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Novel combination of celecoxib and proteasome inhibitor MG132 provides synergistic antiproliferative and proapoptotic effects in human liver tumor cells

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Key words: celecoxib, MG132, HCC, ER stress response, TRB3, apoptosis, UPR, proteasome, COX-2

Abbreviations: HCC, hepatocellular carcinoma; ER, endoplasmic reticulum; ESR, endoplasmic reticulum stress response; UPR, unfolded protein response; XPB1, X-box binding protein 1; IRE1, inositol-requiring ER-to-nucleus signal kinase 1; CHOP, CAAT/enhancer-binding protein homologous transcription factor; ATF4, activating transcription factor 4; ASK1, apoptosis signal-regulating kinase 1; JNK, c-jun-N-terminal kinase; tribbles homolog 3 (TRB3); MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; NF-κB, nuclear transcription factor-κB; CLX, celecoxib; MG, MG132; COX-2, cyclooxygenase-2; COXIB, COX-2 inhibitor; PIs, proteasome inhibitors

Molecular targeted therapy has shown promise as a treatment for advanced hepatocellular carcinoma (HCC). Celecoxib (Celebrex®) exhibits antitumor effects in human HCC cells, and its mechanism of action is mediated either by its ability to inhibit cyclooxygenase 2 (COX-2) or by a number of various other COX-2 independent effects. Proteasome inhibitors (PIs) can exert cell growth inhibitory and apoptotic effects in different tumor cell types, including HCC cells. The present study examined the interaction between celecoxib and the PI MG132 in two human liver tumor cell lines HepG2 and HA22T/VGH. Our data showed that each inhibitor reduced proliferation and induced apoptosis in a dose-dependent manner in both cell lines. Moreover, the combination of celecoxib with MG132 synergistically inhibited cell viability and increased apoptosis, as documented by caspase 3 and 7 activation, PARP cleavage, and downregulation of Bcl-2. Celecoxib and MG132, both alone and synergistically in combination, induced expression of the endoplasmic reticulum (ER) stress genes *ATF4, CHOP, TRB3* and promoted the splicing of XBP1 mRNA. Knockdown of TRB3 mRNA expression by small interference RNA significantly decreased combination-induced cell death in HA22T/VGH cells, whereas it increased combination-induced cell death in HepG2 cells, suggesting that activation of the ER stress response might have either a detrimental or a protective role in liver tumor cell survival. In conclusion, our data indicate that combination treatment with celecoxib and MG132 resulted in synergistic antiproliferative and proapoptotic effects against liver cancer cells, providing a rational basis for the clinical use of this combination in the treatment of liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third most common cause of death from cancer.[1,2] Although the clinical diagnosis and management of early-stage HCC has improved, survival of HCC patients remains dismal due to the lack of adequate therapies. Conventional chemotherapies are generally ineffective, hence development of novel agents to enhance the effectiveness of treatment is mandatory. Recently, molecular targeted therapy, which acts on specific deregulated signal transduction pathways, has shown promise as a treatment for advanced HCC.[3]

There is compelling evidence that COX-2 has a role in hepatocarcinogenesis, as selective COX-2 inhibitors (COXIBs) show antiproliferative and proapoptotic effects in human HCC cell lines,[4,5] suggesting that COXIBs might be effective in HCC treatment.

The effects of COXIBs on tumor growth are likely to be multifactorial as they may use both COX-2 and non-COX-2 molecular

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targets to mediate their antitumor activities.[6] According to recent reports the mitogen-activated protein kinases (MAPK) MEK/ERK and nuclear transcription factor-κB (NF-κB), among others, are targets for COXIBs, and combinations of COXIBs with MEK/ERK inhibitors result in synergistic antitumor effects.[7,8] In addition, it has recently been discovered that the selective COX-2 inhibitor celecoxib (Celebrex®) causes apoptosis due to induction of ER stress in a COX-2 independent manner.[9-12]

Proteasome inhibitors (PIs) are currently considered to be promising anticancer drugs. Preclinical evidence indicates that bortezomib (Velcade®), the only PI that has undergone clinical trials, is effective in the treatment of hematological and solid malignancies.[13] Moreover, bortezomib has been approved by the FDA for the treatment of multiple myeloma.[14] The search for more effective as well as more selective proteasome inhibitors continues unabated. Recently, the discovery of a new proteasome inhibitor, designanted PI-083, has been described.[15] This agent exhibited antitumor effects against diverse epithelial neoplasms, including those of breast, ovarian, lung, prostate and myeloma cells.[15]

In previous studies, we and others have shown that PIs, such as MG132 and bortezomib, have antitumor activity in human liver cancer cells, suggesting the potential application of PIs in the treatment of liver cancer.[16,17]

At the molecular level PIs, similar to celecoxib, are known to trigger endoplasmic reticulum stress response (ESR).[18-20] However, the two types of inhibitors seem to activate ESR through different mechanisms.

Accumulation of unfolded protein in the ER is responsible for the induction of ESR, otherwise known as the unfolded protein response (UPR).[21,22] Cells initially adapt to the accumulation of unfolded proteins by inducing the expression of ER-resident molecular chaperones such as the Ca^{2+}-binding glucose-regulated protein 78 (GRP78).[23] Moreover, one of the ER protein sensors, inositol-requiring ER-to-nucleus signal kinase 1 (IRE1) has an endoribonuclease activity which removes 26 nucleotides from X-box binding protein 1 (XPB1) mRNA, thus resulting in a new transcription factor that induces the expression of chaperone genes.[24] However, if adaptation is not sufficient, the apoptotic response is initiated, which is primarily the induction of CAAT/enhancer-binding protein homologous transcription factor (CHOP),[25] activation of the apoptosis signal-regulating kinase 1 (ASK1)/c-jun-N-terminal kinase (JNK) pathway,[26] and cleavage of caspases 7, 9 and 12.[27] In addition, the tribbles homolog 3 (TRB3) has been identified as a novel target of CHOP in ESR and it seems to be involved in CHOP-dependent cell death as a second messenger.[28] Overall, the ESR system can be viewed as a "yin-yang" principle that regulates cell survival versus cell death.[29]

A novel concept in cancer chemoprevention and treatment is the use of combination therapy. A combination therapy consisting of COXIBs combined with agents that specifically modulate relevant biochemical targets may take advantage of synergistic antitumor effects, and could reduce the toxicity associated with the intake of COXIBs. Indeed, several clinical trials are under way based on combinations of COXIBs with conventional anticancer treatments (chemotherapy) and with novel molecular targeting compounds. In particular, cytotoxic synergy between celecoxib and the PI bortezomib, has been demonstrated in glioblastoma cells.[10] To our knowledge there are no studies on this type of combination treatment in HCC cells. Therefore, in the studies presented in this report, we focused on the development of combined molecular targeted therapies against HCC by testing the efficacy of the combination of the COX-2 inhibitor celecoxib with the proteasome inhibitor MG132 to determine if this resulted in synergistic antiproliferative and proapoptotic effects against liver cancer cells. The effects of celecoxib in combination with MG132 on the growth of two HCC cell lines were assessed with regard to cell viability, apoptosis and ER stress response.

Results

Combination of celecoxib with the proteasome inhibitor MG132 synergistically reduces cell viability and induces apoptosis in HCC cells. We first assessed the effects of celecoxib (CLX) and MG132 (MG) on the viability of two human HCC cell lines using the MTS assay. As shown in **Figure 1A**, CLX and MG effectively reduced the viability of both cell lines. After 48 h of exposure to the compounds, the IC_{50}s of CLX were 86 and 90 μM in HepG2 and HA22T/VGH cells, respectively; the IC_{50}s of MG were 0.69 and 0.47 μM in the same cells. Since COX-2 mRNA expression is undetectable in HepG2 cells,[5] the growth-inhibitory activity of CLX would appear to be largely COX-2 independent in these cells.

We next investigated by MTS assays the cytotoxic effects of the combination of CLX and MG in the HCC cells (**Fig. 1B**); CalcuSyn software was used to determine the type of interaction between the agents (**Table 1 and Fig. 2**). The combination of CLX and MG significantly increased the efficacy of treatment compared to the single agents. For example, 50 μM CLX plus 0.25 μM MG significantly inhibited cell growth in both cell lines, by 73% in HepG2 and 85% in HA22T/VGH cells. Even 25 μM CLX plus 0.1 μM MG significantly reduced, by 41%, cell growth in HA22T/VGH cells (**Fig. 1B**). **Table 1** presents the combination indexes (CI) observed after the treatment with combinations of the two drugs and indicates their synergy. In both cell lines, stronger synergy occurred when CLX was combined with the lower concentrations of MG. Normalized isobolograms for non-constant ratio drug combinations were also constructed for the HepG2 and HA22T/VGH cells treated with CLX and MG (**Fig. 2**). All combination data points plotted below the line expected for additivity in both HepG2 and HA22T/VGH cells, indicating synergistic effects (**Fig. 2**). Overall, the data indicated also that HA22T/VGH cells are more sensitive to the drug combination than HepG2 cells.

Treatment of HepG2 and HA22T/VGH cells with up to 50 μM CLX had negligible effects on the induction of apoptosis as evaluated by flow cytometry analysis of DNA stained with propidium iodide and by determination of the percentage of events which accumulated in the $subG_1$ position (**Fig. 3A**). Treatment with 0.5 μM MG increased the amount of apoptotic HepG2 and HA22T/VGH cells to 28.7 ± 4.1% and 4.6 ± 1.6%,

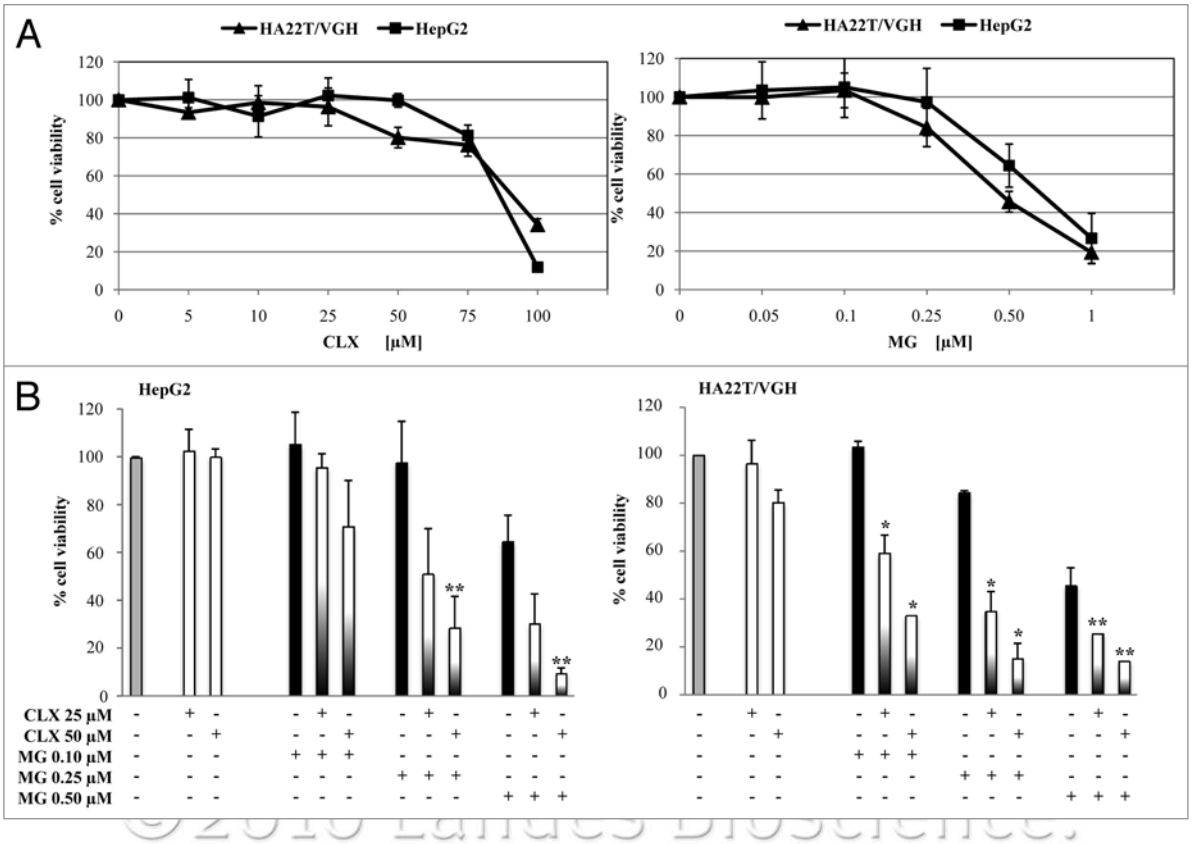

Figure 1. Effect of CLX and MG individually and in combination on growth of HCC cells. Cell growth was assessed by the MTS assay. (A) HepG2 and HA22T/VGH cells were treated for 48 h with the indicated concentrations of CLX and MG. (B) HepG2 and HA22T/VGH cells were incubated for 48 h in the presence of the indicated concentrations of CLX and MG alone or in combination. The combination treatment was found to be synergistic as described in "Materials and Methods". Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments, each of which was performed in triplicate. *p < 0.05; **p < 0.005 versus each agent alone.

respectively. However, the combination of CLX and MG significantly increased apoptosis in HA22T/VGH cells compared to treatment with either agent used alone ($p < 0.05$), whereas in HepG2 cells, only a minor effect was observed (**Fig. 3A**).

The activation and expression of apoptosis-related proteins were analyzed to determine whether apoptotic events were induced by the combination of CLX and MG in HCC cells. The combinatory effects of CLX and MG on the induction of cellular apoptosis corresponded with the activation of caspase 3 and 7 (**Fig. 3B**), with an increase in fold induction in both cell lines. However, a more pronounced induction was observed in HA22T/VGH cells. Caspase activation was associated with a concurrent cleavage of poly(ADP-ribose) polymerase (PARP), a known caspase 3 substrate and a biochemical marker of apoptosis. PARP cleavage was observed only when the cells were exposed to combination treatment (**Fig. 3C**), suggesting a synergistic induction of apoptosis. Interestingly, PARP cleavage was already evident in HA22T/VGH cells when 0.25 μM MG was used in combination with 50 μM CLX, but not in HepG2 cells, again indicating that HA22T/VGH cells are more sensitive to the combination of the two agents than HepG2 cells.

Furthermore, we analyzed the expression levels of the anti-apoptotic protein Bcl-2. Treatment of HCC cells with CLX or MG alone for 24 h decreased Bcl-2 expression levels. The combined treatment further increased this effect (**Fig. 3C**), resulting in a greater inhibition of expression levels of the protein than when either inhibitor was used alone (**Fig. 3C**). This suggests that Bcl-2 may also be involved in mediating the effect of the inhibitors in liver cancer cells.

Table 1. CI of the combination of celecoxib and MG132, indicating synergism according to the CalcuSyn software in HepG2 and HA22T/VGH cells

	HepG2			HA22T/VGH		
	MG132 (μM)			MG132 (μM)		
	0.10	0.25	0.50	0.10	0.25	0.50
Celecoxib (μM) 10	0.299	0.440	0.760	0.192	0.360	n.d.
25	0.400	0.464	0.784	0.164	0.318	0.586
50	0.359	0.469	0.718	0.126	0.260	0.513

Note: CI < 0.1 indicates very strong synergism; CI = 0.1 to 0.3 indicates strong synergism; and CI = 0.3 to 0.85 indicates synergism. n.d. = not determined.

Combination drug treatment stimulates stress-activated MAPKs. To further investigate the mechanisms of induction of apoptosis induced by CLX and MG, we tested their effects on stress-sensitive mitogen-activated protein kinases (MAPKs) p38 and JNK which are required for initiation of the apoptotic program

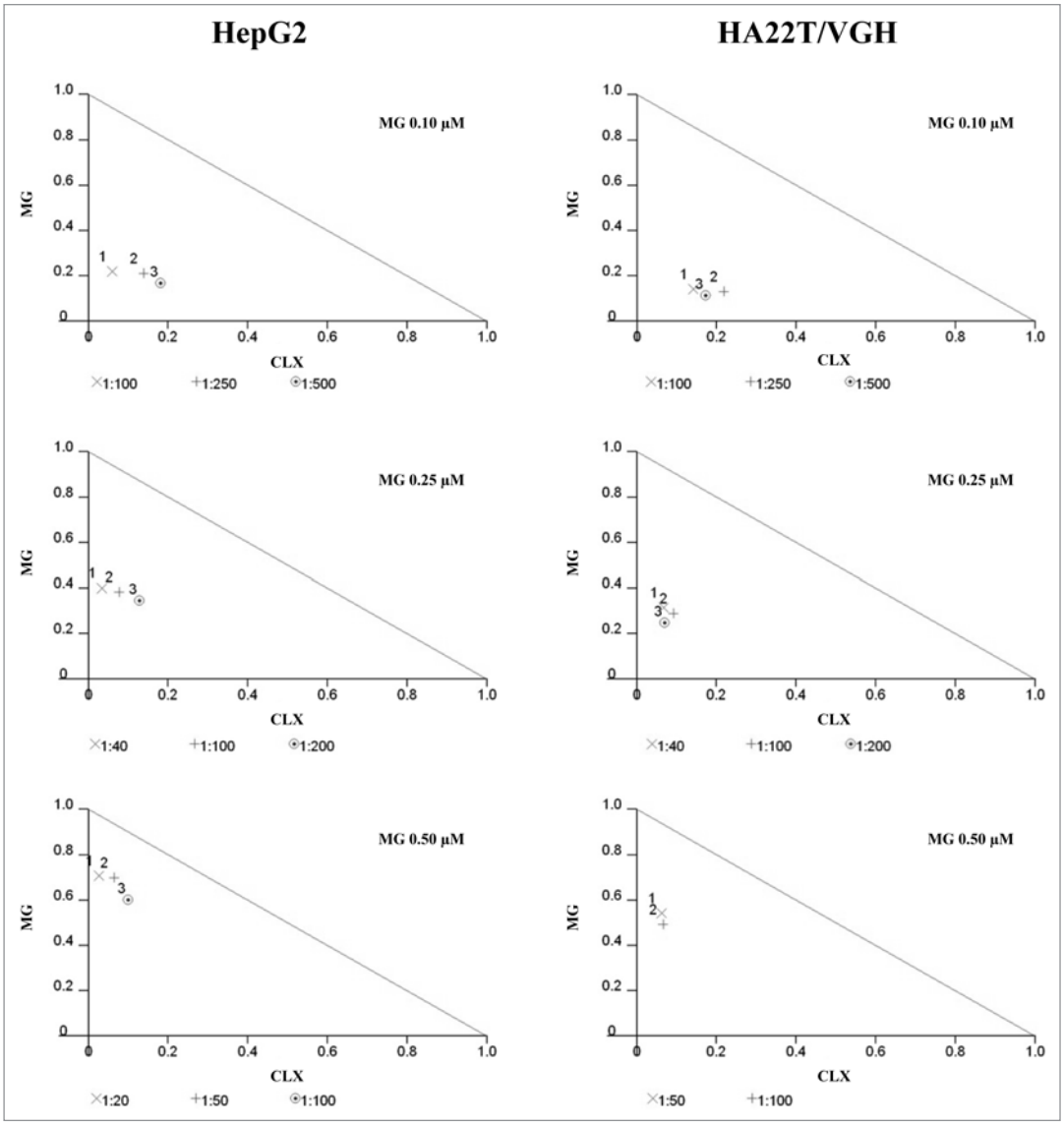

Figure 2. Normalized isobolograms of the combination of CLX and MG in HepG2 and HA22T/VGH cells. Cells were treated the indicated concentration of MG in combination with CLX at 10, 25 and 50 μM. Diagonal line is the additivity line. Data points below the additivity line correspond to a synergistic effect. The concentration ratios of the experimental drugs combinations used (MG:CLX ratio) are indicated below each isobologram. Data were analyzed by the CalcuSyn program.

in response to a variety of stressful conditions. Furthermore, both JNK and p38 MAPK have been linked to the pathogenesis and progression of HCC.[30,31] Treatment with CLX inhibited phosphorylation of p38 MAPK and JNK1/2 in both cell lines, whereas treatment with MG, in agreement with other reports,[32,33] promoted phosphorylation of p38 MAPK and JNK1/2 in a dose-dependent manner in both cell lines (**Fig. 4**).

Exposure to the two drugs resulted in a considerable increase in the phosphorylation level of p38 MAPK and JNK1/2 in both cell lines, and this was associated with activation of ATF-2 and c-Jun proteins, well known downstream substrates of these two MAPKs (**Fig. 4A**).

To test directly whether p38 MAPK and JNK activation were essential for cell death induced by the drug combination, we determined whether these effects could be suppressed by selective p38 MAPK and JNK1/2 inhibitors, i.e., SB203580 and SP600125, respectively. Cells were pretreated for 1 h with the MAPK inhibitors and then, in their presence, the cells were treated with combination. Treatment in the presence of SB203580 or SP600125 did not suppress, and even slightly facilitated, cell death induced by the combination (**Fig. 4B**). Activation of these kinases thus appeared not to be involved in combination-induced cell death in HCC cells, but suggested, as reported by others, that activation of the p38 MAPK and JNK pathways was associated with the protective response against the drug treatment.[33]

Combination drug treatment synergistically activates ER stress response. Accumulating evidence suggests that the endoplasmic reticulum (ER) is also an important apoptotic control point.[34] Activating transcription factor 4 (ATF4), CHOP and the spliced form of XBP1(sXBP1) are transcription factors

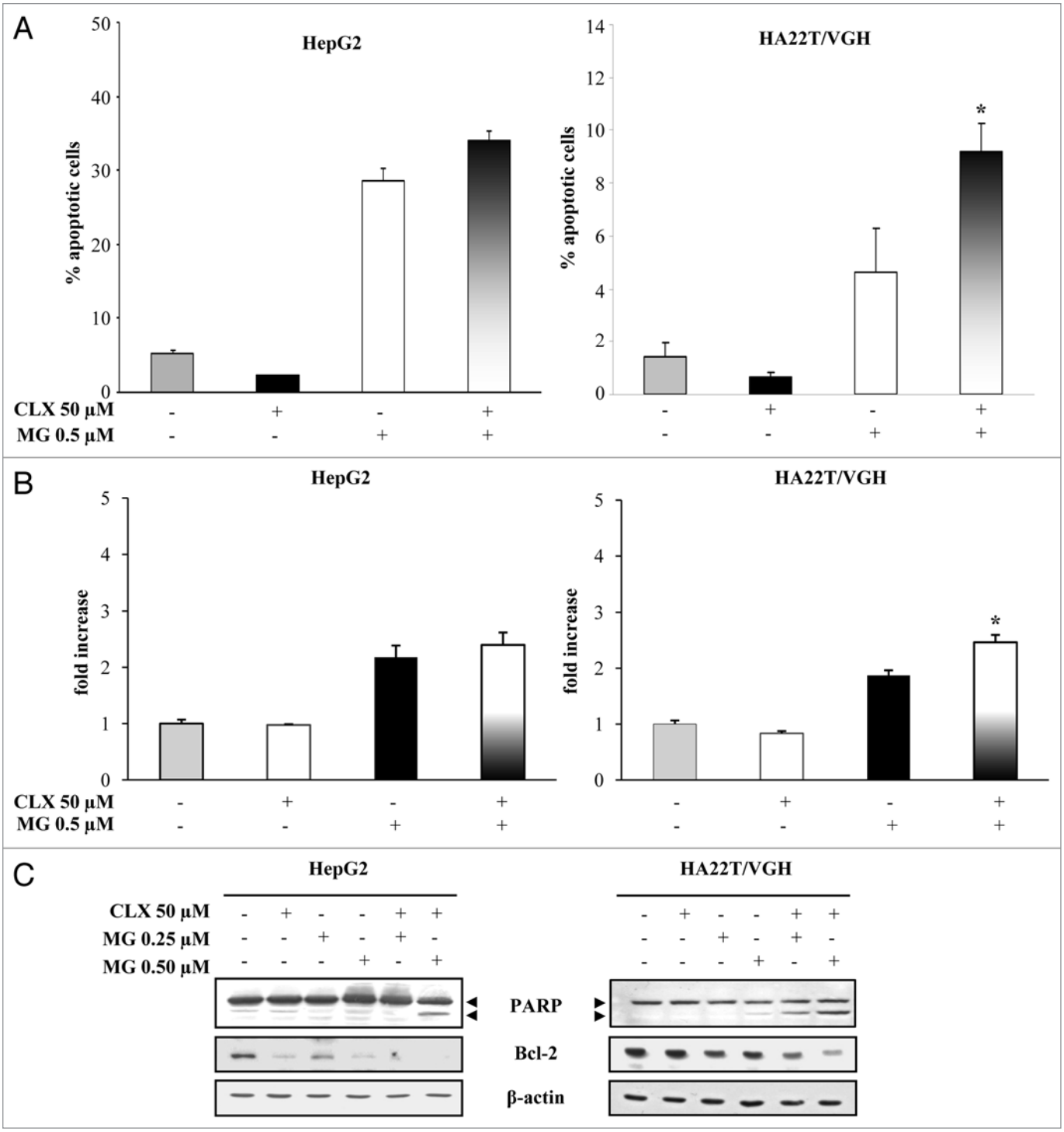

Figure 3. Effect of CLX and MG individually and in combination on induction of apoptosis in HCC cells. (A) Apoptosis was determined after 24 h in HepG2 and HA22T/VGH cells treated with each agent alone or in combination. Cell death was determined by flow cytometry analysis of subgenomic DNA. Data are expressed as percentages of apoptotic cells and are the mean ± SD of three separate experiments. *$p < 0.05$ versus each agent alone. (B) Cells were treated for 24 h with CLX and MG alone or in combination and the levels of caspase activity in the cells were measured by the Caspase-Glo® 3/7 assay. Data are expressed as fold increase of untreated cells and are the mean ± SD of two separate experiments, each of which was performed in duplicate. *$p < 0.05$ versus each agent alone. (C) Induction of PARP cleavage and levels of Bcl-2 protein were analyzed by western blotting. Cells were treated with the indicated concentrations of CLX and MG and their combinations and then harvested after 24 h. The data represent two independent experiments with comparable outcomes. Arrowheads indicate the 115 kDa and 85 kDa form of PARP.

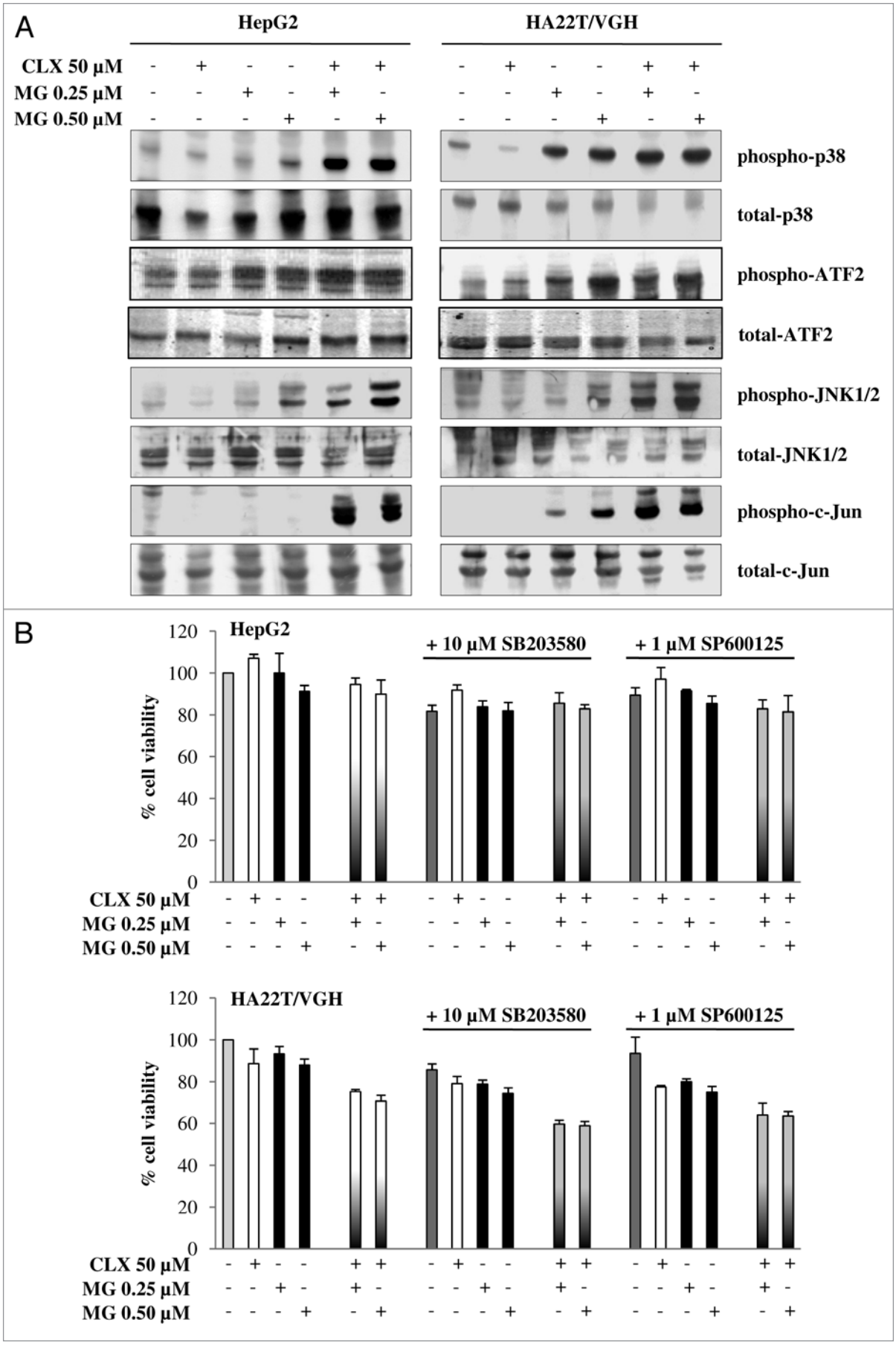
A
HepG2
HA22T/VGH
CLX 50 μM
MG 0.25 μM
MG 0.50 μM
phospho-p38
total-p38
phospho-ATF2
total-ATF2
phospho-JNK1/2
total-JNK1/2
phospho-c-Jun
total-c-Jun
B
HepG2
+ 10 μM SB203580
+ 1 μM SP600125
% cell viability
HA22T/VGH

Figure 4 (Opposite page). Combination of CLX and MG activates p38 MAPK and JNK. (A) HepG2 and HA22T/VGH cells were treated with CLX and MG alone and in combination at the indicated concentration for 24 h. After treatment cells were harvested and lysed, and equal amounts of extracted protein were analyzed for total and phospho-p38, ATF2, JNK1/2 and c-Jun expression by western blotting. The data represent two independent experiments with comparable outcomes. (B) Cell growth was assessed by MTS assays. HepG2 and HA22T/VGH cells were treated with CLX and MG alone and in combination at the indicated concentration for 24 h. The cells were also exposed to the MAPK p38 inhibitor SB203580 (10 μM) or to the JNK1/2 inhibitor SP600125 (1 μM) for 1 h and then co-exposed to CLX and MG alone and in combination for a further 24 h. Data are expressed as the percentage of control cells and are the means ± SD of two separate experiments, each of which was performed in triplicate.

whose expression are induced during the ER stress response and participate in ER-mediated apoptosis. TRB3 is also induced under stressful conditions, such as fasting, nutrient starvation and ER stress.

Since CLX and MG are known to trigger ER stress response through different mechanisms, we hypothesized that their combination might lead to enhanced ER stress, resulting in increased cell death. Therefore, cells were treated for 6 and 24 h with either drug alone, or with both in combination, and the levels of the mRNAs encoding the ER stress markers ATF4, CHOP, TRB3, as well as the induction of XBP1 mRNA splicing were examined by RT-PCR analysis.

After 6 h treatment with CLX, an induction of ATF4, CHOP and TRB3 mRNA expression was detected in HA22T/VGH cells but not in HepG2 cells, whereas after treatment with MG, expression of all mRNAs was enhanced in both cell lines (**Fig. 5A**). Furthermore, at this time, treatment with an individual drug did not induce XBP1 mRNA splicing in either cell lines (**Fig. 5B**). Comparable results were obtained at 24 h except for the induction of XBP1 mRNA splicing in HA22T/VGH cells treated with MG (**Fig. 5B**).

When combinations of the two drugs were used, a significant synergistic increase in all mRNAs was observed in HepG2 cells, especially 24 h following the combination treatment (**Fig. 5A**). With regards to XBP1 mRNA splicing, combination induced-splicing was observed at 6 h only in HA22T/VGH cells, but at 24 h in both cell lines (**Fig. 5B**).

Taken all together, these results indicate that CLX combined with MG caused stronger ESR induction than either drug by itself, and that the ESR is activated earlier in HA22T/VGH cells than in HepG2 cells.

TRB3 has different roles in the two HCC cell lines. As described previously, a synergistic increase of CHOP mRNA expression was observed after treatment with combinations of the two drugs in both cell lines. It is now well known that TRB3 is a downstream target of the ATF4/CHOP pathway, activated as part of the ESR.[28] In agreement in our experiments, we observed that concomitant with increase in ATF4 and CHOP mRNAs a clear increase in the TRB3 mRNA expression was observed especially in HepG2 cells treated with the combination. However, the role of TRB3 in apoptosis is controversial. Therefore, to examine the role of TRB3 in combination-induced cell death, in both HCC cell lines, we first transiently transfected HCC cells with siRNA specific for TRB3 for 72 h and then we analyzed the effect on cell growth by MTS assays after 24 h of treatment with each drug alone, or in combination. The transfection of TRB3 siRNA into HCC cells effectively reduced the endogenous mRNA compared to transfection with control siRNA (**Fig. 6A**). Knockdown of TRB3 did not affect cell death in the single-drug treatments in either cell line (**Fig. 6B**). However, knockdown of TRB3 significantly increased cell death in HepG2 cells treated with the combination of CLX and MG, whereas in HA22T/VGH cells knockdown of TRB3 expression significantly prevented the ER stress-induced cell death induced by the combination. These results suggest that activation of ER stress response, through TRB3, has an important role in regulating cell survival versus cell death in HCC cells.

Discussion

The overall survival rate for most HCC patients is grim, due to the lack of adequate therapy, therefore there is an urgent need to develop novel therapeutic strategies. Targeted therapies have entered the field of anti-cancer treatment and are being used alone or in combination with conventional chemotherapy drugs.

Prior work by our group has demonstrated that selective COX-2 inhibitors (COXIBs) and proteasome inhibitors show antitumor activities against HCC cells.[5,8,16] Several ongoing clinical trials are investigating the therapeutic benefits of COXIBs in oncology. However, a major concern in the development of treatment schedules with selective COXIBs is their safety profile. Combination therapy, which may allow dose reduction, may take advantage of synergistic growth inhibitory effects against cancer cells and could reduce the toxicity associated with COXIBs intake. In this context, several combinations of COXIBs with other chemotherapeutic drugs and with novel molecular targeting compounds have been investigated.[35] However, to our knowledge, there is very little information about such strategies in HCC.

The present studies were designed to examine whether the combination of celecoxib (CLX) and MG132 (MG) interacts in a synergistic manner to cause cell growth inhibition and cell death in liver cancer cells. We demonstrated for the first time that CLX and MG caused synergistic cell growth inhibition and a synergistic increase in apoptosis in both cell lines. We also observed that combination induced phosphorylation of p38 MAPK and JNK in a dose-dependent fashion. However, activation of these stress sensitive protein kinases did not appear to be involved in combination-induced cell death in HCC cells, but rather, as reported by others, their activation seem to be associated with a protective response against the drug treatment.[33]

At the molecular level, proteasome inhibitors and celecoxib exert antitumor activity via different mechanisms, however both are known to trigger endoplasmic reticulum stress response (ESR), which kills cancer cells.[9-12,18-20]

The important role of ESR in tumor cell growth and survival has been recently recognized.[36] The ER stress pathway, such as that mediated by the ATF6/XBP1 system, seems to be essential

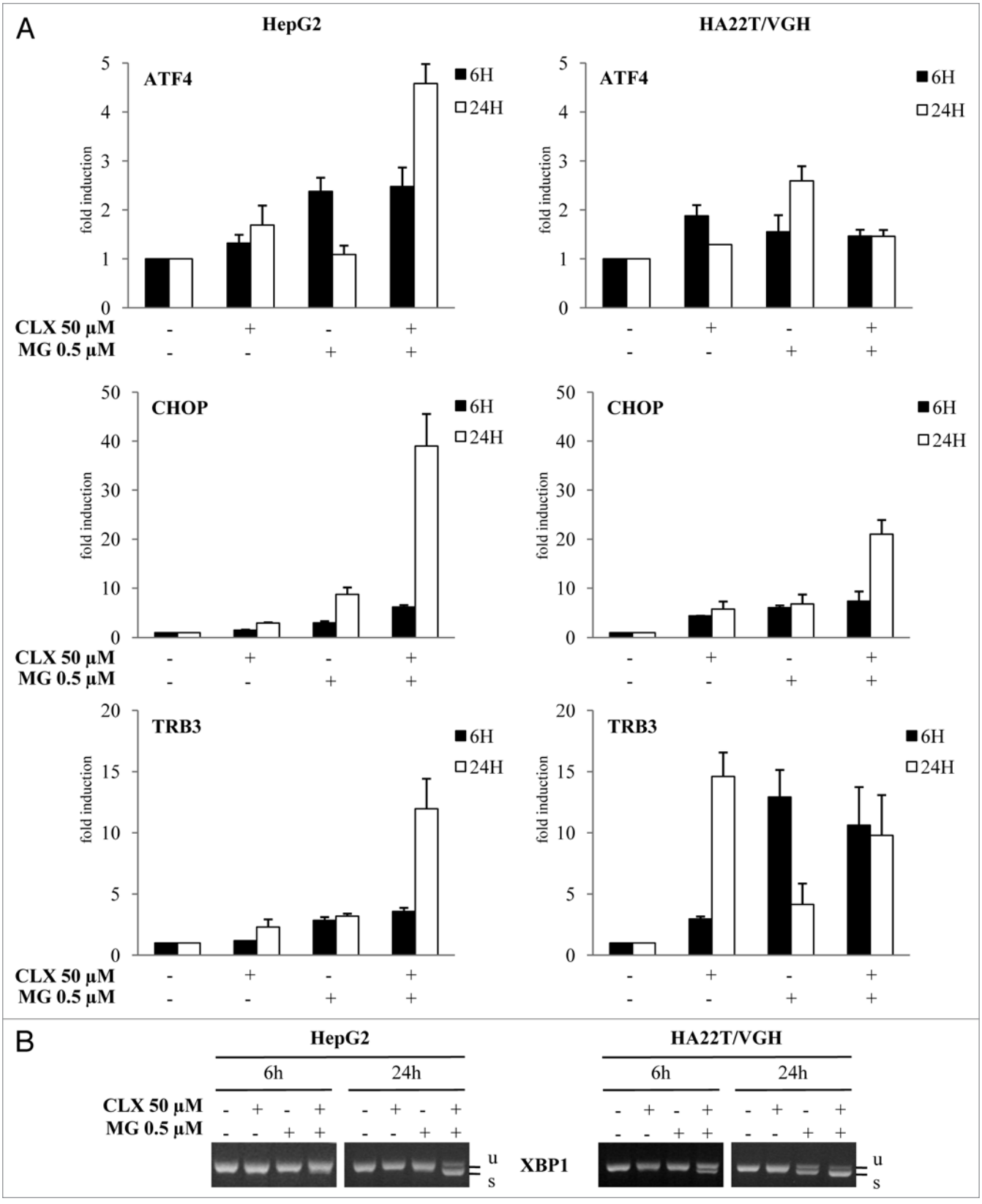

Figure 5. Treatment with combination of CLX and MG activates ER stress response. (A) Cells were treated with CLX and MG alone or in combination for the indicated times, and then mRNA expression levels of genes involved in the ER stress response were analyzed by quantitative real-time PCR. Histograms indicate the mean ± SD of fold induction relative to vehicle-treated control samples (-) arbitrarily set at 1.0. (B) Cells were treated with CLX and MG alone or in combination for the indicated times, and then XBP1 mRNA splicing was analyzed by RT-PCR analysis. (u) and (s) indicate the unspliced and spliced forms of XBP1 mRNA, respectively.

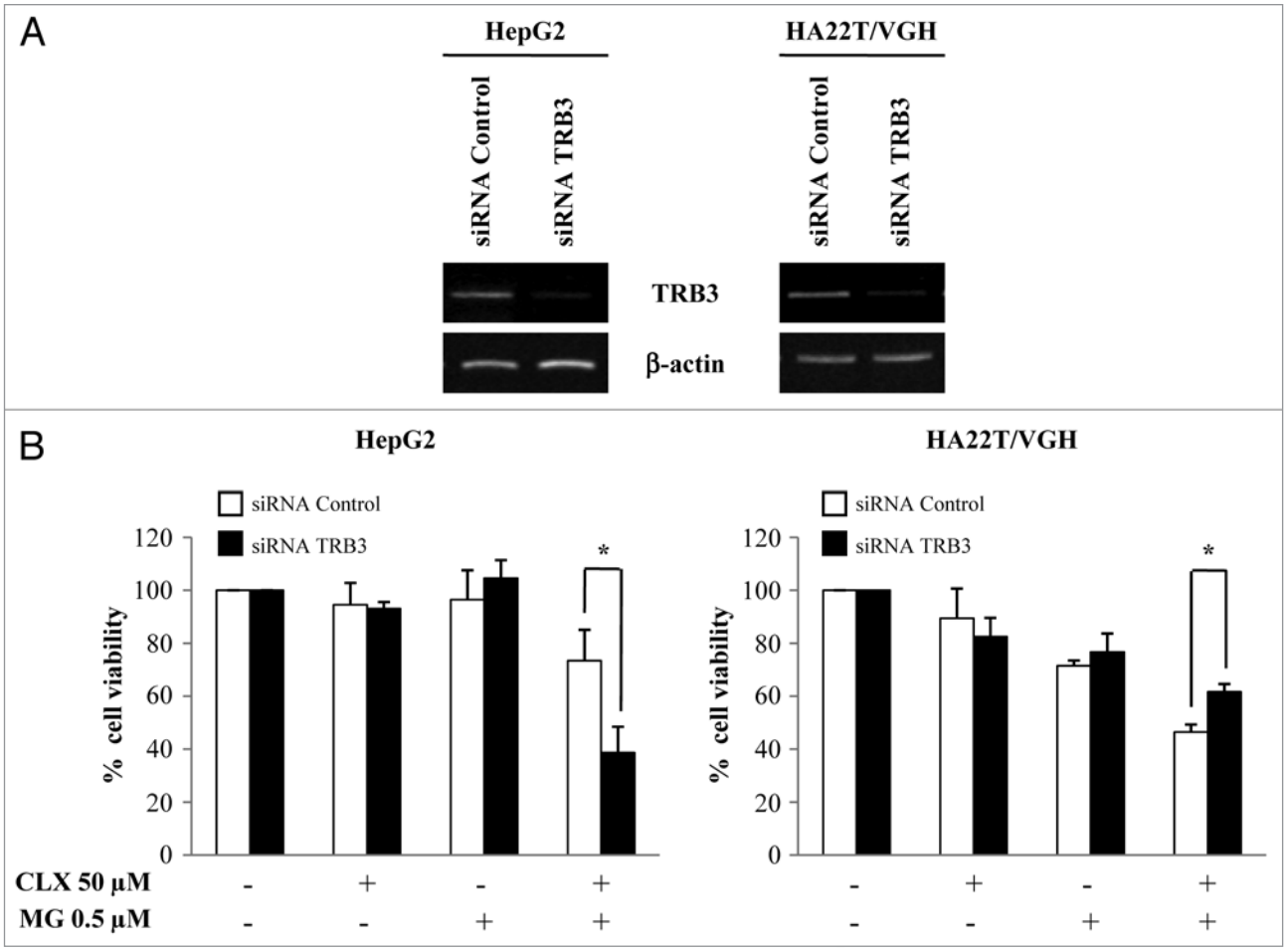

Figure 6. TRB3 siRNA transfection has different effects on cell viability. (A) Cells were transfected with a control siRNA (siRNA Control) or TRB3-selective siRNA (siRNA TRB3). 96 h after transfection TRB3 mRNA expression was analyzed by RT-PCR. (B) Cells were transfected with a control siRNA or TRB3-selective siRNA. 72 h after transfection, cells were treated or untreated for 24 h with CLX and MG alone or in combination and then cell viability was determined by MTS assays. Results are expressed as the percentage of cell viability relative to the vehicle-treated cells and are the means ± SD of three separate experiments, each of which was performed in triplicate. *$p < 0.05$.

for hepatocarcinogenesis.[37] XBP1 mRNA splicing, resulting in the activation of the XBP1 product, occur in HCC tissues with increased histological grading, while its elevation is not observed in non-cancerous livers or in well-differentiated HCC tissues.[37] Moreover, more aggressive HCC tumors appear to harbor chronic ER stress, as shown by elevated expression levels of ER stress response markers. Indeed, most tumor cells appear to harbor chronic ER stress. Chronic exposure to a mild stress can lead to adaptation, which supports cell survival within a hostile tumor microenvironment and also contributes to the chemoresistance of cancer cells. However, the type of ESR has been demonstrated to be dependent on the duration and strength of the stress state. Indeed, if ER stress becomes intense and persistent, its protective function is overwhelmed and its pro-apototic function becomes dominant, thus initiating cell death.[38] Overall, ESR can be seen as an "yin-yang" principle that regulates cell survival versus cell death.[29]

Since ESR is one of the major mechanisms mediating apoptotic cell death, we investigated the effects of treatment with drugs, used either alone or in combination, on ESR induction. Our results showed that combination induces synergistic ER stress response in HCC cells, although there were some differences between the two cell lines investigated.

We demonstrated that treatment with a combination synergistically promotes CHOP upregulation, and also splicing of XBP1 mRNA in both cell lines. CHOP activity represses the promoter of the *bcl-2* gene, thus downregulating the anti-apoptotic protein Bcl-2.[39] Accordingly, we found that Bcl-2 protein is downregulated in combination-treated cells. This result indicates that downregulation of the anti-apoptotic proteins Bcl-2 might be involved in combination-induced cell death.

The inducible ER stress gene TRB3 is a downstream target of CHOP, and it is induced via the ATF4–CHOP pathway.[28] Studies indicate that TRB3 is also involved in many biological processes, including insulin resistance (IR), and the regulation of cell growth and differentiation. However, the role of TRB3 in apoptosis is controversial. Recent results showed that the pro-apoptotic and tumor growth-inhibiting activity of cannabinoids relies on upregulation of TRB3,[40] whereas others have shown that TRB3 protects cells from the apoptosis triggered by

ATF4 overexpression.[41] Therefore, in certain conditions endogenous TRB3 can act as a proapoptotic or as a prosurvival protein. Our results demonstrated that knockdown of TRB3 in HA22T/VGH cells significantly reverted combination-induced cell growth inhibition, whereas it increased combination-induced cell death in HepG2 cells, suggesting that ESR activation might have either a detrimental or a protective role in HCC cell survival. These results might be explained by the fact that in our study we used two human HCC cell lines that have different characteristics of differentiation, biological behavior and genetic defects,[42] and therefore the role of TRB3 could be dependent on various cellular features. In addition, ESR has been demonstrated to be dependent on the duration and strength of the stress state, and our results indicated that ESR is activated earlier and with lower combination doses in the poorly-differentiated HA22T/VGH cells than in the well-differentiated HepG2 cells, therefore suggesting a different threshold level of ESR in the two cell lines. HepG2 cells seem to be less responsive to stress-induced cell death in combination treatment. This might be due to the upregulation of TRB3 under combination conditions, which to some extent might counteract, as reported by others,[41] the proapoptotic effect triggered by ATF4 overexpression observed in these cells after combination-induced ER stress induction. However, this is a futile attempt by the cells because the proapoptotic signaling pathway and the tumor-growth inhibiting activity become predominant.

In summary, our results indicate for the first time that combination treatment with CLX and MG results in synergistic antiproliferative and proapoptotic effects against liver cancer cells, providing a rational basis for the clinical use of this combination in the treatment of HCC.

Materials and Methods

Reagents and cell culture. MG132 and SB203580 were purchased from Alexis Biochemical (Lausen, CH). SP600125 was purchased from Calbiochem (Milan, Italy). The COX-2 inhibitor celecoxib was a gift of Pfizer Corporation (New York, USA). All reagents were dissolved in dimethyl sulfoxide (DMSO). HepG2 and HA22T/VGH cell lines used in this study were of a low passage number and were maintained as previously described.[43] The two cell lines have different characteristics of differentiation, biological behavior and genetic defects.[42] In particular, expression of COX-2 mRNA is undetectable in HepG2 cells, whereas its expression is very strong in HA22T/VGH cells.[5]

Evaluation of cell viability by MTS assay. Cells (5 x 10^3/well HA22T/VGH and 2 x 10^4/well HepG2) in complete medium, containing 10% (v/v) Fetal Bovine Serum (FBS), were distributed into each well of 96-well microtiter plates and then incubated overnight. For combined treatment, cells were treated with COX-2 inhibitor celecoxib and with proteasome inhibitor MG132 for the indicated time. At the end of treatment with various concentrations of the inhibitors, MTS assays were performed with the CellTiter Aqueous OneSolution kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Cytotoxicity was expressed as a percentage of the absorbance measured in the control cells. Values were expressed as means ± standard deviation (SD) of three separate experiments, each performed in triplicate.

Evaluation of apoptosis by flow cytometry. After 24 h of treatment, cells were collected and prepared as a single cell suspension by trypsinization, and stained with propidium iodide (Invitrogen Carlsbad, CA) and the percentage of apoptotic cells was determined as described previously.[16]

Caspase activity assays. Cells (2 x 10^4/well) were treated with the test drugs during incubation in 96-well plates containing complete culture medium. After 24 h, the levels of caspase activity in the cells were measured by the Caspase-Glo® 3/7 assay (Promega, Milan, Italy) according to the manufacturer's instructions. Results were expressed as fold induction over the control. Values were expressed as means ± SD of two separate experiments, each performed in duplicate.

Western blot analysis. At the time of harvest, cell lysates were obtained and western blotting performed as described previously,[16] with primary antibodies raised against β-actin (Sigma-Aldrich Srl., Milan, Italy), Bcl-2, PARP, JNK1/2 (p46/54), phospho-JNK1/2 (p46/54), p38, phospho-p38, ATF2, phospho-ATF2, c-Jun (which detects 43/48 kDa bands) and phospho-c-Jun (all from Cell Signaling Technologies Inc., Beverly, MA, USA).

siRNA transfection. HA22T/VGH cells (2 x 10^5) and HepG2 cells (5 x 10^5) were transfected with TRB3 and Non-Correlated (NC) siRNA SMARTPool ON-Target plus (Dharmacon Inc., Lafayette, CO, USA) according to the manufacturer's instructions. In brief, cells were seeded onto 60 mm dishes in medium without antibiotics, and 24 h later the transfection of siRNAs was carried out with DharmaFECT 4 (Dharmacon Inc.). All transfections were carried out with 20 μM duplex siRNA in medium without FBS and antibiotics. After 48 h, cells were split into 96-well plates to perform MTS assays and into 35 mm dishes for TRB3 mRNA expression analysis.

Reverse transcription-PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) as recommended by the supplier. Reverse transcriptase-polymerase chain reaction (RT-PCR) was then performed using the Superscript One-step RT-PCR kit (Invitrogen, Carlsbad, CA). The amount of RNA amplified was quantified and equalized using primers to β-actin as an internal control. The following sense and antisense primers were used, respectively, to amplify human XBP1 (5'-CCT TGT AGT TGA GAA CCA GG-3' and 5'-GGG GCT TGG TAT ATA TGT GG-3'; 441-bp product), human TRB3 (5'-GCC ACT GCC TCC CGT CTT G-3' and 5'-GCT GCC TTG CCC GAG TAT GA-3'; 538-bp product) and human β-actin (5'-CAC CAC ACC TTC TAC AAT GAG C-3' and 5'-GAG GAT CTT CAT GAG GTA GTC AGT C-3'; 322-bp product). PCR reactions were performed using the following parameters: 95°C for 5 min, 94°C for 30 sec, 57°C for XBP1, 60°C for β-actin and 52°C for TRB3 for 30 sec, and 72°C for 1 min followed by a final extension step of 72°C for 8 min. The number of cycles (35 cycles for XBP1, 30 cycles for TRB3, 25 cycles for β-actin) was adjusted to allow detection in the linear range. Finally, PCR products were analyzed by electrophoresis on agarose gel and photographed.

Quantitative RT-PCR analysis. First-strand cDNA was synthesized using ImProm-II Reverse Transcription System (Promega, Madison, USA). Quantitative PCR was performed using Roche Light Cycler system and reagents following instructions of the manufacturer. PCR reaction mixes were assembled using the SYBR Green Premix Ex Taq (Perfect Real Time) (Takara Bio Inc., Japan). Each sample was analyzed in duplicate and the experiment was repeated twice. Results were analyzed using RelQuant (Roche Diagnostics, Mannheim, Germany) and expressed as a ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5'-GGG AAG CTC ACT GGC ATG GCC TTC C-3' and 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'; 322-bp product) of control values (untreated cells).

Primers used and conditions of annealing were: human ATF4 5'-TGG GGA AAG GGG AAG AGG TTG TAA-3' and 5'-AGT CGG GTT TGG GGG CTG AAG-3', 436-bp product, 57°C for 10 sec; TRB3 5'-GCC ACT GCC TCC CGT CTT G-3' and 5'-GCT GCC TTG CCC GAG TAT GA-3', 538-bp product, 50°C for 6 sec; CHOP 5'-GCG TCT AGA ATG GCA GCT GAG TCA TTG CC-3' and 5'-GCG TCT AGA TCA TGC TTG GTG CAG ATT C-3', 509-bp product, 57°C for 10 sec.

Statistical analysis. Statistical analysis was performed using Student's two-tailed t test. The criterion for statistical significance was $p < 0.05$. For the synergistic activity, data were analyzed using CalcuSyn software version 2.0 (Biosoft, Cambridge, UK) to determine if the combination of celecoxib and MG132 was additive or synergistic. When CI = 1, effects were additive. When CI < 1.0, effects were synergistic. CI < 0.1 indicates very strong synergism as defined by the CalcuSyn manual.

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