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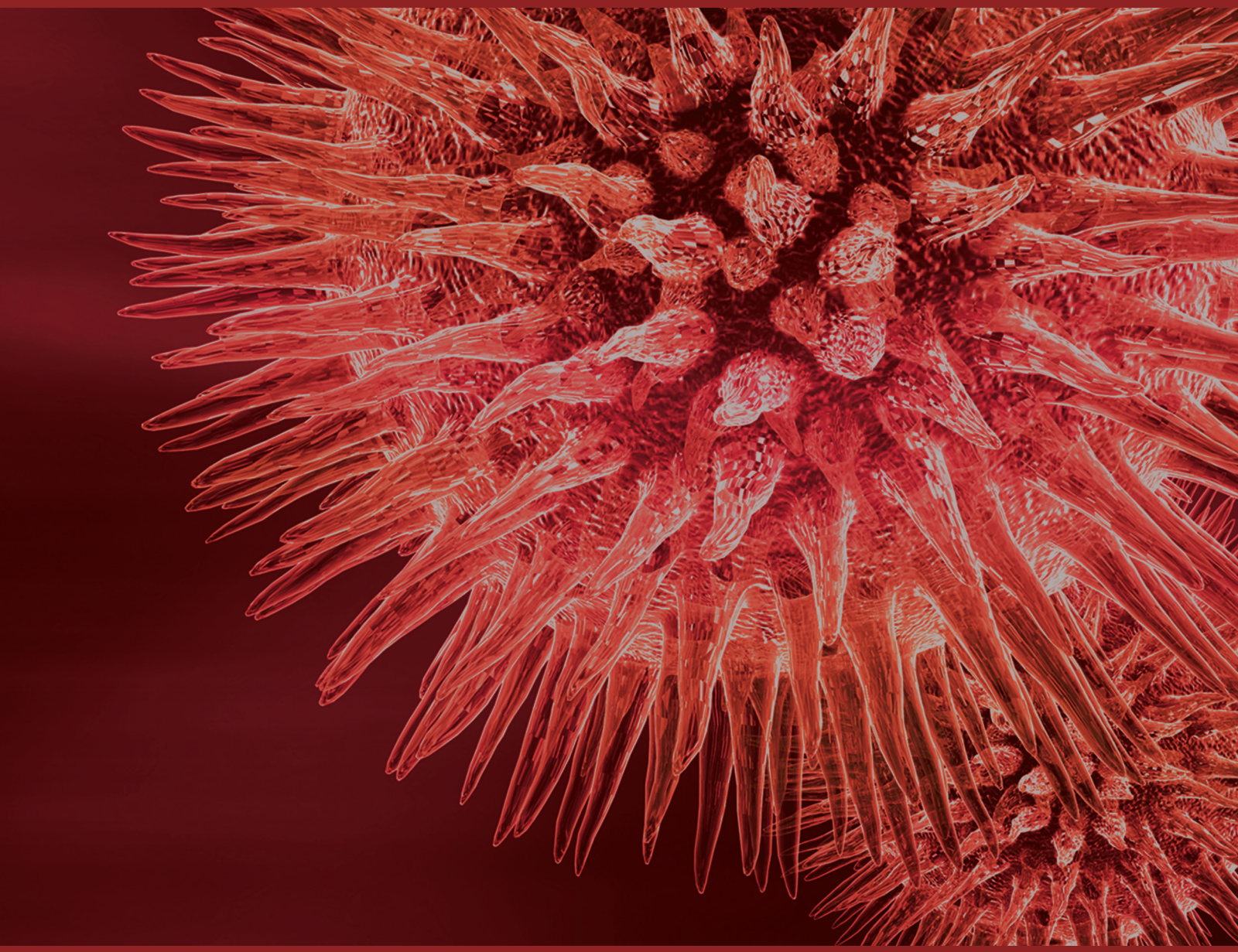
Drug Resistance in Hematologic Malignancies: Induction Mechanisms, Genetics, and Therapeutics

Guest Editors: Fenghuang Zhan, Maurizio Zangari, and Lugui Qiu



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Editorial

Drug Resistance in Hematologic Malignancies: Induction Mechanisms, Genetics, and Therapeutics

Fenghuang Zhan,[1] Maurizio Zangari,[2] and Lugui Qiu[3]

[1]*Department of Internal Medicine, The University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA 52242, USA*
[2]*Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA*
[3]*State Key Laboratory of Experimental Hematology, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Tianjin 300020, China*

Correspondence should be addressed to Fenghuang Zhan; fenghuang-zhan@uiowa.edu

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Hematopoietic malignancies are neoplastic tumors affecting the blood and lymphatic circulatory systems and are commonly referred to as liquid tumors. Malignancies of the blood and lymphatic systems are known to affect a whole host of cell types derived from lymphoid and myeloid progenitors including leukocytic and lymphatic cells and cells that make up the bone marrow microenvironment (Table 1). Due to the variety of cells affected, hematological malignancies of one tissue type can often result in complications with the other hematologic systems.

Hematological malignancies account for approximately 10% of all newly diagnosed neoplasms within the United States. Of the 150,000+ newly diagnosed cases of hematological neoplasms within the US, lymphomas account for 51% of the new cases followed by leukemia and myeloma (Table 1). A common characteristic of hematological malignancies is the presence of chromosomal translocations, a trait not typically observed in solid tumors [1]. This among other characteristics makes hematological malignancies a unique class of neoplasms resulting in a unique set of challenges for treatment and the prevention of relapse.

Science and medicine have focused intently over the last 20–30 years on developing treatment regimens that efficiently and effectively target and destroy cancer cells. Although our understanding of hematological malignancies has improved exponentially, resulting in greatly increased life expectancies and improved quality of life, our ability to abrogate tumor relapse has evaded us. One shortcoming to past and current therapies are centered on the tumor's innate ability to adapt and remain one step ahead of treatment regimens. The next generation of antiproliferative drugs offers a wide spectrum of therapeutic choices. Some of the more common therapies include tyrosine kinase inhibitors like Gleevec and histone deacetylase inhibitors like vorinostat, whereas there is a wealth of new therapeutics available as immunomodulatory drugs, monoclonal antibodies, antibody conjugates, and proteasome inhibitors like Velcade. This next generation of therapies represents a new hope in controlling hematological malignancy relapse and development of refractory disease.

A major underlying complication of hematological malignancies is the development of refractory disease upon patient relapse, resulting in a decreased life expectancy and quality of life [2, 3]. More recently, researchers have turned to novel mediators of drug resistance to try and explain the increase in refractory disease within hematological malignancies. Although researchers are focusing their attention on a wide variety of potential mechanisms of action, current research is centered on a small number of common themes. A few of the more widely studied areas today in drug resistance focus on the contributions of cancer stem cells (CSCs), inducers of drug efflux pump expression and inhibitors of apoptosis [4].

CSCs were originally documented and described in leukemia as a rare population of cells with limitless self-renewal capabilities [5]. Recently, CSCs have been identified in a growing number of solid tumors. A common feature of stem cells is their increased resistance to chemo- and

Table 1: Types of hematological malignancies and rate of diagnosis for all newly diagnosed neoplasms within the United States.

Hematological malignancy	Percent of hematological malignancies	Percent of malignancy
Lymphomas	51%	
Hodgkin's Lymphoma		14%
Non-Hodgkin's Lymphoma		86%
Leukemias	33%	
Acute lymphoblastic leukemia (ALL)		11%
Acute myelogenous leukemia (AML)		36%
Chronic lymphoblastic leukemia (CLL)		30%
Chronic myelogenous leukemia (CML)		11%
Other		11%
Myelomas	15%	
Total		**100%**

(National Cancer Institute 2007–2011 rates http://seer.cancer.gov/ and The Leukemia and Lymphoma Society 2014 rates http://www.lls.org/).

radiotherapy [6, 7]. CSCs show plasticity and heterogeneity [8]. The plasticity indicates an equilibrium between a cell with a stem cell phenotype and mature terminally differentiated tumor cells, while the heterogeneity includes both intraclonal heterogeneity and distinct molecular mechanisms which are essential for tumor development and progression [8]. Unfortunately current chemotherapeutic treatments mainly focus on debulking tumor cells while CSCs escape these conventional therapies. Nevertheless, the upsurge in the development of drugs to target CSC-related pathways, such as Akt, Wnt/β-catenin, Notch, and Hedgehog, is showing promising preclinical and clinical results in hematological malignancies [9]. We expect that targeting CSCs will surely lead this field to evolve for overcoming drug resistance in the next few years.

Drug resistance is a universal problem with current therapies for hematologic malignancies, but very little is known about the molecular mechanisms. This special issue of *BioMed Research International* focuses on drug resistance in hematologic malignancies, induction mechanisms, genetics, and therapeutics. It is our goal that the reader leaves with an improved understanding of the underlying mechanisms of drug resistance in hematological malignancies and the discoveries focusing on drug responsiveness resulting in improved quality of life and increased life expectancy.

Fenghuang Zhan
Maurizio Zangari
Lugui Qiu

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Review Article

Smoldering Multiple Myeloma

Minjie Gao, Guang Yang, Yuanyuan Kong, Xiaosong Wu, and Jumei Shi

Department of Hematology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China

Correspondence should be addressed to Xiaosong Wu; wux163@163.com and Jumei Shi; shijumei@hotmail.com

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Smoldering multiple myeloma (SMM) is an asymptomatic precursor stage of multiple myeloma (MM) characterized by clonal bone marrow plasma cells (BMPC) ≥ 10% and/or M protein level ≥ 30 g/L in the absence of end organ damage. It represents an intermediate stage between monoclonal gammopathy of undetermined significance (MGUS) and symptomatic MM. The risk of progression to symptomatic MM is not uniform, and several parameters have been reported to predict the risk of progression. These include the level of M protein and the percentage of BMPC, the proportion of immunophenotypically aberrant plasma cells, and the presence of immunoparesis, free light-chain (FLC) ratio, peripheral blood plasma cells (PBPC), pattern of serum M protein evolution, abnormal magnetic resonance imaging (MRI), cytogenetic abnormalities, IgA isotype, and Bence Jones proteinuria. So far treatment is still not recommended for SMM, because several trials suggested that patients with SMM do not benefit from early treatment. However, the Mateos et al. trial showed a survival benefit after early treatment with lenalidomide plus dexamethasone in patients with high-risk SMM. This trial has prompted a reevaluation of early treatment in an asymptomatic patient population.

1. Introduction

Smoldering multiple myeloma (SMM) was first described in 1980 in the New England Journal of Medicine (NEJM) [1]. Its definition varied, but the universal recognition was that SMM exceeded the limits of the definition of monoclonal gammopathy of undetermined significance (MGUS) and had no end-organ damage. In 2003 the International Myeloma Working Group (IMWG) provided the criteria that SMM was defined as clonal bone marrow plasma cells (BMPC) ≥ 10% and/or M protein level ≥ 30 g/L and lack of end organ damage (CRAB—hypercalcemia, renal failure, anemia, and bone lesions) [2]. SMM accounts for about 15% of all the patients with newly diagnosed MM [3]. The risk of progression to symptomatic MM is markedly higher in SMM compared to MGUS, 10% per year versus 1% per year, respectively [4]. Currently, patients with SMM are not treated until the development of MM symptoms. In the past, some trials used alkylating agents such as melphalan to evaluate the effect of early treatment on patients with SMM [5–7]. They caused obvious toxicity and failed to show a significant benefit. With the introduction of novel agents, investigators attempted early treatment with novel agents such as thalidomide for patients with SMM, but early treatment still did not result in improved survival benefit [8–11]. However, Mateos et al. reported the results of their phase III trial and showed that early treatment for patients with high-risk SMM improved overall survival [12]. This prompted a reconsideration of treatment of SMM and the definition the high-risk SMM. This purpose of this review is to summarize and evaluate the prognostic factors predicting progression to active MM, to discuss early treatment of patients with SMM, and to provide directions for further investigations.

2. Definition of SMM

In 1980, Kyle and Greipp first introduce the concept of smoldering (asymptomatic) multiple myeloma (SMM) in defining six myeloma patients in whom the percentage of plasma cells and level of M protein were higher than those seen in MGUS and fulfilled the criteria for diagnosis of MM but have no anemia, hypercalcemia, and lytic bone lesions. These patients remained stable without specific therapy for five or more years [1]. At the same time, Alexanian et al. use the term indolent multiple myeloma (IMM) in defining 20 patients who were asymptomatic from their low tumor mass disease, had

a hemoglobin greater than 10 g/dL, and showed no recurrent infection, painful compression fractures, or more than 3 lytic bone lesions [13]. In 1988, Alexanian et al. made changes to the definition of SMM and IMM, distinguishing SMM from IMM [14]. Before 2003, many studies used different criteria to define asymptomatic patients with myeloma. In 2003 IMWG defined SMM as BMPC ≥ 10% and/or M protein level ≥ 30 g/L and lack of end organ damage (CRAB—hypercalcemia, renal failure, anemia, and bone lesions) [2].

3. Predictors of Progression to Active MM

Most of the patients diagnosed with SMM will progress to symptomatic MM. However, SMM is not a uniform disease and patients with SMM do not progress to symptomatic MM at the same rate. Hence, it is important to define the risk of progression. A number of parameters have been described to predict risk of progression to symptomatic MM (Table 1).

3.1. The Level of Serum M Protein and the Percentage of Bone Marrow Plasma Cells. The level of serum M protein and the percentage of BMPC were associated with the risk of progression from SMM to active disease. Kyle et al. divided patients with SMM into three prognostic groups by the percentage of BMPC and level of serum M protein (group 1: BMPC ≥ 10% and serum M protein ≥ 3 g/dL; group 2: BMPC ≥ 10% but serum M protein < 3 g/dL; group 3: serum M protein ≥ 3 g/dL but BMPC < 10%). The median time to progression (TTP) in groups 1, 2, and 3 were 2, 8, and 19 years, respectively [15]. Kastritis et al. reported the median TTP for patients with BMPC ≥ 10% and serum M protein ≥ 3 g/dL was 19 months versus 73 months for patients with BMPC ≥ 10% but serum M protein < 3 g/dL. They identified that 8% of patients had BMPC ≥ 60% at diagnosis of SMM and these patients had a median TTP of 15 months [16]. Rajkumar et al. studied 655 patients with SMM and found that the median TTP to active myeloma was significantly shorter for patients with BMPC ≥ 60%, as compared with those with BMPC < 60% ($P < 0.001$) [17]. It was suggested that SMM should be defined with an upper limit of BMPC and patients with BMPC ≥ 60% should not be considered as asymptomatic MM and should receive therapy immediately [16–18].

3.2. Immunophenotyping and Immunoparesis. The proportion of immunophenotypically aberrant plasma cells (defined as elevated expression of CD56, the absence of CD45 and/or CD19, and decreased expression of CD38) within the bone marrow plasma cells (aPCs/BMPC) ≥ 95% and immunoparesis (reduction of one or more uninvolved immunoglobulin isotypes below the lower limit of normal) were risk factors for progression. Pèrez-Persona et al. reported that a risk of progression to active MM at 5 years of 4%, 46%, and 72%, respectively, for patients with none, 1, or 2 risk factors (aPCs/BMPC ≥ 95% and immunoparesis) [19]. Over time, Pèrez-Persona et al. reported that in the evolving SMM arm (defined as an increase in the level of serum M protein of at least 10% during the first 6 months of follow up, or a progressive and constant increase of the M component until overt MM developed), the 3-year progression rate for patients with aPCs/BMPC ≥ 95% was 46% versus 8% for those patients with aPCs/BMPC < 95% ($P = 0.01$). This was also observed in the nonevolving SMM arm, in which the 3-year progression rate was 15% for patients with aPCs/BMPC ≥ 95%, whereas no progression was observed in the aPCs/BMPC < 95% arm ($P = 0.01$) [20].

3.3. Serum-Free Light-Chain Ratio. The serum immunoglobulin FLC ratio was an independent predictor of progression to active MM. Larsen et al. concluded that a high serum-free light-chain (FLC) ratio ≥ 100 was a biomarker of early progression to active MM. The median TTP in the FLC ratio ≥ 100 arm was 15 months versus 55 months in the FLC < 100 arm ($P < 0.0001$) [21]. Kastritis et al. reported that abnormal FLC ratio was one of the most significant factors for progression, reporting that the median TTP in the FLC ratio ≥ 8 arm was 55 months versus 73 months in the FLC ratio < 8 arm ($P < 0.005$). The median TTP for patients with a FLC ratio ≥ 100 was 18 versus 73 months for patients with FLC ratio < 100 [16]. Dispenzieri et al. constructed a risk-stratification model based on the risk factors (BMPC ≥ 10%; serum M protein ≥ 3 g/dL; and FLC ratio > 8). The 5-year progression rates for the presence of one, two, or three risk factors were 25%, 51%, and 76%, respectively [22].

3.4. Circulating Peripheral Blood Plasma Cells (PBPC). Detection of circulating peripheral blood plasma cells (PBPC) helped to predict disease course in patients with SMM. Witzig et al. detected the number of PBPC in 57 patients with SMM and found that the median TTP for patients with abnormal PBPC (defined as an increase in number or proliferative rate of PBPC) was 0.75 years versus 2.5 years for those patients without abnormal PBPC ($P < 0.01$) [23]. Bianchi et al. concluded that high PBPC (defined as absolute PBPC > 5000×10^6/L and/or >5% cytoplasmic immunoglobulin (Ig) positive plasma cells per 100 peripheral blood mononuclear cells) could predict the evolution of SMM to active MM. The progression rate at two years for patients with high PBPC was 71% versus 25% without high PBPC ($P = 0.001$). Corresponding progression rate at 3 years were 86% versus 35%, respectively ($P < 0.001$) [24].

3.5. Pattern of Serum M Protein Evolution. Based on the pattern of changes in serum M protein, SMM was classified into evolving SMM and nonevolving SMM. Evolving SMM was characterized by a progressive increase in serum M protein, a previously recognized MGUS, and a significantly higher proportion of IgA type. Nonevolving SMM was characterized by stable M protein until disease progression. The median TTP for patients with evolving SMM was 1.3 years versus 3.9 years for those with nonevolving SMM ($P = 0.007$) [25].

3.6. Novel Imaging Assessments. Moulopoulos et al. assessed the prognostic significance of magnetic resonance imaging (MRI) in patients with asymptomatic myeloma and reported that the median TTP for patients with abnormal MRI was 16 months versus 43 months for those with normal MRI ($P < 0.01$) [26]. Hillengass et al. analyzed the prognostic significance of focal lesions in whole-body MRI in patients

Table 1: Studies predicting risk of progression of SMM to symptomatic MM.

Author [year]	Included number and criteria	Risk factors	Outcome
Kyle et al. [2007] [15]	276 IMWG	Group 1 (BMPC ≥ 10% and M protein ≥ 30 g/L); group 2 (BMPC ≥ 10% and M protein < 30 g/L); group 3 (BMPC < 10% and M protein ≥ 30 g/L)	TTP: group 1: 2 y; group 2: 8 y; group 3: 19 y
Kastritis et al. [2013] [16]	96 IMWG	Risk factor 1: M protein ≥ 30 g/L; risk factor 2: extensive BM infiltration ≥ 60%; risk factor 3: FLC ratio ≥ 100; risk factor 4: abnormal MRI	TTP: with risk factor 1 was 2 y (versus 8 y without risk factor 1); with risk factor 2 was 15 m (versus 90 m without risk factor 2); with risk factor 3 was 18 m (versus 73 m without risk factor 3); with risk factor 4 was 15 m (versus not reached without risk factor 4).
Rajkumar et al. [2011] [17]	655 IMWG	BMPC ≥ 60%	2-y progression rate: 95%; TTP: 7 m
Pérez-Persona et al. [2007] [19]	93 IMWG	Group 1: neither aPCs/BMPC ≥ 95% nor immunoparesis; group 2: aPCs/BMPC ≥ 95% or immunoparesis; group 3: aPCs/BMPC ≥ 95% and immunoparesis	5-y progression rate: group 1: 4%; group 2: 46%; group 3: 72%
Pérez-Persona et al. [2010] [20]	61 IMWG	aPCs/BMPC ≥ 95%	3-y progression rate: for evolving SMM with aPCs/BMPC ≥ 95% was 46% (versus 8% with aPCs/BMPC < 95%); for nonevolving SMM with aPCs/BMPC ≥ 95% was 15% (versus no progressions with aPCs/BMPC < 95%)
Larsen et al. [2013] [21]	586 IMWG	FLC ratio ≥ 100	TTP: FLC ratio ≥ 100: 15 m; FLC ratio < 100: 55 m
Dispenzieri et al. [2008] [22]	273 IMWG	BMPC ≥ 10%; M protein ≥ 30 g/L; FLC ratio ≥ 8; low-risk: 1 risk factor; intermediate-risk: 2 risk factor; high-risk: 3 risk factor	5-y progression rate: low-risk: 25%; intermediate-risk: 51%; high-risk: 76%
Witzig et al. [1994] [23]	57 BMPC > 10% without CRAB	Abnormal PBPC (an increase in number or proliferative rate of PBPC)	TTP: with abnormal PBPC was 0.75 y (versus 2.5 y without abnormal PBPC)
Bianchi et al. [2013] [24]	91 IMWG	High PBPC (absolute PBPC > 5000 × 10^6/L and/or >5% cytoplasmic Ig positive PC per 100 PBMC)	2-y progression rate: with high PBPC was 71% (versus 25% without high PBPC); 3-y progression rate: with high PBPC was 86% (versus 34% without high PBPC)
Rosiñol et al. [2003] [25]	53 BMPC > 10%, M-protein > 30 g/L or light chain > 1 g, hemoglobin > 100 g/L, without CRAB	Evolving SMM (a progressive increase in M protein, a previously recognized MGUS and a significant higher proportion of IgA type)	TTP: with evolving SMM was 1.3 y (versus 3.9 y with nonevolving SMM)
Moulopoulos et al. [1995] [26]	38 BMPC > 10%, hemoglobin > 105 g/L, normocalcemia, M protein < 45 g/L, and no lytic bone lesion	Abnormal MRI	TTP: with abnormal MRI was 16 m (versus 43 m with normal MRI)
Hillengass et al. [2010] [27]	149 IMWG	Focal lesions > 1	2-y progression rate: 0 or 1 focal lesion: 20%; >1 focal lesion: 70%
Neben et al. [2013] [28]	248 IMWG	del (17p13), t(4;14), +1q21 and hyperdiploidy	TTP: with del (17p13) was 2.04 y (versus 5.62 y without del (17p13)); with t(4;14) was 2.91 y (versus 5.71 y without t(4;14)). 3-y progression rate: with +1q21 was 43% (versus 27% without +1q21); with hyperdiploidy was 35% (versus 29% without hyperdiploidy)

Table 1: Continued.

Author [year]	Included number and criteria	Risk factors	Outcome
Rajkumar et al. [2013] [29]	351 IMWG	Low-risk: no detectable abnormalities; Standard-risk: t(11;14), MAF translocations, other/unknown IgH translocations, or monosomy 13/del (13q); Intermediate-risk: trisomies alone; High-risk: t(4;14)	TTP: High-risk: 28 m; Intermediate-risk: 34 m; Standard-risk: 55 m; Low-risk: not reached

PC: plasma cells; PBPC: peripheral blood plasma cells; PBMC: peripheral blood mononuclear cells; Ig: immunoglobulin; MRI: magnetic resonance imaging; FLC: serum free light chain; BMPC: bone marrow plasma cells; aPCs/BMPC: aberrant plasma cells within the bone marrow plasma cells; TTP: median time to progression; CRAB: hypercalcemia, renal failure, anemia, and bone lesions; IMWG: International Myeloma Working Group; m: month; y: year.

with SMM and founded that the detection of more than one focal lesions was the adverse prognostic factors for progression into SMM ($P < 0.001$). The 2-year progression rate for patients with 0 or 1 focal lesion was 20% versus 70% for those patients with >1 focal lesion [27]. Kastritis et al. reported that abnormal marrow signal of MRI of the spine was associated with a significant factor for progression to symptomatic myeloma (median 15 months, $P = 0.001$) [16].

3.7. Cytogenetic Abnormalities. Neben et al. analyzed the impact of chromosomal aberrations on progression in patients with SMM and found that the presence of del (17p13), t(4;14), +1q21 and hyperdiploidy predicted shorter TTP. The median TTP for patients with del (17p13) was 2.04 years (versus 5.62 years without del (17p13), $P = 0.001$), and with t(4;14) was 2.91 years (versus 5.71 years without t(4;14), $P = 0.003$). The progression rate at three years for patients with +1q21 was 43% (versus 27% without +1q21, $P = 0.02$), and 35% for those with hyperdiploidy (versus 29% without hyperdiploidy, $P = 0.016$) [28]. Rajkumar et al. reported that the median TTP to SMM was 28 months with t(4;14), 34 months with trisomies alone, 55 months with t(11;14), MAF translocations, other/unknown IgH translocations, monosomy 13/del (13q) without other abnormalities, and those with both trisomies and IgH translocations, but a median TTP was not reached in patients without detectable abnormalities, $P = 0.001$. There was a trend to shorter TTP with deletion 17p (median TTP, 24 months) [29].

3.8. IgA Isotype. Whether IgA isotype was a harmful prognostic factor for progression of SMM to active MM was unclear. Weber et al. assessed the clinical features in 101 patients with SMM and found that IgA isotype was present in 23 of 101 patients. There was a trend that the median TTP for patients with IgA isotype was shorter than those with IgG isotype (21 months versus 36 months, $P = 0.21$) [30].

3.9. Bence Jones Proteinuria. The predictive role for Bence Jones proteinuria was controversial and of uncertain importance. Dimopoulos et al. reported that Bence Jones proteinuria represented an independent prognostic factor. Bence Jones protein > 50 mg/d was present in 28 of 95 patients. The median TTP for patients with Bence Jones protein > 50 mg/d was 19 months versus 29 months for those with Bence Jones protein ≤ 50 mg/d ($P = 0.02$) [31].

4. Results of Interventional Therapeutic Trials

SMM has no obvious symptoms and may require different treatment strategies. For patients with SMM, it is not clear whether it is better to immediately treat after diagnosis or to delay treatment until symptomatic MM develops. Many investigators performed clinical trials to determine whether early treatment using conventional and novel agents resulted in improved clinical outcomes for patients with SMM, when compared with deferred treatment (Table 2).

4.1. Melphalan and Prednisone. Three small randomized studies [5–7] compared early treatment with melphalan and prednisone with deferred therapy until disease progression. All three trials showed no significant improvement in TTP or overall survival (OS).

4.2. Bisphosphonates. Several trials compared single agent bisphosphonate with observation in patients with SMM. Martin et al. performed a single-arm pilot study to assess the effects of pamidronate on 12 patients with SMM or IMM and suggested that pamidronate treatment reduced bone turnover but had no significant antitumour effect [32]. Two random trials also compared pamidronate with observation in patients with SMM and founded that pamidronate decreases the development of skeletal events but did not delay disease progression and improve OS [33, 34]. Musto et al. evaluated zoledronic acid versus observation in patients with SMM and founded that zoledronic acid reduced the rate of skeletal-related events but did not influence the natural history of SMM [35].

4.3. Thalidomide. Three nonrandomized, phase II trials evaluating thalidomide-based treatment suggested that long-term thalidomide therapy was poorly tolerated and most of the patients discontinued treatment due to peripheral neuropathy [8–10]. A randomized study compared combination therapy consisting of thalidomide and zoledronic acid with zoledronic acid alone in patients with SMM. The overall response rate after one year in the thalidomide plus zoledronic acid arm was 37% versus 0% in the zoledronic acid alone arm ($P = 0.0004$). The median TTP was significantly longer in the thalidomide plus zoledronic acid arm than in the zoledronic acid alone arm (2.4 versus 1.2 years, $P = 0.02$). The one-year progression-free survival (PFS) in the thalidomide

Table 2: Clinical trials for patients with SMM.

Author [year]	Trial design	Therapy	Number and type of patients	Outcome
Hjorth et al. [1993] [7]	RCT	Initial versus deferred MP	50 stage I MM (DSS)	No difference in RR, response duration, or OS
Riccardi et al. [2000 and 1994] [5, 6]	RCT	Initial versus deferred MP	145 stage I MM (DSS)	No difference in RR or OS
Rajkumar et al. [2003] [8]	Single-arm phase 2	Thalidomide	16 SMM or IMM	11 of 16 patients responded to therapy
Martín et al. [2002] [32]	Single-arm pilot	Pamidronate	12 SMM or IMM	Reduces bone turnover but has no antitumour effect
Weber et al. [2003] [9]	Single-arm phase 2	Thalidomide	28 SMM	RR was 36% and median time to remission was 4.2 m
Musto et al. [2008] [35]	RCT	Zoledronic acid versus observation	163 SMM	No difference in PFS and TTP; reduce skeletal-related events
Barlogie et al. [2008] [10]	Phase 2	Thalidomide with monthly pamidronate	76 SMM	4-y OS and PFS were 91% and 60%, respectively
D'Arena et al. [2011] [33]	RCT	Pamidronate versus observation	177 SMM	No difference in PFS, TTP, or OS; reduce skeletal events
Witzig et al. [2013] [11]	RCT	Thalidomide plus zoledronic acid versus zoledronic acid	68 SMM	Significant difference in TTP and PFS; no difference in OS
Mateos et al. [2013] [12]	RCT	Lenalidomide plus dexamethasone versus observation	119 high-risk SMM	TTP: treatment: not reached; observation: 21 m; 3-y OS: treatment: 94%; observation: 80%

MP: melphalan and prednisone; DSS: Durie and Salmon stage; RR: response rate; OS: overall survival; PFS: progression-free survival; RCT: randomized controlled trial; IMM: indolent multiple myeloma; m: month; y: year.

plus zoledronic acid arm was 86% versus 55% in the zoledronic acid alone arm ($P = 0.0048$). There was no difference in OS between the arms [11].

4.4. Lenalidomide. Early treatment for patients with high-risk SMM delayed disease progression and increased OS. Mateos et al. evaluated treatment in patients with high-risk SMM. High-risk MM was defined as BMPC $\geq$ 10% and a monoclonal component (IgG $\geq$ 3 g/dL, IgA $\geq$ 2 g/dL, or Bence Jones $>$ 1 g/24 h) or only one of the two criteria described above, plus aPCs/BMPC $\geq$ 95%, with decrease of one or two uninvolved immunoglobulins $\geq$25%, without CRAB. They randomly assigned 119 patients with high-risk SMM to lenalidomide-dexamethasone treatment or observation. Lenalidomide-dexamethasone treatment significantly delayed disease progression and improved OS. The three-year PFS for patients in the treatment arm was 77% versus 30% for those in the observation arm ($P < 0.001$). 94% of the patients in the treatment arm versus 80% in the observation arm were alive at three years ($P = 0.03$) [12].

4.5. Other Agents. Some phase II trials are ongoing to determine whether the use of agents such as siltuximab (anti-IL-6 mAb), elotuzumab, MLN9708 (ixazomib), or BHQ880 (anti-DKK1 neutralizing Ab) is active in high-risk SMM [36]. The results will help provide more evidence and effective strategy to early treatment for patients with high-risk SMM.

5. Summary and Future

Currently, treatment is not recommended for SMM based on data derived from several small randomized controlled trials. However, the recent Mateos et al. trial showed improved OS with lenalidomide and dexamethasone treatment among patients with high-risk SMM. This suggested that high-risk SMM should be targeted for early intervention. However, the Mateos et al. trial had a small sample size. Much larger trials that select patients with high-risk SMM are needed to provide more evidence. The Mateos et al. trial used a combination regimen; therefore, whether the benefit was due to lenalidomide is unclear. More data were needed to isolate the effect of lenalidomide, and more trials were needed to evaluate the effect of other novel drugs which are not as expensive as lenalidomide on high-risk SMM. In addition, the criteria for high-risk SMM should be established. Mateos et al. used two separate criteria to identify high-risk SMM, one defined by Kyle et al. and another by Pèrez-Persona et al. This method was validated to identify patients who progress to active MM within almost 2 years after diagnosis. However, such criteria limit the general applicability of this approach because flow cytometry was required. Currently, a number of parameters have been described to predict risk of progression to symptomatic MM, prompting the following questions: (1) what probability of 2-year progression to symptomatic MM will be defined as high risk? (2) Which predictors should be used alone or in combination to identify high-risk SMM? Currently, there is no consensus, but it is generally accepted

Table 3: Risk factors predicting high-risk SMM.

Risk factors	Patients with risk factors accounting for the population of SMM	Probability of 2-year progression to symptomatic MM
Bone marrow plasma cells ≥ 60%	2–10%	90%
Serum-free light-chain ratio ≥ 100	15%	80%
Abnormal magnetic resonance imaging (>1 focal lesion)	15%	70%
High peripheral blood plasma cells[a]	15%	70%

[a]Defined as absolute peripheral blood plasma cells > 5000×10^6/L and/or >5% cytoplasmic immunoglobulin (Ig) positive plasma cells per 100 peripheral blood mononuclear cells.

that patients with BMPC ≥ 60%, FLC ratio ≥ 100, or abnormal MRI (>1 focal lesion) are at high risk and need to be treated immediately. After reviewing all of the data, patients with BMPC ≥ 60%, FLC ratio ≥ 100, or abnormal MRI (>1 focal lesion) have ≥70% probability of progression to active MM (Table 3). If indeed there is a 70% probability of progression, we recommend that high PBPC also be considered as a high-risk factor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Minjie Gao and Guang Yang contributed equally to this work.

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Research Article

Synergistic Activity of Carfilzomib and Panobinostat in Multiple Myeloma Cells via Modulation of ROS Generation and ERK1/2

Lu Gao, Minjie Gao, Guang Yang, Yi Tao, Yuanyuan Kong, Ruixue Yang, Xiuqin Meng, Gongwen Ai, Rong Wei, Huiqun Wu, Xiaosong Wu, and Jumei Shi

Department of Hematology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China

Correspondence should be addressed to Xiaosong Wu; wux163@163.com and Jumei Shi; shijumei@hotmail.com

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Relapse of disease and subsequent resistance to established therapies remain as major challenges in the treatment of multiple myeloma (MM). New therapeutic options are needed for these extensively pretreated patients. To explore an optimized combinational therapy, interactions between the irreversible proteasome inhibitor carfilzomib exhibiting a well-tolerated side-effect profile and histone deacetylase inhibitor (HDACi) panobinostat (LBH589) were examined in MM cells. Coadministration of carfilzomib and LBH589 led to a synergistic inhibition of proliferation in MM cells. Further studies showed that the combined treatment synergistically increased mitochondrial injury, caspase activation, and apoptosis in MM cells. Lethality of the carfilzomib/LBH589 combination was associated with the reactive oxygen species (ROS) generation and ERK1/2 inactivation. In addition, the free radical scavenger N-acetylcysteine (NAC) could block carfilzomib and LBH589-induced oxidative stress and the subsequent apoptosis. Together, these findings argue that the strategy of combining carfilzomib and LBH589 warrants attention in MM.

1. Introduction

Multiple myeloma (MM) is a B-cell malignant disorder characterized by clonal proliferation of plasma cells in the bone marrow and osteolytic bone lesions [1]. Although new therapeutic options have been introduced and overall survival rate has improved in the management of MM, the disease remains incurable and almost all patients show disease relapse and develop drug resistance because of rapid regrowth of chemotherapy-refractory MM cells. This indicates that efficacious novel therapies are still needed for the patients with relapsed/refractory MM. Recently, data from present studies showed that the combination of proteasome inhibitor and histone deacetylase inhibitor (HDACi) resulted in synergistic inhibition of MM cell growth and might be an effective therapy for such patients [2, 3].

Carfilzomib, a second-generation selective, irreversible proteasome inhibitor of the chymotrypsin-like activity of the proteasome, shows antimyeloma effects [4]. Carfilzomib has been approved for the treatment of relapsed/refractory MM by the US Food and Drug Administration. It is well-tolerated in humans, especially a low incidence of peripheral neuropathy, and has activity against bortezomib-resistant myeloma cells, which makes it particularly suitable for use in combinational strategies [5]. Previous clinical trial has shown that replacing bortezomib with carfilzomib is safe and effective for MM patients failing bortezomib-containing combination regimens [6].

Panobinostat (LBH589), a highly potent HDACi, displays antitumor activity against a range of malignancies, particularly hematological diseases, such as MM, cutaneous T-cell lymphoma, Hodgkin lymphoma, and chronic myelogenous leukemia [7, 8]. LBH589 has shown activity against drug-resistant cancer cell. LBH589 in combination with other therapies has shown synergistic antitumor efficacy by preclinical studies [8]. Some phase I/II clinical trials have been conducted to investigate the safety and efficacy of LBH589 in combination with other agents [9–11].

Present studies have demonstrated that the proteasome inhibitor bortezomib/HDACi combination has a powerful antimyeloma activity on MM cells including cells that are highly resistant to cytotoxic drugs [12, 13]. Clinical trials

further confirm such activity in relapsed/refractory MM patients [3, 9]. However, a considerable part of patients in these clinical trials could not tolerate such therapy because of serious side effects and discontinued treatment [9]. This constrains its application to some extent and an optimized proteasome inhibitor/HDACi combination with lesser side effects is therefore needed. The second-generation proteasome inhibitor carfilzomib has a well-tolerated side-effect profile and potent antimyeloma activity. Thus, the carfilzomib/HDACi combination may represent an optimized proteasome inhibitor/HDACi combination therapy for MM patients if synergistic interactions between them exist. The purpose of the present study is to determine whether the combination of carfilzomib and LBH589 could have a synergistic activity on MM cells. Our results indicate that carfilzomib and LBH589 interact in a highly synergistic manner in all four tested MM cells and that events involve triggering reactive oxygen species (ROS) generation and inhibiting ERK1/2 pathway. Thus, our research provides a basis for clinical evaluation of this possible optimized combination of proteasome inhibitor and HDACi in relapsed/refractory MM patients.

2. Materials and Methods

2.1. Cells. The human MM cell lines RPMI 8226, OPM2, U266, and H929 were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium (Invitrogen, Frederick, USA) containing 10% fetal bovine serum, 1% penicillin (100 units/mL), and 1% streptomycin (100 mg/mL).

2.2. Reagents. Carfilzomib was purchased from Onyx Pharmaceuticals (South San Francisco, USA). LBH589 was purchased from Merck & Co., Inc. (Rahway, USA). These agents were dissolved in dimethyl sulfoxide. N-Acetylcysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, USA) and prepared in double-distilled water before use. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Mashikimachi, Japan). Cell apoptosis kit was obtained from BD Pharmingen (Franklin Lakes, USA). JC-1 Mitochondrial Membrane Potential Assay kit was from Beyotime Institute of Biotechnology (Haimen, China).

2.3. Cell Survival Assay. MM cells were seeded into 96-well plates at a density of 2×10^5 cells per well and treated with different concentrations of carfilzomib and/or LBH589 for 48 h. CCK-8 was added into each well for an additional 2 h at 37°C. The optical density was measured at 450 nm using a microplate reader and the cell survival rate was expressed as the absorbance relative to that of controls.

2.4. Assessment of Cell Apoptosis. After different drug treatments, RPMI 8226 cells were stained with Annexin V (BD Pharmingen, Franklin Lakes, USA) and propidium iodide (PI) (BD Pharmingen, Franklin Lakes, USA) according to the manufacturer's instructions. In our studies, the early apoptotic cells displayed Annexin V^+/PI^- staining and the late apoptotic cells displayed Annexin V^+/PI^+ staining.

2.5. Analysis of Mitochondrial Membrane Potential. The changes in mitochondrial membrane potential ($\Delta\Psi m$) were measured by flow cytometry using JC-1 staining according to the manufacturer's instructions. Briefly, RPMI 8226 cells were stained with 1X JC-1 working solution for 20 min at 37°C. Then cells were washed with JC-1 staining buffer and analyzed by flow cytometry.

2.6. Western Blot Analysis. Cells were lysed in lysis buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) on ice for 30 min. Proteins (30 μg) were subjected to 10% or 12% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% bovine serum albumin for 1 h and probed with primary antibodies overnight at 4°C, followed by treatment with appropriate secondary antibodies. Primary antibodies were as follows: caspase-9, cleaved caspase-8, cleaved caspase-3, phospho-p44/42 MAPK (phospho-ERK1/2), p44/42 MAPK (ERK1/2), p38 mitogen-activated protein kinase, phospho-p38 mitogen-activated protein kinase, and β-actin antibodies. All were from Cell Signaling Technology (Beverly, USA).

2.7. Cell Cycle Distribution Analysis. Cells were collected and washed with ice cold phosphate buffered saline (PBS), fixed in 75% ethanol at −20°C for 16 h, and stained at 37°C for 15 min with PI containing 50 mg/mL RNase (BD Pharmingen, Franklin Lakes, USA) followed by flow cytometric analysis.

2.8. Measurement of ROS Generation. Cells were pretreated with or without NAC for 2 h at 37°C and then incubated with various drugs for indicated times. Then the cells were washed with PBS, resuspended in RPMI-1640 medium containing 10 μM of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime, Haimen, China), and incubated at 37°C for 20 min. Fluorescence intensity was assessed using a flow cytometer (BD, San Diego, USA).

2.9. Statistical Analysis. All data were expressed as mean ± standard deviation (SD). Statistical significance of differences in multiple comparisons was determined by one-way ANOVA. $P < 0.05$ was considered significant. Combination index (CI) was calculated using median dose effect analysis in conjunction with a commercially available software program (CalcuSyn, Biosoft).

3. Results

3.1. Concomitant Treatment with Carfilzomib and LBH589 Results in a Synergistic Inhibition of MM Cells Survival In Vitro. To assess what effect the combination of carfilzomib and LBH589 would have on MM cell survival, RPMI 8226, OPM2, U266, and H929 cells were incubated with increasing concentrations of carfilzomib and/or LBH589 for 48 h, after which cytotoxicity was evaluated by CCK-8 assay. As shown in Figure 1(a), compared to individual exposure (excepting

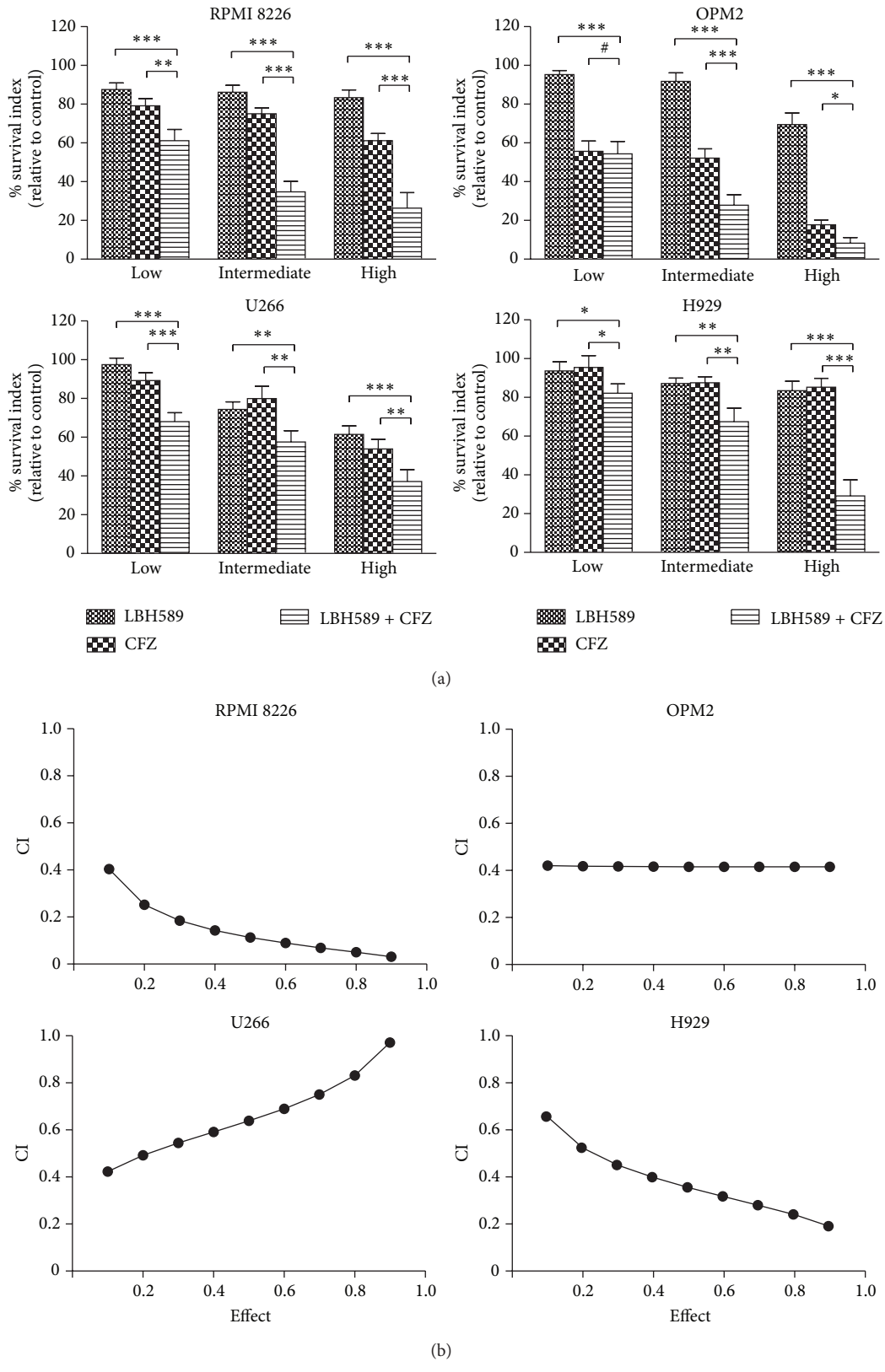

Figure 1: Coadministration of carfilzomib and LBH589 induced a synergistic inhibition of proliferation in MM cells. (a) All cell lines were incubated for 48 h with carfilzomib and/or LBH589 (low: 2 nM LBH589, 20 nM carfilzomib; intermediate: 4 nM LBH589, 40 nM carfilzomib; and high: 6 nM LBH589, 60 nM carfilzomib) followed by CCK-8 assay. Data represent the mean ± SD for three separate experiments performed in triplicate. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{\#}P > 0.05$. CFZ, carfilzomib. (b) CI values were calculated using median dose effect analysis. CI values < 1.0 denote synergistic interactions.

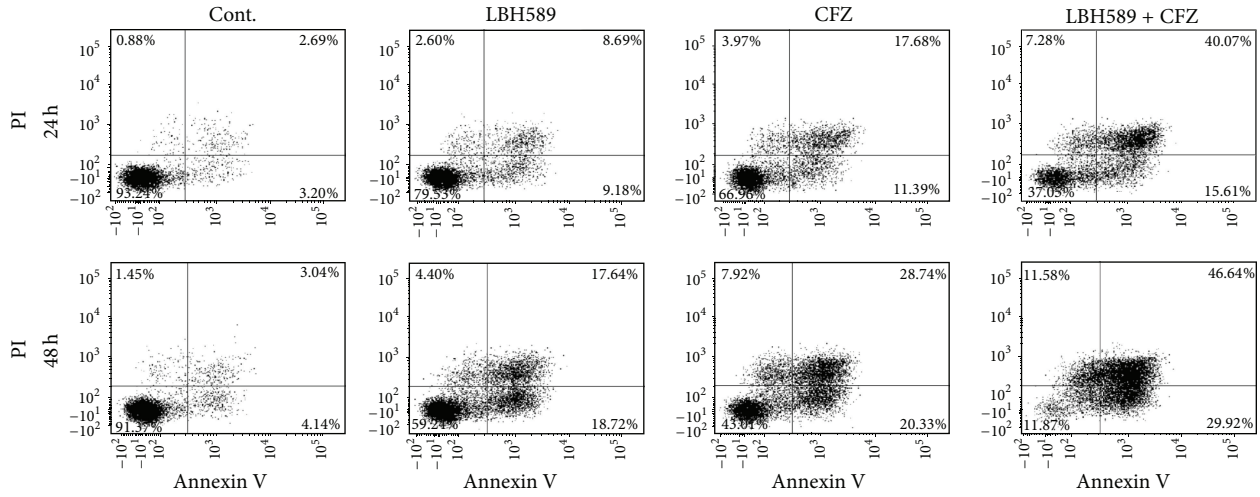

Figure 2: Combination of carfilzomib and LBH589 induced enhanced apoptosis in MM cells. RPMI 8226 cells were exposed to carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h (upper panel) or 48 h (low panel) followed by Annexin V-FITC/PI double staining and flow cytometry analysis. The percentage of apoptotic cells (24 h) in Cont., LBH589, CFZ, and LBH589 + CFZ group was 6.7% ± 0.9%, 18.8% ± 2.8%, 30.0% ± 3.5%, and 56.6% ± 8.8%*, respectively. The value (48 h) was 6.8% ± 1.5%, 35.9% ± 3.8%, 48.3% ± 3.8%, and 81.5% ± 5.0%*, respectively. *$P < 0.05$ versus control group; $n = 3$. The data shown are representative of three independent experiments. Cont., control. CFZ, carfilzomib.

individual exposure of OPM2 cells to low concentration of carfilzomib), combined exposure to low, intermediate, and high concentrations of carfilzomib and LBH589 induced a more significant decrease in the growth of all four tested MM cell lines ($P < 0.05$). The median dose effect analysis for all tested MM cells exposed to carfilzomib and LBH589 yields CI values which were substantially less than 1.0, indicating a synergistic interaction (Figure 1(b)).

3.2. Concomitant Treatment with Carfilzomib and LBH589 Effectively Induces Apoptosis, Mitochondrial Injury, and Caspase Activation in MM Cells. Annexin V/PI double staining was performed to determine the apoptosis of RPMI 8226 cells exposed to carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h or 48 h. Compared with the control, individual treatment with LBH589 only caused a moderate increase in the percentage of Annexin V^+ cells (18.8% ± 2.8% versus 6.7% ± 0.9% for 24 h and 35.9% ± 3.8% versus 6.8% ± 1.5% for 48 h). The values were relatively high in carfilzomib treated RPMI 8226 cells (30.0% ± 3.5% for 24 h and 48.3% ± 3.8% for 48 h). On the other hand, MM cells exposed to carfilzomib and LBH589 exhibited a much higher percentage of Annexin V^+ cells (56.6% ± 8.8% for 24 h and 81.5% ± 5.0% for 48 h), indicating that the combination resulted in a significant induction of apoptosis (Figure 2).

The reduction of ΔΨm is a vital event in the initiation of apoptotic cascade. The combined treatment of carfilzomib (40 nM) and LBH589 (4 nM) induced 35.0% ± 3.0% loss of ΔΨm in RPMI 8226 cells at 24 h, as represented by the cells with decreased JC-1 red fluorescence, whereas the loss of ΔΨm was only 20.3% ± 4.0% for carfilzomib (40 nM) and 16.3% ± 2.9% for LBH589 (4 nM) at 24 h. Moreover, a more significant loss of ΔΨm was observed in the combinational treatment (62.4% ± 5.0%) compared with those treated with carfilzomib (40.1% ± 4.6%) or LBH589 (20.8% ± 3.1%) alone at 48 h (Figure 3(a)). To further confirm that the combined treatment with carfilzomib and LBH589 did trigger classical apoptosis in MM cells, caspase activation, another pivotal event associated with the activation of apoptotic cell death, was examined by Western blot analysis. As shown in Figure 3(b), a clear cleavage of caspase-8, caspase-9, and caspase-3 was observed in RPMI 8226 cells after incubation with both carfilzomib (40 nM) and LBH589 (4 nM) for 24 h. In contrast, only modest cleavage of all three caspase proteins was detected in RPMI 8226 cells after 24-hour treatment of carfilzomib (40 nM) or LBH589 (4 nM) alone. These findings indicate that combined treatment of MM cells with carfilzomib and HDACi LBH589 potently induces ΔΨm loss and caspase activation, events associated with activation of the apoptotic program.

3.3. The Effects of Carfilzomib and/or LBH589 on Cell Cycle Distribution. Cell cycle analysis was performed in U266 cells exposed to carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h. Compared with the control, treatment with carfilzomib resulted in G_1-G_0 arrest accompanied by a decrease in S phase cell population ($n = 3$, $P < 0.01$). Individual treatment with LBH589 had little effect on cell cycle distribution. Similarly, neither G_1-G_0 arrest nor G_2-M arrest was observed in combined treatment ($n = 3$, $P > 0.05$) despite the synergistic induction of apoptosis caused by combined treatment (Figure 4). Similar results were observed in RPMI 8226 cells after treatment with carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h (data not shown).

3.4. Combined Exposure of MM Cells to Carfilzomib and LBH589 Induces ROS Generation. Previous studies have reported that cytotoxicity induced by bortezomib/HDACi

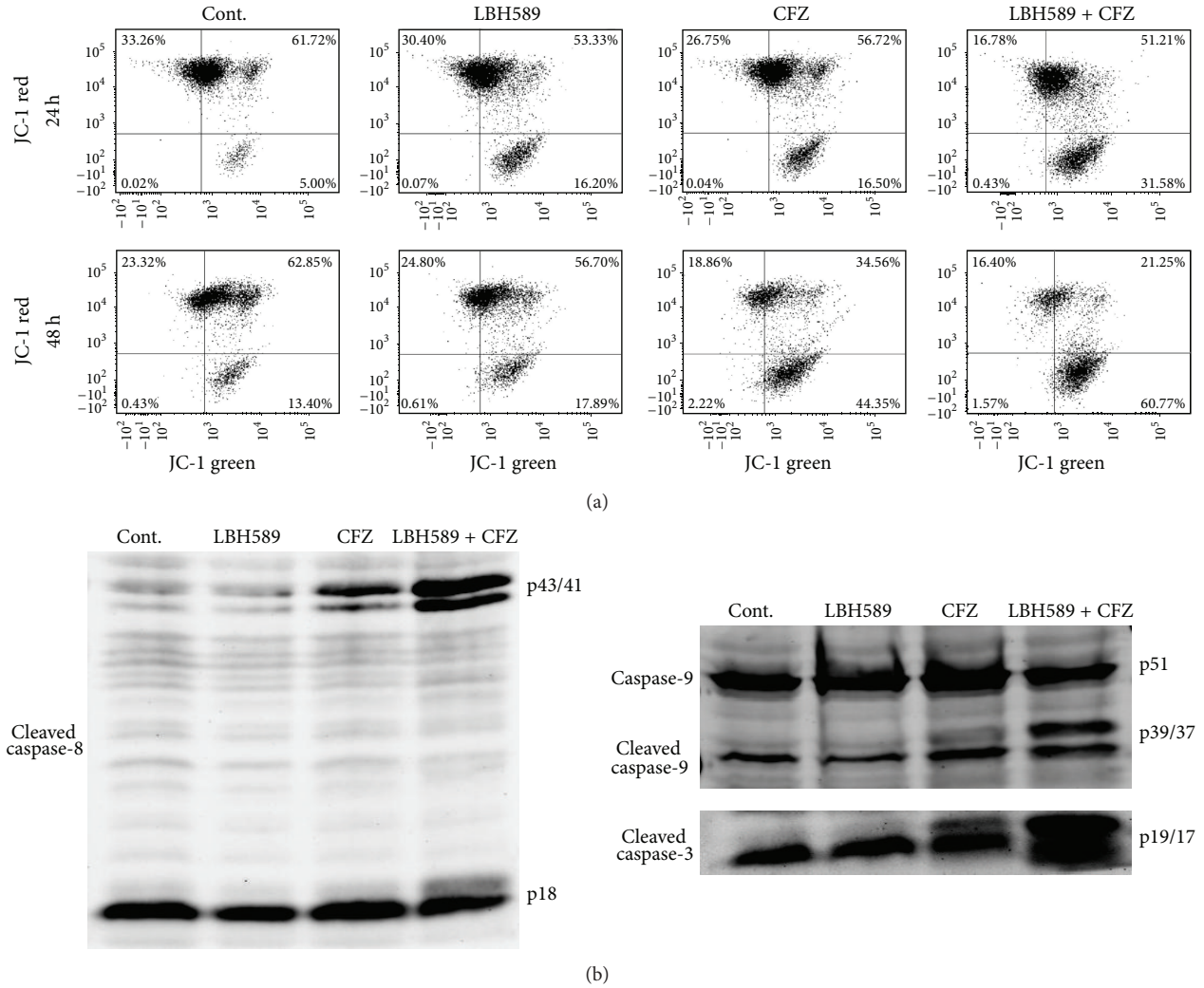

Figure 3: Concomitant treatment with carfilzomib and LBH589 synergistically resulted in mitochondrial injury and caspase activation. (a) RPMI 8226 cells were treated with carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h (upper panel) or 48 h (low panel), after which JC-1 staining was performed. $\Delta\Psi$m was assessed by flow cytometry. Only JC-1 green positive (lower right quadrant) cells were analyzed for the loss of $\Delta\Psi$m. The loss of $\Delta\Psi$m (24 h) in Cont., LBH589, CFZ, and LBH589 + CFZ group was 5.3% ± 2.1%, 16.3% ± 2.9%, 20.3% ± 4.0%, and 35.0% ± 3.0%***, respectively. The value (48 h) was 12.2% ± 3.5%, 20.8% ± 3.1%, 40.1% ± 4.6%, and 62.4% ± 5.0%***, respectively. $^{***}P < 0.001$ versus control group; $n = 3$. (b) RPMI 8226 cells were treated with carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h. Then, caspase-9 (p51) and cleaved caspase-8 (p43/41, p18), caspase-9 (p39/37), and caspase-3 (p19/17) were monitored by Western blot analysis. Cont., control. CFZ, carfilzomib.

combination originates from ROS generation [2, 14]. Studies were therefore performed to investigate whether such mechanism is also responsible for carfilzomib and LBH589-induced cytotoxicity. As shown in Figure 5(a), individual treatment with carfilzomib (40 nM) or LBH589 (4 nM) had modest effect on ROS levels in RPMI 8226 cells, whereas the combined treatment resulted in a marked increase in ROS generation, which was substantially abrogated by the free radical scavenger NAC (15 mM). In addition, 9 hours of combined treatment with carfilzomib (40 nM) and LBH589 (4 nM) induced the most obvious ROS generation in RPMI 8226 cells (Figure 5(b)). To assess the importance of ROS generation in carfilzomib and LBH589-induced apoptosis, RPMI 8226 cells were preincubated with NAC for 2 h and then treated with carfilzomib (40 nM) and LBH589 (4 nM) for 24 h. As expected, NAC significantly ($P < 0.001$) reduced the apoptosis induced by the combined treatment (Figure 5(c)), suggesting that ROS generation plays an important role in carfilzomib and LBH589-mediated cytotoxicity in MM cells.

3.5. Synergistic Induction of Apoptosis after Combined Treatment Involves the ERKI/2 Pathway. To determine the molecular mechanisms underlying the carfilzomib/LBH589 lethality in MM cells, several relevant signaling pathways were investigated. As shown in Figure 5(d), compared with the control, exposure of RPMI 8226 cells to carfilzomib (40 nM) and LBH589 (4 nM) for 24 h markedly decreased the level of

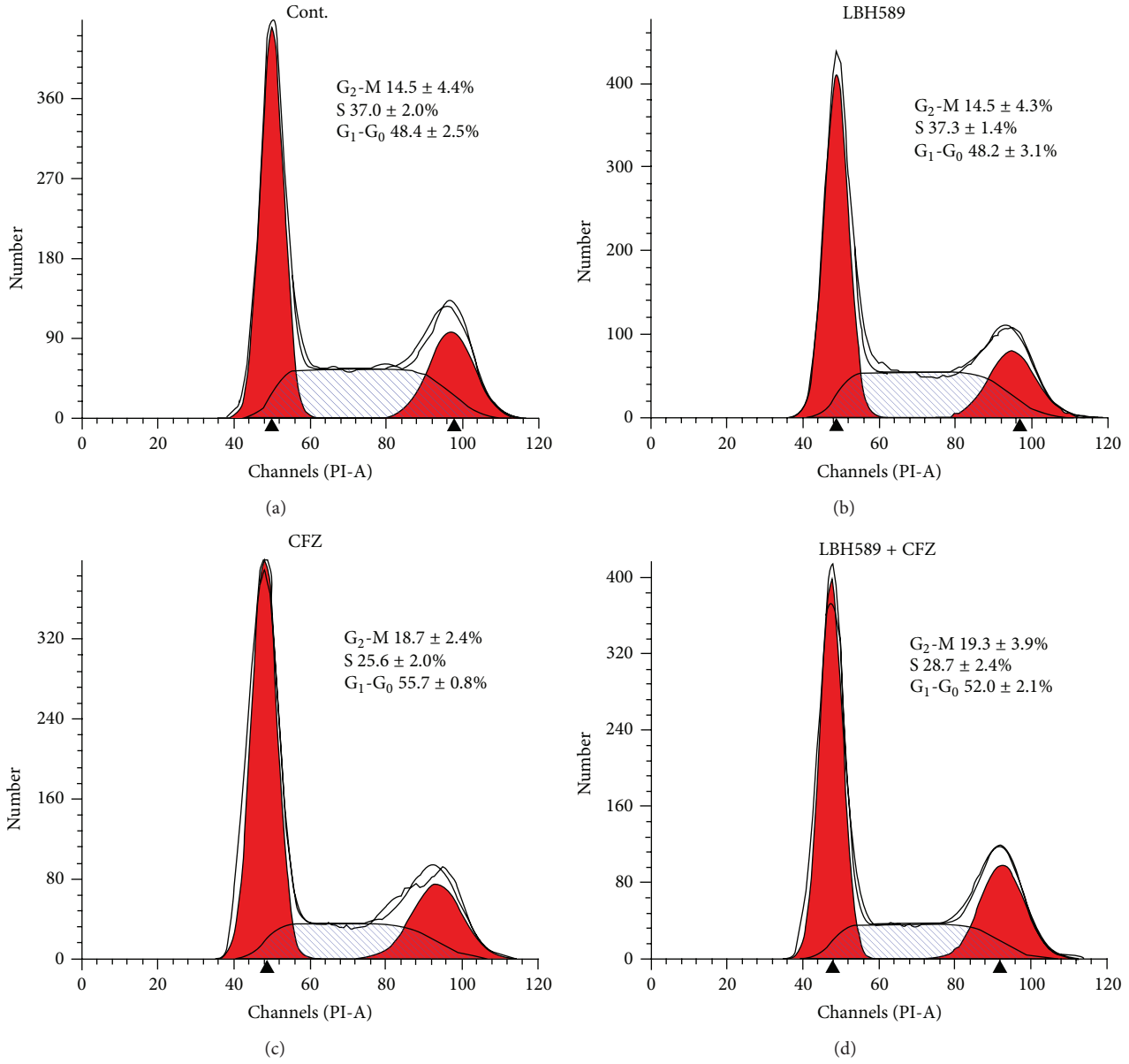

Figure 4: Concomitant treatment with carfilzomib and LBH589 showed no effect on MM cell cycle distribution. MM cells were treated with carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h followed by PI staining and flow cytometry analysis. Data represent the mean ± SD for three separate experiments performed in triplicate. Only the percentage of G_1-G_0 in CFZ group was significantly different from that in control group ($P < 0.01$). The percentage of G_1-G_0 in LBH589 and LBH589 + CFZ group and G_2-M in CFZ, LBH589, and LBH589 + CFZ group was similar to that in control group ($P > 0.05$). Cont., control. CFZ, carfilzomib.

ERK1/2 phosphorylation without obvious changes in the total ERK levels, suggesting that the ERK1/2 pathway, protecting MM cells from apoptosis, was effectively inhibited by the combined treatment. Carfilzomib (40 nM) or LBH589 (4 nM) alone had no significant effect on the phosphorylation of ERK1/2 and total ERK levels. A similar profile was observed in OPM2 cells. In the present study, p38MAPK signaling pathway, another major mechanism involved in the modulation of MM cell apoptosis, was also analyzed. As shown in Figure 5(d), the levels of total p38 and the p38 phosphorylation were much alike amongst different treatments.

4. Discussion

Combinational therapies, with agents that are synergistic when combined, are often required for patients with relapsed and/or refractory MM [15]. Among them, bortezomib/HDACi combination attracts the most attention because of powerful antimyeloma activity. However, side effects of bortezomib/HDACi combination observed in clinical trials confine its application in some MM patients [9]. In the present study, we examine the interactions between carfilzomib and HDACi LBH589 to explore a possible optimized

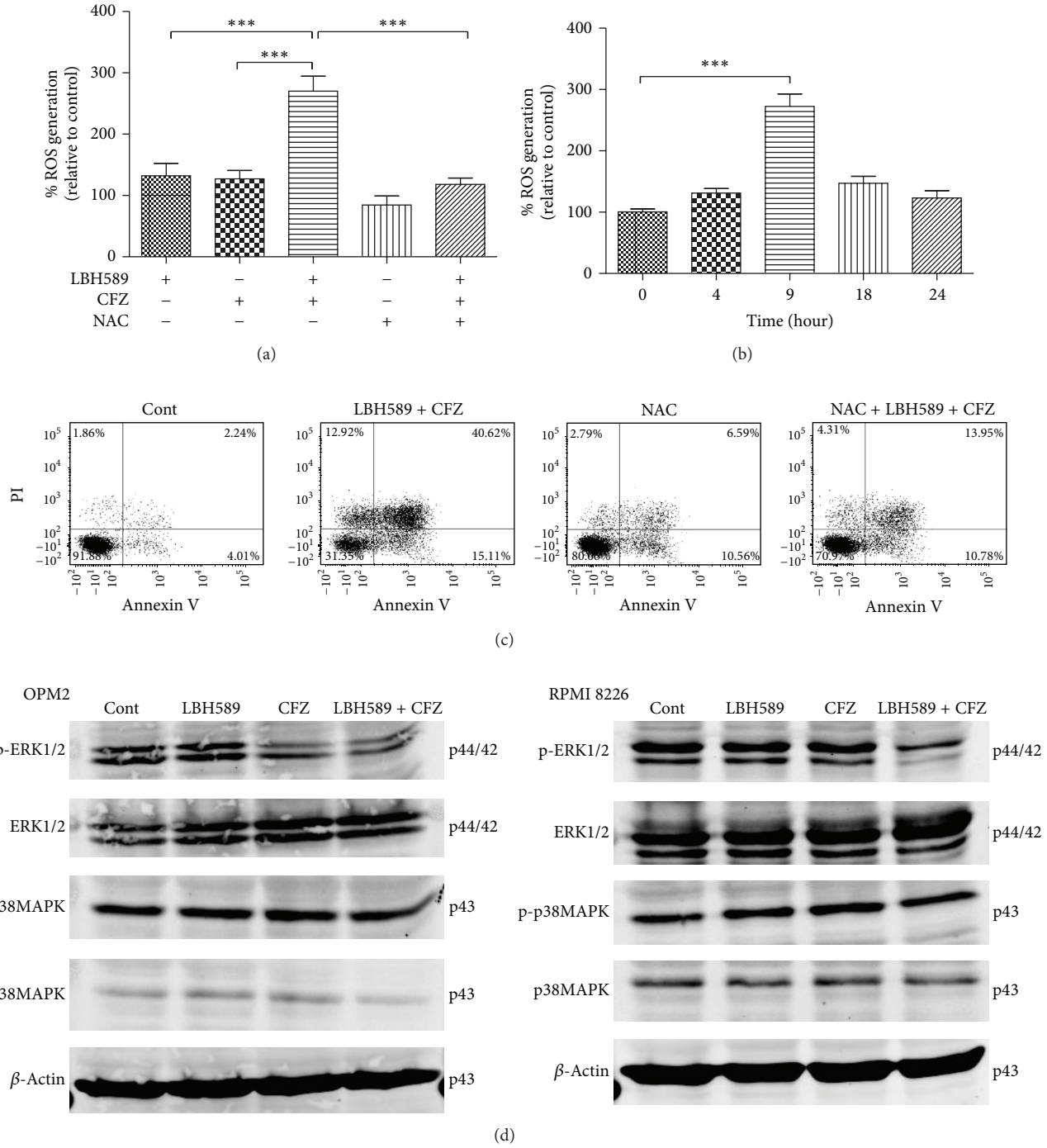

Figure 5: Lethality of the carfilzomib/LBH589 combination in MM cells was associated with the ROS generation and ERK1/2 inactivation. (a) RPMI 8226 cells were pretreated with or without NAC for 2 h at 37°C and then incubated with carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h, after which ROS generation was detected. $^{***}P < 0.001$. $n = 3$. (b) ROS generation in RPMI 8226 cells was monitored 0, 4, 9, 18, and 24 hours after combined treatment with carfilzomib (40 nM) and LBH589 (4 nM). $^{***}P < 0.001$. $n = 3$. (c) RPMI 8226 cells were pretreated with or without NAC for 2 h at 37°C and then incubated with or without carfilzomib (40 nM) and LBH589 (4 nM) for 24 h. Apoptosis rate in Cont., LBH589 + CFZ, NAC, and NAC + LBH589 + CFZ group was 6.5% ± 1.9%, 55.3% ± 9.2%, 12.4% ± 4.2%, and 29.2% ± 4.3%***, respectively. $^{***}P < 0.001$ versus LBH589 + CFZ group. $n = 3$. The data shown are representative of three independent experiments. (d) OPM2 and RPMI 8226 cells were treated with carfilzomib (40 nM) and/or LBH589 (4 nM) for 24 h. Then, Western blot analysis of ERK1/2, p-ERK1/2, p38MAPK, and p-p38MAPK was performed. The levels of β-actin were used as the loading control. Cont., control. CFZ, carfilzomib.

combinational therapy of proteasome inhibitor and HDACi for MM.

We observed a synergistic inhibition of cell proliferation and apoptosis in MM cells after combined treatment with carfilzomib and LBH589. To further confirm the apoptosis, $\Delta\Psi m$ loss and caspase cleavage, events associated with apoptosis activation, were investigated. Our data showed that the levels of cleaved caspase-9, caspase-8, and caspase-3 were markedly increased in MM cells exposed to carfilzomib and LBH589 compared with single drug treatment, suggesting that both intrinsic (caspase-9) and extrinsic (caspase-8) apoptotic pathways were activated after combined treatment. Moreover, the more loss of $\Delta\Psi m$ in both drugs treated cells further indicated the activation of intrinsic apoptotic pathway.

Since cell cycle arrest is often associated with apoptosis and several studies have shown that the proteasome inhibitor/HDACi combination, inducing obvious apoptosis, caused G_1-G_0 or G_2-M arrest [13, 14], cell cycle analysis was performed in the present study. Our data showed that carfilzomib alone induced G_1-G_0 arrest in MM cells, whereas no significant changes in cell cycle distribution were observed after individual treatment with LBH589 or combined treatment. The result was consistent with another study in which the proteasome inhibitor/HDACi combination also failed to induce G_1-G_0 or G_2-M arrest [16]. Reasons for the discrepancy in these studies are unclear, but variations in cell types and different doses of the combined drugs may be responsible for the observed difference.

Previous studies in various tumor cells have indicated that bortezomib or HDACi-induced lethality is related to ROS generation [17–20]. Moreover, lethal effects induced by the combined treatment with proteasome inhibitor and HDACi in leukemia and lymphoma cells have also been demonstrated to proceed through a ROS-dependent mechanism [21, 22]. In the present study, we showed that combined treatment with carfilzomib and LBH589 induced a marked increase in ROS in MM cells and the free radical scavenger NAC attenuated the oxidative stress, as well as the subsequent apoptosis. Thus, our data provide further support for the notion that ROS generation is a crucial factor in proteasome inhibitor/HDACi-mediated lethality.

Activation of ERK1/2 pathway has been shown to protect malignant cells from the lethality of oxidative stress [23] and therefore confers a survival advantage on these cells. Studies have indicated that apoptosis induction of several antitumor drugs, alone or in combination, is associated with inactivation of this cytoprotective pathway. For example, inhibition of ERK1/2 pathway is one of the molecular mechanisms underlying lethality of bortezomib and HDACi combination on T-leukemia/lymphoma cells [24]. In our present study, when carfilzomib was combined with LBH589, a significant decrease in the level of ERK1/2 phosphorylation was observed, whereas phosphorylation of p38 exhibited no change. This suggests that inhibition of the ERK1/2 pathway may be the right mechanism through which carfilzomib and LBH589 combination induces apoptosis.

In summary, our data indicate that carfilzomib and LBH589 combination synergistically induces apoptosis in MM cells, which is accomplished by enhancing ROS generation and decreasing ERK1/2 phosphorylation. Thus, we provide a basis for clinical evaluation of carfilzomib/LBH589 combination in relapsed/refractory MM patients.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Lu Gao, Minjie Gao, and Guang Yang contributed equally to this work.

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Review Article

Constitutive NF-κB Activation Underlines Major Mechanism of Drug Resistance in Relapsed Refractory Diffuse Large B Cell Lymphoma

Francesco Turturro

Department of Lymphoma/Myeloma, MD Anderson Cancer Center, Unit 429, 1515 Holcombe Boulevard, Houston, TX 77030, USA

Correspondence should be addressed to Francesco Turturro; fturturro@mdanderson.org

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Diffuse large B cell lymphoma (DLBCL) is the most common subtype of B cell non-Hodgkin's lymphoma (NHL), encompassing 30–40% of the estimated 70,000 cases of NHL in 2014 in the USA. Despite major improvements with immune-chemotherapy, the fraction of patients who still succumb to a refractory or relapsed disease remains high. This review addresses whether the better understanding of the biology of DLBCL defines new therapeutic avenues that may overcome the emerging resistance of this disease to traditional immune-chemotherapy, such as rituximab in combination with traditional chemotherapy agents. Emerging targeted therapy for relapsed refractory DLBCL encompasses more complex molecular abnormalities involving signaling pathways other than NF-κB as mechanism of resistance to immune-chemotherapy. Our review suggests that NF-κB pathway is an important crossroad where other pathways converge as phenotype of resistance that emerges in patients who fail frontline and salvage immune-chemotherapy. Future efforts should aim at targeting the role of NF-κB resistance in clinical trials, where novel agents like lenalidomide and proteasome inhibitors with established activity in this perspective will be an important component in combination therapy, along with new monoclonal antibody, BTK-inhibitors, and other novel therapy agents.

1. Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of B cell non-Hodgkin's lymphoma (NHL), encompassing 30–40% of the estimated 70,000 cases of NHL in 2014 in USA [1]. Although DLBCL is potentially curable, 30–40% of patients eventually relapse or are primarily refractory and fail to achieve complete remission (CR). Nearly 19,000 affected subjects are projected to die of DLBCL in 2014. As consequence of this incidence, even slight improvement in the outcomes of DLBCL patients has the potential for high impact on their survival, making the study of combination of novel drugs paramount. Historically, the combination regimen CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) established itself as standard of care in a landmark randomized clinical trial in 1993 [2]. Later the "spinoff regimen" CHOP-derivative DA-EPOCH revealed increased efficacy particularly for high grade B-cell lymphoma by modifying the infusion regimen and by adding etoposide [3]. Finally, the addition of targeted therapy with the anti-CD20 monoclonal antibody rituximab has further improved the outcomes of patients with DLBCL [4–6]. Despite major improvements with immune-chemotherapy, the fraction of patients who still succumb to a refractory or relapsed disease remains high. This review will address whether the better understanding of the biology of DLBCL may define new therapeutic avenues that overcome the resistance of this disease to traditional immune-chemotherapy.

2. Heterogeneity of DLBCL

DLBCL revealed itself as a disease more heterogeneous than the 2008 WHO classification had initially defined [7]. In fact, recent studies have established that DLBCL is associated with various genetic alterations and high biologic diversity. This has been studied [8]. The cell of origin (COO) approach based on gene expression profiling (GEP) of DLBCL has defined molecularly the disease better than the morphology.

The prognostic value of COO approach has been investigated and compared to the International Prognostic Index (IPI) in patients treated with anthracycline-based regimens and rituximab [9]. In fact based on retrospective analysis, patients with germinal center B (GCB) DLBCL type have a better prognosis of patients with activated B cell (ABC) type when treated with R-CHOP or R-DA-EPOCH [10, 11], independently of the IPI. Therefore, patients who relapse or are refractory to initial therapy are more likely to belong to the ABC type. For the same reason, ABC type is expected to represent the majority of patients who have poorly responded to salvage chemotherapy like R-DHAP or R-ICE as shown prospectively in the CORAL study [12]. A recent retrospective study has shown that 30% of patients with DLBCL treated with second line therapy are truly nonresponders [13]. In this population immune-chemotherapy-based third line therapy yields a response rate (RR) of 20% with median overall survival (OS) of only 4 months [13]. Furthermore, patients who have relapsed after autologous stem cell transplant have median OS of 9 months [14]. In this group, patients who received novel agents (lenalidomide, non-rituximab monoclonal antibodies, tyrosine kinase inhibitors, and radioimmunotherapy) had a longer median OS of 11.3 months as compared to 6.6 months for patients treated with conventional cytotoxic chemotherapy [14].

3. Relevance and Heterogeneity of the Constitutive NF-κB Activation in DLBCL

The NF-κB family of proteins is a group of transcription factors (RelA, RelB, c-Rel, NF-κB1, and NF-κB2) that are kept inactive by a group of inhibitory cytoplasmic proteins, the IκB kinase complex [15, 16]. The molecular hallmark of the ABC-type of DLBCL is the constitutive pathogenic activation of the nuclear factor-κB (NF-κB) pathway to which several mechanisms converge to ultimately promote cell proliferation and protection from apoptosis [8]. Three proteins—CARD11, BCL10, and MALT1—form a signaling complex (CBM) leading to the activation of NF-κB pathway following antigen stimulation of the B cell receptor (BCR) [17]. Furthermore, MYD88 encodes an adaptor protein that activates NF-κB and JAK2/STAT3 signaling pathways through stimulation of the toll-like and interleukin receptors [18, 19]. The driver-nature of the mutations affecting all the mentioned genes leads to gain-of-function that promotes cell survival and prevents apoptosis [18]. A recent study has established that activating mutations involving at least one of the four genes involving the NF-κB pathway (MYD88, CD79A/B, and CARD11) are present in 30–40% of 161 patients affected with DLBCL, independently from their COO phenotype [20]. These mutations are distributed with at least one mutation present in 39% of patients with the ABC, 23% with the GCB, and 23% in the indeterminate group of the COO phenotype [20]. Interestingly, patients harboring at least 1 mutation targeting one of the four genes of the NF-κB pathway "mutated" as compared to patients in the ABC-DLBCL group without mutations "unmutated" had a 3-year overall survival (OS) of 66.7% versus 26.1%, respectively ($P = 0.0337$) [20].

Although historically it has been recognized that NF-κB pathway is engaged in ABC-type DLBCL through chronic active BCR signaling, a recent work has shown that the members of the NF-κB transcription factors (NF-κB1, NF-κB2, RELA, RELB, and REL) are expressed in 88% of tissue derived from both ABC and GCB type of DLBCL [21]. In this study, the tissues from 188 patients with DLBCL were evaluated by immunohistochemistry (IHC) and validated in a subset with gene expression profiling. Furthermore, no significant differences regarding the expression of the different NF-κB family of transcription factors were detected between the two COO subtypes. This suggests that upregulation of NF-κB signaling pathway is of relevance not only in the ABC subtype, but also in the GCB counterpart [21]. Odqvist et al. made the case for NF-κB pathway to be more broadly engaged in DLBCL than initially estimated and potentially responsible for the resistance to the traditional cytotoxic immunochemotherapy. This study also established that REL expression has a significant favorable clinical impact in patient treated with R-CHOP and identified a subgroup of patients with superior outcome (5-year OS of 73.7% versus 59.7% in REL-positive and REL-negative patients, resp.; $P = 0.0041$) [21]. This finding may be relevant in defining prospectively patients with favorable versus unfavorable prognosis in response to frontline R-CHOP, based on the expression of the members of the NF-κB family of transcription factors. To further support our hypothesis that has driven the current review, more work has been recently published favoring the role of NF-κB pathway as mechanism of resistance. Gene expression profiling in tissue prospectively collected from 51 patients with DLBCL treated with immunochemotherapy has identified 31 genes whose expression changes were strongly associated with copy number aberrations or gains of chromosome 2p15 and 18q12.2 and unfavorable survival [22]. The 2p15 abnormality (amplification) that harbors the gene COMMD1 and expression of the COMMD1 protein by IHC were associated with inferior progression free survival (PFS) as compared to patients without the amplification ($P = 0.010$ and $P = 0.003$, resp.) [22]. COMMD family of proteins plays a distinct and nonredundant role in NF-κB signaling [23]. More recently, it has been shown that NR4A1 (Nur77) expression was significantly associated with poor survival in patients with aggressive large B-cell lymphoma [24]. *In vitro* overexpression of this putative tumor suppressor gene induced apoptosis in lymphoma cells [24]. Previous work had shown that NR4A1 (Nur77) blocks NF-κB activation [25]. More interesting, NF-κB signaling pathway is activated in EBV-positive DLBCL in both the elderly and nonelderly de novo DLBCL, and it seems to have an impact on the outcome of this patient population [26, 27]. Since MYD88 L265P mutation is a hallmark of lymphoma with lymphoplasmacytic features and activated NF-κB signaling pathway, the higher frequency of MYD88 mutations in the ABC phenotype suggests that this subtype may derive from cells with those features. Furthermore, the presence of serum IgM-paraprotein is more frequently associated with lymphoma with lymphoplasmacytic features. It is tempting to speculate that DLBCL patients who present with immunoblastic features and associated serum monoclonal

IgM may have a higher frequency of MYD88 mutations as a hallmark of a subtype of ABC phenotype. None of the patients with increased levels of IgM was harboring MYD88 mutation [28]. Although NF-κB signaling pathway was not studied in those patients with associated monoclonal IgM, they responded to a combination of bortezomib and lenalidomide, active agents in patients with activated NF-κB signaling pathway [28]. This study suggested that the presence of monoclonal IgM or elevated free heavy IgM chains was associated with favorable response to inhibitors of the NF-κB signaling pathway independently from the presence of MYD88 mutation [29, 30].

4. Pharmacological Attempts to Overcome the Constitutive NF-κB Activation-Mediated Resistance with Novel Agents in DLBCL

The proteasome is an intracellular, multiunit-protease complex that regulates protein degradation and remodeling. Bortezomib, first-in-class drug approved for multiple myeloma and relapsed/refractory mantle zone lymphoma (MCL), binds to the β-subunits of the core of the proteasome and inactivates NF-κB by stabilization of the NF-κB-inhibitor IκB kinase complex [31]. Despite its inhibition of the NF-κB pathway, single agent bortezomib has shown little clinical activity in patients with DLBCL [19]. The addition of bortezomib to R-CHOP in untreated patients with DLBCL resulted in overall response rate of 89% in one study and 86% in another [32, 33]. Although a study of bortezomib combined with EPOCH in patients with relapsed or refractory DLBCL showed overall modest activity, ABC type seemed to benefit dramatically more than CGB type (RR 83% versus 13%, $P < 0.01$, and median OS 10.8 versus 3.4 months, $P = 0.003$) [22]. The PYRAMID study is evaluating prospectively R-CHOP with and without bortezomib in untreated patients with DLBCL [34]. Previously, a phase I/II study of bortezomib with gemcitabine for relapsed or refractory DLBCL showed a very modest RR of 10% [35]. Ixazomib (formerly known as MLN9708) is a selective, orally bioavailable, second-generation proteasome inhibitor that has shorter proteasome dissociation half-life and improved pharmacokinetics, pharmacodynamics, and antitumor activity compared with bortezomib [36]. Furthermore, MLN9708 has a larger blood volume distribution at steady state and greater pharmacodynamics effects in tissue than bortezomib. Finally, MLN9708 showed activity in hematologic preclinical xenograft models and increased correlation between pharmacodynamics responses and improved antitumor activity [36]. To date, MLN9708 in its intravenous (IV) formulation has only been studied in a phase I dose escalation of once weekly in patients with relapsed or refractory follicular lymphoma (FL) and peripheral T-cell lymphoma (PTCL), but not in patients with DLBCL [37].

DLBCL ABC subtype presents with recurrent oncogenic mutations activating both the B-cell receptor (BCR) and MYD88 pathways for driving the NF-κB pathway and favoring cell survival [17]. MYD88 signaling pathway also induces IFNβ, detrimental to ABC-DLBCL survival. The complex IRF4/SPIB sits at the crossroad of the two pathways and promotes ABC-DLBCL survival with interaction with by IRF7, IFNβ, and trans-activation of CARD11 that results in increased NF-κB signaling activity. NF-κB factors transactivate IRF4 by a positive feedback oncogenic loop. Lenalidomide, an immunomodulatory drug (IMID), with activity in B-cell non-Hodgkin's lymphoma (NHL), targets this circuit by downregulating IRF4/SBIP, increasing toxic IFNβ secretion, and decreasing NF-κB activity [17, 38]. In fact, lenalidomide as single agent has shown RR of 35% in 49 patients with relapsed/refractory aggressive NHL [39]. RR was 33% (17 patients) and 41% (45 patients) in two other studies of lenalidomide in combination with rituximab for the treatment of relapsed/refractory DLBCL [40, 41].

The convenience of the availability of an oral agent like MLN9708, with better pharmacokinetic and pharmacodynamic profile than bortezomib, makes this second generation proteasome inhibitor a better candidate for studying it in combination with lenalidomide. The combination of two oral agents will make more convenient the use of MLN9708 and lenalidomide in a population of patient with rituximab resistance. The CORAL study has shown that patients with DLBCL exposed to rituximab as part of the initial therapy had inferior response to salvage therapy versus patients who did not receive rituximab (51% versus 83%, $P < 0.001$), supporting the hypothesis that those patients may have acquired resistance to rituximab [12]. It has been shown that NF-κB signaling pathway modulates the response to rituximab and chemosensitization of the NHL B-cell [42]. Finally, a recent article has shown that combined lenalidomide, low dose dexamethasone, and rituximab overcome the rituximab resistance in patients with indolent lymphoma and MCL [43]. In this context, an oral combination therapy targeting dysregulation of NF-κB may be more effective in a population of DLBCL refractory to salvage immune-chemotherapy with R-DHAP, RICE, or similar cisplatin-Ifosfamide-etoposide-based regimens, particularly in view of the most recent data [44]. In fact, only 44% (64/145) of patients were able to be transplanted after crossing over RICE/R-DHAP as 3rd line of therapy [44].

Certainly, the biology of relapsed/refractory DLBCL is complex and involves molecular abnormalities other than NF-κB as mechanism of resistance to immune-chemotherapy that are not covered in this review [8]. We have shown that NF-κB signaling pathway seems to be at an important crossroad where other pathways converge as "resistance-phenotype" in patients who fail frontline and salvage immune-chemotherapy. In this context, lenalidomide and new generation proteasome inhibitors may represent a new platform in combination with new monoclonal antibody, BTK inhibitors, and other novel therapy agents in future studies [8, 45].

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Extramedullary Manifestation in Multiple Myeloma Bears High Incidence of Poor Cytogenetic Aberration and Novel Agents Resistance

Xiaoyan Qu, Lijuan Chen, Hairong Qiu, Hua Lu, Hanxin Wu, Hongxia Qiu, Peng Liu, Rui Guo, and Jianyong Li

Department of Hematology, Jiangsu Province Hospital, First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Jiangsu, Nanjing 210029, China

Correspondence should be addressed to Lijuan Chen; chenljb@126.com

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Extramedullary disease (EMD) in multiple myeloma (MM) patients is an uncommon event and more attention was directed toward the feature of these patients. Cytogenetic aberration is an important characteristic of MM and is associated with patients' outcome. In this study, we aimed to compare the cytogenetic abnormality of patients with and without extramedullary manifestation, and to analyze the clinical outcomes of novel agents in EMD patients. We retrospectively investigated data from 41 MM patients. Our analyses showed del(17p13) in 31% of EMD versus 13% of medullary disease ($P = 0.03$) and amp(1q21) in 55% versus 32% ($P = 0.019$). No differences were shown in del(13q14) and t(4;14). 24/27 patients with EMD at diagnosis responded to the novel agents-containing regimens. However, when relapsed, 70% of patients did not benefit from the sequential use of novel agents as salvage therapy. In 14 patients who developed EMD at relapse phase, only 2 patients responded to novel agents therapy. Median overall survival of patients with extramedullary manifestations was 30 months, in comparison to 104 months for patients without EMD ($P = 0.002$). Patients with extramedullary manifestation bore high incidence of poor cytogenetic aberration and novel agents resistance.

1. Introduction

Multiple myeloma (MM) is a clonal B-cell malignancy characterized by the aberrant proliferation of plasma cells within the bone marrow (BM). However, the disease typically remains confined to the BM [1]. A small number of patients develop extramedullary disease (EMD) at diagnosis, at progression, or during relapse phase.

The reported incidence of EMD in newly diagnosed MM varies from 7% to 18%. Moreover, 6% to 20% of patients develop EMD later in the course of the disease [2–5]. In the past, extramedullary relapse (EMR) was uncommonly encountered in clinic and is not described that often owing to the short life expectancy of patients with MM. Due to the more sensitive imaging techniques and the prolonged patients' survival, the incidence of EMD during disease course is rising [2]. Thus, more attention was directed toward the patients with EMD.

In the case of MM patients, EMD can be present at the time of initial diagnosis or can develop at the time of relapse. Based on the published literature, EMD is a poor prognostic marker in both newly diagnosed and relapsed MM patients and, therefore, is a therapeutic challenge [6–8]. Even in the era of novel agents, EMD was still associated with poor outcome in patients. Increasing EMD cases resistant to novel targeted agents were reported [9–11].

Fluorescence in situ hybridization (FISH) and conventional cytogenetic (CC) studies serve as the cornerstone of risk stratification in MM. These methods are used to distinguish patients who will have an aggressive course and are resistant to therapies from those whose disease will be indolent and slow to relapse [12]. CC abnormalities are seen in a minority of patients with MM because of slow division of neoplastic plasma cells; however, interphase FISH assay is independent of the plasma cell division and has a higher yield to detect genetic aberrations [13]. The deletion of p53 (locus

17p13), immunoglobulin heavy chain (IgH) translocations, t(4;14) (p16.3;q32), t(11;14) (q13;q32), t(14;16) (q32;q23), and 1q21 amplification[amp(1q21)] are more commonly applied for stratification. The t(4;14) abnormality (associated with fibroblast growth factor receptor 3 expression) is detected in approximately 10% to 15% of patients. These patients have an intermediate risk status and tend to be more responsive to bortezomib based therapy [12]. The t(11;14) (q13;q32) is present in up to 20% of patients and confers a favorable prognosis [14]. The tumor suppressor gene, p53, resides at 17p13 locus, and its loss confers survival disadvantage, irrespective of whether novel agents were used [15]. The amp(1q21) is considered a high risk feature and confers bortezomib resistance [16].

Patients with EMD at diagnosis or during disease course were associated with targeted drug resistance. However, whether the adverse effect of EMD on patients was related to cytogenetic aberrations remained unclear. There are only a few studies demonstrating the cytogenetic aberrations in myeloma patients with EMD [17–19]. In this study, we aimed to compare the cytogenetic abnormality of MM patients with EMD and patients without extramedullary manifestations and to analyze the clinical outcomes of novel agents in MM patients with EMD.

2. Materials and Methods

2.1. Patients. We screened our MM database for patients treated at the First Affiliated Hospital of Nanjing Medical University between December 2007 and May 2014 who either presented with EMD at diagnosis or developed EMD at disease progression or relapse. The study has been approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from all human participants.

The EMD was defined as the presence of plasma cell tumor outside the bone marrow, either in the form of soft tissue mass or skeletal EMD, with plasma cell tumors spreading from bone disease or arising in extraosseous organs. The EMD was diagnosed using imaging methods, such as computed tomography (CT), magnetic resonance imaging (MRI), or PET/CT. Biopsies confirmation was carried out whenever possible and the proof of invasion of central nervous system (CNS) was ascertained by positive cytologic findings in cerebrospinal fluid.

Durie and Salmon criteria were used for diagnosis and staging [20]. The International Staging System was also applied to patients [13]. All of the patients received novel agents-based (thalidomide, lenalidomide, and bortezomib) therapy: TAD (thalidomide, doxorubicin, and dexamethasone), TCD (thalidomide, cyclophosphamide, and dexamethasone), TD (thalidomide and dexamethasone), PAD (bortezomib, doxorubicin, and dexamethasone), PCD (bortezomib, cyclophosphamide, and dexamethasone), VTD (bortezomib, thalidomide, and dexamethasone), VD (bortezomib and dexamethasone), MPT (melphalan, prednisone, and thalidomide), RD (lenalidomide and dexamethasone), VAD-T (vincristine, doxorubicin, dexamethasone, and thalidomide), and MOD-T (mitoxantrone, vincristine, dexamethasone, and thalidomide). In this analysis, complete response (CR), very good partial response (VGPR), partial response (PR), stable disease (SD), progressive disease (PD) status, and clinical relapse were defined according to the International Myeloma Working Group Uniform Response Criteria [21].

2.2. Interphase Fluorescence In Situ Hybridization (FISH). Interphase FISH was performed in all cases on bone marrow smears, as described previously [22]. The FISH panel included D13S319 and locus specific identifier (LSI) 13 (RB-1) probes for the detection of 13q14 deletion [del(13q14)] and a 1q21 (CKS1B) probe and 17p13.1 (P53) probe for detection of amp(1q21) and 17p13 deletion [del(17p13)], respectively. An LSI IGHC/IGHV dual-color, break-apart rearrangement probe was used to determine the translocations involving IgH; LSI IGH/CCND1, LSI IGH/FGFK3, and LSI IGH/MAF probes were further used to detect t(11;14) (q13;q32), t(4;14) (p16;q32), and t(14;16) (q32;q23) in patients with 14q32 rearrangement. Fluorescent images were captured with epifluorescence microscope (Leica DRMA2, Germany) equipped with CCD camera (AI Company, UK) and using appropriate filters. Two hundred nuclei were scored for each probe. Bone marrow cells samples of 10 cytogenetically normal individuals served as controls. The cut-off level for positive value of each probe in I-FISH was 10.0%.

2.3. Statistical Analysis. All statistical analyses were performed using SPSS 17.0 software. Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) were plotted and compared by log-rank test. The statistical significance of differences in clinical characteristics between patients was assessed using the χ^2 test. A P value < 0.05 was considered to be statistically significant.

3. Result

3.1. Patient Characteristic. Forty-one MM patients with EMD involvement were collected in this study. Two patients evolved from solitary plasmacytomas. We found evidence of EMD in 9% (27 of 300) of newly diagnosed patients (EMD-1) (Table 1). Fourteen patients developed EMD in the course of the disease (EMD-2) (Table 2): 8 patients (57%) at first relapse and 6 patients (43%) at second and higher relapse. There were 27 men and 14 women with EMD myeloma. The median age was 58 years (range 39–78) of MM patients. According to the Durie-Salmon (D-S) staging system [20], one patient was stage I, three patients were stage II, and the remaining 37 patients were stage III. According to the ISS staging system [13], 14 patients were stage I, 15 patients stage II, and the remaining 12 patients stage III. The monoclonal component was of IgG type in 21 cases, IgA type in 12 cases, IgM type in one case, and light chain type in 7 cases. The result of immunohistochemistry for extramedullary involvement was $CD38^{++}$, $CD138^{++}$, $CD20^{+/-}$, $CD56^{-}$, and ki-67 20%–50%$^{+}$.

For 14 patients who had developed EMD during disease progression or relapse, the median interval between diagnosis of MM and diagnosis of EMD myeloma was 16.5 months;

Table 1: Clinical and FISH data of MM patients with EMD at initial diagnosis.

n		27
Age (median) range in years		60 (39–78)
Sex (male/female)		16/11
Stage (Durie-Salmon)	I	1
	IIA	2
	IIB	1
	IIIA	19
	IIIB	4
Stage (ISS)	I	10
	II	10
	III	7
MM type	IgG	16
	IgA	6
	Light chain	5
FISH result	del(17p13)	6
	del(13q14)	12
	amp(1q21)	12
	t(4;14)	4
	Not available	6
Involved sites	Soft tissues	17
	Lymph nodes	3
	Bone	8
	Abdominal cavity	1
	Pelvic area	1

Table 2: Clinical and FISH data of MM patients with EMD at relapse stage.

n		14
Age (median) range in years		58 (39–78)
Sex (male/female)		11/3
Stage (Durie-Salmon)	IIIA	11
	IIIB	3
Stage (ISS)	I	4
	II	5
	III	5
MM type	IgG	5
	IgA	6
	IgM	1
	Light chain	2
FISH result	del(17p13)	3
	del(13q14)	4
	amp(1q21)	4
	Not available	6
Involved sites	Soft tissues	8
	Central nervous system	4
	Skin	2
	Liver	2
Median time to EMR (month)		16.5 (3–70)

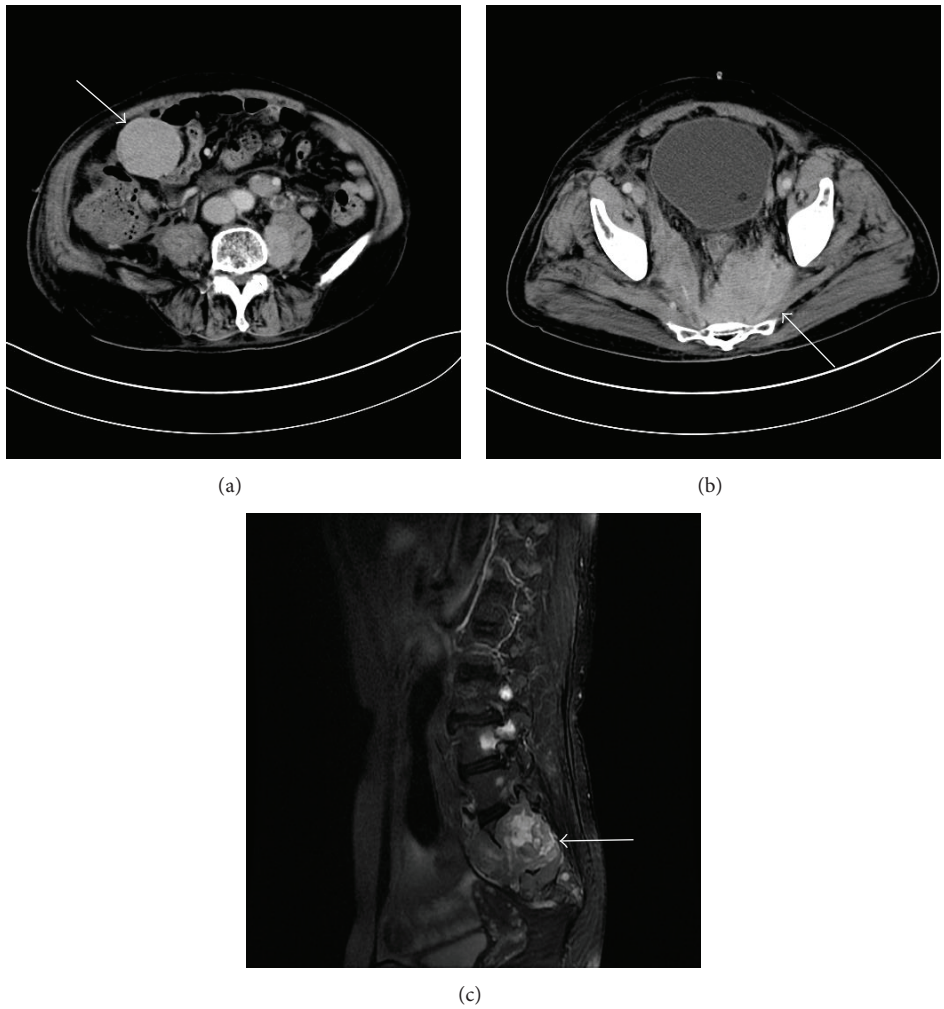
the longest interval was 70 months. The main sites involved in patients with EMD were the soft tissues (25/41 patients, 61%). Other sites included the lymph nodes (3 cases), liver (2 cases), CNS (4 case), skin (2 case), pelvic area (1 case), bone (8 case), and abdominal cavity (1 case). Four patients with EMD indicated involvement at more than one site. The representative CT/MRI scans of two patients with EMD are depicted in Figure 1.

3.2. FISH Results. Among the 41 bone marrow aspirates, 29 were assessable for cytogenetics analysis (lack of plasma cells or FISH failure in 12 samples). In EMD-1 group, baseline cytogenetics were available in 78% of the patients (21/27): del(17p13) in 29% (6/21), del(13q14) in 57% (12/21), amp(1q21) in 57% (12/21), and t(4;14) in 19% (4/21). In EMD-2 group, molecular cytogenetics from initial MM diagnosis were available in 8 patients: del(17p13) in 3 patients, del(13q14) in 4 patients, and amp(1q21) in 4 patients. No patients harbored t(4;14) or t(11;14). In both groups, no patient had t(14;16).

We also analyzed the incidence of cytogenetic aberration in patients without EMD treated in our hospital between December 2007 and May 2014. Molecular cytogenetics were available in 134 patients: del(17p13) in 13% (17/134), del(13q14) in 45% (60/134), amp(1q21) in 32% (43/134), t(11;14) in 21% (28/134), t(4;14) in 12% (16/134), and t(14;16) in 4% (5/134). No differences were shown in the incidence of del (13q14) and t(4;14) between EMD myeloma patients and medullary myeloma patients. However, the incidences of del(17p13) and amp(1q21), when compared with medullary myeloma, are significantly higher ($P = 0.03$ and 0.019, resp.). We did not detect t(11;14) or t(14;16) in patients with EMD.

3.3. Prognosis and Response to Therapy. In EMD-1 group, the regimens used for the initial treatment were TAD (9 patients), TCD (1 patient), TD (2 patients), PAD (3 patients), PCD (5 patients), VTD (3 patients), VD (2 patients), MPT (1 patient), and RD (1 patient). In this group, only one patient received autologous stem cell transplantation. All of these patients received a novel agents-included therapy. After induction therapy, 24/27 (89%) patients responded to the novel agents-containing regimens (CR, VGPR, or PR), and the complete response was 19%. As of July 1, 2014, 10 patients had relapsed. Moreover, all of these patients presented extramedullary relapse with coexisting bone marrow relapse. New agents-based therapy has been used in the relapse setting. However, only 2 patients responded to bortezomib-based therapy and 1 patient responded to lenalidomide and dexamethasone. After a median followup of 14 months (range 5–46 months) from diagnosis, the median duration of PFS of patients was 20 months (Figure 2) and the median OS was 40 months (Figure 3).

In EMD-2 group, the regimens used for the initial treatment were VTD (4 patients), TAD (1 patient), VAD-T (4 patients), TD (1 patient), VD (1 patient), MPT (1 patient), MOD-T (1 patient), and PCD (1 patient). The median interval between diagnosis of MM and EMR was 16.5 months (range 3–70 months). After a median followup of 24 months (range 3–77 months) from diagnosis, the median PFS was 14 months (Figure 2). After extramedullary relapse, bortezomib-based



(a) (b)

(c)

Figure 1: CT/MRI scan images. (a) Enhanced abdominal CT demonstrated soft tissue mass in the right middle abdomen (arrow). (b) Enhanced abdominal CT demonstrated soft tissue mass in the pelvic area (arrow). (c) Fat-suppressed T2-weighted lumbar spine image showed mass (arrow) and the vertebral body and appendix are involved.

regimens were performed on 7 patients, thalidomide-based therapy had been given to 2 patients, and lenalidomide-based regimens were used in 3 patients. One patient received radiotherapy only. One patient refused further therapy. Only two patients who received lenalidomide and dexamethasone achieved further response. However, the duration of response of both patients was short. The OS after EMR of responding patients was only 12 and 17 months, respectively. These patients with EMR bore potential therapeutic difficulties and novel agents resistance. The median OS from diagnosis and from EMR was only 27 months (Figure 3) and 5 months (Figure 4), respectively. However, no differences were shown in the PFS ($P = 0.114$) or OS ($P = 0.076$) between patients with EMD at diagnosis and patients experiencing EMD at relapse phase. We also compared the OS of patients with EMD and patients without EMD. In 134 patients without EMD assessable for cytogenetics, 11 patients were lost to followup. The median OS of 123 patients without EMD was 104 months, in comparison to 30 months for patients with EMD involvement ($P = 0.002$) (Figure 5).

4. Discussion

In this retrospective study, we describe 41 patients with EMD myeloma encountered over the past 7 years at our hospital from 300 patients with MM. The incidence was 14%, which is similar to previous reports [2–5]. Due to the more sensitive imaging techniques and the prolonged patients' survival, the incidence of EMD during disease course is rising [2]. EMD MM, especially EMR, appears to be an uncommon but important phenomenon and needs more emphasis to be put on.

EMD MM appears to have a specific clinical manifestation. The analysis of the presenting features of EMD MM shows they are significantly distinct from the rest of the MM population concerning age, sex, MM subtype, disease stage, and prior history of MGUS [2]. In addition, the disease course is presented differently from patients without EMD. Varettoni et al. [2], using a time-dependent analysis, demonstrated that presence of extramedullary involvement at any time in the course of disease was associated with significantly shorter PFS

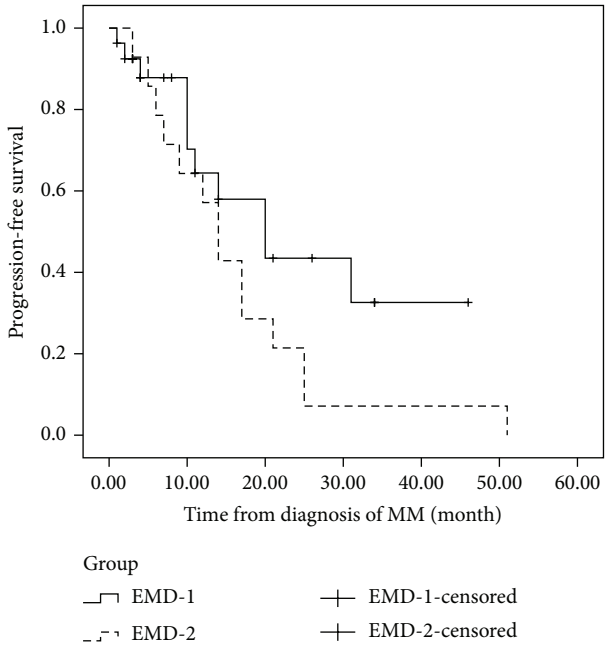


Figure 2: The progression-free survival (PFS) of patients with extramedullary disease at diagnosis and at relapse. The median duration of PFS of patients in EMD-1 and EMD-2 was 20 months and 14 months, respectively ($P = 0.114$).

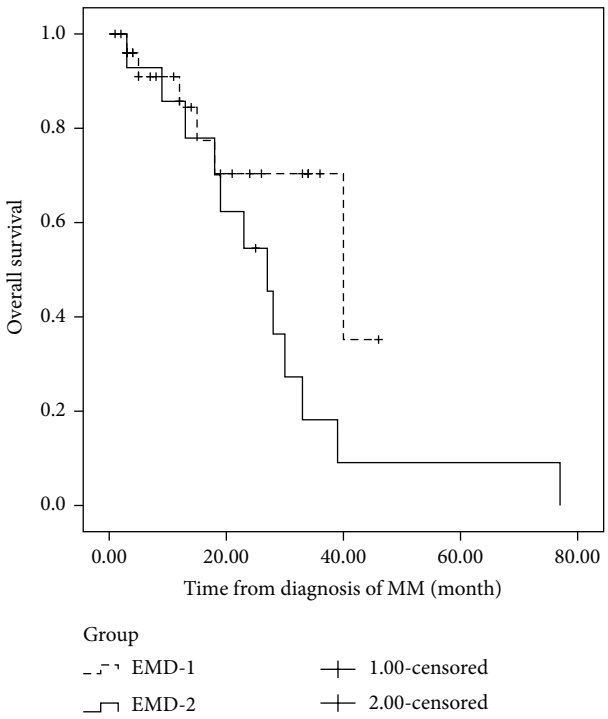


Figure 3: The overall survival (OS) of patients with extramedullary disease at diagnosis and at relapse phase. The OS of patients with extramedullary involvement at initial diagnosis and patients experiencing extramedullary disease at relapse phase was 40 months and 27 months, respectively. No difference was shown in the OS between the two groups ($P = 0.076$).

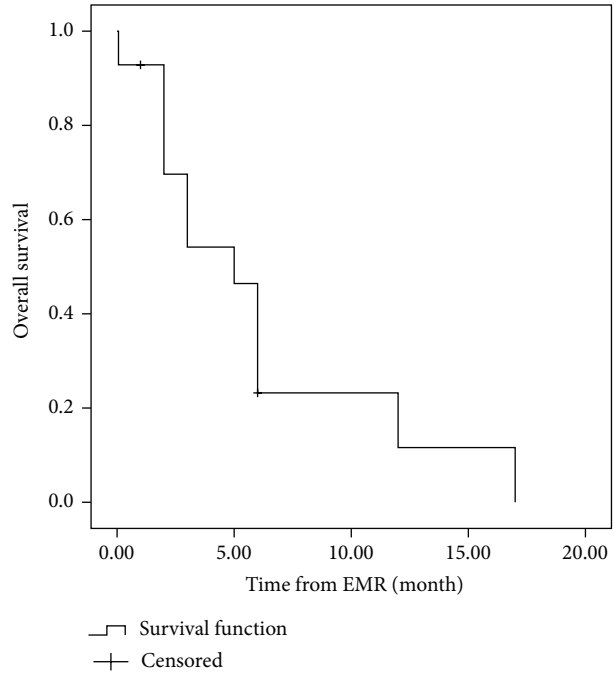


Figure 4: The overall survival (OS) of patients after extramedullary relapse. Median OS of patients in EMD-2 from extramedullary relapse was only 5 months.

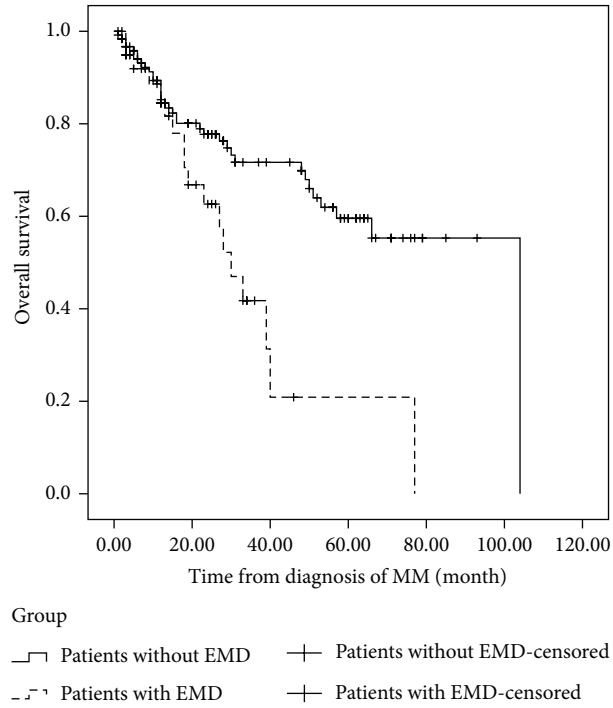


Figure 5: The overall survival (OS) of patients with extramedullary disease (EMD) and patients without extramedullary involvement. In 134 patients without EMD assessable for cytogenetics, 11 patients were lost to followup. The median OS of 123 patients without EMD was 104 months, in comparison to 30 months for patients with EMD involvement ($P = 0.002$).

and OS. Patients presenting EM involvement at diagnosis had significantly shorter PFS as compared with the rest of MM population (18 versus 30 months). A retrospective single-center study of 24 cases demonstrated the median PFS was 2 months and the median OS was 7 months after diagnosis of EMR [7]. Fassas et al. [23] reported the median OS from the time of diagnosis of CNS involvement was only 1.5–2 months.

Even in the era of novel agents, EMD was associated with poor prognosis and drug resistance [6]. Rosiñol et al. [10] reported that none of 11 patients with EMD responded to single-agent thalidomide, as compared with 16 responders among 27 patients without extramedullary involvement. Although 4 of the 11 patients with extramedullary involvement had a serological response, a progression of the soft tissue masses was observed in all of them. Another study also showed, in patients with extramedullary involvement, the use of thalidomide did not improve outcome [3]. With regard to bortezomib efficacy in EMD MM, several case reports showed that patients with EMD responded to bortezomib-based regimens [24, 25]. However, the number of patients studied at present has been small. Bortezomib has extensive tissue penetration; however, data from studies conducted in nonhuman primates have demonstrated that bortezomib cannot penetrate into the CNS or into various regions of the eye [26]. In this study, all of the 27 patients having EMD involvement at diagnosis received novel agents-containing therapy. Three patients receiving thalidomide-containing regimens responded poorly to the induction therapy. Ten patients in EMD-1 group had experienced relapse with EMD. However, only 3 patients benefited from the sequential use of novel agents as salvage therapy. Two patients responded to bortezomib-based therapy and 1 patient responded to lenalidomide and dexamethasone. We treated all of the patients who developed EMD at relapse phase with novel agents-containing therapy. However, only 2 patients obtained short response after lenalidomide-containing therapy. The remaining patients had novel drugs resistance and did not achieve further response.

Hitherto there is no consensus about the best therapeutic choice for EMD patients. In this study, 89% (24/27) patients having EMD involvement at initial diagnosis responded to novel agents-based therapy. When patients developed EMD involvement at relapse phase, 75% (9/12) of patients presented novel agents resistance. Data on the prognostic factors which impact the response of EM involvement in MM are limited. Cytogenetic abnormalities are considered useful factors for prognostication of patients with MM. A series of studies have indicated that patients with t(4;14) may benefit from use of bortezomib, either as induction therapy or long-term treatment [15, 27, 28]. In this study, we did not find differences of t(4;14) between EMD patients and patients without EM involvement. Translocation t(11;14) (q13;q32), which is found in about 15% of patients, appears to be associated with a favorable outcome and therefore is considered neutral with regard to prognosis [29, 30]. However, there was no patient harboring t(11;14) in our study. Due to the low incidence of translocation t(14;16), large series of cases are further needed to confirm the incidence of this abnormality in patients with EMD. Patients with del(17p13) were defined as having high-risk disease; no specific treatment has so far demonstrated a beneficial effect [15]. Two studies demonstrated the incidence of del(17p13) in EMD patients was significantly higher than that in the patients without EMD reported by the published literature data [7, 17]. In this study we compared the incidence of del(17p13) between patients with EMD and patients without EMD. The result showed that the incidence was higher. Also, chromosome 1 amplification was considered an indicator of poor outcome even in the use of novel regimens [31]. To the best of our knowledge, there was no report that studied the chromosome 1 aberration in patients developing EMD. We detected that the incidences of amp(1q21) were also higher in EMD patients when compared to medullary disease. Thus, we think this incidence difference of poor cytogenetic aberration may be one of the causes of novel agents resistance.

5. Conclusions

MM with EM involvement, especially EMR, appears to be an uncommon but important phenomenon. EMD MM appears to have a specific clinical manifestation. In this study, we have demonstrated 41 cases of MM patients presenting extramedullary manifestation. These MM patients confer higher incidence of del(17p13) and amp(1q21) and potential therapeutic difficulties. Patients with extramedullary relapse pattern were resistant to novel targeted agents and were associated with poor prognosis. Further studies are needed to explore the optimal therapeutic strategies to deal with the phenomena.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Inhibition of Nek2 by Small Molecules Affects Proteasome Activity

Lingyao Meng,[1] Kent Carpenter,[2] Alexis Mollard,[1] Hariprasad Vankayalapati,[1] Steven L. Warner,[2] Sunil Sharma,[1,3] Guido Tricot,[4] Fenghuang Zhan,[4] and David J. Bearss[2]

[1] *Center for Investigational Therapeutics, Huntsman Cancer Institute, Salt Lake City, UT 84112, USA*
[2] *Tolero Pharmaceuticals Inc., 2975 Executive Parkway, Suite 320, Lehi, UT 84043, USA*
[3] *Division of Medical Oncology, University of Utah, Salt Lake City, UT 84312, USA*
[4] *Division of Hematology, Oncology, and Blood and Marrow Transplantation, Department of Internal Medicine, University of Iowa, Iowa City, IA 52242, USA*

Correspondence should be addressed to David J. Bearss; dbearss@toleropharma.com

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Background. Nek2 is a serine/threonine kinase localized to the centrosome. It promotes cell cycle progression from G2 to M by inducing centrosome separation. Recent studies have shown that high Nek2 expression is correlated with drug resistance in multiple myeloma patients. *Materials and Methods*. To investigate the role of Nek2 in bortezomib resistance, we ectopically overexpressed Nek2 in several cancer cell lines, including multiple myeloma lines. Small-molecule inhibitors of Nek2 were discovered using an in-house library of compounds. We tested the inhibitors on proteasome and cell cycle activity in several cell lines. *Results*. Proteasome activity was elevated in Nek2-overexpressing cell lines. The Nek2 inhibitors inhibited proteasome activity in these cancer cell lines. Treatment with these inhibitors resulted in inhibition of proteasome-mediated degradation of several cell cycle regulators in HeLa cells, leaving them arrested in G2/M. Combining these Nek2 inhibitors with bortezomib increased the efficacy of bortezomib in decreasing proteasome activity *in vitro*. Treatment with these novel Nek2 inhibitors successfully mitigated drug resistance in bortezomib-resistant multiple myeloma. *Conclusion*. Nek2 plays a central role in proteasome-mediated cell cycle regulation and in conferring resistance to bortezomib in cancer cells. Taken together, our results introduce Nek2 as a therapeutic target in bortezomib-resistant multiple myeloma.

1. Introduction

Nek2 is a serine/threonine protein kinase, belonging to the Nek family of cell cycle regulators [1]. The first member of this family, NIMA, was originally identified as a mutant preventing *A. nidulans* cells from entering mitosis. Thus, "NIM" stands for "never in mitosis" [2]. The Nek family has 11 members (Nek1-11), and Nek2 is the one with the highest sequence identity compared to NIMA [1]. Modern biochemical and proteomic data has shown that Nek2 is a core component of the human centrosome, and similar findings have also been reported for homologues of Nek2 in *Drosophila*, *Xenopus*, and mouse [3–6]. There is substantial evidence that Nek2 plays a key role in centrosome separation and promotion of the cell cycle from G2 to M phase [7–10]. Because the ubiquitin-proteasome system has been previously targeted with the proteasome inhibitor bortezomib in breast cancer [11–13], a few groups began to study and have subsequently reported that Nek2 regulates cell cycle progression in breast cancer cell lines [14–16].

While the effectiveness of proteasome inhibition in breast cancer continues to be evaluated, bortezomib continues to be a mainstay treatment for relapsed refractory MM [17, 18]. In spite of bortezomib's usefulness in improving overall survival in some patients, as many as one-third of relapsed MM patients do not respond to bortezomib and those that do respond often develop resistance [18–20]. For this reason, we sought to identify those genes related to myeloma drug

resistance and disease relapse in a previous report. Among the genes studied, we found that Nek2 most accurately predicted poor prognosis, cell proliferation, and drug resistance in *ex vivo* and *in vitro* models of multiple myeloma [21].

Although several groups have tried to validate Nek2 as a therapeutic target using both small molecules and siRNA, few of them actually achieved efficient inhibition of Nek2 by small molecules [16, 22–25]. In this study, we identify a series of potent and selective inhibitors of Nek2, derived from a kinase-focused library screening approach. This approach provided us with selective, orally available small molecule inhibitors of Nek2, including HCI-2184, HCI-2388, and HCI-2389. All three of the compounds are related and have a pyrimidine scaffold as their core pharmacophore. These compounds inhibited proteasome activity *in vitro* and mitigated bortezomib resistance induced by Nek2 overexpression. Taken together, the data suggest that Nek2 plays an important role in the uncontrolled proliferation of MM cells and introduces Nek2 as a therapeutic target in relapsed refractory MM cells resistant to bortezomib.

2. Materials and Methods

2.1. Generation of Stable Nek2 Overexpressing (OE) Cell Lines. The Nek2 coding sequence was purchased and subcloned from a pCMV6-Entry vector (OriGene). Restriction enzymes AsiSI and XhoI were used to ligate the *NEK2* gene into the pCMV6-GFP vector (OriGene). The correct sequence of pCMV6-NEK2-GFP was verified by sequencing. Plasmid was generated in Top 10 cells (Invitrogen) and the plasmid was purified using the Small Scale Plasmid DNA Purification Kit (QIAGEN). Purified pCMV6-NEK2-GFP was used to transfect HeLa cells in 6-well plates, using Lipofectamine 2000 (Invitrogen). We chose to transfect HeLa cells with the pCMV6-NEK2-GFP plasmid because a previous report indicated the successful transfection of plasmids into NT2/D1 and HeLa cells using Lipofectamine 2000 without visible toxicity [26]. The final concentration of plasmid was 0.4 μg/mL and the cell density was 8×10^5 cells per well. G418 (Invitrogen) was added to DMEM medium with final concentration of 1 mg/mL. HeLa cells were cultured in G418 containing medium for one month. Clones were then isolated and continuously cultured as stable Nek2 OE HeLa cells. The same process was conducted using the pCMV6-GFP vector to establish stable GFP OE HeLa cells.

Other Nek2 OE multiple myeloma cell lines, including ARP1, H929, and KMS28PE cells, were developed as described in our previous report [21]. As with the HeLa cells, the final concentration of plasmid was 0.4 μg/mL and the cell density was 8×10^5 cells per well. Three additional clones of the ARP-1 cell line, Nek2-OE, Nek2-knockdown (KD), and bortezomib-resistant lines were generated as described in our previous report [21].

2.2. Western Blot Analysis. Following the indicated treatments, cells were washed by cold 1 X PBS buffer and were lysed using NP-40 cell lysis buffer (Life Technology). Whole-cell lysates were prepared and subjected to Western blot analysis as described in our previous report [21]. Briefly, after incubation with primary antibodies (Cell Signaling), the blots were probed with HRP-secondary antibodies (abcam). The blots were then detected with an ECL Detection Kit (Amersham).

2.3. Proteasome Isolation and In Vitro Proteasome Activity Assays. The 26S proteasome was isolated from whole-cell lysates by ultracentrifugation as previously described [27]. Proteasome activity was tested either in 96-well plates or 384-well plates using the Proteasome-Glo Trypsin-Like Assay (Promega). The assay was performed according to the vendor's protocol, and the proteasome concentration was optimized to 0.25 μg/mL.

2.4. In Vitro Nek2 Inhibition Assays. Compounds were incubated with human Nek2 kinase (Invitrogen) and then kinase activity was examined by the Kinase-Glo Luminescence Kinase Assay (Promega). The assay was performed according to the manufacturer's protocol in 384-well plates' format using 60 mM Nek2. Twelve different concentrations were set for each compound: 100 μM, 30 μM, 10 μM, 3 μM, 1 μM, 300 nM, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, and 0.3 nM.

2.5. Cell Viability Assays. Cell viability was determined using the ATPlite 1Step Kit (PerkinElmer) in 96-well plates. The assay was performed according to the vendor's protocol. Cell viability was assessed by measuring live cell ATP activity.

2.6. Cell Cycle Analysis. Cell cycle analysis was performed as described [28]. HeLa cells were harvested and resuspended in Krishan's Buffer (0.1% sodium citrate, 50 μg/mL propidium iodide, 20 μg/mL RNase A, and 0.5% NP-40). Flow cytometry was conducted on a FACScan cytometer (Becton, Dickinson and Company). Collected data was analyzed by FlowJo 6.0b software (Tree Star, Inc.).

2.7. Statistical Analyses. Data was tested for statistical significance by unpaired t-tests using the Graph-Pad InStat Software. Data was considered statically significant when $P < 0.05$.

3. Results

3.1. Nek2 Overexpression Induced Bortezomib Resistance in HeLa Cells. We previously reported that bortezomib resistance is accompanied with Nek2 upregulation in MM patients [21]. To confirm this correlation, we used the constructed Nek2-GFP plasmid to transfect HeLa cells, and Nek2 overexpression was first confirmed by Western blot (Figure 1(a)). The lower band in the blots corresponds to endogenous Nek2 whereas the larger band corresponds to the Nek2-GFP plasmid. Increased phosphorylation of PP1-α, a known substrate of Nek2 [29], was also verified by Western blot in Nek2-OE cells (Figure 1(b)).

The two most viable HeLa Nek2-OE clones and HeLa GFP-OE clones were selected for the following experiments. Bortezomib was used to treat these HeLa cells in a 96-well

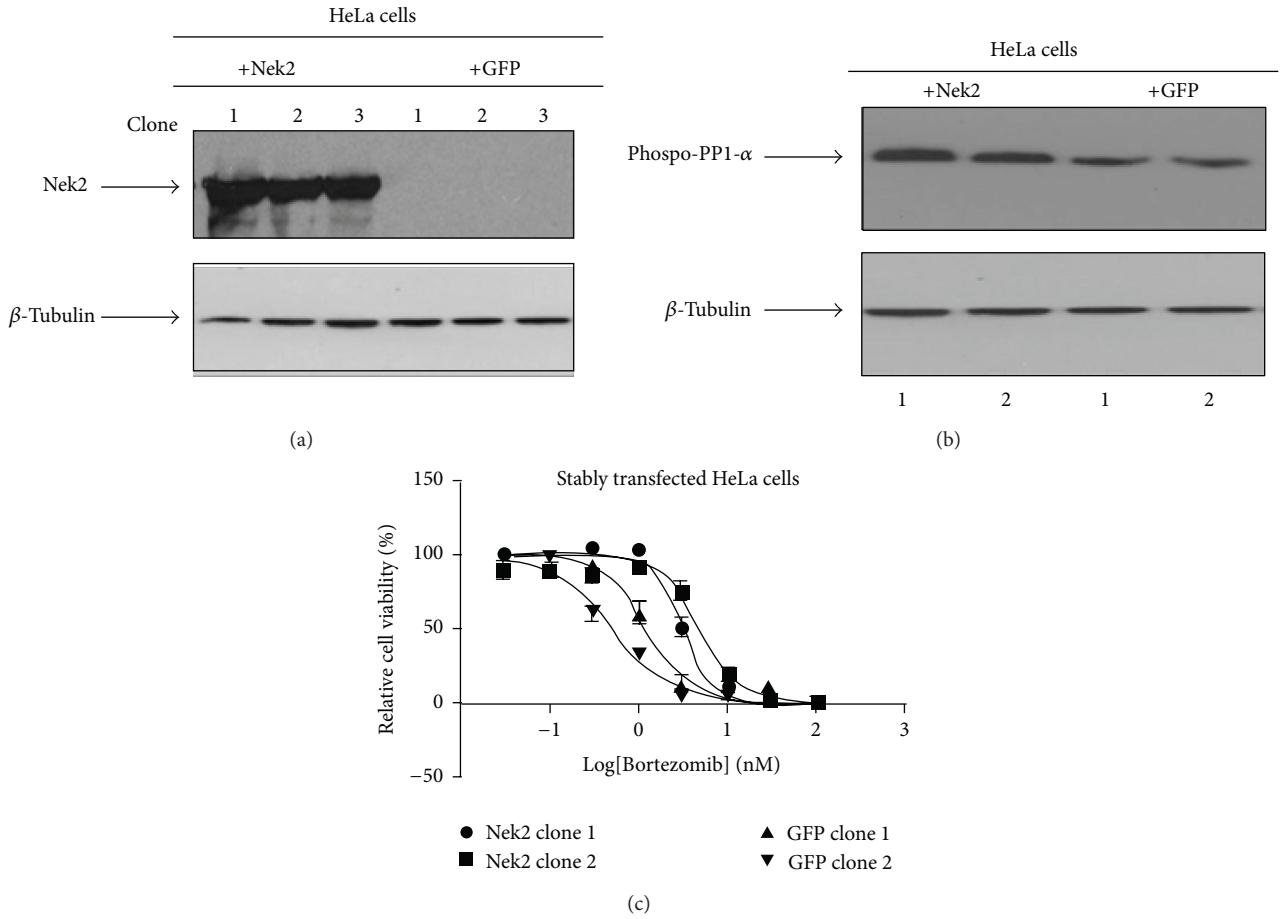


Figure 1: Nek2 overexpression causes HeLa cells to become resistant to bortezomib. (a) The *Nek2* gene was cloned into a GFP expression vector as described in Materials and Methods Section. HeLa cells were then transfected with either the Nek2-GFP plasmid or GFP expression vector alone. Anti-NEK2 antibody was used to confirm NEK2 overexpression as determined by Western blot. (b) Nek2 overexpression increased the level of phosphorylated PP1-α in the two surviving Nek2 transfected clones. (c) Nek2-GFP transfected HeLa cells were resistant to bortezomib treatment compared to GFP-transfected clones. Bortezomib was used to treat HeLa cells with the concentration range from 100 nM to 0.03 nM. Within this range, at any given concentration of bortezomib, Nek2-transfected clones yielded higher cell viability than GFP-transfected clones.

plate under different concentrations (100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 0.3 nM, 0.1 nM, and 0.03 nM) with 0.1% DMSO as control. After 72 hours, cell viability was examined by the ATP lite assay. At every concentration of bortezomib, Nek2-OE clones yielded higher cell viability than GFP clones (Figure 1(c)). These data suggest that bortezomib resistance was induced by Nek2 overexpression in HeLa cells, which is consistent with our previously reported data [21].

3.2. Proteasome Activity Was Significantly Increased by Nek2 Overexpression. Because bortezomib is able to target cancer cells by proteasome inhibition [30], we hypothesized that Nek2 overexpression would increase proteasome activity in transfected cells and subsequently confer bortezomib resistance. To test this hypothesis, the 26S proteasome was isolated by ultracentrifugation from the stable Nek2-OE cells. Three different human MM cell lines, including ARP1, H929, and KMS28PE, were tested. Among them, we tested four verified clones of the ARP-1 cell line, including wild-type, Nek2-OE, Nek2-knockdown (KD), and bortezomib-resistant clones. These cell lines were generated and verified as described in our previous report [21].

In vitro proteasome activity from the isolated proteasome was tested by the Proteasome-Glo Trypsin-Like Assay. For all the studied cell lines, the proteasome activity of the Nek2-OE cells was significantly higher than the control (GFP-treated for HeLa cells and empty vector treated cells for H929, KMS28PE, and ARP-1 cells). Bortezomib resistant ARP-1 cells exhibited the highest proteasome activity (Figures 2(a)–2(d)). These results support our hypothesis and imply that Nek2 overexpression is one of the mechanisms behind increased proteasome activity in bortezomib-resistant MM cell lines.

3.3. Nek2 Inhibitors Reduce the In Vitro Proteasome Activity in Nek2-Expressing Cell Lines. A focused screening library of ~ 2000 compounds was assembled from an in-house collection of previously synthesized kinase inhibitors utilizing a single concentration screening approach in a Nek2 biochemical kinase assay. This yielded four compounds with Nek2 kinase inhibition greater than 80% at 10 μM. These 4 hits were

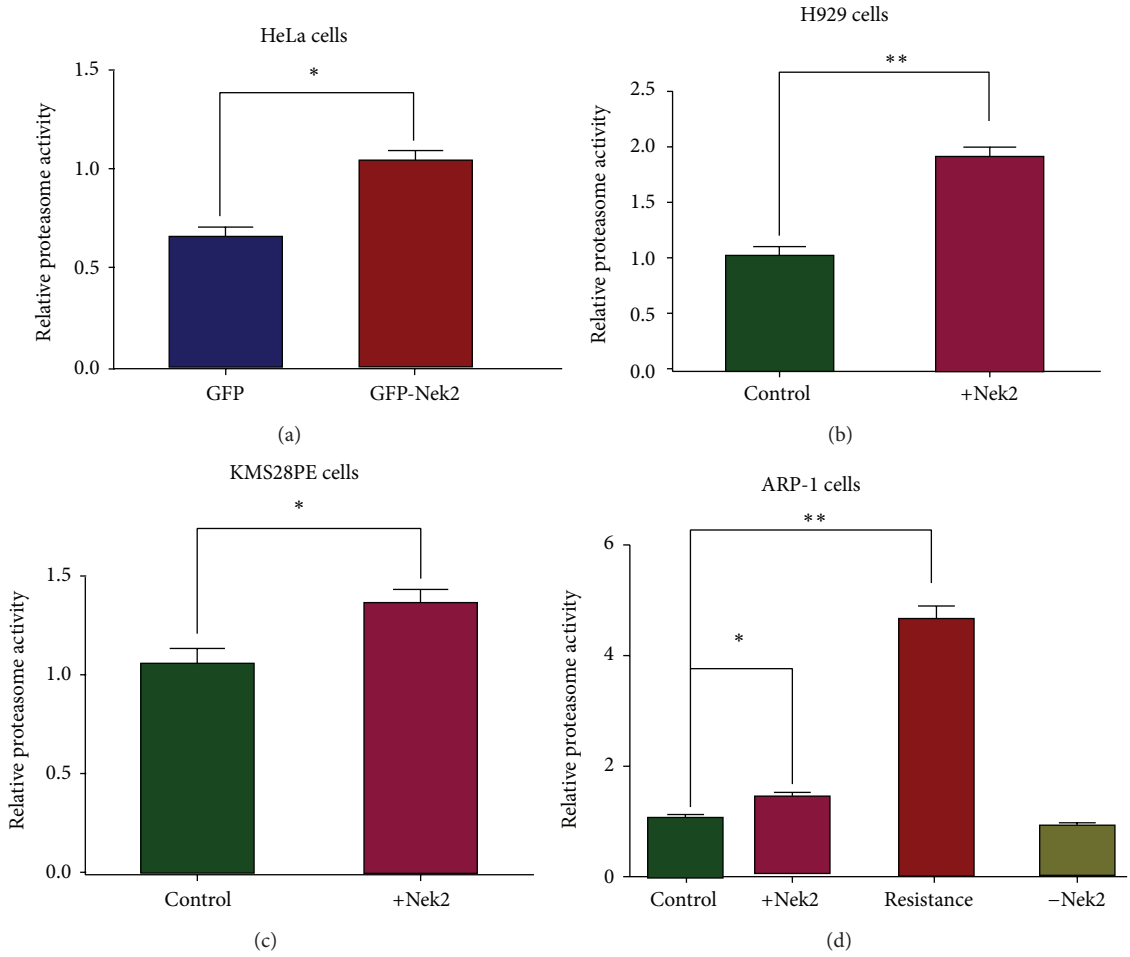
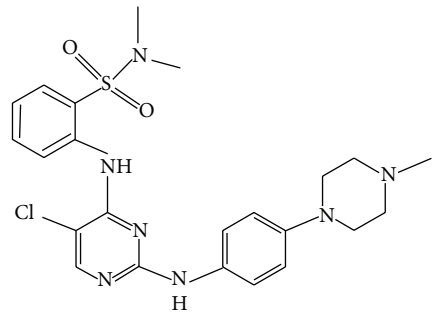


Figure 2: Nek2 overexpression elevates the proteasome activity in multiple cancer cell lines. (a) Proteasome activity is significantly increased in Nek2 overexpressed HeLa cells compared to GFP-transfected control. Proteasome activity was also significantly elevated in H929 (b), KMS28PE (c), and ARP-1 (d) cell lines compared to empty vector transfected (control). For the ARP-1 cell line, Nek-2-OE, NEK-2-KD, and bortezomib-resistant clones were tested in addition to wild-type cells. The 26S proteasome was isolated by ultracentrifugation and the proteasome activity was determined by Proteasome-Glo Assay. (d) For ARP1 cells, the bortezomib-resistant cells (third column in (d)) showed higher proteasome activity. For Figures 2(a)–2(d), $^{*}P < 0.05$, $^{**}P < 0.01$.

filtered by physical property calculations, *in vitro* ADME, and kinase selectivity filters to give one compound, HCI-2184, that was selected for further experiments examining the role of Nek2 in drug resistance. Using the Kinase-Glo assay, we determined the IC_{50} of HCI-2184 and found that it was <100 nM. Structure-based optimization was used to synthesize additional analogues of HCI-2184 and three compounds were selected as potential leads, HCI-2184, HCI-2388, and HCI-2389, all of which yield an average IC_{50} under 50 nM (Figures 3(a)–3(c)). Among them, HCI-2389 was the most potent Nek2 inhibitor. This is most likely due to its irreversible binding mode of action (see Supplementary Figure S1 available online at http://dx.doi.org/10.1155/2014/273180) which was tested by the Kinase-Glo assay with the drug preincubated with Nek2. The Nek2 inhibitory activity of HCI-2389 was significantly increased after preincubation times as short as 0.5 hours (Figure 3(d)).

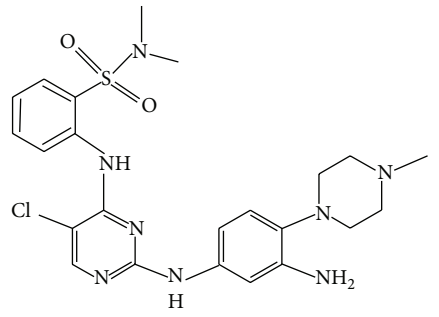
Based on its potency, HCI-2389 was selected to treat the Nek2-OE HeLa cells. We performed Western blots to measure the downstream effects of Nek2 inhibition caused by HCI-2389 treatment. We found that the level of phosphorylated PP1-α was significantly decreased in HeLa cells treated with concentrations as low as 10 nM of HCI-2389 for 72 hours (Figure 3(e)).

Our observation that Nek2 overexpression increased proteasome activity led us to ask whether our Nek2 inhibitors were able to inhibit this increased activity. We tested this hypothesis by isolating the 26S proteasome by ultracentrifugation from multiple Nek2-OE cells as described in Materials and Methods. Interestingly, Nek2 was found to be involved in the proteasome complex (Figure 4(a)), suggesting a possible direct interaction with the proteasome components. In accordance with this notion, the levels of Nek2 associated with the proteasome were proportional to overall Nek2 levels in cells (Figure 4(a)). It is important to note this relationship was not as clear in the KMS28PE cell line, where levels of both endogenous and transfected Nek2 were not as apparent (Figure 4(a)). To test the effect of our Nek2 inhibitors on



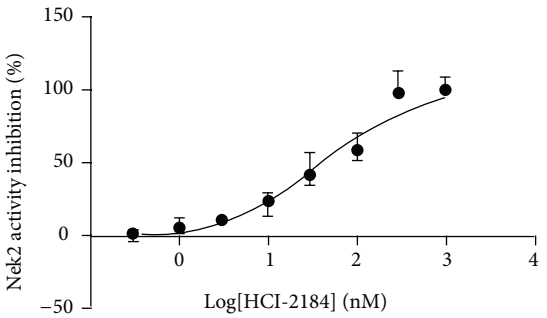
HCI-2184

IC_{50} = 39.90 nM (8 runs)

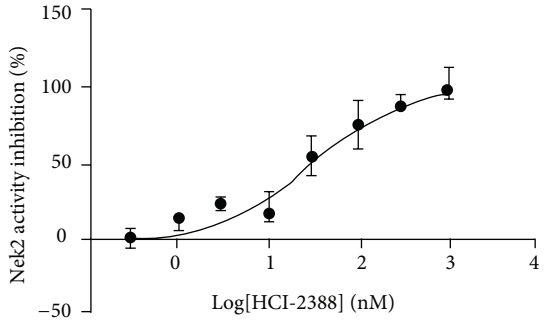


HCI-2388

IC_{50} = 34.27 nM (8 runs)



(a)



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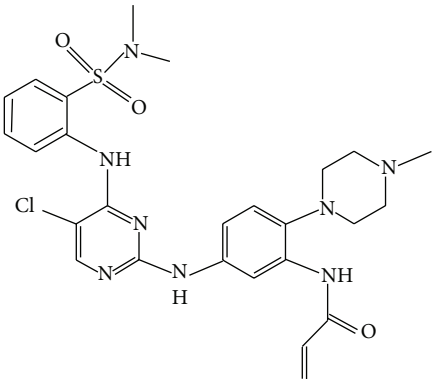
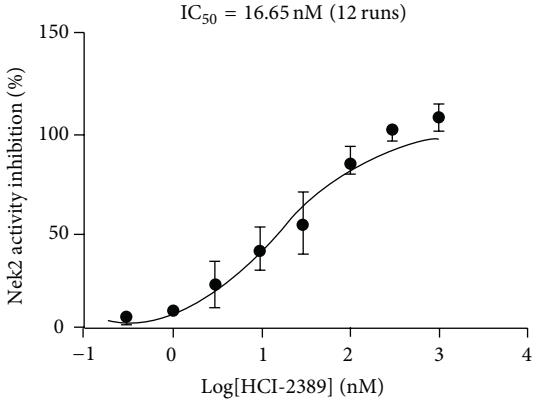
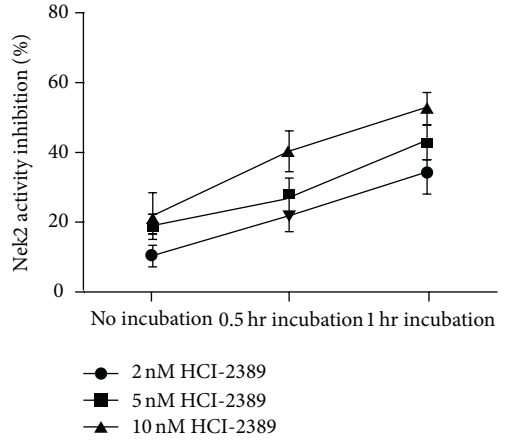


HCI-2389

IC_{50} = 16.65 nM (12 runs)

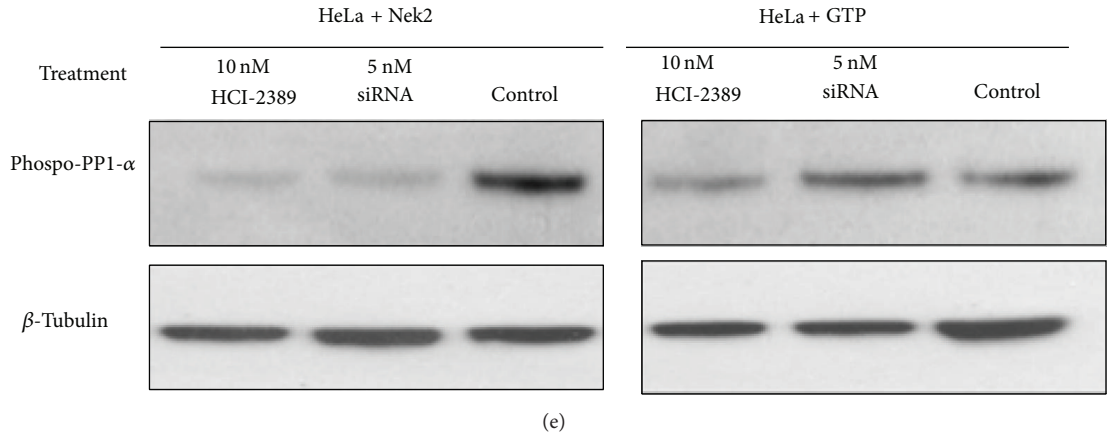


(c)



(d)

Figure 3: Continued.



(e)

Figure 3: Novel Nek2 Inhibitors significantly Inhibit Nek2's activity. (a), (b), and (c), three compounds, HCI-2184, HCI-2388, and HCI-2389 were designed by virtual screening. Synthesized compounds were validated by NMR and MS. The abilities of the three compounds to inhibit Nek2 kinase were tested by Kinase-Glo Assay. (d) HCI-2389 acts as an irreversible Nek2 inhibitor. A 0.5 hr incubation of HCI-2389 and Nek2 kinase increased the ability of HCI-2389 to inhibit Nek2. This effect was more pronounced when HCI-2389 was incubated with Nek2 kinase for 1 hr. (e) 10 nM HCI-2389 treatment for 72 hours greatly decreased the level of phosphorylated PP1-α in both Nek2 overexpressed HeLa cells and GFP controls. The effect was equal to or greater than treatment with 5 nM Nek-2 siRNA.

proteasome activity, the compounds were incubated with the isolated proteasome followed by the Proteasome-Glo assay. Our Nek2 inhibitors inhibited proteasome activity *in vitro* at a level similar to bortezomib (Figures 4(b)–4(e)). Based on these results, we concluded that the Nek2 inhibitors were responsible for the decrease in proteasome activity in the Nek2-OE cancer cell lines tested.

We further studied the effect of Nek2 inhibitors on additional cell lines using the ATP-lite cell viability assay. We treated a large panel of cell lines ($n = 36$) with our Nek2 inhibitors. These 36 cell lines were either responsive ("responsive" was defined as an IC_{50} value of less than 1 μM) to both HCI-2184 and HCI-2389 or nonresponsive to either of the two inhibitors selected for the proteasome activity assay. Data analysis showed that although there was not a strict proportional relation between proteasome activity and Nek2 inhibitor responsiveness, the average proteasome activity of the sensitive cell lines was significantly higher than that of the nonsensitive cell lines (Figure 5).

3.4. The Combination of Bortezomib and Nek2 Inhibitors Reduces Proteasome Activity to a Greater Extent Than Either Drug Alone. We next combined our Nek2 inhibitors with bortezomib in the proteasome-Glo assay to determine whether Nek2 inhibitors could be used in combination with bortezomib. In treated HeLa cells, both HCI-2184 and HCI-2389 significantly increased the effectiveness of bortezomib in inhibiting proteasome activity at concentrations as low as 10 nM (Figure 6(a)). Additionally, dose response studies confirmed that these two Nek2 inhibitors shift the inhibition curve of bortezomib (Figure 6(b)).

For the other three cell lines studied, including H929, KMS28PE, and ARP1, HCI-2389 was also able to increase the efficacy of bortezomib, while HCI-2184 had less of a synergistic effect (Figures 6(c)–6(e)). Again, the irreversible binding of HCI-2389 provides a possible explanation for this difference between compounds.

These results provide evidence that proteasome activity can be inhibited to a greater extent when combining Nek2 inhibitors with bortezomib, compared to bortezomib alone, suggesting that Nek2 is a potential molecular target that might be used in combination with bortezomib to treat MM patients.

3.5. Nek2 Inhibitors Prevented Mitotic Proteins from Being Degraded by Proteasome, Causing G_2/M Phase Arrest. Many proteins are targeted and degraded by the proteasome for mitotic entry as well as mitotic exiting [31–34]. Degradation of Cyclin B and Cdc2 plays a significant role in mitotic regulation [35–38]. Previous research has shown that down-regulation of proteasome activity lead to the accumulation of Cyclin B [39], triggered by the overexpression of Hec1, a substrate of Nek2. As Nek2 overexpression elevated proteasome activity and Nek2 inhibition decreased it, we set out to evaluate the levels of a few key mitotic regulators targeted by the proteasome.

In this experiment, cells were synchronized in mitotic phase, followed by treatment of Nek2 inhibitors for 72 hours. The levels of Cyclin B and Cdc2 were then evaluated by Western blot. Both Cyclin B and Cdc2 were found to be downregulated by Nek2 overexpression (Figure 7(a)), which is consistent with the finding that Nek2 overexpression causes increased proteasome activity. Further, treating the cells with HCI-2389 or Nek2-siRNA successfully inhibited the degradation of Cyclin B and Cdc2. This effect was not as dramatic in the GFP controls, which expressed only basal levels of Nek2.

Cell cycle analysis was performed to examine the effect of Nek2 inhibition on the cell cycle. The stably transfected HeLa cells were treated with 10 nM of HCI-2184 and HCI-2389 for 24 hours and then analyzed by flow cytometry. We

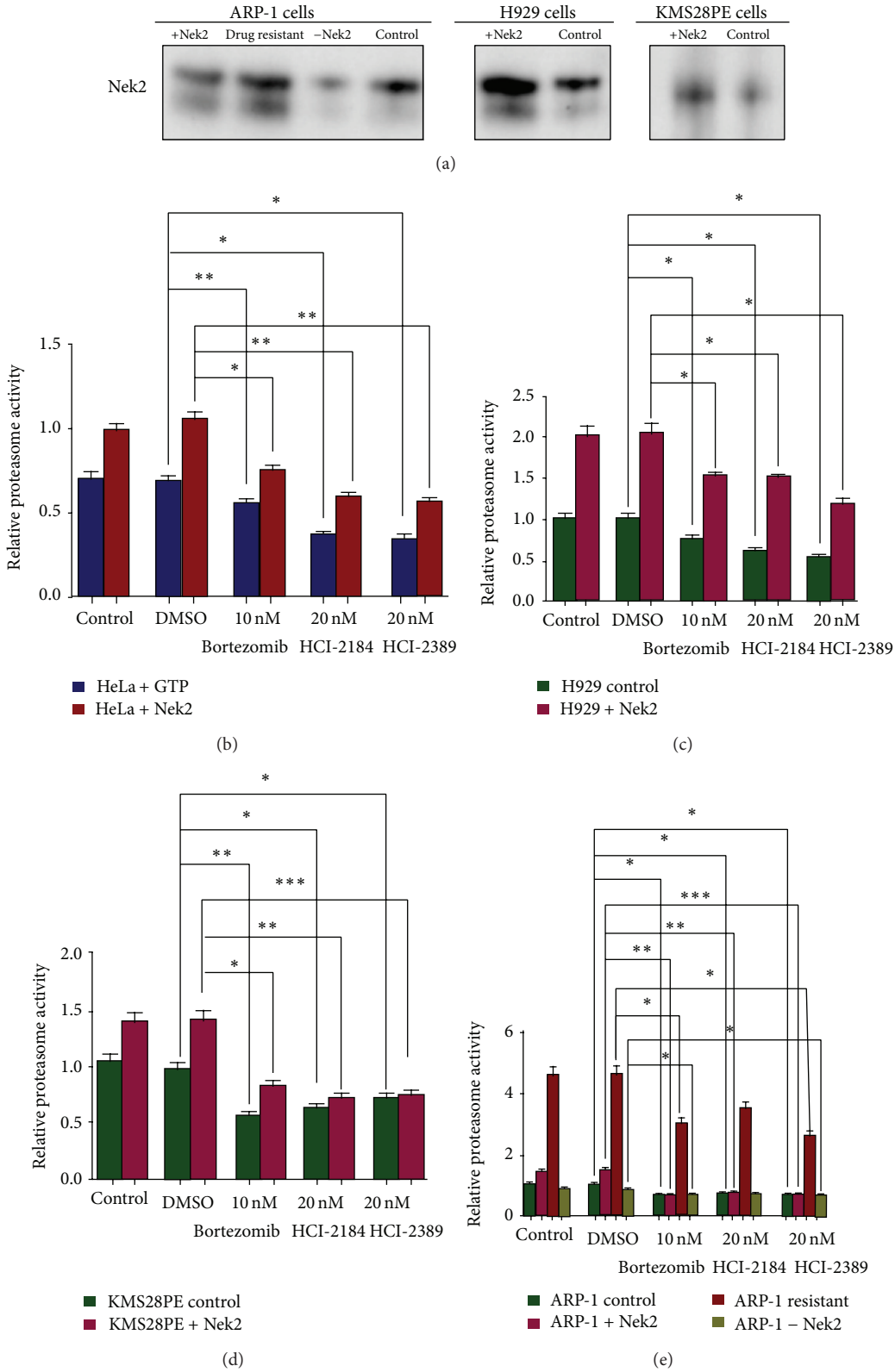


Figure 4: Novel Nek2 inhibitors effectively decrease the proteasome activity *in vitro* for multiple cancer cell lines. (a) Nek2 was found to be involved in the 26S proteasome in cancer cell lines. The 26S proteasome was isolated by ultracentrifugation and the presence of Nek2 in the 26S proteasome was determined by Western blot. (b), (c), (d), and (e), Incubation of HCI-2184 and HCI-2389 significantly inhibits the proteasome activity for HeLa cells (b), H929 cells (c), KMS28PE cells (d), and ARP1 cells (e). (e) The irreversible Nek2 inhibitor HCI-2389 worked better than HCI-2184 in decreasing the proteasome activity for ARP1 cells that are resistant to bortezomib treatment. For Figures 4(b)–4(e), $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

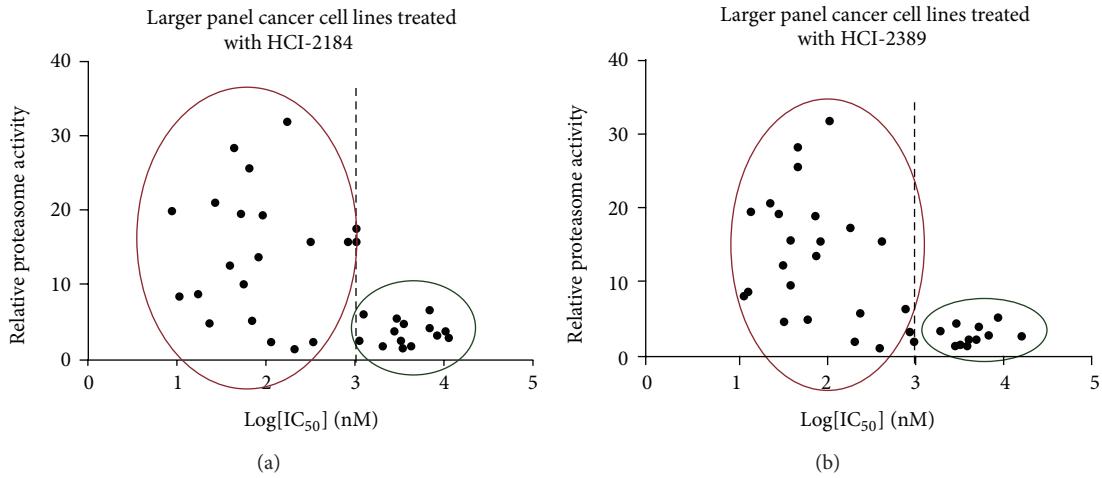


Figure 5: The sensitivity of cancer cell lines to Nek2 inhibitors is correlated with their proteasome activity. (a) Cell lines sensitive to HCI-2184 treatment had, on average, higher proteasome activity compared to resistant cell lines. (b) Cell lines sensitive to HCI-2389 treatment had, on average, higher proteasome activity compared to resistant cell lines. These cancer cell lines were selected from the 150 cell lines in our lab, based on whether or not they were sensitive to both HCI-2184 or HCI-2389. "Sensitive" was defined as an IC_{50} value of 1 μM or lower. The 26S proteasomes were isolated by ultracentrifugation and proteasome activity measured by Proteasome-Glo Assay.

found that almost 50% of the Nek2 inhibitor-treated cells were arrested in G2/M phase (Figure 7(b)). As before, HCI-2184 did not work as well as HCI-2389 in arresting HeLa GFP OE cells, and this is probably because HCI-2389 is a more specific Nek2 inhibitor than HCI-2184. Further research will be needed to elucidate the detailed inhibitory mechanisms of these compounds.

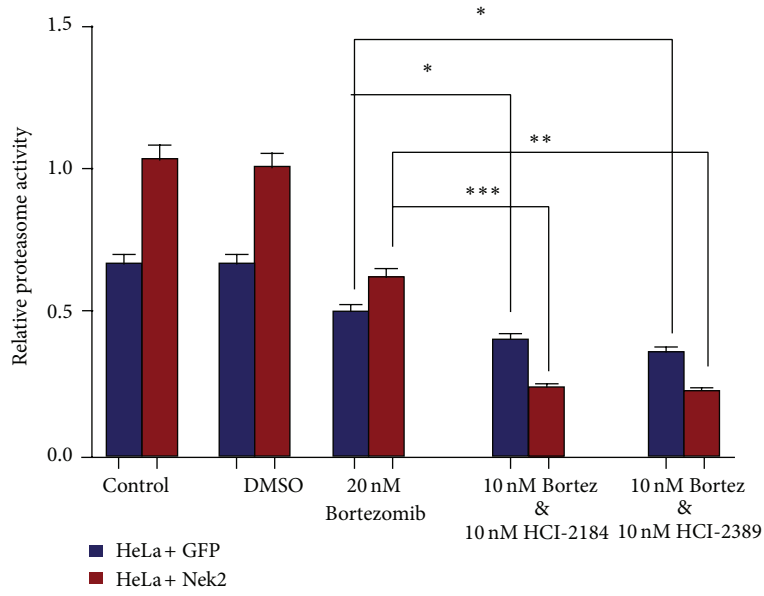
In summary, Cyclin B and Cdc2 were downregulated by Nek2 overexpression and Nek2 inhibition reversed this effect. Nek2 inhibitors, through inhibition of proteasome activity, inhibited Cyclin B and Cdc2 from being degraded. This resulted in cell cycle arrest in G_2/M phase in the Nek2-OE cells.

4. Discussion

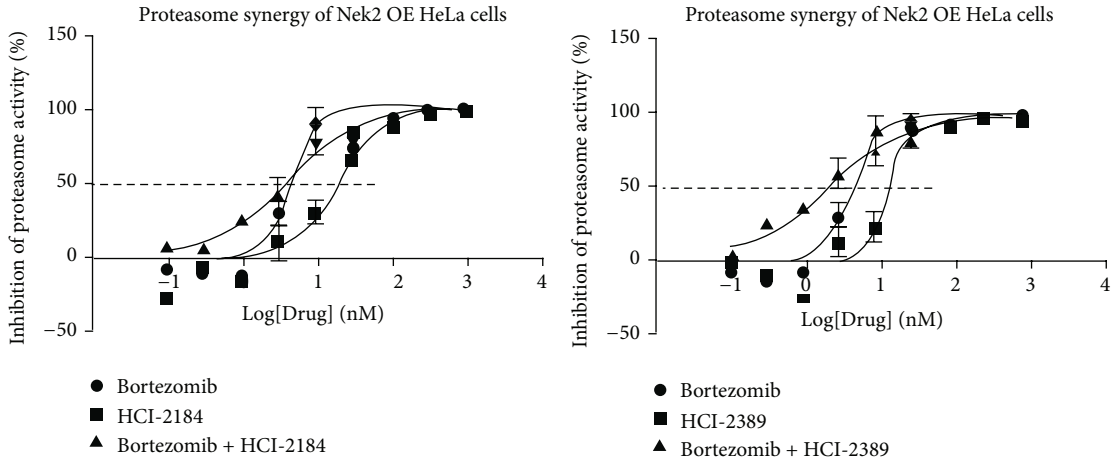
Although progress in the treatment of MM has been made in the past decade [40, 41], myeloma remains largely incurable with current therapeutic strategies. Bortezomib is one of the most effective chemotherapies for MM, but drug resistance remains a crucial problem with bortezomib treatment [17, 19, 20]. Little is known about the molecular mechanisms involved in this resistance. In our previous report, we used gene expression profiling in a variety of MM cases and identified Nek2 as the most significant gene associated with early relapse [21]. Other reports have similarly shown that Nek2 overexpression induces chemotherapeutic resistance *in vitro* [15, 42]. It is clear that there is an urgent need for exploring the mechanism linking the Nek2 kinase to drug resistance and the development of novel Nek2 inhibitors. To our knowledge, this study represents the first link connecting Nek2's biological function of regulating proteasome activity as the mechanism of bortezomib resistance in multiple myeloma. It is also the first to establish highly effective Nek2 inhibitors that successfully inhibit proteasome activity in cancer cell lines.

The 26S proteasome complex is a core component of the ubiquitin-proteasome system (UPS) of protein degradation. Ubiquitination regulates multiple cell cycle aspects including checkpoints control and cell growth progression [18, 43, 44]. The 26S proteasome is essential for the rapid elimination of the cell cycle regulators and the transcription factors such as NF-κB, whose fast degradation is important to the proper cell processes [44, 45]. Cdk1 and Cdk2 drive progression through each cell cycle phase and G2/M transition in particular [38, 46]. The activation of Cdks greatly depends on the availability of their cyclin partners, and cyclin levels are strongly regulated by the UPS [32]. In addition, the UPS has been shown to regulate the Cdk inhibitors such as Wee1 [37, 46]. Studies have shown that two complexes are involved in the UPS regulation of cell cycle: the anaphase-promoting complex or cyclosome (APC/C) and the Skp1/Cullin-1/F-box protein complex (SCF) [34, 47, 48]. These two complexes have different cellular functions and play crucial roles in different cell phases. APC/C regulates the degradation of mitotic cyclins, such as Cyclin B1, and consequently inhibits Cdk1, leading cells to mitotic exit [41, 42].

Previous reports suggested that Nek2 primarily played a role in regulating centrosome separation [7–10]. Overexpression of active Nek2 induces premature splitting of centrosomes, while silencing of Nek2 blocks spindle and chromosome segregation. As centrosome separation is crucial for mitotic entry, Nek2 was thought to participate in cell cycle control. However, compared to other mitotic kinases, Nek2's function is relatively subtle and, in our study, neither suppression nor silencing of Nek2 expression dramatically affected the cell cycle. This has been the major obstacle for studying Nek2's biological function. In this research, we explored Nek2's function and we confirmed the correlation of Nek2 overexpression and bortezomib resistance in HeLa cells. Bortezomib exerts its effects on cancer cells by inhibiting proteasome activity. Subsequently, we hypothesized that



(a)



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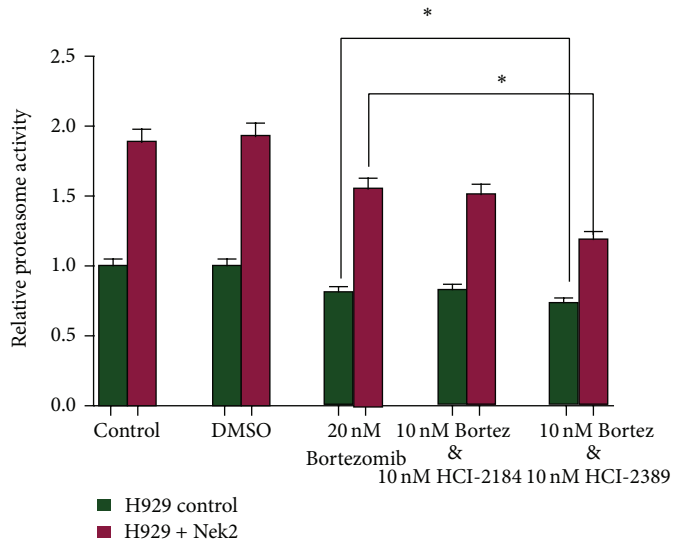

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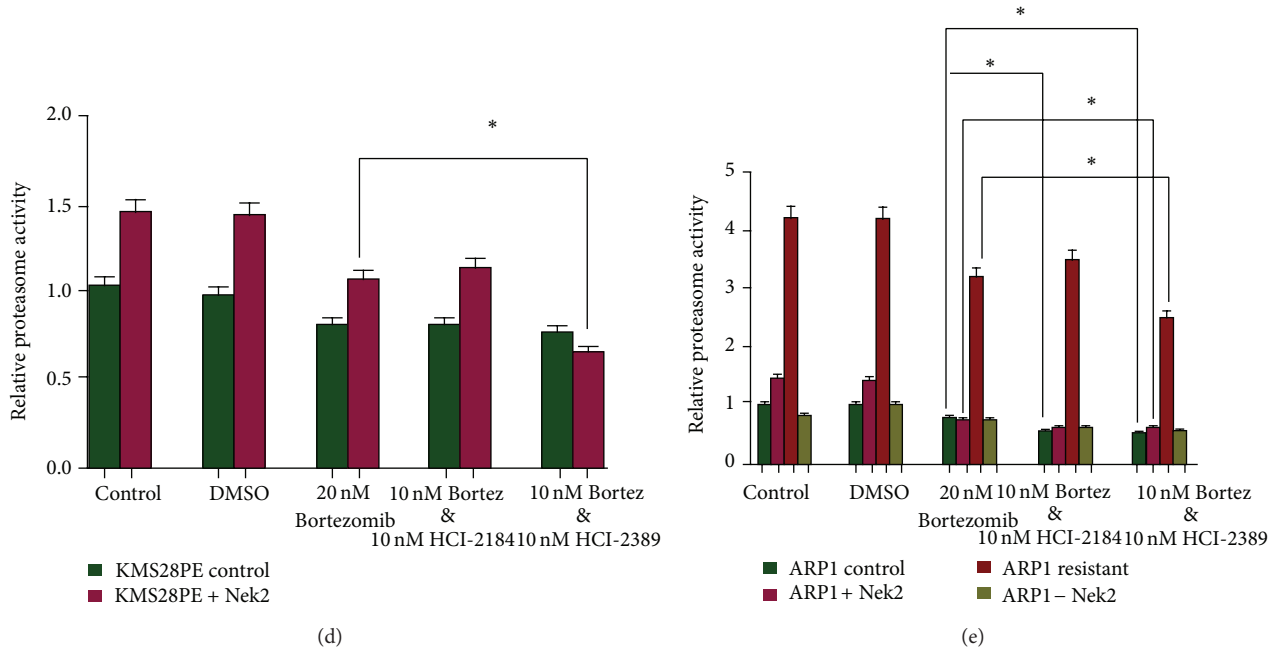

Figure 6: The combination of bortezomib and Nek2 inhibitors reduces proteasome activity to a greater extent than either drug alone. (a) The combination of bortezomib with either HCI-2184 or HCI-2389 significantly increased the effectiveness of bortezomib on Nek2-OE HeLa cells compared to GFP-transfected controls. (b) The combination of Nek2 inhibitors HCI-2184 or HCI-2389 and bortezomib inhibited proteasome activity in Nek2-OE HeLa cells to a greater extent than either drug alone. (c) and (d), the combination of Bortezomib and HCI-2389 decreased proteasome activity compared to untreated or DMSO treated H929 (c) or KMS28PE (d) cells treated with either empty vector (control) or Nek2 overexpressing (+Nek2) cells. (e), the combination of bortezomib and HCI-2389 decreased proteasome activity in empty vector (control), Nek2 plasmid (+Nek2), and Nek2 siRNA knockdown (−Nek2) ARP1 cells. The combination also resulted in a significant decrease in proteasome activity in ARP1 cells resistant to bortezomib (ARP1 resistant). For (a) and (c)–(e), $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

Nek2's role in bortezomib resistance was related to increasing proteasome activity. Using multiple cancer cell lines, we showed that overexpression of Nek2 significantly elevated proteasome activity. Specifically, we found higher proteasome activity in bortezomib resistant ARP1 cells. This elevated *in vitro* proteasome activity is inhibited by our Nek2 inhibitors HCI-2184 and HCI-2389 which rescue drug resistance of Nek2-OE HeLa cells. However, the mechanism of how Nek2 regulates proteasome activity is still unknown and needs further investigation.

Together with the Polo and Aurora kinase families, the NIMA-related protein kinases (Neks) have been called the third family of mitotic kinases [2]. Previous studies suggest that Nek family members influence cell cycle progression by regulating Cyclin B and Cdc2 [2, 49, 50]. Here, we discovered Nek2 overexpression down-regulated both Cyclin B and Cdc2 by increasing activity of the proteasome. This finding may provide more information for further study of Nek2's function in the cell cycle regulation.

By examining the proteasome activity of multiple cancer cell lines, we have identified Nek2 upregulation as a potential mechanism for bortezomib resistance related to proteasome activity elevation. However, for ARP1 cells, the proteasome activity of the bortezomib resistant clone was higher than that of the Nek2 OE clone (Figure 2(c)). Therefore, other mechanisms aside from Nek2 upregulation may be involved in proteasome activity elevation. Further effort is needed to elucidate other proteasome regulators as potential drug targets for MM therapeutics.

Although we have synthesized several potent Nek2 inhibitors with demonstrated activity (Figure 3), these inhibitors need better selectivity to advance them as potential clinical candidates (Supplementary Figure S2).

In summary, we have discovered that high levels of Nek2 expression are at least partly responsible for elevated proteasome activity and subsequent bortezomib resistance in human MM treatment. More excitingly, we have shown that Nek2 inhibition results in proteasome activity suppression and cell cycle arrest. This research provides important knowledge for future studies of Nek2's biological function and provides potential solutions for bortezomib resistance in MM therapy.

5. Clinical Practice Points

Although progress in the treatment of MM has been made in the past decade [40, 41], myeloma remains largely incurable with current therapeutic strategies. Bortezomib is one of the most effective chemotherapies for MM, but drug resistance remains a crucial problem with bortezomib treatment. Little is known about the molecular mechanisms involved in this

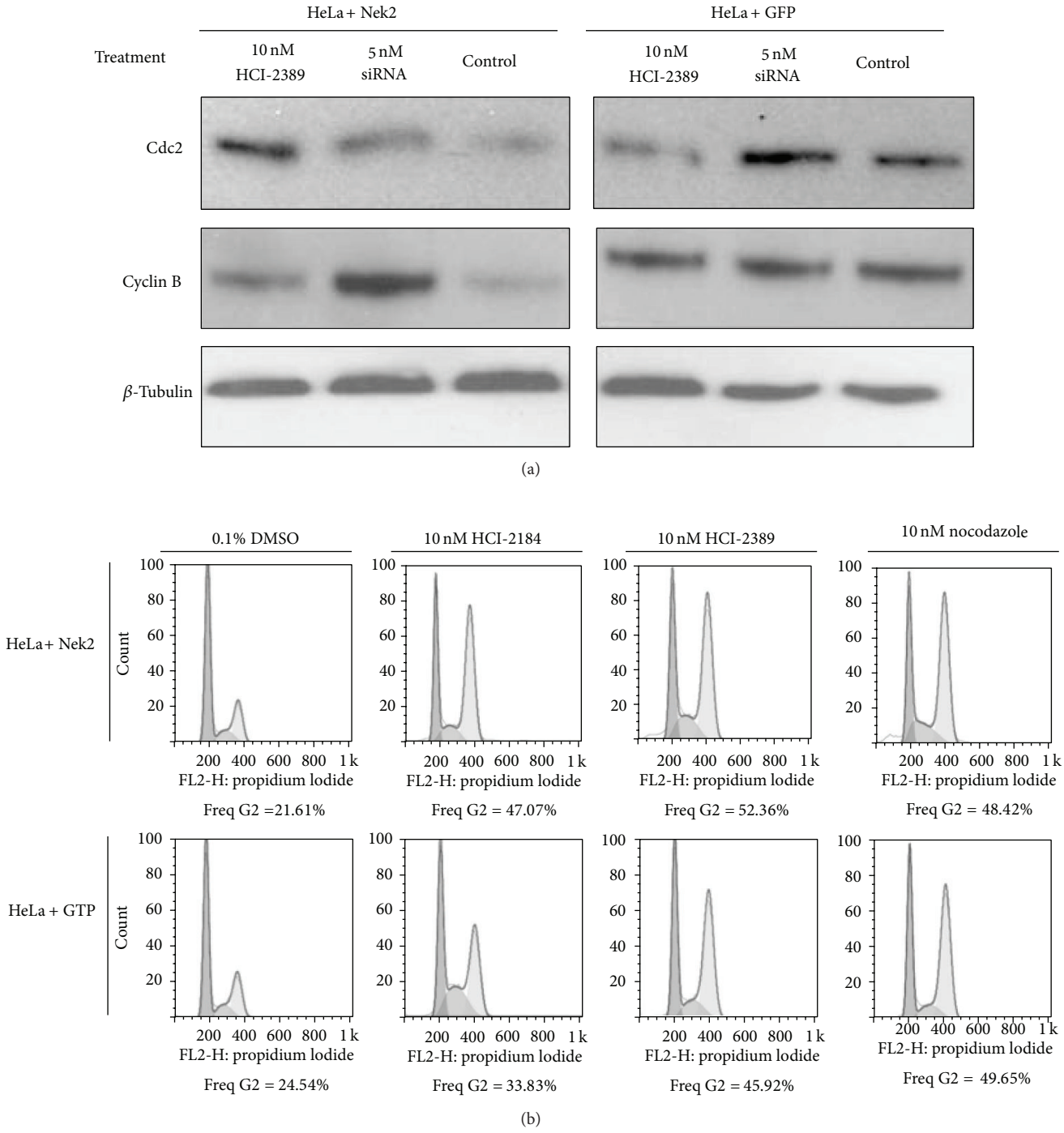

Figure 7: Novel Nek2 inhibitor prevents Cdc2 and Cyclin B from being degraded by the proteasome, catching HeLa cells in G2/M phase. (a) Western blot showed that the degradation of Cdc2 and Cyclin B was inhibited by treatment of 10 nM HCI-2389. This effect was significant in Nek2 overexpressed HeLa cells. (b) Flow cytometry data showed that, treated by 10 nM HCI-2184 or 10 nM HCI-2389, around 50% HeLa cells stayed at G2/M phase. 100 nM nocodazole was used as the positive control.

resistance. In our previous report, we used gene expression profiling in a variety of MM cases and identified Nek2 as the most significant gene associated with early relapse. Other reports have similarly shown that Nek2 overexpression induces chemotherapeutic resistance *in vitro*. It is clear that there is an urgent need for exploring the mechanism linking the Nek2 kinase to drug resistance and the development of novel Nek2 inhibitors. To our knowledge, this study represents the first link connecting Nek2's biological function of regulating proteasome activity as the mechanism of bortezomib resistance in multiple myeloma. It is also the first to establish highly effective Nek2 inhibitors

that successfully inhibit proteasome activity in cancer cell lines.

Conflict of Interests

Lingyao Meng and Alexis Mollard were previously employed by Tolero Pharmaceuticals Inc. Kent Carpenter is currently employed by Tolero Pharmaceuticals Inc. Steven Warner is currently employed by Tolero Pharmaceuticals Inc. David Bearss is the CEO of Tolero Pharmaceuticals Inc. All other authors state that they have no potential conflict of interests.

Acknowledgments

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Research Article

Destabilization of Akt Promotes the Death of Myeloma Cell Lines

Yanan Zhang, Yunfeng Fu, Fan Zhang, and Jing Liu

Department of Hematology, The Third Xiangya Hospital, Central South University, Changsha, China

Correspondence should be addressed to Fan Zhang; zhangfan_ck@sina.com and Jing Liu; jingliu0318@aliyun.com

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Constitutive activation of Akt is believed to be an oncogenic signal in multiple myeloma and is associated with poor patient prognosis and resistance to available treatment. The stability of Akt proteins is regulated by phosphorylating the highly conserved turn motif (TM) of these proteins and the chaperone protein HSP90. In this study we investigate the antitumor effects of inhibiting mTORC2 plus HSP90 in myeloma cell lines. We show that chronic exposure of cells to rapamycin can inhibit mTORC2 pathway, and AKT will be destabilized by administration of the HSP90 inhibitor 17-allylamino-geldanamycin (17-AAG). Finally, we show that the rapamycin synergizes with 17-AAG and inhibits myeloma cells growth and promotes cell death to a greater extent than either drug alone. Our studies provide a clinical rationale of use mTOR inhibitors and chaperone protein inhibitors in combination regimens for the treatment of human blood cancers.

1. Introduction

Constitutive activation of the AGC kinase PKB/Akt is believed to be an oncogenic signal in multiple myeloma and is associated with poor patient prognosis and resistance to available treatment [1, 2]. Constitutive phosphorylation of Akt leads to activation of downstream substrates involved in cell cycle regulation and apoptosis prevention [3]. It is already proved that Akt activation promotes tumor-cell proliferation by phosphorylating and inhibiting the cell-cycle inhibitor $p27^{Kip1}$ and the F-box-containing transcription factor FoxO1 [4–6], as well as the proapoptotic protein BAD [7]. Akt activity also inhibits GSK3 resulting in suppressing the degradation of the antiapoptotic protein Mcl-1 [8, 9]. Extracellular stimulants can activate AKT through both growth factor dependent and growth factor independent ways by mammalian target of rapamycin complex 2 (mTORC2) [10–12].

Mammalian TORC2 is composed of mTOR, Rictor, mitogen-activated protein kinase associated protein 1 (Mapkap1/Sin1), mLST8, protein observed with Rictor (Protor/PRR5), and DEP domain containing mTOR interacting protein (DEPTOR) [13]. Pharmacologic or genetic inhibition of mTORC2 components impairs growth factor dependent Akt S473 phosphorylation and Akt signaling [10, 12, 14, 15]. Mammalian TORC2 also regulates the stability of Akt and cPKC proteins in a growth factor independent manner [16]. Mammalian TORC2 is required for the phosphorylation of Akt and cPKC at the turn motif (TM) site [12, 16]. Mammalian TORC2 interacts with actively translating ribosomes and phosphorylates the TM site of newly synthesized Akt and cPKC polypeptides during translation [17], which promotes the proper folding of newly synthesized Akt or cPKC polypeptides. However, the stability of Akt proteins may be rescued by association with the chaperone protein HSP90 when Akt is lacking TM phosphorylation [16]. Inhibition of HSP90 in $Sin1^{-/-}$ mouse leukemia cells results in the reduction of Akt protein expression and promotes cell death [18].

Because of the crucial role of Akt in multiple myeloma biology, we decided to investigate the idea whether inhibition of both mTORC2 and HSP90 in multiple myeloma cells would decrease Akt expression and inhibit tumor cell proliferation and survival. We tested this novel therapeutic strategy by exploring the effect of rapamycin and 17-AAG in two different human multiple myeloma cell lines on the

Akt expression, cell proliferation, and survival. We show that chronic rapamycin treatment inhibits mTORC2 on both cell lines, and coadministration of rapamycin and 17-AAG inhibits Akt expression and cell survival. These data reveal that combining the chaperone protein inhibitor with mTOR inhibitors can be considered as a promising new antineoplastic strategy.

2. Materials and Methods

2.1. Cell Lines and Culture. Multiple myeloma cell line KM3 was kindly provided by Professor Jian Hou from The Second Shanghai Military Medical University; multiple myeloma cell line U266 was kindly provided by Professor Jiankai Shen from The Second Xiangya Hospital. All cell lines were maintained in RPMI 1640 with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 incubator. Cells were monitored daily and fresh medium was exchanged when needed. Cells were placed in a 6-wells plate; 17-AAG and rapamycin were added to the medium to obtain the desired final concentrations.

2.2. Reagents

2.2.1. Antibodies. Anti-phospho-Akt T450 was purchased from Abcam Technology. Anti-phospho-Akt S473, anti-pan-Akt, anti-phospho-S6 S235/236, and anti-β-actin were purchased from Cell Signaling Technology. Reagents used for flow cytometry were BD Pharmingen FITC Annexin-V Apoptosis Detection Kit I purchased from BD company. All reagents were used at a 1 : 100 dilution of the stock from the same company.

2.2.2. Inhibitors. Both 17-AAG and rapamycin were purchased from Gene Operation.

Stocks were prepared in DMSO at concentration of 40 μmol/L, and 17-AAG was used at a final concentration of 600 nmmol/L; rapamycin was used at a final concentration of 20 nmmol/L. Drugs were diluted in culture medium with DMSO (<0.1%) immediately before use. Diluted drugs were used within 2 hours.

2.3. Determination of Cell Viability and Apoptosis. Cell viability was determined by the trypan blue dye exclusion. For a trypan blue staining, we diluted 1 part of the 0.4% prepared trypan blue to 9 parts of medium, which contained multiple myeloma cells, then mixed them and incubated them for 5 minutes. At last we counted cell percentage which was dyed into blue with optical microscope. Cells were counted in duplicate samples. To estimate apoptosis, control or treated cells were incubated with propidium iodide (PI) and Annexin V-FITC, following the protocol from the kit (FITC Annexin V Apoptosis Detection Kit I, BD Company), and then analyzed by flow cytometry. In brief, cells from 48-hour cultures were washed with ice-cold PBS and resuspended in binding buffer 100 μL. Multiple myeloma cells were first incubated with 5 μL Annexin V-FITC for 15 minutes at 4°C then incubated with 5 μL PI just before analysis. All cells were washed and resuspended in FACS buffer for acquisition on FACSCalibur (BD Bioscience, CA) using CellQuest software (BD Bioscience, CA). Postacquisition analysis was performed with FlowJo software (Treestar, CA). Annexin V-positive and PI-negative cells reflect cells in the early stages of apoptosis, whereas Annexin V-positive and PI-positive cells reflect dead cells or cells at the late stages of apoptosis.

2.4. Immunoblotting. Cells were washed with PBS and lysed in RIPA buffer containing 50 mM Tris-HCI pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 25 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, and 2.5 mM p-nitrophenyl phosphate. Total cell lysates were resolved on 8% SDS-PAGE gels and transferred to an Immobilon-P membrane (Millipore, MA). The resulting blots were blocked with 5% nonfat dry milk and incubated with the antibodies overnight at 4°C as described previously. Antibody dilutions for blots ranged from 1 : 200 to 1 : 4000. Unbound primary antibody was removed by washing with TBS-T containing 0.1% Tween-20 and blots were incubated with anti-rabbit immunoglobulin conjugated with horseradish peroxidase and then developed using an enhanced chemiluminescence kit (Pierce ECL Plus Western Blotting Substrate, Thermo Scientific Pierce) following the manufacturer's instructions. The film was scanned and analyzed with Image-Pro Plus version 6.0 software. Blots were stripped and reprobed with anti-actin antibody (1 : 3000) to ensure equivalent protein loading. Different time points were chosen to determine the effect of the agents on phosphorylated proteins and total proteins (0–48 hours).

2.5. Statistical Analysis. Results are expressed as mean ± SD; the Student's t-test was used to determine the statistical significance of the differences between groups of samples. $P < 0.05$ was considered statistically significant. The number of sample replicates and the number of experimental replicates are indicated in the figure legends.

3. Results

3.1. Chronic Exposure to Rapamycin Inhibits mTORC2 Pathway on U266 and KM3 Cell Lines. mTOR1 regulates various aspects of protein synthesis, which connects mTOR1 to many physiological processes such as nutrient, stress, and hormone signaling [19–22]. In blood cancers mTOR signaling pathway is commonly activated to promote uncontrolled cellular growth and proliferation. During cellular protein translational controls, mTOR1 is one of the rate-limiting signal nodes. And moreover mTORC2 plays an important role in the dynamic interaction between tumor cells and BM microenvironment [23, 24], which is crucial in myeloma pathogenesis and resistance to treatment. Rapamycin is one of the most classical mTORC1 inhibitors. Usually people think that rapamycin (and its analogs) cannot completely inhibit TOR2 pathway in most cells. Recently, it has been proved that chronic exposure of certain kinds of cells to rapamycin can inhibit mTORC2 pathway, but the precise

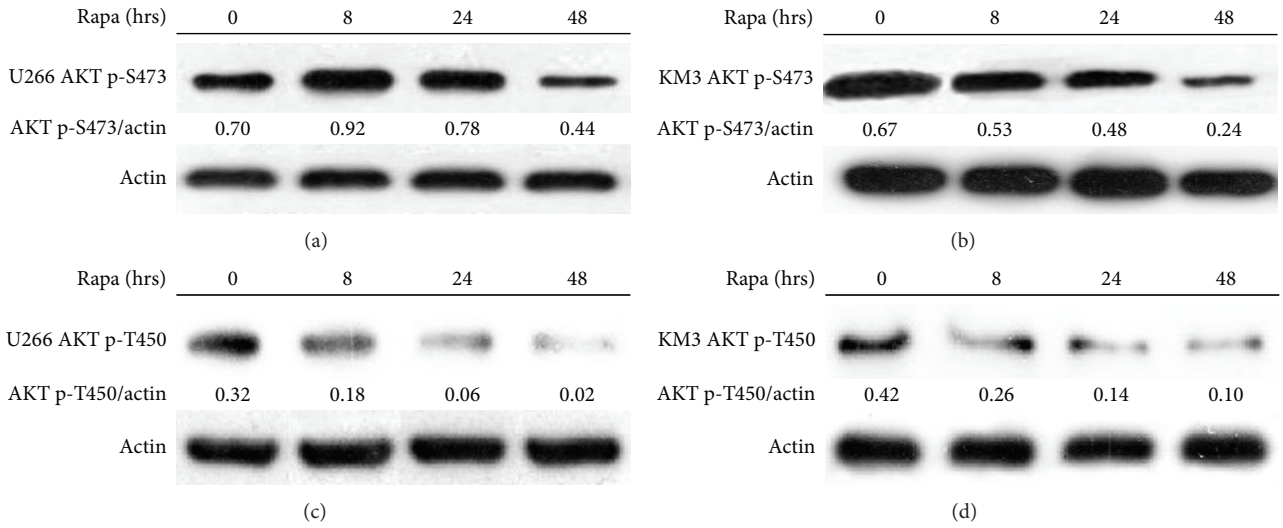

Figure 1: Prolonged exposure to rapamycin also inhibits mTORC2 pathway. (a) U266 cells or (b) Km3 cells were cultured in the presence of 20 nmmol/L rapamycin for the indicated periods of time. Total cellular proteins were assayed by immunoblotting for Akt p-S473 phosphorylation. Actin expression serves as a loading control. The Akt p-S473/actin ratio was calculated by dividing the total pixel volume of Akt by the total pixel volume of actin. (c) U266 cells or (d) Km3 cells were cultured in the presence of 20 nmmol/L rapamycin for the indicated periods of time. Total cellular proteins were assayed by immunoblotting for Akt p-T450 phosphorylation. Actin expression served as a loading control. The Akt p-T450/actin ratio was calculated by dividing the total pixel volume of Akt by the total pixel volume of actin. The results shown are representative of three independent experiments.

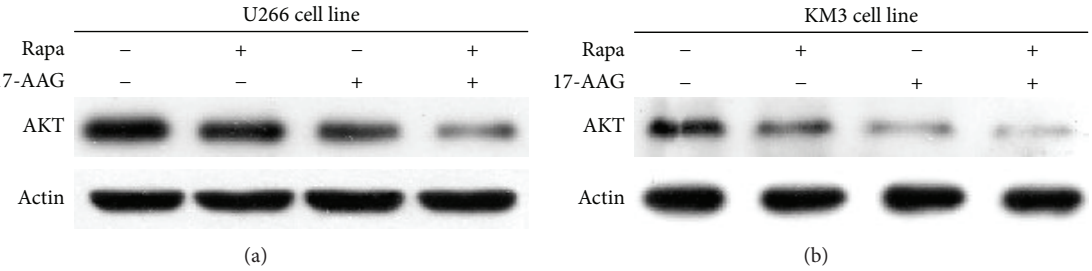

Figure 2: Coadministration of Rapa and 17-AAG destabilizes Akt. (a) U266 cells and (b) KM3 cells were treated with vehicle (Ctrl), 20 nmmol/L rapamycin, 600 nmol/L 17-AAG, or rapamycin plus 17-AAG for 48 h. Total Akt expression was measured by immunoblotting. Actin expression served as a loading control. The results shown are representative of three independent experiments.

mechanism is still unclear [25, 26]. To address whether chronic exposure to rapamycin can inhibit mTORC2 pathway on myeloma cell, we cultured our two myeloma cell lines (U266 and KM3) in the presence of 20 nM rapamycin up to 48 h and harvested the cell lysate at 0 h, 8 h, 24 h, and 48 h. We found that after 48 h treatment rapamycin was able to inhibit Akt S473 phosphorylation, in both cell lines (Figures 1(a) and 1(b)), and Akt T450 phosphorylation (Figures 1(c) and 1(d)), which is the well-known downstream of mTORC2. We also found that rapamycin inhibits mTORC1-dependent S6 S235/236 phosphorylation at 48 h (data not shown) as previously described.

3.2. Combined 17-AAG and Prolonged Rapamycin Treatment on Myeloma Cell Lines Destabilizes Akt. Heat shock protein 90 can protect newly synthesized folded kinases when cells lack TM phosphorylation [27]. As previous results already show that the stability of Akt in mice MEF cells [16] and mice leukemia cells [18] is maintained by the TM phosphorylation and HSP90, we ask what would happen to Akt levels of myeloma cells if we inhibit both mTOR2 and Hsp90. We treated U266 and KM3 cells with combined 17-AAG and prolonged rapamycin for 48 h, which led to a significant decrease in Akt protein level (Figures 2(a) and 2(b)).

3.3. Coadministration of Rapamycin and 17-AAG Promotes Death of Myeloma Cell Lines. Since Akt is critical for the survival of tumor cells, we decided to explore if chronic exposure of cells to rapamycin, the pharmacologic method of mTORC2 inhibition, could synergize with 17-AAG to suppress myeloma cell lines proliferation in vitro. We first compared the cell viability of KM3 cells treated with rapamycin alone, 17-AAG alone, or rapamycin plus 17-AAG with untreated cells. Cell viability was assessed by PI and Annexin-V staining and flow

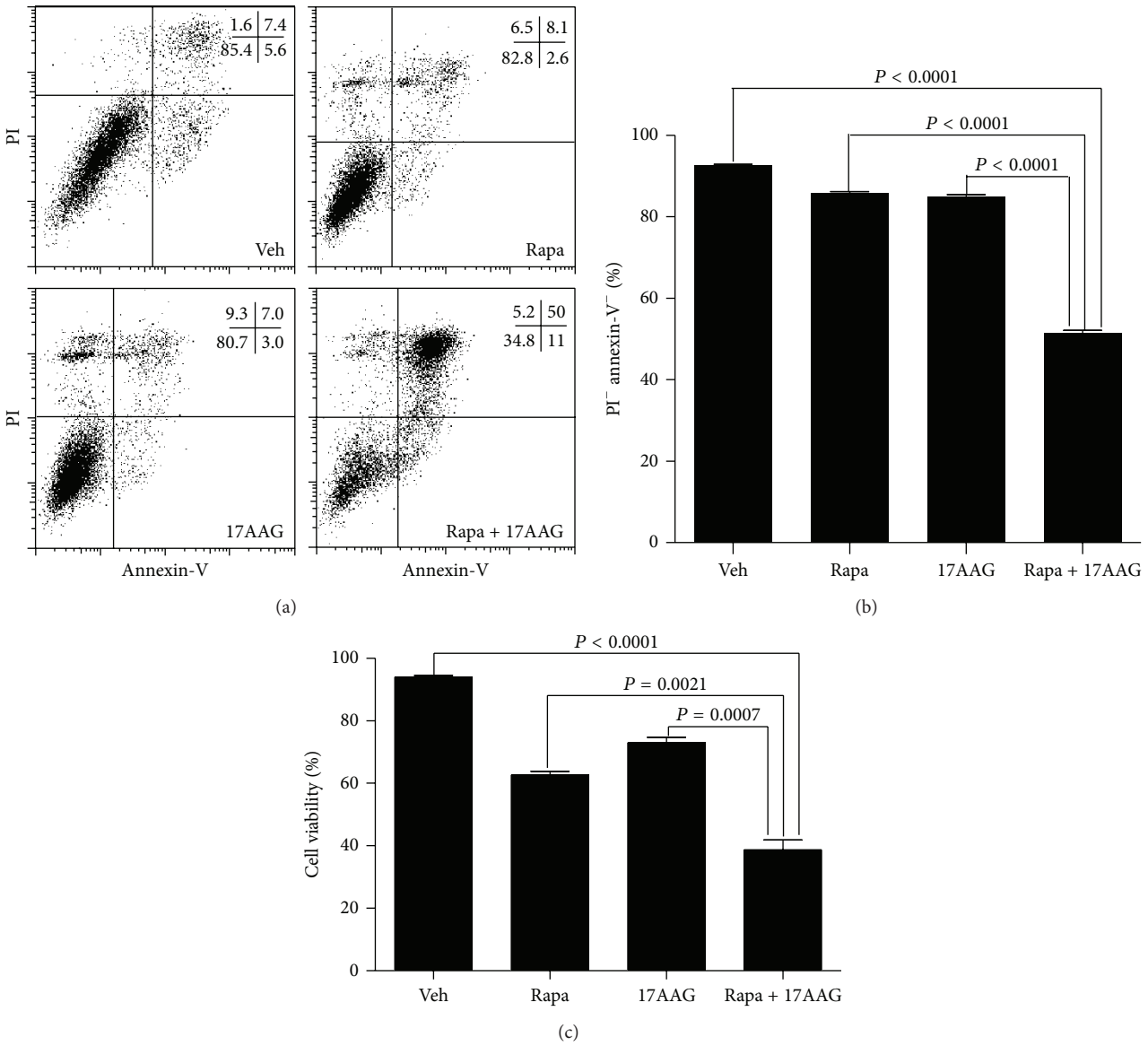

Figure 3: Coadministration of Rapamycin and 17-AAG promotes km3 cell line death. (a) KM3 cells were cultured for 48 h with vehicle (Ctrl), 20 nmmol/L rapamycin, 600 nmol/L 17-AAG, or rapamycin plus 17-AAG, and cells viability was measured by flow cytometry with PI and Annexin-V staining. A representative FACS plot is shown. The numbers in the plot show percentages of the gated populations in each quadrant. (b) It is the average of triplicate samples of data (a) from 1 of 3 independent experiments. (c) KM3 cells were cultured for 48 h with vehicle (Ctrl), 20 nmmol/L rapamycin, 600 nmol/L 17-AAG, or rapamycin plus 17-AAG, and viable cells were determined by trypan blue exclusion assay. The data shown are the average of triplicate samples from 1 of 3 independent experiments. The *P* values shown were calculated by a two-tailed test.

cytometry. We observed that the KM3 cells resulted in a 50% increase in proportion of viable cells after rapamycin and 17-AAG treatment for 48 hrs in comparison with the cells treated with single drugs (Figures 3(a) and 3(b)). We also determined the live cells percentage by trypan blue exclusion, which gives us the same results as FACS (Figure 3(c)).

Consistently, cotreatment of U266 cells with rapamycin plus 17-AAG resulted in a significantly greater inhibition of myeloma cell growth when compared to each drug alone (Figures 4(a), 4(b), and 4(c)). These data show that 17-AAG and prolonged rapamycin treatment act in synergy to inhibit myeloma cell proliferation and survival.

4. Discussion

The AGC kinase PKB/Akt is constitutively activated in human myeloma cell lines and freshly isolated plasmocytes from patients with MM [28] and is considered as an oncogenic signal in MM. It is associated with poor patient prognosis and resistance to available treatment [1, 2]. Therefore, it

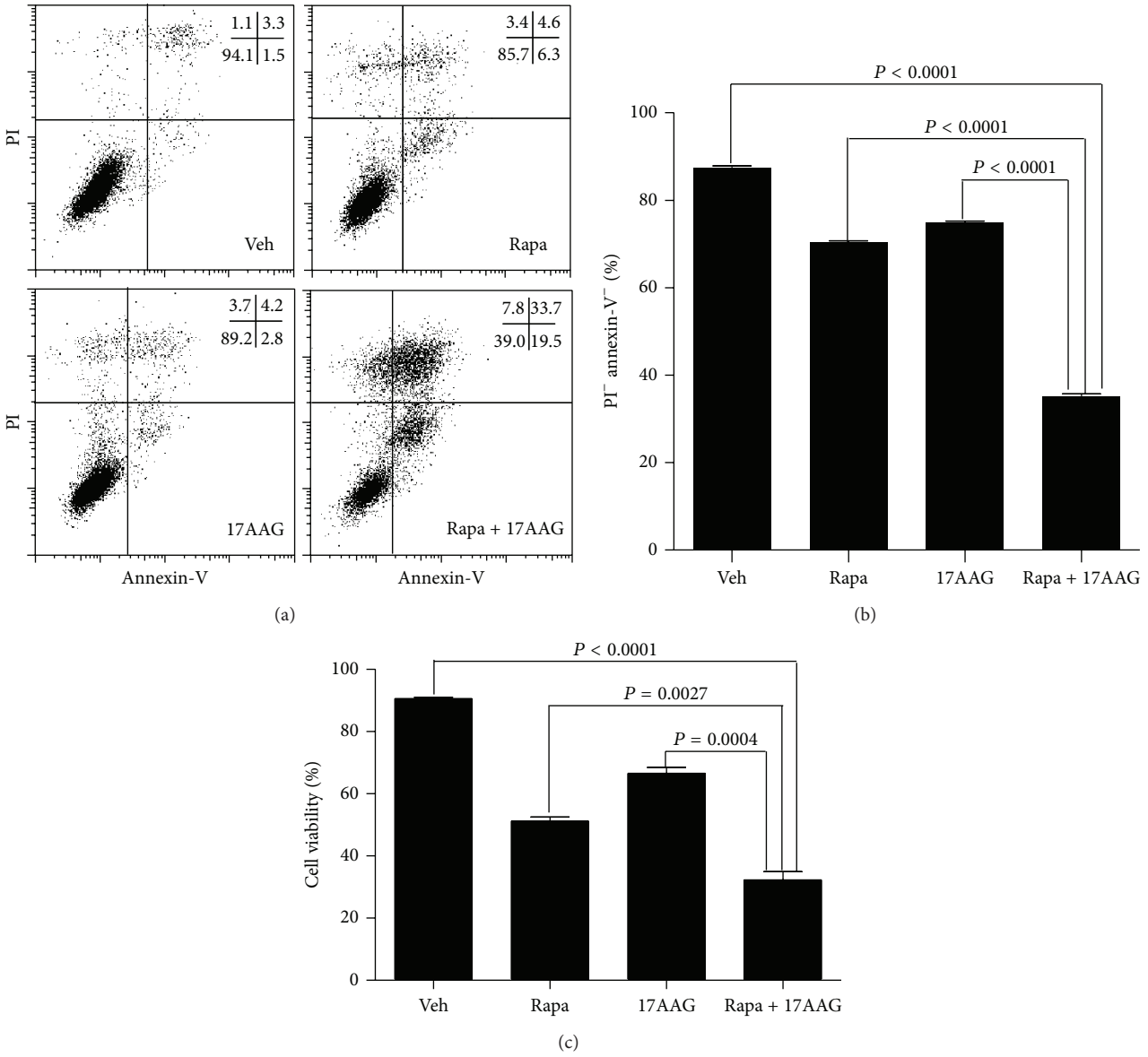

Figure 4: Coadministration of Rapa and 17-AAG promotes U266 cell line death. (a) U266 cells were cultured for 48 h with vehicle (Ctrl), 20 nmmol/L rapamycin, 600 nmol/L 17-AAG, or rapamycin plus 17-AAG, and cells viability was measured by flow cytometry with PI and Annexin-V staining. A representative FACS plot is shown. The numbers in the plot show percentages of the gated populations in each quadrant. (b) It is the average of triplicate samples of data (a) from 1 of 3 independent experiments. (c) U266 cells were cultured for 48 h with vehicle (Ctrl), 20 nmmol/L rapamycin, 600 nmol/L 17-AAG, or rapamycin plus 17-AAG, and viable cells were determined by trypan blue exclusion assay. The data shown are the average of triplicate samples from 1 of 3 independent experiments. The P values shown were calculated by a two-tailed test.

is a logical strategy to include the inhibition of Akt activity in the treatment of MM. Full Akt activation requires phosphorylation at residues of both S473 and T308. mTOR1 regulates the activation of Akt through the phosphorylation of residue T308, but prior studies using TORC1 inhibitors have shown limited effect in complete inhibition of Akt. mTORC2 can phosphorylate Akt HM site at Ser473 through the growth factor dependent pathway, but there are also studies showing that the inhibition of Akt HM phosphorylation does not fully suppress Akt signaling [10, 12, 25, 29]. However, mTORC2 can phosphorylate the TM of Akt (T450) and cPKC proteins [16, 30], which is essential to maintain the stability of Akt and cPKC. In the absence of mTORC2, Akt, and cPKC TM phosphorylation is abolished and the stability of these proteins is reduced [16, 30]. Destabilization of Akt proteins gives us a totally new approach to induce an anticancer effect.

Rapamycin is one of the most classical mTORC1 inhibitors, which has been utilized therapeutically for years as immunosuppressant. Usually people think rapamycin (and its analogs) cannot completely inhibit TOR2 pathway in most cells. Recently, it has been proved that chronic exposure of certain kinds of cells to rapamycin can inhibit mTORC2

pathway, though the precise mechanism is unclear [12, 26]. In our study, after myeloma cell lines were treated in the presence of rapamycin extended to 48 h, two important mTORC2 downstream targets—Akt S473 and T450 phosphorylation—were inhibited, which made us believe that myeloma cells mTORC2 pathway was inhibited after chronic exposure to rapamycin.

When Akt lack TM phosphorylation, HSP90 associates with Akt proteins and rescues the stability of the newly synthesized Akt proteins [16]. In this study, we utilized the ability of chronic rapamycin treatment to inhibit mTORC2 in myeloma cells. We show that the treatment of *myeloma* cells with prolonged rapamycin and HSP90 inhibitor 17-AAG results in the rapid loss of total Akt protein expressed in both myeloma cell lines (Figure 2). Overall, our data demonstrate that the combined inhibition of mTORC2 and HSP90 promotes the destabilization of Akt proteins and increases the capacity of mTOR inhibitors and 17-AAG to elicit an antimyeloma effect.

Due to the critical role of Akt in regulating cell survival, we predicted that prolonged rapamycin and 17-AAG dependent reduction of Akt expression would promote the cell death of *myeloma* cells. Indeed, prolonged rapamycin and 17-AAG treatment induced substantially more cell death than any single drug treatment. These data provide strong in vitro evidence and suggest that the dual inhibition of mTOR plus HSP90 may serve as an effective antimyeloma therapy.

Recently, one of our authors reported that the HSP90 inhibitor 17-AAG induced tumor regression when combined with pharmacologic and genetic inhibition of mTORC2 in a mouse model of leukemia cells [18]. In that study the author first presented in vitro data showing that 17-AAG treatment induced substantial Akt expression reduction and more cell death in $Sin1^{-/-}$ pre-B leukemia cells than wild type pre-B leukemia cells. After reconstitution of human Sin1 in $Sin1^{-/-}$ pre-B leukemia cells, there was increased resistance to 17-AAG mediated cell death. Then in vivo experiments supported these in vitro studies. Authors transplanted $Sin1^{+/+}$ or $Sin1^{-/-}$ p210 BCR-Abl transformed mouse leukemia cells into wild type mice and treated the recipients with 17-AAG or vehicle for five days. There were equivalent numbers of $Sin1^{+/+}$ and $Sin1^{-/-}$ leukemia cells recovered from the bone marrow and spleens of vehicle treated mice indicating Sin1 gene status does not alter leukemia cells growth. However, after 17AGG treatment, $Sin1^{-/-}$ pre-B cell tumor burden was significantly reduced both in the bone marrow and in the spleen while the $Sin1^{+/+}$ leukemia cell numbers were not varied much by 17-AAG. Authors also presented data showing that 17-AAG synergized with rapamycin induced a cytotoxic response causing leukemia cells regression. These data provide strong evidence that the inhibition of mTORC2 sensitizes leukemia cells to 17-AAG.

Our data and leukemia mouse model indicate that inhibition of both mTORC2 and HSP90 will produce a synergistic antitumor effect which is more superior to the inhibition of the mTOR or chaperon pathway alone. The directly targeted mTORC2 inhibitors are currently under development but have not yet been approved for clinical use. Rapamycin (and its analogues) cannot directly inhibit mTORC2 pathway, but our research indicates that chronic rapamycin treatment may block mTORC2 complex assembly in myeloma cell lines U266 and KM3 as in many other cell types [12, 16, 26, 31]. Therefore it is hopeful to propose an antimyeloma strategy by rapamycin treatment synergized with HSP90 inhibitors such as 17-AAG. Furthermore, considering the immunosuppressive and metabolic side effects of rapamycin, we predict that molecules directly inhibiting mTORC2 will be an important new target which can be used in combination with chaperon inhibitors to achieve better cure outcomes for patients with hematologic malignancies.

Conflict of Interests

All authors declare that there are no competing financial interests.

Authors' Contribution

Yanan Zhang and Yunfeng Fu performed the experiments; Fan Zhang and Jing Liu analyzed the data and wrote the paper. Yanan Zhang and Yunfeng Fu contributed equally to this work.

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