Combination of the mTOR inhibitor Rapamycin and Revlimid™ (CC-5013) has synergistic activity in Multiple Myeloma

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ABSTRACT

Previous studies have demonstrated the *in vitro* and *in vivo* activity of Revlimid™ (CC-5013), an immunomodulatory analogue (IMiD) of thalidomide, in multiple myeloma (MM). In the present study, we have examined the anti-MM activity of rapamycin, a specific mTOR inhibitor, combined with Revlimid™. Based on the Chou-Talalay method, combination indices of < 1 were obtained for all dose ranges of Revlimid™ when combined with rapamycin, suggesting strong synergism. Importantly, this combination was able to overcome drug resistance when tested against MM cell lines resistant to conventional chemotherapy. Moreover the combination, but not rapamycin alone, was able to overcome the growth advantage conferred on MM cells by Interleukin-6 (IL-6), Insulin-like growth factor-1 (IGF-1), or adherence to bone marrow stromal cells (BMSCs). The combination of rapamycin with Revlimid™ induced apoptosis of MM cells. Differential signaling cascades, including the MAPK and PI3-K/Akt pathways, were targeted by these drugs individually and in combination, suggesting the molecular mechanism by which they interfere with MM growth and survival. These studies therefore provide the framework for clinical evaluation of mTOR inhibitors combined with immunomodulatory agents to improve patient outcome in MM.
INTRODUCTION

Interleukin-6 (IL-6) and insulin like growth factor-1 (IGF-1) play a key role in the growth, survival, and drug resistance in multiple myeloma (MM) cells. Furthermore, their secretion in bone marrow stromal cells (BMSCs) is up-regulated by adherence of MM cells. IL-6 and IGF-1 mediate growth of MM cells via activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3’-kinase/Akt kinase (PI3-K/Akt) signaling cascades. Several studies show that PI3-K/Akt signaling mediates growth, survival, migration and cell cycle regulation in MM. Activated Akt in turn phosphorylates downstream target molecules, including forkhead transcription factor (FKHR), glycogen synthase kinase (GSK)-3β, and mammalian target of rapamycin (mTOR). These downstream molecules would therefore serve as rational targets for novel therapeutics in MM. Indeed, specific mTOR inhibitors like rapamycin have induced cytoreduction and G1 arrest observed in MM cells. In vitro and in vivo studies have demonstrated marked anti-MM activity of CCI-779 (Wyeth Ayerst, PA, USA) an analogue of rapamycin. CCI-779 is currently under evaluation in phase I and II studies both in solid tumors, and mantle cell lymphoma. A phase II study in renal cell carcinoma has just been completed, and a phase III trial is currently accruing patients. To date, however, there are no clinical trials in MM.

Previous preclinical studies have demonstrated that Thalidomide and immunomodulatory derivatives (IMiDs) like Revlimid™ (CC-5013, linalidomide) can overcome conventional drug resistance and have in part delineated their molecular mechanisms of action. Furthermore, these in vitro observations have translated into clinical trials of these agents, which have resulted in significant responses even in MM
patients with relapsed, refractory disease. Here we have studied the effect of combining Revlimid™ with rapamycin, based upon rational targeting of differential signaling cascades mediating MM cell growth and survival.
MATERIALS AND METHODS

MM derived cell lines

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 resistant (Dox 40) and melphalan resistant (LR5) RPMI-8226 human MM cells were kindly provided by Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). OPM 2 cell line was obtained from Dr. Lief Bergsagel (Cornell University, NY) and SB645 was obtained from Dr. Kishimoto (Osaka University, Japan). All MM cell lines were cultured in RPMI-1640 media (Sigma Chemical, St Louis, MO) containing 10% fetal bovine serum, 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO).

Revlimid™ (CC-5013) and Rapamycin

Revlimid™ (CC-5013) was obtained from Celgene Corporation (Warren, NJ) and Rapamycin was obtained from Calbiochem (San Diego, CA). The drugs were dissolved in DMSO (Sigma) at a concentration of 200 millimol/L (200mM) and stored at -20°C until use. Drugs were diluted in culture medium (0.01 to 100 µM for Revlimid™and 0.01 to 100 nM for rapamycin) with < 0.1% DMSO immediately before use. Diluted drugs were used within 4 hours.
Bone Marrow Stromal Cells (BMSCs)

Bone marrow aspirates were subjected to Ficoll Paque gradient and mononuclear cells (MNC) were separated. The MNC were placed in 25mm² culture flasks in RPMI-1640 media (Sigma Chemical, St Louis, MO) containing 20% fetal bovine serum, 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), 100 U/mL penicillin, and 100µg/mL streptomycin (GIBCO). Once confluent, the cells were trypsinized and passaged as needed. For the experiments, BMSC were incubated in 96 well culture plates (approximately 5000 to 10,000 BMSC/well) for 24 hrs. The media was washed off and MM cells were added to the wells (2 X 10⁴ cells/well) and incubated with media alone, or with differing concentrations of CC-5013, rapamycin or a combination of the two drugs for 48 hours at 37°C.

Proliferation assays

DNA synthesis was measured by tritiated thymidine uptake [³H-TdR] (Perkin Elmer, Boston, MA) as previously described. Briefly, MM cells (2-3 × 10⁴ cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media or varying concentrations of Revlimid™, rapamycin or a combination of the two drugs for 48 hours at 37°C. For evaluation of the effect of growth factors, recombinant IL-6 (10 ng/mL) or IGF-1 (50 ng/mL) were added to the wells at the beginning of the incubation to obtain the final concentration indicated. Cells were pulsed with ³H-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; the 96 well plates were incubated at 37°C for 4 hours, followed by 100 µL isopropanol that contained 0.04 HCl. Absorbance readings at a wavelength of 570 nm (with correction using readings at 630nm) were taken on a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

**Cell cycle analysis and detection of apoptosis**

MM cells (1 × 10⁶) were cultured for 48 hours in media alone, or with varying concentrations of CC5013, rapamycin or a combination of the two drugs. The cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), fixed with 70% ethanol for 1 hour, and pretreated with 10 µg/mL of RNAse (Sigma) for 1 hour. Cells were stained with propidium iodide (PI; 5 µg/mL; Sigma), and cell cycle profile was determined by using the RXP cytomics software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL), as in prior studies.

In addition to identifying sub-G1 cells by cell cycle analysis as described above, apoptosis was also confirmed by using annexin V/PI staining. MM cells were cultured in media or with 1nmol/L of rapamycin or 1µmol/L of CC-5013 or the combination at 37°C for 24, 48 or 72 hours. Cells were then washed twice with ice-cold PBS and re-suspended
(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; MBL) and PI (5 µg/mL) for 15 minutes at room temperature. Annexin V+PI- apoptotic cells were enumerated by using the Epics flow cytometer.

**Western blotting**

MM cells were cultured with of Revlimid™ (1 µmol/L), rapamycin (1 nmol/L) or a combination of the two drugs (at same respective doses); 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 apoptosis related proteins, cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with antibodies against cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase 8 (Cell Signaling Technology, MA). The membrane was stripped and reprobed with anti-actin Ab (Santa Cruz Biotech) to ensure equivalent protein loading.

To characterize growth signaling, immunoblotting was also done with anti-phospho MAPK Ab (Santa Cruz Biotechnologies); as well as anti phospho STAT3, anti-phospho MEK, phospho P70s kinase, phospho-Akt, and anti phospho p65 NFκB (Cell Signaling Technology, MA). Antigen-antibody complexes were detected by using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Blots were stripped and reprobed with anti-actin Ab (Santa Cruz Biotech) to ensure equivalent protein loading.
Detection of apoptosis in patient myeloma cells

Bone marrow aspirates were subjected to Ficoll Paque gradient and mononuclear cells were separated. The mononuclear cells were suspended in RPMI-1640 media containing 20% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100µg/mL streptomycin. The cells were placed in a 24 well plate at a concentration of 2 X 10^6 cells /mL; CC-5013 and rapamycin were added to the medium to obtain the desired final concentrations. The patient cells were cultured for 48 hours and then harvested. After washing twice with PBS, they were stained with FITC conjugated CD38 monoclonal antibody and PE conjugated Apo 2.7 antibody. The cells were analyzed on RXP Cytomics software on an Epics flow cytometer. The percentage of CD38 bright cells that were Apo 2.7 positive was the percentage of apoptotic myeloma cells.

Isobologram analysis

The interaction between Revlimid™ and rapamycin was analyzed using CalcuSyn software program (Biosoft, Ferguson, MO) to determine if the combination was additive or synergistic. Data from cell viability assay (MTT) were expressed as fraction of cells killed by the individual drugs or the combination in drug-treated versus untreated cells. This program is based upon the Chou-Talalay method which calculates a combination index (CI), and analysis is performed based on the following equation: CI = (D1/(Dx)1 + (D)2/(Dx)2 + (D)1(D2)/(Dx)1(Dx)2, where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination, and (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2 that have the same x effect when used alone. When CI = 1, this
equation represents the conservation isobologram and indicates additive effects. CI < 1.0 indicate synergism.
RESULTS

Effect of Revlimid™ (CC-5013), rapamycin, and combination on DNA synthesis of MM cell lines.

The effect of Revlimid™ and rapamycin, both alone and combined on DNA synthesis of MM cell lines (MM.1S, OPM2, and SB645) was determined by measuring $^3$H-TdR uptake during the last 8 hrs of 48-hr cultures. Rapamycin (0.01 to 100 nmol/L), CC-5013 (0.01 to 100 µmol/L), and combinations of these agents inhibited $^3$H-TdR uptake of MM.1S, OPM2, and SB645 cells in a dose-dependent fashion (Figure 1A). Rapamycin (nM) and CC-5013 (µM) were used in fixed dose combinations. Fifty percent inhibition (IC$_{50}$) of proliferation in all 3 cell lines was seen at a dose of between 0.1-1nmol/L rapamycin when combined with 0.1-1µmol/L of CC-5013, a 10 fold lower dose than when either agent is used alone. The effects of these drugs on proliferation were confirmed using MTT assays.(Figure 1B)

Effect of the combination of Revlimid™ and rapamycin on DNA synthesis of MM cells resistant to conventional chemotherapy.

To examine whether these drugs were active against cell lines resistant to conventional therapies, we similarly studied doxorubicin resistant Dox40 cells, melphalan resistant LR5 cells, and dexamethasone resistant MM.1R cells. The combination of Revlimid™ and rapamycin inhibited DNA synthesis of Dox40, LR5 and MM.1R cells in a dose-dependent fashion, albeit at higher concentrations compared to the drug sensitive cell lines. (Figure 1C) Dex resistant MM1.R cells were most sensitive, suggesting a lack of cross-resistance to combined therapy versus dexamethasone.
We next studied the effect of individual drugs and combinations on normal peripheral blood mononuclear cells by MTT assays. No cytotoxicity was noted at even the highest dose of rapamycin (100nMol/L) when combined with Revlimid™ (100 µMol/L). (Figure 1D) In time course experiment to determine the time of maximal drug effect, cytotoxicity was minimal at 24 hrs and maximal at 48 hrs. (Figure 2)

The combination of Revlimid™ and rapamycin is synergistic in MM cell lines.

Addition of increasing doses of rapamycin to Revlimid™ resulted in increased cytotoxicity at every dose of Revlimid™ examined. The interaction between rapamycin and Revlimid™ was analyzed using CalcuSyn software program to determine whether this combination had additive or synergistic cytotoxicity. Based upon the Chou-Talalay method, to calculate a combination index (CI), we generated an isobologram of varying concentrations of rapamycin with Revlimid™. (Figure 3A) At doses ranging from 0.1-100 µmol/l of CC-5013 combined with 0.1-100 nmol/l of rapamycin, CI ranged from 0.526-0.015, suggesting that this combination was highly synergistic. (Figure 3B)

The combination of Revlimid™ and rapamycin overcomes the protective effects of growth factors and BMSCs.

We next examined whether these drugs could overcome the MM cell growth and survival induced by IL-6 and IGF-1. MM1.S cells were cultured with increasing doses of Revlimid™, rapamycin, and combinations of these drugs, in the presence of IL-6 (10 ng/mL) or IGF-1 (50 ng/mL). Rapamycin alone was not able to overcome the protective effects of these growth factors (Figure 4A, 4B). However, rapamycin combined with Revlimid™ triggered a marked inhibition of DNA synthesis, suggesting that the combination overcomes the protective effects of these growth factors.
The effect of this drug combination on DNA synthesis of MM1.S cells in the microenvironment was similarly examined (Figure 4 C). Combined Revlimid™ and rapamycin inhibited \(^3\)H-TdR uptake of MM1.S cells cultured in the presence of BMSCs in a dose-dependent fashion.

**Effect of Revlimid™, rapamycin, and combination therapy on cell cycle profile and apoptosis of MM cell lines**

In order to further characterize the cytotoxic effect of these drugs on MM cell lines, we next performed cell cycle analysis on MM1.S cells cultured with media alone, Revlimid™ (1 µmol/L), rapamycin (1 nmol/L), or a combination of these agents for 12 to 48 hrs. The combination of Revlimid™ and rapamycin increased the sub-G1 MM.1S cell population at 48 hrs. (data not shown) Annexin V/PI staining confirmed 33.9% annexin V+/PI- cells after 48 hrs of exposure to combined therapy. (Figure 5A) An increase in PARP and caspase 8 cleavage was induced at 24hrs by combined therapy demonstrated by western blot analysis. (Figure 5B)

**Effect of Revlimid™ and rapamycin on growth signaling in MM1.S cells.**

To delineate the mechanisms of cytotoxicity of combined Revlimid™ and rapamycin against MM cells, we incubated MM1.S cells with Revlimid™ (1 µmol/L), rapamycin (1 nmol/L), or this drug combination for 2, 4, 6, and 8 hrs; cell lysates were prepared as previously described. Revlimid™ inhibited the MAPK pathway, demonstrated by inhibition of pMEK1/2 and pERK, in a time dependent fashion. In contrast, no inhibition of the MAPK pathway was noted with rapamycin. Conversely, at higher rapamycin doses, an up-regulation of the MAPK pathway was seen, evidenced by increased phosphorylation of MEK1/2 and ERK. The combination of rapamycin with
Revlimid™ triggered a time dependent inhibition of MAPK. (Figure 6A) Rapamycin, resulted in a time dependent inhibition of pSTAT3, which was unaffected by CC-5013. pP65 NFκB, was down-regulated by either of these drugs alone or combined. No change in pAkt was induced (data not shown) by either agent alone or when combined. p70S6kinase was completely inhibited by rapamycin alone and combined with Revlimid™, but Revlimid™ alone had no effect (Figure 6B).

**The combination of Revlimid™ with rapamycin induces apoptosis of patient myeloma cells**

Combined Revlimid™ and rapamycin therapy induced a dose dependent increase in apoptosis of patient MM cells, evidenced by APO2.7 staining of CD 38+ BM mononuclear cells on flow cytometry. Four MM patients’ cells were studied, and representative data from one patient is shown in Figure 7.
DISCUSSION

Our previous data suggests that targeting the PI3-K/Akt pathway may have direct apoptotic effects and anti-proliferative effects on tumor cells, besides overcoming cell adhesion mediated drug resistance.\textsuperscript{14,16} In this report, we have studied the effect of combining Revlimid™ with rapamycin on MM cells. Our study demonstrates a strong synergism between two orally bio-available drugs which have shown \textit{in vitro} activity in MM. Furthermore, both rapamycin and Revlimid™ can be combined at doses well below those that are pharmacologically achievable, making this an attractive combination to test in the clinic. Because a dose of 1 nMol of rapamycin plus 1 \( \mu \)Mol of CC-5013 resulted in peak inhibition of proliferation, we used these doses for subsequent studies of cell cycle, apoptosis, and signaling. This rapamycin dose translates to 15-fold lower than that used for immunosuppression in the transplant setting, coupled with a 5 fold lower dose of CC-5013 than that used in the single agent phase II Revlimid™ trial in relapsed/refractory MM patients.\textsuperscript{24}

Our data also demonstrates that rapamycin alone was not able to overcome the protective effects of IL-6, IGF-1, and tumor cell adherence to BMSCs, whereas the combination of rapamycin with Revlimid™ inhibited MM cell growth in the BM milieu. This study therefore, for the first time, demonstrates the role of mTOR inhibitors not only on tumor cells directly but also on their BM microenvironment. IGF-1 mediated protection from rapamycin-induced apoptosis has been similarly noted in rhabdomyosarcoma cells.\textsuperscript{26} Therefore, although rapamycin alone and other mTOR inhibitors show potent anti-tumor activity \textit{in vitro}, it may be necessary to combine rapamycin with agents able to overcome protective effects of growth factors.
in the tumor milieu in future clinical trials. To this end, Stromberg et al 27 have recently demonstrated that rapamycin can sensitize MM cells to dexamethasone-induced apoptosis in MM cells, suggesting that this combination was able to overcome the protective effects of both IL-6 and IGF-1.

Although most studies have reported TOR inhibition with rapamycin or its analogues to result in a cytostatic response with G1 growth arrest, 19 some reports demonstrate tumor cell apoptosis, as in our study. 26,28 This effect may be cell specific, and enhanced when the two agents are combined. In MM, apoptosis was induced by higher doses of rapamycin alone, as well as combined Revlimid™/rapamycin.

We have delineated downstream signaling cascades targeted by Revlimid™ and rapamycin alone and in combination. As shown previously, Revlimid™ inhibited activation of MAPK pathway signaling 23 whereas rapamycin either did not affect, or at higher doses upregulated this pathway. Importantly, the combination of CC-5013 and rapamycin, inhibited MAPK signaling. STAT3 phosphorylation was not affected by CC-5013, but was inhibited in a time dependent manner by rapamycin. Combined therapy also blocked STAT3 phosphorylation in a time-dependent manner. Rapamycin-induced downregulation of pSTAT 3 has been previously demonstrated in other cell systems, including B cell lymphomas and glial cells 29,30 In MM, STAT signaling mediates survival, 10 and there is increasing evidence to suggest cross talk between the MAPK and PI3kinase/Akt pathways mediating MM cell growth. Importantly, specific inhibition of PI3kinase/Akt abrogated IGF-1 induced MAPK activation and proliferation of MM cells. 15 Furthermore, Qiang et al were able to demonstrate, that the PI3K inhibitor LY294002, completely blocked protection against Dex-induced apoptosis by IGF-1.
Rapamycin only partially blocked protection, whereas specific MAPK inhibitors had no effect. Taken together, these observations suggest that interrupting the PI3K pathway is a promising therapeutic strategy. Rapamycin acts down-stream of the PI3K pathway, as demonstrated by inhibition of p70S6 kinase and pSTAT3. Revlimid™, on the other hand, inhibits the MAPK pathway. Therefore, combining a MAPK inhibitor such as Revlimid™ with an mTOR inhibitor like rapamycin may enhance anti-tumor response.

The role of NF-κB as a therapeutic target in MM has been previously described. In MM, targeting of NF-κB can overcome the growth and survival advantage conferred by MM cell-BMSC interaction and cytokines in the BM milieu. In this study, we demonstrate a time dependent down-regulation of p65NF-κB induced by either rapamycin and CC-5013 or by combined therapy, highlighting the role this drug combination may play in overcoming drug resistance.

mTOR inhibition as an anti-cancer strategy has been previously tested, and ongoing clinical trials are currently evaluating CCI-779, the rapamycin ester analogue. Another mTOR inhibitor undergoing in vitro and in vivo testing is RAD001. Although these inhibitors have shown pre-clinical promise, their role as single agents in phase II studies have resulted in only modest responses. Our data supports the notion that mTOR inhibitors will have maximum benefit when rationally combined with other agents, based upon inhibiting multiple signaling cascades. We show that the combination of rapamycin and Revlimid™ overcomes the protective effects of IL-6, IGF-1, and BMSCs, whereas rapamycin alone does not. The two drugs act via interruption of different signaling cascades, further supporting the role of combining these agents in clinical practice. Importantly, we have also demonstrated their activity in MM cells.
resistant to conventional chemotherapy, further suggesting potential clinical testing in the setting of relapsed, refractory myeloma. These preclinical studies therefore provide the rationale for evaluating combined rapamycin and Revlimid™ therapy to overcome conventional drug resistance and improve patient outcome in MM.
FIGURE LEGENDS

**Figure 1**: Effect of Revlimid™, rapamycin, and their combination on proliferation of MM cell lines and PBMCs. Panel A shows a time and dose dependent inhibition of proliferation in sensitive MM cell lines (MM1.S, OPM2, SB645), demonstrated by measuring thymidine [³H-TdR] uptake during the last 8 hrs of 48-hr cultures, in the presence of Revlimid™ (µmol/l), rapamycin (nmol/l), or combined in fixed dose treatment. Panel B confirms time and dose dependent inhibition of proliferation demonstrated by MTT in MM1.S cells. Inhibition of DNA synthesis as measured by ³H-TdR uptake, in resistant MM cell lines treated with CC5013 and rapamycin is shown in panel 1C. Panel 1D demonstrates the lack of toxicity of combined therapy on PBMCs, demonstrated by MTT assay.

**Figure 2**: Time course for inhibition of proliferation of MM1.S cells, demonstrated by ³H-TdR uptake, after fixed dose treatment with combined Revlimid™ (0.01 – 100 µmol/l) and rapamycin (0.01 – 100 nmol/l). Maximal dose dependent inhibition of DNA synthesis was seen 48 hrs.

**Figure 3**: The combination of Revlimid™ and rapamycin is synergistic in myeloma cell lines. Panel A demonstrates dose-effect curve and isobologram. Panel B tabulates combination indices (CI) generated from the isobologram at increasing concentrations of rapamycin and Revlimid™.

**Figure 4**: The combination of Revlimid™ and rapamycin overcomes the protective effect of IL-6, IGF-1, and adherence to the bone marrow microenvironment. Rapamycin-induced cytotoxicity is abrogated in the presence of cytokines, but the combination of
Revlimid™ with rapamycin overcomes the protective effects of IL-6 (Panel 4A) and IGF-1. (Panel 4B) Combined therapy was also effective in inhibiting DNA synthesis in MM1.S cells adherent to BMSCs. (Panel 4C)

**Figure 5:** The combination of Revlimid™ and rapamycin induces apoptosis in MM1.S cells. Panel A demonstrates an increase in the apoptotic cells (Annexin +, PI negative) after exposure to Revlimid™ (1 µmol/l) and rapamycin (1 nmol/l) for 48 hrs, associated with caspase 8 and PARP cleavage. (Panel 5B)

**Figure 6:** The combination of Revlimid™ and rapamycin affects growth and survival signaling pathways in MM1.S cells. MM1.S cells were treated with Revlimid™ (1 µmol/L), rapamycin (1 nmol/L), or combined therapy for up to 8 hrs. Panel A demonstrates down regulation of the MAPK pathway by CC-5013 and STAT pathway by rapamycin. Rapamycin either does not affect or upregulates MAPK pathway, evidenced by phosphorylation of pERK and pMEK1/2. The combination of Revlimid™ and rapamycin inhibits both pathways, demonstrated by down-regulation of pERK, pMEK1/2, and pSTAT. These agents, both alone and in combination, inhibit pP65NFκB. Panel B demonstrates the complete inhibition of p70S6 kinase phosphorylation by either rapamycin alone or combination therapy, whereas Revlimid™ alone has no effect.

**Figure 7:** Effect of Revlimid™, rapamycin, and combination on patient myeloma cells. A dose dependent increase in percentage of apoptotic patient MM cells, evidenced by Apo 2.7 staining of bright CD38 positive cells, was noted after exposure to combination Revlimid™ and rapamycin treatment.
BIBLIOGRAPHY


Figure 2

Time Course

Percentage of control

Drug Conc

- 24 hrs
- 48 hrs
Figure 3

A

Dose-effect curve

Conservative Isobologram

B

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Figure 4

A

B

C
Figure 5

A

Apoptosis

- CC-5013
- Rapamycin
- Combination

Percentage Annexin + PI cells

Time (hrs)

0

48

B

Cleaved PARP

Cleaved Caspase 8

Control

CC5013

Rapamycin

Combination
Figure 6

A

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4 hrs 8 hrs
Figure 7

Drug Conc

Percentage of apoptotic CD38+ cells

- Rev
- Rapa
- Combo
Combination of the mTOR inhibitor Rapamycin and Revlimid\textsuperscript{TM} (CC-5013) has synergistic activity in multiple myeloma

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