Combination of Proteasomal Inhibitors Lactacystin and MG132 Induced Synergistic Apoptosis in Prostate Cancer Cells

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Abstract

The proteasome inhibitor Velcade (bortezomib/PS-341) has been shown to block the targeted proteolytic degradation of short-lived proteins that are involved in cell maintenance, growth, division, and death, advocating the use of proteasomal inhibitors as therapeutic agents. Although many studies focused on the use of one proteasomal inhibitor for therapy, we hypothesized that the combination of proteasome inhibitors Lactacystin (AG Scientific, Inc., San Diego, CA) and MG132 (Biomol International, Plymouth Meeting, PA) may be more effective in inducing apoptosis. Additionally, this regimen would enable the use of sublethal doses of individual drugs, thus reducing adverse effects. Results indicate a significant increase in apoptosis when LNCaP prostate cancer cells were treated with increasing levels of Lactacystin, MG132, or a combination of sublethal doses of these two inhibitors. Furthermore, induction in apoptosis coincided with a significant loss of IKKα, IKKβ, and IKKγ proteins and NFκB activity. In addition to describing effective therapeutic agents, we provide a model system to facilitate the investigation of the mechanism of action of these drugs and their effects on the IKK–NFκB axis.

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Introduction

Removal of androgens induces apoptosis and drives androgen-sensitive prostate cancer into remission. Despite androgen depletion therapy and remission, prostate cancer survivors may develop a subclass of androgen-refractory cancer that is often more aggressive than the primary cancer [1]. This and other transformations have been associated with an increase in the prosurvival and proliferative activities of NFκB [2,3]. As a result, the regulation, processing, and disruption of NFκB are being explored as chemotherapeutic targets in cancer [4,5]. Of the members of the NFκB family, p65 is one of the most studied transcription factors, as it is involved in short-term and long-term cell survival and is active at both the nuclear and mitochondrial levels [4–8]. The transcriptional activity of p65 is regulated by inhibitor of κB (IκB) proteins that bind to the Rel Homology Domain of NFκB and prevent the translocation and transcriptional activity of NFκB [9]. To disrupt the binding of IκB to NFκB and to increase NFκB activity, IκB kinase proteins IKKα, IKKβ, and IKKγ are required. These proteins are located throughout the cytosol as either trimers of α, β, and γ subunits; dimers of β and γ; or monomers of IKKα [10,11]. Once phosphorylated, IKK proteins increase the transcriptional activity of NFκB directly and/or indirectly. For instance, phosphorylation of IκBα by IKKβ results in the immediate ubiquitination and degradation of IκBα by the 26S proteasome, thereby unmasking the nuclear localization signal of NFκB [4,12,13]. IKKα is a multifunctional protein responsible for the phosphorylation of IKKβ, phosphorylation of IκBα, direct phosphorylation of NFκB, and finally, phosphorylation of the nuclear histone H3 through CBP/p300 [14,15]; these cumulative effects facilitate the transcriptional activity of NFκB [7,16,17]. The activation of IKK proteins is necessary for the translocation of p65 into the nucleus [8] and the transcriptional activation of p65 in the nucleus. The process of NFκB activation and subsequent repression of apoptotic proteins may be deregulated when IKK proteins themselves or regulatory proteins upstream of IKK activation are blocked, missing, or mutated [3,18–21].

Of the drugs used for cancer therapy, proteasome inhibitors have shown promise in disrupting regulatory proteins, thereby reducing and/or eliminating various neoplastic cancers. The theory behind the use of proteasomal inhibitors is that, of all the cellular proteins involved in maintenance, differentiation, immunity, growth, division, and death, approximately 70% to 90% is eventually degraded by multicatalytic proteasomes [22–26]. By interfering with the normal function of proteasomes, cellular stress is induced, leading to cell cycle arrest and/or cell death [27–29]. Many compounds that either interfere or block proteasomal function have been identified. These include β-lactones such as *clasto*-Lactacystin, peptidyl aldehydes such as MG132 (carbobenzoxy-L-Leu–L-Leu–L-Leu), and peptidyl boronates such as Velcade (bortezomib/PS-341). Lactacystin (AG Scientific, Inc., San Diego, CA) binds covalently, whereas MG132 (Biomol International, Plymouth Meeting, PA) and Velcade

bind reversibly to the N-terminal Thr residue of the $\beta1$ subunit within the 26S proteasome [30,31]. Aside from the anti-proteasomal activity of Lactacystin and MG132, these compounds are known inhibitors of other proteases such as cathepsin A, cathepsin B, and calpain 1 [32,33], respectively. Regardless of the latter's protease activity, the primary effect of the inhibitors is directed on the proteasome. Proteasomal inhibitors increase the lifespan of short-lived proteins and block the modification of proteins involved in signaling pathways, resulting in changes that inhibit cell survival and proliferation. In early studies, Velcade was reported to induce DNA damage, arrest cellular cycling at the G1/S or G2/M phase, and reduce $NF\kappa B$ activity [34–37]. When used to treat cancer cells, Velcade induced a dose-dependent cleavage of polyadenosine-5′-diphosphate-ribose polymerase (PARP), significant apoptosis, expression of p21^{WAF1}, and G2/M cell cycle arrest [38]. Based on these beneficial effects, Velcade is being tested in phase I to phase III clinical trials for a variety of solid tumors. Unfortunately, several side effects, including incomplete tumor regression, have been noted at the doses required for the induction of apoptosis [33,39–42], thus necessitating the identification of other drugs that are effective at lower doses.

Although proteasomal inhibitors are being tested in cancer therapy, available data on prostate cancer are sparse. Therefore, our goal was to examine the effect of proteasomal inhibitors on prostate cancer. We hypothesized that a combination of proteasome inhibitors (e.g., MG132 and Lactacystin) would induce more apoptosis at lower doses of the drugs compared to the use of either inhibitor alone. The following results highlight the advantages of using combination therapy, as the inhibitors synergistically induce apoptosis. We also demonstrate that the increase in apoptosis is associated with a decrease in proteins associated with the IKK–$NF\kappa B$ axis.

Materials and Methods

Cell Culture and Experimental Design

LNCaP and PC3 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 (Hyclone, Logan, UT) containing 10% FBS, 0.5% penicillin–streptomycin (0.05 U/ml) (InVitrogen Corporation, Carlsbad, CA), and 0.1% Fungizone (0.25 μg/ml) (InVitrogen Corporation). All experiments were conducted with the same stock media for 24 hours, but with an FBS concentration of 7.5%. Cells were treated with increasing concentrations of Lactacystin in Experiment 1 and with increasing concentrations of MG132 in Experiment 2. In Experiment 3, cells were treated with 5.0 μM Lactacystin, 250 μM MG132, or their combination. The activity of $NF\kappa B$ and p53 was determined in Experiments 4 and 5, respectively. After LNCaP cells were transfected with a plasmid-based $NF\kappa B$-Luciferase or $p53$-Luciferase reporter construct, they were treated as in Experiment 3.

Induction of apoptosis was measured using an M30 Apoptosense kit (Peviva AB, Bromma, Sweden), which measured a neoepitope of cytokeratin 18 generated during apoptosis.

Cell extracts were separated on Tris–glycine gels (10–15%) and were probed for IKKα, IKKβ, IKKγ, IκBα, IκB$\alpha_{(S32/36)}$, phospho-p53$_{(S15)}$, p21^{WAF1}, caspase-3, XIAP, $NF\kappa B1$ (p50), and β-actin. Positive signals were developed using ECL-Plus (Amersham Biosciences Corp., Pittsburg, PA) and captured on a digital imager (Alpha Innotech, San Leandro, CA). Digitized signals were normalized against β-actin to control for differences in protein loading.

p53 and NFκB Activity Assays

Luciferase reporter activity was measured using a Luciferase reporter assay (Promega, Madison, WI). In each experiment, 3.5×10^6 cells were suspended in an electroporation buffer (120 mM KCl, 0.15 mM CaCl$_2$, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl$_2$, 50 mM glutathione, and 2 mM ATP, pH 7.6), transferred to a 4-mm electroporation cuvette, and mixed with 20 μg of a $NF\kappa B$-Luciferase firefly luciferase reporter construct (BD Biosciences Clontech, Palo Alto, CA) or 20 μg of a $p53$-Luciferase firefly luciferase reporter construct (Panomics, Inc., Redwood, CA) . The cells were electroporated using a BTX-830 electroporator (Gentronics, San Diego, CA) with 200-mV voltage, 10-millisecond pulse length, and two pulses at 500-millisecond intervals. After the cells had rested in the cuvette for 10 minutes, they were dispersed in RPMI media. The cells were seeded at 2.5×10^5 cells/well of a 12-well culture plate and allowed to adhere for 24 hours before treatment with the proteasome inhibitors.

Statistical Analysis

Apoptosense and actin-corrected densitometries of Western blot analysis data were analyzed by the GLM procedure of SAS (SAS Institute, Inc., Cary, NC) [43], and means were compared for $NF\kappa B$ and p53 activity data by Duncan's multiple range test ($P < .01$) only when a significant probability value of .05 was detected in the analysis of variance.

Results

Proteasomal Inhibitors MG132 and Lactacystin Induce Apoptosis

Treatment of LNCaP cells with Lactacystin induced apoptosis (greater than five-fold) at the lowest dose (5 μM) tested (Figure 1A). The apoptotic response continued linearly up to 12.5 μM Lactacystin ($P < .0001$; $R^2 = 0.956$), indicating that Lactacystin is an effective apoptotic agent in LNCaP prostate cancer cells. No appreciable cell death was observed with lower doses of MG132, whereas 0.5 μM induced an approximately two-fold apoptosis (Figure 1B). Further increases in MG132 resulted in a curvilinear response ($P < .0001$; $R^2 = 0.985$), leading to a 13-fold increase in apoptosis at the 3.00-μM dose. To test the hypothesis that the combination of the two proteasomal inhibitors would provide apoptotic advantage, LNCaP cells were treated with suboptimal doses of Lactacystin (5 μM) and MG132 (250 nM). Treatment with Lactacystin or MG132 alone confirmed the above results, whereas the combination yielded a highly significant
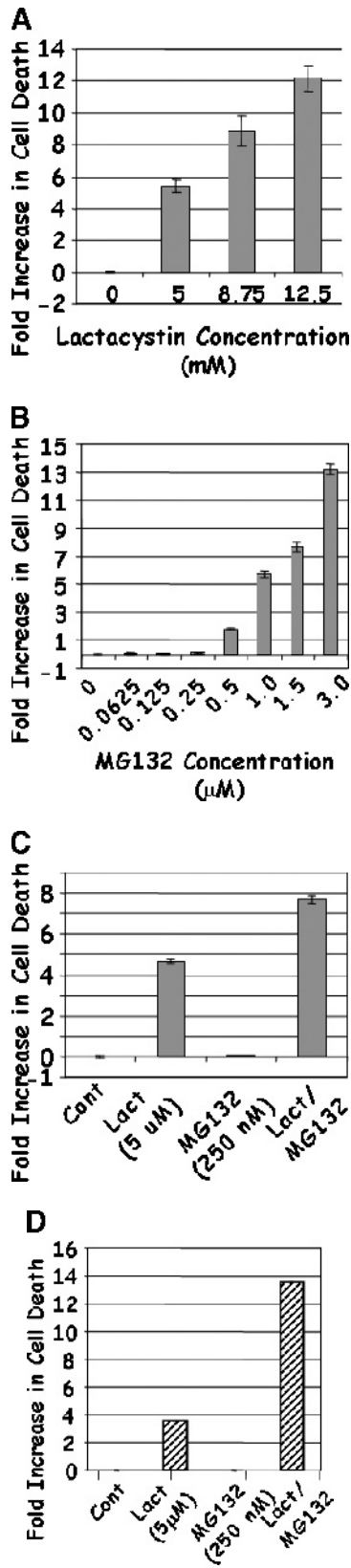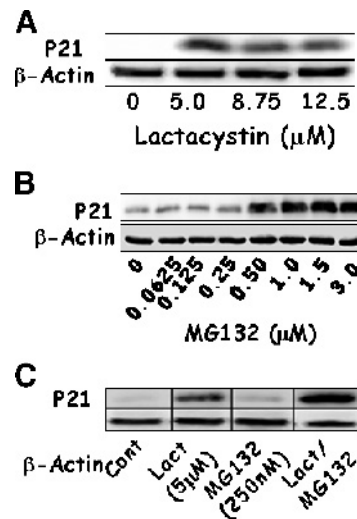
Figure 1. *LNCaP cells were treated with 5.0 to 12.5 μM Lactacystin (panel A; Experiment 1), 0.06 to 3.0 μM MG132 (panel B; Experiment 2), or their combination (panel C; Experiment 3). PC3 cells were treated as in Experiment 3, and apoptosis was measured (panel D). Results are fold inductions of M30 activity. Values represent observations from two experiments with three to four replicates each.*

increase in apoptosis (7.7-fold), indicating that this regimen may have therapeutic advantage (Figure 1*C*).

The above results demonstrated that the proteasomal inhibitors Lactacystin and MG132 induced apoptosis in androgen-responsive LNCaP cells. As androgen-independent prostate cancer is a major concern in patients and is more difficult to treat, it is of interest to determine whether these proteasomal inhibitors are capable of inducing apoptosis in androgen-independent prostate cancer cells. Treatment of PC3 cells with the same suboptimal dose of Lactacystin induced apoptosis (Figure 1*D*). Furthermore, similar to LNCaP, treatment of PC3 with 250 nM MG132 induced very little or no apoptosis, whereas combination of Lactacystin and MG132 resulted in synergistic induction of apoptosis (10-fold increase compared to cells treated with Lactacystin alone). These results indicate that the proteasomal inhibitors Lactacystin and MG132 induced apoptosis in both androgen-responsive and androgen-independent prostate cancer cells, an important therapeutic advantage of these drugs.

To confirm the results of apoptosis, the expression of p21[WAF1] and caspase-3 was determined because an increase in active caspase-3 was associated with an increase in DNA cleavage and the expression of p21[WAF1] in a p53-dependent manner. This not only facilitated apoptosis, but also induced cell cycle arrest. Expression of p21[WAF1] increased with each dose of Lactacystin or MG132 (Figure 2, *A* and *B*). The lowest dose of Lactacystin tested was enough to significantly increase p21[WAF1], whereas 500 nM MG132 was required to elicit a similar response. Combined treatment significantly increased the levels of p21[WAF1] beyond that of either proteasome inhibitor alone (Figure 2*C*). A similar analysis showed that Lactacystin activated caspase-3, as noted by the presence of proteolytic fragments (Figure 3), whereas activation was not significant with MG132 alone. Combined treatment synergistically increased the processing of the 35-kDa procaspase to the 17- and 12-kDa active fragments.

Figure 2. *LNCaP cells were treated with increasing concentrations of Lactacystin (panel A; Experiment 1), MG132 (panel B; Experiment 2), or their combination (panel C; Experiment 3) for 24 hours. Expressions of p21 and β-actin were evaluated using Western blot analysis.*
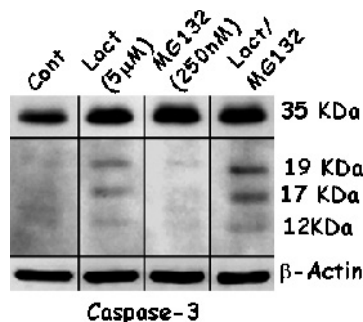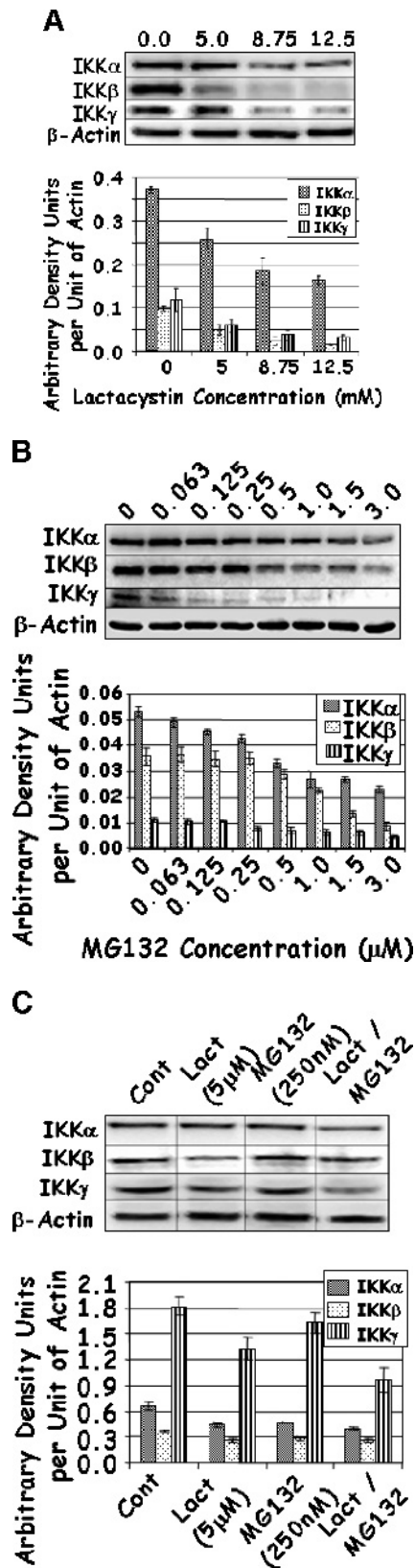
Figure 3. *LNCaP cells were treated with Lactacystin (Lact), MG132, or their combination for 24 hours. Expression levels of caspase-3, cleaved products of caspase-3, and β-actin were evaluated using Western blot analysis. Procaspase signals were obtained after 15 seconds of exposure, and signals from the cleaved caspase-3 fragments were obtained after a 3-minute exposure to a radiological film.*

Proteasomal Inhibitors Decrease the Level of IKK Proteins

To further investigate the mechanism of action of these drugs, the IKK−NFκB axis was investigated starting with the expression of IKKα, IKKβ, and IKKγ. A dose-dependent decrease in the levels of each of the IKK proteins was observed with increasing concentrations of Lactacystin (Figure 4*A*); however, marginal reduction was observed for IKKα at the lower levels of Lactacystin. Where the addition of 5 μM Lactacystin reduced IKKα by 30%, the addition of 8.75 and 12.5 μM Lactacystin reduced IKKα by 50% and 56%, respectively. Although not to the same magnitude, this trend was similar for IKKβ and IKKγ. Results obtained with MG132 were similar to those of Lactacystin, except that IKK proteins decreased significantly only when treated with 0.25 to 3.0 μM MG132 (Figure 4*B*). In combination experiments, IKK proteins responded to the individual drugs as in the previous groups. However, the level of IKKγ was significantly reduced when compared to that of either proteasome inhibitor alone (Figure 4*C*). Together, these experiments demonstrated a significant decrease in all three IKK family members, which coincided with significant apoptosis with increasing concentrations or with a combination of Lactacystin and MG132.

Lactacystin and MG132 Affect IκBα and NFκB Proteins

As changes in the function of IKK may affect the activity of NFκB through their effects on IκBα and the proteolytic modifications of p105 to p50, the levels of total and phosphorylated IκBα and NFκB1 (p50) were determined. Increasing concentrations of Lactacystin significantly increased the phosphorylation of IκBα, although it had no effect on the levels of total IκBα (Figure 5*A*). At first glance, these results seemed contradictive to this established molecular pathway, as the decrease in IKK should have resulted in a reduction in the phosphorylated form of IκBα. Under control conditions, phosphorylation of IκBα led to ubiquitination and degradation of IκBα by the 26S proteasome. However, inhibition of proteasomal activity by Lactacystin resulted in an increase in phosphorylated IκBα. Similarly, treatment with higher doses of MG132 increased IκBα phosphorylation (Figure 5*B*). Unlike Lactacystin, however, treatment with higher doses of

Figure 4. *LNCaP cells were treated with increasing concentrations of Lactacystin (panel A; Experiment 1), MG132 (panel B; Experiment 2), or their combination (panel C; Experiment 3) for 24 hours. Immunoblots were analyzed for the expression of IKKα, IKKβ, IKKγ, and β-actin. The signal was captured in digital image analysis units, and arbitrary density units are expressed per actin value. Values are averages of three replicates from two experiments.*
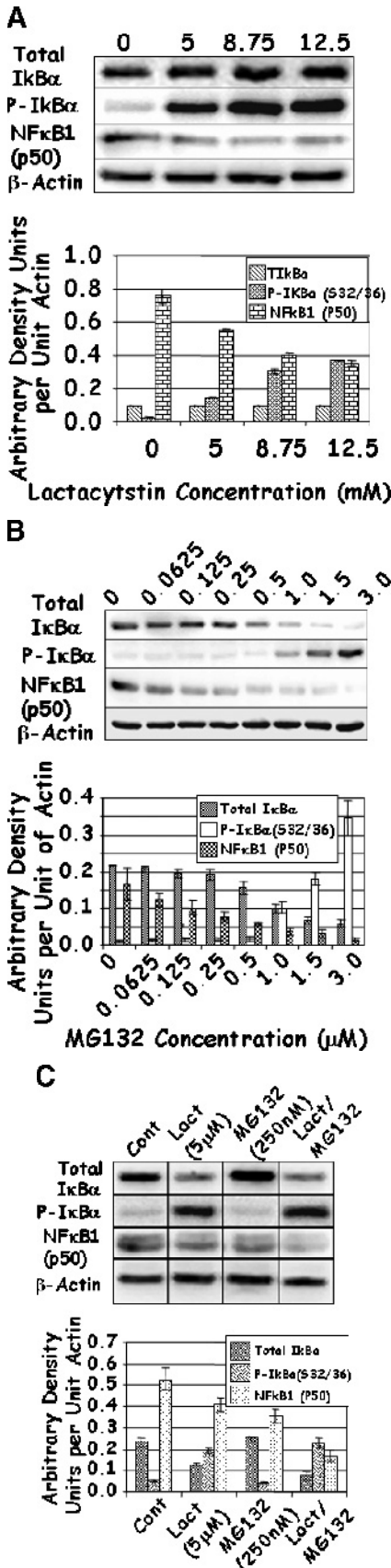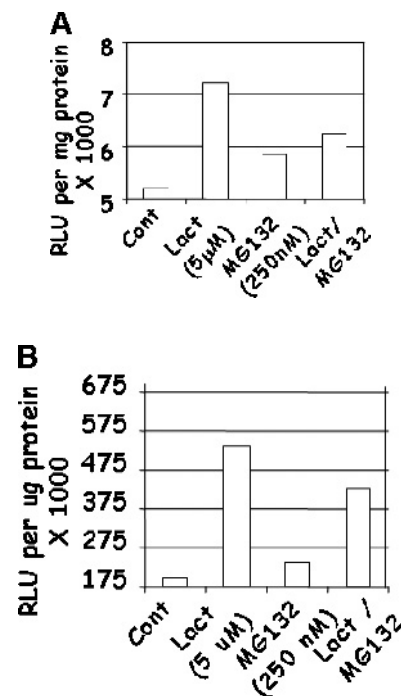
Figure 5. *LNCaP cells were treated with increasing concentrations of Lac-tacystin (panel A; Experiment 1), MG132 (panel B; Experiment 2), or their com-bination (panel C; Experiment 3) for 24 hours. The effect of treatment on total IκBα (IκBα), phosphorylated IκBα$_{S32/36}$ (P-IκBα), and NFκB1 (p50) was ana-lyzed by Western blot analysis. Values are averages of at least three replicates from two experiments.*

MG132 resulted in an unexplainable decrease in total levels of IκBα (Figure 5*B*). Finally, combined treatment with Lacta-cystin and MG132 resulted in a significant reduction in total IκBα when compared to either individual treatment (Figure 5*C*); phosphorylation of IκBα increased significantly in cells treated with both inhibitors. These results indicate that treatment with proteasomal inhibitors Lactacystin and MG132 not only reduced the levels of IKK, but prolonged the half-life and levels of phos-phorylated IκBα.

A similar analysis of NFκB1 (p50) demonstrated a steady de-crease in its levels with increasing concentrations of Lactacystin and/or MG132 (Figure 5), possibly attributed to the decrease in IκBα observed above. It is known that NFκB1 (p105) is pro-cessed to p50 by the proteasome in response to phosphoryla-tion by IKKα. Thus, it is likely that the decrease in NFκB1 (p50) was due to proteasomal inhibition. Given the reduction in both IKK proteins and p50, it was of interest to determine whether these drugs affected the activity of NFκB.

Proteasomal Inhibitors Increase NFκB and p53 Activity

As NFκB is a key target of IKK proteins, the effects of Lacta-cystin and MG132 on the transcriptional activity of NFκB and p53 were determined. LNCaP cells were transfected with *NFκB*-Luciferase or *p53*-Luciferase constructs and treated with Lactacystin and MG132, individually or together. Treat-ment with Lactacystin induced significant NFκB-Luciferase activity (*P* < .0201; R^2 = 0.78), whereas MG132 increased NFκB-Luciferase activity to a lesser extent (Figure 6*A*). These results were contrary to expectations, as IKK levels were

Figure 6. *LNCaP cells were treated with a combination of Lactacystin and MG132 for 24 hours. NFκB (panel A; Experiment 4) and p53 (panel B; Experiment 5). Luciferase activity was expressed as relative light units (RLU) per microgram of cellular protein. Values are averages of two experiments with three replicates per treatment.*

reduced in these cells (Figure 4*C*). The significance of these results is discussed below.

p53-Luciferase activity in Lactacystin- and MG132-treated cells (Figure 6*B*) agrees with p21^{WAF1} expression data (Figure 2*C*). Although the combination of Lactacystin and MG132 increased p53 activity ($P < .0001$; $R^2 = 0.96$), it was lower than that of Lactacystin alone. This was at variance with the expression of p21^{WAF1} in these cells. This was probably due to the transcriptional regulation of the *p53* construct by other members of the p53 protein family (such as p73).

Discussion

It is known that the proteasome is responsible for degrading 70% to 90% of all cellular proteins. The proteasome serves as a regulatory body that modifies proteins to render them functional (e.g., NFκB: p105 to p50), or that degrades proteins (e.g., p21^{WAF1} or active caspase-3) when they are no longer needed [44–46]. Although the proteasomal inhibitor Velcade is being tested in clinical trials, to date, there has been no report on the concurrent use of more than one class of proteasome inhibitors in the treatment of cancer. Therefore, the current study was designed to test the hypothesis that the combination of small doses of two different proteasome inhibitors would significantly induce apoptosis in prostate cancer when compared to the use of one proteasome inhibitor alone. Results from a series of experiments in this study indicate that the combination of Lactacystin and MG132 facilitates a high degree of cell death by inducing apoptosis, while simultaneously decreasing the expression of prosurvival proteins.

Cancer cells express a plethora of prosurvival proteins that override death-promoting signals in normal cells. Therefore, the goal of this study is to design therapy geared toward promoting the survival of death-inducing proteins. This is achieved by inhibiting the function of proteasomes. Our results showed a 39% increase in apoptosis when LNCaP cells were concurrently treated with Lactacystin and MG132. This effect may be due to changes in both the level and activity of proapoptotic and antiapoptotic proteins. Inhibitor-induced decrease in IKK proteins and processing of p105 to p50 may lead to a decrease in the function of prosurvival proteins, such as XIAP, BCL$_2$, BCL$_{XL}$, and MCL-1. Moreover, stabilization and expression of proapoptotic proteins in treated cells induced higher apoptosis and overcame the protection of survival proteins. These two scenarios are supported by the present results. Tang et al. [47] overexpressed caspase-3 in MCF-7 cells and observed a caspase-3–mediated cleavage of IKKβ when MCF-7 and HeLa cells were treated with TNFα. As observed, increased caspase-3 activity in treated cells may have led to an enhanced proteolytic cleavage of IKKβ. Despite the reduction in IKK proteins and contrary to expectations, phosphorylation of IκBα increased in Lactacystin- and MG132-treated cells due to the inhibition of proteasomal activity. The increase in Lactacystin-mediated IκBα phosphorylation was likely responsible for the observed increase in NFκB activity. Surprisingly, increased NFκB activity in Lactacystin-treated cells coincided with enhanced apoptosis, providing an interesting model that can be used to further explore the mecha-

nisms involved in apoptotic response, including proapoptotic functions of NFκB.

Many short-lived proteins are known to induce apoptosis. Activated caspase-3 induces DNA damage through the cleavage of PARP and BRCA1, which signals ATM and ATR to directly phosphorylate p53, thereby increasing the stability and transcriptional activity of p53 [48,49]. Our results demonstrate increased p53 transcriptional activity in Lactacystin-treated cells correlating with apoptosis. Although MG132, by itself, did not increase transcriptional activity, a combination of Lactacystin and MG132 resulted in lower luciferase activity. These results are similar to other observations in which increased levels of Velcade were used to treat a variety of cancers. Williams and McConkey [50] reported an increase in not only the stability of nuclear MDM2-P53, but also in the ability of the complex to bind a p53 DNA consensus sequence. The increase in p53 activity observed in proteasomal inhibitor–treated cells is significant in light of the report that p53 repressed the expression of IKKα by competitively sequestering ETS-1 from the IKKα promoter [51]. This may explain the observed decrease in IKKα and the increase in p21^{WAF1}, which may be responsible for the decreased activity of NFκB. The high degree of NFκB activity in proteasome inhibitor–treated LNCaP cells may be due to the crosstalk between NFκB and p53 [52,53]. Furthermore an NFκB-binding site has been demonstrated in the *p53* gene, suggesting that an increase in NFκB activity could increase the level of p53 protein expression [54].

Conclusion

Arresting proteasome function with Velcade has certainly proven to be beneficial in arresting the progression of tumorigenesis. Preclinical and clinical trials demonstrate the need to improve the efficiency and efficacy of the drug [35,38,55–58]. The present study suggests that the treatment of prostate cancer with two chemically distinct proteasome inhibitors may be superior to the use of any one proteasome inhibitor alone. In this preliminary report, the effectiveness of proteasomal inhibitors is the sum total of its effects on both the apoptotic and the antiapoptotic pathways in cancer cells.

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