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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Thalidomide (Thal) was originally used in Europe for the treatment of morning sickness in the 1950s but was withdrawn from the market in the 1960s because of reports of teratogenicity and phocomelia associated with its use.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.

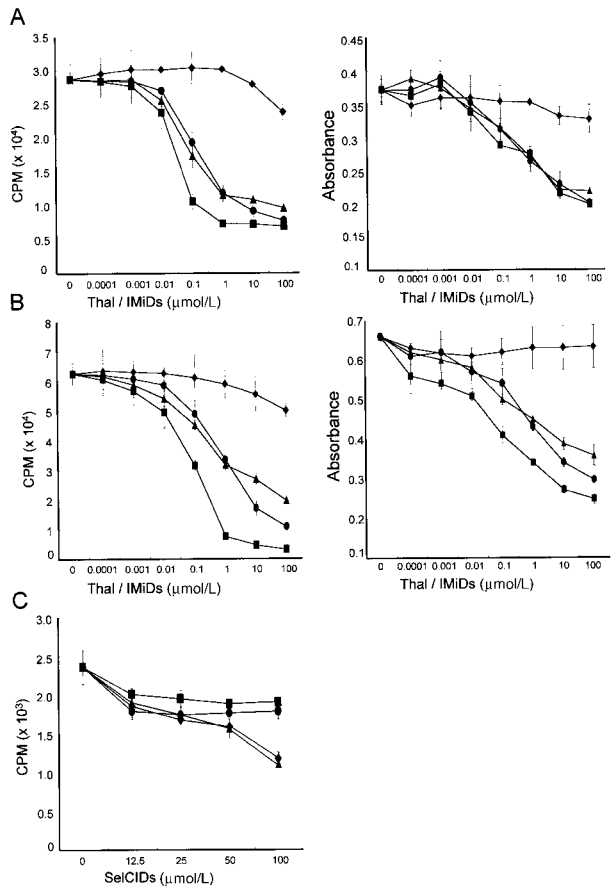

Figure 1. Effect of Thal and analogs on DNA synthesis of MM cell lines and patient cells. MM.1S (A) and Hs Sultan (B) cells were cultured with increasing concentrations (0.0001-100 μM) of Thal (◆), IMiD1 (■), IMiD2 (●), and IMiD3 (▲). (C) MM.1S cells were cultured with increasing concentrations (12.5-100 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 ($P < .001$).

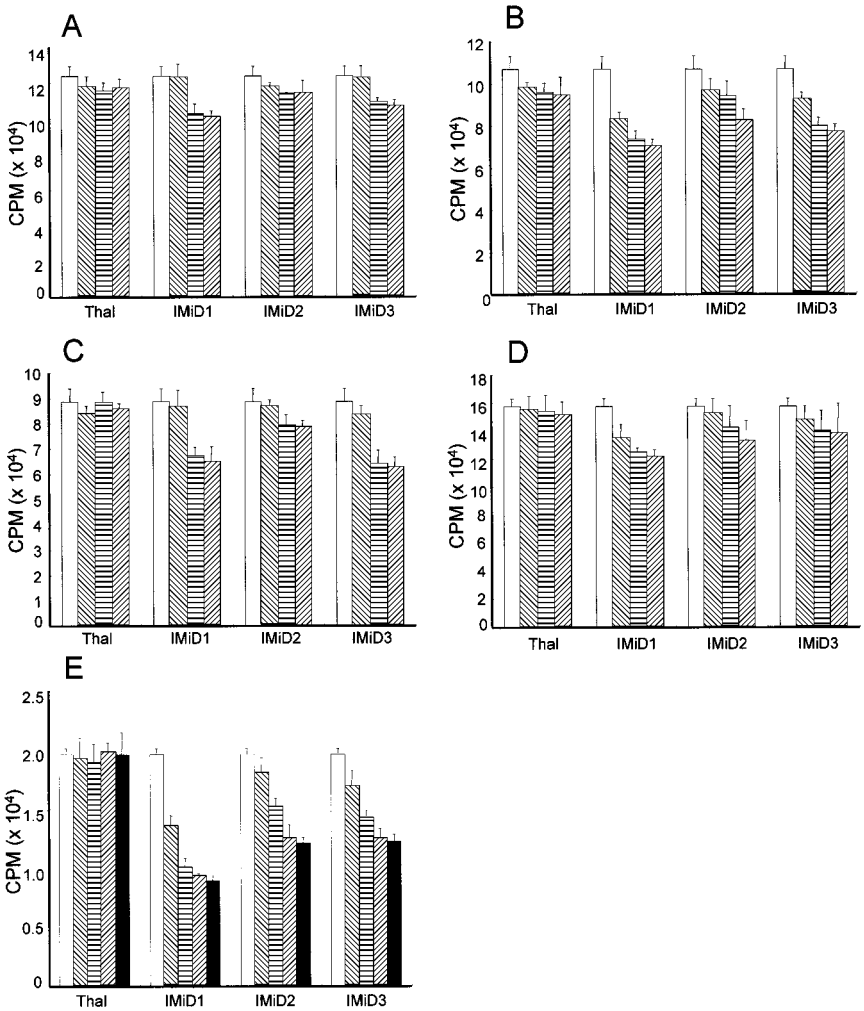

Figure 2. Effect of Thal and analogs on DNA synthesis of MM cells resistant to conventional therapy. Dox-resistant Dox6 (A) and Dox 40 (B), Mit-resistant (MR20; C), and Mel-resistant (LR5; D) cells were cultured with control media (□) or 1 μmol/L (▨), 10 μmol/L (▤), 100 μmol/L (■) of Thal, IMiD1, IMiD2, 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.

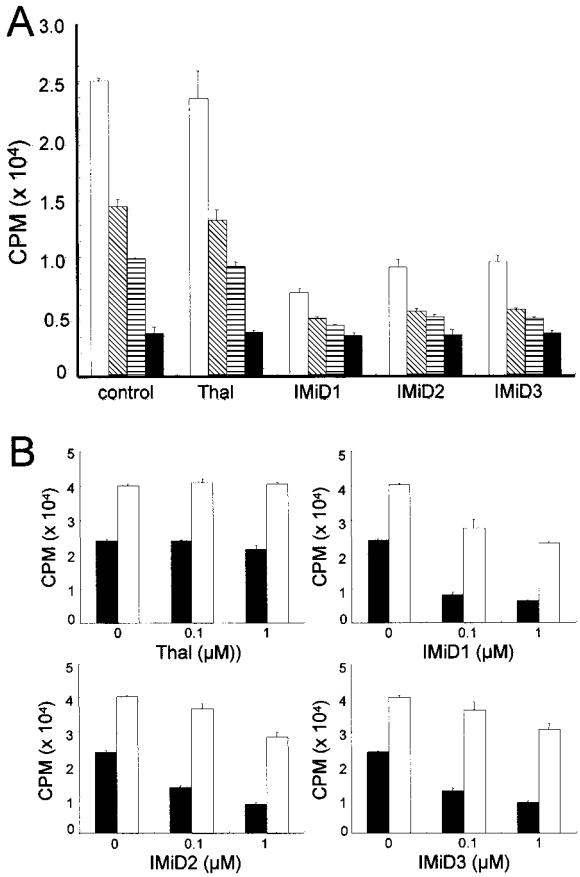

Figure 3. Effect of Dex and IL-6 on response of MM cells to Thal and the IMiDs. (A) MM.1S cells were cultured with 1.0 μM Thal, IMiD1, IMiD2, or IMiD3 in control media alone (□) or with 0.001 (▨), 0.01 (▤), and 0.1 μmol/L (■) Dex. (B) MM.1S cells were cultured in control media alone and with 0.1 and 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

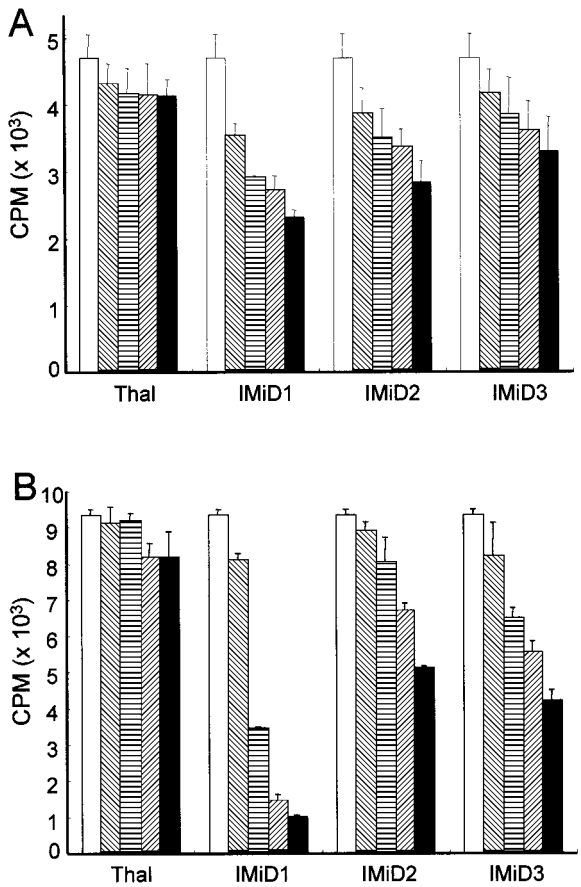

Figure 4. Effect of Thal and analogs on DNA synthesis of patient MM cells. MM cells from patient 1 (A) and patient 2 (B) were cultured with control media (□) or with 0.1 μmol/L (▨), 1.0 μmol/L (▤), 10 μmol/L (▨), and 100 μmol/L (■) Thal, IMiD1, IMiD2, or IMiD3. In each case, ^{3}H-TdR uptake was measured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

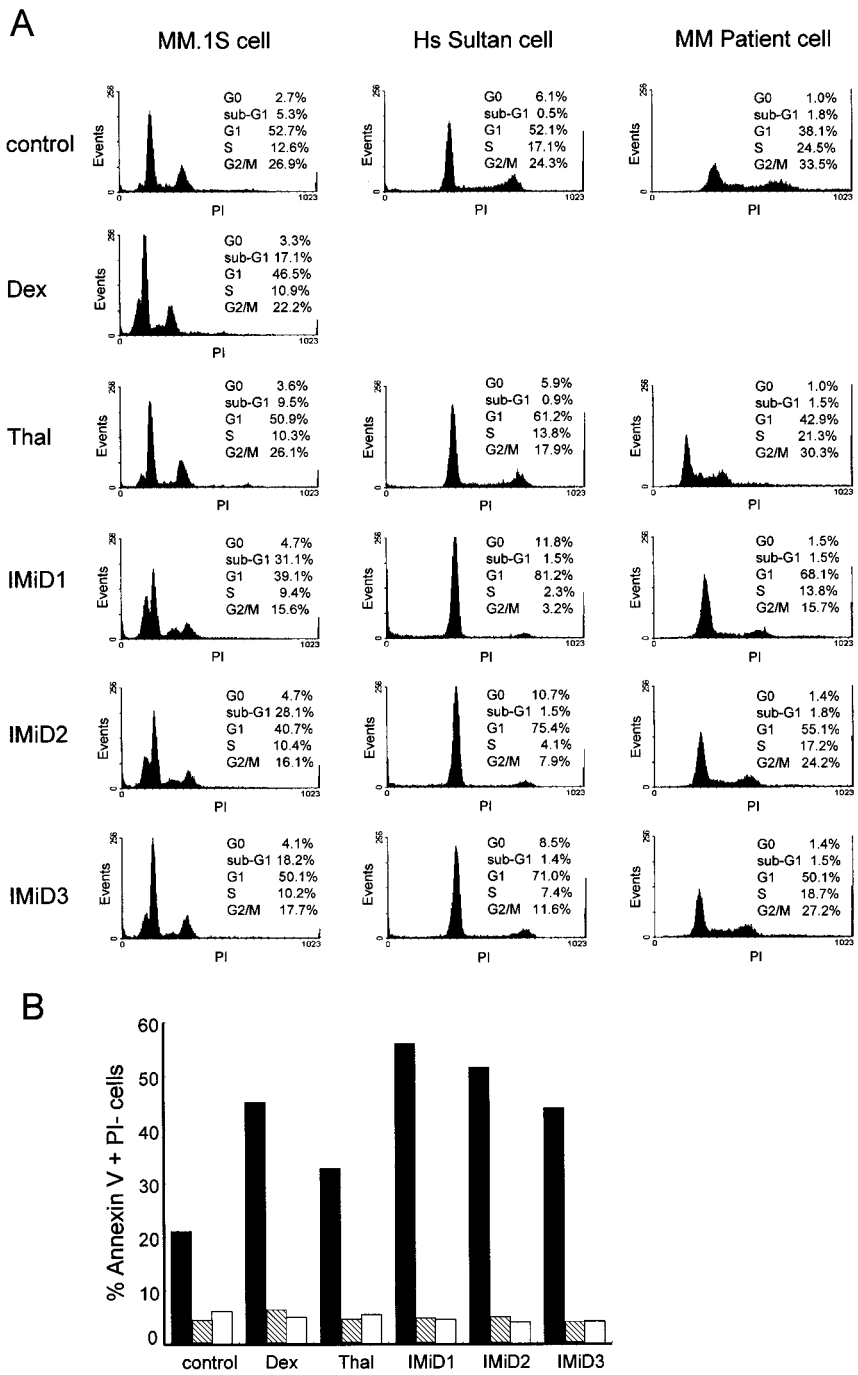

Figure 5. Effect of Thal and analogs on cell cycle profile of MM cell lines and patient MM cells. (A) MM.1S cells, Hs Sultan cells, and patient MM cells were cultured with 10 μmol/L of Thal or 1 μmol/L of IMiD1, IMiD2, or IMiD3 for 72 hours. Cultures in media control alone served as a negative control 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

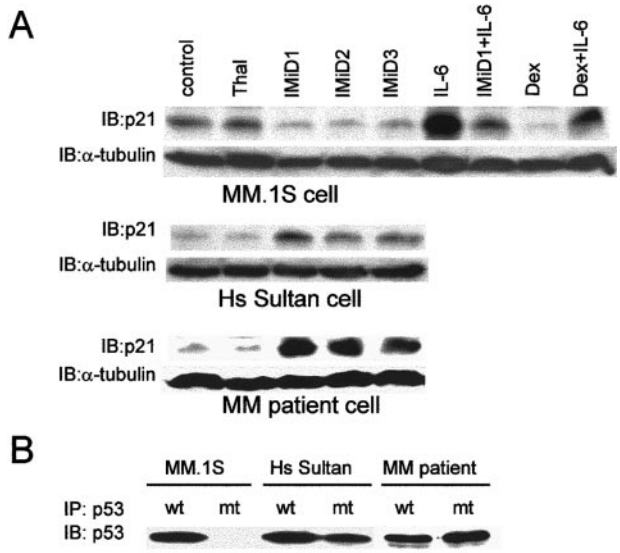

Figure 6. Effect of Thal and analogs on p21 expression in MM cell lines and patient cells. (A) MM.1S cells were cultured with 10 μmol/L of Thal, IMiD1, IMiD2, and IMiD3 for 48 hours. MM.1S cells were also cultured with IL-6 (50 ng/mL) alone and with IMiD1, 10 μmol/L Dex, and Dex plus IL-6. Cells were 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.

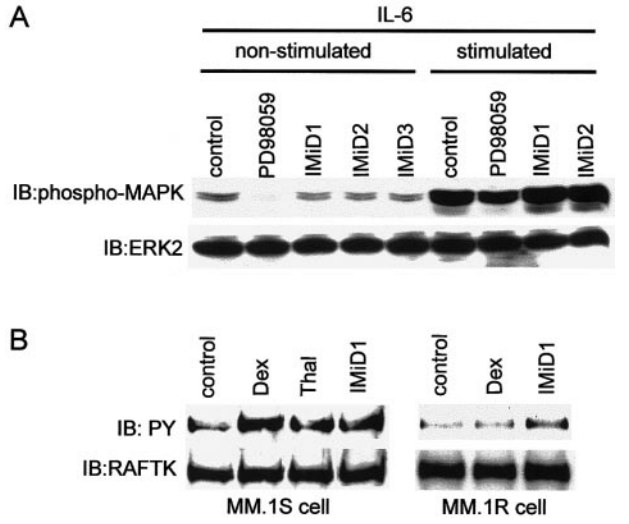

Figure 7. Effect of Thal and analogs on growth and apoptotic signaling in MM.1S and MM.1R cells. (A) MM.1S cells were cultured in media, with 50 μmol/L of PD98059 and with 10 μmol/L of IMiD1, IMiD2, or IMiD3 for 48 hours. Cells were then triggered with 50 ng/mL of IL-6 for 10 minutes, lysed, transferred 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.

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