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Response to Reviewers: Reviewer 2: We have modified the description of the results presented in the Figure 3A and discussed these apparent inconsistencies in the Discussion.

In our experiments, the failure to detect caspase activation, even in the presence of PARP cleavage in the Huh-6 cell line, may not result from the experimental time periods, since we have tested caspases activation from 6 hours to 48 hours with comparable results. Moreover, we think that the caspase activation, which is barely detectable in the Huh-6 cell line may not result from methodical limitations or possible interference of the drugs on caspase activity since we have already successfully measured caspase activation after DHMEQ alone and DHMEQ-CLX treatment in the HA22T/VGH cell line. However, we agree with the referee that it could result from caspase-independent apoptosis in the Huh-6 cell line. Therefore, now we have added two more references suggesting that this possibility may occur in liver cells.

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The novel NF-κB inhibitor DHMEQ synergizes with Celecoxib to exert antitumor effects on human liver cancer cells by a ROS-dependent mechanism.

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Running Title: DHMEQ synergizes with Celecoxib to induce apoptosis in HCC

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Abstract

In a previous work of ours dehydroxymethylepoxyquinomicin (DHMEQ), an inhibitor of NF-κB, was shown to induce apoptosis through Reactive Oxygen Species (ROS) production in hepatoma cells. The present study demonstrated that DHMEQ cooperates with Celecoxib (CLX) to decrease NF-κB DNA binding and to inhibit cell growth and proliferation more effectively than treatment with these single agents alone in the hepatoma cell lines HA22T/VGH and Huh-6. ROS production induced by the DHMEQ-CLX combination in turn generated the expression of genes involved in endoplasmic reticulum (ER) stress and silencing TRB3 mRNA significantly decreased DHMEQ-CLX-induced cell growth inhibition. Moreover, the DHMEQ-CLX combination was associated with ~~an increase in caspase activation,~~ induction of PARP cleavage and down-regulation of the anti-apoptotic proteins Bcl-2, Mcl-1 and survivin, as well as activated Akt. CD95 and CD95 ligand expression increased synergistically in the combination treatment, which was reversed in the presence of NAC. Knockdown of CD95 mRNA expression significantly decreased DHMEQ-CLX-induced cell growth inhibition in both cell lines. These data suggest that the DHMEQ-CLX combination kills hepatoma cells via ROS production, ER stress response and the activation of intrinsic and extrinsic apoptotic pathways.

Keywords: DHMEQ, Celecoxib, NF-κB, CD95/CD95L, liver cancer cells

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Several strategies have been suggested for the treatment of HCC patients but, unfortunately, it still has a high lethality. Therefore, novel approaches are required to contrast this tumor

The nuclear transcription factor-κB (NF-κB) has been implicated in carcinogenesis because it plays a critical role in cell survival, inflammation and cell growth. Recent studies indicate that NF-κB is essential for promoting inflammation-associated cancers and it is therefore a potential target for cancer prevention [1]. Several reports have indicated that NF-κB is constitutively activated in a variety of cancer cells, including hepatocellular carcinoma [2].

Dehydroxymethylepoxyquinomicin (DHMEQ) is a novel NF-κB inhibitor which induces apoptosis and cell-cycle arrest in several cancer cell types [3-5]. We previously demonstrated that DHMEQ promotes ROS generation in human liver cells and that oxidative stress induces ER stress response, DNA damage and release of cytochrome *c* with activation of the caspase cascade [6].

There are two isoforms of cyclooxygenases (COXs), COX-1 and COX-2, the latter being induced by a variety of stimuli. COX-2 is markedly elevated in many types of tumour, including HCC, as selective COX-2 inhibitors (COXIBs) show anti-proliferative and pro-apoptotic effects in human liver cancer cells [7,8] suggesting that COXIBs might be effective in HCC treatment. Accumulating evidence suggests that COXIBs inhibit cell proliferation through a COX-2-independent mechanism [9-11]. The molecular mechanism underlying CLX-mediated apoptosis seems to be associated with the induction of ER stress response through calcium [12] and with the down-regulation of the anti-apoptotic protein survivin [13]. CLX induces the expression of functional death receptors, such as CD95, and a rapid down-regulation of myeloid cell leukemia-1 (Mcl-1) protein, suggesting the activation of intrinsic and extrinsic apoptosis pathways in HCC [14]. CLX has been reported to act synergistically with different drugs in promoting the apoptosis of human liver tumor cells [15]. There

are two different apoptotic pathways: death receptor and mitochondrial [16,17]. Ligation of cell surface death receptors, including CD95 with its specific ligand, triggers a death receptor apoptotic pathway. Fas-associated Death Domain (FADD) is first recruited to the death receptor, followed by the association with pro-caspase 8, which is activated by cleavage. Activation of the mitochondrial apoptotic pathway depends on the release of cytochrome *c* and consequently the activation of caspase-3/7, -9, which in turn cleaves PARP and inhibits anti-apoptotic proteins, such as survivin. Cytotoxic drugs that cause DNA damage either trigger cell death mediated by mitochondria or induce apoptosis in a CD95-dependent manner. The exact mechanisms by which the two cascades are simultaneously activated by cytotoxic drugs are not known. However, it has been reported that the two death pathways may cross-talk, depending on the cell type or stimuli. Cross-talk may depend on Bid protein cleavage, which transduces an apoptotic signal from the cytoplasmic membrane to mitochondria [18] and also on ROS production in mitochondria, which in turn induces the release of cytochrome *c* and CD95 aggregation and activation of the FADD-caspase-8 cascade [19].

Therefore, the present study was set up to investigate whether CLX can potentiate the antitumor effects of DHMEQ against human liver cancer cells. We observed synergistic antitumor effects with the DHMEQ-CLX combination. Moreover, we demonstrated that DHMEQ and CLX interact to increase the activity of the intrinsic and extrinsic apoptotic pathways through CD95 activation and down-regulation of Mcl-1. These pro-apoptotic effects are mediated by the production of ROS, resulting in ER stress response.

2. Materials and Methods

2.1 Reagents and cell culture

DHMEQ was synthesized as previously described [20]. NAC was purchased from Sigma-Aldrich (Milan, Italy). Celecoxib was a gift from the Pfizer Corporation (New York, USA) and was dissolved in dimethyl sulfoxide (DMSO). The human liver cancer cell lines Huh-6, HA22T/VGH and Huh-7 used in this study were a gift from Prof. Massimo Levrero (Department of Internal Medicine, Sapienza University, Rome, Italy). They have different characteristics of differentiation, biological behaviour and genetic defects [21]. Cells were maintained as previously described [6].

2.2 Cell growth and BrdU incorporation assays

MTS assays were performed using the CellTiter Aqueous OneSolution kit (Promega Corporation, Madison, WI, USA) as previously reported [6]. Cell proliferation assays were performed using a colorimetric immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) as previously reported [6]. Data were expressed as the percentage of control cells and were the means ± SD of three separate experiments, each of which was performed in triplicate.

2.3 NF-κB p65 transcription factor assay

Cytoplasm and nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Belgium) according to the manufacturer's instructions. The DNA-binding capacity of NF-κB (p65 subunit) was measured using the Trans-AM^{TM} kit (Active Motif, Belgium) as previously reported [6].

2.4 Detection of apoptosis

The DeadEnd Colorimetric TUNEL System technique was performed as suggested by the supplier (Promega). The number of apoptotic cells was determined by counting the percentage of brown-colour

positive cells. At least 100 cells from two different cell preparations were counted for each condition. Cells were visualized with an Axioskop microscope (Zeiss, Germany).

2.5 Western blot analysis

Whole cellular lysates were obtained using RIPA buffer (Cell Signaling Technologies Inc., Beverly, MA, USA) and western blotting was performed as previously described [21], with primary antibodies raised against survivin (Abcam Limited, Cambridge, UK), β-actin (Sigma-Aldrich Srl, Milan, Italy), PARP, phospho-Akt, Bcl-2 (Cell Signaling Technologies Inc., Beverly, MA, USA), CD95 and Mcl-1 (Santa Cruz Biotechnology Inc, Heidelberg, Germany).

2.6 Caspase activity assays

Caspase activities were measured by the Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 assays (Promega, Milan, Italy) as previously reported [6].

2.7 Semiquantitative-PCR (sq-PCR) and quantitative Real Time PCR (q-PCR)

Total RNA was isolated using TryRYzol reagent (Invitrogen Carlsbad, CA) as recommended by the supplier. Semiquantitative PCR was performed using specific 5' and 3' primers which generated specific bands (Table I). The expression of selected genes was quantified by quantitative Real Time PCR (q-PCR) using SYBR Green fluorescence (Qiagen, Milan, Italy) on StepOnePlus (Applied Biosystem, Carlsbad, CA, USA). Primers were purchased from Qiagen (Milan, Italy) and amplified as recommended. Relative expression was calculated using the comparative C_t method. Expression of the gene of interest was expressed as fold induction compared with the control (DMSO) and was corrected with the quantified expression level of β-actin. The results shown were the means ± SD of two experiments, each performed in triplicate.

2.8 siRNA transfection

HA22T/VGH and Huh-6 cells were transfected with CD95 small interfering RNA (siRNA), TRB3 siRNA and Non Correlated (NC) siRNA (Qiagen) as previously reported [6]. 72 hours after transfection, cells were left untreated or treated for 24 hours with DHMEQ and CLX either alone or in combination, then cell growth was determined by MTS assays.

2.9 Evaluation of Reactive Oxygen Species production

The intracellular accumulation of ROS was evaluated using the fluorescent probe 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCFDA) (Invitrogen Carlsbad, CA, USA; cat. n. C-400) and observed under a fluorescence microscope.

2.10 Statistical analysis

Statistical analysis was performed using Student's T test (two-tailed). The criteria for statistical significance was $p<0.05$. Drug combination studies were designed according to Chou and Talalay [22]. CalcuSyn software (Biosoft) was used to calculate the combination index (CI). CI < 1 indicated synergy, a CI about 1 indicated an additive effect and CI > 1 indicated antagonism.

3. Results

3.1 DHMEQ-CLX combination synergistically inhibits cell growth, NF-κB p65 DNA-binding capacity, and cell proliferation.

DHMEQ has been reported to reduce NF-κB p65 DNA-binding capacity and cell growth in human liver cancer cells [6]. There is increasing evidence that CLX has an anti-cancer activity, associated with its ability to decrease cell survival in HCC [14]. Moreover, it has been reported that the anti-inflammatory activity of CLX depends on the inhibition of NF-κB p65 activation and translocation into the nucleus [10,23].

To determine the potential of DHMEQ to inhibit cell growth, we examined its effects on three human liver cancer-cell lines by MTS assay. After 72 hours of exposure to different concentrations of DHMEQ the IC_{50} values were 12.5 μg/ml ± 2.5 in Huh-6 cells, 32.5 μg/ml ± 2.8 in HA22T/VGH cells and 29.8 μg/ml ± 2.3 in Huh-7 cells, respectively. HA22T/VGH cells were the least sensitive and Huh-6 cells the most sensitive to DHMEQ treatment. The MTS assay was also utilized to assess the effects of DHMEQ-CLX combination on cell growth. Cells were exposed to increasing concentrations of DHMEQ in the presence of CLX (50, 70 μM) for different time periods. After 24, 48 (data not shown) and 72 hours (Fig. 1A) the dose-response curve demonstrated that the combination of DHMEQ and CLX suppressed cell growth significantly more than when each drug was used individually. Synergistic growth inhibition following DHMEQ and CLX combination treatment occurred in HA22T/VGH and Huh-6 cell lines (Fig. 1A). A calculated CI value of 0.8 was found for DHMEQ 5 μg/ml in Huh-6 cells combined with CLX 70 μM, while a value of 0.7 was found for DHMEQ 10, 20 μg/ml in Huh-6 and 30, 40 μg/ml in HA22T/VGH cells combined with CLX 70 μM.

Therefore, we focused on the ability of CLX to potentiate the anti-cancer mechanisms of DHMEQ in HA22T/VGH and Huh-6 cells as resistant and sensitive models, respectively.

First, we tested the effects of the DHMEQ-CLX combination on NF-κB p65 transcriptional activity. Figure 1B shows that combination significantly decreased the nuclear localized p65 subunit more than either of the drugs used alone in both cell lines. Moreover, the DHMEQ-CLX combination dose-dependently decreased the amount of the active form of p65 subunit contained in nuclear extracts in both cell lines (data not shown).

The BrdU assay was performed to study the effects of the combination treatment on cell proliferation. In Figure 1C the results show that the DHMEQ-CLX combination affected cell proliferation in both cell lines significantly more than when each drug was used individually.

Taken together, these results suggest that CLX potentiated the anti-proliferative effects caused by DHMEQ in both cell lines, through NF-κB inhibition.

3.2 CLX potentiates DHMEQ-induced apoptosis.

Suppression of NF-κB inhibits the growth of tumor cells, therefore we evaluated apoptosis in liver cancer cells treated with DHMEQ and CLX alone or in combination using the TUNEL assay (Fig. 2A). Significantly higher dose-dependent increases in the percentage of apoptosis were observed in cells treated with DHMEQ-CLX than with DHMEQ alone in both cell lines (Fig. 2B). To understand the mechanism by which the combination induced apoptosis in liver cancer cells we determined the effects of DHMEQ-CLX on the activation of different caspases. Caspase-8, -9 and the effectors caspase-3/7 were activated by combination drug treatments in HA22T/VGH cells, although caspase activities were not significantly different than when DHMEQ was used alone~~in both cell lines, suggesting the activation of extrinsic and intrinsic apoptotic pathways~~ (Fig. 3A). Instead, in Huh-6 cells caspase activities were barely detectable. Staurosporin (500 nM) was used as a positive control for caspase 3/7 activation, resulting in 3.7 ± 0.8 and 3.0 ± 0.3 fold inductions in HA22T/VGH and Huh-6 cells respectively (data not shown). As NF-κB is known to induce the expression of anti-apoptotic proteins

[24], we determined the levels of the anti-apoptotic proteins survivin and Bcl-2 as target genes of NF-κB, as well as the cleavage of PARP, which is a known caspase-3/7 substrate and a biochemical marker of apoptosis. Treatment with the DHMEQ-CLX combination significantly reduced the levels of survivin and Bcl-2 proteins in both cell lines, and PARP was more effectively cleaved in response to combination treatment than by individual drug treatments (Fig. 3B). Caspase activity is regulated by multiple proteins, including the Bcl-2 family member~~s~~ Mcl-1. Mcl-1 protects cells from apoptosis induction through the blockage of cytochrome *c* release from mitochondria by interacting with pro-apoptotic members of the Bcl-2 family [25,26]. Therefore, we analysed protein levels of Mcl-1 and observed the down-regulation of Mcl-1 protein levels in DHMEQ-CLX-treated cells (Fig. 3C).

COX-2 has been shown to promote survival by up-regulating the level of Mcl-1 through the PI3K/Akt pathway [27]. Therefore, to determine the role of this signaling pathway in DHMEQ-CLX-induced apoptosis, the expression level of phospho-Akt protein was analysed in HA22T/VGH cells by western blotting. As shown in Figure 3C, phosphorylation of Akt protein was down-regulated on combination treatment.

Taken all together these data indicate that the DHMEQ-CLX combination induced events associated with both extrinsic and intrinsic apoptotic pathways in each cell line, which correlated with the down-regulation of mitochondrial anti-apoptotic proteins and Akt signaling.

3.3 Combination of DHMEQ and CLX triggers the extrinsic apoptotic pathway.

CLX has been reported to trigger the extrinsic apoptotic pathway through the increased expression of CD95, with a down-regulation of Mcl-1 in HepG2 and Huh-7 cells [14]. Since we observed that caspase-8 was activated after ~~combination~~ DHMEQ treatment, we investigated the expression of CD95 and CD95L to further dissect the signaling pathways involved in DHMEQ-CLX-induced apoptosis.

q-PCR revealed a significant up-regulation of CD95 mRNA on combination treatment (Fig. 4A) and sq-PCR showed an induced expression of CD95L mRNA in DHMEQ and DHMEQ-CLX treated cells (Fig. 4B). CD95 protein levels were analysed by western blotting (Fig. 4C) and were significantly enhanced on combination treatment in both cell lines. These results suggest that the DHMEQ-CLX combination induces apoptosis through involvement of the CD95/CD95L extrinsic apoptotic pathway.

3.4 CD95 siRNA transfection significantly reverts DHMEQ-CLX-induced cell growth inhibition.

To examine the role of CD95 in DHMEQ-CLX induced cell growth inhibition, HA22T/VGH and Huh-6 cells were first transiently transfected with CD95 siRNA for 72 hours and then MTS assays were performed. The transfection of CD95 siRNA into HA22T/VGH and Huh-6 cells effectively reduced endogenous mRNA by 80% more than the control siRNA (Fig. 4). As shown in Figure 4D knockdown of CD95 significantly reverted the inhibition of cell growth induced by DHMEQ-CLX treatment in both cell lines. These results suggest that activation of extrinsic apoptotic pathway, through CD95 up-regulation, contributes to DHMEQ-CLX-induced cell growth inhibition.

3.5 DHMEQ-CLX combination triggers ER stress response.

CLX triggers ER stress through elevated levels of the ER stress indicators [28]. DHMEQ induces the expression of CHOP and TRB3 mRNAs and the splicing of XBP1 mRNA, which are essential for apoptosis through ROS generation [6]. Therefore, we investigated the potential contribution of ER stress to the synergy observed between DHMEQ and CLX. As shown in Figure 5A, individual drug treatments resulted in the splicing of XBP1 mRNA. Furthermore, a higher dose of the DHMEQ-CLX combination induced the complete splicing of XBP1 mRNA (Fig. 5A). Moreover, the combination treatment decreased GRP78 mRNA, indicating an aggravated ER stress response. q-PCR results showed that CHOP and TRB3 mRNAs were significantly up-regulated after combination

treatment in both cell lines (Fig. 5B). These results indicated that the DHMEQ-CLX combination better induces increased ER stress response than treatment with either of the drugs alone.

3.6 TRB3 siRNA transfection significantly reverts DHMEQ-CLX-induced cell growth inhibition.

TRB3, a crucial factor mediating ER stress response, is a transcriptional target of CHOP and is involved in CHOP-dependent cell death as a second messenger during ER stress [29]. To examine the role of TRB3 in DHMEQ-CLX induced cell growth inhibition, HA22T/VGH cells were first transiently transfected with TRB3 siRNA for 72 hours and then MTS assays were performed. Transfection of TRB3 siRNAs into HA22T/VGH cells effectively reduced the endogenous mRNA by more than 90% more than the control siRNA (Fig. 5C left). As shown in Figure 5C (right) knockdown of TRB3 significantly reverted the inhibition of cell growth induced by DHMEQ-CLX treatment. These results suggest that activation of ER stress response through TRB3 mRNA up-regulation contributes to DHMEQ-CLX-induced cell growth inhibition.

3.7 Effects of DHMEQ and CLX combination on oxidative stress.

Under oxidative stress ROS are produced. High levels of ROS induce cell death, which often involves apoptosis through caspase activation. DHMEQ dose-dependently induces ROS generation by mitochondria in HA22T/VGH cells [6]. Therefore, we investigated the possible role of combination treatment on ROS production. Cells were treated with each drug alone or with the DHMEQ-CLX combination for 24 hours and thereafter ROS were evaluated using the cell-permeable fluorescent probe, H_2DCFDA. Treatment of HA22T/VGH cells with drug combinations increased ROS generation to a greater extent than treatment with either drug by itself (Fig. 6). These results indicated that the drug combination induces ROS production.

3.8 The ROS scavenger NAC prevents DHMEQ-CLX-induced cell growth inhibition, ER stress response and CD95/CD95L expression.

To determine whether DHMEQ-CLX induces apoptosis via ROS generation, we tested the effects of NAC on cell growth, on ER stress response and on the activation of the extrinsic apoptotic pathway in both cell lines. For these purposes, cells were pre-treated with NAC (2 mM) for 2 hours and subsequently, in the presence of NAC, treated with different concentrations of DHMEQ-CLX for 24 additional hours. In HA22T/VGH cells NAC significantly decreased the DHMEQ-CLX-induced inhibition of cell viability, whereas in Huh-6 cells NAC completely abrogated the DHMEQ-CLX-induced reduction in cell viability, leading to a cell viability which was not different from untreated controls (Fig. 7A). In addition, cell morphology in both cell lines was observed after 24 hours of DHMEQ-CLX treatment by light microscopy with or without the presence of NAC (Fig. 7B). As shown in Figure 7B, in the presence of NAC there was a reduction in the numbers of floating and shrunk cells, suggesting a recovery of cell vitality.

To examine the relationship between ER stress response and oxidative stress after DHMEQ-CLX treatment, the induction of CHOP and TRB3 mRNAs, as well as the splicing of XBP1 mRNA were examined in the presence of NAC. Treatment with NAC abrogated the induction of CHOP and TRB3 mRNAs and XBP1 mRNA splicing in HA22T/VGH cells (Fig. 7C). Finally, pre-treatment of the cells with NAC significantly blocked the DHMEQ-CLX-induced expression of CD95 (Fig. 7D left) and CD95L mRNAs (Fig.7D right) in both cell lines.

Taken together, these results indicate that the oxidative stress induced by the combination treatment is able to induce cell growth reduction in liver cancer cell lines and that ER stress response and CD95/CD95L extrinsic pathway activation cause apoptosis downstream of the oxidative stress induced by DHMEQ-CLX treatment .

4. Discussion

NF-κB is a major stress-inducible anti-apoptotic transcription factor [24] which is frequently activated in many types of cancer, including HCC [2]. The NF-κB inhibitor DHMEQ has been reported to induce apoptosis in liver cancer cells through the induction of oxidative stress, mainly via the mitochondrial pathway [6].

The tumorigenic potential of COX-2 over-expression has frequently been associated with resistance to apoptosis in certain types of tumors, including HCC. Celecoxib, a selective COX-2 inhibitor, induces antitumor effects in HCC [7,10] and inhibits NF-κB nuclear translocation [10,23]. ROS interact with NF-κB signaling pathways in many ways. In fact, the transcription of NF-κB-dependent genes influences the levels of ROS in the cell, and in turn, the levels of NF-κB activity are also regulated by ROS levels. Depending on the context, ROS can both activate and inhibit NF-κB signaling [30]. Interestingly, transformed cells are thought to use ROS signals to stimulate both cell proliferation and tumor progression. However, high levels of ROS beyond a certain threshold increase the vulnerability of cancer cells to undergo apoptosis. Thus, agents that either promote ROS generation or decrease the levels of antioxidants have the potential to kill cancer cells with little effect on normal cells [31]. Combinations of drugs for the treatment of tumors have been suggested as a promising therapeutic strategy, both to maximize pharmaceutical efficacy and to minimize the adverse events associated with the individual components. Our studies have demonstrated that the NF-κB inhibitor DHMEQ in combination with CLX causes apoptosis in human liver cancer cells through the induction of oxidative stress. In this study we presented data indicating that ROS induction is crucial in the DHMEQ-CLX-mediated anti-cancer effects. In fact, the drug combination increased ROS generation 24 hours after exposure, more than either drugs used alone. Different cancer cells possess different basal levels of ROS and endogenous scavengers. More aggressive cells possess higher levels of endogenous ROS and need these elevated levels of ROS to undergo apoptosis [32]. We observed that

HA22T/VGH and Huh-6 cells have different sensitivities to the DHMEQ-CLX combination treatment, likely due to different basal levels of ROS, with higher threshold levels required in HA22T/VGH cells to undergo apoptosis. Consequently, pre-treatment with NAC, a glutathione (GSH) precursor, only partially reverts the effect of DHMEQ-CLX in HA22T/VGH cells, whereas in Huh-6 cells NAC completely reverts cells growth inhibition.

Oxidative stress may activate an ER stress response which, through up-regulation of ER stress genes *CHOP* and *TRB3* and XBP1 mRNA splicing, can promote apoptosis [33]. ER stress response is a major mechanism that mediates apoptotic cell death and is used as an anti-cancer strategy [34]. Our results showed that the DHMEQ-CLX combination induces a significant ER stress response and that this response is an event downstream of the oxidative stress in human liver cancer cells. These results are in accordance with other authors who reported that ER stress response could be induced through ROS generation [35] and with our previous results with DHMEQ treatment in liver cancer cells [6]. ER stress response starts with GRP78 mRNA induction, which is a survival signal [36]. We observed that GRP78 was inhibited by the DHMEQ-CLX combination in HA22T/VGH cells, but not in Huh-6 cells, much more than when the drugs were used individually. Suppression of GRP78 can also promote ROS generation and activates the extrinsic apoptotic pathway in HCC cells [37]. Moreover, we observed that the DHMEQ-CLX combination promoted significant CHOP and TRB3 mRNAs induction as well as splicing of XBP1 mRNA in a ROS-dependent manner. Furthermore knockdown of TRB3 in HA22T/VGH cells significantly reverted DHMEQ-CLX induced cell growth inhibition. At present it is not clear how ER stress response induces apoptosis in HCC. The caspase-8 pathway has been reported to induce ER stress response and this is associated with a release of cytochrome *c* from mitochondria in human liver cancer cells [38]. We recently demonstrated that DHMEQ induces the release of cytochrome *c* from mitochondria and caspase activation [6]. In the present study we documented the activation of caspase-9 and caspase-8 by DHMEQ-CLX combination treatment in HA22T/VGH cells

but not in Huh-6 cells. ~~In addition, activation of the intrinsic apoptotic pathway became evident by the activation of the effector caspases-3/7 and by~~ However, we observed the cleavage of PARP, a well-known caspase-3/7 target in both cell lines. Indeed it has been previously demonstrated that during apoptosis, PARP cleavage could be an event either dependent or independent of caspase activation depending on the cell type and/or apoptotic stimuli [39,40]. Moreover, ~~. Moreover~~in our system~~,~~ the levels of survivin and Bcl-2, two anti-apoptotic proteins, were reduced by DHMEQ-CLX treatment. Mitochondrial integrity is regulated by pro-apoptotic and anti-apoptotic members of the Bcl-2 family, such as Mcl-1. Mcl-1 protects cells from apoptosis induction through the blockage of cytochrome *c* release from mitochondria by interacting with the pro-apoptotic Bcl-2 family member [25,26]. Our results showed that Mcl-1 protein is down-regulated after DHMEQ-CLX treatment, in accordance with previous studies demonstrating a decrease in Mcl-1 in HCC cells treated with CLX [14]. Mcl-1 basal expression is dependent on a functional phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway, and treatment of HCC cells with a specific PI3 kinase inhibitor leads to both decreased Mcl-1 expression and a sensitization towards chemotherapeutic drug-induced apoptosis [~~39~~41]. In the present study, DHMEQ and CLX individually attenuated the presence of the activated form of Akt, which was further abrogated by the combination. These results are in agreement with previous studies [4~~0~~2,4~~1~~3] and suggest that Akt inhibition may contribute to the antitumor effect of CLX alone or in combination with other drugs. Recent reports have indicated that CLX results in CD95 up-regulation and Mcl-1 down-regulation and this is a critical checkpoint at which the extrinsic and the intrinsic apoptotic pathways converge in HCC [14]. Moreover, ROS generation can increase CD95 activation in HCC [4~~2~~4] and our previous results ~~showed~~ demonstrated that DHMEQ induces the activation of an intrinsic death pathway in a ROS-dependent manner, with down-regulation of survivin and release of cytochrome *c* in human liver cancer cells [6]. In the present study, we ~~demonstrated~~ document that the DHMEQ-CLX combination treatment increased CD95 protein and mRNA expression in both cell lines

more than when either treatment was used alone and knockdown of CD95 expression in HA22T/VGH and Huh-6 cells reverted DHMEQ-CLX-induced cell growth inhibition. In addition, the DHMEQ-CLX combination triggered CD95L mRNA expression and, as a consequence, it contributed to apoptosis induced by CD95/CD95L signaling. In agreement with Huang et al [435] and Bauer et al [446], quenching of ROS blocked the induction of CD95 and CD95L mRNA expression in both cell lines. These results suggest that ROS induce CD95/CD95L expression and in turn apoptosis, although we cannot exclude the possibility that CD95/CD95L signaling could further promote ROS generation, as in many other cancer cells. CD95/CD95L signaling enhances the apoptotic effect of many chemotherapeutic drugs against various cancer cells, including HCC [457].Our results also demonstrated that DHMEQ inducesd CD95L mRNA expression and in combination with CLX increases the expression of CD95/CD95L signalling, suggesting an enhancement of the apoptotic response on combination treatment in HCC.

Taken together our results indicate that the main signal responsible for the DHMEQ-CLX combination antitumor effects in human liver cancer cells is ROS production. The combination promotes ER stress response and the activation of intrinsic and extrinsic apoptotic pathways, the latter through CD95/CD95L increased expression. Our findings show that the DHMEQ-CLX combination is more effective against liver cancer cells than either of the drugs used alone.

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Conflict of interest

None declared.

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Figure Legends

Fig. 1. *The combination of DHMEQ-CLX inhibits cell growth, NF-κB p65 DNA-binding capacity and cell proliferation.* A) Cell growth was assessed by MTS assays. Cells were treated with the indicated concentrations of DHMEQ and CLX either alone or in combination for 72 hours. *p<0.05 *versus* each agent alone. B) Cells were treated for 24 hours with DHMEQ and CLX either alone or in combination. The NF-κB active form (p65 subunit) contained in the nuclear fraction was measured by Trans-AM assays. Data are expressed as percentages of p65 binding inhibition of the control cells and are the means ± SD of three separate experiments. **p<0.01 *versus* each agent alone C) Cell proliferation was assessed by BrdU assay. Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours. *p<0.05 and **p<0.01 *versus* each agent alone.

Fig. 2. *DHMEQ-CLX combination induces apoptosis.* A) Cells were treated for 24 hours with different concentrations of DHMEQ (μg/ml) and CLX (70 μM) either alone or in combination and apoptotic cells were visualized by TUNEL staining as described in the Materials and Methods section. B) Bar charts show the percentage of DHMEQ-CLX-induced apoptotic cells. Data are expressed as the means ± SD of two separate experiments. *p<0.05, **p<0.01 *versus* each agent alone.

Fig. 3. *Effect of DHMEQ-CLX combination ~~induces activation of~~on caspase~~s~~ activities, and ~~down-regulation of~~expression levels of anti-apoptotic proteins and PARP ~~-~~cleavage ~~of PARP~~*. A) Cells were treated for 24 hours with DHMEQ and CLX either alone or in combination, and caspase activation was determined by the caspase assays. Data are expressed as fold increases of untreated cells and are the means ± SD of three separate experiments, each of which was performed in duplicate. B) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours. The induction of PARP cleavage and changes in Bcl-2 and survivin expression were analysed by western blotting. The data

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shown represent two independent experiments with comparable outcomes. Arrowheads indicate the 85 kDa form of PARP. C) HA22T/VGH cells were treated with DHMEQ and CLX either alone or in combination for 24 hours and equal amounts of extracted proteins were analyzed for Mcl-1 and phospho-Akt expression by western blotting. The data shown represent two independent experiments with comparable outcomes.

Fig. 4. *DHMEQ-CLX combination induces CD95/CD95L expression levels and CD95 siRNA transfection significantly reverts DHMEQ-CLX-induced cell growth inhibition.* A) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours and then mRNA expression levels of CD95 were assessed by q-PCR analysis. ***p*<0.01 *versus* each agent alone. B) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours and then mRNA expression levels of CD95L were assessed by sq-PCR analysis. The data shown represent three independent experiments with comparable outcomes. C) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours. CD95 protein expression levels were analysed by western blotting. The numbers represent the ratio of the relevant protein normalized with β-actin, with vehicle-treated control samples (-) arbitrarily set at 1.0. The data shown represent two independent experiments with comparable outcomes. D) Cells were transfected with a Non Correlated siRNA (siRNA NC) or CD95 siRNA (siRNA CD95). 72 hours after transfection cells were treated for 24 hours and cell growth was determined by MTS assays. The data shown are the means ± SD of three separate experiments. **p*< 0.05, ***p*<0.01 *versus* siRNA NC.

Fig. 5. *DHMEQ-CLX triggers ER stress response and knockdown of TRB3 significantly reverts DHMEQ-CLX-induced cell growth inhibition.* A) Cells were treated for 24 hours with DHMEQ and CLX either alone or in combination and then mRNA expression levels of target genes were analyzed

by RT-PCR analysis. For XBP1 mRNA, the spliced form is indicated by an arrowhead. The numbers represent the ratio of the relevant mRNA normalized with β-actin, with vehicle-treated control samples (-) arbitrarily set at 1.0. The data shown represent three independent experiments with comparable outcomes. B) TRB3 and CHOP mRNAs expression were analysed by q-PCR in both cell lines. The results shown are the means ± SD of two experiments each performed in triplicate. *p< 0.05, **p< 0.01 *versus* each agent alone. C) HA22T/VGH cells were transfected with a Non Correlated siRNA (siRNA NC) or TRB3 siRNA (siRNA TRB3). 72 hours after transfection cells were treated for 24 hours and cell growth was determined by MTS assays. The data shown are the means ± SD of three separate experiments. *p< 0.05 *versus* control siRNA.

Fig. 6. *DHMEQ-CLX combination induces ROS generation.*

HA22T/VGH cells were treated with DHMEQ (30 μg/ml) and CLX (70 μM) either alone or in combination for 24 hours. Intracellular ROS levels were evaluated by H_2DCFDA-based fluorescence staining. Data are representative of three different experiments with comparable outcomes.

Fig. 7. *Antioxidant NAC reverts DHMEQ-CLX-induced cell growth inhibition, ER stress response and CD95/CD95L activation.*

A) Cells were pre-treated with NAC for 2 hours before and during DHMEQ and CLX exposure at the indicated concentrations for 24 hours. Cell growth was assessed by MTS assays. *p<0.05, **p<0.01 *versus* each drug alone or in combination in the absence of NAC. B) HA22T/VGH and Huh-6 cells respectively, were pre-treated with NAC for 2 hours before and during DHMEQ and CLX exposure at the indicated concentrations for 24 hours, then cell morphology was observed under light microscopy. C) HA22T/VGH cells were treated with DHMEQ and CLX either alone or in combination for 24 hours with or without pre-treatment with NAC for 2 hours. mRNA expression was analysed by sq-PCR. For

XBP1 mRNA, the spliced form is indicated by an arrowhead. The data shown represent two independent experiments with comparable outcomes. D) Cells were untreated or pre-treated with NAC (2 mM) for 2 hours and then, in its presence, cells were treated for 24 hours with a combination of DHMEQ (30 μg/ml in HA22T/VGH cells or 10 μg/ml in Huh-6 cells) and CLX (70 μM). mRNA expression levels were analysed in both cell lines by q-PCR analysis of CD95 (left) and by sq-PCR of CD95L (right). $**p<0.01$ *versus* combination treatment in the absence of NAC.

The novel NF-κB inhibitor DHMEQ synergizes with Celecoxib to exert antitumor effects on human liver cancer cells by a ROS-dependent mechanism.

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Running Title: DHMEQ synergizes with Celecoxib to induce apoptosis in HCC

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Abstract

In a previous work of ours dehydroxymethylepoxyquinomicin (DHMEQ), an inhibitor of NF-κB, was shown to induce apoptosis through Reactive Oxygen Species (ROS) production in hepatoma cells. The present study demonstrated that DHMEQ cooperates with Celecoxib (CLX) to decrease NF-κB DNA binding and to inhibit cell growth and proliferation more effectively than treatment with these single agents alone in the hepatoma cell lines HA22T/VGH and Huh-6. ROS production induced by the DHMEQ-CLX combination in turn generated the expression of genes involved in endoplasmic reticulum (ER) stress and silencing TRB3 mRNA significantly decreased DHMEQ-CLX-induced cell growth inhibition. Moreover, the DHMEQ-CLX combination was associated with induction of PARP cleavage and down-regulation of the anti-apoptotic proteins Bcl-2, Mcl-1 and survivin, as well as activated Akt. CD95 and CD95 ligand expression increased synergistically in the combination treatment, which was reversed in the presence of NAC. Knockdown of CD95 mRNA expression significantly decreased DHMEQ-CLX-induced cell growth inhibition in both cell lines. These data suggest that the DHMEQ-CLX combination kills hepatoma cells via ROS production, ER stress response and the activation of intrinsic and extrinsic apoptotic pathways.

Keywords: DHMEQ, Celecoxib, NF-κB, CD95/CD95L, liver cancer cells

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Several strategies have been suggested for the treatment of HCC patients but, unfortunately, it still has a high lethality. Therefore, novel approaches are required to contrast this tumor

The nuclear transcription factor-κB (NF-κB) has been implicated in carcinogenesis because it plays a critical role in cell survival, inflammation and cell growth. Recent studies indicate that NF-κB is essential for promoting inflammation-associated cancers and it is therefore a potential target for cancer prevention [1]. Several reports have indicated that NF-κB is constitutively activated in a variety of cancer cells, including hepatocellular carcinoma [2].

Dehydroxymethylepoxyquinomicin (DHMEQ) is a novel NF-κB inhibitor which induces apoptosis and cell-cycle arrest in several cancer cell types [3-5]. We previously demonstrated that DHMEQ promotes ROS generation in human liver cells and that oxidative stress induces ER stress response, DNA damage and release of cytochrome *c* with activation of the caspase cascade [6].

There are two isoforms of cyclooxygenases (COXs), COX-1 and COX-2, the latter being induced by a variety of stimuli. COX-2 is markedly elevated in many types of tumour, including HCC, as selective COX-2 inhibitors (COXIBs) show anti-proliferative and pro-apoptotic effects in human liver cancer cells [7,8] suggesting that COXIBs might be effective in HCC treatment. Accumulating evidence suggests that COXIBs inhibit cell proliferation through a COX-2-independent mechanism [9-11]. The molecular mechanism underlying CLX-mediated apoptosis seems to be associated with the induction of ER stress response through calcium [12] and with the down-regulation of the anti-apoptotic protein survivin [13]. CLX induces the expression of functional death receptors, such as CD95, and a rapid down-regulation of myeloid cell leukemia-1 (Mcl-1) protein, suggesting the activation of intrinsic and extrinsic apoptosis pathways in HCC [14]. CLX has been reported to act synergistically with different drugs in promoting the apoptosis of human liver tumor cells [15]. There

are two different apoptotic pathways: death receptor and mitochondrial [16,17]. Ligation of cell surface death receptors, including CD95 with its specific ligand, triggers a death receptor apoptotic pathway. Fas-associated Death Domain (FADD) is first recruited to the death receptor, followed by the association with pro-caspase 8, which is activated by cleavage. Activation of the mitochondrial apoptotic pathway depends on the release of cytochrome *c* and consequently the activation of caspase-3/7, -9, which in turn cleaves PARP and inhibits anti-apoptotic proteins, such as survivin. Cytotoxic drugs that cause DNA damage either trigger cell death mediated by mitochondria or induce apoptosis in a CD95-dependent manner. The exact mechanisms by which the two cascades are simultaneously activated by cytotoxic drugs are not known. However, it has been reported that the two death pathways may cross-talk, depending on the cell type or stimuli. Cross-talk may depend on Bid protein cleavage, which transduces an apoptotic signal from the cytoplasmic membrane to mitochondria [18] and also on ROS production in mitochondria, which in turn induces the release of cytochrome *c* and CD95 aggregation and activation of the FADD-caspase-8 cascade [19].

Therefore, the present study was set up to investigate whether CLX can potentiate the antitumor effects of DHMEQ against human liver cancer cells. We observed synergistic antitumor effects with the DHMEQ-CLX combination. Moreover, we demonstrated that DHMEQ and CLX interact to increase the activity of the intrinsic and extrinsic apoptotic pathways through CD95 activation and down-regulation of Mcl-1. These pro-apoptotic effects are mediated by the production of ROS, resulting in ER stress response.

2. Materials and Methods

2.1 Reagents and cell culture

DHMEQ was synthesized as previously described [20]. NAC was purchased from Sigma-Aldrich (Milan, Italy). Celecoxib was a gift from the Pfizer Corporation (New York, USA) and was dissolved in dimethyl sulfoxide (DMSO). The human liver cancer cell lines Huh-6, HA22T/VGH and Huh-7 used in this study were a gift from Prof. Massimo Levrero (Department of Internal Medicine, Sapienza University, Rome, Italy). They have different characteristics of differentiation, biological behaviour and genetic defects [21]. Cells were maintained as previously described [6].

2.2 Cell growth and BrdU incorporation assays

MTS assays were performed using the CellTiter Aqueous OneSolution kit (Promega Corporation, Madison, WI, USA) as previously reported [6]. Cell proliferation assays were performed using a colorimetric immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) as previously reported [6]. Data were expressed as the percentage of control cells and were the means ± SD of three separate experiments, each of which was performed in triplicate.

2.3 NF-κB p65 transcription factor assay

Cytoplasm and nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Belgium) according to the manufacturer's instructions. The DNA-binding capacity of NF-κB (p65 subunit) was measured using the Trans-AM^{TM} kit (Active Motif, Belgium) as previously reported [6].

2.4 Detection of apoptosis

The DeadEnd Colorimetric TUNEL System technique was performed as suggested by the supplier (Promega). The number of apoptotic cells was determined by counting the percentage of brown-colour

positive cells. At least 100 cells from two different cell preparations were counted for each condition. Cells were visualized with an Axioskop microscope (Zeiss, Germany).

2.5 Western blot analysis

Whole cellular lysates were obtained using RIPA buffer (Cell Signaling Technologies Inc., Beverly, MA, USA) and western blotting was performed as previously described [21], with primary antibodies raised against survivin (Abcam Limited, Cambridge, UK), β-actin (Sigma-Aldrich Srl, Milan, Italy), PARP, phospho-Akt, Bcl-2 (Cell Signaling Technologies Inc., Beverly, MA, USA), CD95 and Mcl-1 (Santa Cruz Biotechnology Inc, Heidelberg, Germany).

2.6 Caspase activity assays

Caspase activities were measured by the Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 assays (Promega, Milan, Italy) as previously reported [6].

2.7 Semiquantitative-PCR (sq-PCR) and quantitative Real Time PCR (q-PCR)

Total RNA was isolated using TRYzol reagent (Invitrogen Carlsbad, CA) as recommended by the supplier. Semiquantitative PCR was performed using specific 5' and 3' primers which generated specific bands (Table I). The expression of selected genes was quantified by quantitative Real Time PCR (q-PCR) using SYBR Green fluorescence (Qiagen, Milan, Italy) on StepOnePlus (Applied Biosystem, Carlsbad, CA, USA). Primers were purchased from Qiagen (Milan, Italy) and amplified as recommended. Relative expression was calculated using the comparative C_t method. Expression of the gene of interest was expressed as fold induction compared with the control (DMSO) and was corrected with the quantified expression level of β-actin. The results shown were the means ± SD of two experiments, each performed in triplicate.

2.8 siRNA transfection

HA22T/VGH and Huh-6 cells were transfected with CD95 small interfering RNA (siRNA), TRB3 siRNA and Non Correlated (NC) siRNA (Qiagen) as previously reported [6]. 72 hours after transfection, cells were left untreated or treated for 24 hours with DHMEQ and CLX either alone or in combination, then cell growth was determined by MTS assays.

2.9 Evaluation of Reactive Oxygen Species production

The intracellular accumulation of ROS was evaluated using the fluorescent probe 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCFDA) (Invitrogen Carlsbad, CA, USA; cat. n. C-400) and observed under a fluorescence microscope.

2.10 Statistical analysis

Statistical analysis was performed using Student's T test (two-tailed). The criteria for statistical significance was $p<0.05$. Drug combination studies were designed according to Chou and Talalay [22]. CalcuSyn software (Biosoft) was used to calculate the combination index (CI). CI < 1 indicated synergy, a CI about 1 indicated an additive effect and CI > 1 indicated antagonism.

3. Results

3.1 DHMEQ-CLX combination synergistically inhibits cell growth, NF-κB p65 DNA-binding capacity, and cell proliferation.

DHMEQ has been reported to reduce NF-κB p65 DNA-binding capacity and cell growth in human liver cancer cells [6]. There is increasing evidence that CLX has an anti-cancer activity, associated with its ability to decrease cell survival in HCC [14]. Moreover, it has been reported that the anti-inflammatory activity of CLX depends on the inhibition of NF-κB p65 activation and translocation into the nucleus [10,23].

To determine the potential of DHMEQ to inhibit cell growth, we examined its effects on three human liver cancer-cell lines by MTS assay. After 72 hours of exposure to different concentrations of DHMEQ the IC_{50} values were 12.5 μg/ml ± 2.5 in Huh-6 cells, 32.5 μg/ml ± 2.8 in HA22T/VGH cells and 29.8 μg/ml ± 2.3 in Huh-7 cells, respectively. HA22T/VGH cells were the least sensitive and Huh-6 cells the most sensitive to DHMEQ treatment. The MTS assay was also utilized to assess the effects of DHMEQ-CLX combination on cell growth. Cells were exposed to increasing concentrations of DHMEQ in the presence of CLX (50, 70 μM) for different time periods. After 24, 48 (data not shown) and 72 hours (Fig. 1A) the dose-response curve demonstrated that the combination of DHMEQ and CLX suppressed cell growth significantly more than when each drug was used individually. Synergistic growth inhibition following DHMEQ and CLX combination treatment occurred in HA22T/VGH and Huh-6 cell lines (Fig. 1A). A calculated CI value of 0.8 was found for DHMEQ 5 μg/ml in Huh-6 cells combined with CLX 70 μM, while a value of 0.7 was found for DHMEQ 10, 20 μg/ml in Huh-6 and 30, 40 μg/ml in HA22T/VGH cells combined with CLX 70 μM.

Therefore, we focused on the ability of CLX to potentiate the anti-cancer mechanisms of DHMEQ in HA22T/VGH and Huh-6 cells as resistant and sensitive models, respectively.

First, we tested the effects of the DHMEQ-CLX combination on NF-κB p65 transcriptional activity. Figure 1B shows that combination significantly decreased the nuclear localized p65 subunit more than either of the drugs used alone in both cell lines. Moreover, the DHMEQ-CLX combination dose-dependently decreased the amount of the active form of p65 subunit contained in nuclear extracts in both cell lines (data not shown).

The BrdU assay was performed to study the effects of the combination treatment on cell proliferation. In Figure 1C the results show that the DHMEQ-CLX combination affected cell proliferation in both cell lines significantly more than when each drug was used individually.

Taken together, these results suggest that CLX potentiated the anti-proliferative effects caused by DHMEQ in both cell lines, through NF-κB inhibition.

3.2 CLX potentiates DHMEQ-induced apoptosis.

Suppression of NF-κB inhibits the growth of tumor cells, therefore we evaluated apoptosis in liver cancer cells treated with DHMEQ and CLX alone or in combination using the TUNEL assay (Fig. 2A). Significantly higher dose-dependent increases in the percentage of apoptosis were observed in cells treated with DHMEQ-CLX than with DHMEQ alone in both cell lines (Fig. 2B). To understand the mechanism by which the combination induced apoptosis in liver cancer cells we determined the effects of DHMEQ-CLX on the activation of different caspases. Caspase-8, -9 and the effectors caspase-3/7 were activated by combination drug treatments in HA22T/VGH cells, although caspase activities were not significantly different than when DHMEQ was used alone (Fig. 3A). Instead, in Huh-6 cells caspase activities were barely detectable. Staurosporin (500 nM) was used as a positive control for caspase 3/7 activation, resulting in 3.7 ± 0.8 and 3.0 ± 0.3 fold inductions in HA22T/VGH and Huh-6 cells respectively (data not shown). As NF-κB is known to induce the expression of anti-apoptotic proteins [24], we determined the levels of the anti-apoptotic proteins survivin and Bcl-2 as target genes

of NF-κB, as well as the cleavage of PARP, which is a known caspase-3/7 substrate and a biochemical marker of apoptosis. Treatment with the DHMEQ-CLX combination significantly reduced the levels of survivin and Bcl-2 proteins in both cell lines, and PARP was more effectively cleaved in response to combination treatment than by individual drug treatments (Fig. 3B). Caspase activity is regulated by multiple proteins, including the Bcl-2 family member Mcl-1. Mcl-1 protects cells from apoptosis induction through the blockage of cytochrome *c* release from mitochondria by interacting with pro-apoptotic members of the Bcl-2 family [25,26]. Therefore, we analysed protein levels of Mcl-1 and observed the down-regulation of Mcl-1 protein levels in DHMEQ-CLX-treated cells (Fig. 3C).

COX-2 has been shown to promote survival by up-regulating the level of Mcl-1 through the PI3K/Akt pathway [27]. Therefore, to determine the role of this signaling pathway in DHMEQ-CLX-induced apoptosis, the expression level of phospho-Akt protein was analysed in HA22T/VGH cells by western blotting. As shown in Figure 3C, phosphorylation of Akt protein was down-regulated on combination treatment.

Taken all together these data indicate that the DHMEQ-CLX combination induced events associated with both extrinsic and intrinsic apoptotic pathways in each cell line, which correlated with the down-regulation of mitochondrial anti-apoptotic proteins and Akt signaling.

3.3 Combination of DHMEQ and CLX triggers the extrinsic apoptotic pathway.

CLX has been reported to trigger the extrinsic apoptotic pathway through the increased expression of CD95, with a down-regulation of Mcl-1 in HepG2 and Huh-7 cells [14]. Since we observed that caspase-8 was activated after DHMEQ treatment, we investigated the expression of CD95 and CD95L to further dissect the signaling pathways involved in DHMEQ-CLX-induced apoptosis.

q-PCR revealed a significant up-regulation of CD95 mRNA on combination treatment (Fig. 4A) and sq-PCR showed an induced expression of CD95L mRNA in DHMEQ and DHMEQ-CLX treated cells (Fig. 4B). CD95 protein levels were analysed by western blotting (Fig. 4C) and were significantly enhanced on combination treatment in both cell lines. These results suggest that the DHMEQ-CLX combination induces apoptosis through involvement of the CD95/CD95L extrinsic apoptotic pathway.

3.4 CD95 siRNA transfection significantly reverts DHMEQ-CLX-induced cell growth inhibition.

To examine the role of CD95 in DHMEQ-CLX induced cell growth inhibition, HA22T/VGH and Huh-6 cells were first transiently transfected with CD95 siRNA for 72 hours and then MTS assays were performed. The transfection of CD95 siRNA into HA22T/VGH and Huh-6 cells effectively reduced endogenous mRNA by 80% more than the control siRNA (Fig. 4). As shown in Figure 4D knockdown of CD95 significantly reverted the inhibition of cell growth induced by DHMEQ-CLX treatment in both cell lines. These results suggest that activation of extrinsic apoptotic pathway, through CD95 up-regulation, contributes to DHMEQ-CLX-induced cell growth inhibition.

3.5 DHMEQ-CLX combination triggers ER stress response.

CLX triggers ER stress through elevated levels of the ER stress indicators [28]. DHMEQ induces the expression of CHOP and TRB3 mRNAs and the splicing of XBP1 mRNA, which are essential for apoptosis through ROS generation [6]. Therefore, we investigated the potential contribution of ER stress to the synergy observed between DHMEQ and CLX. As shown in Figure 5A, individual drug treatments resulted in the splicing of XBP1 mRNA. Furthermore, a higher dose of the DHMEQ-CLX combination induced the complete splicing of XBP1 mRNA (Fig. 5A). Moreover, the combination treatment decreased GRP78 mRNA, indicating an aggravated ER stress response. q-PCR results showed that CHOP and TRB3 mRNAs were significantly up-regulated after combination

treatment in both cell lines (Fig. 5B). These results indicated that the DHMEQ-CLX combination better induces increased ER stress response than treatment with either of the drugs alone.

3.6 TRB3 siRNA transfection significantly reverts DHMEQ-CLX-induced cell growth inhibition.

TRB3, a crucial factor mediating ER stress response, is a transcriptional target of CHOP and is involved in CHOP-dependent cell death as a second messenger during ER stress [29]. To examine the role of TRB3 in DHMEQ-CLX induced cell growth inhibition, HA22T/VGH cells were first transiently transfected with TRB3 siRNA for 72 hours and then MTS assays were performed. Transfection of TRB3 siRNAs into HA22T/VGH cells effectively reduced the endogenous mRNA by more than 90% more than the control siRNA (Fig. 5C left). As shown in Figure 5C (right) knockdown of TRB3 significantly reverted the inhibition of cell growth induced by DHMEQ-CLX treatment. These results suggest that activation of ER stress response through TRB3 mRNA up-regulation contributes to DHMEQ-CLX-induced cell growth inhibition.

3.7 Effects of DHMEQ and CLX combination on oxidative stress.

Under oxidative stress ROS are produced. High levels of ROS induce cell death, which often involves apoptosis through caspase activation. DHMEQ dose-dependently induces ROS generation by mitochondria in HA22T/VGH cells [6]. Therefore, we investigated the possible role of combination treatment on ROS production. Cells were treated with each drug alone or with the DHMEQ-CLX combination for 24 hours and thereafter ROS were evaluated using the cell-permeable fluorescent probe, H_2DCFDA. Treatment of HA22T/VGH cells with drug combinations increased ROS generation to a greater extent than treatment with either drug by itself (Fig. 6). These results indicated that the drug combination induces ROS production.

3.8 The ROS scavenger NAC prevents DHMEQ-CLX-induced cell growth inhibition, ER stress response and CD95/CD95L expression.

To determine whether DHMEQ-CLX induces apoptosis via ROS generation, we tested the effects of NAC on cell growth, on ER stress response and on the activation of the extrinsic apoptotic pathway in both cell lines. For these purposes, cells were pre-treated with NAC (2 mM) for 2 hours and subsequently, in the presence of NAC, treated with different concentrations of DHMEQ-CLX for 24 additional hours. In HA22T/VGH cells NAC significantly decreased the DHMEQ-CLX-induced inhibition of cell viability, whereas in Huh-6 cells NAC completely abrogated the DHMEQ-CLX-induced reduction in cell viability, leading to a cell viability which was not different from untreated controls (Fig. 7A). In addition, cell morphology in both cell lines was observed after 24 hours of DHMEQ-CLX treatment by light microscopy with or without the presence of NAC (Fig. 7B). As shown in Figure 7B, in the presence of NAC there was a reduction in the numbers of floating and shrunk cells, suggesting a recovery of cell vitality.

To examine the relationship between ER stress response and oxidative stress after DHMEQ-CLX treatment, the induction of CHOP and TRB3 mRNAs, as well as the splicing of XBP1 mRNA were examined in the presence of NAC. Treatment with NAC abrogated the induction of CHOP and TRB3 mRNAs and XBP1 mRNA splicing in HA22T/VGH cells (Fig. 7C). Finally, pre-treatment of the cells with NAC significantly blocked the DHMEQ-CLX-induced expression of CD95 (Fig. 7D left) and CD95L mRNAs (Fig.7D right) in both cell lines.

Taken together, these results indicate that the oxidative stress induced by the combination treatment is able to induce cell growth reduction in liver cancer cell lines and that ER stress response and CD95/CD95L extrinsic pathway activation cause apoptosis downstream of the oxidative stress induced by DHMEQ-CLX treatment .

4. Discussion

NF-κB is a major stress-inducible anti-apoptotic transcription factor [24] which is frequently activated in many types of cancer, including HCC [2]. The NF-κB inhibitor DHMEQ has been reported to induce apoptosis in liver cancer cells through the induction of oxidative stress, mainly via the mitochondrial pathway [6].

The tumorigenic potential of COX-2 over-expression has frequently been associated with resistance to apoptosis in certain types of tumors, including HCC. Celecoxib, a selective COX-2 inhibitor, induces antitumor effects in HCC [7,10] and inhibits NF-κB nuclear translocation [10,23]. ROS interact with NF-κB signaling pathways in many ways. In fact, the transcription of NF-κB-dependent genes influences the levels of ROS in the cell, and in turn, the levels of NF-κB activity are also regulated by ROS levels. Depending on the context, ROS can both activate and inhibit NF-κB signaling [30]. Interestingly, transformed cells are thought to use ROS signals to stimulate both cell proliferation and tumor progression. However, high levels of ROS beyond a certain threshold increase the vulnerability of cancer cells to undergo apoptosis. Thus, agents that either promote ROS generation or decrease the levels of antioxidants have the potential to kill cancer cells with little effect on normal cells [31]. Combinations of drugs for the treatment of tumors have been suggested as a promising therapeutic strategy, both to maximize pharmaceutical efficacy and to minimize the adverse events associated with the individual components. Our studies have demonstrated that the NF-κB inhibitor DHMEQ in combination with CLX causes apoptosis in human liver cancer cells through the induction of oxidative stress. In this study we presented data indicating that ROS induction is crucial in the DHMEQ-CLX-mediated anti-cancer effects. In fact, the drug combination increased ROS generation 24 hours after exposure, more than either drugs used alone. Different cancer cells possess different basal levels of ROS and endogenous scavengers. More aggressive cells possess higher levels of endogenous ROS and need these elevated levels of ROS to undergo apoptosis [32]. We observed that

HA22T/VGH and Huh-6 cells have different sensitivities to the DHMEQ-CLX combination treatment, likely due to different basal levels of ROS, with higher threshold levels required in HA22T/VGH cells to undergo apoptosis. Consequently, pre-treatment with NAC, a glutathione (GSH) precursor, only partially reverts the effect of DHMEQ-CLX in HA22T/VGH cells, whereas in Huh-6 cells NAC completely reverts cells growth inhibition.

Oxidative stress may activate an ER stress response which, through up-regulation of ER stress genes *CHOP* and *TRB3* and XBP1 mRNA splicing, can promote apoptosis [33]. ER stress response is a major mechanism that mediates apoptotic cell death and is used as an anti-cancer strategy [34]. Our results showed that the DHMEQ-CLX combination induces a significant ER stress response and that this response is an event downstream of the oxidative stress in human liver cancer cells. These results are in accordance with other authors who reported that ER stress response could be induced through ROS generation [35] and with our previous results with DHMEQ treatment in liver cancer cells [6]. ER stress response starts with GRP78 mRNA induction, which is a survival signal [36]. We observed that GRP78 was inhibited by the DHMEQ-CLX combination in HA22T/VGH cells, but not in Huh-6 cells, much more than when the drugs were used individually. Suppression of GRP78 can also promote ROS generation and activates the extrinsic apoptotic pathway in HCC cells [37]. Moreover, we observed that the DHMEQ-CLX combination promoted significant CHOP and TRB3 mRNAs induction as well as splicing of XBP1 mRNA in a ROS-dependent manner. Furthermore knockdown of TRB3 in HA22T/VGH cells significantly reverted DHMEQ-CLX induced cell growth inhibition. At present it is not clear how ER stress response induces apoptosis in HCC. The caspase-8 pathway has been reported to induce ER stress response and this is associated with a release of cytochrome *c* from mitochondria in human liver cancer cells [38]. We recently demonstrated that DHMEQ induces the release of cytochrome *c* from mitochondria and caspase activation [6]. In the present study we documented the activation of caspase-9 and caspase-8 by DHMEQ-CLX combination treatment in HA22T/VGH cells

but not in Huh-6 cells. However, we observed the cleavage of PARP, a well-known caspase-3/7 target in both cell lines. Indeed it has been previously demonstrated that during apoptosis, PARP cleavage could be an event either dependent or independent of caspase activation depending on the cell type and/or apoptotic stimuli [39,40]. Moreover, in our system the levels of survivin and Bcl-2, two anti-apoptotic proteins, were reduced by DHMEQ-CLX treatment. Mitochondrial integrity is regulated by pro-apoptotic and anti-apoptotic members of the Bcl-2 family, such as Mcl-1. Mcl-1 protects cells from apoptosis induction through the blockage of cytochrome *c* release from mitochondria by interacting with the pro-apoptotic Bcl-2 family member [25,26]. Our results showed that Mcl-1 protein is down-regulated after DHMEQ-CLX treatment, in accordance with previous studies demonstrating a decrease in Mcl-1 in HCC cells treated with CLX [14]. Mcl-1 basal expression is dependent on a functional phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway, and treatment of HCC cells with a specific PI3 kinase inhibitor leads to both decreased Mcl-1 expression and a sensitization towards chemotherapeutic drug-induced apoptosis [41]. In the present study, DHMEQ and CLX individually attenuated the presence of the activated form of Akt, which was further abrogated by the combination. These results are in agreement with previous studies [42,43] and suggest that Akt inhibition may contribute to the antitumor effect of CLX alone or in combination with other drugs. Recent reports have indicated that CLX results in CD95 up-regulation and Mcl-1 down-regulation and this is a critical checkpoint at which the extrinsic and the intrinsic apoptotic pathways converge in HCC [14]. Moreover, ROS generation can increase CD95 activation in HCC [44] and our previous results demonstrated that DHMEQ induces the activation of an intrinsic death pathway in a ROS-dependent manner, with down-regulation of survivin and release of cytochrome *c* in human liver cancer cells [6]. In the present study, we document that the DHMEQ-CLX combination treatment increased CD95 protein and mRNA expression in both cell lines more than when either treatment was used alone and knockdown of CD95 expression in HA22T/VGH and Huh-6 cells reverted DHMEQ-CLX-induced cell

growth inhibition. In addition, the DHMEQ-CLX combination triggered CD95L mRNA expression and, as a consequence, it contributed to apoptosis induced by CD95/CD95L signaling. In agreement with Huang et al [45] and Bauer et al [46], quenching of ROS blocked the induction of CD95 and CD95L mRNA expression in both cell lines. These results suggest that ROS induce CD95/CD95L expression and in turn apoptosis, although we cannot exclude the possibility that CD95/CD95L signaling could further promote ROS generation, as in many other cancer cells. CD95/CD95L signaling enhances the apoptotic effect of many chemotherapeutic drugs against various cancer cells, including HCC [47].Our results also demonstrated that DHMEQ induced CD95L mRNA expression and in combination with CLX increases the expression of CD95/CD95L signalling, suggesting an enhancement of the apoptotic response on combination treatment in HCC.

Taken together our results indicate that the main signal responsible for the DHMEQ-CLX combination antitumor effects in human liver cancer cells is ROS production. The combination promotes ER stress response and the activation of intrinsic and extrinsic apoptotic pathways, the latter through CD95/CD95L increased expression. Our findings show that the DHMEQ-CLX combination is more effective against liver cancer cells than either of the drugs used alone.

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Conflict of interest

None declared.

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Figure Legends

Fig. 1. *The combination of DHMEQ-CLX inhibits cell growth, NF-κB p65 DNA-binding capacity and cell proliferation.* A) Cell growth was assessed by MTS assays. Cells were treated with the indicated concentrations of DHMEQ and CLX either alone or in combination for 72 hours. *p<0.05 *versus* each agent alone. B) Cells were treated for 24 hours with DHMEQ and CLX either alone or in combination. The NF-κB active form (p65 subunit) contained in the nuclear fraction was measured by Trans-AM assays. Data are expressed as percentages of p65 binding inhibition of the control cells and are the means ± SD of three separate experiments. **p<0.01 *versus* each agent alone C) Cell proliferation was assessed by BrdU assay. Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours. *p<0.05 and **p<0.01 *versus* each agent alone.

Fig. 2. *DHMEQ-CLX combination induces apoptosis.* A) Cells were treated for 24 hours with different concentrations of DHMEQ (μg/ml) and CLX (70 μM) either alone or in combination and apoptotic cells were visualized by TUNEL staining as described in the Materials and Methods section. B) Bar charts show the percentage of DHMEQ-CLX-induced apoptotic cells. Data are expressed as the means ± SD of two separate experiments. *p<0.05, **p<0.01 *versus* each agent alone.

Fig. 3. *Effect of DHMEQ-CLX combination on caspase activities, and expression levels of anti-apoptotic proteins and PARP cleavage.* A) Cells were treated for 24 hours with DHMEQ and CLX either alone or in combination, and caspase activation was determined by the caspase assays. Data are expressed as fold increases of untreated cells and are the means ± SD of three separate experiments, each of which was performed in duplicate. B) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours. The induction of PARP cleavage and changes in Bcl-2 and survivin expression were analysed by western blotting. The data shown represent two independent experiments

with comparable outcomes. Arrowheads indicate the 85 kDa form of PARP. C) HA22T/VGH cells were treated with DHMEQ and CLX either alone or in combination for 24 hours and equal amounts of extracted proteins were analyzed for Mcl-1 and phospho-Akt expression by western blotting. The data shown represent two independent experiments with comparable outcomes.

Fig. 4. *DHMEQ-CLX combination induces CD95/CD95L expression levels and CD95 siRNA transfection significantly reverts DHMEQ-CLX-induced cell growth inhibition.* A) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours and then mRNA expression levels of CD95 were assessed by q-PCR analysis. $^{**}p<0.01$ *versus* each agent alone. B) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours and then mRNA expression levels of CD95L were assessed by sq-PCR analysis. The data shown represent three independent experiments with comparable outcomes. C) Cells were treated with DHMEQ and CLX either alone or in combination for 24 hours. CD95 protein expression levels were analysed by western blotting. The numbers represent the ratio of the relevant protein normalized with β-actin, with vehicle-treated control samples (-) arbitrarily set at 1.0. The data shown represent two independent experiments with comparable outcomes. D) Cells were transfected with a Non Correlated siRNA (siRNA NC) or CD95 siRNA (siRNA CD95). 72 hours after transfection cells were treated for 24 hours and cell growth was determined by MTS assays. The data shown are the means ± SD of three separate experiments. $^{*}p< 0.05$, $^{**}p<0.01$ *versus* siRNA NC.

Fig. 5. *DHMEQ-CLX triggers ER stress response and knockdown of TRB3 significantly reverts DHMEQ-CLX-induced cell growth inhibition.* A) Cells were treated for 24 hours with DHMEQ and CLX either alone or in combination and then mRNA expression levels of target genes were analyzed by RT-PCR analysis. For XBP1 mRNA, the spliced form is indicated by an arrowhead. The numbers

represent the ratio of the relevant mRNA normalized with β-actin, with vehicle-treated control samples (-) arbitrarily set at 1.0. The data shown represent three independent experiments with comparable outcomes. B) TRB3 and CHOP mRNAs expression were analysed by q-PCR in both cell lines. The results shown are the means ± SD of two experiments each performed in triplicate. *p< 0.05, **p< 0.01 *versus* each agent alone. C) HA22T/VGH cells were transfected with a Non Correlated siRNA (siRNA NC) or TRB3 siRNA (siRNA TRB3). 72 hours after transfection cells were treated for 24 hours and cell growth was determined by MTS assays. The data shown are the means ± SD of three separate experiments. *p< 0.05 *versus* control siRNA.

Fig. 6. *DHMEQ-CLX combination induces ROS generation.*

HA22T/VGH cells were treated with DHMEQ (30 μg/ml) and CLX (70 μM) either alone or in combination for 24 hours. Intracellular ROS levels were evaluated by H_2DCFDA-based fluorescence staining. Data are representative of three different experiments with comparable outcomes.

Fig. 7. *Antioxidant NAC reverts DHMEQ-CLX-induced cell growth inhibition, ER stress response and CD95/CD95L activation.*

A) Cells were pre-treated with NAC for 2 hours before and during DHMEQ and CLX exposure at the indicated concentrations for 24 hours. Cell growth was assessed by MTS assays. ∗p<0.05, ∗∗p<0.01 *versus* each drug alone or in combination in the absence of NAC. B) HA22T/VGH and Huh-6 cells respectively, were pre-treated with NAC for 2 hours before and during DHMEQ and CLX exposure at the indicated concentrations for 24 hours, then cell morphology was observed under light microscopy. C) HA22T/VGH cells were treated with DHMEQ and CLX either alone or in combination for 24 hours with or without pre-treatment with NAC for 2 hours. mRNA expression was analysed by sq-PCR. For XBP1 mRNA, the spliced form is indicated by an arrowhead. The data shown represent two

independent experiments with comparable outcomes. D) Cells were untreated or pre-treated with NAC (2 mM) for 2 hours and then, in its presence, cells were treated for 24 hours with a combination of DHMEQ (30 μg/ml in HA22T/VGH cells or 10 μg/ml in Huh-6 cells) and CLX (70 μM). mRNA expression levels were analysed in both cell lines by q-PCR analysis of CD95 (left) and by sq-PCR of CD95L (right). $**p<0.01$ *versus* combination treatment in the absence of NAC.

February 7, 2012

Dear Professor Schwab,

Please find enclosed the revised manuscript by Lampiasi N., Azzolina A., Umezawa K., Montalto G., Mc Cubrey James A., Cervello M. entitled “The novel NF-κB inhibitor DHMEQ synergizes with Celecoxib to exert antitumoral effects on human liver cancer cells by a ROS-dependent mechanism” for consideration for publication in Cancer Letters.

Following your advice, we have modified the manuscript as suggested by the reviewer. As you will see, we have now modified the description of the results presented in Figure 3A and we have discussed the dissonant results obtained also by adding two new references.

We hope that this revised manuscript will meet the standards of the reviewers and that you will find it suitable for the readership of Cancer Letters.

Thank you for your consideration and selection of helpful reviewers. We look forward to receiving your final decision.

Sincerely yours,

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phone +39 0916809513 fax: +39 0916809548 email: lampiasi@ibim.cnr.it

Figure(s)

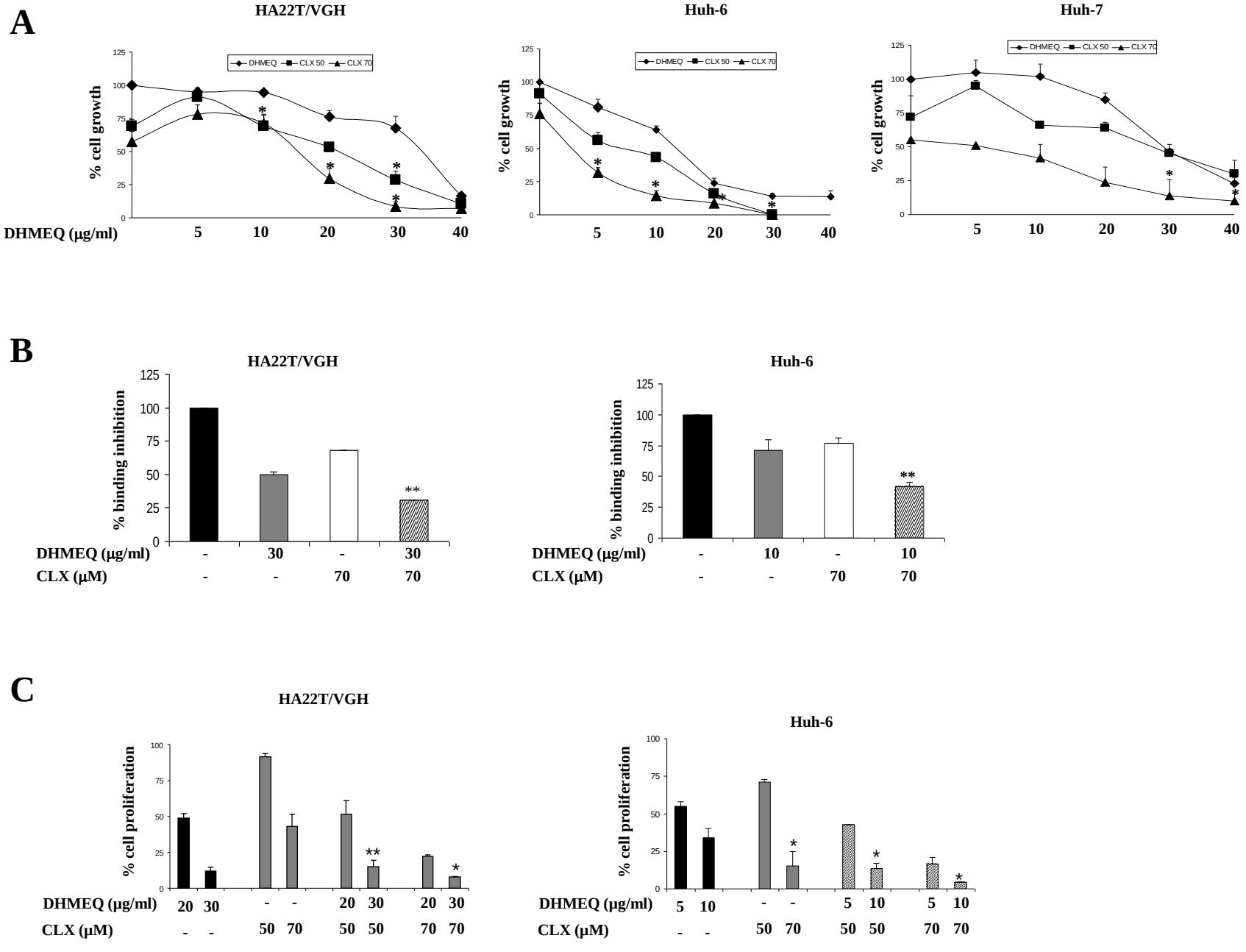
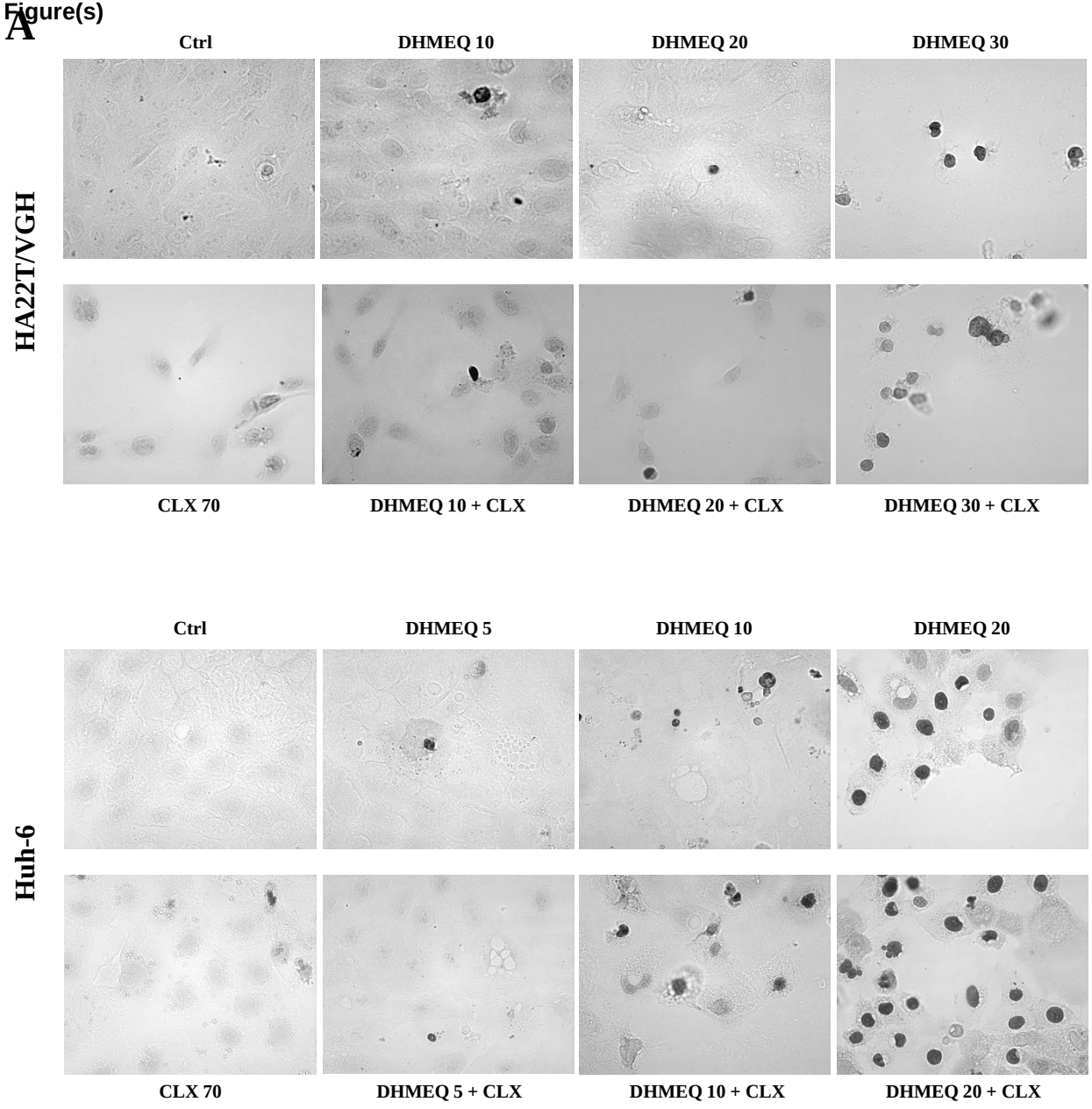


Figure 1

Figure(s)



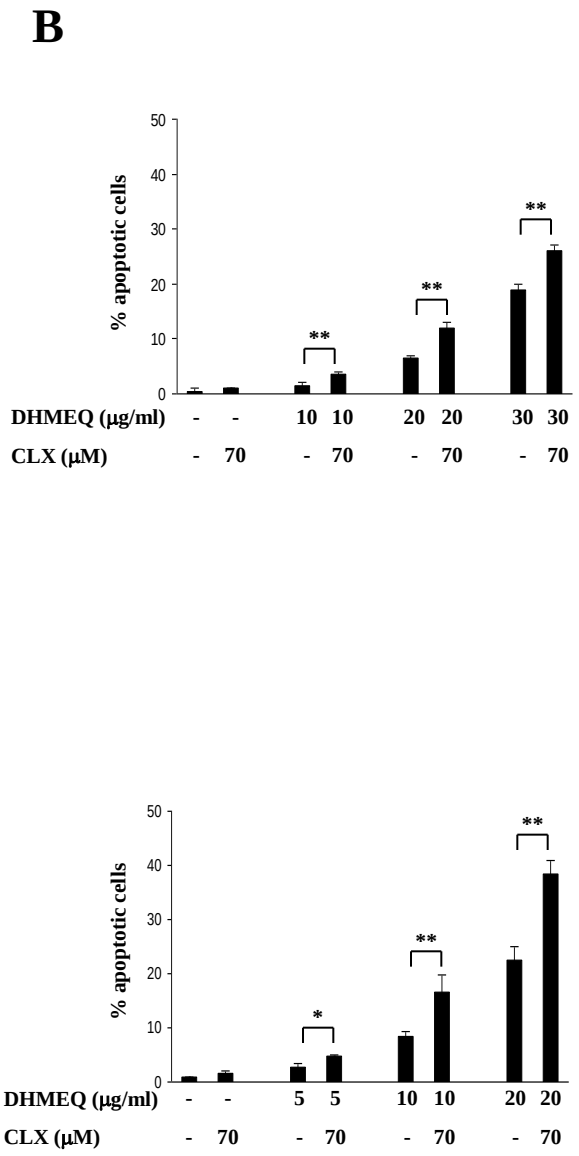


Figure 2

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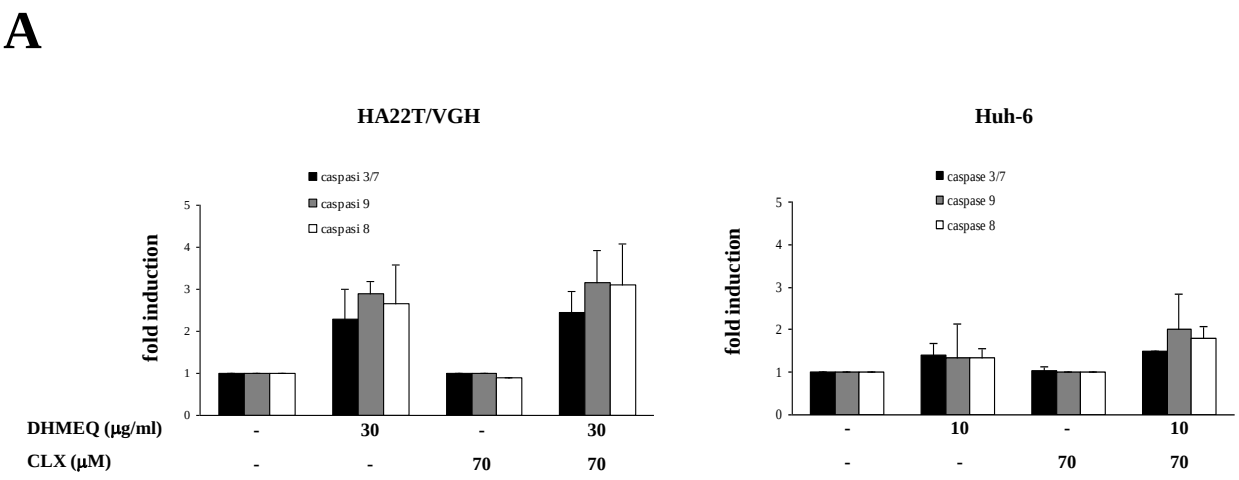

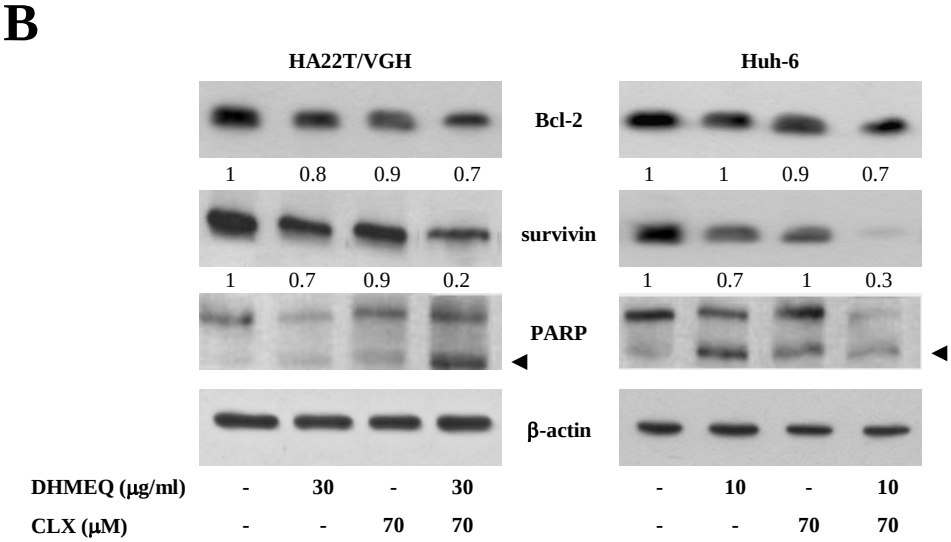

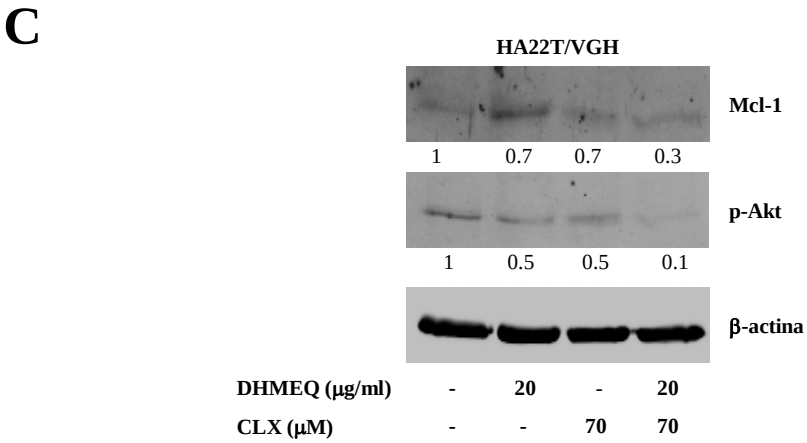

Figure 3

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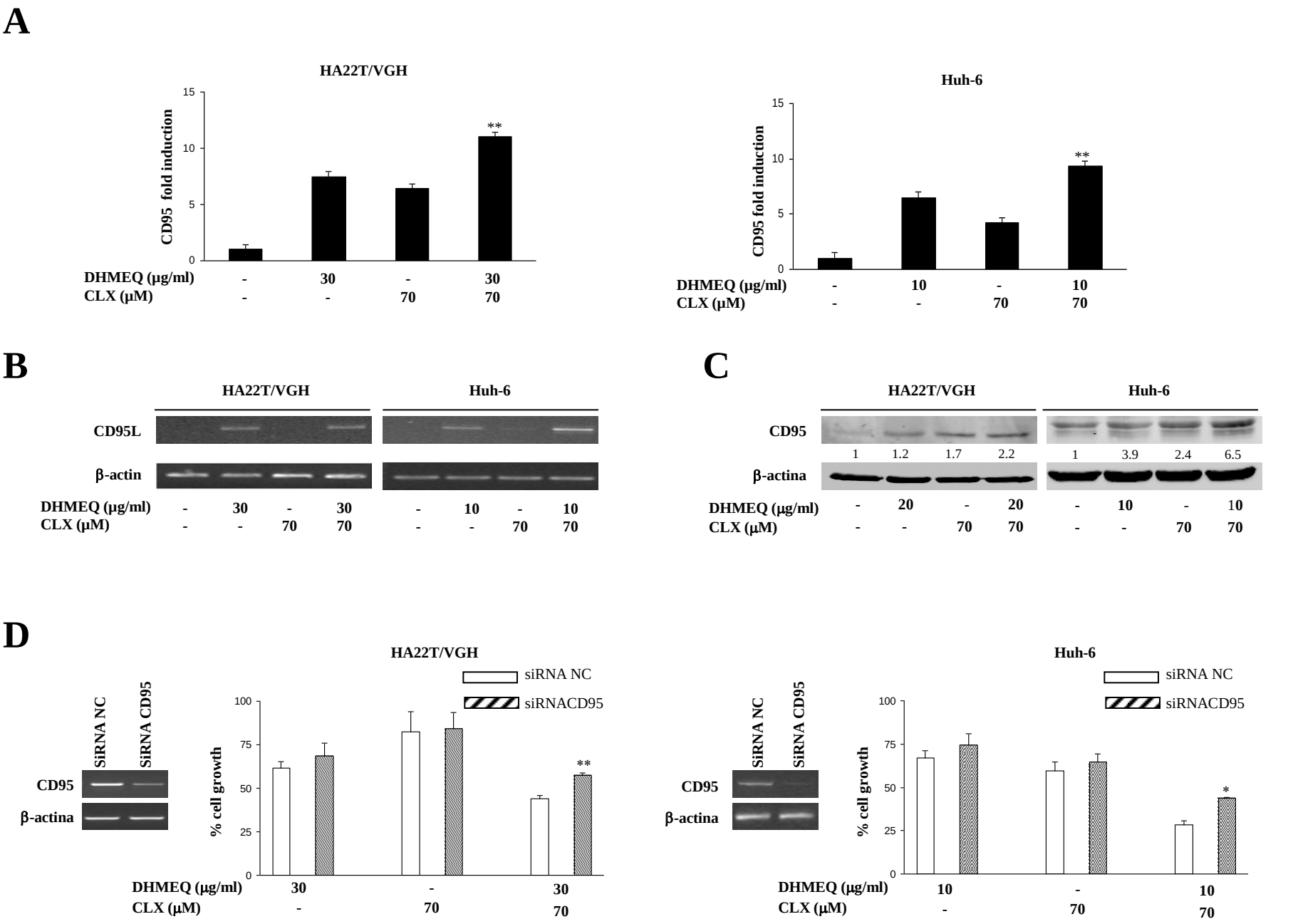

Figure 4

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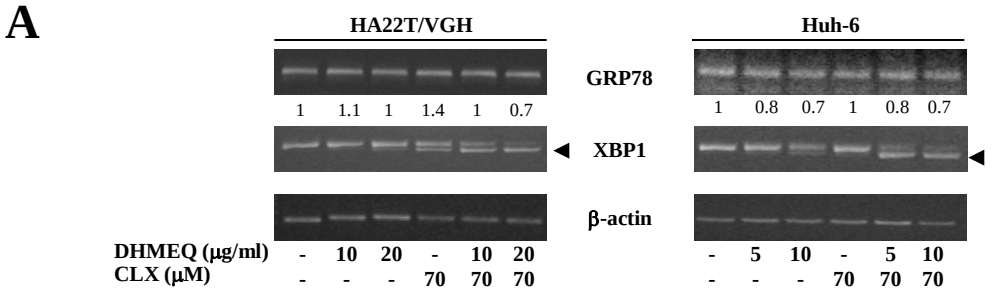

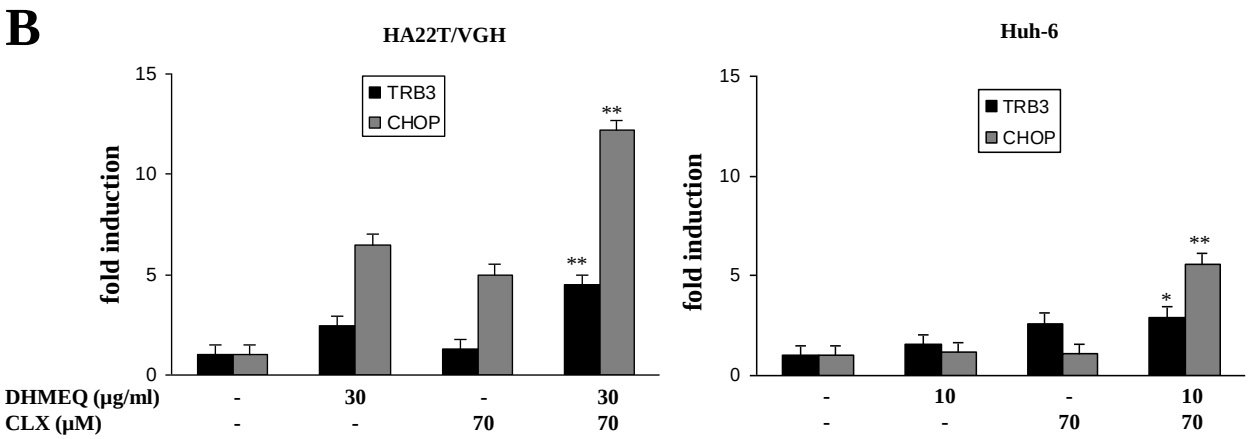

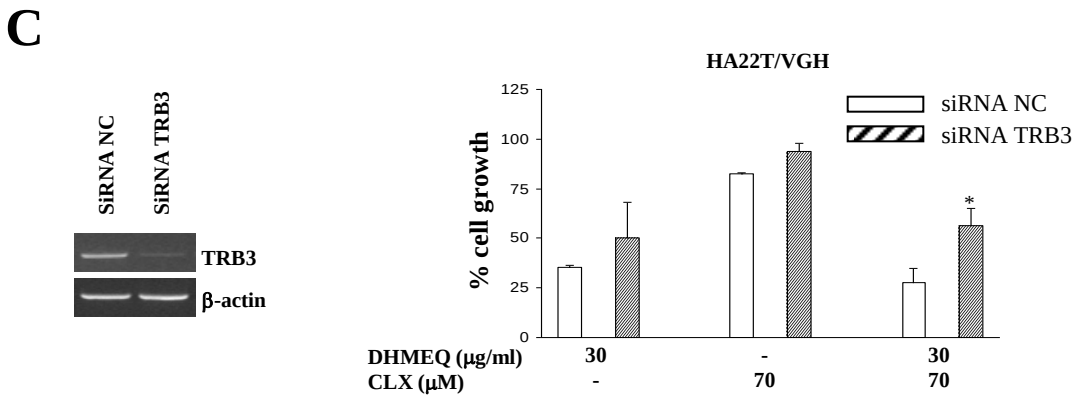

Figure 5

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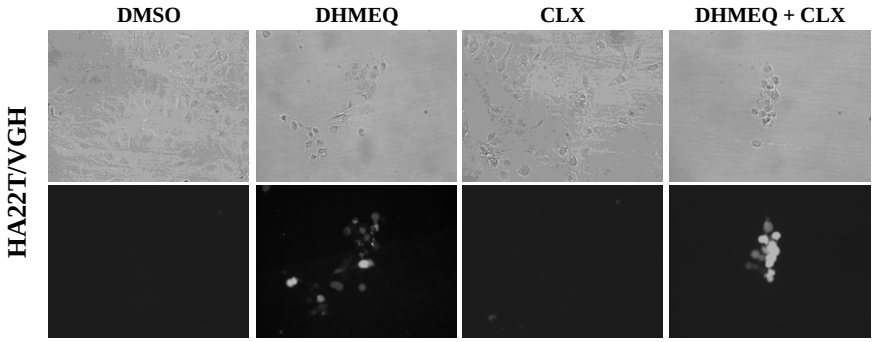

Figure 6

Figure(s)

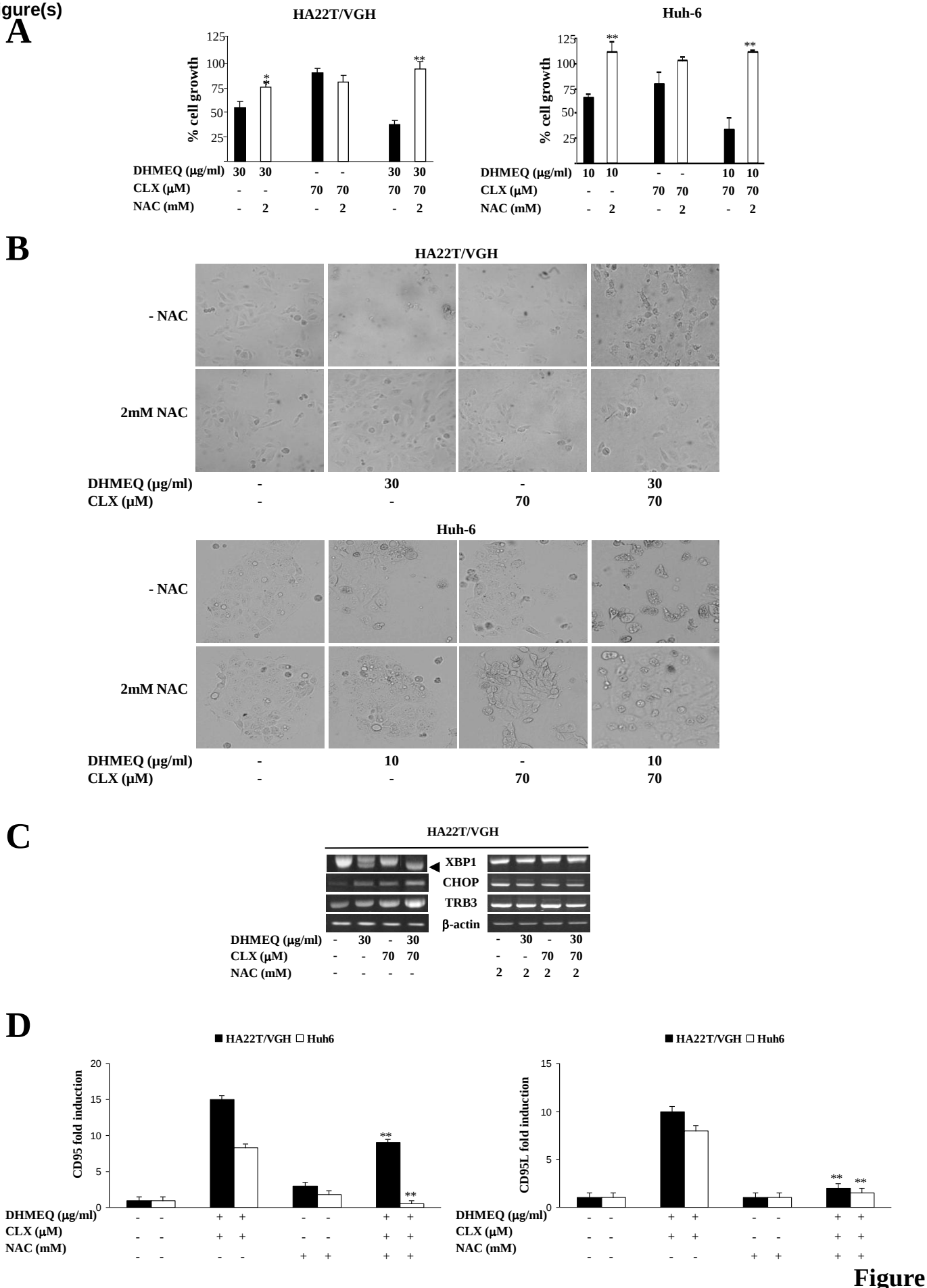

Figure 7

Table(s)

GENE	FORWARD PRIMER	REVERSE PRIMER	bp	TA (°C)	N° of cycles
GRP78	GACATCAAGTTCTTGCCGTT	CTCATAACATTTAGGCCAGC	259	58	40
XBP1	CCTTGTAGTTGAGAACCAGG	GGGGCTTGGTATATATGTGG	441	57	35
CD95L	ATGTTTCAGCTCTTCCACCTACAGA	CCAGAGAGAGCTCAGATACGTTGAC	498	60	40
CD95	ATGGCCAATTCTGCCATAAG	GACAAAGCCACCCCAAGTTA	371	58	38
CHOP	ATGGCAGCTGAGTCATTGCC	TCATGCTTGGTGCAGATTC	509	58	30
TRB3	GCCACTGCCTCCCGTCTTG	GCTGCCTTGCCCGAGTATGA	538	58	30
β-ACT	CACCACACCTTCTACAATGAGC	GAGGATCTTCATGAGGTAGTCAGT	175	60	22

Conflict of interest

None declared.