Co-administration of simvastatin and cytotoxic drugs is advantageous in myeloma cell lines

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We have evaluated the potential application of simvastatin (Sim) combined with conventional cytotoxic drugs for the treatment of multiple myeloma. RPMI 8226 and U266 myeloma cells seeded in culture plates were treated with Sim (5 and 10 μM, respectively) combined with melphalan (Mel; 25 and 20 μM, respectively) or dexamethasone (Dex; 1 μM). We assessed cell cycle (propidium iodide staining and flow cytometric analysis), cell morphology, viability (WST1), total cell count and cell death (Trypan blue exclusion). Sim significantly enhanced the anti-myeloma activity of cytotoxic agents *in vitro* ($p < 0.05$). Incubation of U266 and RPMI 8226 with Sim prior to Mel increased the cytotoxicity in an additive manner, whereas the exposure of U266 to combined Sim and Dex resulted in a synergistic amplification of the individual effects. Combined application of Dex and Sim to RPMI 8226 cells resulted in antagonistic activity. The possible roles of Ras and phosphoinositol 3-kinase are discussed. *Anti-Cancer Drugs* 15:79–84 © 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:79–84

Keywords: combined chemotherapy, dexamethasone, hydroxymethylglutaryl-CoA reductase, melphalan, statin

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Sponsorship: Merck & Co. (Rahway, NJ) generously provided simvastatin for this study.

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Received 10 July 2003 **Revised form accepted** 10 September 2003

Introduction

Multiple myeloma is a progressive disease characterized by the accumulation of malignant plasma cells in the bone marrow [1]. Despite the continuous expansion of data regarding signaling pathways involved in viability and proliferation of myeloma cells, their genetic heterogeneity undermines the understanding of the disease's pathogenesis and impedes the promotion of effective treatment. Previous studies demonstrated that the genetic background alters therapeutic responses [2,3]. Moreover, the mutator phenotype typical of cancer cells continuously promotes the evolvement of a heterogeneous cell population and, when exposed to chemotherapy, more than one mechanism of multidrug resistance can be selected for [4]. Specifically, multiple myeloma cells are known to acquire a chemoresistant phenotype that includes drug efflux pumps, changes in apoptotic threshold and increased ability to detoxify or metabolize drug agents [5]. As a result, multiple myeloma is an incurable malignancy with an ongoing 5-year survival rate for the past two decades. The classic combination of melphalan (Mel) and prednisone is still the standard treatment for most patients, and a novel approach is desperately needed [6].

In the pursuit for more potent treatment, combined chemotherapy is utilized thereby enhancing anti-tumor effectiveness of currently available chemotherapeutic drugs, overcoming drug resistance mechanisms and reducing normal tissue toxicity.

We recently demonstrated anti-myeloma activity of simvastatin (Sim), a hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor widely prescribed for hypercholesterolinemia [7]. It was also reported that Sim has an additive effect when combined with cytosine arabinoside for the treatment of acute myelogenous leukemia *in vitro* [8]; we hypothesized it may also be advantageous in the treatment of myeloma. The present work was designed to assess the potential benefit of Sim administration combined with conventional cytotoxic drugs [Mel and dexamethasone (Dex)] used for treatment of multiple myeloma.

Materials and methods

Cell lines

Multiple myeloma cell lines U266 and RPMI 8226 were purchased from the ATCC (Rockville, MD), and cultured in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (FCS) and antibiotics (Biological Industries, Beit Haemek, Israel). Twenty-four hours prior to the experiments, 3×10^6 cells were seeded in 10 ml fresh media.

0959-4973 © 2004 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000109323.56830.0f

Reagents

Mel (#M-2011) and Dex (#D-8893) were purchased from Sigma (St Louis, MO). Stock solutions of 2.5 mM Dex in ethanol and 10 mM Mel in ethanol were prepared. The drugs were administered to cell lines at a final concentration of 1 μM Dex and 25 or 20 μM Mel to RPMI 8226 and U266, respectively. Sim was provided by Merck (Rahway, NJ). Sim stock solution of 10 mM in dimethyl sulfoxide (DMSO) was used for cell treatments maintaining 0.1% DMSO in all. Respective dose curves and IC_{50} were established previously in our laboratory for each cell line, and accordingly we used 10 μM Sim for U266 and 5 μM Sim for RPMI 8226 (alone or in combinations with cytotoxic drugs). All cells were exposed to identical concentrations of DMSO.

Culture conditions

Myeloma cells were seeded at an initial cell concentration of 2×10^5/ml in 100 μl medium in 96-well culture plates. Drugs at indicated concentrations were added alone or in combination with Sim and incubated at 37°C for 5 days. Cells treated with solvent only (DMSO) were considered a control.

Viability and proliferation assays

WST1 assay. WST1 cell proliferation reagent (# 1644807; Roche, Mannheim, Germany) measures the metabolic activity of viable cells, based on the cleavage of the tetrasolium salt WST1 by mitochondrial dehydrogenases in viable cells. Assessment of viability was performed according to the manufacturer's instructions. Absorbency was measured by a scanning multiwell spectrophotometer (ELISA reader model 'Sunrise') (Tecan, Austria) and results were plotted on a linear scale. Cell survival was estimated as a percentage of the value of solvent-treated (DMSO) controls. At least three separate experiments in hexaplicates were conducted and results are expressed as the mean ± SE.

Dye exclusion assay. Total cell counts as well as the respective proportion of viable and dead cells were enumerated by Trypan blue dye exclusion using a hematocytometer and a phase-contrast microscope. Experiments were performed 3 times to determine mean ± SE percentage of viable and dead cells.

Cell-cycle analysis

Multiple myeloma cells (2×10^6) were cultured in six-well culture dishes with media and drugs in the presence or absence of Sim for 5 days. Thereafter cells were harvested, sedimented by centrifugation at 300*g* and resuspended in 50 μl PBS. Cell perforation and DNA propidium iodide (PI) labeling were performed according to the manufacturer's instructions with the DNA-Prep System Kit (#6607055; Beckman Coulter, Miami, FL). DNA content was examined by flow cytometry employing a FACS Epics-XL (Beckman Coulter).

Statistical analysis

In the figures, the data are presented as the mean values for a 95% confidence interval. Multiple statistical comparisons were performed using ANOVA in a univariate linear model. Other statistical comparisons were completed using Student's paired *t*-test; $p < 0.05$ was considered statistically significant. A significant interaction between treatments was regarded as an indication of a synergistic mode of action between drugs in combined administration. Lack of significant interaction was concluded to be the result of an additive effect.

Results

Sim induces cell-cycle arrest after 72 h treatment

In order to determine the time needed for incubation of multiple myeloma cell lines with Sim prior to the addition of cytotoxic drugs, we assessed its effect on the progression of the cells cycle. RPMI 8226 as well as U266 responded to Sim administration with an arrest (G_2/M and G_1, respectively) following 72 h treatment. Three independent experiments conducted in duplicate were analyzed (data not shown). Based on these results we decided to supplement the multiple myeloma cell lines with pre-determined doses of Mel and Dex after a 72-h treatment with the respective IC_{50} dose of Sim. The cell lines were then incubated for an additional 48 h with the combined drugs (Sim and Mel/Dex).

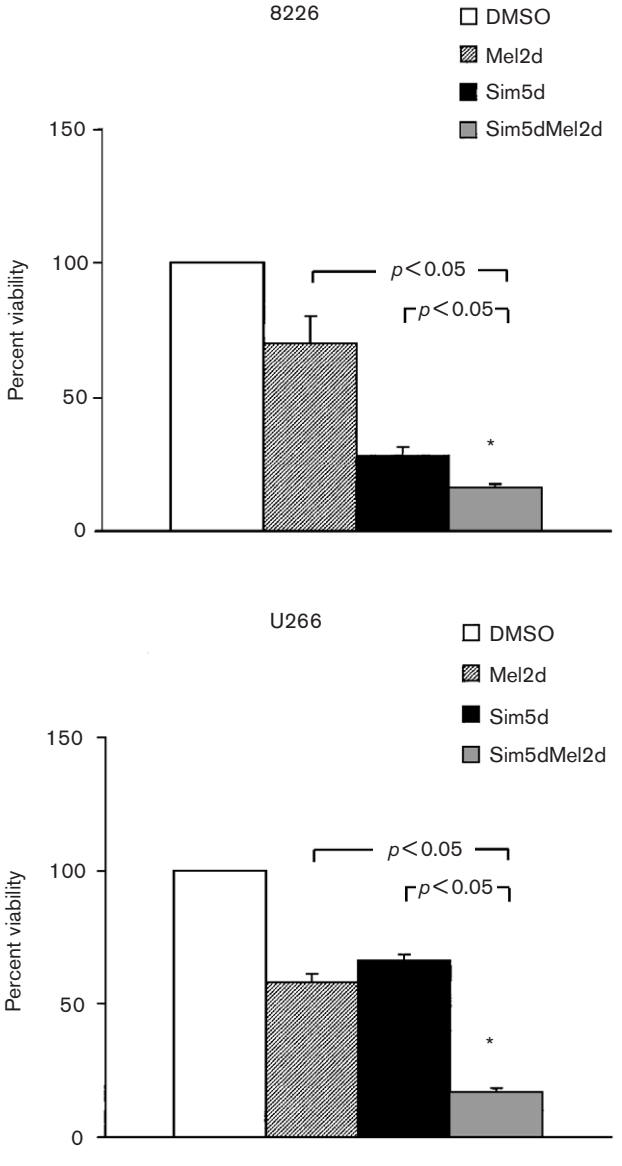
An additive anti-myeloma effect is attributed to combined Sim and Mel

RPMI 8226 and U266 exhibited reduced viability with either Sim or Mel treatment ($p < 0.05$). (Fig. 1). Combined Sim and Mel reduced viability of both cell lines to an extent that is statistically different than demonstrated with either drug alone ($p < 0.05$) (Fig. 1). Assessment of the drugs' influence on cell proliferation and cell death (Fig. 2) indicated that the diminished viability could be attributed to the cumulative effect of reduced proliferation and elevated cell death. Indeed, following the combined treatment, both cell lines were characterized with lower total cell counts as well as a smaller fraction of viable cells ($p < 0.05$ U266; $p < 0.01$ RPMI 8226). Simultaneous administration of Sim and Mel displayed an additive inhibitory effect on the growth of both cell lines.

Co-administration of Sim with Dex induces a synergistic anti-myeloma effect on U266, and counteracts the proliferative effect of Dex on RPMI 8226

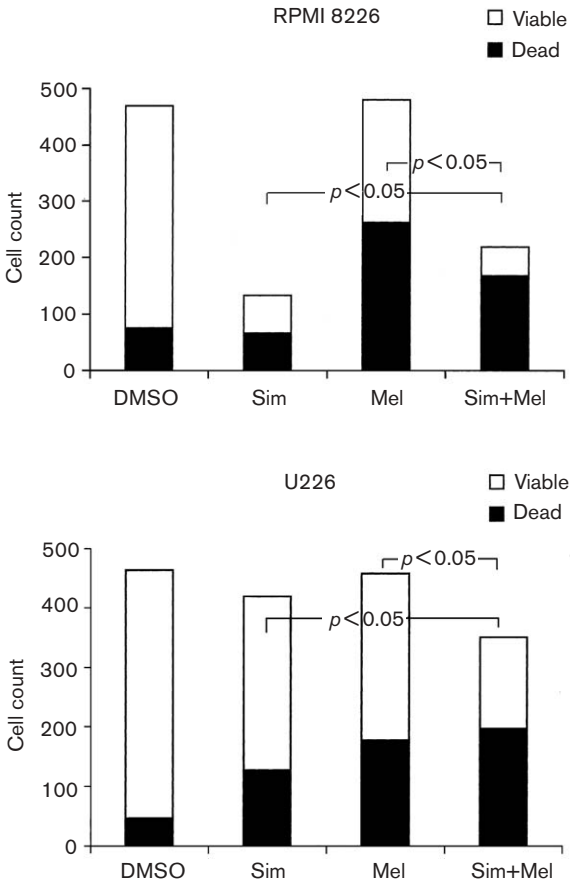
Our studies indicate that U266 is unresponsive to Dex, whereas RPMI 8226 responds to its administration with enhanced proliferation, primarily following a 5-day exposure to this drug ($p < 0.05$) (Fig. 3). The enhanced viability was attributed to elevated total cell counts in 5-day treated cells ($p < 0.05$) and diminished cell death (2- and 5-day Dex treatments) ($p < 0.05$) (Fig. 4). Upon combined exposure to Dex and Sim, both cell lines

Fig. 1



Viability of multiple myeloma cell lines treated with Sim, Mel and combinations. RPMI 8226 (top) and U266 (bottom) cultured in 96-well tissue dishes in the presence of DMSO, Sim (5 or 10 μM, respectively, for 5 days), Mel (25 or 20 μM, respectively, for 2 days) and Sim combined with Mel were assayed with WST1 proliferation reagent (see Materials and methods) for mitochondrial metabolic activity, indicative of cell viability. Hexaplicates of each treatment were averaged in each experiment and cell survival was estimated as a percentage of the value of DMSO-treated cells. The graph bars represent the mean viability of three separate experiments conducted for each cell line and SEs between experiments are indicated. Difference between treatments of separate drugs and their combined effect is depicted (respective *p* value). Assessment of interaction between drugs indicated an additive effect (*).

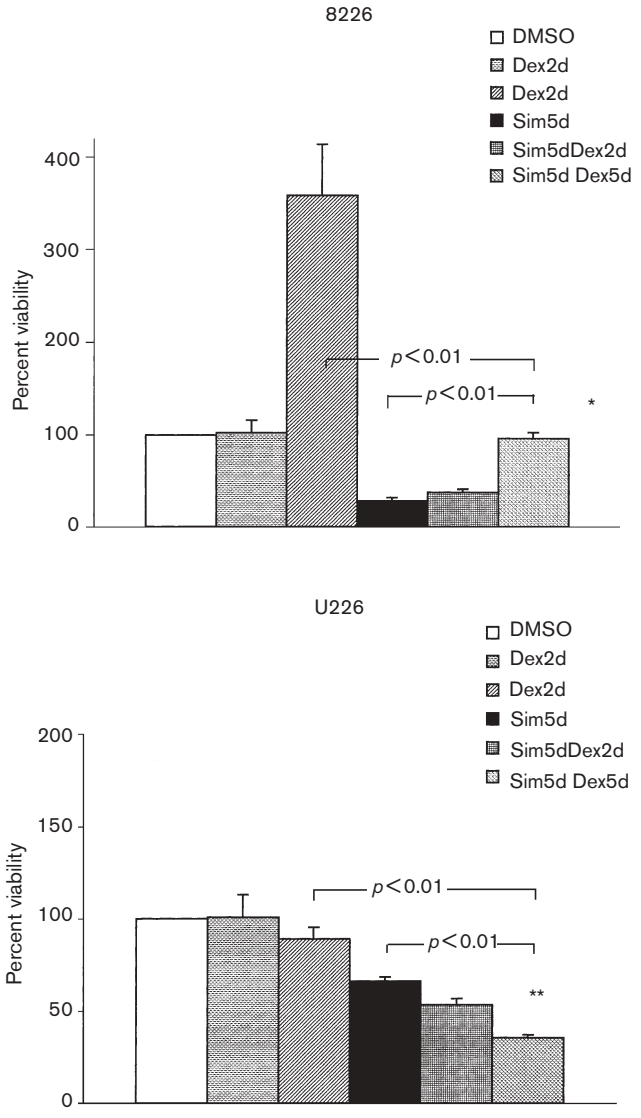
Fig. 2



Cell count of multiple myeloma cell lines treated with Sim, Mel and combinations. RPMI 8226 (top) and U266 (bottom) cultured in 96-well tissue dishes in the presence of DMSO, Sim (5 or 10 μM, respectively, for 5 days), Mel (25 or 20 μM, respectively, for 2 days) and Sim combined with Mel were counted and assessed for viability by dye exclusion with Trypan blue (see Materials and methods). Total cell counts as well as the respective proportion of viable (white upper portion of bars) and dead cells (black lower portion of bars) were enumerated. Experiments were performed 3 times and their mean values are represented in these graphs. Difference between treatments of separate drugs and their combined effect is depicted (respective *p* value).

demonstrated reduced viability compared to Dex-only-treated cell lines ($p < 0.05$). This phenomenon can be primarily explained by diminished cell counts ($p < 0.05$) (Fig. 4). Moreover, this effect was evident in cells treated with Dex for both 2 and 5 days. In the case of U266, the combined treatment was also more effective than exposure to Sim alone, apparent in reduced viability accounted for by smaller cell counts ($p < 0.05$) (Figs 2 and 4). The growth inhibitory effect of the drug combinations (Sim and 5-day Dex) was characterized by a synergistic mode of action indicated in both viability assay as well as cell counts ($p < 0.01$). In comparison to U266, the anti-myeloma contribution of Sim co-administration with Dex in RPMI 8226 was more limited. Although the Dex-induced proliferation was significantly compromised, the total cell count was still elevated compared to cells treated with Sim alone ($p < 0.05$) (Figs 2 and 4), which resulted in similar viability levels in cells

Fig. 3



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treated with the combined drugs or Sim alone. In conclusion, the drugs in combined administration displayed an additive mode of cell growth inhibition primarily manifested in Sim's negating effect on Dex-induced proliferation, thereby contradicting the majority of Dex's influence.

Discussion

Multiple myeloma is a malignant proliferation of plasma cells, which fail to undergo apoptosis spontaneously. Undisputable evidence exists that anti-tumor drugs trigger apoptosis and modulation of the cells' propensity to respond to cytotoxic agents by apoptosis is continuously explored [9]. We studied the potential advantage of combined chemotherapy of Sim and Mel/prednisone, the most commonly used drugs in multiple myeloma treatment.

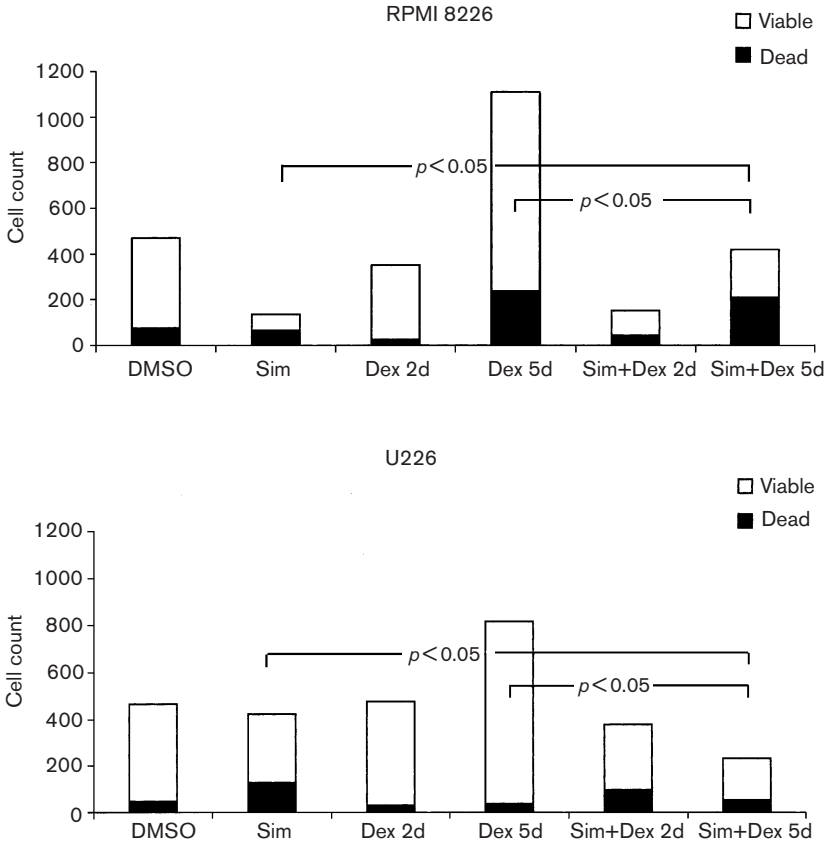
The main findings of our study are that Sim significantly enhanced the anti-myeloma activity of cytotoxic agents *in vitro*. Incubation of U266 and RPMI 8226 with Sim prior to Mel increased the cytotoxicity in an additive manner, whereas the exposure of U266 to combined Sim and Dex resulted in a synergistic amplification of the drugs individual effects.

Combined chemotherapy is a modality extensively studied and clinically employed that allows side-stepping drug resistance by addressing several cellular mechanisms simultaneously as well as lowering drug dosages and thereby toxicity through synergistic manipulation of cell sensitivity [10]. Various rationales exist for designing drug combinations which may increase the efficiency of cell kill. Recent studies have demonstrated an association between drug resistance and failure to undergo apoptosis [10,11]. With the emerging contention that most chemotherapeutic agents in current practice induce cell death through the mechanisms of apoptosis, it is conceivable that the synergistic effects of drug combination would evolve from the compound activation of the apoptotic machinery as well.

The death-inducing characteristics of Mel and Sim administered separately to RPMI 8226 and U266 have been previously demonstrated [2,7]. The alkylating agent Mel, known to induce DNA damage and cell death, is most often instigated upon progression of cells to the G_2/M phase [2,12,13]. U266 and RPMI 8226 treated with Sim were arrested in phases G_1 and G_2/M, respectively. It was therefore expected that the cell lines pre-treated with Sim might differ in their response to the addition of Mel. Yet, both cell lines responded with an enhanced cell kill upon combined administration in an additive manner. This may suggest that the targeting of the cells at multiple cycle phases or the cumulative assault of apoptotic machinery (or combination) was advantageous. The combination of drugs with specificities for different phases of the cell cycle has been previously recognized as an efficacious strategy [9].

Contrary to Mel, Dex is known to induce cell death regardless of cell cycle [12]. In addition, it was also established that RPMI 8226 and U266 are resistant to

Fig. 4



Cell count of multiple myeloma cell lines treated with Sim, Dex and combinations. RPMI 8226 (top) and U266 (bottom) cultured in 96-well tissue dishes in the presence of DMSO, Sim (5 or 10μM respectively, for 5 days), Dex (1 μM for 2 days) and Sim combined with Dex were counted and assessed for viability by dye exclusion with Trypan blue (see Materials and methods). Total cell counts as well as the respective proportion of viable (white upper portion of bars) and dead cells (black lower portion of bars) were enumerated. Experiments were performed 3 times and their mean values are represented in these graphs. Difference between treatments of separate drugs and their combined effect is depicted (respective *p* value).

Dex, and even respond by elevated proliferation as was evident in our work [14–16]. We aimed to exploit this stimulatory effect of Dex, and speculated that pre-treatment with Sim might sensitize the cells to its administration via the simultaneous disruption of cell-cycle signaling and delivery of a potent stimulation, a combination already shown to bypass the block to cell death as well as enhance the drugs' cytotoxicity in other circumstances [9]. In fact, whereas our findings demonstrated that Sim only antagonized Dex's proliferative effect on RPMI 8226, their combined application to U266 resulted in a synergistic amplification of their cytotoxicity.

The antagonistic effect of Dex in combined therapy has been previously observed with several mevalonate pathway inhibitors [17–19]. The action of Dex is yet to be elucidated, although it was suggested by Newton *et al.* that it may be attributed to a disruption of the cells' apoptotic pathway [17].

Extensive research has been done on the drug resistance mechanisms in multiple myeloma, and particularly resistance to Mel and Dex [20–22]. The phosphoinositol 3-kinase (PI3K)/Akt pathway was repeatedly singled out as a major contributing protective stimulus and its inhibition abrogated the cells drug resistance [6,21]. A recent publication has shown that PI3K is normally incorporated in cholesterol-dependent caveolae and co-precipitates with caveolin [23]. Therefore, we speculate that Sim by lowering cellular cholesterol levels causes abrogation of caveolae and hampers activation of PI3K, thereby sensitizing the cells to cytotoxic agents.

Signaling via PI3K/Akt is initiated by multiple stimuli, including interleukin (IL)-6 and insulin growth factor (IGF)-I [3]. It was also described that IL-6 and IGF-I signaling in myeloma cells necessitates functional caveolae [24]. IL-6, clearly the most important cytokine in myeloma biology, is predominantly produced in a paracrine fashion, although U266 is characterized by an

autocrine loop [21,25]. It not only triggers multiple myeloma cell growth via the MAPK signaling cascade [26], but also blocks dexamethasone-induced apoptosis via activation of PI3K/Akt signaling [27]. Furthermore, it was shown that reduced IL-6 sensitizes myeloma and specifically U266 to Dex [2,16]. Indeed, we have previously demonstrated that Sim treatment of U266 depleted the levels of secreted IL-6 [7]. Taken together, it can be stipulated that Sim administration to U266 both inhibits the PI3K pathway, and diminishes IL-6 and its protective signaling cascade in face of Dex exposure.

The cell lines used in this study vary considerably. Whereas U266 is distinguished by wild-type Ras and a mutant p53, RPMI 8226 overexpresses K-Ras and c-*myc*. The difference in Ras function may present a contributing factor to the diminished response of RPMI 8226 to combined Sim and Dex compared to U266. In fact, it is accepted that Ras signaling confers protection from apoptosis by both Dex and Mel [2,28,29].

A growing number of reports indicate an additive or even synergistic cytotoxicity of combined farnesyl transferase inhibitors (FTI) and chemotherapeutic agents in cancer cell lines [30,31]. Yet, to the best of our knowledge, this is the first report regarding the interaction between statins and Mel or Dex in multiple myeloma. Enhanced anti-malignant activity of combined Sim and cytosine arabinoside was demonstrated in acute myeloid leukemia *in vitro* [8]. In this work the authors assessed the drug combination's effect on proliferation, whereas we assayed the cell kill potential of combined Sim and Mel/Dex as well. Due to Sim's minute side-effects and the enhanced efficacy of currently available myeloma treatment there is potential advantage in combined application and further research is warranted.

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