Dexamethasone Synergizes with Lenalidomide to Inhibit Multiple Myeloma Tumor Growth, But Reduces Lenalidomide-Induced Immunomodulation of T and NK Cell Function

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Abstract: To determine the effect of dexamethasone on the antimyeloma effects of lenalidomide, we tested *in vitro* proliferation, tumor suppressor gene expression, caspase activity, cell cycling, and apoptosis levels in a series of multiple myeloma (MM) and plasma cell leukemia cell lines treated with lenalidomide and dexamethasone, alone or in combination. The effect of dexamethasone on the immunomodulatory activities of lenalidomide such as T cell and natural killer (NK) cell activation was measured *via* interleukin [IL]-2 production, and interferon-γ and granzyme B production respectively. Lenalidomide inhibited proliferation in most cell lines tested, and this effect was enhanced by dexamethasone. This effect was observed in MM cells containing the high-risk cytogenetic abnormalities t(4;14), t(14;16), del17p, del13, and hypodiploidy. Mechanistically, lenalidomide plus dexamethasone synergistically induced expression of the tumor suppressor genes *Egr1, Egr2, Egr3, p15, p21,* and *p27* in MM cell lines and MM patient cells. The combination activated caspases 3, 8, and 9 and induced cell cycle arrest and apoptosis. Lenalidomide alone increased T cell production of IL-2, and NK cell production of interferon-γ and granzyme B. Notably, dexamethasone antagonized these immunostimulatory effects of lenalidomide in a dose-dependent manner. These data further elucidate the mechanism of action of lenalidomide and dexamethasone in MM, and suggest that use of low-dose dexamethasone with lenalidomide may retain the antiproliferative effect of lenalidomide while permitting greater immunomodulatory effects of this combination regimen.

Keywords: Myeloma, lenalidomide, dexamethasone, proliferation, immunomodulation.

INTRODUCTION

Lenalidomide is an immunomodulatory agent that is approved for use in combination with dexamethasone in patients with multiple myeloma (MM) who have received at least one prior therapy. Recently reported evidence from a randomized trial conducted in patients with newly diagnosed MM suggests that overall survival was prolonged when dexamethasone was given less frequently (low-dose dexamethasone regimen defined as 40 mg/day on days 1, 8, 15, and 22 of each 28-day cycle) in combination with standard lenalidomide, compared with standard dexamethasone therapy (40 mg/day on days 1–4, 9–12, and 17–20 of each 28-day cycle) in combination with standard lenalidomide.[1]

Lenalidomide has multiple effects on myeloma cells and their microenvironment which may contribute to its anticancer effects [1], including direct antiproliferative effects on myeloma cells [2], increasing T cell cytokine production [3], and immunomodulatory effects *via* natural killer (NK) cell activity [4]. The mechanism of direct myeloma tumor growth inhibition by lenalidomide has been shown, in some cases, to involve upregulation of the tumor suppressor gene *p21* [1, 2], and activation of caspases [5, 6]. Although the antiproliferative effects of lenalidomide play a key role in the antimyeloma mechanism, the immunomodulatory effects of lenalidomide also contribute to its anticancer activity. Lenalidomide co-stimulates T cells to proliferate, produce several cytokines including interleukin (IL)-2, IL-5, IL-10 and interferon (IFN)-γ, and increase expression of CD40 ligand [3, 7-9]. The immunomodulatory effects of lenalidomide also involve NK cell activity and antibody-dependent cellular cytotoxicity. In purified human NK cells, lenalidomide enhances the production of IFN-γ and granzyme B [4], as well as enhancing NK cell-mediated lysis of both MM cell lines and MM patient cells [10].

As with other glucocorticoids, dexamethasone targets steroid receptors and affects many biological processes including glucose and protein metabolism, and inflammation. Dexamethasone is a potent anti-inflammatory agent, which is used in various therapeutic areas, including dermatology, hematology, and endocrinology. The anti-inflammatory effects of dexamethasone are produced *via* several mechanisms, including inhibition of phospholipase A2, release of arachidonic acid from cellular lipids, and inhibition of prostaglandin H2 synthase or cyclooxygenase-2 expression [11]. Dexamethasone's anti-proliferative activity involves upregulation of p21 protein expression and inhibition of CDK2 activity and retinoblastoma (Rb) phosphorylation [12]. The anti-inflammatory and antiproliferative properties of dexamethasone have made it part of the conventional therapy regimen for MM along with alkylating agents and anthracyclines.

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Although the individual effects of lenalidomide and dexamethasone on proliferation and immunomodulation have been studied, the combination effects of lenalidomide together with dexamethasone have not been thoroughly explored, nor has the mechanism of this combination been examined. Therefore, we conducted a series of experiments in various myeloma cell lines to determine the effects of dexamethasone on the antiproliferative, apoptotic and immunomodulatory activity of lenalidomide. In addition, tumor suppressor gene expression and caspase activity were assessed in MM cell lines and MM patient cells to identify any synergistic effects of combining lenalidomide with dexamethasone.

MATERIALS AND METHODS

Multiple Myeloma and Plasma Cell Leukemia Cell Lines

Human cell lines EJM, L363, KMS-12-BM, KMS-12-PE, Karpas-620, SKMM2, JJN-3, OPM-2, and LP-1 (DSMZ, Braunschweig, Germany), and cell lines RPMI-8226 (lenalidomide-resistant), U266 B1, and NCI-H929 (ATCC, Manassas, VA) were obtained.

Cell Proliferation Assay

Cell proliferation was assessed by the ^{3}H-thymidine incorporation assay. Briefly, cells were cultured in 96-well cell culture plates in the presence or absence of lenalidomide, dexamethasone, or both. Each well contained 6000 cells/80 µL cell culture medium (Roswell Park Memorial Institute (RPMI)-1640 + 10–20% fetal bovine serum (FBS), 1% pen/strep/1% L-glutamine). Compound dilutions were made in 10× the required final concentration, and 10 µL of each compound was added to the cells in triplicate. The cells were treated with lenalidomide at 0.0001–100 µM final, or with dexamethasone at 0.000098–0.4 µM (Calbiochem, San Diego, CA), in a final concentration of 0.2% dimethyl sulfoxide (DMSO) for all samples. Cells were grown at 37°C in a humidified incubator at 5% CO_2 for 72 hours in the presence of the test compounds. One microcurie of ^{3}H-thymidine (GE Healthcare, Fairfield, CT) was added to each well for the final 6 hours of culture. The cells were harvested onto UniFilter-96 GF/C filter plates (PerkinElmer, Waltham, MA) using a cell harvester (Tomtec, Hamden, CT), and the plates were allowed to dry overnight. A total of 25 µL/well of Microscint™-20 (PerkinElmer) was added and the plates were analyzed in TopCount NXT (PerkinElmer). Each well was counted for 1 minute. The percentage inhibition of cell proliferation was calculated by averaging all triplicates and normalizing to the DMSO control (0% inhibition). Final cumulative half-maximal inhibitory concentrations (IC_{50}) were calculated using non-linear regression and sigmoidal dose-response, constraining the top to 100% and bottom to 0% and allowing variable slope, using GraphPad Prism version 5.01. SEM (standard error of the mean) was calculated from the individual IC_{50s} of each replicate.

ATP Production Assay

For each cell line, 40 µL of cells were seeded in each well of a 384-well flat, clear bottom, black polystyrene, TC-Treated plate (Corning Inc. Life Sciences, Lowell, MA) at an optimized density to ensure that the cell growth was within the linear detection range after 4 days in culture. For single-agent treatment, lenalidomide or dexamethasone was serially diluted 3-fold for 9 concentrations and added to the cells with the final DMSO concentration of 0.2%. The maximum concentrations for lenalidomide and dexamethasone were 30 µM and 1 µM, respectively. For combination, lenalidomide and dexamethasone were titrated orthogonally in 3-fold dilution for 9 series and added to the cells. The 384-well plate was then sealed with Breathe-Easy™ sealing membrane (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C. After 96 hours, the cell viability was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp., Madison, WI) following the manufacturer's instructions. Background subtracted luminescence counts were converted to percentages of cell viability with respect to DMSO treated control cells. Dose response curves were generated using XLfit4 (IDBS, Surrey, UK) by fitting the percentage of control data at each concentration using a four-parameter logistic model/sigmoidal dose-response model: (y = (A+((B-A)/(1+((C/x)^D))))); where A = minimum, B = maximum, C = IC_{50}, and D = Hill slope. Average half-maximal inhibitory concentration (IC_{50}) and SEM (standard error of the mean) was calculated from the individual IC_{50s} of each replicate.

Gene Expression Analysis

Myeloma cell lines (1×10^6 cells/sample) were cultured in 2 mL RPMI-1640 medium + 10% FBS with 0.2% DMSO, and lenalidomide (1 or 10 µM) with or without dexamethasone (800 nM) for 6 hours and 24 hours. Ficoll-purified peripheral blood mononuclear cells from a newly diagnosed MM patient (Proteogenex, Culver City, CA) were thawed and resuspended in 40% original freezing medium, 40% conditioned media, 20% autologous donor serum (ProteoGenex) and 1 ng/mL recombinant IL-6 (R&D Systems, Minneapolis, MN). Patient cells were seeded in a 48-well plate at 1×10^6 cell in 0.5 mL and treated with 0.2% DMSO, 10 µM lenalidomide, 800 nM dexamethasone or 10 µM lenalidomide/800 nM dexamethasone for 24 hours. Conditioned media was prepared by co-culturing 5×10^5 HS-5 human bone marrow stromal cells (ATCC) with 1×10^5 NCI-H929 MM cells (ATCC) in 4 mL RPMI-1640 media with 10% premium FBS for 24 hours and media was spun to remove cells. After compound treatment, cells were harvested and total RNA was purified with RNAeasy (Qiagen, Valencia, CA). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed. Gene expression assays were performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and analyzed using the relative quantification method for the early growth response (Egr) genes *Egr1*, *Egr2* and *Egr3*, and the cyclin-dependent kinase (CDK) inhibitors *p15*, *p16*, *p21*, and *p27*. For each sample, a GAPDH control was used for normalization. For each gene, samples within each experiment were normalized to a control (0.2% DMSO only) for each time point and cell line. Gene expression results are expressed as 2^{-ddCt}.

Western Blot Analysis

Protein extracts made from 5 x 10^6 cells were subjected to 4–12% Bis-Tris Criterion XT gel (Bio-Rad Laboratories, Hercules, CA) and transferred onto a 0.45 µM nitrocellulose

criterion membrane. Membranes were probed with primary rabbit phospho-antibodies pp-RbSer807/811, pp-RbSer608 and anti-p21 and Egr1 antibodies (Cell Signaling Technology Inc., Danvers, MA), mouse anti-p27 antibody (BD Biosciences, San Jose, CA) and actin antibody (Sigma-Aldrich) followed by Alexa-conjugated secondary antibody (Invitrogen Corporation, Carlsbad, CA). Immunoreactive bands were visualized and quantified with Odyssey Infrared Imaging (LI-COR Biosciences, Lincoln, NE).

Caspase Assay

Myeloma cells (0.5×10^6–1×10^6 cells/sample) were cultured in 4 mL RPMI-1640 + 10–20% FBS with 0.2% DMSO. Caspases 3, 8, and 9 fluorescent-based assays (R&D Systems) were performed after 24 and 48 hours in cells from various myeloma cell lines incubated with lenalidomide (1 or 10 μM) with or without dexamethasone (1 μM). Staurosporine (10 μM; Alexis Biochemicals, Plymouth Meeting, PA) was used as a positive control for caspase activation; positive control data were collected after 24 hours only. Cell lysates were made and all samples were normalized to protein concentration using the BCA Protein Assay (Thermo Scientific, Rockford, IL). Caspase activity was measured by fluorescence wavelengths 420/50 nm (excitation) and 530/25 nm (emission) (FLx800 Fluorescence Microplate Reader; BioTek Instruments Inc., Winooski, VT).

Cell Cycle Analysis

Myeloma cell lines were treated with lenalidomide (1 or 10 μM), dexamethasone (0.8 or 1 μM), or both for 72 hours; lenalidomide was added once daily. Cell cycle profiling was performed using BD Cycletest™ Plus (BD Biosciences) and analyzed on a FACSCanto™ flow cytometer (BD Biosciences).

Apoptosis Analysis

Cell lines LP-1 (lenalidomide-sensitive) and RPMI-8226 (lenalidomide-resistant) were treated with lenalidomide (1 or 10 μM), dexamethasone (1 μM), or both for 72 hours. Apoptotic cells were stained with propidium iodide and annexin V, and analyzed using a FACSCanto™ flow cytometer (BD Biosciences).

T Cell IL-2 Production

Human T cells were isolated from leukocyte units by negative selection using CD16, CD33, CD56, CD14, CD19, and human leukocyte antigen class II antibodies (Invitrogen). The T cells were plated at 2.5×10^5 cells/well in 96-well plates pre-coated with anti-CD3 monoclonal antibody (2.5 μg/mL). Lenalidomide (0.0016–10 μM) was added alone or in combination with dexamethasone (0.3–10 nM). The plates were incubated for 48 hours at 37°C. Supernatants from two donors were then harvested and analyzed in the human IL-2 enzyme-linked immunosorbent assay (R&D Systems).

NK Cell IFN-γ Production

NK cells were isolated from leukocyte units by a 30-minute incubation with RosetteSep® cocktail (StemCell Technologies, Inc., Vancouver, BC, Canada) followed by Ficoll-Hypaque density gradient centrifugation. The CD56+ NK cells were isolated to approximately 85% purity as determined by flow cytometry. Flat-bottom plates were coated with 100 μg/mL of human immunoglobulin (Ig)G (Sigma-Aldrich) overnight at 4°C, and unbound IgG was removed. The NK cells were plated at 2×10^5 cells/well in 96-well plates, and 10 ng/mL of IL-2 (R&D Systems) and 0–10 μM lenalidomide were added. After 48 hours, supernatants were harvested and analyzed for levels of IFN-γ (R&D Systems) and granzyme B (Cell Sciences, Inc., Canton, MA).

Calculation of Synergy/Additive Effects

To evaluate the combinatory effect of lenalidomide and dexamethasone on MM cell lines, data from the two independent experiments were analyzed by comparing the combinatory response against the theoretical additive response of the two agents. The expected additive effect of two agents (A and B) was calculated using the fractional product method [13]: $(fu)A,B = (fu)A \times (fu)B$; where fu = fraction unaffected by treatment.

A synergism of a combination is determined when the observed fraction unaffected in combination is less than $(fu)A,B$, whereas an additive effect is determined when the observed fraction unaffected in combination equals $(fu)A,B$.

RESULTS

Antiproliferative Activity

Lenalidomide and Dexamethasone Inhibit the Growth of MM Cells

The effects of lenalidomide alone, dexamethasone alone, and lenalidomide plus dexamethasone on the growth of MM cells were evaluated with a 72-hour ^{3}H-thymidine incorporation assay. The growth inhibition effects of lenalidomide and dexamethasone as single agents were determined in 10 MM cell lines. As shown in Table **1**, lenalidomide alone was most potent in the Karpas-620, KMS-12-BM, and NCI-H929 cells. Dexamethasone alone induced inhibition at sub-micromolar concentrations in most MM cells tested. The antiproliferative effects of lenalidomide plus dexamethasone ranged from non-additive (Karpas-620, KMS-12-BM, and EJM) to partially-additive (NCI-H929, U266 B1, JJN-3, SKMM2, and RPMI-8226) and additive (LP-1 and OPM-2). Non-additive effects indicate that the combination has no greater effect than either lenalidomide or dexamethasone alone, whichever was greater.

As no synergies were detected by the thymidine incorporation assay, an ATP production assay was performed to assess all viable cells after a longer, 96 hour, drug treatment period. The ATP assay is a less sensitive assay because it measures all viable cells, including arrested cells, whereas the thymidine incorporation assay measures only those cells undergoing active DNA synthesis. The growth inhibition effects of lenalidomide and dexamethasone as single agents were determined in seven MM cell lines. Lenalidomide alone was most potent in the KMS-12-BM and NCI-H929 cells (Table **2**). Dexamethasone alone induced inhibition at sub-micromolar concentrations in OPM-2, NCI-H929, and SKMM2 cells. Although the individual inhibitory concentrations were higher in the ATP assay compared with the thymidine incorporation assay, lenalidomide plus dexamethasone produced synergistic effects on ATP inhibition

Table 1. Inhibition of Proliferation in Myeloma Cell Lines Treated with Lenalidomide and Dexamethasone for 72 Hours, as Measured by the ^{3}H-Thymidine Incorporation Assay (n=3)

Cell Line	Lenalidomide IC_{50} in µM (SEM)	Dexamethasone IC_{50} in µM (SEM)	Combination Effect
Karpas-620	0.046 (0.005)	0.041 (0.02)	Non-additive
KMS-12-BM	0.057 (0.01)	0.051 (0.05)	Non-additive
NCI-H929	0.08 (0.03)	0.0043 (0.001)	Partially additive
LP-1	2.1 (1.1)	0.018 (0.005)	Additive
U266 B1	3.7 (0.9)	0.19 (0.05)	Partially additive
JJN-3	9.9 (6.0)	0.061 (0.02)	Partially additive
SKMM2	18 (4.0)	0.0077 (0.0008)	Partially additive
OPM-2	32 (17)	0.02 (0.0054)	Additive
EJM	99 (64)	> 1.0	Non-additive
RPMI-8226	>100	0.0054 (0.0019)	Partially additive

Table 2. Inhibition of Myeloma Cell Lines Treated with Lenalidomide and Dexamethasone for 96 Hours, as Measured by the ATP Production Assay (n=2)

Cell Line	Lenalidomide IC_{50} in µM (SEM)	Dexamethasone IC_{50} in µM (SEM)	Effect	High Risk Cytogenetic Feature
LP-1	> 30	> 1	Synergy	del13
OPM-2	3 (1.0)	0.069 (0.012)	Synergy	t(4;14), hypodiploid
NCI-H929	1.78 (0.9)	0.04 (0.008)	Synergy	del13, hypodiploid
KMS-12-PE	0.74 (n=1)	> 1	Synergy	del(13)(q11), 4(11;14), hypodiploid
U266 B1	> 30	> 1	Non-additive	del13, hypodiploid
L363	> 30	> 1	Non-additive	del(17p)(p12)
SKMM2	> 30	0.012 (0.0006)	Non-additive	del13, hypodiploid

in the LP-1, OPM-2, NCI-H929, and KMS-12-PE cells. The discrepancy between the combination effects observed with the two assays can be attributed to the longer duration of drug treatment in the ATP assay. LP-1 cells treated with constant lenalidomide and titrated dexamethasone (Fig. **1A**) or constant dexamethasone and titrated lenalidomide (Fig. **1B**) synergistically inhibited cell viability. Similar results were seen in OPM-2 cells, where treatment with constant lenalidomide and titrated dexamethasone (Fig. **1C**) or constant dexamethasone and titrated lenalidomide (Fig. **1D**) synergistically inhibited cell viability.

Lenalidomide and Dexamethasone Enhance Expression of Tumor Suppressor Genes

Upregulation of the tumor suppressor gene *p21* in MM has been described as a mechanism of action of both dexamethasone [1, 12] and lenalidomide [2]. The effects of lenalidomide and dexamethasone, alone or in combination, on tumor suppressor gene expression were evaluated by qRT-PCR in 6 MM cell lines and in cells taken from a MM patient. The results from all cell lines are summarized in Table **3** and show that lenalidomide and dexamethasone alone can enhance expression of tumor suppressor genes *p15*, *p21*, *Egr1*, *Egr2*, and *Egr3* differentially in different cell lines. The combination of lenalidomide and dexamethasone synergistically induced gene expression of *p15* in Karpas-620 cells, *p21* in NCI-H929, LP-1, U266 B1, JJN-3 and RPMI-8226, and *p27* expression in NCI-H929 and RPMI-8226. Lenalidomide and dexamethasone also synergistically enhanced *Egr1* in Karpas-620 and JJN-3 cells, *Egr2* in JJN-3 and RPMI-8226, and *Egr3* in Karpas-620, NCI-H929 and JJN-3. Gene expression of *Egr1*, *Egr2*, and *Egr3* were synergistically induced by the combination in MM patient cells. After 24 hours, LP-1 cells treated with lenalidomide or dexamethasone as single agents enhanced *p21* expression 9-fold and 12-fold, respectively, whereas the combination enhanced *p21* expression up to 65-fold (Fig. **2A**). JJN-3 cells treated with both lenalidomide and dexamethasone synergistically enhanced expression of *Egr1*, *Egr2*, *Egr3*, and *p21* (Fig. **2B**). MM patient cells treated with lenalidomide or dexamethasone as single agents enhanced *Egr1*, *Egr2*, and *Egr3* expression up to 1.5-fold compared with DMSO, whereas the combination synergistically enhanced *Egr1*, *Egr2*, and *Egr3* expression 2.5-, 2.7-, and 2-fold, respectively after 24 hour drug treatment (Fig. **2C**). Similar results were seen in MM patient cells incubated for 6 hours (data not shown). Synergistic upregulation of *Egr1* and p21 in LP-1 and JJN-3 cells, respectively (Fig. **3A**), and p27 in NCI-H929 cells (Fig. **3B**) was observed at the protein level.

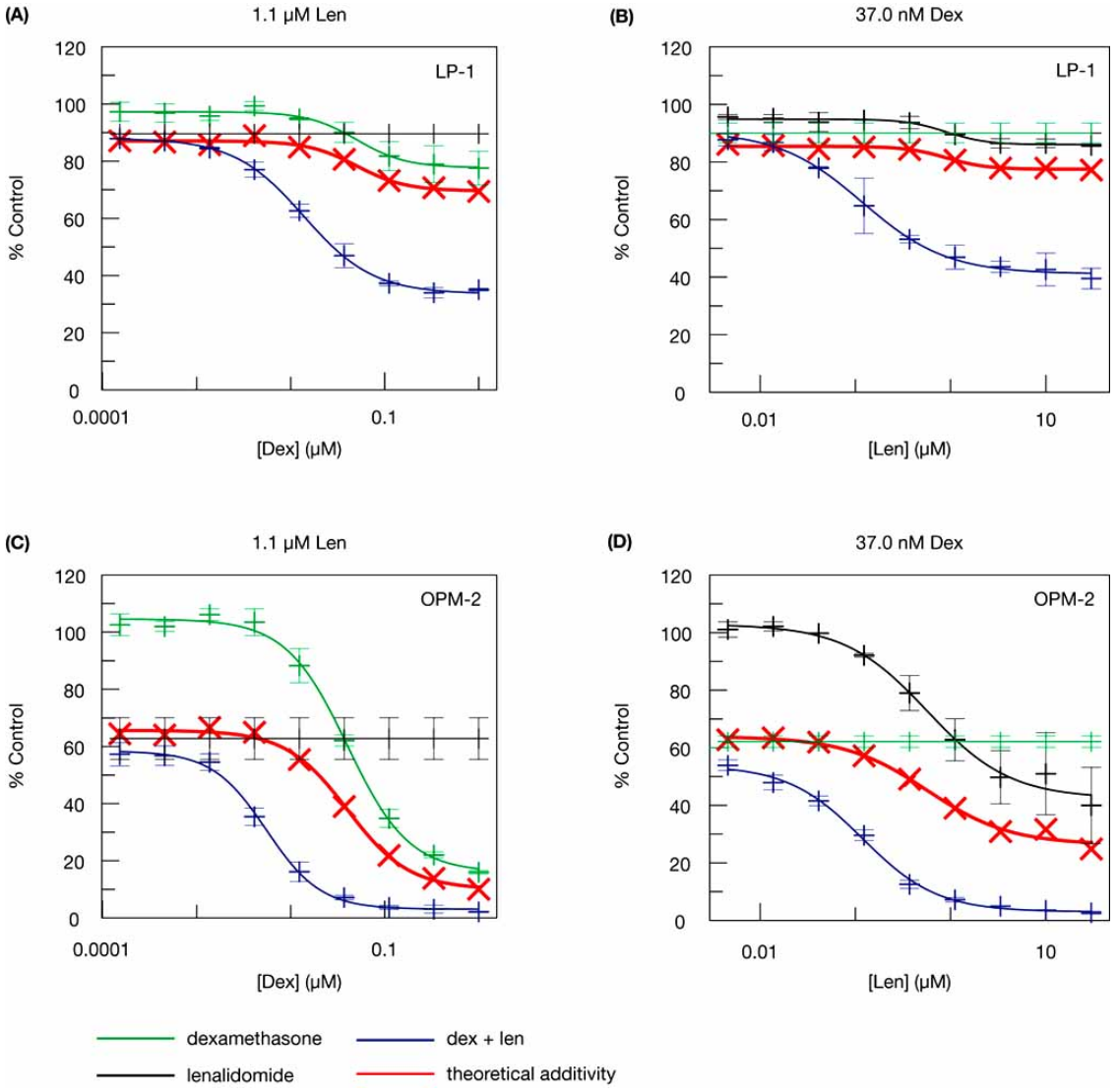

Fig. (1). Combination of lenalidomide and dexamethasone causes synergistic inhibition of proliferation on multiple myeloma cell lines LP-1 and OPM-2. (**A**) LP-1 cells were treated with 1.1 µM of lenalidomide alone, dexamethasone alone at nine concentrations with the highest concentration 1 µM, and combination 1.1 µM of lenalidomide with dexamethasone at different concentrations for 96 hours. (**B**) LP-1 cells were treated with 37 nM of dexamethasone alone, lenalidomide alone at nine concentrations with the highest concentration 100 µM, and combination 37 nM of dexamethasone with lenalidomide at different concentrations for 96 hours. (**C**) OPM-2 cells were treated with 1.1 µM of lenalidomide alone, dexamethasone alone at nine concentrations with the highest concentration 1 µM, and combination 1.1 µM of lenalidomide with dexamethasone at different concentrations for 96 hours. (**D**) OPM-2 cells were treated with 37 nM of dexamethasone alone, lenalidomide alone at nine concentrations with the highest concentration 100 µM, and combination 37 nM of dexamethasone with lenalidomide at different concentrations for 96 hours. The *y*-axis is the fraction unaffected calculated as percentage of positive control. Lines shown are lenalidomide alone (black), dexamethasone alone (green), lenalidomide plus dexamethasone (blue), and the predicted additive effect (red).

Table 3. Induction of Tumor Suppressor Genes in Myeloma Cell Lines

Cell Line	Time (hour)	Lenalidomide	Dexamethasone	Combination*
Karpas-620	6	*Egr3*	-	***Egr1, p15***
	24	*Egr1, p15, p21*	-	***Egr1, Egr3****, p15, p21*
NCI-H929	6	*Egr2, Egr3*	*Egr1, Egr2, p15*	*Egr1, p15,* ***p21***
	24	-	-	***Egr3, p27***
LP-1	6	*Egr2, Egr3*	*p21*	***p21***
	24	*p21*	*p21*	***p21***

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