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-XL mRNA in a dose-dependent manner and up-regulated that of Bcl-XS. 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 β-catenin mRNAs. The combination of DHMEQ with cisplatin produced unexpected significant decrease in c-IAP-2 and Bcl-Xs mRNAs as well as additive decrease (IL-6, NAIP and, after 16 h, Bcl-Xs) 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.

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 growth assays. To test the effects of the agents, the cells were seeded at 2x10^5 cells/well onto 96-well plates and then incubated overnight. At time 0, medium was replaced with fresh complete medium and DHE, cisplatin or combinations thereof were added in concentrations as indicated. At the end of treatment, 15 μl of a commercial solution (obtained from Promega Corporation, Madison, WI, USA) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate were added. The plates were incubated for 2 h in a humidified atmosphere at 37˚C in 5% CO₂. The bioreduction 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.

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 biotinylated 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 by 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 β-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 β-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-XL, IL-6, c-IAP-1, NAIP, XIAP, Bax and β-catenin), 60˚C (COX-2, cyclin D1 and c-myc) or 62˚C (Bcl-2, survivin and livin-a) 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 β-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 the other mRNAs were 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 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 preG₀-G₁ position. The occurrence of apoptosis was determined by evaluating the percentage of events accumulated in the sub-G₀-G₁ position. The data were analyzed with CellQuest software by subtracting control cells from the cell population stained 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.
IL-6: 5'-ATGAACTCCTTCTCCACAAGCGC-3' (sense) and 5'-GAAGAGCCCTCAGGCTGGACTG-3' (antisense); Bax: 5'-TGCTTCAGGGTTTCATCCAG-3' (sense) and 5'-GGCGGCAATCATCCTCTG-3' (antisense); c-IAP-1: 5'-TGACTTTTCCTGTGAACTCT-3' (sense) and 5'-GCCTTTCATTCGTATCATCAAGA-3' (antisense); c-IAP-2: 5'-ATGAACATCTAGAAAACAGC-3' (sense) and 5'-CCTGTCCTTTAATTCTATCA-3' (antisense); NAIP: 5'-AAATGTGAATTTCTTGGAGT-3' (sense) and 5'-TTTTGAAGCAATAGACAGATC-3' (antisense); XIAP: 5'-GCAGGGTTTCTTTATACGTGG-3' (sense) and 5'-TGTCCCTTCTGTCTAAGACAGATC-3' (antisense); survivin: 5'-GCATGGGTGCCCCGACGTTG-3' (sense) and 5'-GCTCCGGCCAGAGGCCTCAA-3' (antisense); livin-1: 5'-GTCCCTGCTCTGCTTAC-3' (sense) and 5'-CAGGGAGCCCACTCTCTG-3' (antisense); P-glycoprotein: 5'-GCCTGGCAGCTGGGAAGACAAATTACAAAATT-3' (sense) and 5'-CAGACAGCAGCTGACAGTCCAAGAAGACT-3' (antisense); Cyclin D1: 5'-GGATGCTGGAGGTCTGCGAGGAAC-3' (sense) and 5'-GAGAGGAAGCGTGAGGCGGT-3' (antisense); \( \beta \)-actin: 5'-TCACCCACACTGTGCCCATCTACGA-3' (sense) and 5'-CAGCGGAACCGCTATTGCCAATGG-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 cytotoxicity was determined by calculating the interaction index (I) according to the classic isobologram equation (19): 

\[ I = \frac{(D_1)_{1/2 + (D_2)_{1/2}}}{D_x_{1/2} + D_x_{1/2}}, \]

where \( D_x \) is the concentration of one compound alone required to produce the effect (in this case 50% inhibition of cell growth) and \( (D_1)_{1/2} \) and \( (D_2)_{1/2} \) are the concentration of both compounds that produce the same effect.
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 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;
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-XL and some IAPs) have been shown to be transcriptional targets of NF-κB (12,13,20-24).

DHMEQ down-regulated Bcl-XL 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-XS (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 β-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-XL (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-XL (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-XL (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
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 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-XL, and the pro-apoptotic Bcl-Xs. 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/β-catenin (31-33) and, indeed, β-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-Xs and NAIP expression after 16 h. Its combination with DHMEQ (5 μg/ml) resulted in reduction in c-IAP-2, but also in Bcl-Xs mRNA levels. The combination produced substantially
additive effects of decrease (IL-6, NAIP and Bcl-XL) 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 previous studies on other tumor cell types (25,26), the anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci USA 98: 8662-8667, 2001.


References


