Antitumor effects of the novel NF-κB inhibitor dehydroxymethylepoxyquinomicin on human hepatic cancer cells: Analysis of synergy with cisplatin and of possible correlation with inhibition of pro-survival genes and IL-6 production

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Abstract. We tested the novel NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) in the hepatic cancer (HCC) HepG2, HA22T/VGH and HuH-6 cells. The sensitivity to the cell growth inhibitory and apoptotic effects of the agent increased along with the levels of constitutively activated NF-κB, which were low in HepG2 and higher in HA22T/VGH and HuH-6. In HA22T/VGH, DHMEQ exhibited synergy with cisplatin. In the same cells, DHMEQ exerted dose-dependent decreases in the nuclear levels of activated NF-κB and attenuated NF-κB activation by cisplatin. It down-regulated Bcl-X_L mRNA in a dose-dependent manner and up-regulated that of Bcl-X_S. It also decreased interleukin 6 (IL-6), NAIP and, after 16 h of exposure to the higher concentration tested (10 μg/ml), c-IAP-1 mRNA levels. At 10 μg/ml it caused significant increase in Bax, XIAP, cyclin D1 and ß-catenin mRNAs. The combination of DHMEQ with cisplatin produced unexpected significant decrease in c-IAP-2 and Bcl-X_S mRNAs as well as additive decrease (IL-6, NAIP and, after 16 h, Bcl-X_L) or increase (XIAP at 8 h) in gene expression. HA22T/VGH produce IL-6; in agreement with the results on mRNA, DHMEQ inhibited such a process. HA22T/VGH lack the IL-6 receptor alpha chain, ruling out that in these cells the antitumor effects of DHMEQ may be attributed to an interference with a growth stimulatory autocrine loop based on IL-6. However, the use of DHMEQ in HCC might be beneficial to contrast the adverse systemic effects of the released cytokine.

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Introduction

Hepatocellular carcinoma (HCC) is a quite frequent and highly aggressive tumor, which in the advanced stages responds very poorly to currently available therapies (1). Like in other tumor cell types, an imbalance between unrestrained cell proliferation and low ability to perform apoptosis appears to be a major unfavourable feature of HCC. Recent studies have indicated that a relevant adverse factor in this cancer is the over-expression of anti-apoptotic factors such as the Bcl-2 family members and the IAPs (inhibitory of apoptosis proteins) (2-7). Human IAPs include c-IAP-1, c-IAP-2, NAIP, XIAP, survivin and ML-IAP (known also as livin-α) and are endowed with a remarkable ability of blocking cell death from many different triggers, through inhibition of the key effector caspases as well as by other means (8-11).

The regulatory mechanisms of IAP expression are far from to be fully elucidated, but it has been shown that at least c-IAP-1, c-IAP-2 and XIAP can be up-regulated by nuclear factor-κB (NF-κB) (12,13). This, in turn, is frequently constitutively activated in HCC (14,15); thus, its inhibition might be of help to antagonize the IAPs as well as other target genes involved in this cancer. We studied the antitumor effects of the novel NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ), alone or in combination with the conventional anticancer agent cisplatin, in the HCC cell lines HepG2, HA22T/VGH and HuH-6 endowed with different levels of NF-κB. DHMEQ inhibits the nuclear translocation of NF-κB and has already shown good effects in different *in vitro* and *in vivo* tumor models (16-18). To our knowledge, its activity on HCC has not been investigated yet.

Materials and methods

Agents. Dehydroxymethylepoxyquinomicin (DHMEQ) was kindly provided by Dr Kazuo Umezawa, Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan. Cisplatin was purchased from Sigma-Aldrich Srl, Milan, Italy.

Cell culture. The hepatocellular carcinoma HepG2, HA22T/VGH and HuH-6 cell lines were kindly provided by Professor Massimo Levrero (Laboratory of Gene Expression, Fondazione Andrea Cesalpino, University of Rome 'La Sapienza', Rome, Italy) and cultured in Roswell Park Memorial Institute (RPMI)-1640 (HyClone Europe Ltd., Cramlington, UK) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin (all reagents were from HyClone Europe) in a humidified atmosphere at 37˚C in 5% CO_2. Cells having a narrow range of passage number were used for all experiments.

Cell growth assays. To test the effects of the agents, the cells were seeded at $2x10^4$ cells/well onto 96-well plates and then incubated overnight. At time 0, medium was replaced with fresh complete medium and DHMEQ, cisplatin or combinations thereof were added in concentrations as indicated. At the end of treatment, 15 μl of a commercial solution (obtained fron Promega Corporation, Madison, WI, USA) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate were added. The plates were incubated for 2 h in a humidified atmosphere at 37˚C in 5% CO_2. The bio-reduction of the MTS dye was assessed by measuring the absorbance of each well at 490 nm. Cytotoxicity was expressed as a percentage of the absorbance measured in the control cells.

Evaluation of cell death by flow cytometry. Cells were washed twice with ice-cold PBS and then suspended at $1x10^6$/ml in a hypotonic fluorochrome solution containing propidium iodide 50 μg/ml in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40. After 1 h of incubation in this solution the samples were filtered through nylon cloth, 40 μm mesh, and their fluorescence was analyzed as single-parameter frequency histograms using a FACSort instrument (Becton-Dickinson, Montain View, CA, USA). The data were analyzed with CellQuest™ software (Becton-Dickinson). Cell death was determined by evaluating the percentage of events accumulated in the preG$_0$-G$_1$ position. The occurrence of apoptosis was evaluated also by studying phosphatidylserine exposure on cell surface. The cells were resuspended at $1x10^6$/ml in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 μM NaCl and 2.5 mM $CaCl_2$), incubated with FITC-conjugated annexin V (Becton-Dickinson) for 10 min and then analyzed by flow cytometry. The results were analyzed using the CellQuest software by subtracting control cells from the cell population stained with FITC-conjugated annexin V.

NF-κB activation. EMSAs were performed using an EMSA 'Gel-Shift' kit from Panomics, Inc. (Redwood City, CA, USA). In brief, nuclear extracts of exponentially growing cells were prepared using the manufacturer's protocol for the Panomics nuclear extraction kit. The nuclear extracts were then incubated with a biotin-tagged NF-κB probe (p65 subunit) or with non-specific and specific competitors supplied with the kit for 30 min at 15˚C, according to the manufacturer's protocol. The extracts were electrophoresed using a 6% polyacrylamide gel at 4˚C and transferred to Hybond™-N$^+$ nylon membrane (Amersham Biosciences, Little Chalfont, UK). The blots were developed using the detection kit provided in the EMSA kit and visualized using Hyperfilm™ ECL (Amersham) for 5 min. The DNA-binding capacity of NF-κB (p65 subunit) was measured in the nuclear extracts of HA22T/VGH cells using the TransAM™ NF-κB and Nuclear Extract™ Kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the determination is based on a 96-well plate to which an oligonucleotide containing the NF-κB consensus binding site (5'-GGGACT TTCC-3') has been immobilized. The activated NF-κB contained in extracts specifically binds to this nucleotide. By using an antibody that is directed against an epitope on p65 that is accessible only when NF-κB is bound to its target DNA, the NF-κB bound to the oligonucleotide is detected. Addition of a secondary antibody conjugated to horseradish peroxidase provides sensitive colorimetric readout that is quantified by densitometry. The specificity of the assay is confirmed by contemporaneous incubations in presence of an excess of the non-immobilized consensus oligonucleotide, as a competitor, or of a mutated consensus oligonucleotide. The results were expressed as arbitrary units (one unit is the DNA binding capacity shown by 1 μg of whole cell extract from HeLa cells stimulated with TNF-α)/μg protein of nuclear extracts.

Evaluation of gene expression by semi-quantitative RT-PCR. Total RNA was isolated from $1x10^6$ HA22T/VGH cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR was then performed using the one-step protocol of the Ready-to-go RT-PCR beads kit (Amersham). Quantification and equalization of the amount of cDNA was achieved using primers to amplify ß-actin as an internal control. Briefly, we first determined the conditions in which the amount of each RT-PCR product was directly proportional to that of the template RNA. The first-strand oligo(dT) primer and the appropriate set of oligonucleotide primers for the different factors or ß-actin were added individually to each dissolved bead in a total volume of 50 μl. First strand cDNAs were obtained after 30 min at 42˚C. Following inactivation at 95˚C for 5 min, PCR amplification was then performed under the following reaction conditions: 94˚C for 1 min, 50˚C (c-IAP-2), 55˚C (P-glycoprotein), 58˚C (Bcl-X_L, IL-6, c-IAP-1, NAIP, XIAP, Bax and ß-catenin), 60˚C (COX-2, cyclin D1 and c-myc) or 62˚C (Bcl-2, survivin and livin-α) for 1 min, 72˚C for 1 min and a final extension at 72˚C for 5 min. We used 15 cycles of amplification for ß-actin, 25 cycles for IL-6 and 30 cycles for the other mRNAs. All PCR products (10 μl) were analyzed by electrophoresis on 1.5% (w/v) agarose gel, photographed and quantified by densitometric scanning. The sequence of primers used in the RT-PCR was as follows: ß-catenin: 5'-CTGATTTGATGGAGTTGGAC-3' (sense) and 5'-CTGCTA CTTGTTCTTGAGTG-3' (antisense); COX-2: 5'-GAGAAA ACTGCTCAACACCG-3' (sense) and 5'-GCATACTCTGTT GTGTTCCC-3' (antisense); c-myc: 5'-AGGCAGACGGAGC TGGAGCC-3' (sense) and 5'-ATGTCTTGCGCGCAGC CTG-3' (antisense); Bcl-2: 5'-ATGGCGCACCGTGGGA GAA-3' (sense) and 5'-TGTGGCCCAGATAGGCACC-3' (antisense); Bcl-X_L: 5'-TTGGACAATGGACTGGTTTGA-3' (sense) and 5'-GTAGAGTGGATGGTCAGTG-3' (antisense);

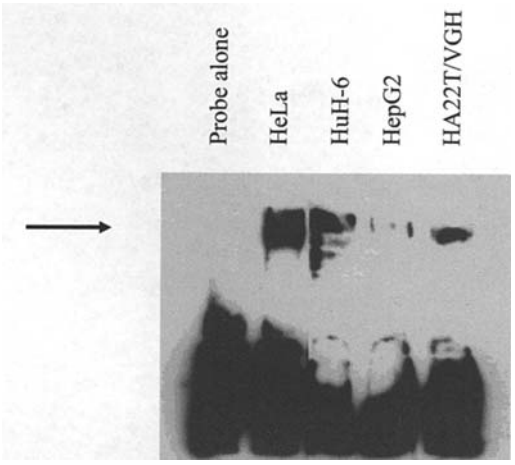

Figure 1. Baseline levels of NF-κB in nuclear extracts of HCC cell lines. NF-κB (p65 subunit) activity was examined by EMSA as described in Materials and methods. An extract of HeLa cells stimulated with TNF-α was used as a positive control. Two repeat experiments gave very similar results.

IL-6: 5'-ATGAACTCCTTCTCCACAAGCGC-3' (sense) and 5'-GAAGAGCCCTCAGGCTGGACTG-3' (antisense); Bax: 5'-TGCTTCAGGGTTTCATCCAG-3' (sense) and 5'-GGCG GCAATCATCCTCTG-3' (antisense); c-IAP-1: 5'-TGACTT TTCCTGTGAACTCT-3' (sense) and 5'-GCCTTTCATTCG TATCAAGA-3' (antisense); c-IAP-2: 5'-ATGAACATACT AGAAAACAGC-3' (sense) and 5'-CCTGTCCTTTAATTCT TATCA-3' (antisense); NAIP: 5'-AAATGTGAATTTCTTC GGAGT-3' (sense) and 5'-TTTTGAAGCAATAGACAG ATC-3' (antisense); XIAP: 5'-GCAGGGTTTCTTTATAC TGG-3' (sense) and 5'-TGTCCCTTCTGTTCTAACAG-3' (antisense); survivin: 5'-GCATGGGTGCCCCGACGTTG-3' (sense) and 5'-GCTCCGGCCAGAGGCCTCAA-3' (antisense); livin-α: 5'-GTCCCTGCCTCTGGGTAC-3' (sense) and 5'-CAG GGAGCCCACTCTGCA-3' (antisense); P-glycoprotein: 5'-GCCTGGCAGCTGGAAGACAAATADACAAAATT-3' (sense) and 5'-CAGACAGCAGCTGACAGTCCAAGAACA GGACT-3' (antisense); Cyclin D1: 5'-GGATGCTGGAGGT CTGCGAGGAAC-3' (sense) and 5'-GAGAGGAAGCGTGT GAGGCGGT-3' (antisense); ß-actin: 5'-TCACCCACACTG TGCCCATCTACGA-3' (sense) and 5'-CAGCGGAACCGC TCATTGCCAATGG-3' (antisense).

Analysis of interleukin 6 production. The presence of extracellular human IL-6 protein was determined using a high-sensitivity ELISA kit (Amersham). With this assay the minimum detectable concentration of human IL-6 is 1.4 pg/ml. Briefly, HA22T/VGH cells were seeded at $1x10^4$ cells/well onto 96-well plates and incubated overnight. The medium was then aspirated and, after two washes with RPMI medium, the cells were incubated in fresh complete medium containing different concentrations of DHMEQ, cisplatin or their combinations. After 8 h of incubation, the medium was collected to measure the extracellular content of IL-6.

Analysis of synergistic cytotoxicity. Synergistic cytototoxicity was determined by calculating the interaction index (I) according to the classic isobologram equation (19): $I = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where *Dx* is the concentration of one compound alone required to produce the effect (in this case 50% inhibition of cell growth) and $(D)_1$ an $(D)_2$ are the concentration of both compounds that produce the same effect.

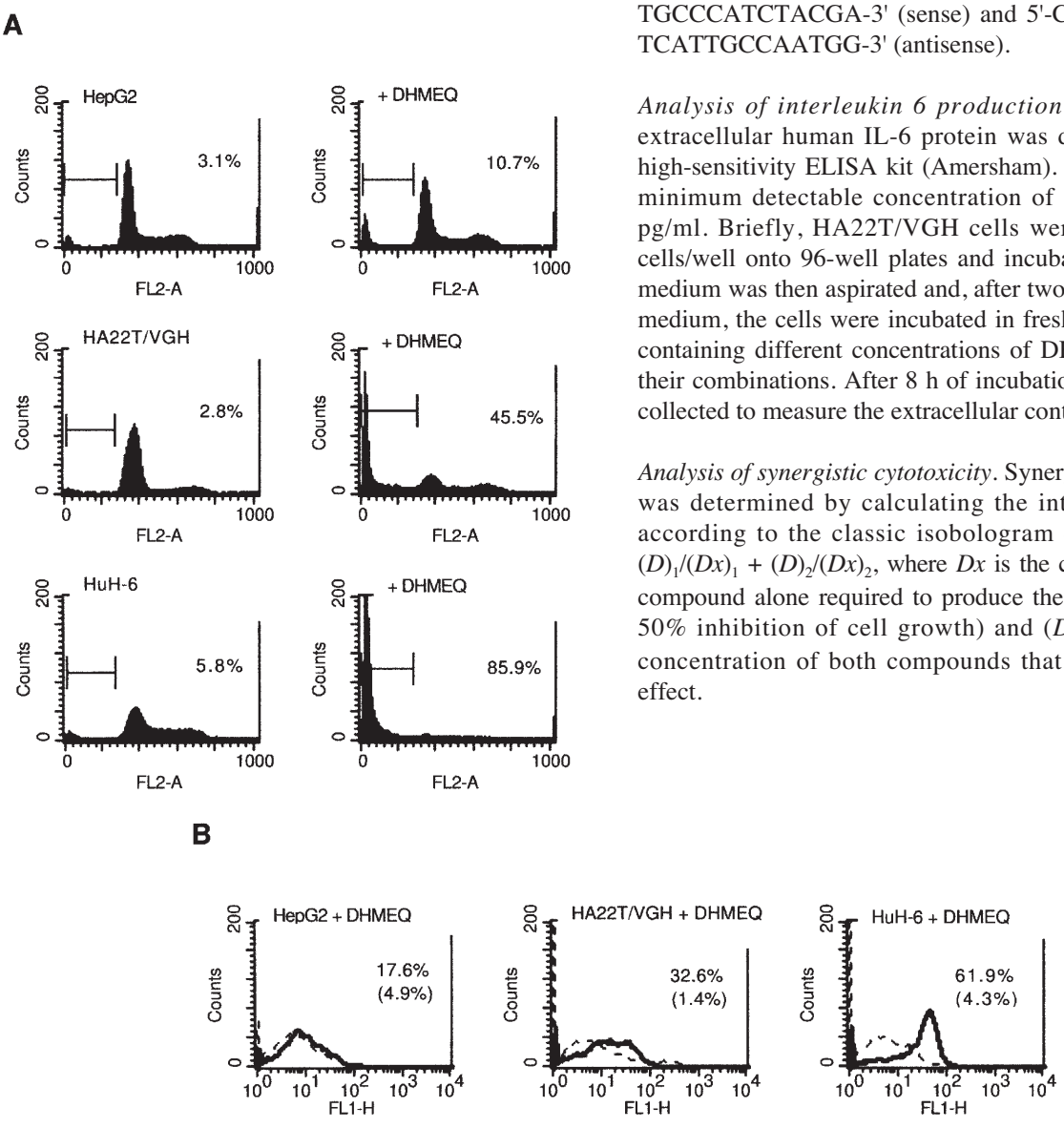

Figure 2. Representative example of flow cytometry analysis of apoptosis. The cells were treated with DHMEQ 10 μg/ml or not for 48 h. Shown in (A) are the profiles of propidium iodide-stained DNA; indicated in the panels are the percentages of events in the $preG_0$-G_1 position. In (B), the cells were stained with FITC-labeled annexin V; indicated in the panels are the percentages of positive treated cells and, in parenthesis, the percentages of positive control cells. For further details see Materials and methods. Two repeat experiments gave very similar results.

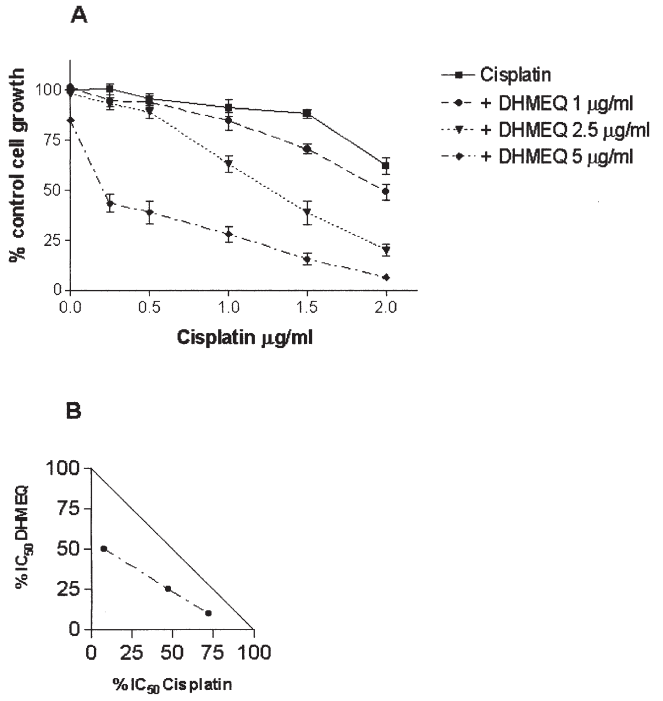

Figure 3. Synergistic cytotoxicity of DHMEQ in combination with cisplatin. (A), HA22T/VGH cells were incubated with different concentrations of DHMEQ, cisplatin or combination thereof for 72 h and cell growth was assessed by the MTS dye reduction assay. Data are expressed as % of control cell growth and are the mean ± SE of three separate experiments, each of which was performed in quadruplicate. (B), The relative isobolographic analysis of synergistic cytotoxicity is presented.

Statistical analysis. Results are given as means ± SE. The significance of differences between means was evaluated by Student's t-test for unpaired samples.

Results

Cell NF-κB content and antitumor effects of DHMEQ, alone or in combination with cisplatin. We preliminarily evaluated the baseline activity of NF-κB in nuclear extracts of the cells by EMSA (Fig. 1): the level was low in HepG2 and higher in

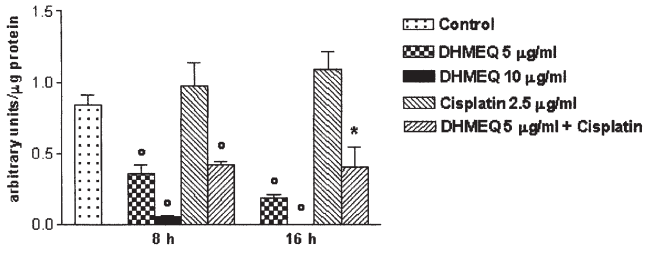

Figure 5. NF-κB (p65 subunit) DNA binding capacity in nuclear extracts of HA22T/VGH cells. The cells were treated for 8 or 16 h with DHMEQ, cisplatin or their combination. Results are expressed as arbitrary units/μg protein of HA22T/VGH nuclear extracts and the mean ± SE of three (six for the control) independent observations. *P<0.05 versus control; °P<0.01 versus control. For further details see Materials and methods.

HA22T/VGH and especially HuH-6. The antitumor effects of DHMEQ were therefore studied by MTS assays: after 72 h of treatment the concentrations which caused 50% inhibition of cell growth were 15.5 μg/ml in HepG2, 9.9 μg/ml in HA22T/VGH and 6.1 μg/ml in HuH-6. DHMEQ induced also different extents of apoptosis in the cell lines (Fig. 2).

The cells were also treated with combinations of different concentrations of DHMEQ and cisplatin and MTS assays were performed. The effects were additive in HepG2 and HuH-6 (data not shown) and synergistic in HA22T/VGH: in these cells, the interaction index (I) for 50% inhibition of cell growth was 0.82 at DHMEQ 1 μg/ml, 0.72 at DHMEQ 2.5 μg/ml, 0.58 at DHMEQ 5 μg/ml (Fig. 3). The synergy between DHMEQ and cisplatin in HA22T/VGH was confirmed in long-term colony forming assays (data not shown) and by determining cell death (Fig. 4).

Effect of DHMEQ, alone or in combination with cisplatin, on the nuclear levels of activated NF-κB. To investigate the mechanism of DHMEQ and its synergy with cisplatin, we analyzed the nuclear levels of activated NF-κB in the treated HA22T/VGH cells. DHMEQ induced clear dose-dependent reductions in the activated factor (42% and 6% of the control 8 h after administering DHMEQ 5 and 10 μg/ml, respectively;

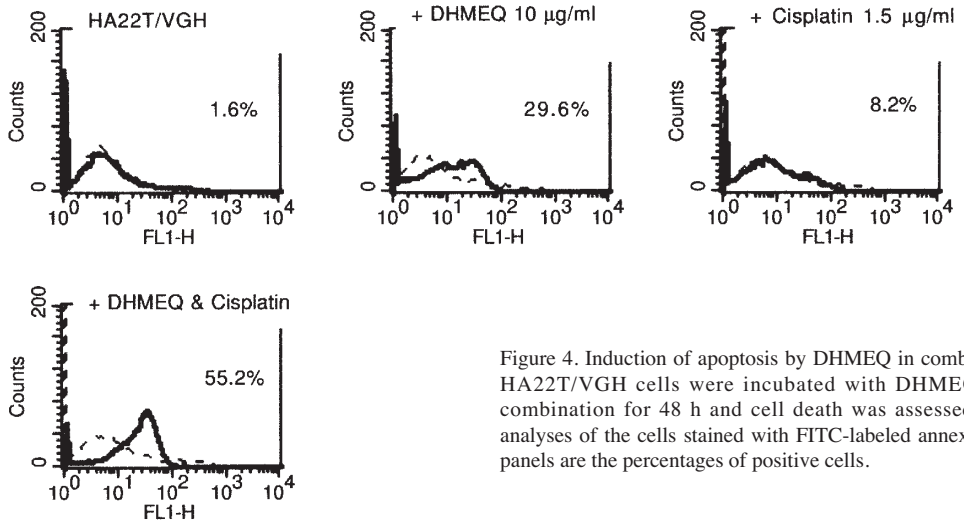

Figure 4. Induction of apoptosis by DHMEQ in combination with cisplatin. HA22T/VGH cells were incubated with DHMEQ, cisplatin or their combination for 48 h and cell death was assessed by flow cytometry analyses of the cells stained with FITC-labeled annexin V. Indicated in the panels are the percentages of positive cells.

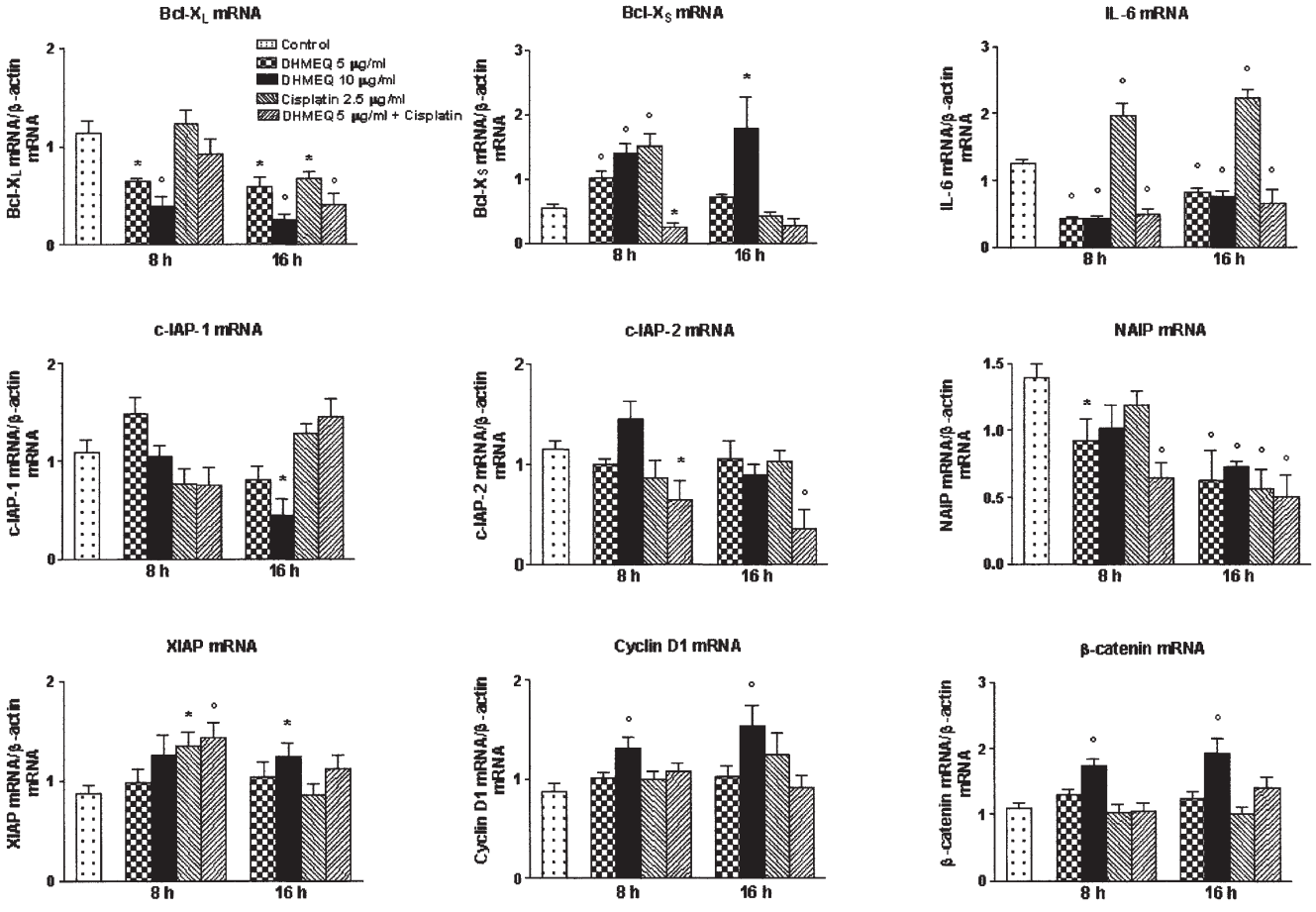

Figure 6. Effects of DHMEQ, alone or in combination with cisplatin, on gene expression in HA22T/VGH cells. The cells were treated for 8 or 16 h with DHMEQ, cisplatin or their combination. The level of expression of the different mRNAs was assessed by RT-PCR as reported in Materials and methods. Data are expressed as arbitrary units (relevant mRNA/ß-actin mRNA) and are the mean ± SE of five independent (ten for the control) observations; in the case of IL-6 mRNA data are the mean ± SE of four independent (eight for the control) observations. *P<0.05 versus control; °P<0.01 versus control.

22% and 0% of the control 16 h after DHMEQ 5 and 10 μg/ml, respectively) (Fig. 5). Cisplatin caused minor, non-significant, increases (115% and 130% of the control at 8 and 16 h, respectively) in activated NF-κB; the levels of the factor were significantly lower (49% and 48% of the control at 8 h and 16 h, respectively) when cisplatin was combined with DHMEQ 5 μg/ml.

Effect of DHMEQ, alone or in combination with cisplatin, on gene expression in HA22T/VGH cells. Further, gene expression in HA22T/VGH cells was investigated by specific RT-PCR procedures (Figs. 6 and 7). The cells lacked Bcl-2 (not shown), but showed detectable levels of the transcripts of different other tumor promoting genes, some of which (like *IL-6, COX-2, c-myc, mdr-1* (P-glycoprotein), *Bcl-X$_L$* and some *IAPs*) have been shown to be transcriptional targets of NF-κB (12,13,20-24).

DHMEQ down-regulated Bcl-X$_L$ mRNA levels in a dose-dependent manner (57% and 51% of the control with 5 μg/ml and 34% and 23% of the control with 10 μg/ml, at 8 and 16 h, respectively) and up-regulated those of Bcl-X$_S$ (187% and 133% of the control with 5 μg/ml and 259% and 329% of the control with 10 μg/ml, at 8 and 16 h, respectively). It decreased also IL-6 (33% and 66% of the control with 5 μg/ml and 34% and 60% of the control with 10 μg/ml, at 8 and 16 h, respectively), NAIP (65% and 44% of the control with 5 μg/ml and 72% and 52% of the control with 10 μg/ml, at 8 and 16 h, respectively) and, after 16 h of exposure to the higher dose, c-IAP-1 (40% of the control) mRNA levels. At 10 μg/ml, it caused also significant increases in Bax (155% of the control at 8 h with p<0.01, not shown) XIAP (143% of the control at 16 h), cyclin D1 (150% and 176% of the control, at 8 and 16 h, respectively) and ß-catenin (157% and 176% of the control, at 8 and 16 h, respectively) mRNAs.

For cisplatin (2.5 μg/ml), it decreased at 16 h Bcl-X$_L$ (59% of the control) and NAIP (40%) expression. It up-regulated IL-6 (160% and 179% of the control, at 8 and 16 h respectively) and XIAP (154% of the control at 8 h) mRNAs. The combination of DHMEQ 5 μg/ml with cisplatin 2.5 μg/ml produced significant decrease in c-IAP-2 (55% and 30% of the control, at 8 and 16 h, respectively) and Bcl-X$_S$ (49% of the control at 8 h) mRNAs; the decrease was not seen with the single agents. The combination gave also substantially additive decrease in Bcl-X$_L$ (36% of the control at 16 h), IL-6 (39.8% and 52.7% of the control at 8 and 16 h, respectively), and NAIP (46% and 36% of the control, at 8 and 16 h, respectively) mRNAs, as well an additive increase in XIAP (163% of the control at 8 h) mRNA. Finally, DHMEQ

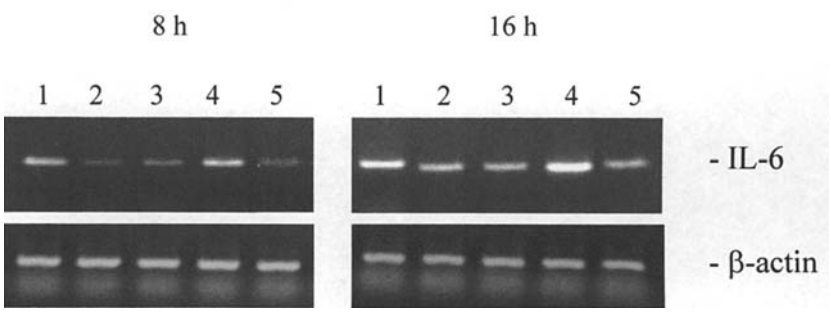

Figure 7. Representative analysis of the effect of DHMEQ, alone or in combination with cisplatin, on IL-6 mRNA expression in HA22T/VGH cells. The cells were treated for 8 or 16 h with DHMEQ, cisplatin or their combination. IL-6 mRNA expression was assessed by RT-PCR as reported in Materials and methods. RT-PCR with ß-actin primers was performed as a control for the same amount of RNA. Lane 1, control; lane 2, DHMEQ 5 μg/ml; lane 3, DHMEQ 10 μg/ml; lane 4, cisplatin 2.5 μg/ml; lane 5, DHMEQ 5 μg/ml plus cisplatin 2.5 μg/ml.

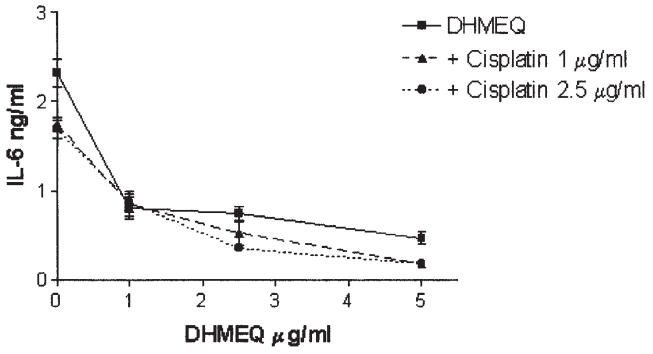

Figure 8. Effects of DHMEQ, alone or in combination with cisplatin, on IL-6 production by HA22T/VGH cells. The cells were treated for 8 h with the agents and then IL-6 release was determined as reported in Materials and methods. Data are the mean ± SE from an experiment performed in triplicate. A repeat experiment gave very similar results.

and cisplatin, alone or in combination, determined only minor and non-significant variations of the mRNAs of the other genes tested (*survivin, livin, COX-2, c-myc, mdr-1*, data not shown) at the time intervals (8 or 16 h of exposure) considered.

DHMEQ inhibition of IL-6 production by HA22T cells does not explain the antitumor effects of the agent or its synergy with cisplatin. On the basis of previous studies (25,26), we have considered the possibility that the antitumor effects of DHMEQ and its synergy with cisplatin might be attributed, at least in part, to the ability of the agent to interfere with an autocrine/paracrine loop based on the production of the possible growth and pro-survival factor IL-6. In fact, HA22T/VGH (Fig. 8), but not HepG2 cells (27) or HuH-6 (personal unpublished data), release IL-6; in agreement with the results on mRNA, DHMEQ was able to interfere, also in the presence of cisplatin, with such production (Fig. 8). Nevertheless, by immunocytometry and ELISA assays we have ascertained that HA22T/VGH cells do not express at the cell surface the α chain of the IL-6 receptor or release it in soluble form; in addition, exposure to a neutralizing anti-IL-6 antibody for up to 7 days or the use of an efficient anti-IL-6 siRNA did not affect the growth of HA22T/VGH cells (Notarbartolo *et al*, unpublished data), thus excluding a role of the autologous IL-6 in DHMEQ antitumor effects.

Discussion

We are studying possible strategies to overcome the resistance to drugs and apoptosis characterizing cancer with a poor prognosis such as HCC. NF-κB is often constitutively activated in HCC (14,15) and inhibition of the transcription factor might be of help to antagonize the IAPs and other target genes implicated in the biology of this tumor. Moreover, interfering with NF-κB has often been shown to increase the tumor cell response to different NF-κB activating chemotherapeutic drugs (28-30).

We tested the activity of the novel NF-κB inhibitor DHMEQ, alone or in combination with cisplatin, in three HCC cell lines: the sensitivity to the antitumor effects of the compound positively correlated to higher basal amounts of activated NF-κB, implying the specificity of the mechanism involved (16). In addition, in HA22T/VGH cells the combination of DHMEQ with cisplatin was synergistic. In these cells, DHMEQ exerted clear dose-dependent decrease in the nuclear levels of activated NF-κB (p65 subunit) and reduced the nuclear activated factor also in presence of cisplatin.

The NF-κB inhibition was accompanied by decrease in the mRNAs of some IAPs (c-IAP-1 and NAIP) and of IL-6 and by a noticeable inversion of the ratio between the anti-apoptotic isoform of Bcl-X, Bcl-X_L, and the pro-apoptotic Bcl-X_S. Other possible NF-κB target genes were not significantly influenced by DHMEQ, but this is not surprising if the multiplicity of the factors which may regulate their expression is considered. For example, *COX-2, c-myc* and *mdr-1* can be transcriptionally up-regulated by Tcf/ß-catenin (31-33) and, indeed, ß-catenin mRNA was increased by treatment with DHMEQ. The mRNA of pro-apoptotic Bax was also elevated by the agent, along with up-regulation of other genes such as XIAP and cyclin D1, which instead promote cell proliferation and survival. For cisplatin, it up-regulated XIAP mRNA after 8 h and decreased Bcl-X_L and NAIP expression after 16 h. Its combination with DHMEQ (5 μg/ml) resulted in reduction in c-IAP-2, but also in Bcl-X_S, mRNA levels. The combination produced substantially

additive effects of decrease (IL-6, NAIP and Bcl-X_L) or of increase (XIAP at 8 h) in gene expression.

We have considered the possibility that, similarly to previous studies on other tumor cell types (25,26), the anti-tumor effects of DHMEQ and in particular its synergy with cisplatin might be, at least partly, due to an interference of the agent with an autocrine/paracrine loop based on the possible growth and pro-survival factor IL-6. In contrast to HepG2 and HuH-6, HA22T/VGH cells exhibited an abundant production of IL-6 protein and, in accordance with the results on mRNA, DHMEQ was able to inhibit such a process. However, by a thorough analysis of the status of the IL-6 receptor and of the response to specific anti-IL-6 approaches, we excluded that such effect is involved in the anti-tumor activity of the agent on HA22T/VGH cells and the reasons why DHMEQ synergized with cisplatin only in these cells deserve to be further investigated. On the other hand, since release of IL-6 by tumor cells is frequent in HCC, especially in its more advanced stages (34), our results indicate that the use of DHMEQ in this tumor might be beneficial also to contrast the adverse systemic effects (e.g., fatigue and cachexia) of the cytokine.

In conclusion, our present results underline the possible therapeutic validity of interfering with constitutively activated NF-κB in HCC, supporting the possible use of the novel agent DHMEQ, possibly combined with cisplatin, in this cancer. At a molecular level, the compound induced clear pro-apoptotic and anti-inflammatory changes, which, like in other contexts (13,35,36), involved also some IAPs. There were also some opposite, likely counterbalancing, influences on the mRNAs of other genes regulating cell proliferation and survival. A more complete understanding of the mechanisms regulating the transcription of the different IAPs is needed, also to exploit more fully the potential of their inhibition in the therapy of HCC.

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