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Inhibitor of Apoptosis Proteins (IAPs) as therapeutic targets in Multiple Myeloma (MM)

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Abstract

The inhibitor of apoptosis (IAP) proteins plays a critical role in the control of apoptotic machinery, and has been explored as a therapeutic target. Here, we have examined the functional importance of IAPs in multiple myeloma (MM) by using a Smac-mimetic LCL161. We observed that LCL161 was able to potently induce apoptosis in some MM cell lines but not in others. Examining the levels of XIAP, cIAP1 and cIAP2 post LCL161 treatment indicated clear down regulation of both XIAP activity and cIAP1 levels in both the sensitive and less sensitive (resistant) cell lines. cIAP2, however, was not down regulated in the cell line resistant to the drug. siRNA mediated silencing of cIAP2 significantly enhanced the effect of LCL161 indicating the importance of down regulating all IAPs simultaneously for induction of apopotsis in MM cells. LCL161 induced marked up regulation of the Jak2/Stat3 pathway in the resistant MM cell lines. Combining LCL161 with a Jak2 specific inhibitor resulted in synergistic cell death in MM cell lines and patient cells. In addition, combining LCL161 with death inducing ligands clearly showed that LCL161 sensitized MM cells to both FAS-L and TRAIL.

Keywords

Myeloma; Apoptosis; IAP

Introduction

Abnormalities in the apoptosis (programmed cell death) machinery are common in various cancers including MM and are an important basis of resistance to existing therapeutic options (1–3). Tumor cells evade apoptosis through numerous mechanisms which include abnormal activation of signaling events that lead to increased proliferation and decreased

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apoptosis signals and/or altered balance between anti-apoptotic and pro-apoptotic proteins (1–4). Inhibitors of apoptosis (IAP) proteins originally identified in baculovirus, are endogenous inhibitors of programmed cell death that are aberrantly expressed in a wide variety of tumors (5). Subsequent sequence homology searches led to the identification of eight related proteins in humans, namely, cIAP1, cIAP2, XIAP, survivin, NAIP, ML-IAP, and BRUCE (5, 6). All members of the IAP family are characterized by the presence of the Baculovirus inhibitor of apoptosis repeat (BIR) domains (5, 6). Among the IAPs, XIAP, cIAP1 and c1AP2 are the three well-studied proteins that are expressed in a wide variety of tumors. Recently, it has been shown that XIAP is the only member of the IAP family that binds and inhibits the activation of caspases 9 and 3 (7). cIAP1 and cIAP2 on the other hand are integral members of the Tumor Necrosis Factor (TNF-α) pathway where they associate with Receptor interacting protein (RIP) and TNF receptor associated factor (TRAF) and modulate both the canonical and noncanonical NF-κB pathways (8, 9). cIAP1 and cIAP2 ubiquitinate RIP1 through their RING domains. In the absence of cIAP1 and cIAP2, RIP1 is not ubiquitinated, which signals RIP1 to form a complex with the death receptor complex activating caspase 8 and extrinsic apoptosis (8, 9). In cells primed to undergo apoptosis, Second Mitochondria-derived Activator of Caspases (Smac), a mitochondrial pro-apoptotic protein is released into the cytosol where it binds to IAP proteins relieving their inhibition of caspases and promoting apoptosis (10–12). Tumor cells can evade the pro-apoptotic effects of Smac by aberrantly expressing high levels of the IAP proteins. Smac mimetics are compounds that bind the IAPs at Smac binding sites and promote apoptosis. These compounds have been shown to sensitize tumors to chemotherapies in a wide variety of tumors (5).

MM cell lines typically express high levels of XIAP (13), and may be driven at least in part by cytokines IL6 and IGF-1, both present at high levels in myeloma microenvironment. IL6 and IGF1 up regulate XIAP by activating the NF-κB, MAPK and PI3K signaling pathways that are commonly aberrant in MM and other tumors (14). XIAP down regulation by siRNA leads to increased drug sensitivity in myeloma cell lines and decreased tumor formation in BPD/SCID mice (13). Given this, XIAP presents an attractive therapeutic target in myeloma and XIAP inhibitors need to be investigated for their potential as anti-MM agents as single agents and in combination with existing therapies. cIAP1 and cIAP2 on the other hand have been suggested to have a tumor suppressor role since mutations in these two IAPs have been seen in some MM cell lines and patient cells which results in activation of the NF-KB pathway (15, 16). However, in another study it has been shown that IAP expression increased after chemotherapy in MM patients in association with multidrug resistance protein and correlated positively with poor prognosis (17). Moreover, a Smac-mimetic LBW242 was shown to induce cell death in MM cell lines and patient cells, though the ability of the molecule to inhibit the IAPs and the mechanism of action of the drug was not investigated in detail (18). Thus, the precise functions of the IAPs in MM are still not very clear. Here, we have investigated the importance of the IAP proteins by using an orally bio-available Smac mimetic LCL161 in both MM cell lines and patient cells. We observed potent activity of LCL161 in a subset of MM cell lines and patient cells. We have identified cIAP2 and Jak2/Stat3 pathway as factors contributing to resistance to single agent LCL161.

Combining LCL161 with Jak2 inhibitors leads to synergistic cell death in both MM cell lines and patient cells.

Materials and Methods

Multiple myeloma cell lines and patient cells

Cell lines MM1S, MM1R, OPM2, RPMI8226 and U266 were kindly provided by Dr. Jonathan Keats (TGen, Phoenix, AZ). H929 was purchased from ATCC (Manassas, VA). DOX40 was kindly provided by Dr. William Dalton's laboratory (Moffitt Cancer Center, Tampa, FL). RM43 was kindly provided by Dr. Diane Jelinek's laboratory (Mayo Clinic, Rochester, MN). All cell lines except RM43 were cultured in RPMI 1640 media (Mediatech Inc., Manassas, VA) containing 10% fetal bovine serum (Mediatech, Inc.), 2 mM L-glutamine (Invitrogen, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). RM43 was cultured in the same media but in the presence of 1ng/ml of IL6. Freshly obtained bone marrow aspirates from patients were collected with informed consent and were processed to obtain myeloma cells or stromal cells as previously described (19–21). All patient cells were cultured in RPMI 1640 media that contained 10% fetal bovine serum, 2 mM L-glutamine (GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Drugs

LCL161 was synthesized and provided by Novartis (Basel, Switzerland) under a Material Transfer Agreement (MTA). TG101209 was purchased from Selleckchem (Houston, TX). SD1029 was purchased from EMD Millipore (Billerica, MA). Recombinant human TRAIL and Fas-L were purchased from R&D systems (Minneapolis, MN). Pan-caspase inhibitor Q-VD-OPH, caspase 9 inhibitor Ac-LEHD-CMK and caspase 8 inhibitor Z-IETD-FMK were purchased from EMD Millipore.

MTT and proliferation assays

Cytotoxic effects of LCL161 alone or in combination was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasodium bromide (MTT) (Chemicon International Inc., Temecula, CA) colorimetric assay. The effect of LCL161 on the proliferation of MM cells when cultured alone or when cultured together with either bone marrow stromal cells (BMSCs) or tumor promoting cytokines (IL6, IGF, VEGF) was examined by performing tritiated thymidine uptake assays (19–21).

Apoptosis, mitocapture and cytochrome c release assays

Apoptosis induction by LCL161 in both MM cell lines and patient cells was measured by annexin V/PI staining as described elsewhere (19–21). Briefly, cells were washed twice in Annexin Binding Buffer (ABB) (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Following this, 100μl cells (10 cells/ml) were stained with 3μl of annexin V-FITC (Caltag, Burlingame, CA) for 15 minutes at room temperature. Then, cells were again washed with ABB and resuspended in 500μl ABB containing 5μl of 1mg/ml propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) and run on a Canto flow cytometer (BD Biosciences, San Jose, CA). The MitoCapture Apoptosis Detection Kit and cytochrome c release assay kit

(Calbiochem, San Diego, CA, USA) was used to measure disruption of mitochondrial membrane potential per the manufacturer's recommendations. The MitoCapture cationic dye exhibits distinct red fluorescence in healthy cells in contrast to green fluorescence in cells undergoing apoptosis as shown by increase in % cells in the FITC channel with increasing LCL161 dose and time. Intact viable cells express cytochrome c in the mitochondria while apoptotic cells release cytochrome c into the cytoplasm.

Western Blotting

Cells were lysed in RIPA buffer (50mM HEPES (pH 7.4), 150mM NaCl, 1% Triton X-100, 30mM sodium pyrophosphate, 5mM EDTA) containing Halt Phosphatase Cocktail (Thermo Fisher Scientific, Rockford, IL), 1mM phenylmethyl-sulfonyl-fluoride (PMSF) (Thermo Fischer) and protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO). Protein lysate concentrations were measured using BCA assay (Thermo Fisher). Equal amounts of protein were loaded on Tris-Glycine gels and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). All antibodies except the following were purchased from Cell Signaling Technology (Danvers, MA). cIAP1 antibody was purchased from Epitomics (Burlingame, CA). p50, p52, RelB and cRel antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Antigen-antibody complexes were detected using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

siRNA transfection

cIAP2 and scrambled siRNAs were purchased from Life Technologies (Grand Island, NY). 1μM of either siRNAs was transfected using the Amaxa Nucleofector Technology (Kit C-Protocol: X-005) from Lonza (Basel, Switzerland) following the manufacturer's protocol. Following this, LCL161 (2.5μM) was added or cells were left untreated. 72hrs later, apoptosis was measured by Annexin V/PI staining and cIAP2 knockdown was confirmed by western blotting.

ELISA array

Human cytokine ELISA plate array was purchased from Signosis, Inc. (Santa Clara, CA). MM1S and MM1R cells were left untreated or treated with LCL161 (2.5μM) for indicated time points. Conditioned media was collected and ELISA array was performed following the vendor's protocol.

ELISA

Human IP-10, IL-6 and TNF-α quantikine ELISA kits were purchased from R&D systems. MM1S and MM1R cells were left untreated or treated with indicated doses of LCL161 for indicated time points. Conditioned media was collected and ELISA was performed following the vendor's protocol.

Statistical Analysis

Effects of combination of LCL161 with TG101209, SD-1029, Fas-L or TRAIL was examined using the CalcuSyn software (Biosoft, Ferguson, MO). This program calculates a combination index (CI) value based on the Chou-Talalay method. The program uses the

equation $CI=(D)1/(Dx)1+(D)2/(Dx)2+(D)1(D)2/(Dx)1(Dx)2$, 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 (22). A CI of <1.0 indicates synergy, 1.0 indicative additive effect and >1 indicates antagonistic effect of the drugs under investigation. Statistical significance of differences observed between scrambled siRNA plus LCL161 treated and cIAP2 siRNA plus LCL161 treated cells was evaluated using the student t test. Error bars in all experiments represent mean ± Standard Deviation (SD)

Results

LCL161 induces cytotoxicity and inhibits proliferation of a subset of MM cell lines

When MM cell lines were incubated with indicated concentrations of LCL161 for 72hrs and viability measured, we observed heterogeneity among MM cell lines in their sensitivity levels to the drug (Figure 1A). The IC50 values are in the range of 2.5μM–5μM for the sensitive cell lines and 15μM-NR (Not reached) for the resistant cell lines. This heterogeneity was also seen when proliferation of these cell lines was examined after drug treatment (Figure 1B).

Next, we examined baseline expression of IAP proteins in the MM cell lines to examine if differences between cell lines could predict for sensitivity to LCL161. XIAP and cIAP1 are expressed at fairly similar levels in all cell lines examined (Figure 1C). cIAP2, however, is not expressed in H929 (sensitive) and OPM2 (resistant) cell lines and expressed at very low levels in RPMI8226 (resistant) cell line (Figure 1C). Thus, baseline levels of IAPs do not appear to predict for sensitivity to LCL161.

LCL161 is able to overcome protective effects of components of the tumor microenvironment

The close interaction between MM plasma cells and the cellular components of the bone marrow microenvironment has been documented to be important for disease progression and drug resistance (23, 24). We co-cultured MM1S cells with or without bone marrow stromal cells derived from MM patients, followed by incubation with LCL161. LCL161 is able to overcome the tumor promoting effects of the stromal cells and inhibit the proliferation of MM cells at levels comparable to when cultured alone (Figures 1D). Similarly, LCL161 is also able to overcome the tumor promoting effects of three most commonly implicated cytokines in MM, namely, IL-6 and VEGF and inhibit the proliferation of MM cells when cultured with each of the three cytokines (Figures 1E).

LCL161 induced cell death is primarily driven by activation of the extrinsic apoptotic pathway

Since IAP inhibitors have been shown to trigger cell death by inducing apoptosis or necroptosis (25, 26), we wanted to examine if LCL161 induced cell death through one or both of these pathways. To address this, we treated H929 or MM1S cells with various concentrations of LCL161 for indicated time points. LCL161 treatment caused an increase in apoptotic cells in both the cell lines with a more remarkable increase in H929 than in

MM1S cells (Figures 2A and B). XIAP inhibits caspases 9 and 3, which act downstream of the mitochondria and the cIAPs inhibit caspase 8 which could act independent of the mitochondria (Type 1 cells (27)) to trigger cell death. To understand if LCL161 induced cell death in MM is completely independent of the mitochondria (Type 1 cells) or involves further activation of mitochondrial pathway of apoptosis (Type II cells (27)), we treated both H929 and MM1S cells with various concentrations of LCL161 for indicated time points following which we measured mitochondrial membrane potential alterations and cytochrome c release from the mitochondria. In both H929 and MM1S cells, we observed disruption of the mitochondrial transmembrane potential and cytochrome c release, both indicative of Type II cell death (Figures 2C–F). We also observed Bid cleavage post LCL161 treatment **(data not shown)** indicative of type II cell death (27). To understand if LCL161 induced necroptosis and also to understand the importance of extrinsic and intrinsic pathways of apoptosis in LCL161 induced cell death, we treated H929 and MM1S cells with LCL161 in combination with caspase 8, 9 or pan-caspase inhibitors. Using pan-caspase inhibitor in combination with LCL161 protected cells from LCL161 induced cell death (Figures 2G and H), indicating the lack of necroptosis induction by LCL161 in MM cells. In addition, the results also indicated that the extrinsic pathway was essential and the intrinsic pathway was involved but not essential for the ability of LCL161 to induce apoptosis. Given the importance of the extrinsic pathway in LC161 induced apoptosis, we then examined the baseline expression levels of the members of the death receptor, ligands and proteins involved in the death receptor signaling. We did not observe any correlation between their expression levels and sensitivity to LCL161 (Figure S1). Next, we examined the ability of LCL161 to induce apoptosis in primary plasma cells from MM patients. Like with cell lines, we observed heterogeneity in the response patterns observed in patient cells to LCL161 (Figures 2I). Importantly, LCL161 was able to induce potent apoptosis in about half of the patients examined at clinically achievable doses **(maximum clinically achievable dose: 7.5μM).**

LCL161 inhibits the IAPs and causes activation of caspases

Next, we performed western blots to understand the mechanism of action of LCL161. Incubating H929, MM1R, MM1S and U266 cells with indicated concentrations of LCL161 showed marked down regulation of cIAP1 in all the four cell lines (Figures 3A–C and S2A). XIAP, unlike cIAPs, is not degraded but inhibited by Smac-mimetic treatment (28). XIAP inhibition as measured by up-regulation of caspases 9 and 3 was also blocked in all the four cell lines examined (Figures 3A–C and S2A). cIAP2, however was down regulated only in the two sensitive cell lines H929 and MM1R (Figures 3A and B) but not in the resistant cell lines MM1S and U266 (Figures 3C and S2A). Examining caspase 8, which is mainly inhibited by the cIAPs, indicated more pronounced activation in H929 and MM1R (Figures 3A and B) than in MM1S and U266 (Figures 3C and S2A). Thus, lack of down regulation of cIAP2 could be a contributing factor to resistance observed to LCL161.

LCL161 modulates the canonical and noncanonical NF-κB pathways

cIAPs are required for the ubiquitination of RIP and NIK and are therefore key regulators of both the canonical and noncanonical NF-κB pathways (8, 9). We therefore examined the activated levels of both arms of the NF- κB pathway and observed clear activation of the

canonical pathway in all the four MM cell lines examined as demonstrated by up regulation of pIκB and p-p65 (Figures 3D–F and S2B). The noncanonical pathway, however, was activated only in the resistant cells as demonstrated by the up regulation of p52 and RelB in MM1S and U266 (Figures 3F and S2B) but not in H929 and MM1R cells (Figures 3D and E). The canonical and non-canonical NF-KB pathways regulate the expression of multiple genes that could promote or inhibit apoptosis. The observation that non-canonical NF-KB pathway up regulation occurred in the resistant cells but not the sensitive cells suggested that this could be a contributing factor to resistance to LCL161.

IAP inhibition modulates the Jak2/Stat3 pathway

Next, we wanted to examine if IAP inhibition modulated the activated states of other aberrantly regulated signaling cascades implicated in MM, namely the PI3K/Akt, Mek/Erk and Jak2/Stat3 pathways. In H929 and MM1R cells, we observed no significant differences in pAkt, pErk and pStat3 (Figures 3F and G). In MM1S and U266 cells, we observed up-regulation of pStat3 post LCL161 treatment (Figures 3H and S2C). Taken together, examining the mechanism of action of LCL161 indicated two main contributing factors to resistance observed in drug resistant MM cells, namely, the lack of down regulation of cIAP2 and up-regulation of pStat3.

Knockdown of cIAP2 sensitizes MM cells to LCL161

To understand if the lack of down regulation of cIAP2 contributed to resistance to LCL161, we performed knockdown experiments in MM1S cells using siRNAs specific to cIAP2. We examined cIAP2 levels and cell viability 72hrs post incubation with siRNA alone or in combination with LCL161. cIAP2 was down regulated in cells treated with LCL161 alone, cIAP2 siRNA alone and more markedly in cells treated with both the drug and the siRNA (Figure 4A). cIAP2 knockdown by itself was unable to trigger cell death in MM1S cells. However, cIAP2 knockdown in combination with LCL161 was able to significantly (p value <0.05) decrease the viability of MM1S cells when compared to when treated with LCL161 alone (Figure 4A). This experiment clearly showed that simultaneous inhibition of all the three IAPs is required for maximal induction of apoptosis in MM cells.

pStat3 is up regulated by LCL161 through secreted factor(s)

From the experiments shown in figure 3, it is clear that LCL161 caused activation of the noncanonical NF-κB pathway and the Jak2/Stat3 pathway in drug resistant MM cell lines. It is well known that the NF-κB pathway regulates the transcription of multiple cytokines and anti-apoptotic proteins and the Jak2/Stat3 pathway is an important mediator of cytokine mediated signaling (29). We therefore hypothesized that the activated noncanonical NF-κB pathway produced one or more cytokines, which ultimately led to up regulation of the Jak2/Stat3 pathway. To test this, we treated MM1S cells for indicated time points with conditioned media obtained after incubating cells with LCL161 for 6 hours. We then examined pStat3 levels and observed that the conditioned media was able to increase pStat3 levels (Figure 4B). This suggested that pStat3 up regulation was at least partially caused by the release of secreted factor(s) stimulated by LCL161 treatment. In order to know if the secreted factor was a cytokine and to identify the cytokine(s) responsible for Stat3 activation, we performed an ELISA array using conditioned media obtained after treating a

resistant cell line MM1S (Figure 1A) and a sensitive cell line MM1R (Figure 1A) with LCL161 for 24hrs. The results indicated differential expression of IP-10 between untreated and drug treated media in both MM1S and MM1R cells (Figures 4C). There were no significant differences in both the levels of other cytokines examined between untreated and drug treated media and the expression patterns of cytokines between the two cell lines (Figures 4C). To explore more into the kinetics of IP-10 regulation, we performed ELISA for IP-10 using supernatants from MM1S and MM1R cells that were either untreated or treated with LCL161. The kinetics of IP-10 regulation was fairly similar between cell lines except that the extent of up regulation of IP-10 was more pronounced and prolonged in MM1S when compared to MM1R (Figure 4D). To understand if IP-10 modulated pStat3 levels, we cultured MM1S cells with IP-10 (25ng/ml) and examined pStat3 levels. We did not observe any change in pStat3 levels **(data not shown).** We also checked to see if blocking IP-10 by using a neutralizing antibody sensitized resistant cells to LCL161 treatment and observed no difference in the sensitivity levels **(data not shown).**

LCL161 synergizes with inhibitors of Jak2 kinase

Targeting the Jak2/Stat3 pathway using small molecule inhibitors has shown promising activity in MM (30, 31). To understand the functional significance of Jak2/Stat3 pathway upregulation in contributing to resistance to LCL161, we used a Jak2 inhibitor (TG101209) in combination with LCL161. We treated DOX40 and MM1Scells with several doses of LCL161, TG101209 or the drug combination and observed additive or synergistic cell death when the drugs were used in combination. The concentrations at which maximum synergy was observed is shown (Figure 5A). We then performed western blots to understand the mechanism of action of the drug combination. TG101209 was able to inhibit pStat3 levels alone and more importantly this inhibition was also observed when used in combination with LCL161 (Figure 5B). cIAP1, as expected was down regulated by LCL161 and the combination. XIAP and cIAP2 were more potently down regulated when cells were treated with the drug combination suggesting the importance of the Jak2/Stat3 pathway in regulating these IAPs. Importantly, the drug combination triggered more pronounced activation of caspases 8, 9 and 3 and PARP cleavage than either of the drugs alone (Figure 5B). Similar results were observed when LCL161 was used in combination with another Jak2 inhibitor SD1029 (Figures 5C). Examining LCL161/TG101209 combination on patient myeloma cells showed promising and more potent anti-MM activity than when used as single agents as observed by annexin/PI or apo 2.7 staining (Figures 5D and E). LCL161/TG101209 combination resulted in marked down regulation of pStat3, cIAP1, cIAP2 and XIAP when used on patient 5 (Figure 5F). LCL161/SD1029 combination was also able to induce more pronounced apoptosis when used in combination on primary cells from a MM patient (Figure 5G) through the down regulation of cIAP2, XIAP and pStat3 (Figure 5H).

LCL161 sensitizes cells to TRAIL and Fas-L treatment

Death inducing ligands (DIL) such as TRAIL and Fas-L induce apoptosis through the extrinsic pathway of apoptosis (32). Abnormal levels of the IAP proteins have been documented to be a major resistance factor to single agent DIL treatment reducing the impact of these agents inspite of their minimal toxicity profiles (33, 34). We therefore

examined if LCL161 sensitized to TRAIL and Fas-L treatment and observed pronounced synergy in their ability to induce cytotoxicity in MM (Figures 6A and B).

Discussion

IAP inhibition in cancer therapeutics is now an area of extensive research in a wide variety of tumors. In MM, while the role of XIAP as an anti-apoptotic protein is well established the functions of cIAP1 and cIAP2 are still unclear. In this study, we examined the functional importance of the IAPs in MM by using an orally available Smac-mimetic LCL161 and noted potent activity in a subset of MM cell lines. Since inactivating mutations in cIAP1/2 and TRAF3 have been reported in MM and implicated to be critical factors involved in stabilizing NIK, we examined if these could contribute to resistance observed to LCL161 (15, 16). None of the cell lines used here have mutated cIAP1/2. H929 and OPM2 have been shown to express wild type TRAF3 while all the other cell lines used have mutated TRAF3. Thus, mutational status of TRAF3 alone does not predict for sensitivity to LCL161, which is expected because TRAF3 has been shown to be not essential for cIAP1/2 mediated inhibition of the noncanonical NF-κB pathway (35). Likewise, there was no correlation between the cytogenetic status and drug sensitivity in both MM cell lines and patient cells.

By performing western blots, we observed potent and significant down regulation of cIAP1 by LCL161 in all cell lines tested irrespective of their sensitivity to the drug. The down regulation was very pronounced and rapid with almost complete knockdown of cIAP1 observed as early as 2hr of drug treatment **(data not shown).** cIAP2, however, was down regulated in the sensitive cell line MM1R but not in the resistant cell lines MM1S and U266. Examining the kinetics of cIAP2 down regulation in MM1S cells showed potent down regulation at earlier time points (2–8hr) after which there was a gradual increase with levels reaching close to baseline by 24hrs of drug treatment **(data not shown).** Furthermore, using a siRNA to cIAP2, we were able to show that cIAP2 down regulation sensitized cells to LCL161 treatment. These results are consistent with observations made in other studies where cIAP2 down regulation was non-existent and contributed to resistance to Smac-mimetic treatment (36, 37).

Additionally, we also observed that LCL161 activated the canonical NF-κB pathway in both the sensitive and resistant MM cell lines and the noncanonical NF-κB pathway in only the resistant cell lines. In tumor systems, this could lead to increased transcription and secretion of TNF-α, which then potentiates cell death in the absence of cIAP1/2 (38). IL-6 is a tumor promoting cytokine that is increased in the MM microenvironment (39, 40). IL-6 is down stream of the NF-κB pathway and among other things has been shown to induce Jak2/Stat3 pathway (39, 40). We therefore measured TNF-α and IL-6 levels in supernatents obtained from cells untreated or treated with LCL161. We did not observe detectable amounts of TNF-α or IL-6 in both sensitive and resistant cells **(data not shown).**

Our cytokine array experiment identified IP-10 to be the only cytokine differentially expressed between untreated and drug treated cells. This was observed in both sensitive and resistant cell lines and did not contribute to up regulation of pStat3 and resistance to LCL161 **(data not shown).** However, increase in IP-10 levels might have pronounced

clinical implications. IP-10 is induced during pro-inflammatory conditions by interferon-γ (IFN-γ) and is a chemo-attractant for activated T helper cells (Th1), dendritic cells and natural killer (NK) cells, thus modulating both innate and acquired immune responses (41). IP-10 is secreted from various cells including keratinocytes, monocytes, stromal and endothelial cells (41, 42). The role of IP-10 in cancer is still unclear with reports suggesting either tumor promoting or tumor inhibiting functions (41). LCL161 induced transient up regulation of IP-10could be important for acute inflammation and cell death. However, prolonged increase in IP-10 might lead to chronic inflammation with deleterious effects. Our studies have not been able to identify the cytokine(s) induced by LCL161 that caused up regulation of pStat3. In addition to cytokines, intercellular communication occurs through the release of microvesicles and exosomes that contain molecular mediators like miRNAs, proteins and lipids (43). It remains to be seen whether LCL161 induced pStat3 is mediated by such factors.

NF-κB controls the expression of various genes including the Bcl2 family of anti-apoptotic proteins (44). Examining the Bcl2 family of anti-apoptotic proteins post LCL161 treatment showed no obvious change or a slight increase in expression levels in the drug sensitive and the drug resistant cell lines respectively. Studies using LCL161 in combination with a pan Bcl2 family inhibitor such as obatoclax might therefore show synergistic activity in a MM setting. Furthermore, LCL161, by modulating the NF-κB pathway was shown to potentiate the anti-tumor activity of T cells and NK-cells without affecting their viabilities at concentrations comparable to ones used in our study (45, 46). However, using LCL161 with a cancer vaccine reduced the ability of dendritic cells to cross present the antigen and activate antigen specific immune response (45). In another study, it was shown that the IAP inhibitor BV6 increased bone metastasis and tumor growth (47). Using an antiresorptive agent in combination with BV6 was able to overcome this effect (47).

Our studies here have validated IAPs as therapeutic targets in MM. Results shown here provide evidence for clinical evaluation of LCL161 in combination with a Jak2 inhibitor in MM patients. During clinical trials, the effects of LCL161 on immune cells must be examined closely with plans to counteract adverse events like drug induced bone metastasis or chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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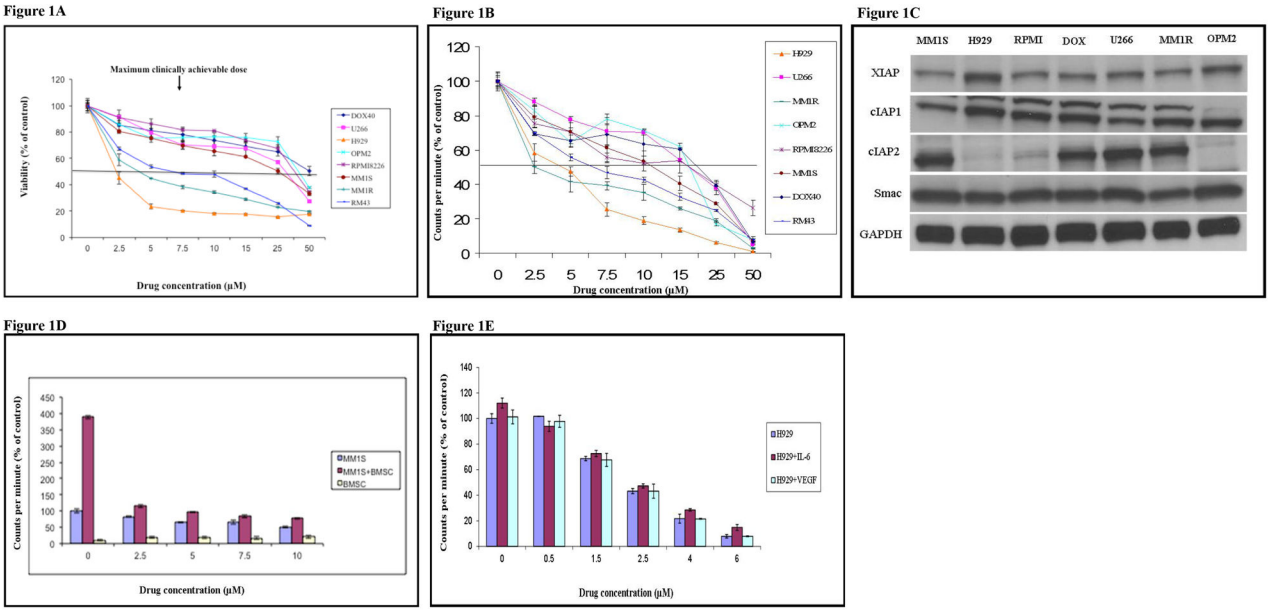

Figure 1.

LCL161 causes **A)** dose dependent cytotoxicity and **B)** dose dependent inhibition of proliferation of MM cell lines after 72hrs of treatment. **C)** Baseline expression profile of IAPs and Smac in a panel of MM cell lines showed no correlation between their expression and sensitivity to LCL161. **D) and E)** LCL161, after 72hrs of treatment with indicated doses, is able to overcome the protective effects of tumor promoting bone marrow stromal cells and cytokines (IL-6 and VEGF) and inhibit proliferation of MM1S and H929 cells respectively

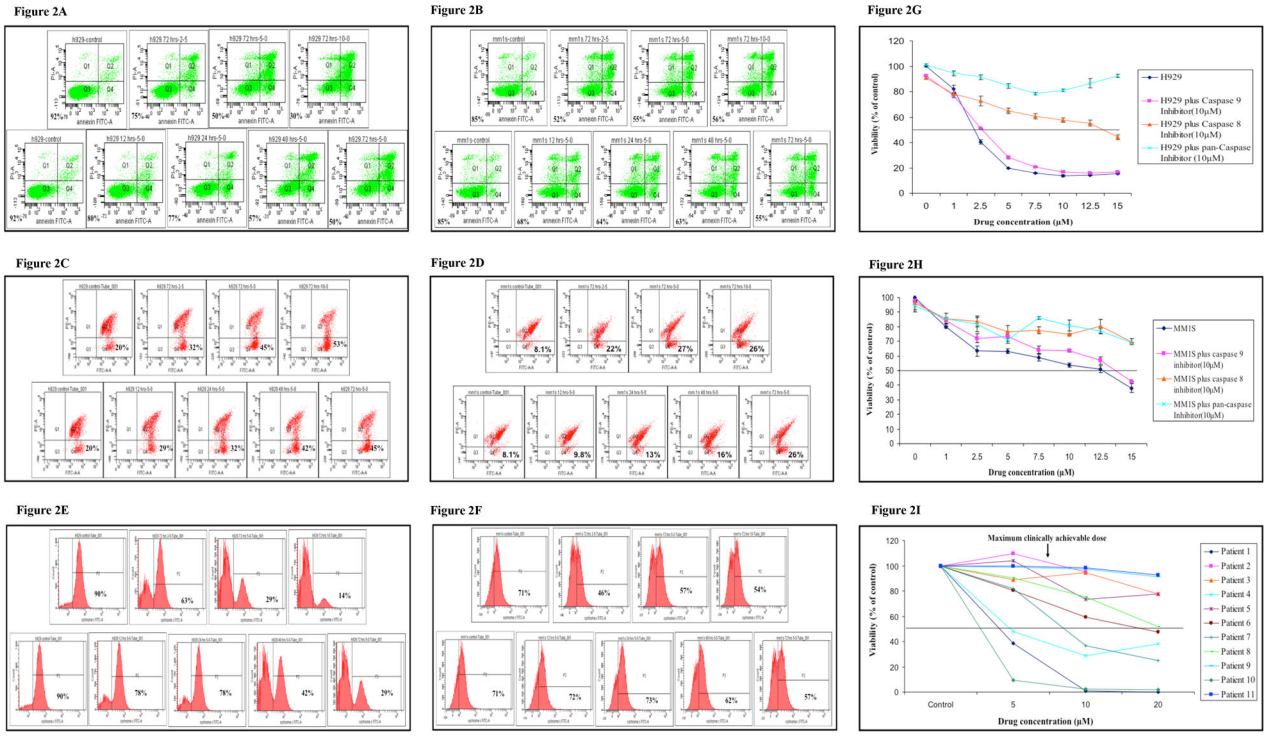

Figure 2.

LCL161 induces pronounced dose and time dependent **A)** increase in apoptosis, **C)** disruption in mitochondrial membrane potential and **E)** increase in cytochrome c release in H929 cells and less pronounced dose and time dependent **B)** increase in apoptosis, **D)** disruption in mitochondrial membrane potential and **F)** increase in cytochrome c release in MM1S cells. We incubated **G)** H929 or **H)** MM1S cells with indicated doses of LCL161 for 72hrs alone or in the presence of 10μM of either caspase 9 inhibitor (Ac-LEHD-CMK), caspase 8 inhibitor (Z-IETD-FMK) or pan-caspase inhibitor (Q-VD-OPH) and measured the cytotoxicity induced by LCL161. **I)** Primary plasma cells from MM patients were incubated with indicated doses of LCL161 for 72hrs. Apoptosis was measured by annexin V/PI staining. Data is presented as decrease in viability (% of control)

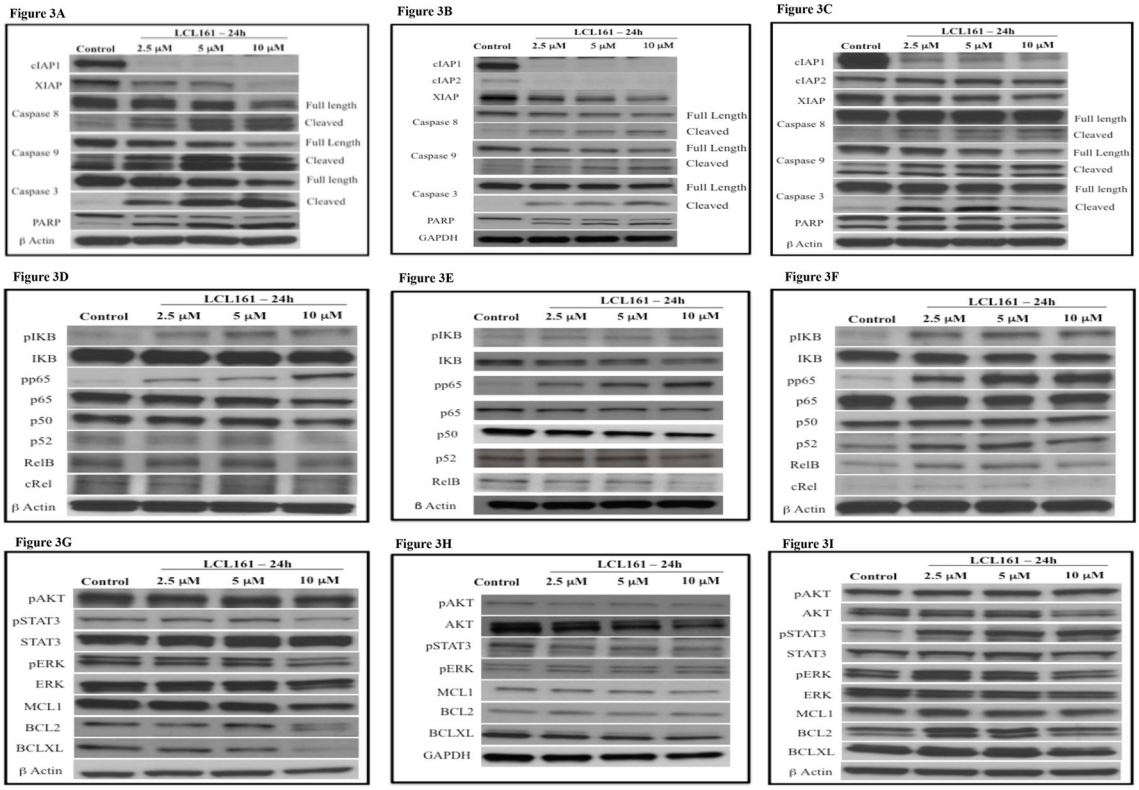

Figure 3.

We incubated **A)** H929, **B)** MM1R and **C)** MM1S cells with indicated doses of LCL161 and measured expression levels of the IAPs, caspases 9, 8 and 3 and PARP. We incubated **D)** H929, **E)** MM1R and **F)** MM1S cells with indicated doses of LCL161 and examined levels of both the canonical and noncanonical NF-κB pathway proteins. We incubated **G)** H929, **H)** MM1R and **I)** MM1S cells with indicated doses of LCL161 and checked expression levels of pAkt, pStat3, pErk, Bcl2, Bcl-Xl and Mcl1.

Figure 4A

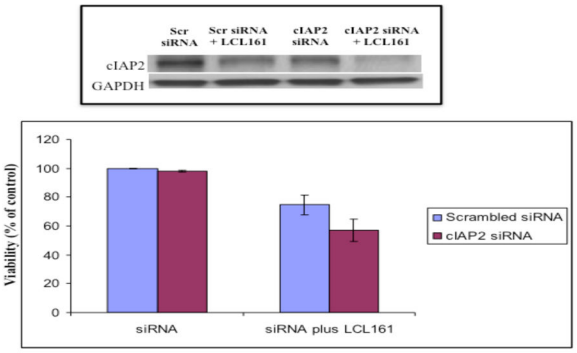

Figure 4B

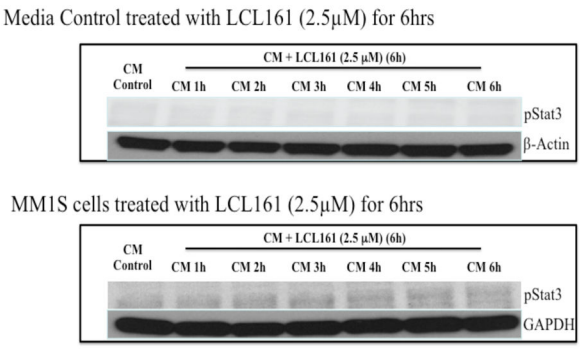

Figure 4C

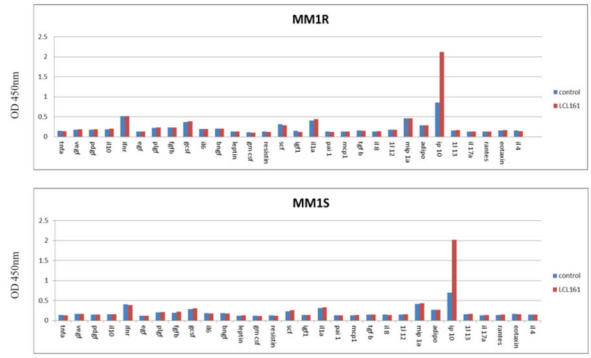

Figure 4D

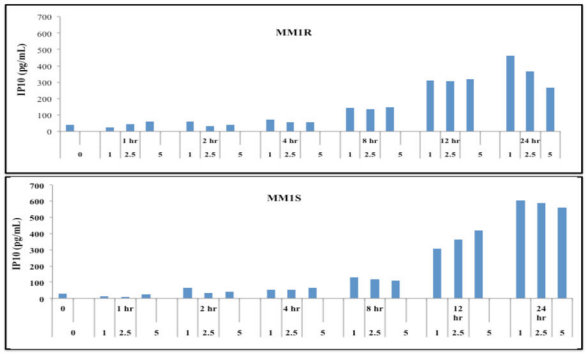

Figure 4.

A) MM1S cells were transfected with cIAP2 siRNA or scrambled siRNA. Cells were then left untreated or treated with 2.5μM of LCL161 for 72hrs. Subsequently, apoptosis was measured by annexin V/PI staining and cIAP2 knockdown was confirmed by western blotting. **B)** MM1S cells or culture media (negative control) were either untreated or treated with 2.5μM of LCL161 for 6hrs. Conditioned media (CM) was taken and fresh MM1S cells were cultured with the CM for indicated time points. Lysates were made and pStat3 expression level was examined by western blots. **C)** MM1R or MM1S cells were left untreated or treated for indicated time points with 1μM or 2.5μM of LCL161 respectively. Conditioned media was obtained and levels of various cytokines measured using an ELISA cytokine array. **D)** MM1R or MM1S cells were left untreated or treated with indicated doses of LCL161 for indicated time points. Conditioned media was obtained and used for measuring IP-10 levels by ELISA.

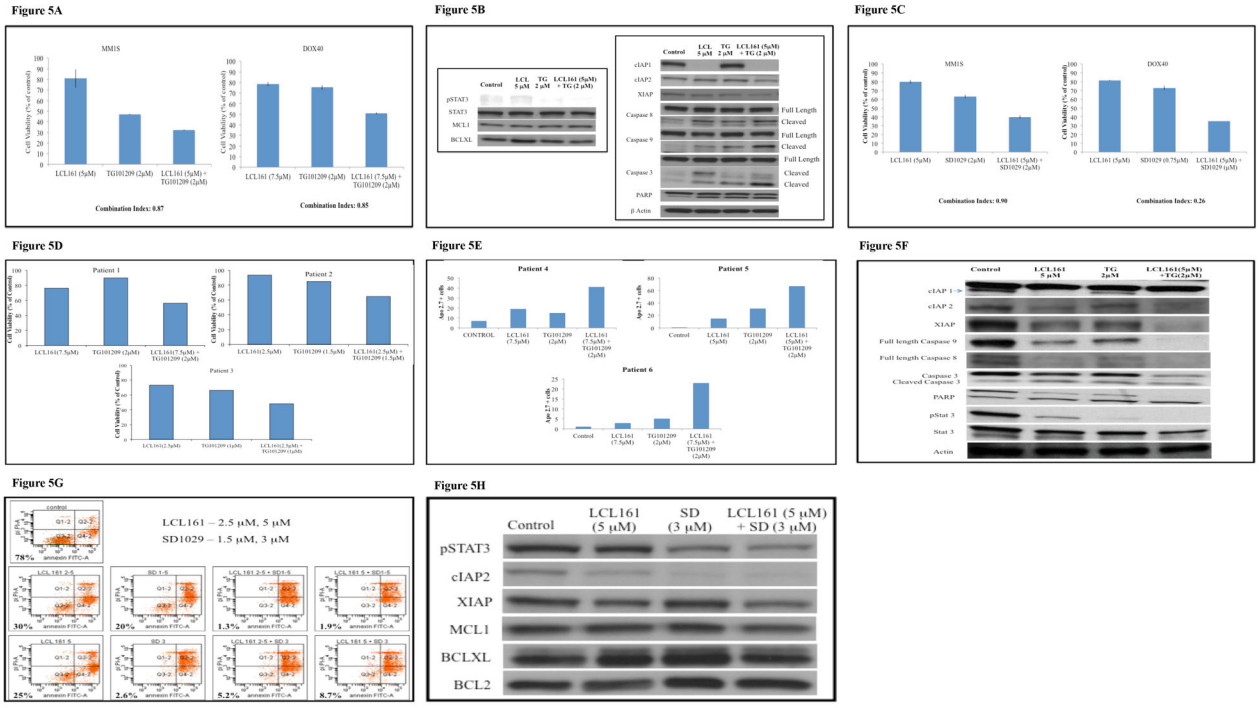

Figure 5.

A) MM1Sor DOX40 cells were treated with various concentrations of LCL161 for 72hrs, various concentrations of TG101209 for either 40hrs (MM1S) or 48hrs (DOX40) or both the drugs in combination. MTT assays were performed. The concentrations at which maximum synergy was observed is shown in the figures. CI values are indicated below the graphs. **B)** MM1S cells were treated with 5μM LCL161, 2μM TG101209 or the combination for 24hrs. Lysates were made and expression levels of indicated proteins measured by western blots. **C)**MM1S or DOX40 cells were treated with various concentrations of LCL161 for 72hrs, various concentrations of SD1029 for either 40hrs (MM1S) or 48hrs (DOX40) or the combination. MTT assays were performed. The concentrations at which maximum synergy was observed is shown in the figures. CI values are indicated below the graphs. **D) and E)** Primary plasma cells from MM patients were treated with indicated concentrations of LCL161, TG101209 or the combination. Apoptosis was measured by **D)**annexin V/PI staining or **E)**apo 2.7 staining. **F)**Primary plasma cells from MM patient 5 used in figure 5E were incubated with indicated concentrations of LCL161, TG101209 or the combination for 24hrs. Western blots were performed to examine expression levels of pStat3 and IAPs. **G)**Primary plasma cells from a MM patient were treated with indicated concentrations of LCL161, SD1029 or the combination. Apoptosis was measured by annexin V/PI staining. Data is presented as decrease in viability (% of control). **H)** Primary plasma cells from the same patient used in figure 5G were incubated with indicated concentrations of LCL161, SD1029 or the combination for 24hrs. Western blots were performed to examine expression levels of pStat3 and IAPs.

Figure 6A

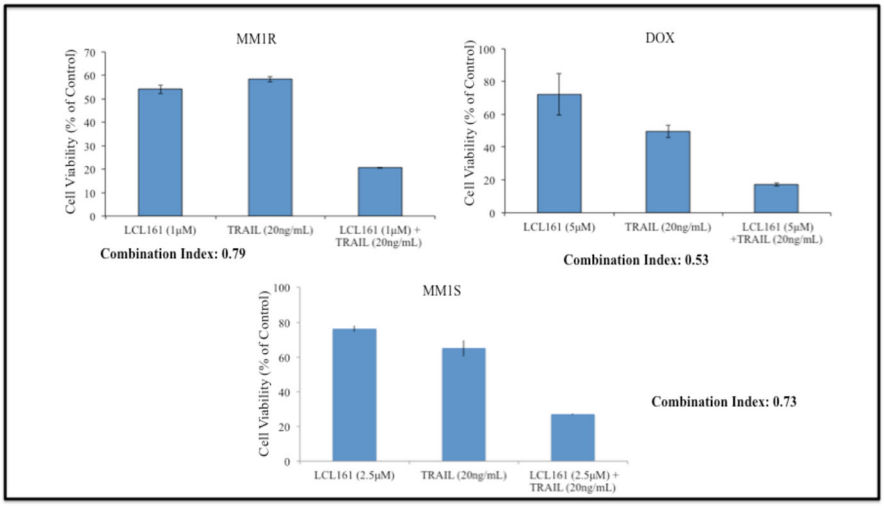

Figure 6B

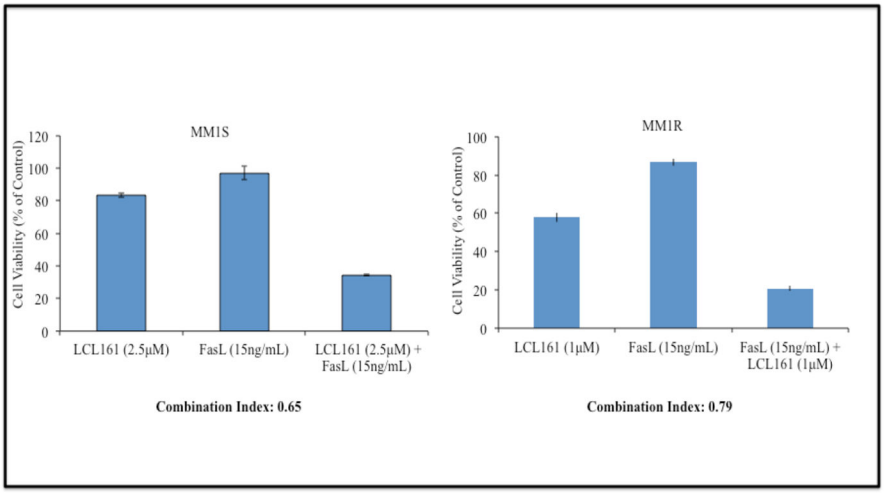

Figure 6.

A) MM1S, MM1R or DOX40 cells were treated with various concentrations of LCL161 for 72hrs, various concentrations of TRAIL for 48hrs or the combination. MTT assays were performed. The concentrations at which maximum synergy was observed is shown in the figures. CI values are indicated below the graphs. **B)** MM1S or MM1R cells were treated with various concentrations of LCL161 for 72hrs, various concentrations of Fas-L for 48hrs or the combination. MTT assays were performed. The concentrations at which maximum synergy was observed is shown in the figures. CI values are indicated below the graphs.