MMTV-ErbB2 transgenic mice [47]. Its mechanism of action is complex and involves cell cycle, angiogenesis, and growth factors [62, 63]. Moreover, results of preclinical and clinical studies about breast cancer treatment remain controversial

[64, 65], but in preventive setting, the ability of gefitinib to inhibit the proliferation at the early stages of breast cancer and also in the normal adjacent epithelium [66] could be the rationale for using this compound in prevention trial.

5.3. COX-2 Inhibitors. The inducible isoenzyme COX-2 is expressed in invasive and in situ breast cancers cells [67], and several epidemiological studies have shown the inverse relationship between nonsteroidal, anti-inflammatory drugs (NSAIDs) and cancer incidence [68, 69]. COX-2 is the main target of NSAIDs, and despite the mechanism by which it contributes to tumor formation is not fully understood, it is possible to hypothesize an involvement of a multidisciplinary process which involves proliferative stimulation, mutagen production, and apoptosis inhibition. The COX-1 and COX-2 pathway, which converts arachidonic acid to prostaglandin, is involved in the development and growth of several different neoplastic lesions [70], and it is frequently overexpressed not only in invasive breast cancer but also in adjacent intraductal neoplasia; therefore, it might be an early event in mammary tumorigenesis [71]. A meta-analysis, published in 2001, demonstrated that NSAIDs were associated with a 20% reduction of breast cancer risk, and the same results were confirmed in a more recent publication [72, 73]. These data suggested the chemopreventive potential (including breast cancer) of anti-inflammatory drugs.

Celecoxib, a selective COX-2 inhibitor, reduced the incidence and multiplicity of DMBA-induced mammary tumors in rat models by 68 and 86%, respectively [74]. Nimesulide, another selective COX-2 inhibitor, significantly reduced the incidence and multiplicity of PhIP and NMU-induced rat mammary tumors [75]. Similar effects were observed with aspirin, but the level of evidence for both of agents on breast cancer incidence is, at present, too small to justify their use solely as a preventive therapy and insufficient to make any recommendations.

5.4. Bisphosphonates. Bisphosphonates, the drugs of choice for the treatment of osteoporosis, act on the mevalonate pathway [76] and for this reason are currently of considerable interest in the treatment and prevention of breast cancer. Their mechanism of action involved osteoclasts, and in particular, they are able to inhibit their activity [77]. Thus they have proven efficacy in control of breast cancer bone metastases and also in bone loss induced by other treatment as AIs [78]. Previous studies showed the antiangiogenic, antiproliferative, and proapoptotic effect of these drugs [79]. Moreover, interestingly recent two cohort studies showed a reduction of 30% in breast cancer incidence in bisphosphonates users [80, 81], irrespective of hormonal status. Bisphosphonates are generally well tolerated, but randomised prevention trials with composite endpoints in women with osteopenia and increased risk of a new breast cancer are required to fully investigate the risk-benefit profile of these drugs.

5.5. Poly(ADP-Ribose) Polymerases Inhibitors. Poly(ADP-ribose) polymerases (PARPs) are a family of enzymes that play a key role in the repair of DNA damage [82]. In particular, the most important seems to be PARP enzymes (PARP-1 and PARP-2) [83, 84]. Their role was recently considerable of interest in oncology treatment and prevention.

A key role for PARP-1 and PARP-2 is maintaining genomic integrity, in particular, repair of single strand DNA lesions and breaks using the base excision repair (BER) pathway. The inhibition of these enzymes leads to accumulation of DNA single-strand breaks, which can lead to DNA double-strand breaks at replication forks [85, 86]. Normally, these breaks are repaired and a key component of this mechanism comprises the tumour-suppressor proteins BRCA1 and BRCA2. In BRCA mutated cells, this DNA repair ability is lost and the aberrations drive to carcinogenesis. Consequently, the requirement for a BRCA mutation to be present for a PARP inhibitor to be effective constitutes a synthetic lethal strategy selectively affecting mutant tumour cells comprising BRCA1-BRCA2, which are 1000-fold more sensitive than others [87–89]. Recent preclinical studies have shown encouraging results, and at present PARP inhibitors are usually studied in combination with other cytotoxic agents [90, 91]. The only published study with a PARP inhibitor as a single-agent treatment is a phase I trial with olaparib in patient with BRCA-associated cancer, which showed a good efficacy to inhibit PARP activity and that it has few side effects compared conventional chemotherapy [92].

The efficacy of a particular risk group as the mutation carriers and the relative good tolerability make these agents well suited for cancer prevention. Further investigations should be proposed in BRCA mutation carriers to assess the ability of this class of agents to prevent cancer, evaluate the safety profile, and reduce the incidence of breast cancer.

5.6. Metformin. There is increasing evidence that presence of hyperinsulinemia and insulin resistance increased breast cancer risk, worsen, the prognosised and partly explained the obesity-breast cancer risk association in postmenopausal women [93–98]. Several epidemiological and observational studies have confirmed the relationship between insulin levels and cancer induction [99, 100]. Insulin may promote tumorigenesis via a direct effect on epithelial tissues, or indirectly by affecting other modulators, such as insulin-like growth factors, sex hormones, and adipokines [101, 102]. Thus, there is a great interest in exploring the possibility that antidiabetic therapies, which lower insulin levels, could decrease breast cancer incidence or its related mortality.

Metformin, a biguanide derivative, is the most commonly used drug worldwide to treat type II diabetes. It is generally well tolerated with low toxicity and a very low cost. Epidemiological studies have shown a significant risk reduction in cancer incidence and mortality in diabetic patients on metformin, relative to other antidiabetic drugs, including positive results specifically in breast cancer [103–105]. It may impact cancer through a direct (insulin-independent) activation of AMPK-mTOR pathway mechanism or indirect effect (insulin-dependent) reducing hepatic gluconeogenesis obtaining lower circulating insulin levels with inhibition of proliferation cells and protein synthesis over increase apoptosis (Figure 5).

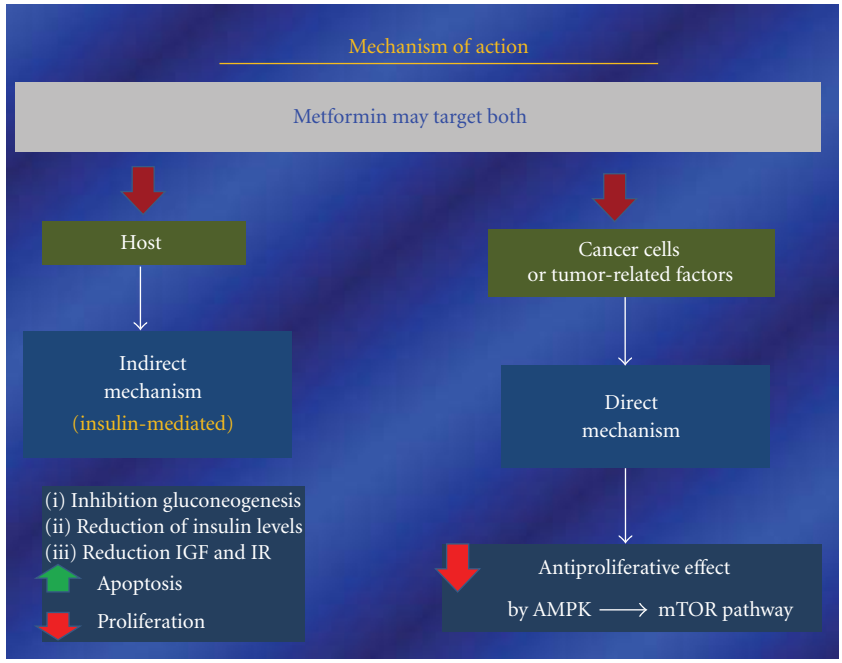

Figure 5: Metformin anticancer mechanism of action.

Several preclinical studies have confirmed these effects of metformin in vitro and in vivo and showed a significant reduction of both breast epithelial cell proliferation and protein synthesis [106, 107]. [108] In particular, it is confirmed that metformin produces a significant repression of cell proliferation, and moreover, they also found that this effect was different in human breast cancer cell lines if related to either positive or negative ERs. They in fact detected a complete cell growth repression in ER-positive cell lines, although only a partial inhibition was detected in ER-negative phenotypes. These data suggest that, although ER-negative cells are not as sensitive as ER-positive ones, both of them show a reduction in cell growth under metformin treatment. These data make metformin an intriguing compound for treatment and prevention of breast cancer. Several important phase II and III studies are actually ongoing in the world in order to confirm and clarify these promising settings [109].

6. New Molecular Targets for Breast Cancer Chemoprevention

Many molecularly pathways and the correlated targeted drugs are actually in development for advanced cancer therapy, and they have potential activity and tolerability also in cancer chemoprevention setting. The identification of new potential molecular targets and the development of agents aimed at these targets within cancer have already had a significant impact on advanced cancer therapy and provide a wealth of opportunities for chemoprevention.

6.1. Microenvironment and Its Molecular Targets. There is substantial evidence that together with the epithelial cells alterations the microenvironment dysfunction is crucial for carcinogenesis, and this makes the microenvironment an interesting target for breast cancer chemoprevention. In particular, there are many excellent publications which consider microenvironment as a good target for cancer therapy, but the application of chemoprevention to control the tumour microenvironment during the early stages of carcinogenesis is not yet adequately analyzed. We will briefly explain a recent progress that indicates that the effects of chemopreventive agents on the microenvironment are an important aspect of their preventive action and that many classes of agents, which showed to have significant chemopreventive actions on epithelia, also have similar useful actions on the microenvironment.

Many molecular targets inside the microenvironment with the correlated drugs are summarized in the Table 2.

These transcription factors and their associated regulatory proteins are an ideal target of chemoprevention, and in particular three attractive pathways as the nuclear factor κB (NFκB), hypoxia-inducible factor 1α (HIF-1α), and PI3κ-mTOR are analyzed in this section.

Nuclear factor-κB pathway plays important roles in the control of cell proliferation, differentiation, apoptosis, inflammation, stress response, cell signaling transduction, and other physiological processes, but it is also critically involved in the processes of development and progression of cancers [110–113]. NF-kappaB is critically involved in the processes of oxidative stress response. Oxidative stress is defined as an increase in intracellular reactive oxygen species (ROS) such as H_2O_2, superoxide, and hydroxyl radical and. ROS in cells are increased in response to agents that also activate NFkappaB. These findings suggest that oxidative stress activates NF-kappaB activity in the cells [114, 115].

Table 2: Molecular targets and chemopreventive agents in the microenvironment.

Molecular targets	Chemopreventive agents
Oestrogen receptors	Tamoxifen; raloxifene; aroxifene
Akt and NFκB	Curcumin; N-acetyl cysteine; silibinin; xanthohumol; deguelin; EGCG; resveratrol
NRF2-KEAP1	Sulphoraphane; oltipraz
COX2	Rofecoxib; celecoxib; EGCG
COX1/2	Aspirin and other NSAIDs
Histone deacetylases	Sulphoraphane
TGFβ pathway	CDDO-Imidazolide
HIF1α	WGCG; resveratrol; apigenin; sulphoraphane
STATs	CDDO-Imidazolide
VEGF	Sulphoraphane; EGCG; fenretinide

Some of the specific targets in the microenvironment and specific agents that interact with these targets: CDDO: 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; COX2: cyclooxygenase 2; EGCG: epigallocatechin-3-gallate; HIF1α: hypoxia-inducible factor 1α; KEAP1: kelch-like ECH-associated protein 1; NFκB: nuclear factor κB; NSAIDs: nonsteroidal anti-inflammatory drugs; STATs: signal transducers and activators of transcription; TGFβ: transforming growth factor-β; VEGF: vascular endothelial growth factor.

Moreover, NFκB is activated not only by the ROS but also by various carcinogen and tumor promoters [116], and these are the reasons why NFκB is overexpressed and activated in various cancers, especially in the poorly differentiated.

Experimental studies have shown that natural antioxidant compounds including isoflavones, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), curcumin, epigallocatechin-3-gallate (EGCG), rosveratrol, curcumin and others seems to be able to inhibit the activity of NF-kappaB and the growth of cancer cells and also to induce apoptosis, suggesting that NF-kappaB could be a target for cancer prevention [117–121].

Similarly, HIF-1—a master regulator in the control of tissue homeostasis, crucial in adaptive responses to tissue oxygenation including energy status, glucose, and iron metabolism as well as growth factor signaling [122, 123]—is a key target for the prevention and treatment of cancer.

Recent experimental evidence in fact suggests that HIF-1 is a key player in carcinogenesis. Interest in the role of HIF-1 in cancer has grown exponentially over the last two decades, as this factor activates the transcription of many genes that code for proteins involved in several pathways intimately related to cancer [124–126].

Tumors are invariably less well oxygenated than the normal tissues from which they arise. Hypoxia-inducible factor-1 (HIF-1) plays a central role in the adaptation of tumor cells to hypoxia by activating the transcription of genes, which regulate several biological processes.

For these reasons, HIF-1 is considered a potential target for cancer therapy, and, recently, many efforts to develop new HIF-1-targeting agents have been made [127–130]. Interestingly, they are recently identified by increased HIF-1 expression (relative to adjacent normal tissue) in 13 tumor types, including lung, prostate, breast, and colon carcinoma. Moreover, HIF-1 was also overexpressed in preneoplastic and premalignant lesions, such as colonic adenoma, breast ductal carcinoma in situ, and prostate intraepithelial neoplasia. These data show that overexpression of HIF-1 may occur very early in carcinogenesis, before histologic evidence of angiogenesis or invasion [131], and suggest that HIF-1 might be a biomarker of carcinogenesis and a suitable target for cancer chemoprevention. Because HIF-1 seems to have an important function in carcinogenesis, HIF-1 inhibitors may be considered a source of potential cancer chemopreventive agents. Several, approved anticancer drugs (e.g., topotecan, imatinib mesylate, trastuzumab, NS398, celecoxib, and ibuprofen) inhibit HIF-1 activity [127]. Moreover, also several natural products (e.g., resveratrol, genistein, apigenin, and berberin) have also been found to inhibit the activity of this transcription [129]. In this setting it is important to say that: however, the use of HIF-1 inhibitors in cancer chemoprevention might be associated with toxicity. An excessive inhibition of HIF-1 may produce adverse effects, as HIF-1 regulates many cellular processes under physiologic conditions [125, 132]. Therefore, although HIF-1 inhibitors may represent a useful source of chemopreventive agents, the potential toxicity associated with these agents should be considered carefully, especially when chemopreventive interventions are aimed at preventing cancer in healthy populations.

The mammalian target of rapamycin (mTOR) is a signaling kinase of the phosphatidylinositol 3-kinase/protein kinase B or PI3K pathway that mediates cell growth and metabolism and coordinates cell cycle progression in response to genetic, epigenetic, and environmental conditions. Pathways involved in mTOR signaling are dysregulated in precancerous human tissues, including breast cancer, and is associated with the development of resistance to endocrine therapy [133–135] and to the anti-human epidermal growth factor receptor-2 (HER2) monoclonal antibody trastuzumab [136, 137]. Rapamycin and the rapalogues have been used in clinical trials for many cancer types. Phase I trials have demonstrated that mTOR inhibitors are fairly well tolerated with the most frequent drug-related toxic effects being acne-like maculopapular rash, mucositis, and stomatitis, all of which were reversible on discontinuation of treatment [138].

Rapamycin and its analogues, the "rapalogues," decrease tumor growth in many xenograft models, including those

with breast cancer cell lines [139, 140]. Thus, preclinical data have confirmed the antitumor activity of rapamycin and the rapalogues and have suggested that patients with breast cancer may especially respond to mTOR inhibitors. Phase I-II clinical trials have demonstrated that everolimus (RAD-001), an mTOR inhibitor with demonstrated preclinical activity against breast cancer cell lines, has been shown to reverse Akt-induced resistance to hormonal therapy and trastuzumab. It has promising clinical activity in women with HER2-positive, HER2-negative, and estrogen receptor-positive breast cancer when combined with HER2-targeted therapy, cytotoxic chemotherapy, and hormonal therapy, respectively.

The involvement of mTOR pathways in precancerous lesions makes the mTOR signaling an intriguing target for chemopreventive intervention. Thus, several recent preclinical studies explored also the possibility of a chemopreventive action through the mTOR inhibition. In one of this, rapamycin showed chemopreventive activity against mammary gland tumors in transgenic mice, bearing activated ErbB2 (HER-2/neu) receptor either alone (NeuYD) or with VEGF expression [141], where it dramatically inhibited tumor formation in NeuYD mice.

These results seem to suggest the mTOR inhibition as a possible chemopreventive strategy against metachronous tumors or recurrence in high-risk patients, whose primary tumors overexpressed ErbB-2, or in patients showing dysregulation of the PI3K/AKT/mTOR signaling pathway.

Another recent preclinical study evaluated chemopreventive effects of rapamycin in a transgenic mouse model of human breast carcinogenesis [142], where it significantly inhibited growth of mammary intraepithelial neoplasia outgrowths, invasive tumor incidence, and tumor burden.

Finally, some natural products, such as epigallocatechin gallate (EGCG), caffeine, curcumin, and resveratrol, have been found to inhibit mTOR as well and are actually under investigations in this setting.

7. Conclusions

In conclusion, the success of chemopreventive approach depends on a tumor-specific risk model for identifying high-risk subjects, increasing preclinical drug test over the development novel and more safety chemopreventative agents, and identifying new surrogate endpoint using molecular pathways and new targets of drugs activity.

Safety is a very important point to take into account, because several large randomized prevention trials in several cancers have shown that major adverse events can prevent widespread public acceptance of active chemoprevention agents.

Despite the success of action showed for example in endocrine intervention is a promising starting point in order to continue to evolve with the rapid integration of molecular approaches into research and clinical practice. It is urgent to find active agents in other fields as nonhormone-responsive lesions. The personalized approaches in advanced cancer therapy and the evolution of molecularly targeted will streamline chemoprevention research and facilitate the development of rational, effective, and safe preventive drugs, involving different pathways and with the ability to modify carcinogenesis in early phases.

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Review Article

Colorectal Cancer Chemoprevention by Mesalazine and Its Derivatives

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Patients with inflammatory bowel disease (IBD) face an increased lifetime risk of developing colorectal cancer (CRC). Independent factors associated with increased risk include long disease duration, extensive colonic involvement, young age at onset of IBD, severity of inflammation, primary sclerosing cholangitis, backwash ileitis, and a family history of CRC, thus emphasising the role of intestinal inflammation as an underlying mechanism. This notion is also supported by the demonstration that the use of certain drugs used to attenuate the ongoing mucosal inflammation, such as mesalazine, seems to associate with a reduced incidence of colitis-associated CRC. In the last decade, work from many laboratories has contributed to delineate the mechanisms by which mesalazine alters CRC cell behaviour. In this paper, we review the available experimental data supporting the ability of mesalazine and its derivatives to interfere with intracellular signals involved in CRC cell growth.

1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), the major forms of inflammatory bowel diseases (IBDs) in humans, are chronic inflammatory disorders of the gastrointestinal tract [1]. In UC, inflammation is restricted to the mucosa and extends proximally from the rectum to involve all or part of the colon. CD is typically a patchy, transmural, inflammatory disease that can affect the gastrointestinal tract anywhere from the mouth to the anus. Both IBDs are characterized by episodes of remission and exacerbations in which the patient experiences abdominal pain, diarrhea, blood in the stool, and systemic symptoms [1]. Patients with long-standing disease and severe inflammatory lesions involving the entire colon have increased risk of developing colorectal cancer (CRC) [2–4]. A family history of CRC, the presence of primary sclerosing cholangitis, backwash ileitis, and, in some studies, young age at onset of colitis increase further the risk of IBD-associated CRC [5–8]. CRC complicating the natural history of IBDs accounts for only 1-2% of all cases of CRC [9]. Nonetheless, chronic colitis is among the top high-risk conditions for CRC, and IBD patients are 6 times more likely to develop CRC than the general population [10].

Unlike sporadic CRC in which the dysplastic precursor is usually the adenomatous polyp, IBD-associated dysplasia can be both polypoid and flat, localized or diffuse, and this probably reflects the fact that carcinogenesis in the inflamed colon follows a different sequence of genetic alterations than that observed in sporadic CRC [11]. The goal of CRC screening and surveillance colonoscopy in IBD population is detection of premalignant changes early enough that intervention can prevent complications of invasive cancer. Unfortunately, however, surveillance programs have not substantially prolonged survival in IBD patients, due to the technical limitations of recognizing dysplastic lesions in flat, normal-appearing mucosa [12]. This clearly suggests the need of alternative strategies of chemoprevention.

Over the last decades, the advent of sophisticated techniques of molecular biology has contributed to the identification of key steps in the process of colon carcinogenesis, thereby facilitating the development of new drugs that efficiently target malignant cells. Some of these compounds, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and selective inhibitors of cyclooxygenase (COX)-2, are effective in attenuating the growth and diffusion of CRC cells [13, 14].

However, their use in the chemopreventive programs of IBD-related CRC is not justified because the administration of NSAIDs and COX2-inhibitors in IBD patients associates with high risk of disease flare-ups [15, 16].

Mesalazine or 5-aminosalicylic acid (5-ASA) is a drug widely used in IBDs, mainly UC, for the treatment of mild relapses and maintenance of remission. Mesalazine is structurally related to NSAIDs, but unlike these compounds, it is safe and free of serious adverse effects. Epidemiological observations indicate that mesalazine can be also chemopreventive for IBD-associated CRC [4, 17, 18], even though one study has documented no benefit [19]. Moreover studies conducted in experimental models of carcinogenesis have shown that the drug has many targets in cancer cells and modulates multiple biological pathways that sustain CRC [20]. In this paper we review the available experimental data that demonstrate the ability of mesalazine and its derivatives to interfere with intracellular signals involved in CRC cell growth.

2. Mesalazine Inhibits CRC Cell Growth and Survival

The process of carcinogenesis is facilitated by a complex and dynamic interaction between genes and environmental factors that ultimately affects cell growth and survival. As a matter of fact, apoptosis progressively decreases and proliferation increases in the sequential steps from normal colonic mucosa to dysplasia and CRC. Therefore, compounds that inhibit cell proliferation and/or enhance cell apoptosis could find a place in the therapeutic armamentarium for CRC. The initial demonstration that mesalazine can block the growth and promote apoptosis of CRC cells comes from *ex vivo* studies in patients with colonic adenoma. Reinacher-Schick et al. showed that mesalazine administered orally to patients with sporadic polyps increased the apoptotic rate and decreased proliferation of cancer cells [21]. Bus et al. demonstrated that rectal administration of mesalazine in patients with sporadic CRC enhanced apoptosis of tumor cells [22]. These observations were supported by studies in the mouse model of colitis-associated CRC, induced by administration of azoxymethane (AOM) followed by repeated oral administration of dextran sulfate sodium (DSS), which showed that mesalazine reduced the number and size of neoplasms [23, 24].

It is conceivable that the anti-neoplastic properties of mesalazine are in part linked to the ability of the drug to target mucosal immune cells and inhibit expression and/or activity of various molecules involved in colon carcinogenesis (e.g., COX-2, inducible nitric oxide synthase, reactive oxygen species and nuclear factor-kB). However, mesalazine could also regulate directly the activity of neoplastic cells. Indeed, it has been shown that mesalazine inhibits the growth and enhances the apoptosis of several CRC cell lines in a time- and dose-dependent manner [25]. The anti-mitogenic effect of mesalazine is also seen in COX-2-deficient cells raising the possibility that mesalazine inhibits CRC cell growth via both COX-2-dependent and independent mechanisms [26].

Mesalazine-induced antiproliferative effect is associated with modulation of replication checkpoints in CRC cells, which ultimately alter cell cycle progression. In this context, we have shown that increasing concentrations of mesalazine (i.e., 5–30 mM) cause a progressive accumulation of CRC cells in S phase, thereby decreasing the percentage of cells in G2/M and G0/G1 [27]. Mesalazine-treated cells exhibit a reduced expression of the phosphatase CDC25A, but not CDC25B or CDC25C, and inactivation of CDK2, a target of CDC25A [27]. Since CDK2 binds to either cyclin E or cyclin A and regulates the G1/S transition and S phase progression, respectively [28], mesalazine-induced CDK2 inactivation could be responsible for cell cycle block in S-phase. The exact mechanism by which mesalazine down-regulates CDC25A expression is not yet known. It is unlikely that CHK1 and CHK2, two upstream kinases that phosphorylate and promote degradation of CDC25A are involved in the negative regulation of CDC25A, because mesalazine down-regulates CDC25A, expression in CHK1- or CHK2-deficient cells [27]. By contrast, we showed that cells treated with mesalazine displayed enhanced ubiquitination and proteasome-dependent degradation of CDC25A [27]. These findings together with the demonstration that mesalazine does not affect CDC25A RNA expression strongly suggest that CDC25A is posttranscriptionally down-regulated in mesalazine-treated cells. These results are consistent with those published by Luciani and colleagues, who show that exposure of CRC cells to mesalazine causes a reversible accumulation of cells in S-phase [29]. These authors showed that such an effect was p53 independent and related with the activation of proteins involved in the ATM- and Rad3-related kinase- (ATR-) dependent S-phase checkpoint response (e.g., Chk1, RAD17) [29]. Further work by other researchers has confirmed the ability of mesalazine to interfere with cell cycle progression [30, 31] and shown that CRC cells can accumulate in various phases of the cell cycle in relation to the dose and time of exposure to the drug.

3. Effects of Mesalazine on Replication Fidelity

Both sporadic and IBD-related CRC are characterized by a very similar frequency of the two major types of genomic instability, namely, chromosomal instability (CIN) and microsatellite instability (MIN) [32]. CIN is characterized by atypical segregation of chromosomes and abnormal DNA content (aneuploidy), with consequent loss of whole (or part of) chromosomes and function of critical tumor suppressor genes (e.g., *adenomatous polyposis coli*, *p53*). MIN is characterized by an increased rate of point mutations and is dependent on defects in the mismatch repair system, a mechanism involved in repairing DNA base-pair mismatches, which occur in the normal DNA replication. During this process, frameshift mutations, called microsatellites, tend to accumulate. Microsatellites occur mainly in intronic DNA sequences thus resulting in no gene function alteration. However, when microsatellites are located in exonic gene regions, there can be a shift in the codon reading frame which results in a loss of protein function [33]. Studies performed by Gasche

and Campregher demonstrated that mesalazine improves replication fidelity in cultured CRC cells independently of the presence of mismatch repair system [34, 35]. The mechanism by which mesalazine inhibits the generation of frameshift mutations remains unknown, even though it could in part rely on the ability of the drug to slow down DNA replication and cell division [29], because cell cycle regulation is one of the defense mechanisms that allow cells to either repair DNA damage or eventually undergo apoptosis thus safeguarding the integrity of the genome [36].

4. Mesalazine Inhibits Wnt/β-Catenin Pathway and EGFR Activation

An intriguing possibility that emerges from the available experimental data is that the antitumoral properties of mesalazine reflect the ability of the drug to target several pathways that are both early and common in colorectal carcinogenesis. The Wnt/β-catenin pathway, which is constitutively activated in the majority of CRC, is one of such targets. In this pathway, Wnt binds to the transmembrane Frizzled receptor, leading to the activation of the cytoplasmic disheveled (Dsh) protein. Dsh forms a complex with the β-catenin degradation complex, which consists of the *APC* gene product, glycogen synthase kinase-3β (GSK-3β), axin, and β-catenin. In the absence of Wnt, GSK-3β phosphorylates β-catenin, thereby promoting its ubiquitination and degradation [37]. In response to Wnt signals, β-catenin is no longer targeted for degradation, and it accumulates in the cytoplasm and subsequently translocates to the nucleus, where it associates with the transcriptional enhancers of the lymphoid enhancer-binding factor/Tcf family and stimulates the expression of genes involved in tumor progression [37]. Bos and colleagues showed that mesalazine inhibits the Wnt/β-catenin pathway in *APC*-mutated CRC cells with intact β-catenin [38]. Mesalazine increases β-catenin phosphorylation and consequently reduces nuclear accumulation of β-catenin and expression of Wnt/β-catenin target genes (e.g., cyclin D1, c-met, and c-Myc) [38]. The mechanism by which mesalazine promotes β-catenin phosphorylation remains to be ascertained, but it is known that the drug inhibits the activity of the protein phosphatase (PP)2A [38], a known regulator of the β-catenin phosphorylation status in CRC cells [39].

Another important target of mesalazine in CRC cells is epidermal growth factor receptor (EGFR), the activation of which is followed by a range of intracellular events that eventually stimulate CRC growth and survival [40]. EGFR is overexpressed not only in sporadic CRC but also in the premalignant lesions of IBD-associated carcinogenesis [41, 42]. We showed that mesalazine suppresses EGFR phosphorylation/activation in CRC cells, as a result of its ability to enhance the activity of protein tyrosine phosphatases (PTPs) that control EGFR activation [43]. In particular, mesalazine induces Src homology (SH)-PTP2 to interact with and promote dephosphorylation of EGFR [43].

5. Mesalazine Activates PPAR-γ in CRC Cells

The peroxisome-proliferator-activated receptor gamma (PPAR-γ), a transcription factor belonging to the nuclear hormone receptor superfamily, is highly expressed in the colon where it regulates cellular proliferation, differentiation, and apoptosis [44]. PPAR-γ activation inhibits formation of aberrant crypts foci and development of CRC in mice [45, 46]. Rousseaux et al. showed that PPAR-γ is a target of mesalazine in CRC cells [47]. Specifically, mesalazine enhances PPAR-γ expression, promotes its translocation from the cytoplasm to the nucleus, and increases its interaction with vitamin D3 receptor-interacting protein-205 in CRC cells [47]. In competitive binding studies, mesalazine displaces rosiglitazone and the selective PPAR-γ ligand GW1929 from their binding sites on the PPAR-γ molecule [47]. In immune-deficient mice engrafted with human CRC cells, administration of mesalazine reduces the growth of xenografts, via a PPAR-γ-dependent mechanism [48]. Activation of PPAR-γ by mesalazine is accompanied by induction of the tumor suppressor gene PTEN, activation of caspase-8 and caspase-3, and diminished expression of survivin and X-linked inhibitor of apoptosis protein [49].

6. Antineoplastic Effects of Mesalazine Derivatives

Before generally considering mesalazine as an antitumor compound, it should be taken into consideration that the majority of preclinical studies investigating the role of this drug in CRC growth and survival have been conducted in experimental models using very high doses which are not commonly reached within the gut tissue under standard oral treatment. Therefore, the validation of novel mesalazine derivatives that exhibit similar safety profiles but enhanced anticancer activity is highly desirable.

To this end, we have recently developed several mesalazine derivatives and focused our work on 2-methoxy-5-amino-N-hydroxybenzamide (we termed 2–14), since this compound displayed the more pronounced antimitogenic effect and was ten times more potent than mesalazine in blocking CRC cell growth [50]. Interestingly, 2–14 did not affect the growth of normal colonic intraepithelial lymphocytes and fibroblasts [50]. Unlike mesalazine, 2–14 induced endoplasmic reticulum stress (ERS) in CRC cells thereby promoting downregulation of cyclin D1 and accumulation of cells in G0/G1 phase of the cell cycle [50]. As expected, persistent block of 2–14-treated CRC cells in G0/G1 phase of cell cycle was accompanied by a caspase-dependent cell death [50]. To translate these observations *in vivo*, we assessed the anti-neoplastic effect of 2–14 in a syngeneic CRC model, in which tumors were generated by injecting the murine CRC cell line, CT26, into Balb/c mice. Mice treated with 2–14 showed a remarkable dose-dependent decrease in tumor volume compared to control mice [50]. The anticancer effects of 2–14 was documented regardless of whether the drug was administered subcutaneously or systemically while in the same model mesalazine reduced

the growth of tumors only when given subcutaneously [50]. We further strengthened the *in vivo* anti-neoplastic effect of 2–14 by using the AOM/DSS-induced colitis-associated CRC. Mice given 2–14 developed fewer smaller tumors than control mice and 2–14 treatment was not associated with significant changes in the proliferation rate of normal colonic cells [50]. More recently, we showed that 2–14 sensitized CRC cells to TNF-related apoptosis-inducing-ligand- (TRAIL-) driven death [51]. TRAIL-driven cancer cell apoptosis is mediated by ligand-dependent activation of two functional death receptors (DRs) (i.e., DR4 and DR5) [52]. Treatment of TRAIL-resistant CRC cells (i.e., HT-29 and DLD-1) with 2–14 but not mesalazine upregulated DR5 [51]. Moreover, 2–14 down-regulated the expression of survivin, an antiapoptotic protein that interferes with TRAIL-induced cell death in cancer cells [53]. These *in vitro* data were confirmed in a graft model generated by TRAIL-resistant CRC cells (i.e., CT26) in mice. In this model 2–14 synergized with TRAIL in inhibiting the *in vivo* growth of CT26-derived tumors [51]. In line with our results, Honeder and coworkers have recently showed that mesalazine derivatives, including 2–14, inhibit CRC cell growth [54].

7. Conclusion

In the last decade extensive research has been performed to evaluate the anti-neoplastic action of mesalazine. Although definitive conclusions from these studies cannot be drawn, it seems that when used at very high doses the drug can interfere with critical steps in the process of colon carcinogenesis. Even more promising seem to be the results obtained with mesalazine derivatives. Among these, 2–14 is more potent than mesalazine in inhibiting CRC cell growth and survival, maintains its anti-tumor activity when administered systemically, and does not affect the proliferation of normal gut cells. These observations and the fact that 2–14 is fully synthetic and has a fairly simple structure suggest that this drug could be helpful in designing novel CRC chemoprevention programs. Further experimentation is however needed to define the pharmacokinetic properties of the compound and evaluate the potential effects of 2–14 on vital functions of the host.

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Research Article

Curcumin Attenuates Gastric Cancer Induced by *N*-Methyl-*N*-Nitrosourea and Saturated Sodium Chloride in Rats

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To determine effects of curcumin on *N*-methyl-*N*-nitrosourea (MNU) and saturated sodium chloride (s-NaCl)-induced gastric cancer in rats. Male Wistar rats were divided into 5 groups: control (CO), control supplemented with 200 mg/kg curcumin (CC), MNU + s-NaCl, MNU + s-NaCl supplemented with 200 mg/kg curcumin daily for the first 3 weeks (MNU + s-NaCl + C3W), and MNU + s-NaCl supplemented with curcumin for 20 weeks (MNU + s-NaCl + C20W). To induce stomach cancer, rats except for CO and CC were orally treated with 100 mg/kg MNU on day 0 and 14, and s-NaCl twice-a-week for the first 3 weeks. The experiment was finished and rats were sacrificed at the end of 20 weeks. Cancers were found in forestomachs of all rats in MNU + s-NaCl. The expressions of phosphorylated inhibitor kappaB alpha (phospho-IκBα), 8-hydroxy-2′-deoxyguanosine (8-OHdG), and cyclin D1 significantly increased in MNU + s-NaCl compared with CO. Curcumin treatments for 3 and 20 weeks reduced the cancer incidence resulting in a decrease of phospho-IκBα expression in benign tumor-bearing rats compared with MNU + s-NaCl. Curcumin treatment for 20 weeks also decreased 8-OHdG expression in benign tumor-bearing rats compared with MNU + s-NaCl. Curcumin can attenuate cancer via a reduction of phospho-IκBα and 8-OHdG expressions, which may play a promising role in gastric carcinogenesis.

1. Introduction

Gastric cancer can generate in any part of the stomach. Poorly detected, gastric cancer causes nearly one million annual deaths worldwide [1, 2]. Gastric cancer is closely associated with dietary factors and *Helicobacter pylori* infection. Previous studies have reported that consumption of salty foods and *N*-nitroso compounds and a low intake of fresh fruits and vegetables increases the risk of gastric cancer [3, 4]. Hypertonic NaCl solutions induce gastric cancer in animal models through the enhancement of tissue damage resulting in cell proliferation [5]. Experimental animal models have also provided support for the hypothesis that salt promotes gastric carcinogenesis induced by *N*-nitroso carcinogen, such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) or *N*-methyl-*N*-nitrosourea (MNU) [6–9]. MNU, a mutagen and genotoxic substance, is a potent inducer of cellular stress leading to chromosomal aberrations, point mutations, cell death, and DNA damage [10]. Other studies reported that administration of MNU by oral gavage induced gastric cancer in rats and mice [11, 12]. Thus, the association between saturated NaCl and *N*-nitroso carcinogen could promote gastric carcinogenesis in rats by inflammation, mutation, and compensatory cell proliferation.

A range of stimuli such as reactive oxygen species (ROS) and cytokines from inflammatory response can activate inhibitor kappaB kinase (IKK) complex resulting in inhibitor kappaB (IκB) phosphorylation and proteolysis. Phosphorylation of IκBα elicits IκBα degradation, allowing the nuclear translocation of nuclear factor kappaB (NF-κB) complex and activation of target genes that are involved in the control of cellular proliferation and carcinogenesis such as cyclin D1 [13, 14]. The interaction of ROS with the nucleobases of the DNA strand, such as guanine, leads to the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and structural alteration in DNA. The 8-OHdG is a potential biomarker of oxidative damage of DNA and a factor of initiation and promotion of carcinogenesis [15]. These molecules play

a pivotal role in carcinogenesis and may be targets for therapeutic approaches.

Chemoprevention is promising as a preventive approach for cancers. Curcumin (diferuloylmethane), a polyphenol compound, is an active ingredient of tumeric (*Curcuma longa*). Curcumin has chemopreventive properties. Importantly, curcumin is safe for humans and animals [16]. Curcumin shows beneficial effects in many cancers including colorectal cancer, breast cancer, skin cancer, and oral cancer [17]. Several signalling pathways implicated in carcinogenesis including NF-κB signalling have been modulated by curcumin treatment [18]. However, data concerning the effect of curcumin on *in vivo* study of gastric cancer and key proteins involved in carcinogenesis induced by MNU and saturated NaCl (s-NaCl) have not been confirmed.

Therefore, the present study aims to examine the protective effect of curcumin on gastric cancer in rats induced by MNU and s-NaCl administration. In addition, activation of NF-κB, expressions of oxidative damage of DNA and cell cycle regulator cyclin D1 will be investigated.

2. Methods

2.1. Experimental Design. 6-week-old male Wistar rats (National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand) were used. All experiments and procedures carried out on the animals were approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Rats were housed in a controlled temperature room at 25 ± 1°C under standard conditions (12-h dark-light cycle). Thirty rats were randomized into five groups (six rats each) as follows.

Control rats (CO) were fed citrate buffer, pH 4.5 (1 mL/rat) orally via intragastric tube on days 0 and 14 of the experiment. In addition, rats were fed normal saline (1 mL/rat) orally twice a week for the first 3 weeks of the experiment. Corn oil (2.5 mL/kg) was administrated daily by intragastric tube for 20 weeks.

Control rats supplemented with curcumin (CC) were fed citrate buffer, pH 4.5 and normal saline as previously described. 200 mg/kg curcumin (95% purified curcumin, Cayman Chemical, MI, USA) was dissolved in corn oil and given daily to rats by intragastric tube for 20 weeks.

MNU- and saturated NaCl-induced rats (MNU + s-NaCl) were treated for gastric carcinogenesis by MNU (Sigma-Aldrich, MO, USA) and s-NaCl (Merck, Germany) according to Thong-Ngam et al. [19]. Briefly, rats were fed 100 mg/kg MNU (dissolved in citrate buffer, pH 4.5) via intragastric tube on days 0 and 14 of the experiment. In addition, rats were fed s-NaCl (30% NaCl solution, 1 mL/rat) orally twice weekly for the first 3 weeks of the experiment. Corn oil was administrated daily by intragastric tube for 20 weeks.

MNU- and s-NaCl-induced rats, supplemented with curcumin for 3 weeks (MNU + s-NaCl + C3W), were given with MNU and s-NaCl as previously describe. The 200 mg/kg curcumin was fed daily to rats by intragastric tube during administration of MNU and s-NaCl for the first 3 weeks.

MNU- and s-NaCl-induced rats, supplemented with curcumin for 20 weeks (MNU + s-NaCl+C20W), were induced with MNU and s-NaCl. The 200 mg/kg curcumin was fed daily to rats by intragastric tube for 20 weeks.

2.2. Stomach Tissues Preparation. After 20 weeks, all animals were sacrificed by intraperitoneal injection of Thiopental (Abbott, Italy, 120 mg/kg) after overnight fasting. Then, the stomach was excised and divided into 2 parts symmetrically along the greater and lesser curves. One part was fixed in liquid nitrogen and kept at −80°C for western blot analysis. Another part was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for histological study.

2.3. Histopathological Study. The tissue was fixed with 4% paraformaldehyde fixed and paraffin embedded. Multiple 2 μm-thick histological sections were stained with hemotoxylin and eosin (H&E). The alterations of gastric epithelial cells and the incidence of gastric carcinogenesis were determined by a pathologist. In addition, the cancer incidence was calculated as percentage using the following formula:

$$\text{Cancer incidence} = 100 \times \left(\frac{\text{numbers of cancer-bearing rats}}{\text{numbers of induced rats}}\right). \quad (1)$$

2.4. Western Blot Analysis. The tissue sample (0.05 g) was homogenized in 0.5 mL of ice-cold lysis RIPA buffer (Cell Signalling Technology Inc., MA, USA) with protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Sigma-Aldrich). The homogenate was sonicated for 15 seconds and centrifuged at 11,000 g for 10 minutes at 4°C. The supernatant was retained. Protein concentration was measured with the BCA Protein Assay Kit (Thermo Scientific). The extracted proteins were mixed with loading buffer and boiled for 5 min. Then, the extracted proteins (35 μg/lane) were applied to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membrane (Pall Corporation, FL, USA). The blotted membrane was incubated with 5% nonfat dried milk in TBS (0.02 M Tris, pH 7.6, and 0.15 M NaCl) for 1 hour at room temperature. Then, the membrane was probed with mouse monoclonal anti-phosphorylation of inhibitor kappaB alpha (Phospho-IκBα, Ser32/36) antibody (1 : 1000; Cell Signalling Technology Inc.) overnight at 4°C. Moreover, the membrane was also probed with anti-β-actin antibody (sc-47778, 1 : 5,000; Santa Cruz Biotechnology Inc., CA, USA) for 1 hour at room temperature. After three-time washing in TBS/0.01% Tween-20), the membrane was incubated with goat anti-mouse IgG HRP secondary antibody (1 : 4,000; Cayman Chemical) for 1 hour at room temperature. The bands of protein expression were developed with a commercial chemiluminescence detection kit (Amersham ECL plus western blotting system, GE Healthcare, UK). The luminescence was exposed to film (Fujiflim, Japan). Expression levels of proteins were quantified by ImageJ program (US National Institutes of Health, Bethesda, MA, USA).

Table 1: Histopathological changes of gastric mucosa in the experimental groups.

Group	Normal	Mucosal congestion	Benign papillary growth	Squamous cell carcinoma			Cancer incidence (%)
				Submucosal invasion	Muscle invasion	Serosal invasion	
CO ($N = 6$)	6	—	—	—	—	—	0
CC ($N = 6$)	6	—	—	—	—	—	0
MNU + s-NaCl ($N = 6$)	—	—	—	2	3	1	100
MNU + s-NaCl + C3W ($N = 5$)	—	—	2	2	1	—	60
MNU + s-NaCl + C20W ($N = 6$)	—	1	2	3	—	—	50

The level of phospho-IκBα (Ser32/36) expression was normalized by β-actin density.

2.5. Immunohistochemistry. In this study, we used BenchMark XT Instrument (Ventana, Medical System Inc., AZ, USA). Immunostaining for 8-OHdG or cyclin D1 was performed in paraffin embedded sections by the following processes. Briefly, the tissue sections were deparaffinized with EZ prepTM. After that, the sections were retrieved the antigen (8-OHdG or cyclin D1) with Sodium Chloride Sodium Citrate pH 6.5–7.5 (SSCTM). Next, 1% Hydrogen peroxide (H_2O_2, UltraViewTM Inhibitor) was used to block endogenous peroxidase activity. Then, the primary antibody used for 8-OHdG (1 : 400; Japan Institute for the Control of Aging, Japan) or cyclin D1 (1 : 200; Thermo Scientific, MI, USA) was applied and incubated at 37°C for 60 minutes or 32 minutes, respectively. After that, the goat anti-Mouse IgG (UltraViewTM HRP Multimer) was used as secondary antibody. Color was developed by UltraViewTM DAB chromogen, UltraViewTM H_2O_2, and UltraViewTM copper. Then, the slides were counterstained with Hematoxylin II and Lithium Carbonate. Under light microscope (Nikon E50i, Nikon Corporation, Japan), immunoreactive cells of 8-OHdG and cyclin D1 were defined as those with dark-brown-stained nuclei of gastric epithelial cells. To verify the expressions of 8-OHdG and cyclin D1 in all animals, digital images were taken in high magnification field (400x) from each sample using a microscope equipped with digital camera (Nikon Digital Sight DS-Fi1, Nikon Corporation, Japan). Ten images from two sections per animals were analyzed. The numbers of dark brown stained in nuclei of epithelial cells were counted manually using Point tool in the IMAGE-PRO PLUS software program (version 6.1). A thousand gastric epithelial cells were counted for each rat. The data were shown as the percentage (%) of immunoreactive cells calculating from following equation:

$$\text{The percentage of immunoreactive cells (\%)} = \left(\frac{\text{number of nuclei stained cells}}{\text{number of examined cells}}\right) \times 100\%. \quad (2)$$

2.6. Statistical Analysis. Statistical analyses were conducted using Fisher's exact test for incidence of gastric cancer. Phospho-IκBα, 8-OHdG, and cyclin D1 results were shown as mean ± SD and analyzed with one-way ANOVA and LSD post hoc test. For all comparisons, a P value of less than 0.05 was considered to be statistically significant. All the statistical tests were performed using the computer program SPSS, version 13.0, for Windows (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Gastric Cancer Incidence and Histopathological Studies of MNU and s-NaCl-Associated Gastric Carcinogenesis and Effects of Curcumin. During the whole-study period, there was only one rat death. At 3 weeks, the rat in MNU + s-NaCl + C3W died from aspiration after feeding. The results of autopsy showed congestion and bleeding spots in both lungs. There was hyperkeratosis in forestomach mucosa, but no precancerous lesions or tumor was detected by histological examination. 29 rats survived the 20 weeks of the study.

Gastric cancer incidence was shown in Table 1. There was no squamous cell carcinoma (SCC) in CO and CC groups. In MNU + s-NaCl group, SCC was found in forestomach of all rats (100%). SCC showed tumors scattered in the forestomach. In gross view, the tumor masses were coliform-like (Figure 1(a)). Histopathology of SCC showed dyskeratosis and invasion of cancerous tissue through all layers of stomach wall. The cancerous tissue invaded the submucosal layer (Figure 1(b)), the muscle layer (Figure 1(c)), or the serosa (Figure 1(d)). 3/5 (60%) rats in MNU + s-NaCl + C3W developed SCC. 2 rats in this group showed tumor-like lesions. Papillary growth or tumor-like lesion displayed hyperproliferation of epithelial cells, hyperkeratosis, but no invasion. These lesions were not diagnosed as cancers. In MNU + s-NaCl + C20W group, three rats developed SCC with submucosal invasion. The cancer incidence was 50%. One rat did not exhibit a cancerous lesion. Another 2 rats developed papillary growths.

3.2. Curcumin Supplementation for 3 and 20 Weeks Attenuated Development of Carcinogenesis Associated with Phospho-IκBα Expression. From histopathological results (Table 1), rats in MNU + s-NaCl + C3W and MNU + s-NaCl + C20W groups were divided into subgroup C3W benign group ($N = 2$), C3W cancer group ($N = 3$), C20W benign group ($N = 3$), and C20W cancer group ($N = 3$). Results showed that the expression of phosphorylated IκBα was not significantly different between CO and CC. Compared with CO, phosphorylated IκBα expression in MNU + s-NaCl increased

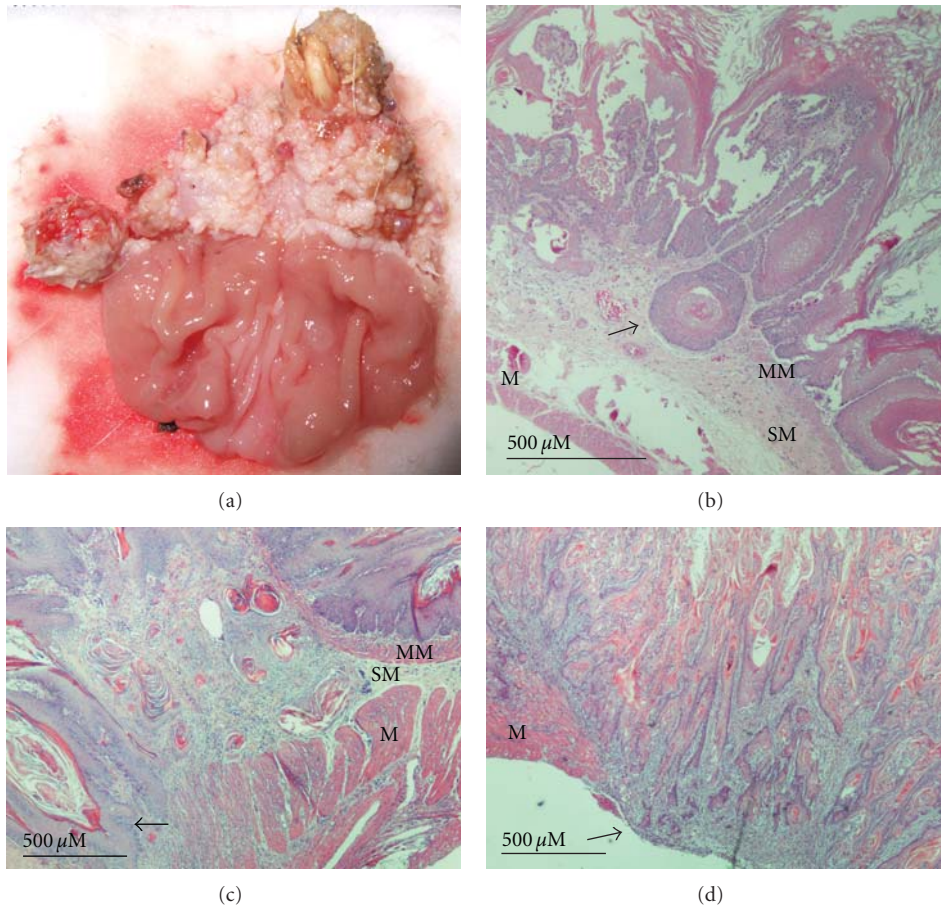

Figure 1: Macroscopic (a) and microscopic (b–d, H&E staining, 40x) appearance of squamous cell carcinoma (SCC) in the forestomachs. The multiple polypoid tumors developed in the stomach after MNU and saturated NaCl administration (a). SCCs in the gastric epithelium show the invasion of cancerous tissue with dyskeratosis through submucosal layer (b), muscle layer (c), or serosa (d). Note: the arrows indicate SCC with submucosal invasion (b), muscle-layer invasion (c), and stomach perforation (d). MM: muscularis mucosae, SM: submucosa, and M: muscle layer.

Table 2: The results of phospho-IκBα-relative expression, 8-OHdG-immunoreactive cells (%), and cyclin D1-immunoreactive cells (%).

Parameters/Group	CO ($N = 6$)	CC ($N = 6$)	MNU + s-NaCl ($N = 6$)	MNU + s-NaCl + C3W ($N = 5$)		MNU + s-NaCl + C20W ($N = 6$)	
				C3W benign ($N = 2$)	C3W cancer ($N = 3$)	C20W benign ($N = 3$)	C20W cancer ($N = 3$)
Phospho-IκBα	$0.51 \pm 0.11^{\dagger}$	$0.54 \pm 0.18^{\dagger}$	$0.82 \pm 0.18^{*}$	$0.46 \pm 0.04^{\dagger}$	0.60 ± 0.29	$0.51 \pm 0.08^{\dagger}$	$1.16 \pm 0.34^{**,\dagger}$
8-OHdG	$44.42 \pm 3.41^{\dagger\dagger}$	$40.39 \pm 3.53^{\dagger\dagger}$	$53.06 \pm 5.96^{**}$	49.28 ± 7.43	48.06 ± 3.47	$44.99 \pm 3.51^{\dagger}$	47.77 ± 2.55
Cyclin D1	$38.58 \pm 5.37^{\dagger\dagger}$	$42.53 \pm 6.90^{\dagger\dagger}$	$54.91 \pm 7.93^{**}$	$4.33 \pm 1.27^{*}$	$54.92 \pm 3.87^{**}$	45.31 ± 6.29	$53.82 \pm 11.01^{**}$

These data were presented as the mean ± SD. *Represented significant difference compared with CO group ($P < 0.05$). **Represented significant difference compared with CO group ($P < 0.01$). †Represented significant difference compared with MNU + s-NaCl group ($P < 0.05$). ††Represented significant difference compared with MNU + s-NaCl group ($P < 0.01$).

significantly ($P = 0.010$). Curcumin supplementations for 3 and 20 weeks in C3W benign and C20W benign groups significantly declined the expression of phosphorylated IκBα compared with MNU + s-NaCl ($P = 0.028$ and $P = 0.031$, resp.) (Table 2). The represented bands of phospho-IκBα and β-actin were shown in Figure 2.

3.3. Curcumin Supplementation for 20 Weeks Attenuated Development of Carcinogenesis Associated with 8-OHdG and Cyclin D1 Expressions. 8-OHdG and cyclin D1 expressions were studied by immunohistochemistry and shown as nuclei-stained cells. The average percentages of immunoreactive cells of all groups were shown in Table 2. From the results, the mean percentages of 8-OHdG and cyclin D1-immunoreactive cells were not significantly different between CO and CC. They were significantly increased in MNU + s-NaCl compared with CO ($P = 0.002$ and $P = 0.000$, resp.). Curcumin supplementation for 20 weeks reduced gastric cancer incidence and significantly decreased 8-OHdG expression in C20W benign group compared with

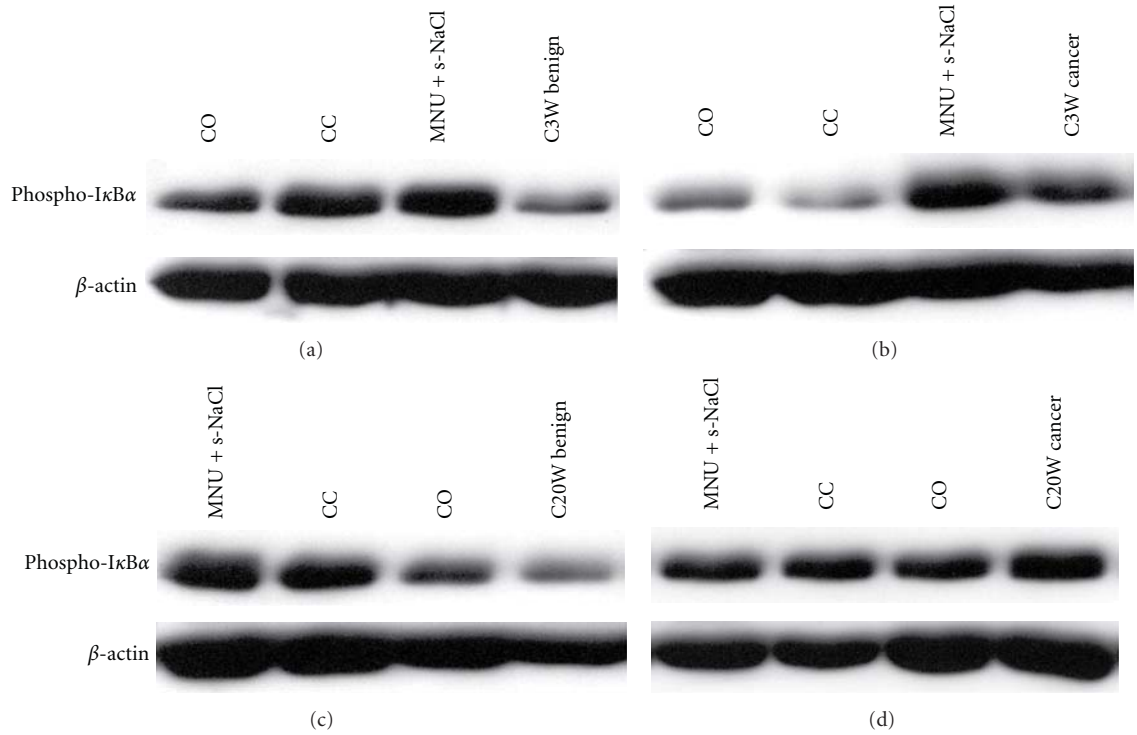

Figure 2: Western blot analysis of phospho-IκBα expression. Curcumin supplementation in C3W benign group declined the expression of phosphorylated IκBα compared with MNU + s-NaCl (a), whereas the expression of phosphorylated IκBα in C3W cancer group did not decrease (b). Curcumin supplementation for 20 weeks in C20W benign decreased the expression of phosphorylated IκBα compared with MNU + s-NaCl (c), whereas the expression of phosphorylated IκBα in C20W cancer group did not decline (d). An antibody for β-actin was used as an internal control.

MNU + s-NaCl ($P = 0.015$) (Table 2). The average percentage of cyclin D1 stained cells in the C20W benign group tended to reduce, but not reach a statistically significant level when compared with MNU + s-NaCl ($P = 0.062$; Table 2). 8-OHdG was expressed primarily in gastric epithelial cells (Figure 2(a)). This expression was increased in the mucosa of SCC in both MNU + s-NaCl and C20W cancer groups (Figures 3(b) and 3(c)). This enhanced expression was limited to a small number of epithelial cells in C20W benign group (Figure 3(d)).

4. Discussion

This study demonstrated that oral gavage of MNU- and s-NaCl-induced a 100% cancer incidence in rats. The histological results showed that curcumin could attenuate the gastric carcinogenesis induced by MNU and s-NaCl in rats. Administration of curcumin in both MNU + s-NaCl + C3W and MNU + s-NaCl + C20W groups showed 40% and 50% reduction of cancer incidence, respectively. These observations indicated that early administration of curcumin (during the first 3 weeks of cancer induction) might prevent the initiation of carcinogenesis. This is in agreement with previous studies. Feeding 0.5 and 2.0% of commercial grade curcumin in the diet during the initiation period (2 weeks before, during, and 1 week after benzo(a)pyrene administration) reduced the number of mice with forestomach tumors [20]. Daily feeding of 1 and 2 g/mL radix curcumae extract solution during MNNG administration for 40 weeks also showed the reduction of tumor incidences in 10% NaCl and MNNG-induced gastric cancer in rats [21].

Phosphorylation of IκBα could imply the activation of NF-κB, which plays a major role in carcinogenesis [13, 14]. In gastric carcinoma patients, NF-κB activation correlated with IκBα phosphorylation and degradation [22, 23]. Our study showed that overexpression of phosphorylated IκBα was associated with cancer. This finding coincided with other reports [24, 25]. Activation of NF-κB appeared to be a key step of keratinocyte transformation into SCC in mice [24]. 49% of prostate adenocarcinoma patients showed NF-κB overexpression that correlated with advanced tumor stage [25]. Curcumin has a chemopreventive property resulting in suppressing NF-κB activation. Many studies confirmed that a pivotal role of curcumin is inhibiting IKK activity with declining IκBα phosphorylation [26, 27]. Our results showed that curcumin supplementations for 3 and 20 weeks significantly decreased phosphorylated IκBα in benign tumor-bearing rats. Curcumin supplementation in this study prevented carcinogenesis by declining IκBα phosphorylation.

8-OHdG is a potential biomarker of oxidative DNA damage and a factor of initiation and promotion of carcinogenesis [15]. In this study, we also showed that 8-OHdG expression significantly increased in the MNU + s-NaCl group compared with CO group. This observation confirmed many previous results obtained from various types of cancers in patients. The level of 8-OHdG expression is

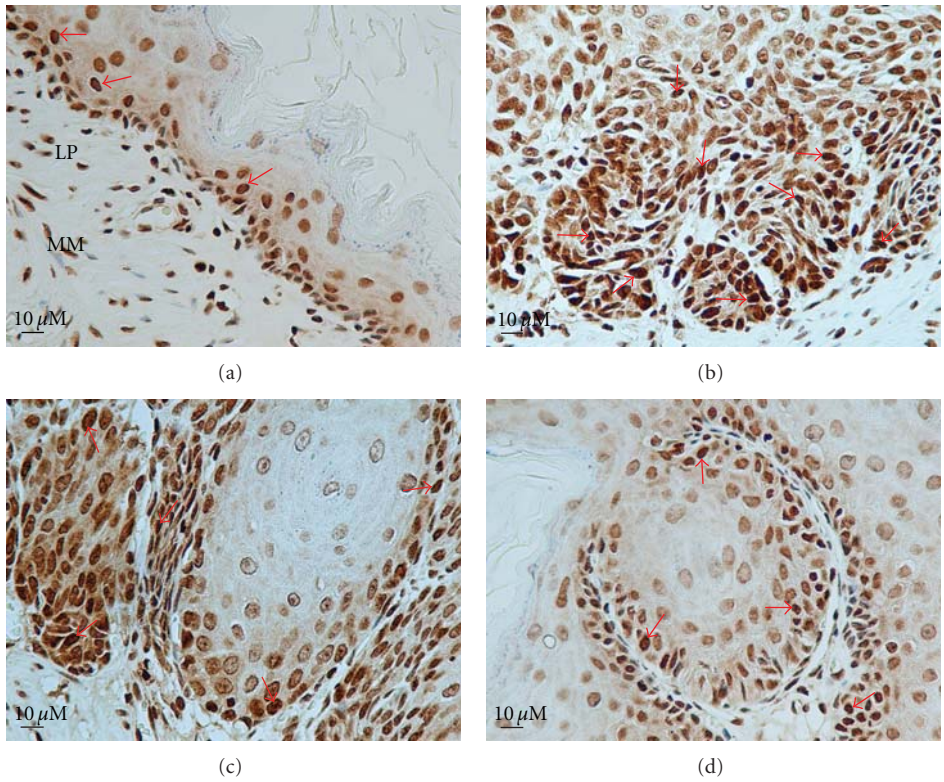

Figure 3: Immunohistochemical staining of 8-OHdG antibodies in the representative tissue specimens (400x): CO group (a), MNU + s-NaCl group (b), C20W cancer group (c), and C20W benign group (d). DAB stained immunoreactive cells (dark brown, arrows); nuclear counterstaining was performed with Hematoxylin II and Bluing reagent. LP: laminar propria and MM: muscularis mucosae.

elevated in colorectal cancer [28], hepatocellular carcinoma [29], oral SCC [30], and gastric cancer patients [31]. Considering the number of reports demonstrating a close relation between 8-OHdG formation and carcinogenicity, including this study, it is likely that 8-OHdG formations might participate in carcinogen-induced forestomach SCC. Curcumin showed a potent scavenger of reactive species (RS), such as superoxide anion, hydroxyl radical, singlet oxygen, nitric oxide, and peroxynitrite [32]. The reduction of RS could prevent the formation of 8-OHdG. This study demonstrated that 200 mg/kg curcumin supplementations for 3 and 20 weeks in MNU + s-NaCl-induced SCCs diminished 8-OHdG expression. In addition, 8-OHdG expression in C20W benign group was significantly reduced compared to MNU + s-NaCl. These results suggested that curcumin administration could protect carcinogenesis against formation of 8-OHdG.

Oxidative damage of DNA as well as deregulation of cell cycle control causes cancer. This study demonstrated that the immunoreactive cells of cyclin D1, the positive cell cycle regulator, significantly increased in MNU + s-NaCl compared with CO. The nuclear accumulation of cyclin D1 is an essential indicator of oncogenicity. Previous studies showed that nuclear immunoreactivity for cyclin D1 positively correlated with tumor cell proliferation of gastric cancer patients [33]. Moreover, cyclin D1 overexpression occurred in patients with oral SCC [34]. Cyclin D1 expression in the C20W benign subgroup tended to decrease, but not reach a statistically significant level when compared with MNU + s-NaCl. This study suggested that curcumin could not prevent carcinogenesis through improvement of dysregulation of cell cycle as shown by cyclin D1 accumulation.

5. Conclusion

MNU and s-NaCl led to increase of phosphorylated IκBα, 8-OHdG, and cyclin D1 that are associated with forestomach carcinogenesis. Concomitant treatment with 200 mg/kg curcumin during the first three weeks or the entire study period (20 weeks) could decrease cancer incidence to 40% and 50%, respectively. This treatment shows a significant reduction of phosphorylated IκBα in benign rats. In addition, curcumin treatment for 20 weeks significantly reduces 8-OHdG expression. Hence, administration of curcumin during the initiation period could attenuate the incidence of cancer via reduction of phospho-IκBα and 8-OHdG expressions.

Acknowledgments

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Research Article

A Standardized Extract of *Ginkgo biloba* Neutralizes Cisplatin-Mediated Reproductive Toxicity in Rats

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The aim of this study was to evaluate the protective effects of *Ginkgo biloba* (GB) against testicular damage and oxidative stress as well as caudal sperm indices in a cisplatin- (CIS-) induced rodent model. Adult male Wistar rats were given vehicle, single i.p. dose of CIS alone (10 mg/kg), GB alone (200 mg g/kg every day for five days), or single dose of CIS followed by GB (50, 100, or 200 mg/kg every day for five days). On day 6, after the first drug treatment oxidative and apoptotic testicular toxicity was evaluated. CIS-treated rats displayed decreased weights of testes and epididymis as well as caudal sperm count and motility. This reproductive toxicity was accompanied with increased germ-cell degeneration in seminiferous tubules and increased germ-cell apoptosis, increased testicular MDA levels and MPO activity, and decreased SOD and CAT activities in testes. Intensive expressions of COX-2, iNOS, and NF-κB p65 in testicular tissues were detected in CIS-treated group. Oral GB administrations at all doses to CIS-treated rats effectively alleviated all of the CIS-induced toxicity in reproductive system. The present results provide further insights into the mechanisms of protection against CIS-induced reproductive toxicity and confirm the essential antioxidant potential of a GB extract.

1. Introduction

Chemotherapy has emerged as an efficient mode of treatment for various carcinogenesis. Effective systemic drugs are increasingly used to treat cancer patients. Cisplatin (cis-diamminedichloroplatinum-II, CIS), one of the most effective and widely prescribed anticancer drugs, is still used in the treatment of many types of solid tumors including testicular cancer [1, 2]. It has been proven highly effective in curing testicular cancer in combination with other drugs even at an advanced stage of the disease [3]. CIS kills cancer cells by forming covalent adducts with the cellular DNA molecules and thereby terminating the vital processes like replication and transcription and inducing apoptosis [4].

In spite of its high efficiency in the treatment of testicular cancer, CIS has severe adverse effects on spermatogenesis and even leads to a condition of azoospermia [5, 6]. Spermatogenesis is a complex process which is highly influenced by hormone molecules and temperature and involves an array of testicular cells such as germ cells, Sertoli cells, Leydig cells, and peritubular cells [7, 8]. Acute exposure to antineoplastic agents like CIS has shown an increase in the frequency of germ-cell apoptosis [9, 10] in experimental animals. Moreover, it can also lead to decreased reproductive organ weights, azoospermia, and degenerated spermatogenic cells [11, 12]. The molecular mechanism by which CIS causes reproductive toxicity and germ-cell apoptosis remains to be elucidated. However, pathogenesis of testicular damage followed by CIS exposure is generally ascribed to oxidative stress mediated by increased free radical generation and depletion of antioxidants. Free radicals have been reported to mediate reactions responsible for a wide range of CIS-induced side effects [9, 11]. Consequently, antioxidants have been shown to protect nonmalignant cells and organs against damage by CIS [9, 11, 13].

Ginkgo biloba (GB) has been used in traditional Chinese medicine for about 5000 years, and it is one of the herbal drugs that is used widely according to its antioxidant

properties and ability to modify vasomotor function, affect ion channels to inhibit activation of platelets and smooth muscle cells [14], stimulate neurotransmitters [15], decrease adhesion of blood cells to endothelium, and modify signal transduction [14]. In addition, GB has been used in the treatment of Alzheimer's disease and cognitive impairment. The major GB components are flavonoglycosides and terpene lactones. GB extract was also reported for many decades to increase peripheral and cerebral blood flow as well as for the treatment of dementia [16]. Furthermore, extract of GB leaves has been shown to have a strong antioxidant that directly scavenges ROS [15].

Inducible nitric oxide synthase (iNOS) is responsible for the formation of high levels of nitric oxide (NO) under oxidative stress resulting in autocytotoxicity [17]. Previous studies have shown that NO along with ROS triggers cell death and the oxidation products of NO can induce lipid peroxidation [18–20]. Transcription factors like NF-kB stimulated under oxidative stress can also induce iNOS expression [21]. Inhibiting NF-kB and thereby iNOS using antioxidants has already proved to be effective in attenuating the CIS-induced testicular injury [20]. Cyclooxygenase-(COX)-2 is an inducible form of COX which plays a physiological role in inflammation and tumor proliferation [22]. COX-2 selective inhibitors have been found to be effective in ameliorating CIS-induced nephrotoxicity in rats [23].

To determine whether or not standardized GB extract could attenuate toxicity and oxidative stress in testicular tissues, this study was designed to assess the preventive role of GB extract on the biochemistry and pathology of CIS-induced testicular abnormalities in rats. The modulating roles of NF-kB, iNOS, and COX-2 in CIS-induced oxidative testicular injury induced were also evaluated in the present work.

2. Materials and Methods

2.1. Animals. Sixty adult male Wistar rats (8 weeks old weighing 120–240 g) were obtained from the Animal House, United Arab Emirates University,UAE. They were kept in polycarbonate cages and supplied with standard pellet diet and tap water under a 12 h light/dark cycle and room temperature of 22–24°C. This study was approved by the Animal Ethics Committee, UAE University,UAE.

2.2. Chemicals and Plant. CIS was purchased from Hospira UK Limited, Warwickshire, UK. GB-leaf extract was obtained from General Nutrition Corporation, Pittsburgh, USA. Thiobarbituric acid, 1,1,3,3-tetramethoxy-propan, phosphoric acid, sulfuric acid and hydrogen peroxide were obtained from Sigma Chemical (St. Louis, MO, USA). The GB extract is standardized to Ginkgo flavonoglycosides (24%) and terpene lactones (6%) which represent the major active contents of GB.

2.3. Experimental Protocol. Rats were divided into six groups ($n = 10$). The groups were treated as follows: control or normal (N) received water (5 mL/kg body weight) for five days and a single intraperitoneal injection (i.p.) of saline on the first day (5mL/kg body weight). Same dose volume (5 mL) was used for all other groups; GB alone (GB) received only 200 mg/kg body weight of GB water extract; CIS alone (CIS) where rats were given CIS as single dose (10 mg/kg; i.p.) on the first day of treatment, a dose that induced testicular toxicity in rats [24]. Rats of the protective groups (CIS+L: 50 mg/kg GB; CIS+M; 100 mg/kg GB; CIS+H: 200 mg/kg GB) received GB for five days 1 h after a single dose of CIS on the first day of treatment. GB was dissolved in water and administrated orally at three-dose levels 50 (low dose; L), 100 (medium dose; M), and 200 (high dose; H) mg/kg body weight. Doses of GB were selected based on previously reported pharmacological properties of this plant [19]. Five days after the administration of CIS or GB, the rats were sacrificed after being anesthetized with diethyl ether. Rats were weighed in regularly, and their testes and epididymis were dissected out and weighed.

2.4. Sperm Motility and Count. Total sperm number was determined by using a Neubauer hemocytometer. Cauda epididymis was dissected out, weighed, immediately minced in 5 mls of physiological saline, and then incubated at 37°C for 30 minutes to allow sperms to leave the epididymal tubules. The percentage of motile sperm was recorded from left cauda epididymis using a phase contrast microscope at 400x magnification. The total number of sperm per gram of cauda (of the right side) was then calculated.

2.5. Biochemistry. Testes were homogenized separately in ice-cold Tris-KCl buffer (150 mmol/L). The w/v ratio of the tissue to the homogenization buffer was (1 : 10 w/v). Aliquots were prepared and used for determination of different biochemical markers.

Supernatants were collected, and assays for lipid peroxidation and CAT were performed. Determination of MDA in testicular homogenate is based on its reaction with thiobarbituric acid (TBA) to form a pink complex with an absorption maximum at 535 nm. CAT activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm and was expressed in units/mg of protein as described by Aebi [25], and total protein was estimated by Lowry's method. Superoxide dismutase (SOD) activity in testicular tissues was determined according to the method described in [26]. This method is based on the ability of SOD to inhibit the auto-oxidation of pyrogallol at alkaline pH. Myeloperoxidase (MPO) activity in testicular tissues was determined as described in [27]. One unit of MPO activity is defined as that which degrades 1 μmol H_2O_2/min at 25°C.

2.6. Histology. For the histological examinations, small pieces of testis were fixed in 10% neutral phosphate-buffered formalin, and the hydrated 5 μm thick sections were stained with hematoxylin and eosin. Sections were examined under a Leica DMRB/E light microscope (Heerbrugg, Switzerland).

2.7. Immunohistochemistry. Terminal deoxynucleotidyl transferase-mediated triphosphate nick-end labeling

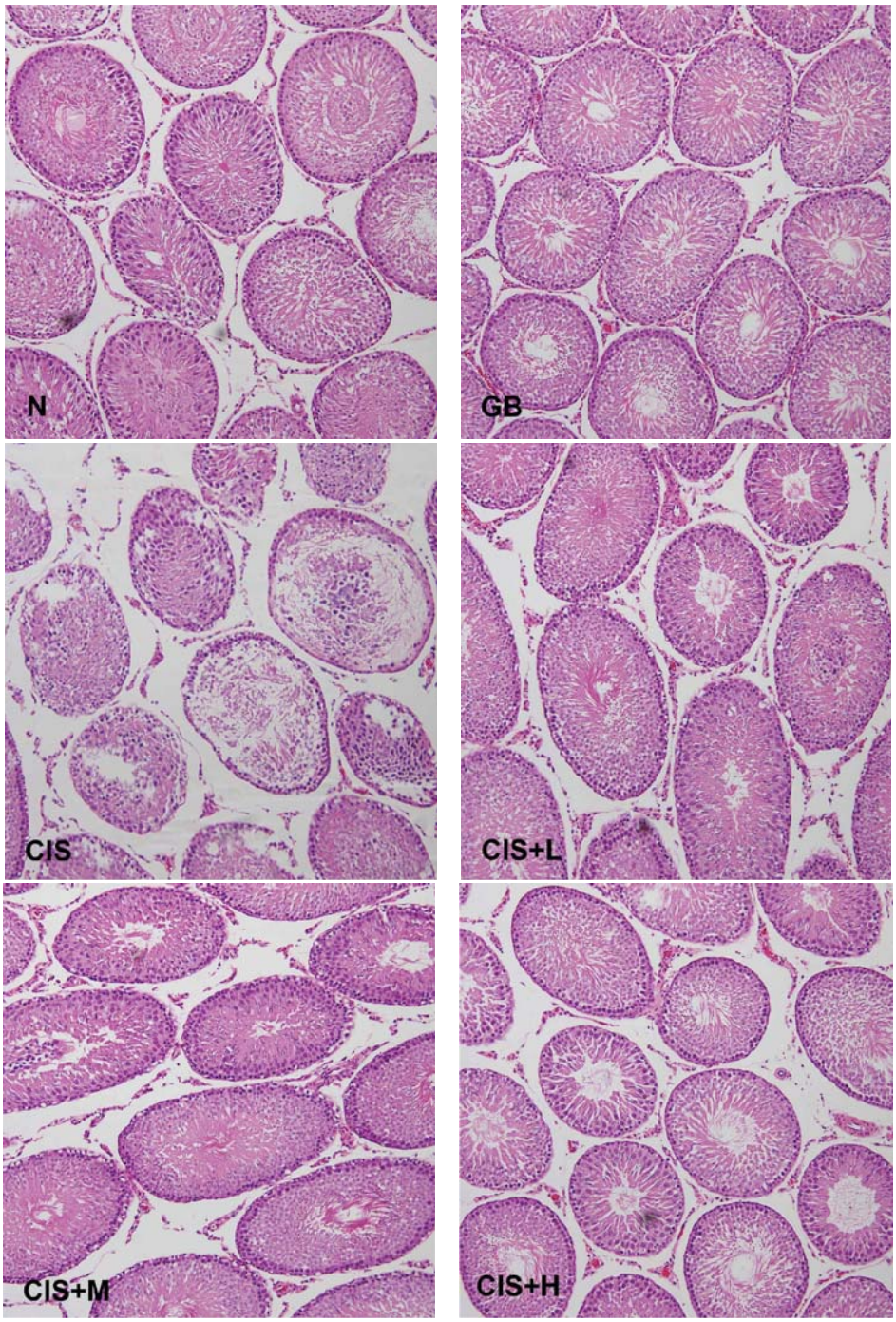

Figure 1: Photomicrograph of cross-sections of the testes of control (N), GB alone (GB), CIS alone (CIS), and the three GB-protected rats (CIS+L: 50 mg/kg GB; CIS+M: 100 mg/kg GB; CIS+H: 200 mg/kg GB). Testes of control and GB alone treated groups show normal arrangement of germ cells and Sertoli cells. However, CIS-treated testes show severely damaged seminiferous tubules. Rats protected with GB were less affected by CIS (H & E 200x).

(TUNEL) technique was used to determine apoptosis. Deparaffinized and gradually hydrated, 4 μm thick sections of testes have been used to assess apoptosis, and TUNEL was performed using the ApopTag Plus Peroxidase in situ Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA, USA). The principle of the method is based on the catalytic activity of terminal deoxynucleotidyl transferase which adds digoxigenin nucleotides to the terminal 3′-OH of DNA molecule and thereby detecting the DNA fragmentation associated with apoptosis. Further immunohistochemical analysis was done using sequential mounted sections of the specimen. Initially the antigens were

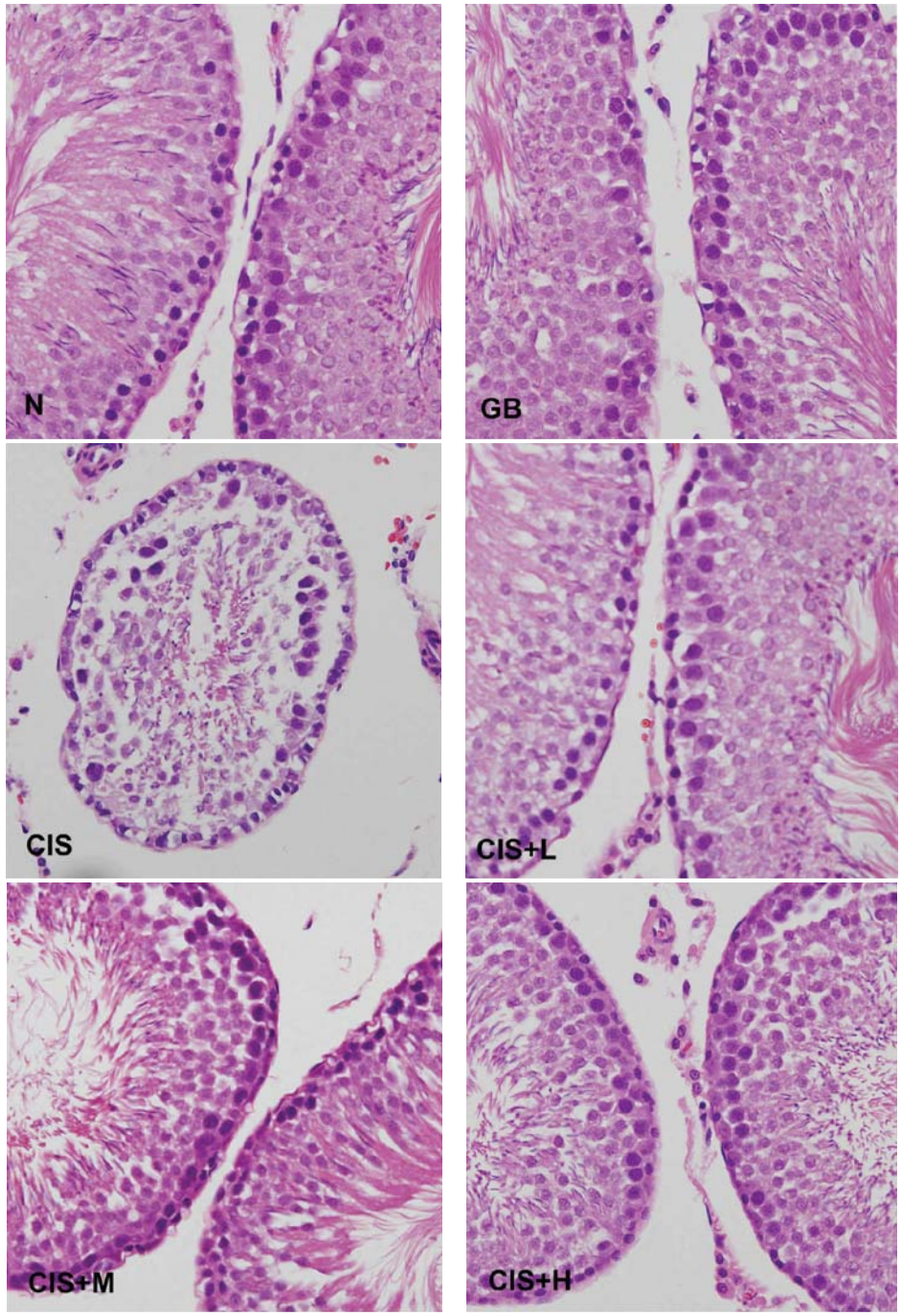

Figure 2: Photomicrograph of cross-sections of the testes of control (N), GB alone (GB), CIS alone (CIS), and the three GB-protected rats (CIS+L: 50 mg/kg GB; CIS+M: 100 mg/kg GB; CIS+H: 200 mg/kg GB). Testes of control and GB alone treated groups show normal arrangement of germ cells and Sertoli cells. However, CIS-treated testes show extensive atrophy of seminiferous tubules and degenerations of germ cells. Rats protected with different GB doses clearly show less degeneration of some tubules and irregular derangement of some germ cells (H & E 400x).

revealed by incubating the sections in a heated water bath for 15 minutes followed by the blocking of the endogenous peroxidase activity with 0.3% H_2O_2 in methanol. Anti-rat primary antibodies (1 : 100 dilution) for COX-2 (Clone SP-21), iNOS (Ab-1) and NF-kB p65 (Rel A, Ab-1) from rabbit were obtained from Thermo Fisher Scientific (Anatomical Pathology, Fremont, USA). The sections were first incubated in primary antibodies overnight at 4°C. After overnight incubation, the slides were washed with PBS, and the sections were incubated with polyvalent biotin-labeled goat anti-rabbit secondary antibody (1 : 200 dilution) for 10 minutes at room temperature. The sections were then stained

Table 1: The effect of the *GB* extracts on testicular epididymal weights and on epididymal sperm count, motility, and abnormality in CIS-treated rats.

Parameters	N	GB	CIS	CIS+L	CIS+M	CIS+H
Testes weight (gm)	3.51 ± 0.06	3.59 ± 0.11	2.99 ± 0.12*	3.49 ± 0.08$^{\#}$	3.52 ± 0.18$^{\#}$	3.65 ± 0.13$^{\#\#\#}$
Epididymis weight (gm)	1.64 ± 0.06	1.53 ± 0.06	1.30 ± 0.04**	1.38 ± 0.05**	1.46 ± 0.08	1.44 ± 0.05
Sperm (count.10^6)/gm of cauda	131.0 ± 7.73	134.9 ± 11.64	83.94 ± 8.03*	94.50 ± 20.25	94.67 ± 7.77	118.78 ± 8.78
Sperm motility (%)	79.125 ± 3.34	68.12 ± 2.64	28.125 ± 1.52***	54.0 ± 5.59$^{***\#\#\#}$	49.0 ± 3.83$^{***\#\#\#}$	50.0 ± 4.2$^{***\#\#\#}$

$^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus CIS, $^{**}P < 0.01$ versus control, $^{\#\#\#}P < 0.001$ versus CIS, $^{***}P < 0.001$ versus control.

Table 2: The effect of the *GB* extracts on oxidative stress parameters associated with CIS treatment in testicular tissues of rats.

Parameters	N	GB	CIS	CIS+L	CIS+M	CIS+H
MDA (nmol/mg protein)	0.97 ± 0.03	0.86 ± 0.08	1.2 ± 0.08***	1.0 ± 0.07^{c}	0.84 ± 0.06^{a}	0.98 ± 0.05^{c}
MPO (mu/mg protein)	16.92 ± 0.34	17.13 ± 0.31	24.65 ± 2.2***	18.88 ± 0.93^{b}	19.69 ± 1.54^{c}	16.93 ± 0.59^{c}
CAT (u/mg protein)	146.79 ± 2.9	144.07 ± 2.03	84.89 ± 3.24***	127.9 ± 3.9^{***a}	118.0 ± 3.64^{***a}	134.4 ± 1.88^{*a}
SOD (u/mg protein)	3.37 ± 0.02	3.37 ± 0.01	3.62 ± 0.05***	3.26 ± 0.08^{a}	3.16 ± 0.09^{a}	3.25 ± 0.06^{a}

Values are expressed as mean ± SEM of eight rats per group. Concentration is expressed as nmol/mg protein for MDA. Activity is expressed as unit/mg protein for CAT and SOD. Activity is expressed as m unit/mg protein for MPO. Significance was determined by one-way analysis of variance followed by Dennett's *t*-test: $^{*}P < 0.05$; $^{***}P < 0.001$ versus control. $^{c}P < 0.05$; $^{b}P < 0.01$; $^{a}P < 0.001$ versus CIS group.

using Universal LSAB plus kit and a DAB plus substrate kit as the chromogen followed by a light counterstaining with hematoxylin. Tissue images were captured by optical microscopy (Olympus DP71).

2.8. Statistical Analysis. Data are expressed as group mean ± SE. The statistical analysis was carried out using ANOVA, with SPSS version 10 (SPSS, Chicago, IL, USA). ANOVA was carried out to detect the differences between all the various groups. When significant differences were detected, analysis of a difference between the means of the treated and control groups was carried out using Dunnett's *t*-test.

3. Results

3.1. Histopathological Effects. Testicular tissues in the control group showed normal arrangement of germinal and Sertoli cells without any histopathological lesions. CIS-treated groups showed moderate to severe testicular atrophy with severe cellular disorganization and degeneration in seminiferous tubules (Figures 1 and 2) and interstitium. CIS treatment also induced depletion of Leydig cells between the tubules. Degenerated Sertoli cells were also observed in the lumen. Animals pretreated with *GB* showed normal testicular morphology with irregular arrangement of germ cells and slight degeneration of seminiferous epithelium and shedding of germ cells in some tubules.

3.2. Effects on Weights of Testes and Epididymis. The weights of testes and epididymis in rats after CIS administration were found to be significantly decreased, compared with the control group (Table 1). No significant changes in the weights of testes and epididymis were found in rats treated with *GB* alone. However, GB caused significant improvements in the weights of testes ($P < 0.05$ and $P < 0.001$) and showed some recovery of epididymal weights of rats of all protected groups (Table 1). The administration of CIS alone or with prior GB administrations at all three tested doses did not alter the body weight of the animals (data not shown).

3.3. Effects on Sperm Motility and Count. After CIS was administered, the caudal sperm count ($P < 0.05$) and motility decreased significantly ($P < 0.001$). Administration of GB attenuated the depletion of sperm counts where there was no significant difference between preventive groups and the control. However, all doses of GB significantly ($P < 0.001$) increased the CIS-induced decreases in sperm motility. Administration of *GB* attenuated the CIS-induced decrease of sperm count. Effect of GB extract on caudal sperm count and motility is given in Table 1.

3.4. Effects on Oxidative Stress on Testicular Tissues. CIS-intoxicated rats, testicular MDA levels, and MPO activity were significantly ($P < 0.001$) increased, and SOD and CAT activities were significantly ($P < 0.001$) decreased indicating the potent oxidative action of CIS on testicular tissues. Coadministration of GB with CIS neutralized these abnormalities in levels of MDA, SOD, CAT, and MPO reflecting the effect of GB against CIS-induced oxidative stress in testes (Table 2).

3.5. Immunohistochemistry. Apoptotic cell death in seminiferous tubules was assessed using TUNEL technique. TUNEL-positive nuclei were observed in brown color in the seminiferous tubules of control and CIS-treated rats (Figure 3). However, testicular tissues exposed to CIS contained high frequency of TUNEL-positive germ cells in contrast to those treated with GB.

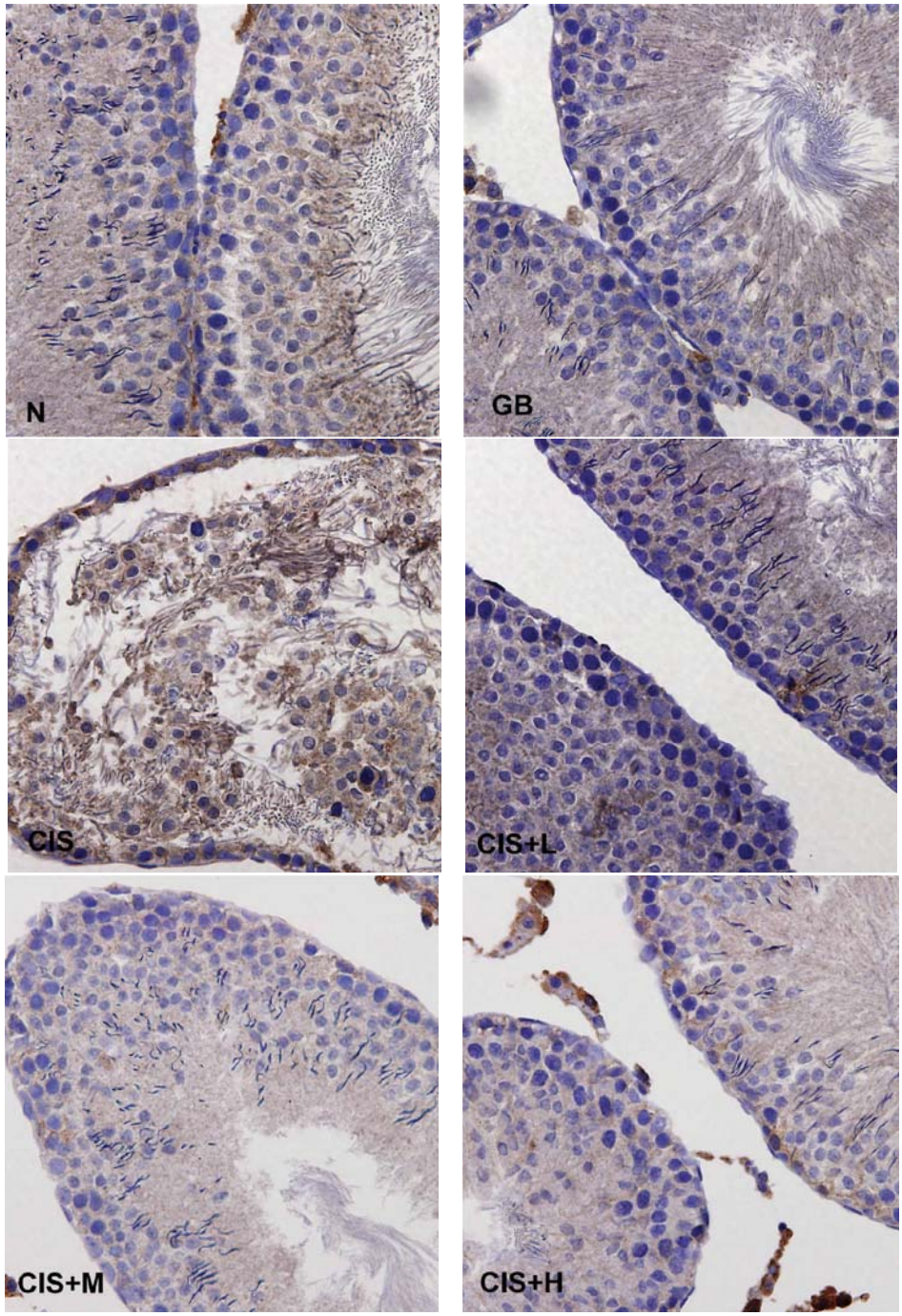

Figure 3: Terminal deoxynucleotidyl transferase-mediated triphosphate nick-end labeling-(TUNEL-) positive cells in seminiferous tubules of rats treated with vehicle (N), GB alone (GB), CIS alone (CIS), and the three GB-protected rats (CIS+L: 50 mg/kg GB; CIS+M: 100 mg/kg GB; CIS+H: 200 mg/kg GB). Photomicrographs show variable levels of apoptosis in different experimental groups Brown staining indicates TUNEL-positive cells. Tissues were counterstained with hematoxylin, 400x.

Immunohistochemical findings showed an increase in the expression of COX-2 (Figure 4), iNOS (Figure 5), and NF-kB (Figure 6) after CIS administration. Coadministration with GB extract markedly reduced the CIS-induced overexpression of COX-2, iNOS, and NF-kB which shows no significant variation when compared to the control.

4. Discussion

CIS is one of the leading anticancer drugs in the chemotherapy treatment of variety of cancer types; it induces a testicular damage, sperm dysfunction, germ-cell apoptosis, and abnormalities in Leydig cells in rats [28–30]. Our previous study

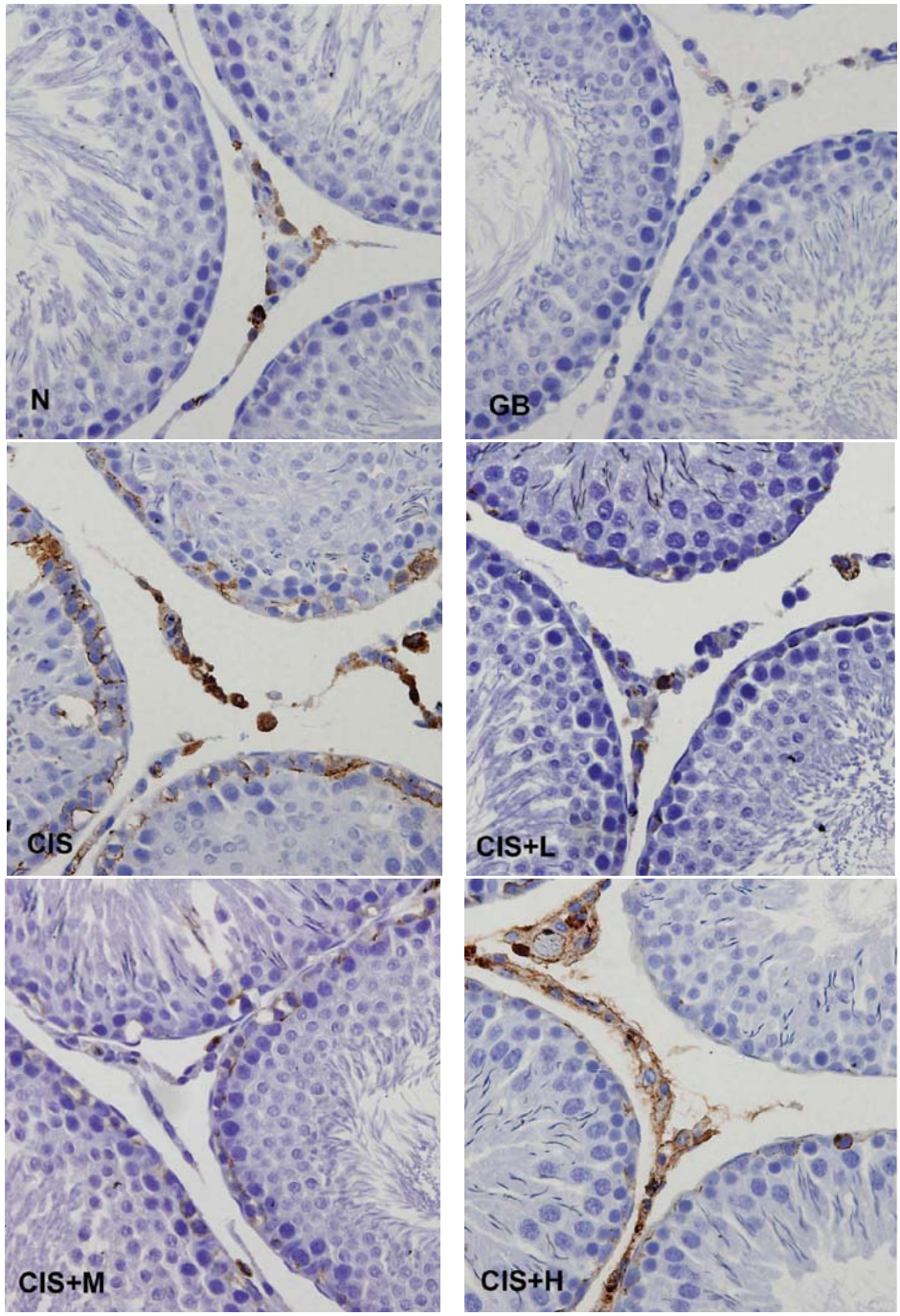

Figure 4: Immunohistochemical staining showing COX-2 expression in control (N), GB alone (GB), CIS alone (CIS), and the three GB-protected rats (CIS+L: 50 mg/kg GB; CIS+M: 100 mg/kg GB; CIS+H: 200 mg/kg GB). Photomicrographs show variable levels of COX-2 expression in different experimental groups. Brown staining indicates COX-2 expression. CIS group show increased levels of COX-2 expression compared to N and all three GB-protected groups. Tissues were counterstained with hematoxylin, 400x.

has shown that CIS impaired rat testicular structure through inflicting oxidative stress and inducing cell apoptosis [9, 24]. Current data shows that pretreatment with GB extract (24% *Ginkgo biloba* flavonoglycoside, 6% terpene lactones) offers protection against the histopathological lesions induced by CIS. Also the increase in apoptotic changes induced by CIS has been found to be decreased in those rats which were treated with different doses of GB extract.

These testicular protective effects of GB were accompanied with restoration of the normal level of CAT, SOD, and

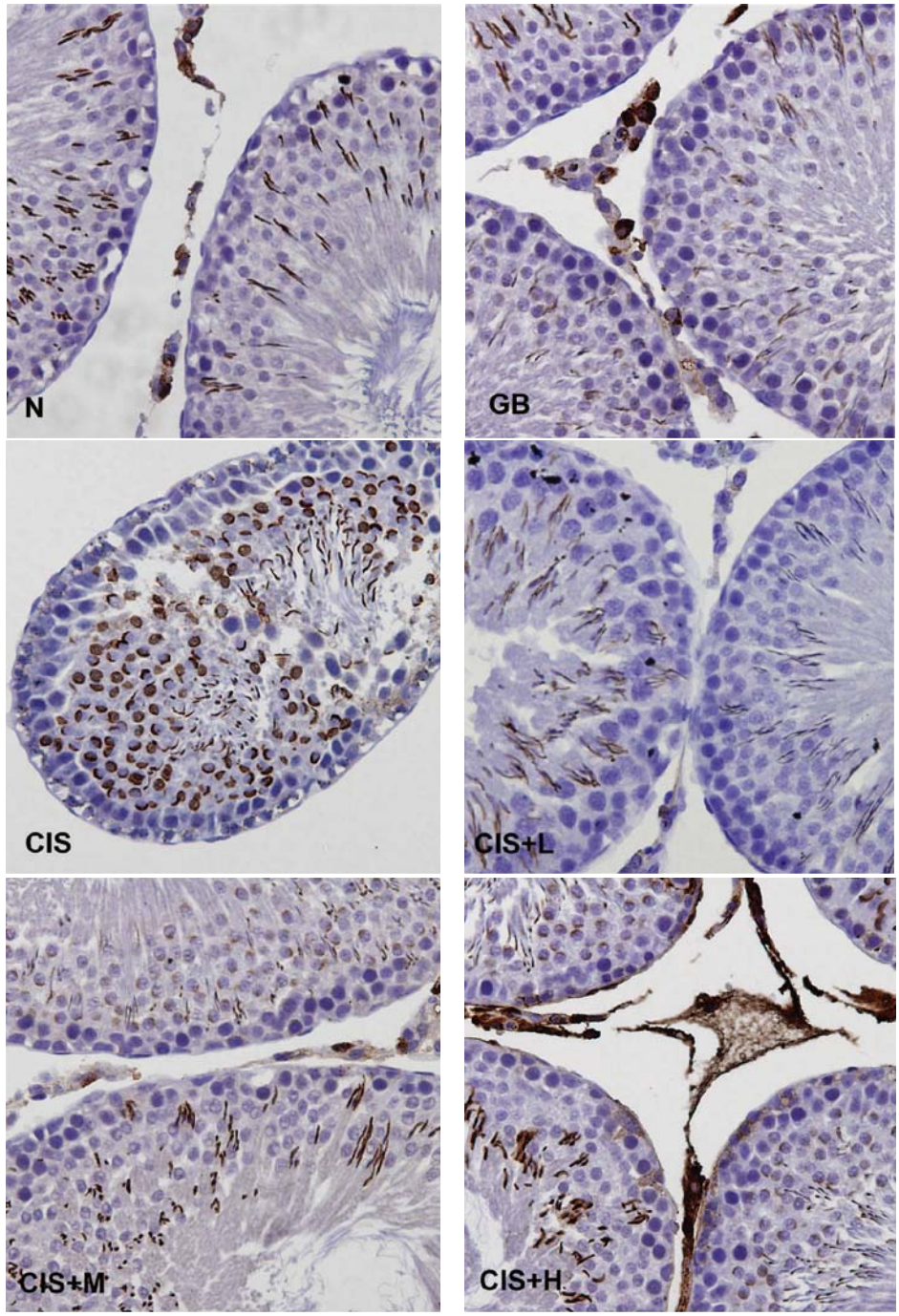

Figure 5: Immunohistochemical staining showing iNOS expression in control (N), GB alone (GB), CIS alone (CIS), and the three GB-protected rats (CIS+L: 50 mg/kg GB; CIS+M: 100 mg/kg GB; CIS+H: 200 mg/kg GB). Photomicrographs show variable levels of iNOS expression in different experimental groups. Brown staining indicates iNOS expression. CIS group shows increased levels of iNOS expression compared to N and the GB-protected groups. Tissues were counterstained with hematoxylin, 400x.

MPO in CIS-treated animals. Normal physiological levels of NF-kB, iNOS, and COX-2 expression have also been maintained by the Coadministration of GB extract.

Recently studies have shown that herbal plants extracts with protective effects against CIS-induced reproductive damages are due to the presence of antioxidant agents [31]. The present investigation illustrates that the administration of GB extract restores the control values of oxidative stress markers. This study provides evidence that the antioxidative properties of GB may contribute to its ability to restore the level of SOD and CAT enzyme and to reduce the MDA content as well as MPO levels in the testicular tissues. The antioxidant activity of GB could be attributed to its active components, namely, flavonoglycoside and terpene lactones.

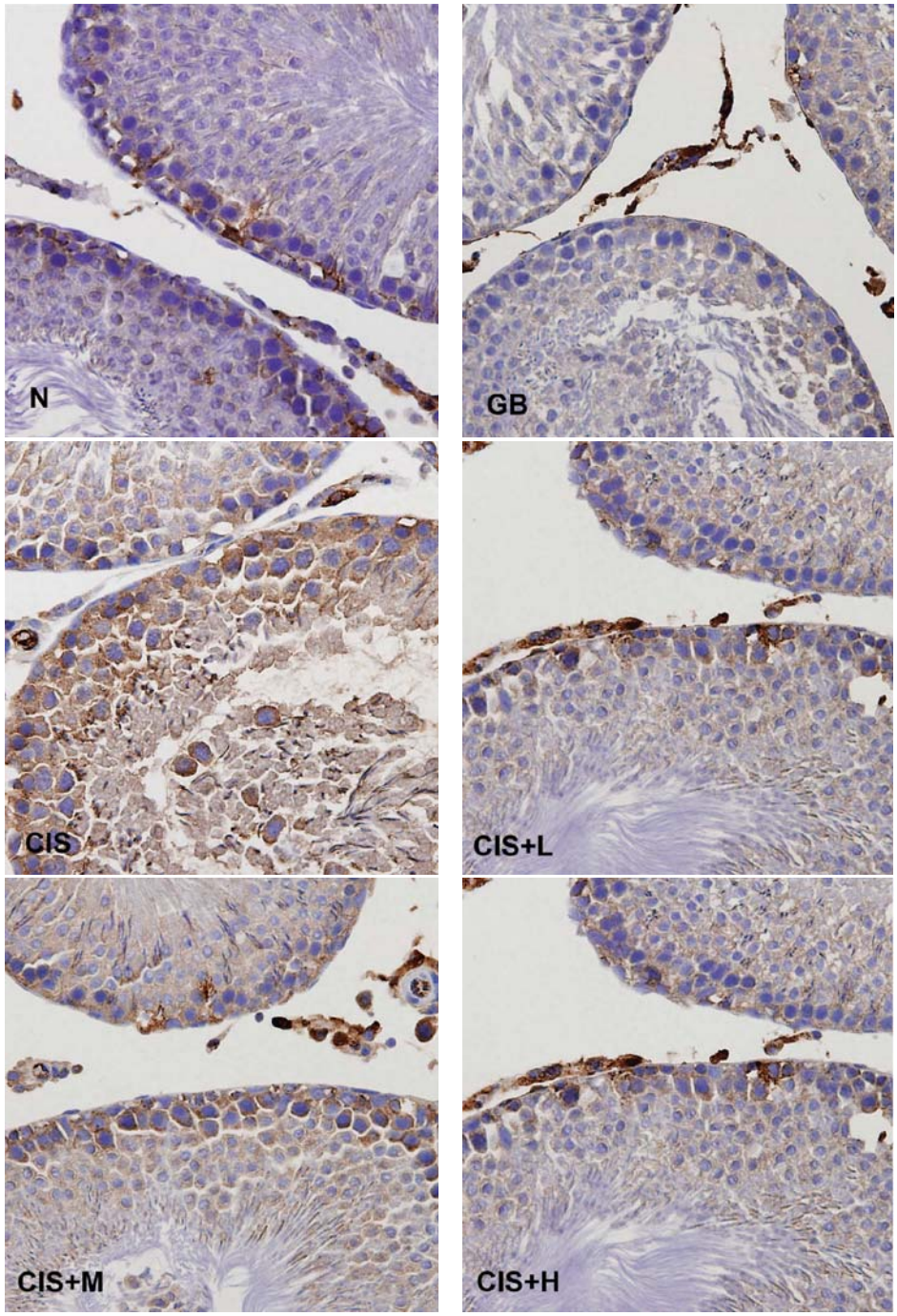

Figure 6: Immunohistochemical staining showing NF-kB expression in control (N), GB alone (GB), CIS alone (CIS), and the three GB-protected rats (CIS+L: 50 mg/kg GB; CIS+M: 100 mg/kg GB; CIS+H: 200 mg/kg GB). Photomicrographs show variable levels of NF-kB expression in different experimental groups. Brown staining indicates NF-kB expression. CIS-treated rats show increased NF-kB levels compared to N and all other GB-protected groups. Tissues were counterstained with hematoxylin, 400x.

The current study also showed an elevated level of NF-kB, iNOS, and COX-2 as a result of CIS treatment. Oxidative stress and subsequent activation of signaling kinases are known to stimulate transcription factors, like NF-kB [32–34]. NF-kB function as a link between oxidative damage and inflammation. This factor transduces oxidative stimuli to nucleus to modulate the expression of many genes involved in inflammatory responses [35, 36]. One such gene is that for iNOS, it is generally thought that excessive NO production due to elevated iNOS can cause cytotoxic effects and has the potential to induce germ-cell apoptosis [20, 37, 38]. According to the data obtained, GB extract reduces the formation of NF-kB and iNOS to the normal level as in untreated rats. Rise in COX-2 levels induced by CIS,

observed during the immunohistochemical analysis, seems to be efficiently reducing in the GB-treated animals.

In conclusion, this study provides evidence that CIS adversely damages testicular tissue and significantly reduces sperm production through increasing oxidative stress and inducing apoptosis and upregulations of NF-kB, iNOS, and COX-2, while GB treatment effectively attenuated these oxidative and apoptosis actions of CIS in testes.

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