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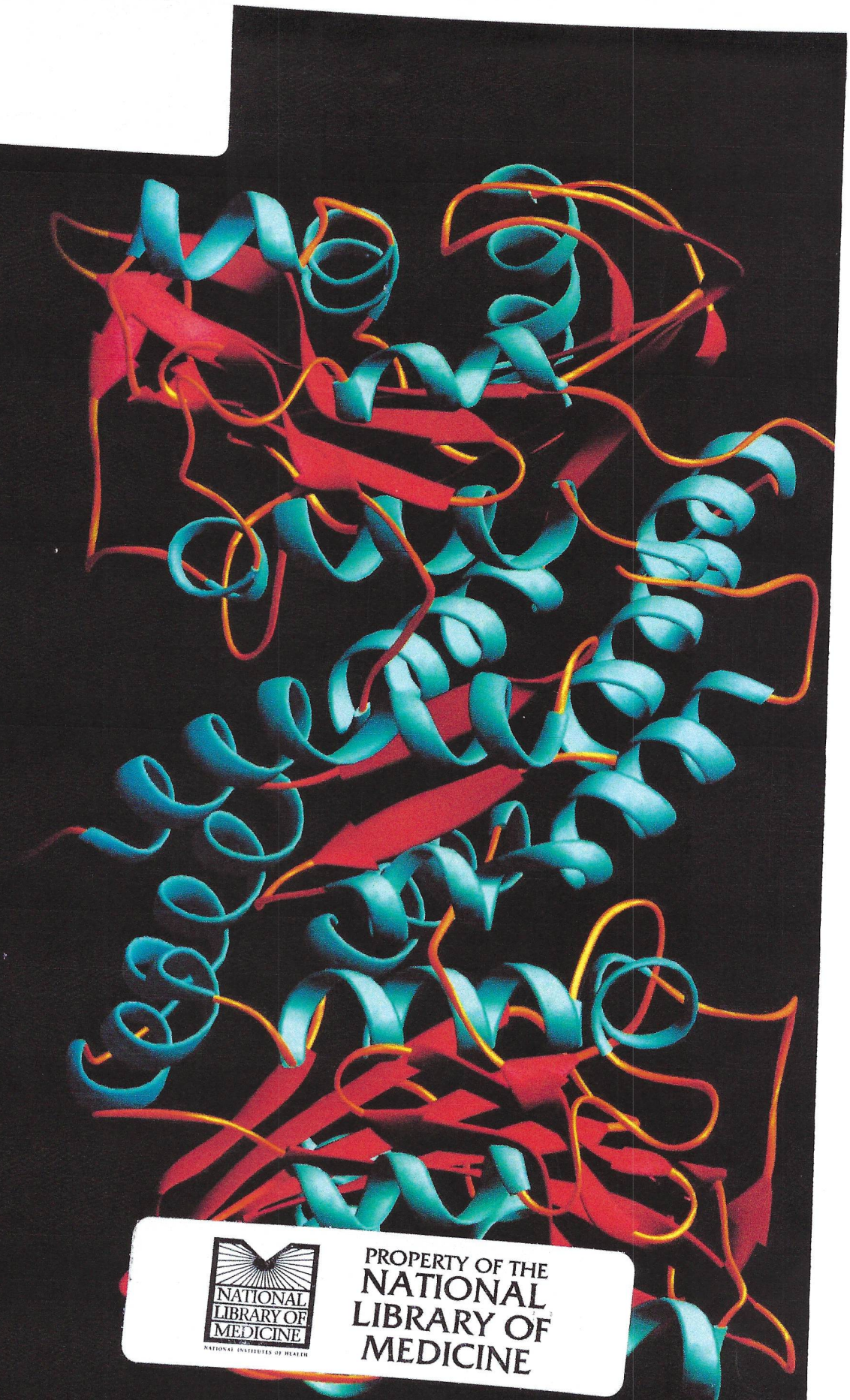

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Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy

Teru Hideshima, Dharminder Chauhan, Yoshihito Shima, Noopur Raje, Faith E. Davies, Yu-Tzu Tai, Steven P. Treon, Boris Lin, Robert L. Schlossman, Paul Richardson, George Muller, David I. Stirling, and Kenneth C. Anderson

Although thalidomide (Thal) was initially used to treat multiple myeloma (MM) because of its known antiangiogenic effects, the mechanism of its anti-MM activity is unclear. These studies demonstrate clinical activity of Thal against MM that is refractory to conventional therapy and delineate mechanisms of anti-tumor activity of Thal and its potent analogs (immunomodulatory drugs [IMiDs]). Importantly, these agents act directly, by inducing apoptosis or G1 growth arrest, in MM cell lines and in patient MM cells that are resistant to melphalan, doxorubicin, and dexamethasone (Dex). Moreover, Thal and the IMiDs enhance the anti-MM activity of Dex and, conversely, are inhibited by interleukin 6. As for Dex, apoptotic signaling triggered by Thal and the IMiDs is associated with activation of related adhesion focal tyrosine kinase. These studies establish the framework for the development and testing of Thal and the IMiDs in a new treatment paradigm to target both the tumor cell and the microenvironment, overcome classical drug resistance, and achieve improved outcome in this presently incurable disease. (Blood. 2000;96:2943-2950)

Introduction

Thalidomide (Thal) was originally used in Europe for the treatment of morning sickness in the 1950s but was withdrawn from the market in the 1960s because of reports of teratogenicity and phocomelia associated with its use. The renewed interest in Thal stems from its broad spectrum of pharmacologic and immunologic effects.[1] Because of its immunomodulatory and antiangiogenic effects, it has been used to effectively treat erythema nodosum leprosum, an inflammatory manifestation of leprosy.[2] Potential therapeutic applications span a wide spectrum of diseases, including cancer and related conditions, infectious diseases, autoimmune diseases, dermatologic diseases, and other disorders such as sarcoidosis, macular degeneration, and diabetic retinopathy.[3] Recent reports of increased bone marrow (BM) angiogenesis in multiple myeloma (MM),[4,5] coupled with the known antiangiogenic properties of Thal,[6] provided the rationale for its use to treat MM.[7] Importantly, Thal induced clinical responses in 32% of MM patients whose disease was refractory to conventional and high-dose therapy,[7] suggesting that it can overcome drug resistance because of its alternative mechanisms of anti-MM activity. Besides alkylating agents and corticosteroids, Thal now, therefore, represents the third distinct class of agents useful in the treatment of MM.

Given its broad spectrum of activities, Thal may be acting against MM in several ways.[8] First, Thal may have a direct effect on the MM cell and/or BM stromal cell to inhibit their growth and survival. For example, free radical–mediated oxidative DNA damage may play a role in the teratogenicity of Thal[9] and may also have anti-tumor effects. Second, adhesion of MM cells to BM stromal cells both triggers secretion of cytokines that augment MM cell growth and survival[10-12] and confers drug resistance[13]; Thal modulates adhesive interactions[14] and, thereby, may alter tumor cell growth, survival, and drug resistance. Third, cytokines secreted into the BM microenvironment by MM and/or BM stromal cells, such as interleukin (IL)-6, IL-1β, IL-10, and tumor necrosis factor (TNF)–α, may augment MM cell growth and survival,[12] and Thal may alter their secretion and bioactivity.[15] Fourth, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor 2 (bFGF-2) are secreted by MM and/or BM stromal cells and may play a role both in tumor cell growth and survival, as well as BM angiogenesis.[5,16] Given its known antiangiogenic activity,[6] Thal may inhibit activity of VEGF, bFGF-2, and/or angiogenesis in MM. However, Singhal et al.[7] observed no correlation of BM angiogenesis with response to Thal, suggesting that it may not be mediating anti-MM activity by its antiangiogenic effects. Finally, Thal may be acting against MM by its immunomodulatory effects, such as induction of a Th1 T-cell response with secretion of interferon gamma (IFN-γ) and IL-2.[17] Already 2 classes of Thal analogs have been reported, including phosphodiesterase 4 inhibitors that inhibit TNF-α but do not enhance T-cell activation (selected cytokine inhibitory drugs [SelCIDs]) and others that are not phosphodiesterase 4 inhibitors but markedly stimulate T-cell proliferation as well as IL-2 and IFN-γ production (immunomodulatory drugs [IMiDs]).[15]

In this study, we have begun to characterize the mechanisms of activity of Thal and these analogs against human MM cells. Delineation of their mechanisms of action, as well as mechanisms of resistance to these agents, will both enhance understanding of MM disease pathogenesis and derive novel treatment strategies.

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Materials and methods

MM-derived cell lines and patient cells

Dexamethasone (Dex)-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL). Doxorubicin (Dox)-, mitoxantrone (Mit)-, and melphalan (Mel)-sensitive and -resistant RPMI-8226 human MM cells were kindly provided by Dr William Dalton (Moffitt Cancer Center, Tampa, FL). RPMI-8226 cells resistant to Dox, Mit, and Mel included Dox 6 and Dox 40 cells, MR20 cells, and LR5 cells, respectively. Hs Sultan human MM cells were obtained from the American Type Culture Collection (Rockville, MD). All MM cell lines were cultured in RPMI-1640 media (Sigma Chemical, St Louis, MO) that contained 10% fetal bovine serum, 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO). Drug-resistant cell lines were cultured with either Dox, Mit, Mel, or Dex to confirm their lack of drug sensitivity. MM patient cells (96% $CD38^+CD45RA^-$) were purified from patient BM samples, as previously described.[18]

Thal and analogs

Thal and analogs (Celgene, Warren, NJ) were dissolved in DMSO (Sigma) and stored at −20°C until use. Drugs were diluted in culture medium (0.0001 to 100 μM) with < 0.1% DMSO immediately before use. The Thal analogs used in this study were 4 SelCIDs (SelCIDs 1, 2, 3, and 4), which are phosphodiesterase 4 inhibitors that inhibit TNF-α production and increase IL-10 production from lipopolysaccharide (LPS)–stimulated peripheral blood mononuclear cells (PBMCs) but do not stimulate T-cell proliferation; and 3 IMiDs (IMiD1, IMiD2, and IMiD3), which do stimulate T-cell proliferation, as well as IL-2 and IFN-γ secretion, but are not phosphodiesterase 4 inhibitors. The IMiDs also inhibit TNF-α, IL-1β, and IL-6 and greatly increase IL-10 production by LPS-stimulated PBMCs.[15]

DNA synthesis

DNA synthesis was measured as previously described.[19] MM cells (3×10^4 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media, Thal, SelCID1, SelCID2, SelCID3, SelCID4, IMiD1, IMiD2, IMiD3, and/or recombinant IL-6 (50 ng/mL) (Genetics Institute, Cambridge, MA) for 48 hours at 37°C. DNA synthesis was measured by [^{3}H]-thymidine (^{3}H-TdR; NEN Products, Boston, MA) uptake. Cells were pulsed with ^{3}H-TdR (0.5 μCi/well) during the last 8 hours of 48-hour cultures, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted by using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). All experiments were performed in triplicate.

Colorimetric assays were also performed to assay drug activity. Cells from 48-hour cultures were pulsed with 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT; Chemicon International Inc, Temecula, CA) to each well for 4 hours, followed by 100 μL isopropanol that contained 0.04 HCl. Absorbance readings at a wavelength of 570 nm were taken on a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Cell cycle analysis

MM cells (1×10^6) cultured for 72 hours in media alone, Thal, IMiD1, IMiD2, and IMiD3 were harvested, washed with phosphate-buffered saline (PBS), fixed with 70% ethanol, and pretreated with 10 μg/mL of RNAse (Sigma). Cells were stained with propidium iodide (PI; 5 μg/mL; Sigma), and cell cycle profile was determined by using the program M software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL), as in prior studies.[20]

Detection of apoptosis

In addition to identifying sub-G1 cells as described above, apoptosis was also confirmed by using annexin V staining. MM cells were cultured in media (0.01% DMSO) or with 10 μmol/L of Thal or 1 μmol/L IMiD1, IMiD2, and IMiD3 at 37°C for 72 hours, with addition of drugs at 24-hour intervals. Cells were then washed twice with ice-cold PBS and resuspended (1×10^6 cells/mL) in binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). MM cells (1×10^5) were incubated with annexin V-FITC (5 μL; Pharmingen, San Diego, CA) and PI (5 μg/mL) for 15 minutes at room temperature. Annexin V+PI− apoptotic cells were enumerated by using the Epics cell sorter (Coulter).

Immunoblotting

MM cells were cultured with 10 μmol/L of Thal, IMiD1, IMiD2, or IMiD3; harvested; washed; and lysed using lysis buffer: 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton-X 100, 30 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 5 μg/mL leupeptin, and 5 μg/mL aprotinin. For detection of p21, cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-p21 antibody (Ab; Santa Cruz Biotech, Santa Cruz, CA). The membrane was stripped and reprobed with anti–alpha tubulin Ab (Sigma) to ensure equivalent protein loading. For detection of p53, cell lysates were prepared from MM cells (2×10^7) with the use of lysis buffer. Lysates were incubated with anti-mutant (mt) or wild-type (wt) p53 monoclonal Abs (Calbiochem, San Diego, CA) and then immunoprecipitated overnight with protein A Sepharose (Sepharose CL-4B; Pharmacia, Uppsala, Sweden). Immune complexes were analyzed by immunoblotting with horseradish peroxidase–conjugated anti-p53 Ab reactive with both mt and wt p53 (Calbiochem).

To characterize growth signaling, immunoblotting was also done with anti-phospho–specific MAPK Ab (New England Biolabs, Beverly, MA) in the presence or absence of IL-6 (Genetics Institute) and/or the MEK 1 inhibitor PD98059 (New England Biolabs), as in prior studies.[21] Antigen-antibody complexes were detected by using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Blots were stripped and reprobed with anti-ERK2 Ab (Santa Cruz Biotech) to ensure equivalent protein loading.

To characterize apoptotic signaling, MM cells were cultured with 100 μmol/L of Thal, IMiD1, IMiD2, or IMiD3; harvested; washed; and lysed in 1 mL of lysis buffer (50 mmol/L Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin), as in prior studies.[22] Lysates were incubated with anti-related adhesion focal tyrosine kinase (RAFTK) Ab for 1 hour at 4°C and then for 45 minutes after the addition of protein G–agarose (Santa Cruz Biotech). Immune complexes were analyzed by immunoblotting with anti-P-Tyr Ab (RC20; Transduction Laboratories, Lexington, KY) or anti-RAFTK Abs. Proteins were separated by electrophoresis in 7.5% SDS-PAGE gels, transferred to nitrocellulose paper, and analyzed by immunoblotting. The antigen-antibody complexes were visualized by chemiluminescence.

Statistical analyses

Statistical significance of differences observed in drug-treated versus control cultures was determined by using the Student *t* test. The minimal level of significance was $P < .05$.

Results

Treatment of MM patients with Thal

Seventeen (39%) of 44 patients with MM treated at our institute responded to Thal (Table 1). This response included 6 men and 11 women. These patients had received a median of 4 (1-9) prior treatment regimens, and 10 patients had a prior high-dose therapy and hematopoietic stem cell transplant. One patient achieved

Table 1. Response to thalidomide in multiple myeloma*

Patient	Sex†	Prior therapies	Prior stem cell transplant	Maximum change M protein‡	Duration of thalidomide therapy (mo)	Maximum daily dose thalidomide	Current status (daily thalidomide dose)
1	M	3	Yes	− 58%(PR)	8.5	200 mg	Continued response (200 mg)
2	F	5	No	− 78%(PR)	6.0	400 mg	Continued response (400 mg)
3	F	1	Yes	+ 16%(SD)	6.5	100 mg	Continued response (100 mg)
4	M	6	No	− 56%(PR)	9.0	200 mg	Continued response (200 mg)
5	F	1	No	− 62%(PR)	5.5	200 mg	Continued response (50 mg)
6	F	5	Yes	− 100%(CR)	13	500 mg	Continued response (50 mg)
7	M	9	Yes	− 54%(PR)	10	800 mg	Progressed (800 mg)
8	F	5	Yes	− 68%(PR)	4.0	200 mg	Continued response, discontinued
9	F	5	No	− 90%(PR)	7.5	400 mg	Continued response (400 mg)
10	M	5	Yes	− 9%(SD)	1.5	400 mg	Progressed
11§	F	4	Yes	− 59%(PR)	5.5	400 mg	Progressed
12§	M	4	Yes	− 64%(PR)	7.0	400 mg	Progressed
13§	F	3	Yes	− 14%(SD)	4.5	400 mg	Progressed
14§	F	2	Yes	− 55%(PR)	4.0	800 mg	Continued response (800 mg)
15	F	1	No	− 31%(SD)	6.0	400 mg	Continued response (400 mg)
16	F	1	No	− 12%(SD)	4.5	400 mg	Progressed
17	M	2	No	− 55%(PR)	6.0	200 mg	Continued response (100 mg)

*As of January 1, 2000.

†Male (M) or female (F).

‡Partial response (PR) is ≥ 50% decrease in M protein; complete response (CR) is absence of M protein on immunofixation and normal bone marrow biopsy; stable disease (SD) is < 50% decrease in M protein; progression is ≥ 25% increase in M protein or progressive clinical disease.

§Also received decadron therapy.

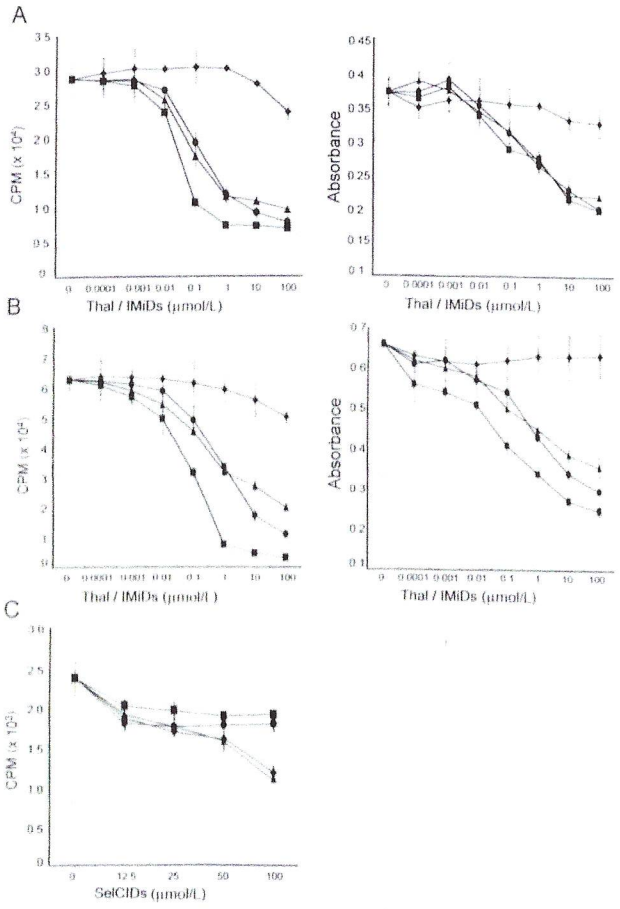

Figure 1. Effect of Thal and analogs on DNA synthesis of MM cell lines and patient cells. MM.1S (A) and Hs Sultan (B) cells were cultured with increasing concentrations (0.0001-100 μM) of Thal (◆), IMiD1 (■), IMiD2 (●), and IMiD3 (▲). (C) MM.1S cells were cultured with increasing concentrations (12.5-100 μM) of SelCID1 (◆), SelCID2 (■), SelCID3 (▲), and SelCID4 (●). In each case ^{3}H-TdR uptake (left panels) or MTT cleavage (right panels) were measured during the last 8 and 4 hours, respectively, of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) or absorbance of triplicate cultures.

complete response (absence of monoclonal protein on immunofixation and normal BM biopsy), 11 patients achieved partial response (> 50% decrease in monoclonal protein), and 5 patients achieved stable disease (< 50% decrease in monoclonal protein). Patients received a median of 400 mg (range, 100-800 mg) maximum dose of daily Thal for a median of 6 months (range, 1.5-13 months). As of January 1, 2000, 11 patients have continued response at a median of 6 months (range, 4-13 months), and 6 patients have progressed at a median of 4.5 months (range, 1.5-10 months).

Effect of Thal and analogs on DNA synthesis by MM cell lines and patient MM cells

The effect of Thal and its analogs, including IMiD1, IMiD2, IMiD3, SelCID1, SelCID2, SelCID3, and SelCID4, on DNA synthesis of MM cell lines (MM.1S, Hs Sultan, U266, and RPMI-8226) was determined by measuring ^{3}H-TdR uptake during the last 8 hours of 48-hour cultures, in the presence or absence of drug at various concentrations. IMiD1, IMiD2, and IMiD3 inhibited ^{3}H-TdR uptake of MM.1S (Figure 1A) and Hs Sultan (Figure 1B) cells in a dose-dependent fashion. Fifty percent inhibition of proliferation of MM.1S cells was noted at 0.01-0.1 μmol/L IMiD1, 0.1-1.0 μmol/L IMiD2, and 0.1-1.0 μmol/L IMiD3 ($P < .001$). Fifty percent inhibition of proliferation of Hs Sultan cells was noted at 0.1 μmol/L IMiD1, 1.0 μmol/L IMiD2, and 1.0 μmol/L IMiD3 ($P < .001$). In contrast, only 15% and 20% inhibition in MM.1S and Sultan cells, respectively, were observed in cultures at even higher concentrations (100 μmol/L) of Thal. No significant inhibition of DNA synthesis of U266 MM cells was noted in cultures with 0.001 to 100 μmol/L Thal or these IMiDs (data not shown). The effects of these drugs on proliferation were confirmed by using MTT assays for MM.1S cells (Figure 1A) and Hs Sultan cells (Figure 1B). Although there was also a dose-dependent inhibition of proliferation of MM.1S cells by SelCIDs, 50% inhibition was observed only at high doses (100 μmol/L) for only 2 of the 4 SelCIDs (SelCIDs 1 and 3, Figure 1C). Further studies, therefore, focused on Thal and the IMiDs.

Effect of Thal and analogs in DNA synthesis of MM cells resistant to conventional therapy

To examine whether there was cross-resistance between Thal and the IMiDs with conventional therapies, RPMI-8226 MM cells resistant to Dox (Dox6 and Dox40 cells), Mit (MR20 cells), or Mel (LR5 cells), and MM.1R cells resistant to Dex were similarly studied. Proliferation of Dox6 and Dox40, MR20, LR5, or MM1.R cells is unaffected by culture with 60 nmol/L and 400 nmol/L Dox, 20 nmol/L Mit, 5 μmol/L Mel, and 1 μmol/L Dex, respectively (data not shown). Importantly, ^{3}H-TdR uptake of Dox6, Dox40, MR20, or LR5 was inhibited in cultures with Thal and the IMiDs in a dose-dependent manner (1-100 μmol/L) versus media alone cultures (Figure 2A-D). For example, 10 μmol/L IMiD1 blocked proliferation of Dox6, Dox40, MR20, and LR5 cells by 20%, 33%, 32%, and 21%, respectively ($P < .001$). The IMiDs similarly inhibited DNA synthesis of MM.1R cells in a dose-dependent fashion, with more than 50% inhibition at more than 1 μmol/L IMiD1 ($P < .001$; Figure 2E). These data suggest independent mechanisms of resistance to Dox, Mit, Mel, and Dex versus Thal and its analogs.

Effect of Dex and IL-6 on response of MM cells to Thal and the ImiDs

To determine whether the effects of Thal and the IMiDs are additive with conventional therapies, we next examined the effect of Dex (0.001-0.1 μmol/L) together with 1 μmol/L Thal or IMiDs on proliferation of Dex-sensitive MM.1S cells. As can be seen in Figure 3A, the IMiDs (1 μmol/L) significantly inhibited ^{3}H-TdR uptake of MM.1S cells (60%-75% block, $P < .01$), and Dex (0.001-0.1 μmol/L) increased this inhibition in a dose-dependent fashion. For example, doses of 0.001 to 0.01 μmol/L Dex added to 1 μmol/L IMiD1 increased the inhibition of proliferation by 35% relative to cultures with 1 μmol/L IMiD1 alone ($P < .01$). Given the additive effects of Dex and the IMiDs, as well as the known role of IL-6 as a growth factor and specific inhibitor of Dex-induced MM cell apoptosis,[19,22,23] we also examined whether exogenous IL-6 could overcome the inhibition of DNA synthesis triggered by Thal and the IMiDs. Figure 3B demonstrates that IL-6 (50 ng/mL) triggers DNA synthesis of MM.1S cells in cultures with media alone, as well as in cultures with the IMiDs (0.1 and 1 μmol/L).

Effect of Thal and analogs on DNA synthesis of patient MM cells

The effect of Thal and the IMiDs on DNA synthesis of patient MM cells was next examined (Figure 4). As was true for MM.1S and Hs Sultan MM cell lines, ^{3}H-TdR uptake of patients' MM cells was also inhibited by IMiDs (0.1-100 μmol/L) in a dose-dependent fashion, whereas the inhibitory effect of Thal, even at 100 μmol/L, was not significant. Fifty percent inhibition of MM patient cells was observed at 100 μmol/L (Figure 4A) and 1 μmol/L (Figure 4B) IMiD1, respectively ($P < .001$).

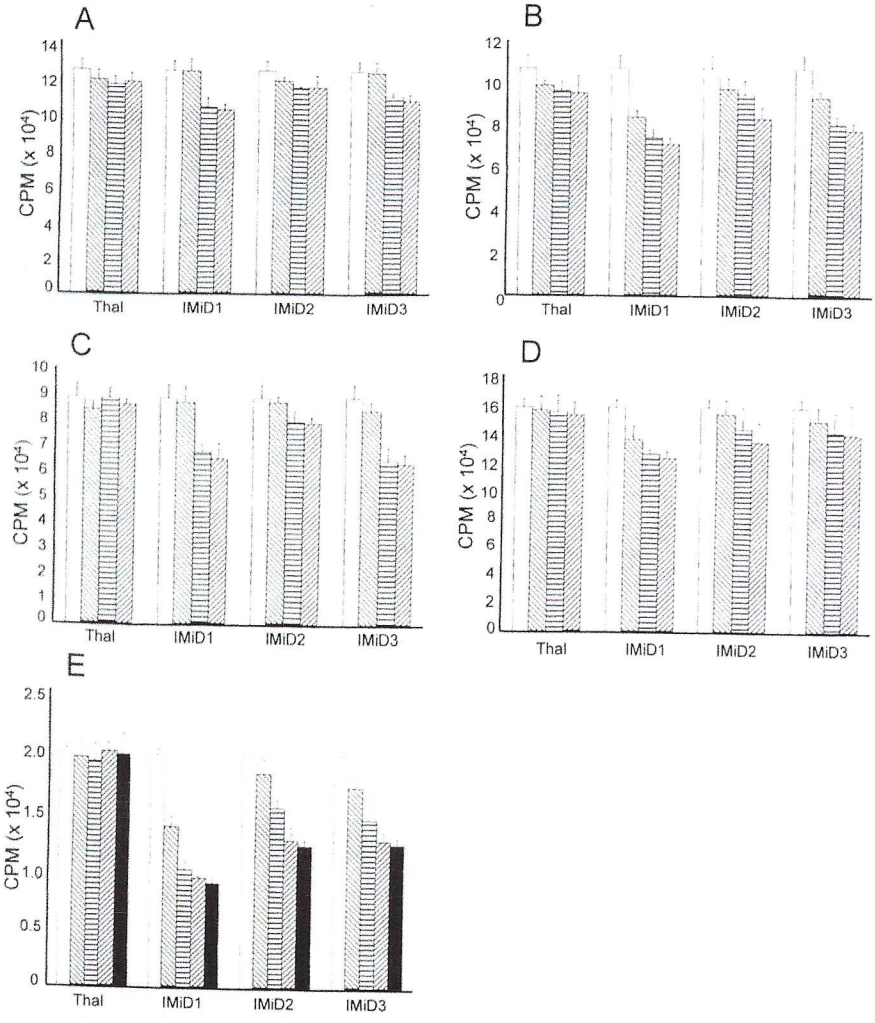

Figure 2. Effect of Thal and analogs on DNA synthesis of MM cells resistant to conventional therapy. Dox-resistant Dox6 (A) and Dox 40 (B), Mit-resistant (MR20; C), and Mel-resistant (LR5; D) cells were cultured with control media (□) or 1 μmol/L (▨), 10 μmol/L (▩), 100 μmol/L (■) of Thal, IMiD1, IMiD2, or IMiD3. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures. (E) Dex-resistant MM.1R cells were cultured in control media (□) or with 0.1 μmol/L (▨), 1 μmol/L (▩), 10 μmol/L (▩), or 100 μmol/L (■) of Thal, IMiD1, IMiD2, or IMiD3.

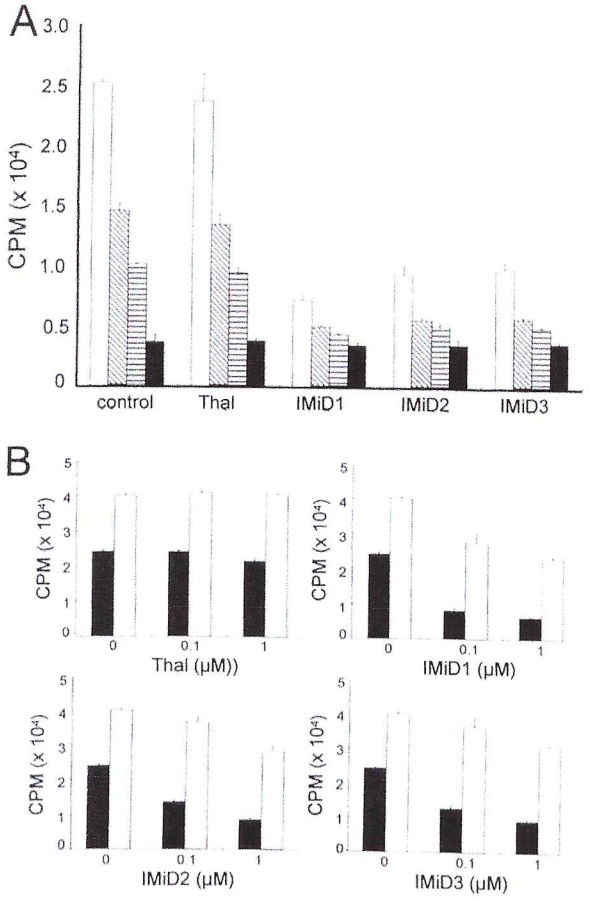

Figure 3. Effect of Dex and IL-6 on response of MM cells to Thal and the IMiDs. (A) MM.1S cells were cultured with 1.0 μM Thal, IMiD1, IMiD2, or IMiD3 in control media alone (□) or with 0.001 (▨), 0.01 (▤), and 0.1 μmol/L (■) Dex. (B) MM.1S cells were cultured in control media alone and with 0.1 and 1.0 μmol/L Thal, IMiD1, IMiD2, or IMiD3 either in the presence (□) or absence (■) of IL-6 (50 ng/mL). In each case, ^{3}H-TdR uptake was measured during the last 8 hours of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures.

Effect of Thal and analogs on cell cycle profile of MM cell lines and patient MM cells

To further analyze the mechanism of Thal- and IMiD-induced inhibition of DNA synthesis and to determine whether these drugs induced apoptosis of MM cells, we first examined the cell cycle profile of MM.1S, Hs Sultan cells, and patient MM cells cultured with media alone, Thal (10 μmol/L), or the IMiDs (1 μmol/L). Cells were harvested from 72-hour cultures and stained with PI. As shown in Figure 5A, all 3 IMiDs, and Thal to a lesser extent, increased sub-G1 MM.1S cells. Induction of apoptosis occurred at the dose-response curve noted for inhibition of proliferation. Twelve-hour cultures with Dex (10 μmol/L) served as a positive control for triggering increased sub-G1 cells. In contrast, no increase in sub-G1 cells was observed in cultures of Hs Sultan cells or of patient MM cells with Thal or the IMiDs. Importantly, Thal and the IMiDs induced G1 growth arrest in both Hs Sultan cells and in AS patient MM cells.

To confirm these results, we performed annexin V staining of cells in these cultures. As can be seen in Figure 5B, the percentage of annexin V–positive cells in cultures of MM.1S cells with Thal, IMiD1, IMiD2, and IMiD3 was 32%, 55%, 51%, and 43%, respectively. Forty-six percent of annexin V staining was observed in cultures with Dex, whereas only 22% annexin V–positive cells were present in cultures with media alone. The percentage of annexin V–positive Hs Sultan cells and AS patient MM cells was 4% to 7%, respectively, under all culture conditions and was not increased by Thal or the IMiDs.

Effect of Thal and analogs on p21 expression in MM cell lines and patient cells

We next correlated these distinct biologic sequelae of Thal and the IMiDs with p21 status in MM.1S versus Hs Sultan and patient MM cells. As can be seen in Figure 6A, p21 expression was down-regulated by the IMiDs, as well as by Dex, in MM1.S cells; and IL-6 overcomes this inhibitory effect. In contrast, the IMiDs up-regulated p21 in Hs Sultan cells and patient MM cells. Immunoblotting with anti-tubulin Ab confirmed equivalent protein loading. Wt-p53 was recognized in MM.1S cells, whereas both wt- and mt-p53 were recognized in Hs Sultan cells and patient MM cells (Figure 6B). These studies further support the observation that Thal and the IMiDs can induce either apoptosis or G1 growth arrest in sensitive MM cells, and they are consistent with Thal and IMiD p53-mediated down-regulation of p21 and susceptibility to p53-mediated apoptosis in MM.1S cells, in contrast to induction of p21 and growth arrest in Hs Sultan cells and patient MM cells, conferring protection from apoptosis.

Effect of Thal and analogs on growth and apoptotic signaling in MM.1S and MM.1R cells

We have previously characterized signaling cascades mediating MM cell growth and apoptosis, as well as the antiapoptotic effect of

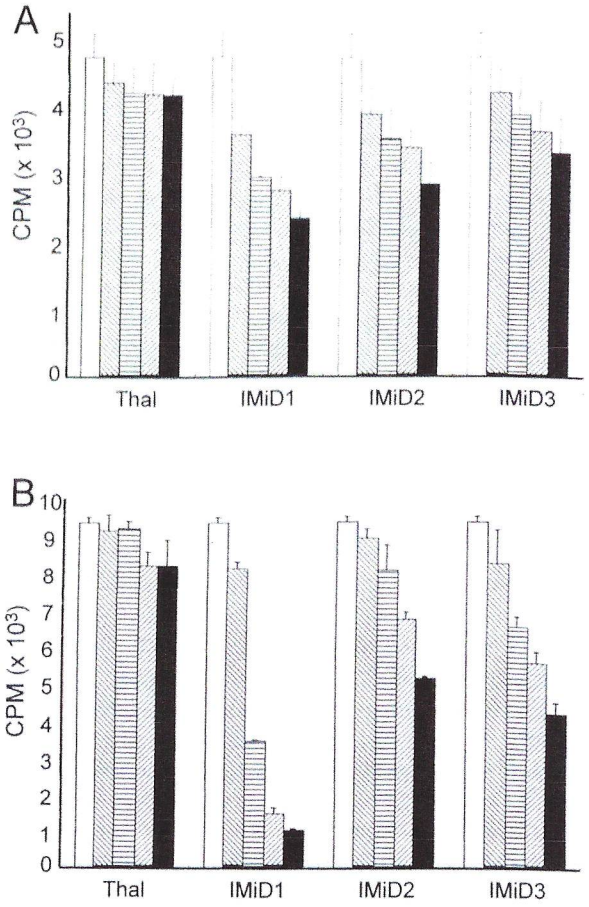

Figure 4. Effect of Thal and analogs on DNA synthesis of patient MM cells. MM cells from patient 1 (A) and patient 2 (B) were cultured with control media (□) or with 0.1 μmol/L (▨), 1.0 μmol/L (▤), 10 μmol/L (▥), and 100 μmol/L (■) Thal, IMiD1, IMiD2, or IMiD3. In each case, ^{3}H-TdR uptake was measured during the last 8 hours of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures.

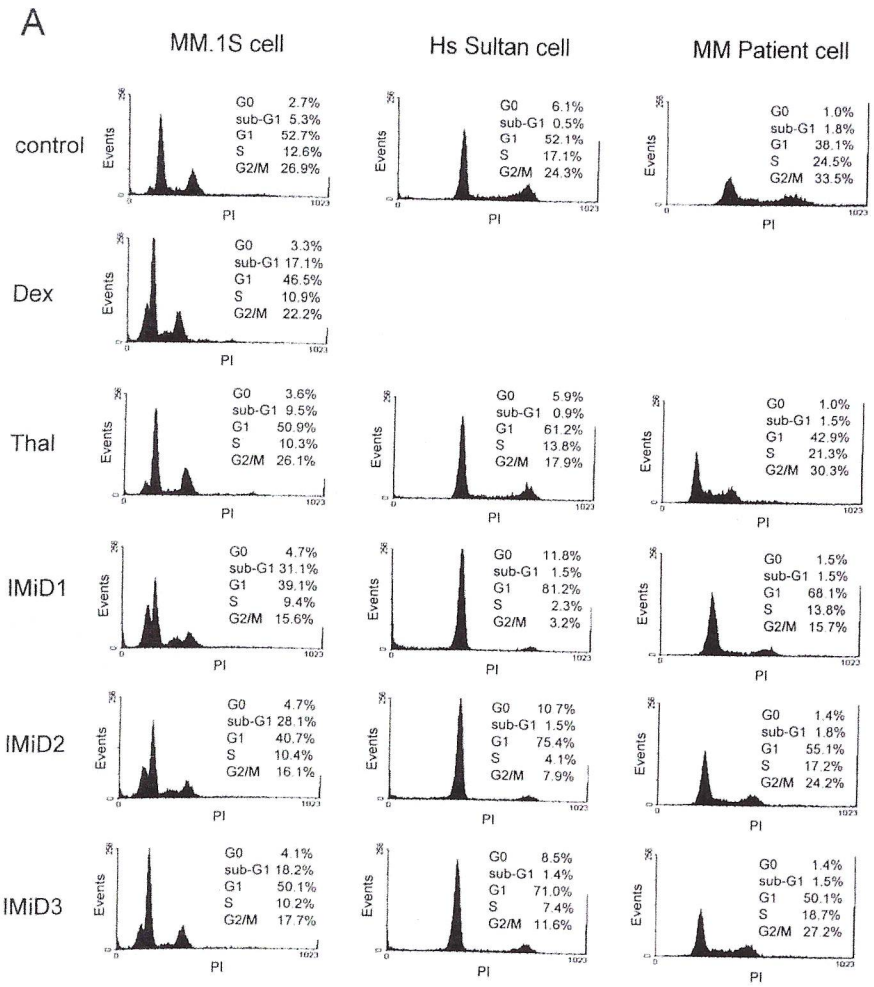

Figure 5. Effect of Thal and analogs on cell cycle profile of MM cell lines and patient MM cells. (A) MM.1S cells, Hs Sultan cells, and patient MM cells were cultured with 10 μmol/L of Thal or 1 μmol/L of IMiD1, IMiD2, or IMiD3 for 72 hours. Cultures in media control alone served as a negative control and 18-hour cultures with 10 μmol/L Dex as positive controls. Cells were then stained with PI, and cell cycle profile was determined by flow cytometric analysis. (B) These MM.1S (■), Hs Sultan (▨), and patient MM (□) cells were also stained with annexin V as an additional assay for apoptosis.

IL-6.[19,22-25] Because we have shown that IL-6–induced proliferation is mediated by the ras-dependent mitogen-activated protein kinase (MAPK) cascade,[19] we next examined the effect of Thal and the IMiDs on tyrosine phosphorylation of MAPK in IL-6–responsive MM.1S cells. Constitutive tyrosine phosphorylation of MAPK in MM.1S cells was down-regulated by the MEK1 inhibitor PD98059 (50 μmol/L), which served as a positive control (Figure 6A), and to a lesser extent by the IMiDs (1 μmol/L; Figure 7A) or Thal (10 μmol/L; data not shown). Treatment of MM.1S cells with IL-6 increased MAPK tyrosine phosphorylation, which was partially blocked by PD98059 but was unaffected by the IMiDs (Figure 7A) or Thal (data not shown). Stripping the blot and reprobing with anti-ERK2 Ab confirmed equivalent protein loading.

The observation that IL-6 can overcome the effects of Thal, the IMiDs, and Dex, coupled with our prior studies delineating signaling cascades mediating Dex-induced apoptosis and the protective effects of IL-6,[22,23,25] suggested that RAFTK activation may be induced during apoptosis triggered by Thal and IMiDs. MM.1S and MM.1R cells were, therefore, next cultured with 1 μmol/L Thal, IMiD1, IMiD2, or IMiD3 for 12 hours. Twelve-hour cultures with Dex (10 μmol/L) served as a positive control for activation of RAFTK. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK Ab and analyzed by immunoblotting with anti-P-Tyr Ab or anti-RAFTK Ab. As can be seen in Figure 7B, Dex induced tyrosine phosphorylation of RAFTK in MM.1S cells but not in MM.1R cells. Importantly, IMiD1 induced RAFTK tyrosine phosphorylation in both MM.1S and MM.1R

cells, correlating with its effects on both Dex-sensitive and Dex-resistant MM cells.

Discussion

This study demonstrates for the first time a direct dose-dependent effect of Thal and these analogs on tumor cells. Thal has demonstrated clinical anti-MM activity at the University of Arkansas[7] and in this study, and Thal at high concentrations (100 μmol/L) resulted in a modest (< 20%) inhibition of in vitro DNA synthesis of MM cells. SelCIDs also induced a dose-dependent inhibition of MM cells, but only 2 of 4 SelCIDs tested achieved 50% inhibition of proliferation, even at 100 μmol/L concentrations. Importantly, all 3 IMiDs tested achieved 50% inhibition of DNA synthesis at concentrations (0.1-1.0 μmol/L) corresponding to serum levels that are readily achievable, both confirming their direct action on tumor cells and suggesting their potential clinical utility. Moreover, the IMiDs inhibited the proliferation of Dox-, Mit-, and Mel-resistant MM cells by 20% to 35%, and of Dex-resistant MM cells by 50%. These in vitro effects correlate with the observed clinical activity of Thal in patients with MM that is refractory to conventional therapies, both at the University of Arkansas[7] and reported in this study, and suggest their clinical utility to overcome drug resistance. Moreover, our studies further suggest that Dex can add to the antiproliferative effect of Thal and the IMiDs in vitro, suggesting the potential utility of coupling these agents therapeutically. Finally, our study also identified MM cells resistant to Thal and the analogs (U266 cells), which, therefore, can be used to study mechanisms of Thal resistance.

Our studies demonstrate that Thal and the IMiDs are acting directly on MM cells, in the absence of accessory BM or T cells. It is also possible that these agents may be mediating their anti-MM effect by cytokines, given their known inhibitory effects on TNF-α, IL-1β, and IL-6.[15] Our prior studies have characterized the growth

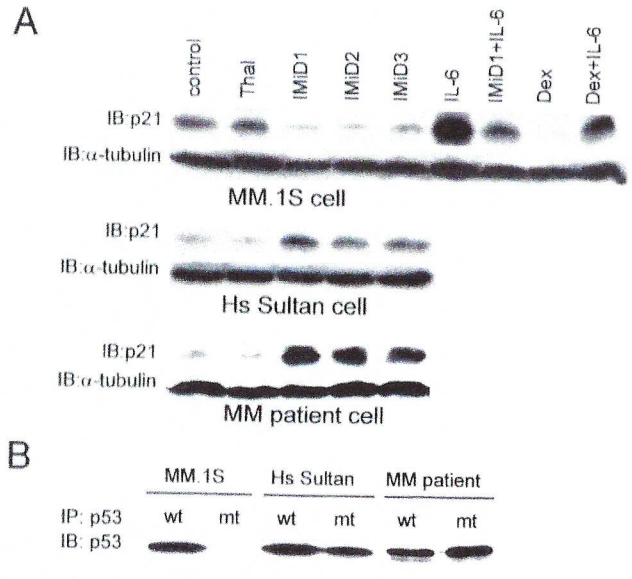

Figure 6. Effect of Thal and analogs on p21 expression in MM cell lines and patient cells. (A) MM.1S cells were cultured with 10 μmol/L of Thal, IMiD1, IMiD2, and IMiD3 for 48 hours. MM.1S cells were also cultured with IL-6 (50 ng/mL) alone and with IMiD1, 10 μmol/L Dex, and Dex plus IL-6. Cells were lysed, subjected to SDS-PAGE, transferred to PVDF membrane, and blotted with anti-p21 Ab. The membrane was stripped and reprobed with anti-α-tubulin Ab. (B) MM.1S, Hs Sultan, and patient MM cells were lysed and immunoprecipitated with wt-p53 and mt-p53 Abs, transferred to PVDF membrane, and blotted with anti-p53 Ab.

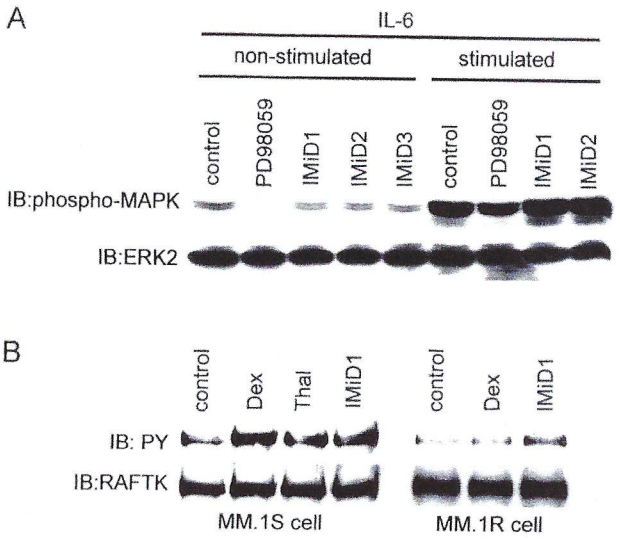

Figure 7. Effect of Thal and analogs on growth and apoptotic signaling in MM.1S and MM.1R cells. (A) MM.1S cells were cultured in media, with 50 μmol/L of PD98059 and with 10 μmol/L of IMiD1, IMiD2, or IMiD3 for 48 hours. Cells were then triggered with 50 ng/mL of IL-6 for 10 minutes, lysed, transferred to PVDF membrane, and blotted with anti-phospho MAPK Ab. Blots were stripped and reprobed with anti-ERK2 Ab. (B) MM.1S and MM.1R cells were treated with Thal (100 μM), IMiD1 (100 μmol/L), or Dex (10 μmol/L) and harvested at 12 hours. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK Ab and analyzed by immunoblotting with anti-P-Tyr Ab or anti-RAFTK Ab.

effects of IL-6 on human MM cells,[12,26] and we, therefore, next determined the effect of exogenous IL-6 on drug activity. Our studies showed that IL-6 can overcome the effect of Thal and the IMiDs on MM cell lines and patient cells, suggesting that these novel drugs may, at least in part, be inhibiting IL-6 production. Our prior studies have further demonstrated that IL-6–induced proliferation of MM cells is mediated through the MAPK cascade and that blockade of this pathway with either MAPK antisense oligonucleotide or the MEK1 inhibitor PD98059 can abrogate this response.[19,21,24] The present study showed constitutive MAPK phosphorylation in MM cells that is inhibited by PD98059 and, to a lesser extent, by the IMiDs. Importantly, IL-6–triggered MAPK tyrosine phosphorylation is also blocked by PD98059 but not by IMiDs. These studies, therefore, suggest that the IMiDs do not work only by directly inhibiting MAPK growth signaling and further support their potential activity in down-regulating IL-6 production. In MM, IL-6 production in tumor cells can either be constitutive or induced, mediating autocrine tumor cell growth.[26,27] In addition, IL-6 is also produced by BM stromal cells in MM, a process that is up-regulated by tumor cell adhesion to BM stromal cells, with related tumor cell growth in a paracrine mechanism.[10,11] Our ongoing studies are, therefore, evaluating the effect of Thal and these analogs on IL-6 production in the BM microenvironment.

Having shown the inhibitory effects of Thal and the IMiDs on ^{3}H-TdR uptake of tumor cells, we next examined their effect on MM cell cycle. Interestingly, these drugs had distinct functional sequelae in MM cells. Specifically, the IMiDs, and to a lesser extent Thal, induced apoptosis of MM.1S cells, evidenced both by increased sub-G1 cells on PI staining and increased annexin V–positive cells. In these cells that have wt p53, these agents (and Dex) down-regulate p21, thereby facilitating G1-to-S transition and susceptibility to apoptosis. This apoptotic effect may correlate with the clinical observation that complete response to Thal is rarely observed. IL-6 overcomes the down-regulation of p21 induced by these agents, consistent with the increase in DNA synthesis triggered by IL-6 even in the presence of these drugs. In

contrast, in Hs Sultan cells (wt and mt p53) and patient cells (wt p53 and mt p53), the IMiDs and Thal induce p21 and related G1 growth arrest, thereby conferring protection from apoptosis, as has been observed in other systems.[28,29] In our prior study,[20] p21 was also constitutively expressed in the majority of MM cells and also inhibited proliferation in both p53-dependent and -independent mechanisms. Previous reports that cells overexpressing p21 protein demonstrate chemoresistance[30] further support the protective effect of G1 growth arrest induced by these agents in Hs Sultan MM cells and patient MM cells. Conversely, the frequent regrowth of progressive MM noted clinically on discontinuation of Thal treatment may correlate with release of drug-related G1 growth arrest. An ongoing clinical trial is correlating response to Thal with laboratory parameters (ie, serum IL-6 or the surrogate marker C reactive protein) and will gain further insights into its mechanisms of in vivo anti-tumor activity.

Finally, our prior studies have characterized apoptotic signaling cascades in MM, as well as the protective effect of IL-6, especially against Dex-induced apoptosis.[22,23,25,31] Specifically, we have shown that Dex down-regulates growth kinases, such as MAPK and $p70^{RSK}$;[23] importantly, it activates RAFTK, which is required for Dex-induced apoptosis and abrogated by IL-6.[22] The current studies show that IMiD1 acts similarly to Dex, because it activates RAFTK and apoptosis in MM.1S cells, sequelae that are blocked by IL-6. Given our prior studies, which demonstrate that apoptosis of MM cells induced by UV irradiation, γ irradiation, and Fas ligation do not involve RAFTK,[22] the current signaling studies, therefore, further support both the ability of the IMiDs to act through distinct signaling cascades to overcome drug resistance, as well as the enhanced anti-tumor activity observed when Thal or the IMiDs are coupled with Dex.

In conclusion, the results of this study, therefore, demonstrate evidence for direct activity of Thal and the IMiDs against human MM cells. To confirm their in vivo mechanism of action, these compounds and SelCIDs will be examined in an animal model. Importantly, these studies provide the framework for the development and testing of a new biologically based treatment paradigm that uses these novel agents, either alone or together with conventional therapies, to target both the tumor cell and its microenvironment, overcome classical drug resistance, and achieve improved outcome in this presently incurable disease.

References

1. Hales BF. Thalidomide on the comeback trail. Nat Med. 1999;5:489-490.
2. Sampaio EP, Kaplan G, Miranda A, et al. The influence of thalidomide on the clinical and immunologic manifestation of erythema nodosum leprosum. J Infect Dis. 1993;168:408-414.
3. Anderson KC. Thalidomide: therapeutic potential in hematologic malignancies. Sem Hematol. 2000;37:1-4.
4. Ribatti D, Vacca A, Nico B, et al. Bone marrow angiogenesis and mast cell density increase simultaneously with progression of human multiple myeloma. Br J Cancer. 1999;79:451-455.
5. Vacca A, Ribatti D, Presta M, et al. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. Blood. 1999;93:3064-3073.
6. D'Amato RJ, Loughman MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci U S A. 1994;91:4082-4085.
7. Singhal S, Mehta J, Desikan R, et al. Anti-tumor activity of thalidomide in refractory multiple myeloma. N Engl J Med. 1999;341:1565-1571.
8. Raje N, Anderson KC. Thalidomide: a revival story. N Engl J Med. 1999;341:1606-1609.
9. Parman T, Wiley MJ, Wells PG. Free radical-mediated oxidative DNA damage in the mechanism of thalidomide teratogenicity. Nat Med. 1999;5:582-585.
10. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates IL-6 secretion. Blood. 1993;82:3712-3720.
11. Chauhan D, Uchiyama H, Akbarali Y, et al. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-κB. Blood. 1996;87:1104-1112.
12. Hallek M, Bergsagel PL, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. Blood. 1998;91:3-21.
13. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood. 1999;93:1658-1667.
14. Geitz H, Handt S, Zwingengerger K. Thalidomide selectively modulates the density of cell surface molecules involved in the adhesion cascade. Immunopharmacology. 1996;32:213-221.
15. Corral LG, Haslett PAJ, Muller GW, et al. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogs that are potent inhibitors of TNF-α. J Immunol. 1999;163:380-386.
16. Bellamy W, Richter L, Frutiger Y, Grogan T. Expression of vascular endothelial growth factor and its receptor in hematological malignancies. Cancer Res. 1999;59:728-733.
17. Haslett PAJ, Corral LG, Albert M, Kaplan G. Thalidomide costimulates primary human T lymphocytes, preferentially inducing proliferation, cytokine production, and cytotoxic responses in the $CD8^+$ subset. J Exp Med. 1998;187:1885-1892.
18. Tai YT, Teoh G, Shima Y, et al. Isolation and characterization of human multiple myeloma cell enriched populations. J Immunol Method. 2000;235:11-19.
19. Ogata A, Chauhan D, Teoh G, et al. Interleukin-6 triggers cell growth via the ras-dependent mitogen-activated protein kinase cascade. J Immunol. 1997;159:2212-2221.
20. Urashima M, Teoh G, Chauhan D, et al. Interleukin-6 overcomes $p21^{WAF1}$ upregulation and G1 growth arrest induced by dexamethasone and interferon-γ in multiple myeloma cells. Blood. 1997;90:279-289.
21. Hideshima T, Chauhan D, Teoh G, et al. Characterization of signaling cascades by human interleukin-6 versus Kaposi's sarcoma-associated herpesvirus encoded viral interleukin-6. Clin Cancer Res. 2000;6:1180-1189.
22. Chauhan D, Hideshima T, Pandey P, et al. RAFTK/PYK2-dependent and independent apoptosis in multiple myeloma cells. Oncogene. 1999;18:6733-6740.
23. Chauhan D, Pandey P, Ogata A, et al. Dexamethasone induces apoptosis of multiple myeloma cells in a JNK/SAP kinase independent mechanism. Oncogene. 1997;15:837-843.
24. Ogata A, Chauhan D, Urashima M, Teoh G, Treon SP, Anderson KC. Blockade of mitogen-activated protein kinase cascade signaling in interleukin-6 independent multiple myeloma cells. Clin Cancer Res. 1997;3:1017-1022.
25. Chauhan D, Kharbanda S, Ogata A, et al. Interleukin-6 inhibits Fas-induced apoptosis and stress-activated protein kinase activation in multiple myeloma cells. Blood. 1997;89:227-234.
26. Anderson KC, Jones RC, Morimoto C, Leavitt P, Barut B. Response of purified myeloma cells to hematopoietic growth factors. Blood. 1989;73:1915-1924.
27. Urashima M, Chauhan D, Uchiyama H, Freeman GJ, Anderson KC. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. Blood. 1995;85:1903-1912.
28. Jung J-M, Brunet JM, Ruan S, et al. Increased level of $p21^{WAF1,CIP1}$ in human brain tumors. Oncogene. 1998;11:2021-2028.
29. Polyak K, Waldman T, He T-C, Kinzler KW, Vogelstein B. Genetic determinants of p53-induced apoptosis and growth arrest. Genes Dev. 1996;10:1945-1952.
30. Wang J, Walsh K. Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. Science. 1996;273:359-361.
31. Chauhan D, Pandey P, Ogata A, et al. Cytochrome-c dependent and independent induction of apoptosis in multiple myeloma cells. J Biol Chem. 1997;272:29995-29997.

EXHIBIT C2

Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy

Teru Hideshima, Dharminder Chauhan, Yoshihito Shima, Noopur Raje, Faith E. Davies, Yu-Tzu Tai, Steven P. Treon, Boris Lin, Robert L. Schlossman, Paul Richardson, George Muller, David I. Stirling, and Kenneth C. Anderson

Although thalidomide (Thal) was initially used to treat multiple myeloma (MM) because of its known antiangiogenic effects, the mechanism of its anti-MM activity is unclear. These studies demonstrate clinical activity of Thal against MM that is refractory to conventional therapy and delineate mechanisms of anti-tumor activity of Thal and its potent analogs (immunomodulatory drugs [IMiDs]). Importantly, these agents act directly, by inducing apoptosis or G1 growth arrest, in MM cell lines and in patient MM cells that are resistant to melphalan, doxorubicin, and dexamethasone (Dex). Moreover, Thal and the IMiDs enhance the anti-MM activity of Dex and, conversely, are inhibited by interleukin 6. As for Dex, apoptotic signaling triggered by Thal and the IMiDs is associated with activation of related adhesion focal tyrosine kinase. These studies establish the framework for the development and testing of Thal and the IMiDs in a new treatment paradigm to target both the tumor cell and the microenvironment, overcome classical drug resistance, and achieve improved outcome in this presently incurable disease. (Blood. 2000;96:2943-2950)

Introduction

Thalidomide (Thal) was originally used in Europe for the treatment of morning sickness in the 1950s but was withdrawn from the market in the 1960s because of reports of teratogenicity and phocomelia associated with its use. The renewed interest in Thal stems from its broad spectrum of pharmacologic and immunologic effects.[1] Because of its immunomodulatory and antiangiogenic effects, it has been used to effectively treat erythema nodosum leprosum, an inflammatory manifestation of leprosy.[2] Potential therapeutic applications span a wide spectrum of diseases, including cancer and related conditions, infectious diseases, autoimmune diseases, dermatologic diseases, and other disorders such as sarcoidosis, macular degeneration, and diabetic retinopathy.[3] Recent reports of increased bone marrow (BM) angiogenesis in multiple myeloma (MM),[4,5] coupled with the known antiangiogenic properties of Thal,[6] provided the rationale for its use to treat MM.[7] Importantly, Thal induced clinical responses in 32% of MM patients whose disease was refractory to conventional and high-dose therapy,[7] suggesting that it can overcome drug resistance because of its alternative mechanisms of anti-MM activity. Besides alkylating agents and corticosteroids, Thal now, therefore, represents the third distinct class of agents useful in the treatment of MM.

Given its broad spectrum of activities, Thal may be acting against MM in several ways.[8] First, Thal may have a direct effect on the MM cell and/or BM stromal cell to inhibit their growth and survival. For example, free radical–mediated oxidative DNA damage may play a role in the teratogenicity of Thal[9] and may also have anti-tumor effects. Second, adhesion of MM cells to BM stromal cells both triggers secretion of cytokines that augment MM cell growth and survival[10-12] and confers drug resistance[13]; Thal modulates adhesive interactions[14] and, thereby, may alter tumor cell growth, survival, and drug resistance. Third, cytokines secreted into the BM microenvironment by MM and/or BM stromal cells, such as interleukin (IL)-6, IL-1β, IL-10, and tumor necrosis factor (TNF)–α, may augment MM cell growth and survival,[12] and Thal may alter their secretion and bioactivity.[15] Fourth, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor 2 (bFGF-2) are secreted by MM and/or BM stromal cells and may play a role both in tumor cell growth and survival, as well as BM angiogenesis.[5,16] Given its known antiangiogenic activity,[6] Thal may inhibit activity of VEGF, bFGF-2, and/or angiogenesis in MM. However, Singhal et al.[7] observed no correlation of BM angiogenesis with response to Thal, suggesting that it may not be mediating anti-MM activity by its antiangiogenic effects. Finally, Thal may be acting against MM by its immunomodulatory effects, such as induction of a Th1 T-cell response with secretion of interferon gamma (IFN-γ) and IL-2.[17] Already 2 classes of Thal analogs have been reported, including phosphodiesterase 4 inhibitors that inhibit TNF-α but do not enhance T-cell activation (selected cytokine inhibitory drugs [SelCIDs]) and others that are not phosphodiesterase 4 inhibitors but markedly stimulate T-cell proliferation as well as IL-2 and IFN-γ production (immunomodulatory drugs [IMiDs]).[15]

In this study, we have begun to characterize the mechanisms of activity of Thal and these analogs against human MM cells. Delineation of their mechanisms of action, as well as mechanisms of resistance to these agents, will both enhance understanding of MM disease pathogenesis and derive novel treatment strategies.

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Materials and methods

MM-derived cell lines and patient cells

Dexamethasone (Dex)-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL). Doxorubicin (Dox)-, mitoxantrone (Mit)-, and melphalan (Mel)-sensitive and -resistant RPMI-8226 human MM cells were kindly provided by Dr William Dalton (Moffitt Cancer Center, Tampa, FL). RPMI-8226 cells resistant to Dox, Mit, and Mel included Dox 6 and Dox 40 cells, MR20 cells, and LR5 cells, respectively. Hs Sultan human MM cells were obtained from the American Type Culture Collection (Rockville, MD). All MM cell lines were cultured in RPMI-1640 media (Sigma Chemical, St Louis, MO) that contained 10% fetal bovine serum, 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO). Drug-resistant cell lines were cultured with either Dox, Mit, Mel, or Dex to confirm their lack of drug sensitivity. MM patient cells (96% $CD38^+CD45RA^-$) were purified from patient BM samples, as previously described.[18]

Thal and analogs

Thal and analogs (Celgene, Warren, NJ) were dissolved in DMSO (Sigma) and stored at −20°C until use. Drugs were diluted in culture medium (0.0001 to 100 μM) with < 0.1% DMSO immediately before use. The Thal analogs used in this study were 4 SelCIDs (SelCIDs 1, 2, 3, and 4), which are phosphodiesterase 4 inhibitors that inhibit TNF-α production and increase IL-10 production from lipopolysaccharide (LPS)–stimulated peripheral blood mononuclear cells (PBMCs) but do not stimulate T-cell proliferation; and 3 IMiDs (IMiD1, IMiD2, and IMiD3), which do stimulate T-cell proliferation, as well as IL-2 and IFN-γ secretion, but are not phosphodiesterase 4 inhibitors. The IMiDs also inhibit TNF-α, IL-1β, and IL-6 and greatly increase IL-10 production by LPS-stimulated PBMCs.[15]

DNA synthesis

DNA synthesis was measured as previously described.[19] MM cells (3×10^4 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media, Thal, SelCID1, SelCID2, SelCID3, SelCID4, IMiD1, IMiD2, IMiD3, and/or recombinant IL-6 (50 ng/mL) (Genetics Institute, Cambridge, MA) for 48 hours at 37°C. DNA synthesis was measured by [^{3}H]-thymidine (^{3}H-TdR; NEN Products, Boston, MA) uptake. Cells were pulsed with ^{3}H-TdR (0.5 μCi/well) during the last 8 hours of 48-hour cultures, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted by using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). All experiments were performed in triplicate.

Colorimetric assays were also performed to assay drug activity. Cells from 48-hour cultures were pulsed with 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT; Chemicon International Inc, Temecula, CA) to each well for 4 hours, followed by 100 μL isopropanol that contained 0.04 HCl. Absorbance readings at a wavelength of 570 nm were taken on a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Cell cycle analysis

MM cells (1×10^6) cultured for 72 hours in media alone, Thal, IMiD1, IMiD2, and IMiD3 were harvested, washed with phosphate-buffered saline (PBS), fixed with 70% ethanol, and pretreated with 10 μg/mL of RNAse (Sigma). Cells were stained with propidium iodide (PI; 5 μg/mL; Sigma), and cell cycle profile was determined by using the program M software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL), as in prior studies.[20]

Detection of apoptosis

In addition to identifying sub-G1 cells as described above, apoptosis was also confirmed by using annexin V staining. MM cells were cultured in media (0.01% DMSO) or with 10 μmol/L of Thal or 1 μmol/L IMiD1, IMiD2, and IMiD3 at 37°C for 72 hours, with addition of drugs at 24-hour intervals. Cells were then washed twice with ice-cold PBS and resuspended (1×10^6 cells/mL) in binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). MM cells (1×10^5) were incubated with annexin V-FITC (5 μL; Pharmingen, San Diego, CA) and PI (5 μg/mL) for 15 minutes at room temperature. Annexin V+PI− apoptotic cells were enumerated by using the Epics cell sorter (Coulter).

Immunoblotting

MM cells were cultured with 10 μmol/L of Thal, IMiD1, IMiD2, or IMiD3; harvested; washed; and lysed using lysis buffer: 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton-X 100, 30 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 5 μg/mL leupeptin, and 5 μg/mL aprotinin. For detection of p21, cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-p21 antibody (Ab; Santa Cruz Biotech, Santa Cruz, CA). The membrane was stripped and reprobed with anti–alpha tubulin Ab (Sigma) to ensure equivalent protein loading. For detection of p53, cell lysates were prepared from MM cells (2×10^7) with the use of lysis buffer. Lysates were incubated with anti-mutant (mt) or wild-type (wt) p53 monoclonal Abs (Calbiochem, San Diego, CA) and then immunoprecipitated overnight with protein A Sepharose (Sepharose CL-4B; Pharmacia, Uppsala, Sweden). Immune complexes were analyzed by immunoblotting with horseradish peroxidase–conjugated anti-p53 Ab reactive with both mt and wt p53 (Calbiochem).

To characterize growth signaling, immunoblotting was also done with anti-phospho–specific MAPK Ab (New England Biolabs, Beverly, MA) in the presence or absence of IL-6 (Genetics Institute) and/or the MEK 1 inhibitor PD98059 (New England Biolabs), as in prior studies.[21] Antigen-antibody complexes were detected by using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Blots were stripped and reprobed with anti-ERK2 Ab (Santa Cruz Biotech) to ensure equivalent protein loading.

To characterize apoptotic signaling, MM cells were cultured with 100 μmol/L of Thal, IMiD1, IMiD2, or IMiD3; harvested; washed; and lysed in 1 mL of lysis buffer (50 mmol/L Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin), as in prior studies.[22] Lysates were incubated with anti-related adhesion focal tyrosine kinase (RAFTK) Ab for 1 hour at 4°C and then for 45 minutes after the addition of protein G–agarose (Santa Cruz Biotech). Immune complexes were analyzed by immunoblotting with anti-P-Tyr Ab (RC20; Transduction Laboratories, Lexington, KY) or anti-RAFTK Abs. Proteins were separated by electrophoresis in 7.5% SDS-PAGE gels, transferred to nitrocellulose paper, and analyzed by immunoblotting. The antigen-antibody complexes were visualized by chemiluminescence.

Statistical analyses

Statistical significance of differences observed in drug-treated versus control cultures was determined by using the Student *t* test. The minimal level of significance was $P < .05$.

Results

Treatment of MM patients with Thal

Seventeen (39%) of 44 patients with MM treated at our institute responded to Thal (Table 1). This response included 6 men and 11 women. These patients had received a median of 4 (1-9) prior treatment regimens, and 10 patients had a prior high-dose therapy and hematopoietic stem cell transplant. One patient achieved

Table 1. Response to thalidomide in multiple myeloma*

Patient	Sex†	Prior therapies	Prior stem cell transplant	Maximum change M protein‡	Duration of thalidomide therapy (mo)	Maximum daily dose thalidomide	Current status (daily thalidomide dose)
1	M	3	Yes	− 58%(PR)	8.5	200 mg	Continued response (200 mg)
2	F	5	No	− 78%(PR)	6.0	400 mg	Continued response (400 mg)
3	F	1	Yes	+ 16%(SD)	6.5	100 mg	Continued response (100 mg)
4	M	6	No	− 56%(PR)	9.0	200 mg	Continued response (200 mg)
5	F	1	No	− 62%(PR)	5.5	200 mg	Continued response (50 mg)
6	F	5	Yes	− 100%(CR)	13	500 mg	Continued response (50 mg)
7	M	9	Yes	− 54%(PR)	10	800 mg	Progressed (800 mg)
8	F	5	Yes	− 68%(PR)	4.0	200 mg	Continued response, discontinued
9	F	5	No	− 90%(PR)	7.5	400 mg	Continued response (400 mg)
10	M	5	Yes	− 9%(SD)	1.5	400 mg	Progressed
11§	F	4	Yes	− 59%(PR)	5.5	400 mg	Progressed
12§	M	4	Yes	− 64%(PR)	7.0	400 mg	Progressed
13§	F	3	Yes	− 14%(SD)	4.5	400 mg	Progressed
14§	F	2	Yes	− 55%(PR)	4.0	800 mg	Continued response (800 mg)
15	F	1	No	− 31%(SD)	6.0	400 mg	Continued response (400 mg)
16	F	1	No	− 12%(SD)	4.5	400 mg	Progressed
17	M	2	No	− 55%(PR)	6.0	200 mg	Continued response (100 mg)

*As of January 1, 2000.

†Male (M) or female (F).

‡Partial response (PR) is ≥ 50% decrease in M protein; complete response (CR) is absence of M protein on immunofixation and normal bone marrow biopsy; stable disease (SD) is ≤ 50% decrease in M protein; progression is ≥ 25% increase in M protein or progressive clinical disease.

§Also received decadron therapy.

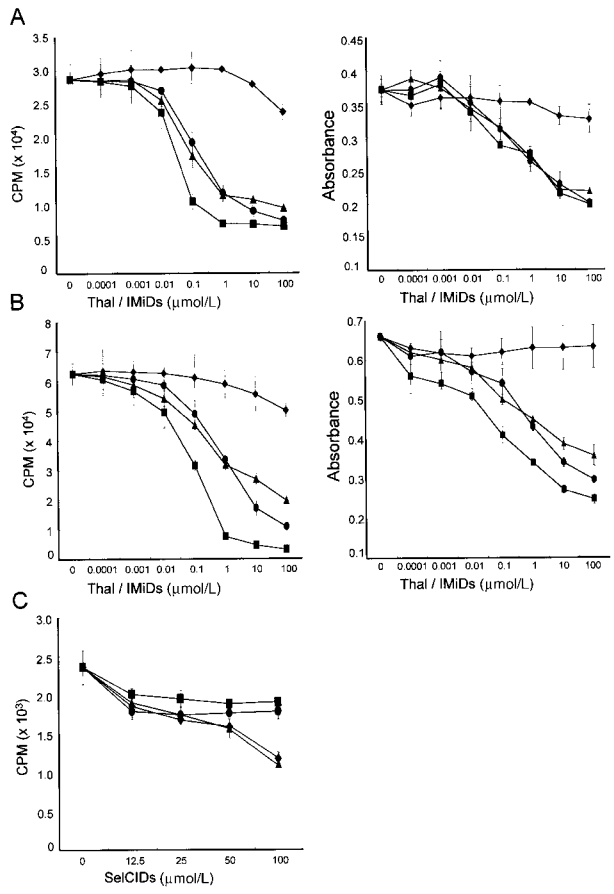

Figure 1. Effect of Thal and analogs on DNA synthesis of MM cell lines and patient cells. MM.1S (A) and Hs Sultan (B) cells were cultured with increasing concentrations (0.0001-100 μM) of Thal (◆), IMiD1 (■), IMiD2 (●), and IMiD3 (▲). (C) MM.1S cells were cultured with increasing concentrations (12.5-100 μM) of SelCID1 (◆), SelCID2 (■), SelCID3 (▲), and SelCID4 (●). In each case ^{3}H-TdR uptake (left panels) or MTT cleavage (right panels) were measured during the last 8 and 4 hours, respectively, of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) or absorbance of triplicate cultures.

complete response (absence of monoclonal protein on immunofixation and normal BM biopsy), 11 patients achieved partial response (> 50% decrease in monoclonal protein), and 5 patients achieved stable disease (< 50% decrease in monoclonal protein). Patients received a median of 400 mg (range, 100-800 mg) maximum dose of daily Thal for a median of 6 months (range, 1.5-13 months). As of January 1, 2000, 11 patients have continued response at a median of 6 months (range, 4-13 months), and 6 patients have progressed at a median of 4.5 months (range, 1.5-10 months).

Effect of Thal and analogs on DNA synthesis by MM cell lines and patient MM cells

The effect of Thal and its analogs, including IMiD1, IMiD2, IMiD3, SelCID1, SelCID2, SelCID3, and SelCID4, on DNA synthesis of MM cell lines (MM.1S, Hs Sultan, U266, and RPMI-8226) was determined by measuring ^{3}H-TdR uptake during the last 8 hours of 48-hour cultures, in the presence or absence of drug at various concentrations. IMiD1, IMiD2, and IMiD3 inhibited ^{3}H-TdR uptake of MM.1S (Figure 1A) and Hs Sultan (Figure 1B) cells in a dose-dependent fashion. Fifty percent inhibition of proliferation of MM.1S cells was noted at 0.01-0.1 μmol/L IMiD1, 0.1-1.0 μmol/L IMiD2, and 0.1-1.0 μmol/L IMiD3 ($P < .001$). Fifty percent inhibition of proliferation of Hs Sultan cells was noted at 0.1 μmol/L IMiD1, 1.0 μmol/L IMiD2, and 1.0 μmol/L IMiD3 ($P < .001$). In contrast, only 15% and 20% inhibition in MM.1S and Sultan cells, respectively, were observed in cultures at even higher concentrations (100 μmol/L) of Thal. No significant inhibition of DNA synthesis of U266 MM cells was noted in cultures with 0.001 to 100 μmol/L Thal or these IMiDs (data not shown). The effects of these drugs on proliferation were confirmed by using MTT assays for MM.1S cells (Figure 1A) and Hs Sultan cells (Figure 1B). Although there was also a dose-dependent inhibition of proliferation of MM.1S cells by SelCIDs, 50% inhibition was observed only at high doses (100 μmol/L) for only 2 of the 4 SelCIDs (SelCIDs 1 and 3, Figure 1C). Further studies, therefore, focused on Thal and the IMiDs.

Effect of Thal and analogs in DNA synthesis of MM cells resistant to conventional therapy

To examine whether there was cross-resistance between Thal and the IMiDs with conventional therapies, RPMI-8226 MM cells resistant to Dox (Dox6 and Dox40 cells), Mit (MR20 cells), or Mel (LR5 cells), and MM.1R cells resistant to Dex were similarly studied. Proliferation of Dox6 and Dox40, MR20, LR5, or MM1.R cells is unaffected by culture with 60 nmol/L and 400 nmol/L Dox, 20 nmol/L Mit, 5 μmol/L Mel, and 1 μmol/L Dex, respectively (data not shown). Importantly, ^{3}H-TdR uptake of Dox6, Dox40, MR20, or LR5 was inhibited in cultures with Thal and the IMiDs in a dose-dependent manner (1-100 μmol/L) versus media alone cultures (Figure 2A-D). For example, 10 μmol/L IMiD1 blocked proliferation of Dox6, Dox40, MR20, and LR5 cells by 20%, 33%, 32%, and 21%, respectively ($P < .001$). The IMiDs similarly inhibited DNA synthesis of MM.1R cells in a dose-dependent fashion, with more than 50% inhibition at more than 1 μmol/L IMiD1 ($P < .001$; Figure 2E). These data suggest independent mechanisms of resistance to Dox, Mit, Mel, and Dex versus Thal and its analogs.

Effect of Dex and IL-6 on response of MM cells to Thal and the ImiDs

To determine whether the effects of Thal and the IMiDs are additive with conventional therapies, we next examined the effect of Dex (0.001-0.1 μmol/L) together with 1 μmol/L Thal or IMiDs on proliferation of Dex-sensitive MM.1S cells. As can be seen in Figure 3A, the IMiDs (1 μmol/L) significantly inhibited ^{3}H-TdR uptake of MM.1S cells (60%-75% block, $P < .01$), and Dex (0.001-0.1 μmol/L) increased this inhibition in a dose-dependent fashion. For example, doses of 0.001 to 0.01 μmol/L Dex added to 1 μmol/L IMiD1 increased the inhibition of proliferation by 35% relative to cultures with 1 μmol/L IMiD1 alone ($P < .01$). Given the additive effects of Dex and the IMiDs, as well as the known role of IL-6 as a growth factor and specific inhibitor of Dex-induced MM cell apoptosis,[19,22,23] we also examined whether exogenous IL-6 could overcome the inhibition of DNA synthesis triggered by Thal and the IMiDs. Figure 3B demonstrates that IL-6 (50 ng/mL) triggers DNA synthesis of MM.1S cells in cultures with media alone, as well as in cultures with the IMiDs (0.1 and 1 μmol/L).

Effect of Thal and analogs on DNA synthesis of patient MM cells

The effect of Thal and the IMiDs on DNA synthesis of patient MM cells was next examined (Figure 4). As was true for MM.1S and Hs Sultan MM cell lines, ^{3}H-TdR uptake of patients' MM cells was also inhibited by IMiDs (0.1-100 μmol/L) in a dose-dependent fashion, whereas the inhibitory effect of Thal, even at 100 μmol/L, was not significant. Fifty percent inhibition of MM patient cells was observed at 100 μmol/L (Figure 4A) and 1 μmol/L (Figure 4B) IMiD1, respectively ($P < .001$).

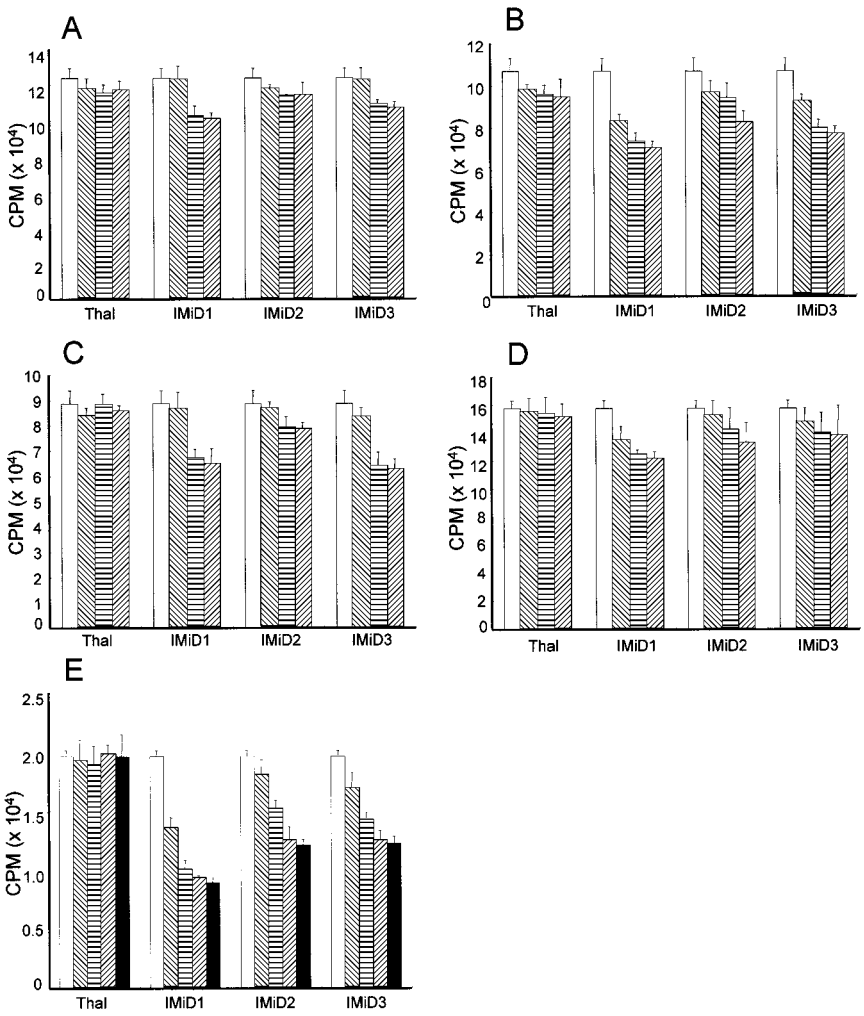

Figure 2. Effect of Thal and analogs on DNA synthesis of MM cells resistant to conventional therapy. Dox-resistant Dox6 (A) and Dox 40 (B), Mit-resistant (MR20; C), and Mel-resistant (LR5; D) cells were cultured with control media (□) or 1 μmol/L (▨), 10 μmol/L (▤), 100 μmol/L (■) of Thal, IMiD1, IMiD2, or IMiD3. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures. (E) Dex-resistant MM.1R cells were cultured in control media (□) or with 0.1 μmol/L (▨), 1 μmol/L (▤), 10 μmol/L (▨), or 100 μmol/L (■) of Thal, IMiD1, IMiD2, or IMiD3.

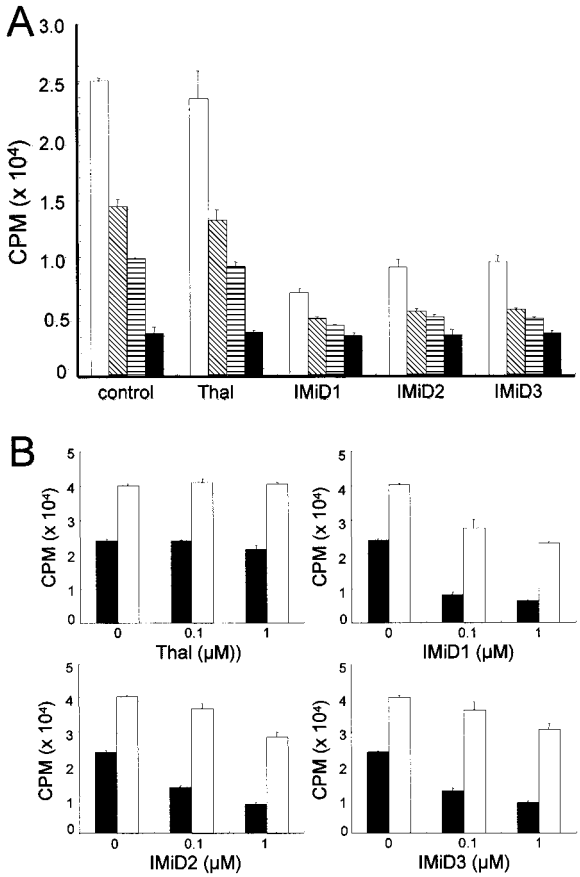

Figure 3. Effect of Dex and IL-6 on response of MM cells to Thal and the IMiDs. (A) MM.1S cells were cultured with 1.0 μM Thal, IMiD1, IMiD2, or IMiD3 in control media alone (□) or with 0.001 (▨), 0.01 (▤), and 0.1 μmol/L (■) Dex. (B) MM.1S cells were cultured in control media alone and with 0.1 and 1.0 μmol/L Thal, IMiD1, IMiD2, or IMiD3 either in the presence (□) or absence (■) of IL-6 (50 ng/mL). In each case, ^{3}H-TdR uptake was measured during the last 8 hours of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures.

Effect of Thal and analogs on cell cycle profile of MM cell lines and patient MM cells

To further analyze the mechanism of Thal- and IMiD-induced inhibition of DNA synthesis and to determine whether these drugs induced apoptosis of MM cells, we first examined the cell cycle profile of MM.1S, Hs Sultan cells, and patient MM cells cultured with media alone, Thal (10 μmol/L), or the IMiDs (1 μmol/L). Cells were harvested from 72-hour cultures and stained with PI. As shown in Figure 5A, all 3 IMiDs, and Thal to a lesser extent, increased sub-G1 MM.1S cells. Induction of apoptosis occurred at the dose-response curve noted for inhibition of proliferation. Twelve-hour cultures with Dex (10 μmol/L) served as a positive control for triggering increased sub-G1 cells. In contrast, no increase in sub-G1 cells was observed in cultures of Hs Sultan cells or of patient MM cells with Thal or the IMiDs. Importantly, Thal and the IMiDs induced G1 growth arrest in both Hs Sultan cells and in AS patient MM cells.

To confirm these results, we performed annexin V staining of cells in these cultures. As can be seen in Figure 5B, the percentage of annexin V–positive cells in cultures of MM.1S cells with Thal, IMiD1, IMiD2, and IMiD3 was 32%, 55%, 51%, and 43%, respectively. Forty-six percent of annexin V staining was observed in cultures with Dex, whereas only 22% annexin V–positive cells were present in cultures with media alone. The percentage of annexin V–positive Hs Sultan cells and AS patient MM cells was 4% to 7%, respectively, under all culture conditions and was not increased by Thal or the IMiDs.

Effect of Thal and analogs on p21 expression in MM cell lines and patient cells

We next correlated these distinct biologic sequelae of Thal and the IMiDs with p21 status in MM.1S versus Hs Sultan and patient MM cells. As can be seen in Figure 6A, p21 expression was down-regulated by the IMiDs, as well as by Dex, in MM1.S cells; and IL-6 overcomes this inhibitory effect. In contrast, the IMiDs up-regulated p21 in Hs Sultan cells and patient MM cells. Immunoblotting with anti-tubulin Ab confirmed equivalent protein loading. Wt-p53 was recognized in MM.1S cells, whereas both wt- and mt-p53 were recognized in Hs Sultan cells and patient MM cells (Figure 6B). These studies further support the observation that Thal and the IMiDs can induce either apoptosis or G1 growth arrest in sensitive MM cells, and they are consistent with Thal and IMiD p53-mediated down-regulation of p21 and susceptibility to p53-mediated apoptosis in MM.1S cells, in contrast to induction of p21 and growth arrest in Hs Sultan cells and patient MM cells, conferring protection from apoptosis.

Effect of Thal and analogs on growth and apoptotic signaling in MM.1S and MM.1R cells

We have previously characterized signaling cascades mediating MM cell growth and apoptosis, as well as the antiapoptotic effect of

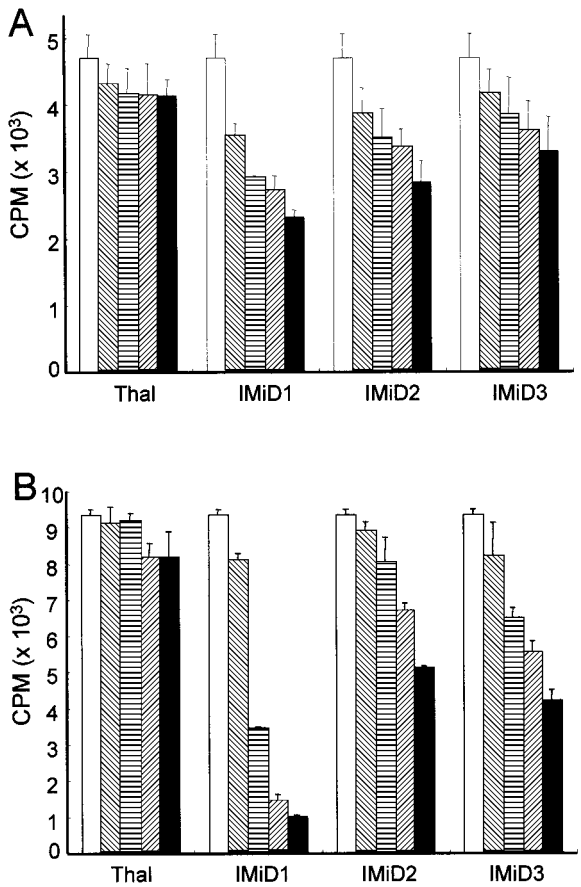

Figure 4. Effect of Thal and analogs on DNA synthesis of patient MM cells. MM cells from patient 1 (A) and patient 2 (B) were cultured with control media (□) or with 0.1 μmol/L (▧), 1.0 μmol/L (▤), 10 μmol/L (▨), and 100 μmol/L (■) Thal, IMiD1, IMiD2, or IMiD3. In each case, ^{3}H-TdR uptake was measured during the last 8 hours of 48-hour cultures. Values represent the mean (± SD) ^{3}H-TdR (cpm) of triplicate cultures.

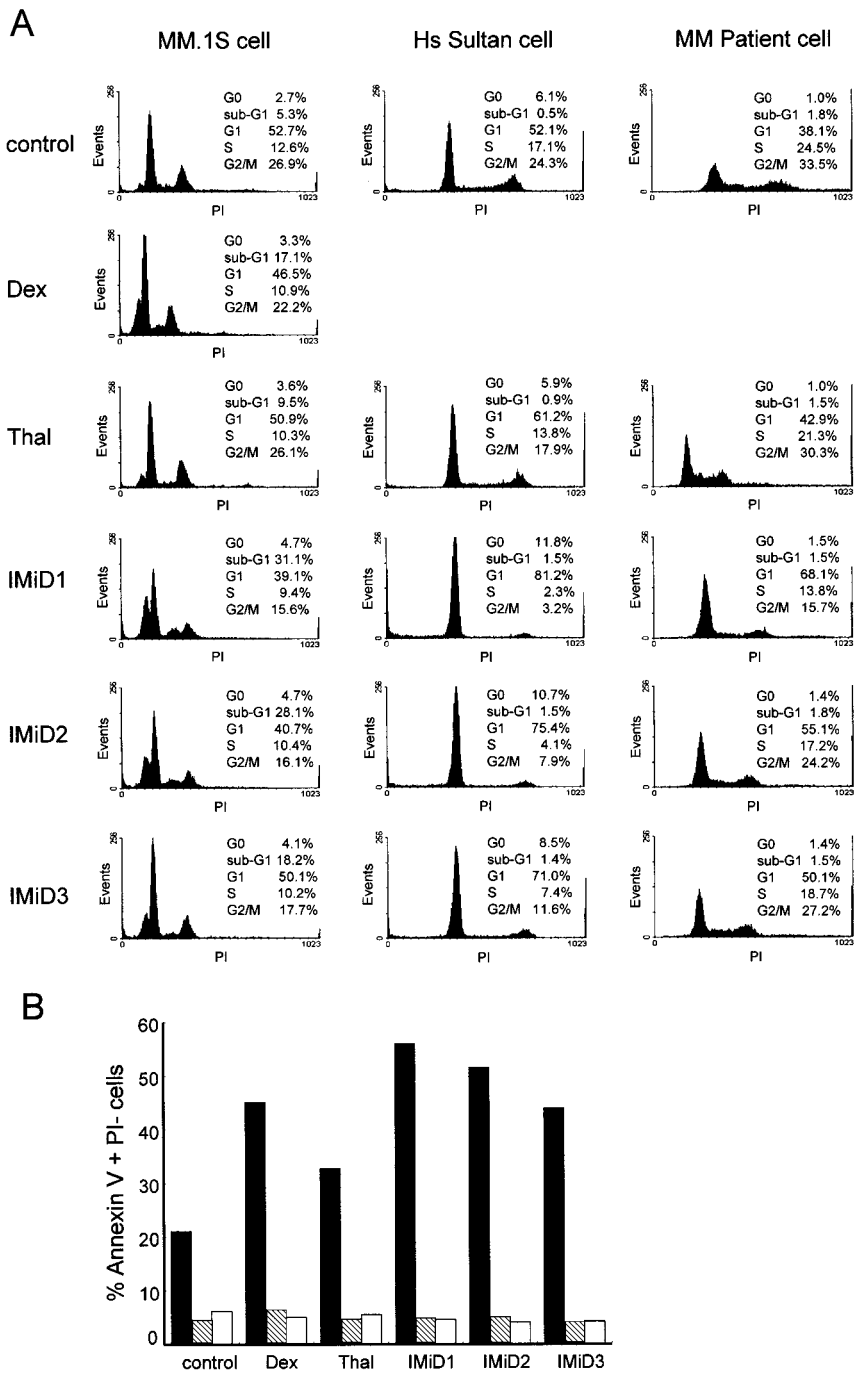

Figure 5. Effect of Thal and analogs on cell cycle profile of MM cell lines and patient MM cells. (A) MM.1S cells, Hs Sultan cells, and patient MM cells were cultured with 10 μmol/L of Thal or 1 μmol/L of IMiD1, IMiD2, or IMiD3 for 72 hours. Cultures in media control alone served as a negative control and 18-hour cultures with 10 μmol/L Dex as positive controls. Cells were then stained with PI, and cell cycle profile was determined by flow cytometric analysis. (B) These MM.1S (■), Hs Sultan (▨), and patient MM (□) cells were also stained with annexin V as an additional assay for apoptosis.

IL-6.[19,22-25] Because we have shown that IL-6–induced proliferation is mediated by the ras-dependent mitogen-activated protein kinase (MAPK) cascade,[19] we next examined the effect of Thal and the IMiDs on tyrosine phosphorylation of MAPK in IL-6–responsive MM.1S cells. Constitutive tyrosine phosphorylation of MAPK in MM.1S cells was down-regulated by the MEK1 inhibitor PD98059 (50 μmol/L), which served as a positive control (Figure 6A), and to a lesser extent by the IMiDs (1 μmol/L; Figure 7A) or Thal (10 μmol/L; data not shown). Treatment of MM.1S cells with IL-6 increased MAPK tyrosine phosphorylation, which was partially blocked by PD98059 but was unaffected by the IMiDs (Figure 7A) or Thal (data not shown). Stripping the blot and reprobing with anti-ERK2 Ab confirmed equivalent protein loading.

The observation that IL-6 can overcome the effects of Thal, the IMiDs, and Dex, coupled with our prior studies delineating signaling cascades mediating Dex-induced apoptosis and the protective effects of IL-6,[22,23,25] suggested that RAFTK activation may be induced during apoptosis triggered by Thal and IMiDs. MM.1S and MM.1R cells were, therefore, next cultured with 1 μmol/L Thal, IMiD1, IMiD2, or IMiD3 for 12 hours. Twelve-hour cultures with Dex (10 μmol/L) served as a positive control for activation of RAFTK. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK Ab and analyzed by immunoblotting with anti-P-Tyr Ab or anti-RAFTK Ab. As can be seen in Figure 7B, Dex induced tyrosine phosphorylation of RAFTK in MM.1S cells but not in MM.1R cells. Importantly, IMiD1 induced RAFTK tyrosine phosphorylation in both MM.1S and MM.1R

cells, correlating with its effects on both Dex-sensitive and Dex-resistant MM cells.

Discussion

This study demonstrates for the first time a direct dose-dependent effect of Thal and these analogs on tumor cells. Thal has demonstrated clinical anti-MM activity at the University of Arkansas[7] and in this study, and Thal at high concentrations (100 μmol/L) resulted in a modest (< 20%) inhibition of in vitro DNA synthesis of MM cells. SelCIDs also induced a dose-dependent inhibition of MM cells, but only 2 of 4 SelCIDs tested achieved 50% inhibition of proliferation, even at 100 μmol/L concentrations. Importantly, all 3 IMiDs tested achieved 50% inhibition of DNA synthesis at concentrations (0.1-1.0 μmol/L) corresponding to serum levels that are readily achievable, both confirming their direct action on tumor cells and suggesting their potential clinical utility. Moreover, the IMiDs inhibited the proliferation of Dox-, Mit-, and Mel-resistant MM cells by 20% to 35%, and of Dex-resistant MM cells by 50%. These in vitro effects correlate with the observed clinical activity of Thal in patients with MM that is refractory to conventional therapies, both at the University of Arkansas[7] and reported in this study, and suggest their clinical utility to overcome drug resistance. Moreover, our studies further suggest that Dex can add to the antiproliferative effect of Thal and the IMiDs in vitro, suggesting the potential utility of coupling these agents therapeutically. Finally, our study also identified MM cells resistant to Thal and the analogs (U266 cells), which, therefore, can be used to study mechanisms of Thal resistance.

Our studies demonstrate that Thal and the IMiDs are acting directly on MM cells, in the absence of accessory BM or T cells. It is also possible that these agents may be mediating their anti-MM effect by cytokines, given their known inhibitory effects on TNF-α, IL-1β, and IL-6.[15] Our prior studies have characterized the growth

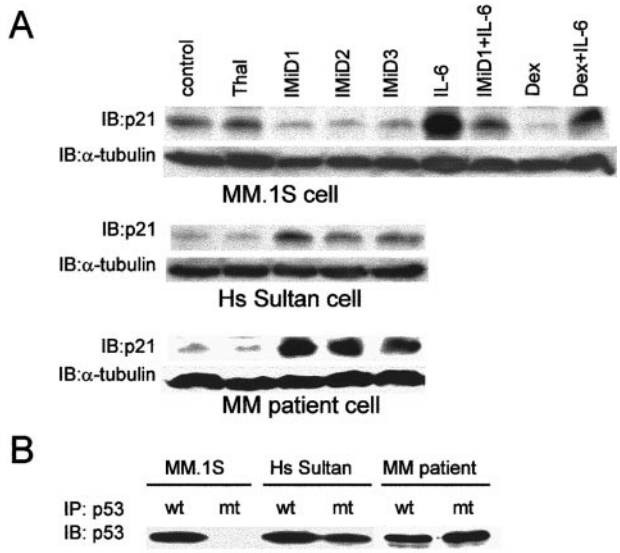

Figure 6. Effect of Thal and analogs on p21 expression in MM cell lines and patient cells. (A) MM.1S cells were cultured with 10 μmol/L of Thal, IMiD1, IMiD2, and IMiD3 for 48 hours. MM.1S cells were also cultured with IL-6 (50 ng/mL) alone and with IMiD1, 10 μmol/L Dex, and Dex plus IL-6. Cells were lysed, subjected to SDS-PAGE, transferred to PVDF membrane, and blotted with anti-p21 Ab. The membrane was stripped and reprobed with anti-α-tubulin Ab. (B) MM.1S, Hs Sultan, and patient MM cells were lysed and immunoprecipitated with wt-p53 and mt-p53 Abs, transferred to PVDF membrane, and blotted with anti-p53 Ab.

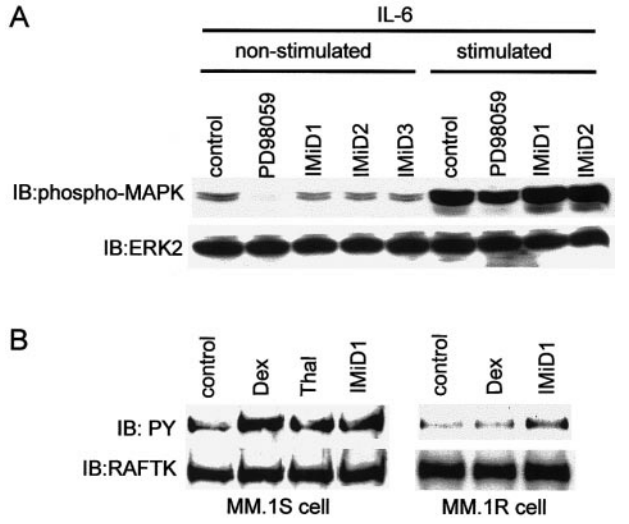

Figure 7. Effect of Thal and analogs on growth and apoptotic signaling in MM.1S and MM.1R cells. (A) MM.1S cells were cultured in media, with 50 μmol/L of PD98059 and with 10 μmol/L of IMiD1, IMiD2, or IMiD3 for 48 hours. Cells were then triggered with 50 ng/mL of IL-6 for 10 minutes, lysed, transferred to PVDF membrane, and blotted with anti-phospho MAPK Ab. Blots were stripped and reprobed with anti-ERK2 Ab. (B) MM.1S and MM.1R cells were treated with Thal (100 μM), IMiD1 (100 μmol/L), or Dex (10 μmol/L) and harvested at 12 hours. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK Ab and analyzed by immunoblotting with anti-P-Tyr Ab or anti-RAFTK Ab.

effects of IL-6 on human MM cells,[12,26] and we, therefore, next determined the effect of exogenous IL-6 on drug activity. Our studies showed that IL-6 can overcome the effect of Thal and the IMiDs on MM cell lines and patient cells, suggesting that these novel drugs may, at least in part, be inhibiting IL-6 production. Our prior studies have further demonstrated that IL-6–induced proliferation of MM cells is mediated through the MAPK cascade and that blockade of this pathway with either MAPK antisense oligonucleotide or the MEK1 inhibitor PD98059 can abrogate this response.[19,21,24] The present study showed constitutive MAPK phosphorylation in MM cells that is inhibited by PD98059 and, to a lesser extent, by the IMiDs. Importantly, IL-6–triggered MAPK tyrosine phosphorylation is also blocked by PD98059 but not by IMiDs. These studies, therefore, suggest that the IMiDs do not work only by directly inhibiting MAPK growth signaling and further support their potential activity in down-regulating IL-6 production. In MM, IL-6 production in tumor cells can either be constitutive or induced, mediating autocrine tumor cell growth.[26,27] In addition, IL-6 is also produced by BM stromal cells in MM, a process that is up-regulated by tumor cell adhesion to BM stromal cells, with related tumor cell growth in a paracrine mechanism.[10,11] Our ongoing studies are, therefore, evaluating the effect of Thal and these analogs on IL-6 production in the BM microenvironment.

Having shown the inhibitory effects of Thal and the IMiDs on ^{3}H-TdR uptake of tumor cells, we next examined their effect on MM cell cycle. Interestingly, these drugs had distinct functional sequelae in MM cells. Specifically, the IMiDs, and to a lesser extent Thal, induced apoptosis of MM.1S cells, evidenced both by increased sub-G1 cells on PI staining and increased annexin V–positive cells. In these cells that have wt p53, these agents (and Dex) down-regulate p21, thereby facilitating G1-to-S transition and susceptibility to apoptosis. This apoptotic effect may correlate with the clinical observation that complete response to Thal is rarely observed. IL-6 overcomes the down-regulation of p21 induced by these agents, consistent with the increase in DNA synthesis triggered by IL-6 even in the presence of these drugs. In

contrast, in Hs Sultan cells (wt and mt p53) and patient cells (wt p53 and mt p53), the IMiDs and Thal induce p21 and related G1 growth arrest, thereby conferring protection from apoptosis, as has been observed in other systems.[28,29] In our prior study,[20] p21 was also constitutively expressed in the majority of MM cells and also inhibited proliferation in both p53-dependent and -independent mechanisms. Previous reports that cells overexpressing p21 protein demonstrate chemoresistance[30] further support the protective effect of G1 growth arrest induced by these agents in Hs Sultan MM cells and patient MM cells. Conversely, the frequent regrowth of progressive MM noted clinically on discontinuation of Thal treatment may correlate with release of drug-related G1 growth arrest. An ongoing clinical trial is correlating response to Thal with laboratory parameters (ie, serum IL-6 or the surrogate marker C reactive protein) and will gain further insights into its mechanisms of in vivo anti-tumor activity.

Finally, our prior studies have characterized apoptotic signaling cascades in MM, as well as the protective effect of IL-6, especially against Dex-induced apoptosis.[22,23,25,31] Specifically, we have shown that Dex down-regulates growth kinases, such as MAPK and $p70^{RSK}$;[23] importantly, it activates RAFTK, which is required for Dex-induced apoptosis and abrogated by IL-6.[22] The current studies show that IMiD1 acts similarly to Dex, because it activates RAFTK and apoptosis in MM.1S cells, sequelae that are blocked by IL-6. Given our prior studies, which demonstrate that apoptosis of MM cells induced by UV irradiation, γ irradiation, and Fas ligation do not involve RAFTK,[22] the current signaling studies, therefore, further support both the ability of the IMiDs to act through distinct signaling cascades to overcome drug resistance, as well as the enhanced anti-tumor activity observed when Thal or the IMiDs are coupled with Dex.

In conclusion, the results of this study, therefore, demonstrate evidence for direct activity of Thal and the IMiDs against human MM cells. To confirm their in vivo mechanism of action, these compounds and SelCIDs will be examined in an animal model. Importantly, these studies provide the framework for the development and testing of a new biologically based treatment paradigm that uses these novel agents, either alone or together with conventional therapies, to target both the tumor cell and its microenvironment, overcome classical drug resistance, and achieve improved outcome in this presently incurable disease.

References

1. Hales BF. Thalidomide on the comeback trail. Nat Med. 1999;5:489-490.
2. Sampaio EP, Kaplan G, Miranda A, et al. The influence of thalidomide on the clinical and immunologic manifestation of erythema nodosum leprosum. J Infect Dis. 1993;168:408-414.
3. Anderson KC. Thalidomide: therapeutic potential in hematologic malignancies. Sem Hematol. 2000;37:1-4.
4. Ribatti D, Vacca A, Nico B, et al. Bone marrow angiogenesis and mast cell density increase simultaneously with progression of human multiple myeloma. Br J Cancer. 1999;79:451-455.
5. Vacca A, Ribatti D, Presta M, et al. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. Blood. 1999;93:3064-3073.
6. D'Amato RJ, Loughman MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci U S A. 1994;91:4082-4085.
7. Singhal S, Mehta J, Desikan R, et al. Anti-tumor activity of thalidomide in refractory multiple myeloma. N Engl J Med. 1999;341:1565-1571.
8. Raje N, Anderson KC. Thalidomide: a revival story. N Engl J Med. 1999;341:1606-1609.
9. Parman T, Wiley MJ, Wells PG. Free radical-mediated oxidative DNA damage in the mechanism of thalidomide teratogenicity. Nat Med. 1999;5:582-585.
10. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates IL-6 secretion. Blood. 1993;82:3712-3720.
11. Chauhan D, Uchiyama H, Akbarali Y, et al. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-κB. Blood. 1996;87:1104-1112.
12. Hallek M, Bergsagel PL, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. Blood. 1998;91:3-21.
13. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood. 1999;93:1658-1667.
14. Geitz H, Handt S, Zwingengerger K. Thalidomide selectively modulates the density of cell surface molecules involved in the adhesion cascade. Immunopharmacology. 1996;32:213-221.
15. Corral LG, Haslett PAJ, Muller GW, et al. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogs that are potent inhibitors of TNF-α. J Immunol. 1999;163:380-386.
16. Bellamy W, Richter L, Frutiger Y, Grogan T. Expression of vascular endothelial growth factor and its receptor in hematological malignancies. Cancer Res. 1999;59:728-733.
17. Haslett PAJ, Corral LG, Albert M, Kaplan G. Thalidomide costimulates primary human T lymphocytes, preferentially inducing proliferation, cytokine production, and cytotoxic responses in the $CD8^+$ subset. J Exp Med. 1998;187:1885-1892.
18. Tai YT, Teoh G, Shima Y, et al. Isolation and characterization of human multiple myeloma cell enriched populations. J Immunol Method. 2000;235:11-19.
19. Ogata A, Chauhan D, Teoh G, et al. Interleukin-6 triggers cell growth via the ras-dependent mitogen-activated protein kinase cascade. J Immunol. 1997;159:2212-2221.
20. Urashima M, Teoh G, Chauhan D, et al. Interleukin-6 overcomes $p21^{WAF1}$ upregulation and G1 growth arrest induced by dexamethasone and interferon-γ in multiple myeloma cells. Blood. 1997;90:279-289.
21. Hideshima T, Chauhan D, Teoh G, et al. Characterization of signaling cascades by human interleukin-6 versus Kaposi's sarcoma-associated herpesvirus encoded viral interleukin-6. Clin Cancer Res. 2000;6:1180-1189.
22. Chauhan D, Hideshima T, Pandey P, et al. RAFTK/PYK2-dependent and independent apoptosis in multiple myeloma cells. Oncogene. 1999;18:6733-6740.
23. Chauhan D, Pandey P, Ogata A, et al. Dexamethasone induces apoptosis of multiple myeloma cells in a JNK/SAP kinase independent mechanism. Oncogene. 1997;15:837-843.
24. Ogata A, Chauhan D, Urashima M, Teoh G, Treon SP, Anderson KC. Blockade of mitogen-activated protein kinase cascade signaling in interleukin-6 independent multiple myeloma cells. Clin Cancer Res. 1997;3:1017-1022.
25. Chauhan D, Kharbanda S, Ogata A, et al. Interleukin-6 inhibits Fas-induced apoptosis and stress-activated protein kinase activation in multiple myeloma cells. Blood. 1997;89:227-234.
26. Anderson KC, Jones RC, Morimoto C, Leavitt P, Barut B. Response of purified myeloma cells to hematopoietic growth factors. Blood. 1989;73:1915-1924.
27. Urashima M, Chauhan D, Uchiyama H, Freeman GJ, Anderson KC. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. Blood. 1995;85:1903-1912.
28. Jung J-M, Brunet JM, Ruan S, et al. Increased level of $p21^{WAF1,CIP1}$ in human brain tumors. Oncogene. 1998;11:2021-2028.
29. Polyak K, Waldman T, He T-C, Kinzler KW, Vogelstein B. Genetic determinants of p53-induced apoptosis and growth arrest. Genes Dev. 1996;10:1945-1952.
30. Wang J, Walsh K. Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. Science. 1996;273:359-361.
31. Chauhan D, Pandey P, Ogata A, et al. Cytochrome-c dependent and independent induction of apoptosis in multiple myeloma cells. J Biol Chem. 1997;272:29995-29997.

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Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy

Teru Hideshima, Dharminder Chauhan, Yoshihito Shima, Noopur Raje, Faith E. Davies, Yu-Tzu Tai, Steven P. Treon, Boris Lin, Robert L. Schlossman, Paul Richardson, George Muller, David I. Stirling and Kenneth C. Anderson

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EXHIBIT C6

[CANCER RESEARCH 61, 3071–3076, April 1, 2001]

The Proteasome Inhibitor PS-341 Inhibits Growth, Induces Apoptosis, and Overcomes Drug Resistance in Human Multiple Myeloma Cells[1]

Teru Hideshima, Paul Richardson, Dharminder Chauhan, Vito J. Palombella, Peter J. Elliott, Julian Adams, and Kenneth C. Anderson[2]

Department of Adult Oncology, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115 [T. H., P. R., D. C., K. C. A]; and Millennium Pharmaceuticals, Inc. Cambridge, Massachusetts 02139 [V. J. P., P. J. E., J. A.]

ABSTRACT

Human multiple myeloma (MM) is a presently incurable hematological malignancy, and novel biologically based therapies are urgently needed. Proteasome inhibitors represent a novel potential anticancer therapy. In this study, we demonstrate that the proteasome inhibitor PS-341 directly inhibits proliferation and induces apoptosis of human MM cell lines and freshly isolated patient MM cells; inhibits mitogen-activated protein kinase growth signaling in MM cells; induces apoptosis despite induction of p21 and p27 in both p53 wild-type and p53 mutant MM cells; overcomes drug resistance; adds to the anti-MM activity of dexamethasone; and overcomes the resistance to apoptosis in MM cells conferred by interleukin-6. PS-341 also inhibits the paracrine growth of human MM cells by decreasing their adherence to bone marrow stromal cells (BMSCs) and related nuclear factor κB-dependent induction of interleukin-6 secretion in BMSCs, as well as inhibiting proliferation and growth signaling of residual adherent MM cells. These data, therefore, demonstrate that PS-341 both acts directly on MM cells and alters cellular interactions and cytokine secretion in the BM millieu to inhibit tumor cell growth, induce apoptosis, and overcome drug resistance. Given the acceptable animal and human toxicity profile of PS-341, these studies provide the framework for clinical evaluation of PS-341 to improve outcome for patients with this universally fatal hematological malignancy.

INTRODUCTION

Proteasome inhibitors represent potential novel anticancer therapy (1, 2). These agents inhibit the degradation of multiubiquitinated target proteins, *i.e.*, cell cycle regulatory proteins such as cyclins and cyclin-dependent kinase inhibitors, and regulate cell cycle progression (3). Proteasome inhibitors induce apoptosis of tumor cells, despite the accumulation of p21 and p27 and irrespective of the p53 wild-type or mutant status (4, 5). Accumulation of Bax induced by proteasome inhibitors can overcome the survival effect of Bcl-2 and increase cytochrome *c*-dependent apoptosis (6). Importantly, proteasome inhibitors can also overcome NF-κB activation and related drug resistance by inhibiting degradation of IκB and the P105 precursor of p50 subunit of NF-κB (7–10). Moreover, proteasome inhibitors are synergistic with Dex[3] in an asthma model (11). Finally, the proteasome inhibitor PS-341 demonstrated marked *in vivo* activity against human prostate cancer (1) and Burkitt's lymphoma (12) in a murine model; produced additive growth delays with 5-fluorouracil, cisplatin, Taxol, and Adriamycin against Lewis lung carcinoma (13); and demonstrated antiangiogenic activity in an orthotopic pancreatic cancer model (14). PS-341 is nearly completing Phase I testing in humans, with an acceptable toxicity profile, and will soon be evaluated for efficacy in Phase II clinical trials.

MM is an incurable hematological malignancy, which affected 13,700 new individuals in the United States in 2000 (15), and novel biologically based therapies are, therefore, urgently needed. There are several characteristics of MM that suggest that it is an ideal candidate for proteasome inhibitor therapy. First, MM cells adhere to BMSCs, which both localizes them in the BM millieu (16) and confers resistance to apoptosis (17). Proteasome inhibitors have been reported to down-regulate cytokine-induced expression of VCAM-1 (18), a major ligand on BMSCs for VLA-4 on MM cells (19), and thereby might inhibit MM cell-BMSC binding and related protection against apoptosis. Second, adherence of MM cells to BMSCs triggers NF-κB-dependent transcription and secretion of IL-6 (19), a MM cell growth and survival factor (20). By virtue of its inhibition of NF-κB activation (7–10), PS-341 can inhibit this synthesis of IL-6. Third, Dex is a major therapy for MM, and PS-341 synergizes with Dex (11). Moreover, resistance to Dex in MM cells is conferred by IL-6 (21–23), and PS-341 may overcome Dex resistance by virtue of its effects on IL-6. In addition, NF-κB has been shown to play a role in the rescue of MM cells from Dex-induced apoptosis by Bcl-2 (24), and PS-341 may also overcome Dex resistance by inhibiting NF-κB activation. Finally, increased angiogenesis has recently been described in MM BM (25), as well as significant *in vitro* and clinical activity of antiangiogenic agents such as thalidomide and its analogues (26, 27). The antiangiogenic effect of PS-341 (14, 28), therefore, represents another potential mechanism of anti-MM activity.

In this study, we examined the effects of PS-341 on human MM cell lines, freshly isolated patient MM cells, as well as MM cells adherent to BMSCs. Given that PS-341 has a favorable toxicity profile, these studies provide the framework for clinical evaluation of PS-341 in patients with MM.

MATERIALS AND METHODS

MM-derived Cell Lines and Patient MM Cells. Dex-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). Dox-, Mit-, and Mel-resistant (Dox40 cells, MR20 cells, and LR5 cells, respectively) RPMI8226 human MM cells were kindly provided by Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). IM-9, U266, ARH-77, and Hs Sultan cells were obtained from American Type Culture Collection (Rockville, MD). All of the MM cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2×10^{-3} M L-glutamine, 100 units/ml penicillin (Pen), and 100 μg/ml streptomycin (Strep; Life Technologies, Inc., Grand Island, NY). Drug-resistant cell lines were cultured with Dox, Mit, Mel, or Dex to confirm their lack of drug sensitivity. Patient MM cells (96% CD38 positive/CD45RA negative) were purified from patient BM samples, as described previously (29).

BMSC Cultures. BM specimens were obtained from patients with MM. Mononuclear cells separated by Ficoll-Hipaque density sedimentation were

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[2] To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. Phone: (617) 632-2144; Fax: (617) 632-2140; E-mail: kenneth_anderson@dfci.harvard.edu.

[3] The abbreviations used are: Dex, dexamethasone; MM, multiple myeloma; BM, bone marrow; BMSC, BM stromal cell; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4; IL, interleukin; NF-κB, nuclear factor κB; Dox, doxorubicin; Mit, mitoxantrone; Mel, melphalan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; Ab, antibody; MAPK, mitogen-activated protein kinase; STAT3, signal transducing and transcription 3; ERK2, extracellular signal-regulated kinase 2; TNF, tumor necrosis factor; EMSA, electrophoretic mobility shift analysis; PBMC, peripheral blood mononuclear cell; HUVEC, human umbilical vein endothelial cell.

used to establish long-term BM cultures, as described previously (27). When an adherent cell monolayer had developed, cells were harvested in HBSS containing 0.25% trypsin and 0.02% EDTA and were washed and collected by centrifugation.

Proteasome Inhibitor. PS-341[1] [pyrazylCONH(CHPhe)CONH(CHisobutyl)B(OH)$_2$; Millennium Predictive Medicine Inc., Cambridge, MA) was dissolved in DMSO and stored at −20°C until use. PS-341 was diluted in culture medium (0.0001–10 × 10^{-3} M) immediately before use. PS-341 and control media contained <0.1% DMSO.

DNA Synthesis. Proliferation was measured as described previously (27). MM cells (3 × 10^4 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media, PS-341, and/or Dex or recombinant IL-6 (Genetics Institute, Cambridge, MA) for 48 h at 37°C. DNA synthesis was measured by [^{3}H]thymidine (NEN Products, Boston MA) uptake. Cells were pulsed with [^{3}H]thymidine (0.5 μCi/well) during the last 8 h of 48-h cultures. All of the experiments were performed in triplicate.

Growth Inhibition Assay. The inhibitory effect of PS-341 on MM and BMSC growth was assessed by measuring MTT dye absorbance of the cells. Cells from 48-h cultures were pulsed with 10 μl of 5 mg/ml MTT to each well for the last 4 h of 48-h cultures, followed by 100 μl of isopropanol containing 0.04 N HCl. Absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale CA).

Cell Cycle Analysis. MM cells and patient MM cells cultured for 0, 4, 6, 8, 12, and 16 h in PS-341 (0.01 × 10^{-6} M) or control media were harvested, washed with PBS, fixed with 70% ethanol, and treated with 10 μg/ml RNase (Roche Diagnostics Corp., Indianapolis, IN). Cells were then stained with PI (Sigma; 5 μg/ml), and cell cycle profile was determined using the program M software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL), as in prior studies (27).

Assays of Apoptosis. MM cells were cultured for 12 h at 37°C in the presence of PS-341 (0.01 × 10^{-6} M). To assay for apoptosis, genomic DNA, extracted using a genomic DNA purification kit (Promega, Madison, WI) was electrophoresed on 2% agarose gel containing 5 μg/ml ethidium bromide and was analyzed under UV light for DNA fragmentation, as in prior studies (27). Additional assays of apoptosis included PI staining for the percentage of sub-G_0/G_1 phase cells and caspase-3 cleavage, as in our prior studies (27).

Immunoblotting. MM cells were cultured with PS-341 and were harvested, washed, and lysed using lysis buffer [50 × 10^{-3} M Tris-HCl (pH 7.4), 150 × 10^{-3} M NaCl, 1% NP40, 5 × 10^{-3} M EDTA, 5 × 10^{-3} M NaF, 2 × 10^{-3} M Na_3VO_4, 1 × 10^{-3} M PMSF, 5 μg/ml leupeptine, and 5 μg/ml aprotinin]. For detection of p21, p27, Bcl-2, Bax, caspase-3, phospho-MAPK, phospho-STAT3, ERK2, or α-tubulin, cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA), and immunoblotted with anti-p21, anti-p27, anti-Bcl-2, anti-Bax, anti-ERK2, anti-caspase-3 (Santa Cruz Biotechnology), or anti-α-tubulin (Sigma) Abs. To characterize the inhibition of growth signaling by PS-341, immunoblotting was also done with anti-phospho-specific MAPK or anti-phospho-specific STAT3 Abs (New England Biolabs, Beverly, MA).

Assays of NF-κB Activation. To analyze the effect of PS-341 on degradation of IκB induced by TNFα (R&D Systems) in MM.1S cells, MM.1S cells were pretreated with control media (0.05% DMSO) or PS-341 (5 × 10^{-6} M) for 2 h. TNFα (5 ng/ml) was then added for the times indicated, and the cells were washed with PBS. Whole-cell extracts were prepared and analyzed by Western blotting using anti-IκBα Ab (Santa Cruz Biotechnology). The antigen-Ab complexes were visualized by ECL.

To assay for NF-κB activation in BMSCs, BMSCs were preincubated with PS-341 (5 × 10^{-6} M for 1 h) before stimulation with TNFα (10 ng/ml) for 10, 20, or 30 min. Cells were then pelleted, resuspended in 400 μl of hypotonic lysis buffer A [20 × 10^{-3} M HEPES (pH 7.9), 10 × 10^{-3} M KCl, 1 × 10^{-3} M EDTA, 0.2% Triton X-100, 1 × 10^{-3} M Na_3VO_4, 5 × 10^{-3} M NaF, 1 × 10^{-3} M PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin], and kept on ice for 20 min. After centrifugation (14,000 × *g* for 5 min) at 4°C, the nuclear pellet was extracted with 100 μl of hypertonic lysis buffer B [20 × 10^{-3} M HEPES (pH 7.9), 400 × 10^{-3} M NaCl, 1 × 10^{-3} M EDTA, 1 × 10^{-3} M Na_3VO_4, 5 × 10^{-3} M NaF, 1 × 10^{-3} M PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) on ice for 20 min. After centrifugation (14000 × *g* for 5 min) at 4°C, the supernatant was diluted to 100 × 10^{-3} M NaCl and subjected to SDS-PAGE. Nuclear extracts were immunoblotted with anti-p65 NF-κB Ab (Santa Cruz Biotechnology). The PVDF membrane was stripped and reprobed with anti-nucleolin Ab (Santa Cruz Biotechnology) to confirm equal loading of protein.

EMSA. Nuclear extracts for EMSAs were carried out as in our previous studies (19). Double-stranded NF-κB consensus oligonucleotide probe (5′-GGGGACTTTCCC-3′, Santa Cruz Biotechnology) was end-labeled with [γ-^{32}P]ATP (50 μCi at 222 TBq/mM; NEN, Boston, MA). Binding reactions containing 1 ng of oligonucleotide and 3 μg of nuclear protein were conducted at room temperature for 20 min in total volume of 10 μl of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 0.5 μg of poly (dI·dC) (Pharmacia, Peapack, NJ)]. For supershift analysis, 1 μg of anti-p65 NF-κB Ab was added 5 min before the reaction mixtures, immediately after the addition of radiolabeled probe. The samples were loaded onto a 4% polyacrylamide gel, transferred to Whatman paper (Whatman International, Maidstone, United Kingdom), and visualized by autoradiography.

Effect of PS-341 on Paracrine MM Cell Growth and Signaling in the BM. Adhesion assays were performed as described previously (19). MM.1S cells were pretreated with PS-341 for 12 h, washed, and labeled with $Na_2{}^{51}CrO_4$ (NEN). Cells were then added to BMSC-coated 96-well plates and incubated for 1 h. After incubation, each well was washed twice with media and lysed with 0.5% NP40, and lysate radioactivity was counted on a gamma counter.

To evaluate growth stimulation and signaling in MM cells adherent to BMSCs, 3 × 10^4 MM.1S cells were cultured in BMSC-coated 96-well plates for 48 h in the presence or absence of PS-341. DNA synthesis was measured as described above. To characterize the signaling in MM cells that is triggered by the adhesion to BMSCs, MM.1S cells (5 × 10^6) were cultured in BMSC-coated 6-well plates for 4 h in the presence or absence of PS-341 (5 × 10^{-6} M). MM.1S cells were harvested, washed with PBS, lysed, subjected to SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-phospho-MAPK and anti-phospho-STAT3 Abs. The Duoset ELISA (R&D System) was used to measure IL-6 in supernatants of 48-h cultures of BMSCs with or without MM.1S cells, in the presence or absence of PS-341.

Statistical Analysis. Statistical significance of differences observed in drug-treated *versus* control cultures was determined using Student's *t* test. The minimal level of significance was $P < 0.05$.

RESULTS

Effect of PS-341 on Growth of MM Cell Lines and MM Patients' Cells. We first determined the effect of PS-341 on growth of MM cell lines (U266, IM-9, and Hs Sultan) cultured for 48 h, either in the presence or absence of PS-341, using MTT assay. As shown in Fig. 1*A*, the growth of these MM cell lines was completely inhibited by PS-341 (0.1 × 10^{-6} M). Fifty % growth inhibition (IC_{50}) in U266, IM-9, and Hs Sultan cells was noted at concentrations of 0.003, 0.006, or 0.02 × 10^{-6} M, respectively. To examine whether there was cross-resistance between PS-341 and conventional therapies, RPMI8226 MM cells resistant to Dox (Dox/40 cells), Mit (MR20 cells), and Mel (LR5 cells) were similarly studied. Proliferation of Dox40, MR20, and LR5 MM cells was unaffected by culturing with 400 nM/liter Dox, 20 nM/liter Mit, and 5 × 10^{-6} M Mel, respectively (data not shown). Importantly, cell growth of Dox40, MR20, and LR5 MM cells was completely inhibited by PS-341 (0.1 × 10^{-6} M; Fig. 1*B*). IC_{50} of PS-341 in RPMI8226, Dox40, MR20, and LR5 was 0.03, 0.04, 0.02, and 0.02 × 10^{-6} M, respectively. Dex-sensitive (MM.1S) and -resistant (MM.1R) MM cell lines were similarly examined. As can be seen in Fig. 1*C*, growth of both cell lines was completely inhibited by PS-341 (0.01 × 10^{-6} M). IC_{50} of PS-341 in MM.1S and MM.1R cells was 0.0015 and 0.003 × 10^{-6} M, respectively. These data demonstrate that PS-341 effectively inhibits the growth of chemoresistant MM cells at pharmacologically achievable doses and can overcome resistance to Dox, Mit, Mel, and Dex. As was true for MM cell lines, cell growth of four patients' MM cells (MM1–4) was also inhibited by PS-341 in a dose-dependent fashion (Fig. 1*D*), with IC_{50}

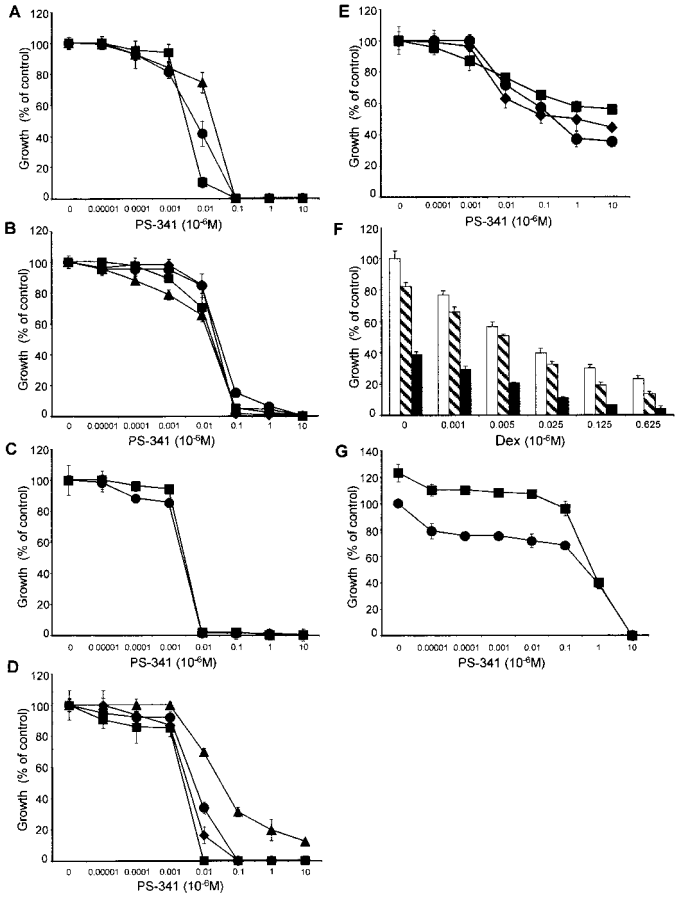

Fig. 1. Effect of PS-341 on growth of MM cell lines and patient MM cells. *A*, U266 (■), Hs Sultan (▲), and IM-9 (●) MM cells; *B*, drug-sensitive RPMI (◆), Dox-resistant (Dox40; ●), Mit-resistant (MR20; ▲), and Mel-resistant (LR5; ■) MM cells; *C*, Dex-sensitive MM.1S (●) and -resistant MM.1R (■) cells; and *D*, patient 1(◆), 2 (●), 3 (▲), and 4 (■) MM cells were cultured with PS-341 (0.00001–10 $\times$ 10^{-6} M). *E*, PBMCs from normal volunteers were cultured with PS-341 (0.0001–10 $\times$ 10^{-6} M). *F*, MM.1S cells were cultured with 0.001–0.625 $\times$ 10^{-6} M Dex in control media (□) and with PS-341 0.0025 (▨) or 0.005 (■) $\times$ 10^{-6} M. *G*, MM.1S cells were cultured in control media alone or with PS-341 (0.00001–10 $\times$ 10^{-6} M) in the presence (■) or absence (●) of IL-6 (50 ng/ml). In each case, cell growth was assessed by MTT assay. Values represent the mean (± SD) of triplicate cultures.

of PS-341 at 0.0035, 0.005, 0.03, and 0.0025 $\times$ 10^{-6} M, respectively. PS-341 similarly inhibited the proliferation of these MM cell lines and patient MM cells, as assessed by [^{3}H]thymidine uptake in 48-h cultures (data not shown). PBMCs from three normal volunteers were also examined for their susceptibility to PS-341. As can be seen in Fig. 1*E*, IC_{50} of PS-341 in these PBMCs is $\geq$0.1 $\times$ 10^{-6} M. These findings are consistent with the reported increased sensitivity to proteasome inhibitors of B chronic lymphocytic leukemia cells relative to normal B lymphocytes (30).

To determine whether PS-341 is additive with conventional therapies, we next examined the effect of Dex together with PS-341 on proliferation of Dex-sensitive MM.1S cells. As can be seen in Fig. 1*F*, MTT assay at 48 h revealed that PS-341 alone (0.0025 and 0.005 $\times$ 10^{-6} M) and Dex alone (0.001 to 0.625 $\times$ 10^{-6} M) each significantly inhibited MM.1S cell growth in a dose-dependent fashion and, furthermore, showed that their growth inhibitory effects are additive.

Given the additive effect of PS-341 and Dex (11), as well as the known role of IL-6 as a growth factor (31) and an inhibitor of Dex-induced apoptosis (32–34), we next examined whether exogenous IL-6 could overcome the growth inhibitory effect that is triggered by PS-341. Although IL-6 (50 ng/ml) triggered a 1.3-fold increase in MM.1S cell growth in cultures with media alone, PS-341 inhibited this response in a dose-dependent fashion (Fig. 1*G*). This result showed that IL-6 does not overcome the inhibitory effect of PS-341 on MM cell growth.

PS-341 Induces Apoptosis and IκBα Degradation and Inhibits p44/42 MAPK Activation in MM Cells. To further analyze the mechanism of PS-341-induced inhibition of DNA synthesis and to determine whether PS-341 induced apoptosis of MM cells, we examined the cell cycle profile of U266 and patient MM cells cultured with media or PS-341 (0.01 $\times$ 10^{-6} M) for 0, 4, 6, 8, 12, and 16 h. After incubation, cells were harvested and stained with PI. As shown in Fig. 2*A*, PS-341 induced a progressive increase in sub-G_0/G_1 phase cells in a time-dependent manner; similar results were observed for RPMI8226 and MM.1S cells (data not shown). To confirm these results, we performed agarose gel electrophoresis using genomic DNA purified from MM cell lines and patient MM cells treated with PS-341 (0.01 $\times$ 10^{-6} M) for 12 h. Apoptosis, evidenced by DNA fragmentation, was induced by PS-341 (Fig. 2*B*).

Apoptosis triggered by PS-341 was further confirmed by cleavage of caspase-3 in U266 cells (Fig. 2*C*) as well as RPMI8226, MM.1S, and patient MM cells (data not shown). Apoptosis occurred despite up-regulation of p21 and p27 in U266, IM9, ARH77, and RPMI8226 cells (Fig. 2*C*). No changes in Bcl-2 or Bax expression were induced by PS-341. These cell lines contain either wild-type p53 or mutant p53 phenotype, and these results confirm prior reports that proteasome inhibitors can induce apoptosis in both settings (1, 4, 5).

To determine whether the apoptotic effect of PS-341 is reversible, RPMI8226 cells were treated with 0.01 $\times$ 10^{-6} M PS-341 for 0, 2, 4, 6, 8, 12, or 24 h. Cells were then washed and cultured in PS-341-free media for 24 h. Cell viability and percentage of apoptotic cells were assessed by trypan blue and PI staining, respectively. Fig. 2*E* demonstrates an irreversible progressive drug exposure time-dependent effect of PS-341 on RPMI 8226 cells: >50% growth inhibition was observed in cultures with PS-341 for $\geq$6 h, with complete abrogation of growth at exposure times of >12 h.

Because PS-341 inhibits TNFα-stimulated activation of NF-κB in primary HUVECs by blocking the degradation of the inhibitor IκBα (33), we next examined whether PS-341 also inhibited degradation of IκBα in TNFα-treated MM cells. Specifically, MM.1S cells were treated with 5 $\times$ 10^{-6} M PS-341 or control media for 1 h and were subsequently stimulated by TNFα (5 ng/ml). As can be seen in Fig. 2*F*, IκBα decreased after stimulation of TNFα in DMSO control media-treated MM.1S cells, but not in PS-341-treated cells. Inhibition of NF-κB activation by PS-341 was further confirmed by EMSA. As can be seen in Fig. 2*G*, activation of NF-κB by TNFα was inhibited by pretreatment with PS-341 (5 $\times$ 10^{-6} M for 1 h). These data indicated that PS-341 inhibits NF-κB activation in MM cells by stabilizing IκBα.

Because we have shown that proliferation of MM cells induced by IL-6 is mediated via the Ras-Raf MAPK cascade (31), we also determined whether PS-341 inhibits the activation of p42/44 MAPK that is triggered by IL-6. As can be seen Fig. 2*H*, tyrosine phosphorylation of p42/44 MAPK that was triggered by IL-6 was inhibited completely by PS-341 pretreatment of MM.1S cells for 2 h, whereas the activation of STAT3 was unaffected. This result demonstrates that PS-341 selectively inhibits the tyrosine phosphorylation of MAPK that is triggered by IL-6 in MM.1S cells.

Effect of PS-341 on Paracrine MM Cell Growth and Signaling in the BM Microenvironment. We next examined the effect of PS-341 on paracrine MM cell growth and signaling in the BM. As shown in Fig. 3*A*, PS-341 inhibited the proliferation of two MM patients' BMSCs in a dose-dependent fashion, with IC_{50} of 5 and

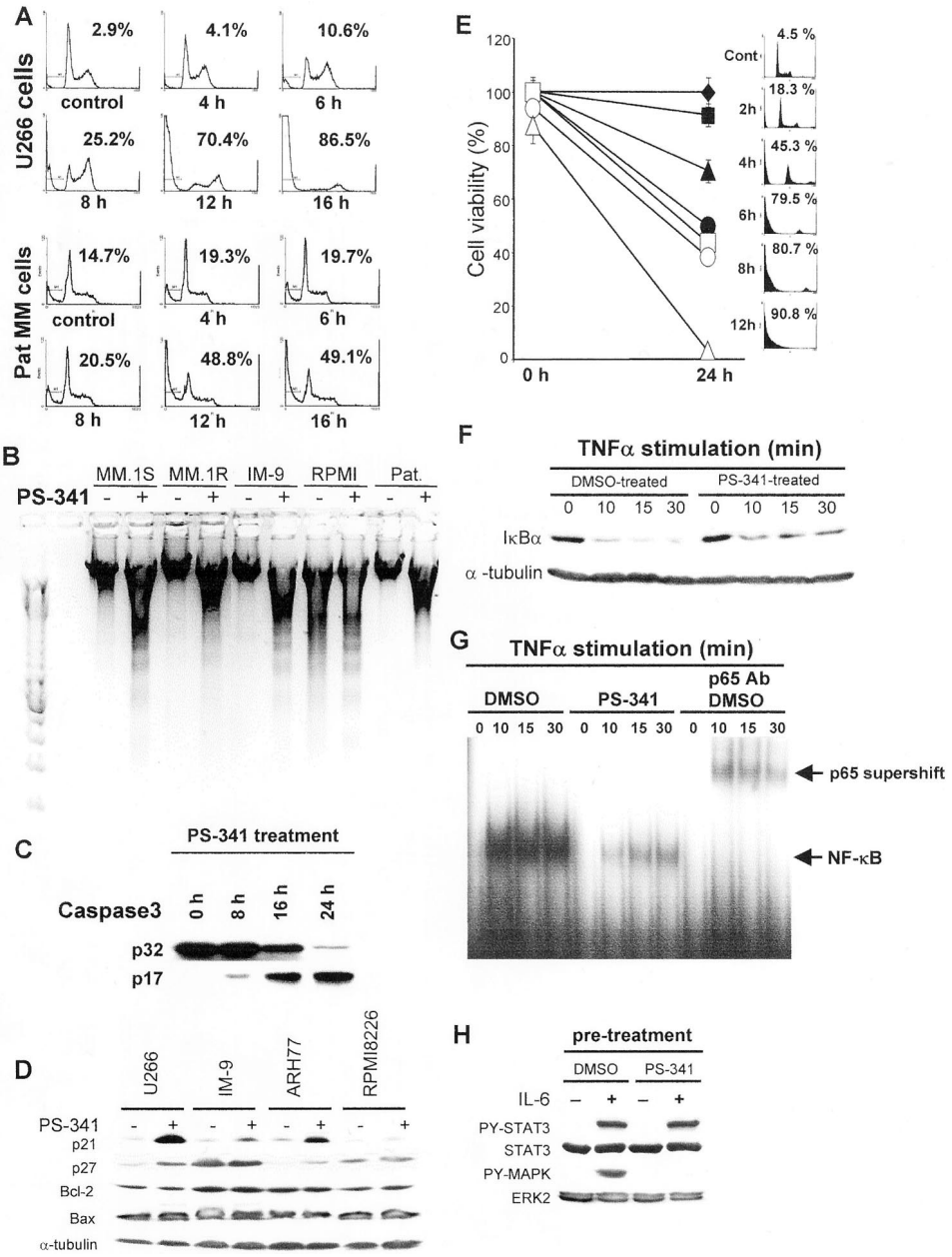

Fig. 2. Effect of PS-341 on cell cycle profile, apoptosis, and signaling cascades triggered by IL-6 in MM cell lines and patient MM cells. *A*, U266 and patient MM cells were treated with PS-341 (0.01×10^{-6} M) for the time intervals indicated. Cell cycle profile was evaluated by PI staining using flow cytometry. *% on each panel*, percentage of sub-G_0/G_1 cells. *B*, genomic DNA was extracted from MM.1S, MM.1R, IM-9, RPMI8226, and patient MM cells treated with either DMSO control (−) or PS-341 (+; 0.01×10^{-6} M for 12 h), and subjected to 2% agarose gel electrophoresis. *C*, U266 cells were incubated with PS-341 (0.01×10^{-6} M), and cell lysates were immunoblotted with anti-caspase-3 Ab. *D*, lysates from U266, IM-9, ARH77, and RPMI8226 MM cells treated with control DMSO (−) or PS-341 (+; 0.01×10^{-6} M for 12 h) were immunoblotted with anti-p21, p27, Bcl-2, Bax, and α-tubulin Abs. *E*, RPMI8226 cells were cultured with control media (◆) or 0.1×10^{-6} M PS-341 for 2 (■), 4 (▲), 6 (●), 8 (□), 12 (○), and 24 (△) h. Cells were washed, resuspended in PS-341-free media, and then stained with trypan blue and PI to assess cell viability and apoptosis. *F*, MM.1S cells were pretreated with either DMSO control or PS-341 (5×10^{-6} M for 1 h) prior to stimulation with TNFα (5 ng/ml) for indicated times. Whole-cell lysates were immunoblotted with anti-IκBα Ab to assess degradation of IκBα. *G*, MM.1S cells were pretreated with either DMSO control or PS-341 (5×10^{-6} M for 1 h) prior to stimulation with TNFα (5 ng/ml) for indicated time intervals. EMSA using nuclear extract was performed as described in "Materials in Methods." *H*, MM.1S cells were pretreated with either DMSO control or PS-341 (5×10^{-6} M for 1 h) before stimulation with IL-6 (50 ng/ml). Whole-cell lysates were immunoblotted with anti-phospho-STAT3, -phospho-MAPK, or -ERK2 Abs.

10×10^{-6} M, respectively. This IC_{50} was more than 170-fold higher than for MM cell lines and patient MM cells.

Adhesion molecules on MM cells mediate their binding to BMSCs, via VLA-4 on the MM surface binding to VCAM-1 on BMSCs, localizing them in the BM millieu (19). Given that PS-341 inhibits transcription of VCAM-1 and expression of VCAM-1 on HUVECs (18), we next determined whether PS-341 altered the adhesion of MM cells to BMSCs. Treatment with PS-341 decreased MM cell to BMSC binding by 50% (Fig. 3*B*). Binding of MM cells to BMSCs triggers NF-κB-dependent transcription and the secretion of IL-6 in BMSCs (19), which mediates paracrine growth and survival of tumor cells. As shown in Fig. 3*C*, the adhesion of MM.1S cells to BMSCs induced a >3-fold increase in IL-6 secretion, which was inhibited to baseline levels by PS-341 (0.01 and 0.1×10^{-6} M). The binding of MM cells to BMSCs also triggered a 2-fold increase in DNA synthesis of MM.1S cells, which was completely abrogated by PS-341 (0.001×10^{-6} M; Fig. 3*D*).

Given our prior studies, which showed that MM-cell binding to BMSCs triggers NF-κB-dependent transcription and secretion of IL-6 in BMSCs, we next examined the effect of PS-341 on NF-κB activation in BMSCs, assessed by p65 NF-κB nuclear translocation. As can be seen in Fig. 3*E*, TNFα (10 ng/ml for 15 min) induced nuclear p65 NF-κB in BMSCs, and PS-341 blocked this response. Finally, PS-341 also blocked the activation of MAPK, but not of STAT3, in MM cells adherent to BMSCs for 4 h (Fig. 3*F*), consistent with its effect of inhibiting tumor cell growth and proliferation.

DISCUSSION

Recent studies have shown that proteasome inhibitors represent potential novel anticancer therapy (1, 2.). These agents inhibit the degradation of multiubiquitinated target proteins, *i.e.*, cell cycle regulatory proteins such as cyclins and cyclin-dependent kinase inhibitors, and regulate cell cycle progression (3). In this study, we demonstrate that the proteasome inhibitor PS-341 not only inhibits growth and induces apoptosis of MM cell lines and patient MM cells resistant

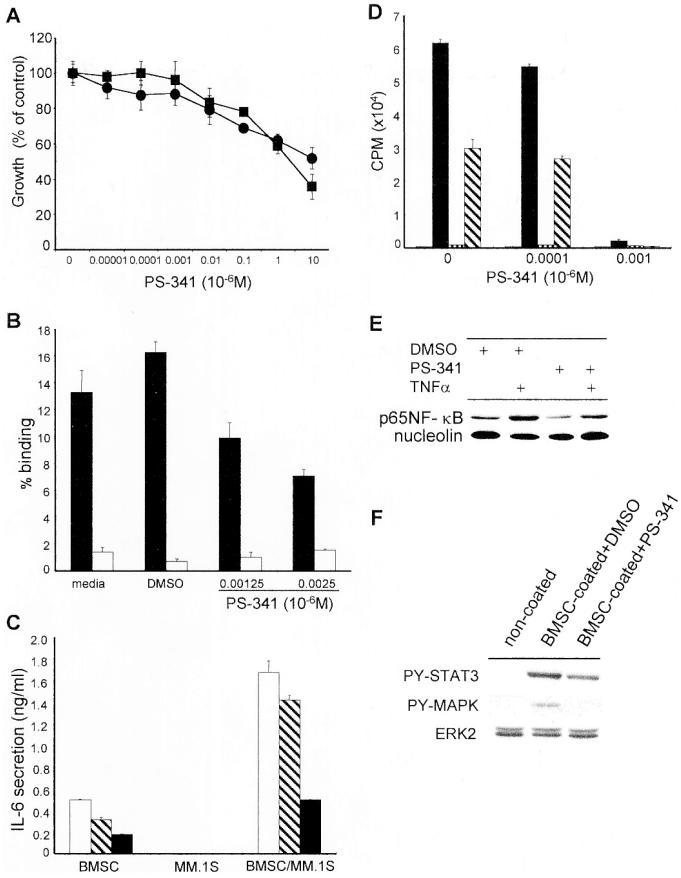

Fig. 3. Effect of PS-341 on paracrine MM.1S and patient MM cell growth and signaling in the BM microenvironment. *A*, growth of MM patient 1 (●) and 2 (■) BMSCs in 48-h cultures with PS-341 ($0.00001–10 \times 10^{-6}$ M) was measured by MTT assay. *B*, ^{51}Cr-labeled MM.1S cells were cultured with either BMSC-coated (■) or noncoated control (□) plates, and percentage of adhesion was assessed at 1 h. *C*, BMSCs or MM.1S cells were cultured alone or together in the absence (□) or presence of 0.01 (▨) or 0.1×10^{-6} M (■) PS-341; IL-6 was measured in supernatants of 48 h cultures by ELISA. *D*, BMSCs (□), MM.1S cells with BMSCs (■), MM.1S cells with 1% paraformaldehyde fixed BMSCs (▥), or MM.1S cells alone (▨) were cultured in the absence or presence of PS-341 (0.0001 and 0.001×10^{-6} M). [^{3}H]Thymidine uptake was measured during the last 8 h of 48-h cultures. *E*, BMSCs were pretreated with PS-341 (5×10^{-6} M for 1 h) and stimulated with TNFα (5 ng/ml). Nuclear translocation of p65-NF-κB was detected by the immunoblotting of nuclear extracts. PVDF membrane was stripped and reprobed with nucleolin. *F*, MM.1S cells were incubated in BMSC-coated plates for 4 h, either in the presence or absence of PS-341 (5×10^{-6} M). MM.1S cells were harvested, lysed, electrophoresed, and immunoblotted with phospho-STAT3, phospho-MAPK, and ERK Abs.

to conventional therapies but also inhibits binding of MM cells to BMSCs with related up-regulation of IL-6 secretion and paracrine MM cell growth. Given the favorable toxicity profile of PS-341, these studies provide the framework for clinical trials of PS-341 in MM.

In this study, we first showed that PS-341 acts directly to inhibit the growth of MM cell lines and patient MM cells, assessed both by MTT assay and DNA synthesis. Growth inhibition of MM cell lines that were sensitive and resistant to Mel, Dox, and Dex was observed at an IC_{50} of $<0.01 \times 10^{-6}$ M PS-341. These data demonstrate that PS-341 effectively inhibits the growth of chemoresistant MM cells at pharmacologically achievable doses and suggests independent mechanisms of resistance to Dox, Mel, and Dex *versus* PS-341. These results are also consistent with the reported increased sensitivity to proteasome inhibitors of B chronic lymphocytic leukemia cells relative to normal B lymphocytes (30).

Given the additive effect of PS-341 and Dex (11), as well as the known effect of IL-6 as a growth factor (31) and an inhibitor of Dex-induced apoptosis (23), we examined whether Dex added to the anti-MM activity of PS-341; and conversely, whether exogenous IL-6 could abrogate its antitumor effects. Importantly, Dex enhanced the inhibitory effects of PS-341 on MM growth, evidenced by MTT assay; moreover, IL-6 cannot protect MM cells against PS-341. These studies suggest the potential therapeutic advantage of combined Dex and PS-341 in treatment protocols for MM.

Our studies further confirmed that PS-341 induced apoptosis of MM cells, evidenced by cell cycle analysis, DNA fragmentation, and caspase 3 cleavage. Apoptosis occurred despite the induction of p21 and p27, and without associated changes in Bcl-2 or Bax expression. Importantly, it occurred in MM cell lines containing either wild-type p53 or mutant p53, confirming prior reports that proteasome inhibitors can induce apoptosis in both settings (1, 4, 5). Moreover, the apoptotic effect of PS-341 was irreversible at all of the intervals examined (2–24 h), further suggesting its potential clinical utility.

In this study, PS-341 inhibited IL-6-triggered activation of p42/44 MAPK, known to mediate proliferation (31), as well as TNF-α-induced activation of NF-κB, known to mediate drug resistance (24). In contrast, the IL-6 induced activation of STAT3 in MM cells was unaffected by PS-341, demonstrating differential susceptibility of protein kinases in the Raf/MEK/MAPK *versus* JAK/STAT3 signaling pathways to PS-341.

Our study demonstrates the importance of studying the effects of novel agents such as proteasome inhibitors not only on the tumor cells directly, but also within the BM microenvironment. Importantly, these studies show that PS-341 also inhibited paracrine growth of MM cells within the BM millieu. First, adhesion of MM cells to BMSCs confers protection against apoptosis (17), and PS-341 inhibits tumor cell binding. Second, PS-341 inhibited MAPK growth signaling (31), even in those MM cells adhering to BMSCs, overcoming the growth-promoting effects of BMSC binding. Third, PS-341 abrogates the NF-κB-dependent up-regulation of IL-6 triggered by tumor to BMSC binding (19), which is of central importance given that IL-6 is the major growth and survival factor for MM cells (20). In previous studies, NF-κB activation conferred protection of tumor cells against apoptosis by modulating transcription targets of the Bcl-2 homologue Bfl/A1, the immediate-early response gene *IEX-1 L*, the inhibitors of apoptosis c-IAP1 and c-IAP2, and TNF receptor-associated factors 1 and 2 (8, 34, 35). These studies, coupled with our prior studies demonstrating that IL-6 can block Dex-induced apoptosis and confer drug resistance in MM cells (21, 23, 32), further suggest that the inhibition of NF-κB activation by PS-341 can overcome drug resistance.

These studies, therefore, demonstrate that the proteasome inhibitor PS-341 both directly induces apoptosis of human MM cells and abrogates paracrine growth of MM cells in the BM via altering cellular interactions and cytokine secretion in the BM millieu. They provide the framework for the clinical investigation of these novel agents to improve outcome for patients with this presently incurable disease.

REFERENCES

1. Adams, J., Palombella, V. J., Sausville, E. A., Johnson, J., Destree, A., Lazarus, D. D., Maas, J., Pien, C. S., Prakash, S., and Elliot, P. J. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res., *59:* 2615–2622, 1999.
2. Dou, Q., and Li, B. Proteasome inhibitors as potential novel anticancer agents. Drug Resist. Updates, *2:* 215–223, 1999.
3. King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. How proteolysis drives the cell cycle. Science (Washington DC), *274:* 1652–1659, 1996.
4. Lopes, U. G., Erhardt, P., Yao, R., and Cooper, G. M. p53-dependent induction of apoptosis by proteasome inhibitors. J. Biol. Chem., *272:* 12893–12896, 1997.
5. Herrmann, J. L., Briones, F., Brisbay, S., Logothetis, C. J., and McDonnell, T. J. Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. Oncogene, *17:* 2889–2899, 1998.

6. Li, B., and Dou, Q. P. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. Proc. Natl. Acad. Sci. USA, *97:* 3850–3855, 2000.
7. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. The ubiquitin proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell, *78:* 773–785, 1994.
8. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB. Science (Washington DC), *274:* 787–789, 1996.
9. Beg, A. A., and Baltimore, D. An essential role for NF-κB in preventing TNF-α induced cell death. Science (Washington DC), *274:* 782–784, 1996.
10. Wang, C. Y., Mayo, M. W., and Baldwin, A. S. TNF-α and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science (Washington DC), *274:* 784–787, 1996.
11. Elliott, P. J., Pien, C. S., McCormack, I. D., Chapman, I. D., and Adams, J. Proteasome inhibition: a novel mechanism to combat asthma. J. Allergy Clin. Immunol., *104:* 1–7, 1999.
12. Orlowski, R. Z., Eswara, J. R., Lafond-Walker, A., Grever, M. R., Orlowski, M., and Dang, C. V. Tumor growth inhibition induced in a murine model of human Burkitt's lymphoma by a proteasome inhibitor. Cancer Res., *58:* 4342–4348, 1998.
13. Teicher, B. A., Ara, G., Herbst, R., Palombella, V. J., and Adams, J. The proteasome inhibitor PS-341 in cancer therapy. Clin. Cancer Res., *5:* 2638–2645, 1999.
14. Harbison, M. T., Bruns, C. J., Bold, R. J., Elliot, P. J., Adams, J., Abbruzzese, J. L., and McConkey, D. J. Proteasome inhibitor PS-341 is effective as an anti-angiogenic agent in the treatment of human pancreatic carcinoma via the inhibition of NF-κB and subsequent inhibition of vascular endothelial growth factor production. Proc. Am. Assoc. Cancer Res., *41:* 71, 2000.
15. Greenlee, R. T., Murray, T., Bolden, S., and Wingo, P. A. Cancer Statistics, 2000. CA Cancer J. Clin., *50:* 7–33, 2000.
16. Teoh, G., and Anderson, K. C. Interaction of tumor and host cells with adhesion and extracellular matrix molecules in the development of multiple myeloma. Hematol. Oncol. Clin. North Am., *11:* 27–42, 1997.
17. Damiano, J. S., Cress, A. E., Hazlehurst, L. A., Shtil, A. A., and Dalton, W. S. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood, *93:* 1658–1667, 1999.
18. Read, M. A., Neish, A. S., Luscinskas, F. W., Palombella, V. J., Maniatis, T., and Collins, T. The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. Immunity, *2:* 493–506, 1995.
19. Chauhan, D., Uchiyama, H., Akbarali, Y., Urashima, M., Yamamoto, K., Libermann, T. A., and Anderson, K. C. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-κB. Blood, *87:* 1104–1112, 1996.
20. Hallek, M., Bergsagel, P. L., and Anderson, K. C. Multiple myeloma: increasing evidence for a multistep transformation process. Blood, *91:* 3–21, 1998.
21. Chauhan, D., Hideshima, T., Pandey, P., Treon, S., Teoh, G., Raje, N., Rosen, S., Krett, N., Husson, H., Avraham, S., Kharbanda, S., and Anderson, K. C. RAFTK/PYK2-dependent and independent apoptosis in multiple myeloma cells. Oncogene, *18:* 6733–6740, 1999.
22. Chauhan, D., Pandey, P., Ogata, A., Teoh, G., Krett, N., Halgren, R., Rosen, S., Kufe, D., Kharbanda, S., and Anderson, K. C. Cytochrome-c dependent and independent induction of apoptosis in multiple myeloma cells. J. Biol. Chem., *272:* 29995–29997, 1997.
23. Chauhan, D., Pandey, P., Hideshima, T., Treon, S., Davies, F. E., Shima, Y., Tai, Y. T., Rosen, S., Avraham, S., Kharbanda, S., and Anderson, K. C. SHP2 mediates the protective effect of interleukin-6 against dexamethasone-induced apoptosis in multiple myeloma cells. J. Biol. Chem., *275:* 27845–27850, 2000.
24. Feinman, R., Koury, J., Thames, M., Barlogie, B., Epstein, J., and Siegel, D. S. Role of NF-κB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by Bcl-2. Blood, *93:* 3044–3052, 1999.
25. Vacca, A., Ribatti, D., Presta, M., Minischetti, M., Iurlaro, M., Ria, R., Albini, A., Bussolino, F., and Dammacco, F. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. Blood, *93:* 3064–3073, 1999.
26. Singhal, S., Mehta, J., Desikan, R., Ayers, D., Roberson, P., Eddlemon, P., Munshi, N., Anaissie, E., Wilson, C., Dhodapkar, M., Zeldis, J., and Barlogie, B. Anti-tumor activity of thalidomide in refractory multiple myeloma. N. Engl. J. Med., *341:* 1565–1571, 1999.
27. Hideshima, T., Chauhan, D., Shima, Y., Raje, N., Davies, F. E., Tai, Y. T., Treon, S. P., Lin, B., Schlossman, R. L., Richardson, P., Muller, G., Stirling, D. I., and Anderson, K. C. Thalidomide and its analogues overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood, *96:* 2943–2950, 2000.
28. Oikawa, T., Sasaki, T., Nakamura, M., Shimamura, M., Tanahashi, N., Omura, S., and Tanaka, K. The proteasome is involved in angiogenesis. Biochem. Biophys. Res. Commun., *246:* 2 43–248, 1998.
29. Tai, Y. T., Teoh, G., Shima, Y., Chauhan, D., Treon, S. P., Raje, N., Hideshima, T., Davies, F. E., and Anderson, K. C. Isolation and characterization of human multiple myeloma cell enriched populations. J. Immunol. Methods, *235:* 11–19, 2000.
30. Masdehors, P., Omura, S., Merle-Beral, H., Mentz, F., Cosset, J. M., Dumont, J., Magdelenat, H., and Delic, J. Increased sensitivity of CLL-derived lymphocytes to apoptotic death activation by the proteasome-specific inhibitor lactacystin. Br. J. Haematol., *105:* 752–757, 1999.
31. Ogata, A., Chauhan, D., Teoh, G., Treon, S. P., Urashima, M., Schlossman, R. L., and Anderson, K. C. Interleukin-6 triggers cell growth via the ras-dependent mitogen-activated protein kinase cascade. J. Immunol., *159:* 2212–2221, 1997.
32. Chauhan, D., Pandey, P., Ogata, A., Teoh, G., Treon, S., Urashima, M., Kharmanda, S., and Anderson, K. C. Dexamethasone induces apoptosis of multiple myeloma cells in a JNK/SAP kinase independent mechanism. Oncogene, *15:* 837–843, 1997.
33. Kalogeris, T. J., Laroux, F. S., Cockrell, A., Ichikawa, H., Okayama, N., Phifer, T. J., Alexander, J. S., and Grisham, M. B. Effect of selective proteasome inhibitors on TNF-induced activation of primary and transformed endothelial cells. Am. J. Physiol, *276:* 856–864, 1999.
34. Chu, Z. L., McKinsey, T. A., Liu, L., Gentry, J. J., Malim, M. H., and Ballard, D. W. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proc. Natl. Acad. Sci. USA, *94:* 10057–10062, 1997.
35. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. NF-κB antiapoptosis: induction of TRAF-1 and TRAF-2 and cIAP2 to suppress caspase-8 activation. Science (Washington DC), *281:* 680–683, 1998.