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Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-kB activation levels and in IAP gene expression

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Abstract

The hepatic cancer HA22T/VGH cell line, which constitutively expresses activated nuclear factor-kappaB (NF-kB), was chosen as a model to examine the antitumor activity of curcumin, also in relationship to its possible influences on the activation of the transcription factor and on the expression of the inhibitory of apoptosis proteins (IAPs) and of other NF-kB target genes. Curcumin exerted cell growth inhibitory and apoptotic effects, related, at least part, to free radical generation and mainly dependent on caspase-9 and -3 activation. The combination of curcumin with cisplatin resulted in a synergistic antitumor activity and that with doxorubicin in additivity or sub-additivity. Curcumin exerted biphasic changes in the levels of NF-kB, with an increase at 8 h after its administration and a decrease at 16 h. For the combinations of curcumin with the other drugs, the levels of the transcription factor were lower than those predicted from the effects of the single agents, especially with a blunting of the remarkable increases in NF-kB activation induced by doxorubicin. Except for Bcl-2, the HA22T/VGH cells expressed different other genes, including the *IAPs*, implicated in cell proliferation and survival. Curcumin determined early changes in COX-2 and c-myc mRNAs, which were down-regulated, and in livin mRNA, which was up-regulated. Later it decreased Bcl-X_L mRNA and increased Bcl-X_S and c-IAP-2 mRNAs. Cisplatin and doxorubicin exerted distinct effects on gene expression. The cytotoxic interactions between curcumin and these agents were accompanied by synergistic (in particular with cisplatin) or additive effects of decrease in the expression of different genes, including *c-myc*, *Bcl-X_L*, *c-IAP-2*, *NAIP* and *XIAP*. However, the combinations attenuated also certain other influences on mRNA expression of the single agents, like, for example, the increases in Bcl-X_s given by curcumin and doxorubicin. Overall, the effects of the drugs, alone or in combination, on

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tumor cell growth, cell death and gene expression did not show a simple relationship to the relative influences on NF-kB activation, inferring that they can be due also to other mechanisms.

Keywords: Hepatocellular carcinoma; Curcumin; NF-kB; Inhibitory of apoptosis proteins; Cisplatin; Doxorubicin

1. Introduction

There is an increasing evidence that the inability of the cells to undergo apoptosis may critically contribute to the genesis and progression of cancer and represent an important cause of tumor drug resistance. This well applies to hepatocellular carcinoma (HCC), a highly aggressive tumor which responds poorly to currently available therapies [1]. Indeed, this cancer frequently shows the over-expression of anti-apoptotic factors like the Bcl-2 family members and the IAPs (inhibitory of apoptosis proteins) [2–6]. IAPs, which in humans include c-IAP-1, c-IAP-2, XIAP, NAIP survivin and livin-α (known also as ML-IAP), have shown a remarkable ability of blocking cell death induced by very many non-related triggers [7–9]. They can in fact bind and potently inhibit key effector caspases. Further, some of them, like XIAP, can trigger the proteasome degradation of pro-apoptotic substrates, like caspases and Smac [7,10,11].

The expression of XIAP and other IAPs can be up-regulated by nuclear factor-kappaB (NF-kB) [12,13], which, in turn, is frequently constitutively activated in HCC [14,15]. Thus, the inhibition of the transcription factor might be of help to antagonize the IAPs as well as other NF-kB target genes (e.g. *COX*-2, *Bcl-X_L* and *c-myc*) involved in the adverse biology of this cancer [3,16–19]. To address this aspect, we have examined the effects of curcumin, alone or in combination with conventional anticancer agents like cisplatin and doxorubicin, on the HCC cell line HA22T/VGH. Curcumin, a polyphenolic compound extracted from rhizomes of *Curcuma* species, has in fact been shown to possess interesting anti inflammatory and antitumor properties, which, at least in part, appear be linked to its ability to suppress the activation of NF-kB [20–22]. Since the HA22T/VGH cell line constitutively expresses activated NF-kB, we thought that it might be an appropriate model to examine the possible relationship of the antitumor activities of curcumin to its influences on the activation of NF-kB as well as on the expression of IAPs and other NF-kB target genes.

2. Materials and methods

2.1. Agents

Curcumin, cisplatin and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich Srl, Milan, Italy, doxorubicin from Pharmacia, Milan, Italy, z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-fmk (z-DEVD-fmk) from Calbiochem, Milan, Italy and z-Ile-Glu-Thr-Asp-fmk (z-IETD-fmk) and z-Leu-Glu (OMe)-His-Asp (OMe)-fmk (z-LEDH-fmk) from Alexis Biochemicals, Laufelfingen, Switzerland.

2.2. Cell culture

HA22T/VGH is a poorly differentiated hepatoma cell line which contains HBV integrants. It was kindly provided by Professor M. Levrero (Laboratory of Gene Expression, Fondazione Andrea Cesalpino, University of Rome 'La Sapienza', Rome, Italy) and was 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.

2.3. Cell growth assays

To test the effects of the agents, the cells were seeded at 2×10^4 cells/well onto 96-well plates and then incubated overnight. At time 0, medium was replaced with fresh complete medium and curcumin, cisplatin, doxorubicin 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-carboxy methoxyphenyl)-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 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.

2.4. Cell proliferation assay

The ability of curcumin, alone or in combination with cisplatin or doxorubicin, to inhibit DNA synthesis was determined by estimating the amount of bromodeoxyuridine (BrdU) incorporation into DNA by a colorimetric immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells were cultured in 96-well plates (2.5×10^3) in the presence of the agents for 24 h. BrdU (10 μM final concentration) was added, and the cells were re-incubated for an additional 24 h. The cells were fixed and incubated with anti-BrdU-POD (100 μl per well) for 2 h at room temperature. The color was developed by the addition of tetramethylbenzidine substrate and measured at 490 nm. The color intensity and the absorbance values directly correlate to the amount of BrdU incorporated into DNA. The results were expressed as percent inhibition of BrdU incorporation over the control.

2.5. Evaluation of cell death by flow cytometry

Cells were washed twice with ice-cold PBS and then resuspended at 1×10^6/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_o-G_1 position. The occurrence of apoptosis was evaluated also by studying phosphatidylserine exposure on cell surface and the penetration of propidium iodide into the cells. The cells were resuspended at 1×10^6/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 (Pharmingen, San Diego, CA) and propidium iodide 5 μg/ml for 10 min and then analyzed by flow cytometry. Finally, the cells were fixed and permeabilized with Cytofix/Cytoperm (Pharmingen) and then washed twice with PBS containing 1% (w/v) bovine serum albumin (BSA). They were incubated on ice for 40 min with FITC-conjugated anti-caspase 3 active form monoclonal antibody (mAb). After two washes with PBS/BSA, the cells were examined by flow cytometry. The results were analyzed using the CellQuest™ software by subtracting the cells stained with a FITC-conjugated negative control mAb from the cell population stained with the anti-caspase 3 Ab.

2.6. NF-kB activation

The DNA-binding capacity of NF-kB (p65 subunit) was measured in the nuclear extracts of HA22T/VGH cells using the Nuclear Extract™ and TransAM™ NF-kB Kits (Active Motif, Carlsbad, CA, 92008, USA) according to the manufacturer's instructions. Briefly, the assay is based on a 96-well plate to which an oligonucleotide containing the NF-kB consensus binding site (5′-GGGACTTTCC-3′) has been immobilized. The activated NF-kB contained in nuclear extracts specifically binds to this nucleotide. By using an antibody that is directed against an epitope on p65 that is accessible only when NF-kB is bound to its target DNA, the NF-kB 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 was 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 HA22T/VGH nuclear extract.

We studied also the nuclear disposition of NF-kB by immunofluorescence. The cells were grown at a density of 2×10^4/well on glass 8-well tissue culture chamber slides (Nunc Inc., Naperville, IL, USA) coated with 2 μg/ml of poly-L-lysine (Sigma-Aldrich). After the treatments, the cells were washed with PBS and fixed with cold methanol for 5 min at −20 °C. The cells were incubated with a 1:100 dilution of mouse monoclonal antibody against NF-kB p65 subunit (clone F-6, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, then with biotin conjugated goat anti-mouse IgG (dilution 1:100, from Pierce Endogen, Rockford, IL, USA) and finally with fluorescein isothiocyanate conjugated to streptavidin (dilution 1:60, from Pierce Endogen) for 30 min. The cells were examined with an Axioskop 2 Zeiss fluorescence microscope. Between each step the cells were washed three times with PBS.

2.7. Evaluation of mRNA expression by semiquantitative RT-PCR

Total RNA was isolated from 1×10^6 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, Little Chalfont, UK). 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), 58 °C (Bcl-X_L, c-IAP-1, NAIP, XIAP and β-catenin), 60 °C (COX-2 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 β-actin 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 identity of the amplification products was confirmed by performing digestion of the amplification products by appropriate restriction enzymes. The sequence of primers used in the RT-PCR was as follows:

β-catenin: 5′-CTGATTTGATGGAGTTGGAC-3′ (sense) and 5′-CTGCTACTTGTTCTTGAGTG-3′ (antisense);

COX-2: 5′-GAGAAAACTGCTCAACACCG-3′ (sense) and 5′-GCATACTCTGTTGTGTTCCC-3′ (antisense);

c-myc: 5′-AGGCAGACGGAGCTGGAGCC-3′ (sense) and 5′-ATGTCTTGCGCGCAGCCTG-3′ (antisense);

Bcl-2: 5′-ATGGCGCACCGTGGGAGAA-3′ (sense) and 5′-TGTGGCCCAGATAGGCACC-3′ (antisense);

Bcl-X_L: 5′-TTGGACAATGGACTGGTTTGA-3′ (sense) and 5′-GTAGAGTGGATGGTCAGTG-3′ (antisense);

c-IAP-1: 5′-TGACTTTTCCTGTGAACTCT-3′ (sense) and 5′-GCCTTTCATTCGTATCAAGA-3′ (antisense);

c-IAP-2: 5′-ATGAACATACTAGAAAACAGC-3′ (sense) and 5′-CCTGTCCTTTAATTCTTATCA-3′ (antisense);

NAIP: 5′-AAATGTGAATTTCTTCGGAGT-3′ (sense) and 5′-TTTTGAAGCAATAGACAGATC-3′ (antisense);

XIAP: 5′-GCAGGGTTTCTTTATACTGG-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′-CAGGGAGCCCACTCTGCA-3′ antisense;

β-actin: 5′-TCACCCACACTGTGCCCATC-TACGA-3′ (sense) and 5′-CAGCGGAACCGCT-CATTGCCAATGG-3′ (antisense).

2.8. Analysis of synergistic cytotoxicity

Synergistic cytotoxicity was determined by calculating the interaction index (I) according to the classic isobologram equation [23]: $I=(D)_1/(Dx)_1+(D)_2/(Dx)_2$,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.

2.9. Statistical analysis

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

3. Results

3.1. Antitumor and cell death effects of curcumin alone or in combination with cisplatin or doxorubicin

The antitumor effects of curcumin on HA22T/VGH cells were first evaluated by MTS assay: after 72 h of treatment the concentrations of the drug which caused 50 and 70% inhibition of cell growth were 39.1 and 79.2 μM, respectively. We determined also BrdU incorporation into DNA and this assay was more sensitive, since the concentrations of curcumin which inhibited of 50 and 70% DNA synthesis after 48 h of treatment were 9.8 and 12.3 μM, respectively. Curcumin induced apoptosis, which was evaluated by flow cytometry analyses of DNA stained with propidium iodide, of annexin V binding and penetration of propidium iodide into the cells and of levels of caspase-3 activation (Fig. 1(A)–(C)). The drug changed also the distribution of the cells in the phases of the cell cycle, with a shift from G_o-G_1 to S and G_2-M (Fig. 1(A)).

In separate experiments, the antioxidant NAC reduced of about 30% cell death from curcumin, suggesting that this process involves, at least partially, an increase in free radical generation (Table 1). Further, the results of co-treatments with the caspase-3 (Z-DEVD-fmk), caspase-9 (Z-LEDH-fmk) or caspase-8 (Z-IETD-fmk) inhibitors indicated that in HA22T/VGH cells apoptosis from curcumin, beside by caspase-3, preferentially occurs through the activation of caspase-9, rather than of caspase-8 (Table 1).

Therefore, the HA22T/VGH cells were treated contemporaneously with curcumin and other agents and cytotoxicity was evaluated by the MTS assay (Fig. 2). There was a synergistic cytotoxicity in combination with cisplatin, while the effects were additive or sub-additive in combination with doxorubicin: the interaction index (*I*) for 50% inhibition of cell growth was 0.69 with cisplatin and 0.90 with doxorubicin at curcumin 25 μM, 0.72 with cisplatin and 0.83 with doxorubicin at curcumin 20 μM, 0.80 with cisplatin and 1.15 with doxorubicin at curcumin 15 μM and 0.84 with cisplatin and 1.17 with doxorubicin at curcumin 10 μM (Fig. 2). A certain synergy between curcumin and cisplatin occurred also in the induction of apoptosis and of caspase-3 activation, while the effects on apoptosis of the combination of curcumin with doxorubicin were sub-additive (Fig. 1(A)–(C)). The effects of sequential treatments were therefore examined (Fig. 3): the results indicated that the cytotoxicity was potentiated only when curcumin preceded cisplatin, likely indicating that it is curcumin which sensitizes the cells to cisplatin. The effects of curcumin and doxorubicin remained substantially additive irrespectively of the sequence used.

3.2. Effect of curcumin, alone or in combination with cisplatin or doxorubicin, on NF-kB activation

NF-kB activation was studied by determining by an ELISA-based assay the DNA-binding capacity of the NF-kB p65 subunit in nuclear extracts of HA22T/VGH cells (Fig. 4). The cells showed a constitutive activation of the transcription factor. Treatment with curcumin induced biphasic changes in NF-kB activation, with an increase (187% of the control, $P<0.01$) at 8 h and a decrease (58% of the control, $P<0.01$) at 16 h. Cisplatin alone caused minor, not statistically significant, increases (117 and 130% of the control at 8 and 16 h, respectively), while the values of its combination with curcumin (116 and 65% of the control at 8 and 16 h, respectively), although not significant versus the control, were lower than those of curcumin alone at 8 h ($P<0.05$) and of cisplatin alone at 16 h ($P<0.05$). Doxorubicin determined sharp increases (639% of the control with $P<0.01$ at 8 h and 288% of the control with $P<0.01$ at 16 h), which were markedly attenuated by co-administration of curcumin (278 and 97% of the control at 8 and 16 h, respectively and both with $P<0.01$ versus doxorubicin alone). We studied also

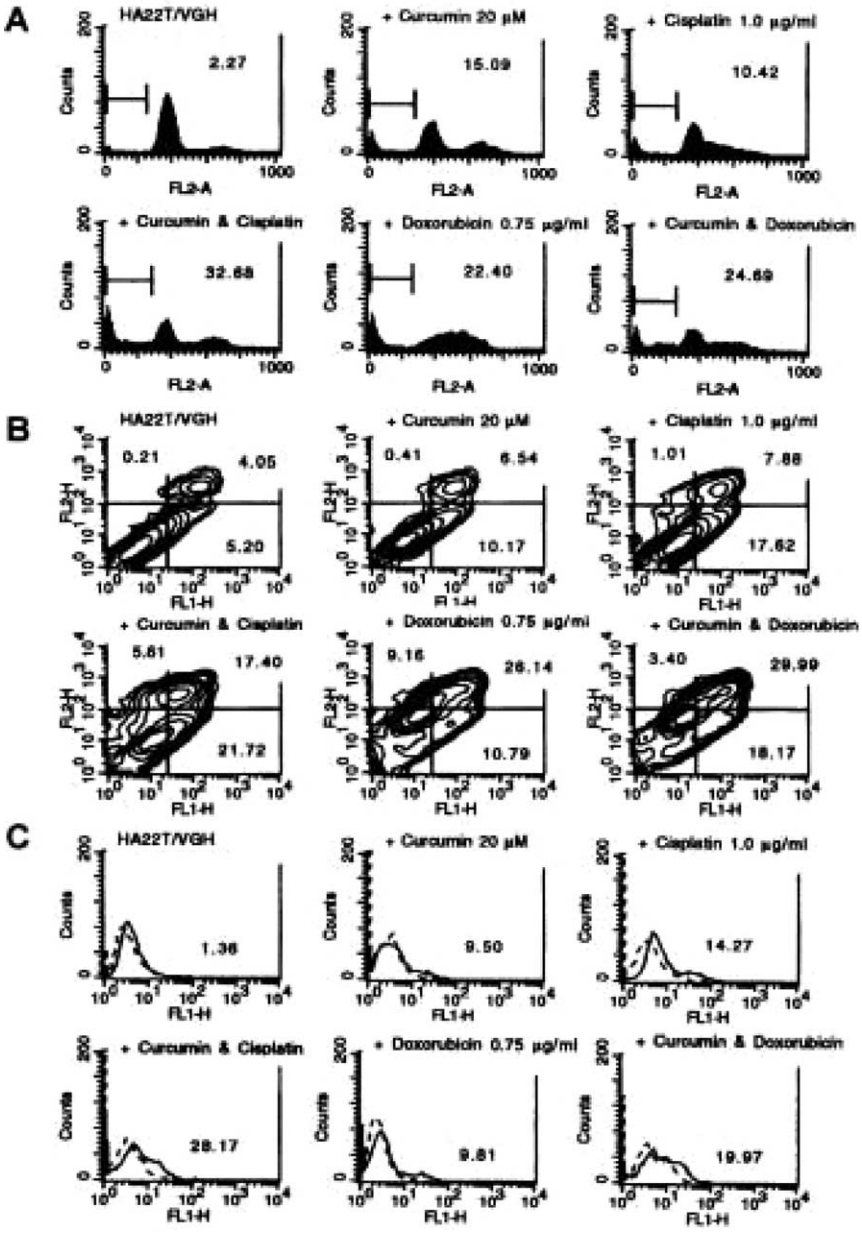

Fig. 1. Representative example of flow cytometry analysis of apoptosis. HA22T/VGH cells were treated with curcumin 20 μM, cisplatin 1 μg/ml, doxorubicin 0.75 μg/ml or combinations thereof for 48 h. Shown in (A) are the profiles of propidium iodide-stained DNA. Numbers in the panels indicate the % of the events in the preG$_o$-G$_1$ position. In (B), the cells were also stained with FITC-labeled annexin V (log fluorescence intensity on the horizontal scale) and propidium iodide (log fluorescence intensity on the vertical scale). Quadrant markers were positioned on the negative control (not shown) and the percentages of positive cells are indicated in the quadrants. Viable cells (annexin V−/propidium iodide−) are in the lower left quadrant; early apoptotic cells are in the lower right quadrant (annexin V+/propidium iodide−); late apoptotic and dead cells (annexin V+/propidium iodide+) are in the upper right quadrant; dead cells (annexin V−/propidium iodide+) are in the upper left quadrant. In (C), the percentages of cells showing activated caspase 3 are reported in each panel. For further details see Section 2.5. Two repeat experiments gave similar results.

Table 1
Effect of NAC or caspase inhibitors on cell death from curcumin

Treatment	Cell death (%)
Control	2.41 ± 0.15
Curcumin	16.77 ± 1.21
NAC 1 mM	1.97 ± 0.08
NAC 2 mM	2.69 ± 0.16
z-DEVD-fmk	3.48 ± 0.14
z-LEHD-fmk	6.52 ± 0.53
z-IETD-fmk	3.02 ± 0.22
Curcumin + NAC 1 mM	11.82 ± 0.76
Curcumin + NAC 2 mM	12.14 ± 0.50
Curcumin + z-DEVD-fmk	5.95 ± 0.42
Curcumin + z-LEHD-fmk	6.64 ± 0.56
Curcumin + z-IETD-fmk	11.68 ± 0.49

Cell death was determined 48 h after the addition of curcumin (20 μM) through flow cytometry analysis of DNA stained with propidium iodide and evaluating the percentage of events accumulated in the preG$_o$-G$_1$ position, as reported in the Section 2.5. NAC was added 1 h before curcumin. z-DEVD-fmk (20 μM), z-LEHD-fmk (2 μM) or z-IETD-fmk (20 μM) were added twice, 1 h before and 24 h after curcumin. Data are the mean ± S.E. of three separate experiments.

the nuclear disposition of NF-kB p65 subunit by immunofluorescence: both after 8 (Fig. 5) and 16 h (not shown) of treatment, the results were in good agreement with those of the ELISA-based assay, in particular showing again the remarkable increase in nuclear NF-kB induced by doxorubicin and the blunting of this phenomenon by curcumin.

3.3. Effect of curcumin, alone or in combination with cisplatin or doxorubicin, on β-catenin, COX-2, c-myc, Bcl-X$_L$, Bcl-X$_S$ and IAP mRNA levels

Except for Bcl-2 (not shown), HA22T/VGH cells showed a baseline expression of different genes, including the *IAPs*, implicated in cell proliferation and survival and mostly considered to be upregulated by NF-kB (Figs. 6 and 7). Curcumin significantly down-regulated the mRNA levels of COX-2 and c-myc (both at 39% of the control) at 8 h and of Bcl-X$_L$ (25% of the control) at 16 h. It up-regulated the mRNAs of livin (192% of the control) at 8 h and of Bcl-X$_S$ (185% of the control) and c-IAP-2 (195% of the control) at 16 h. For cisplatin, it significantly decreased the mRNA levels of β-catenin (53% of the control), c-IAP-1 (54% of the control) and XIAP (61% of the control) at 8 h and of Bcl-X$_L$ (43% of the control) and NAIP (15% of the control) at 16 h. Doxorubicin decreased the mRNA levels of COX-2 (42% of the control at 8 h), c-myc (41 and 36% of the control at 8 and 16 h, respectively), Bcl-X$_L$ (50 and 53% of the control at 8 and 16 h, respectively), c-IAP-1 (46 and 42% of the control, at 8 and 16 h, respectively), c-IAP-2 (29% of the control at 16 h) and NAIP (57 and 23% of the control, at 8 and 16 h, respectively). It increased Bcl-X$_S$ mRNA (152% of the control) at 16 h. With the combination of curcumin with cisplatin or, at a lesser degree, with doxorubicin, there were decreases greater than expected in livin and Bcl-X$_L$ mRNAs at 8 h and in c-myc, c-IAP-2 and XIAP mRNAs at 16 h. However, the association of curcumin with cisplatin annulled the effect on COX-2 or c-IAP-1 mRNAs produced at 8 h by curcumin or cisplatin, respectively. Also, curcumin antagonized the decrease in c-IAP-1 mRNA given by doxorubicin at 16 h. Finally, the decreases in Bcl-X$_L$ mRNA and the increases in Bcl-X$_S$ mRNA seen with the combinations at 16 h were either less marked or annulled with respect to those expected from the activity of the single agents.

4. Discussion

Our current research interests deal with possible strategies able to overcome the resistance to drugs and apoptosis, possibly related to IAP expression, which characterizes a cancer with a so poor prognosis such as HCC. Inhibition of NF-kB might be of help to antagonize the IAPs and other NF-kB target genes in this tumor and in this context we have focused on curcumin. This interesting polyphenolic compound is endowed with different mechanisms which may explain its anti-inflammatory and antitumor properties [20–22]. In particular, it has been frequently reported that curcumin may interfere with NF-kB activation and increase tumor cell response to different NF-kB activating anticancer drugs, including doxorubicin [22,24–26]. Nevertheless, importantly, other studies have indicated that activation of NF-kB may be required for the cytotoxicity of doxorubicin and its analogs, supporting the debated possibility that curcumin may instead diminish their antitumor activities [27,28].

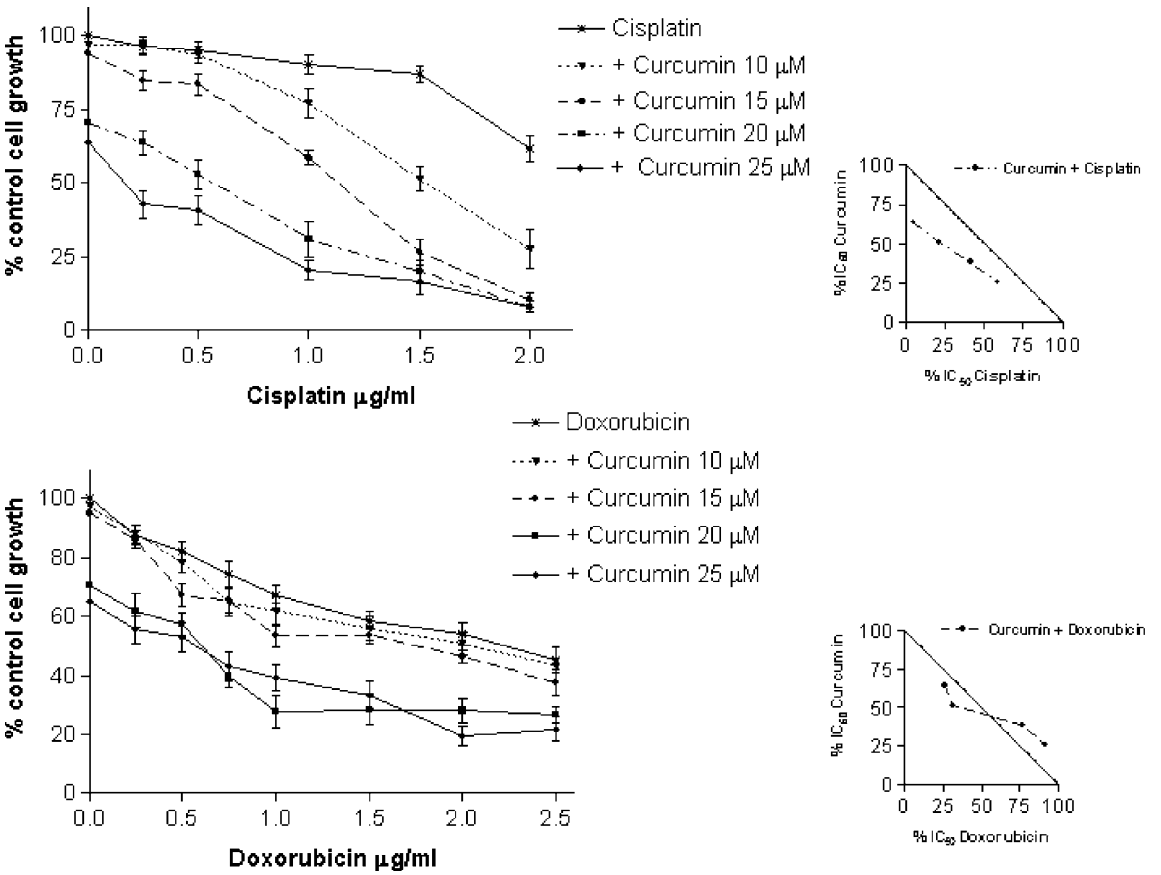

Fig. 2. Synergistic cytotoxicity of curcumin in combination with cisplatin or doxorubicin. The cells were incubated with different concentrations of curcumin, cisplatin, doxorubicin or combination thereof for 72 h and cell growth was assessed by the MTS dye reduction assay. Data are expressed as percent of control cells and are the mean ± S.E. of three separate experiments, each of which was performed in quadruplicate. Panels on the right represent the relative isobolographic analyses of synergistic cytotoxicity.

In the present work, curcumin produced significant growth inhibitory and apoptotic effects in a human HCC cell line constitutively expressing activated NF-kB. The cell death from curcumin appeared to involve, at least in part, a pro-oxidant mechanism and to be chiefly caspase-3 dependent. It occurred preferentially through caspase-9, the initiator caspase of the mitochondrial pathway for apoptosis, rather than by caspase-8, the mediator of the extrinsic pathway. Indeed, previous studies have shown that, depending on the cell models, apoptosis from curcumin can follow different pathways, based or not on increased free radicals and dependent on caspase-9, caspase-8 or both [29–31]. In some circumstances, apoptosis from curcumin appeared to be caspase-independent [31].

Further, curcumin potentiated in a sequence-dependent way the antitumor and apoptotic effects of cisplatin on HA22T/VGH cells, while its effects in combination with doxorubicin were additive or sub-additive. In a study on ovarian cancer cells, an increase in response to cisplatin by curcumin also occurred and was attributed to the ability of curcumin to interfere with the autologous production of the growth factor interleukin 6 by the cells as well as to other less defined mechanisms [32]. Nevertheless, we have seen that HA22T/VGH cells, even though release substantial amounts of interleukin 6, are not responsive to the effects of the cytokine due to the lack of interleukin 6 receptor alpha either in soluble or in membrane-bound form (unpublished observations).

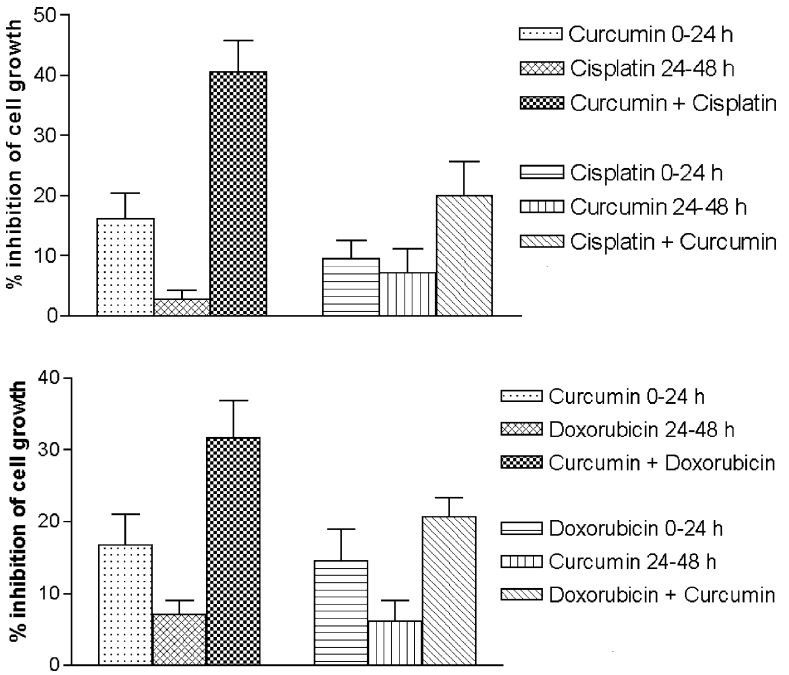

Fig. 3. Dependency of the combinatory antitumor effects on the sequence of administration. HA22T/VGH cells were treated for 24 h with one agent, the medium was aspirated and the cells were washed twice with RPMI medium. The second agent was subsequently added for other 24 h and cell growth was assessed by the MTS assay. Curcumin was applicated at 20 μM, cisplatin at 1.5 μg/ml and doxorubicin at 1.0 μg/ml. Results are the mean ± S.E. of three independent observations.

On the other hand, as said before, different studies have suggested that the mechanism of interaction of curcumin with anticancer drugs may consist in the inhibition of NF-kB [22,25,26].

With reference to this, curcumin exerted biphasic changes in the levels of the activated transcription factor in HA22T/VGH cells, with an increase after 8 h of exposure to the drug and a decrease after 16 h. When curcumin was combined with cisplatin or doxorubicin, the levels of NF-kB remained lower than those predicted from the effects of the single agents, especially with a blunting of the striking increases in NF-kB activation induced by doxorubicin. Other authors have reported that in HCC cells (the Hep3-B ones) curcumin given alone exerts only slight reducing effects on constitutive NF-kB activation, but inhibits its increases in response to doxorubicin [26]. Curcumin may act by inhibiting IkappaBα kinase [20], but, in the whole, the mechanisms by which it prevents NF-kB activation from anticancer drugs remain largely elusive [26].

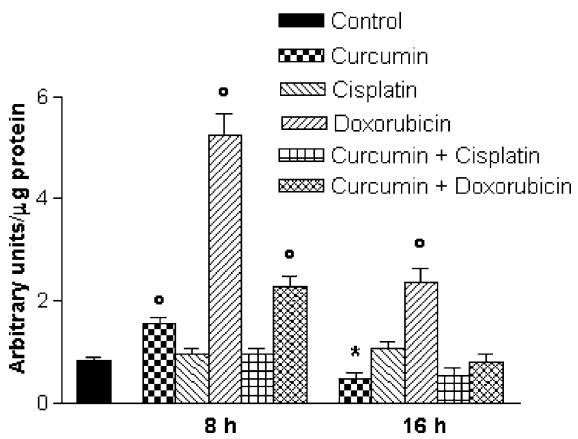

Fig. 4. NF-kB (p65 subunit) DNA binding capacity in nuclear extracts of HA22T/VGH cells. The cells were treated for 8 or 16 h with curcumin 40 μM, cisplatin 2.5 μg/ml, doxorubicin 2.5 μg/ml or combinations thereof. Results are expressed as arbitrary units/μg protein of HA22T/VGH nuclear extracts and are the mean ± S.E. of three (six for the control) independent observations. * = $P < 0.05$ versus control; ° = $P < 0.01$ versus control. For further details on the methodology of the assay see Section 2.6.

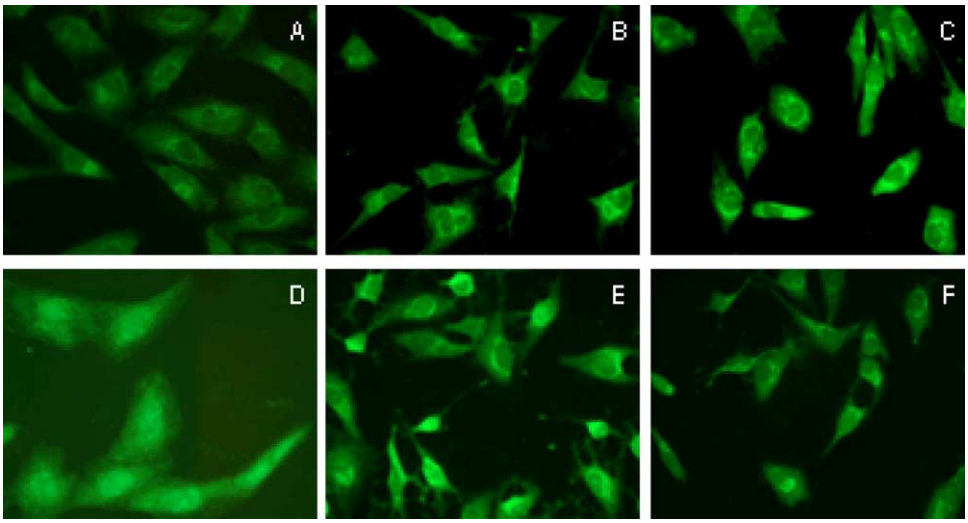

Fig. 5. Immunofluorescence analysis of NF-kB (p65 subunit) localization in the nuclei of HA22T/VGH cells after 8 h of treatment. (A) control; (B) curcumin 40 μM; (C) cisplatin 2.5 μg/ml; (D) doxorubicin 2.5 μg/ml; (E) curcumin 40 μM plus cisplatin 2.5 μg/ml; (F) curcumin 40 μM plus doxorubicin 2.5 μg/ml. A repeat experiment gave very similar results. For further details on the methodology of the assay see Section 2.6.

At the level of gene expression, curcumin decreased the levels of COX-2 and c-myc mRNAs at 8 h. At 16 h, it caused a noticeable inversion of the ratio between the anti-apoptotic isoform of Bcl-X, Bcl-X_L, and the pro-apoptotic one, Bcl-X_S, and also up-regulated c-IAP-2 and livin mRNAs. Effects of decrease in COX-2 and Bcl-X_L expression by curcumin have been previously reported in studies on normal or tumor human cells [20,29,33,34]. It has been also reported that in human renal carcinoma Caki cells undergoing apoptosis from curcumin a down-regulation of c-IAP-1 and XIAP, but not of c-IAP-2, protein levels occurs; the effect on c-IAP-1 appeared to be secondary to caspase activation [29]. We cannot definitively exclude that our results on NF-kB and gene expression following curcumin administration may in part be due to the latter mechanism, but the fact that there were no morphological signs of cytotoxicity and extremely low levels of caspase 3 activation (not shown) at the early times that we took in consideration, makes less likely the hypothesis of their caspase-dependency.

Cisplatin and doxorubicin exerted distinct effects of decrease on gene expression. The results are consistent with previous, also personal, studies which have indicated that down-regulation of IAPs may be caused from these drugs and possibly required for their antitumor activities [35–39]. In addition, the cytotoxic interactions between curcumin and the other agents may be explained by the observation of synergistic (in particular with cisplatin) or additive effects of decrease in the expression of different relevant genes, including *c-myc*, *Bcl-X_L*, *c-IAP*-2, *NAIP* and *XIAP*. Nevertheless, the combinations antagonized also certain other influences on mRNA expression exerted by the single agents, like, for example, the increases in the pro-apoptotic factor Bcl-X_s seen at 16 h after curcumin or doxorubicin.

Overall, the effects of the agents on gene expression did not show a simple relationship to the relative influences on NF-kB activation. Strikingly, curcumin affected above all the strong NF-kB activation from doxorubicin, but the antitumor effects, also in terms of gene expression, were more favorable when it was combined with cisplatin. With regard to this, interestingly, a recent study has shown that, non-canonically and depending on the circumstances, the NF-kB p65 subunit can repress transcription of anti-apoptotic genes, corroborating that doxorubicin may rely on NF-kB activation to exert its antitumor effects [40]. On the other hand it is clear that, apart from NF-kB, curcumin, cisplatin and doxorubicin might have influenced by several other means the gene expression in HA22T/VGH cells. For example, beside NF-kB,

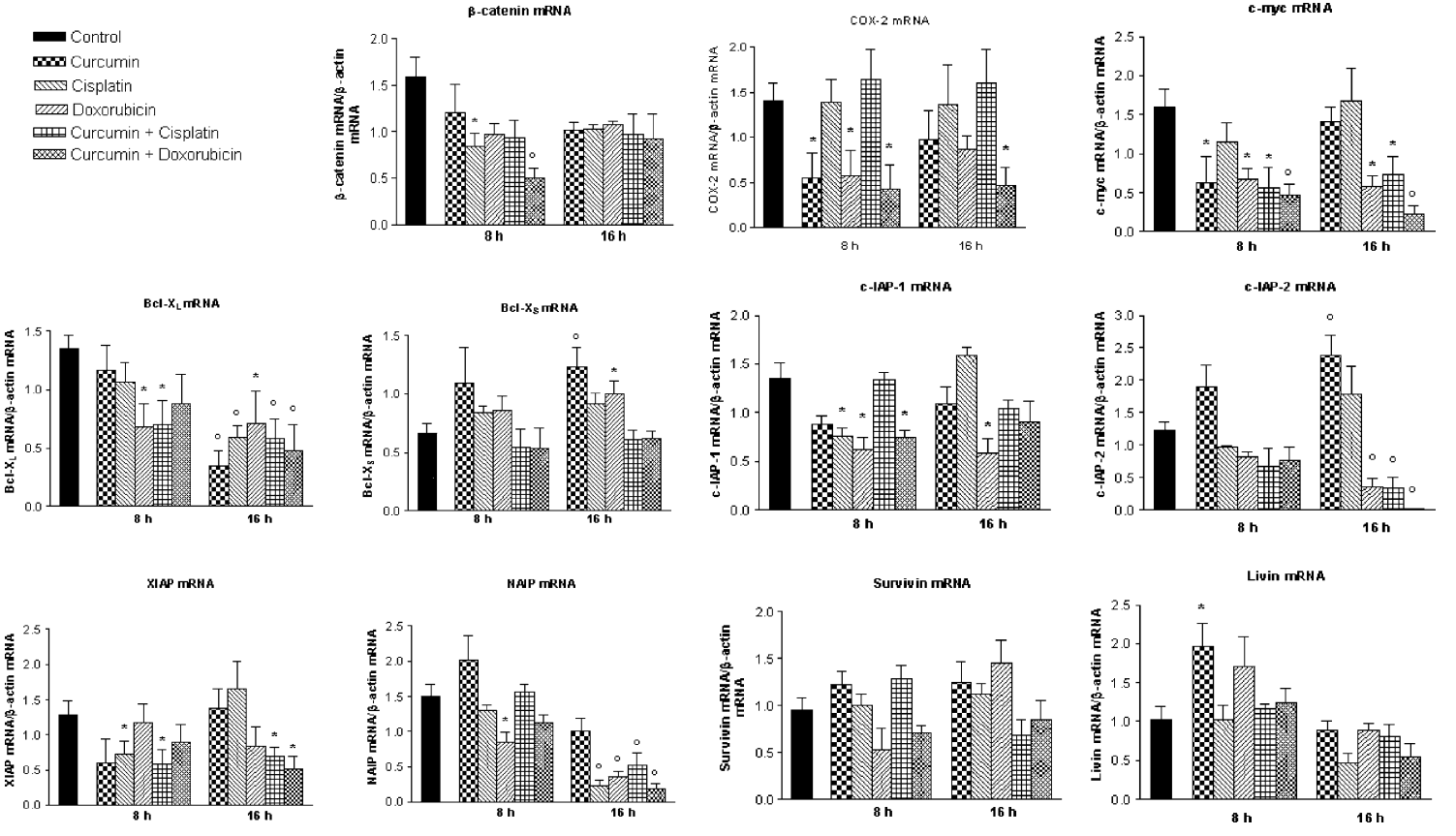

Fig. 6. Effects of curcumin in combination with cisplatin or doxorubicin on the mRNA levels of β-catenin, COX-2, c-myc, Bcl-X_L, Bcl-X_S and IAPs. The cells were treated for 8 or 16 h with curcumin 40 μM, cisplatin 2.5 μg/ml, doxorubicin 2.5 μg/ml or combinations thereof. The level of expression of the different mRNAs was assessed by RT-PCR as reported in the Section 2.7. Data are expressed as arbitrary units (relevant mRNA/β-actin mRNA) and are the mean ± S.E. of four independent (eight for the control) observations. * = $P < 0.05$ versus control; ° = $P < 0.01$ versus control.

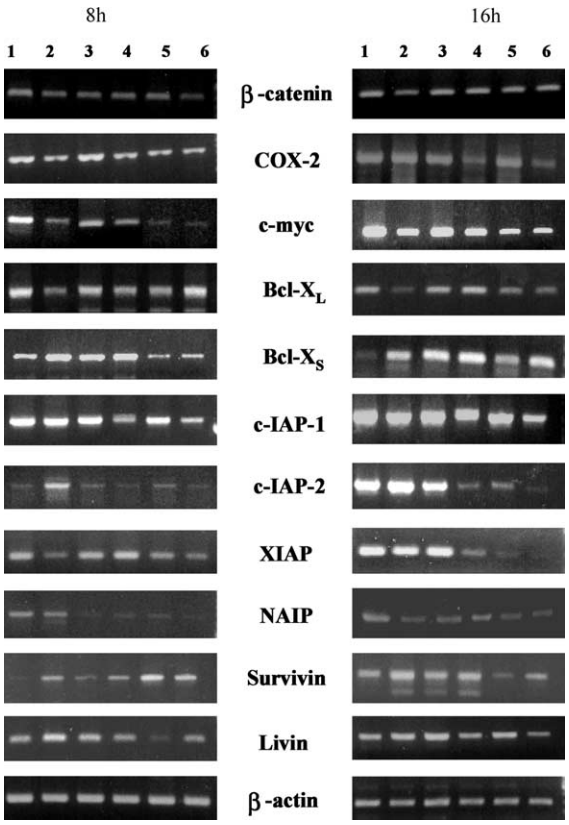

Fig. 7. Representative analysis of mRNA expression in HA22T/VGH cells. The cells were treated for 8 or 16 h. mRNA was isolated and RT-PCR was performed as reported in the Section 2.7. RT-PCR with β-actin primers was performed as a control for the same amount of RNA. Lane 1: control; Lane 2: curcumin 40 μM; Lane 3: cisplatin 2.5 μg/ml; Lane 4: doxorubicin 2.5 μg/ml; Lane 5: curcumin 40 μM plus cisplatin 2.5 μg/ml; Lane 6: curcumin 40 μM plus doxorubicin 2.5 μg/ml.

HCC cells may constitutively express other transcription factors, like AP-1 and STATs, playing a major role in the control of cell proliferation and survival [14]. Curcumin and the other agents may well interact with AP-1 and STATs [21], so that further studies are worthy to be carried out in HA22T/VGH and other HCC cell lines to explain the effects and reciprocal influences of these drugs in a more comprehensive scenario.

In conclusion, the results presented here underline that, together with other genes, the IAPs are promising molecular targets for the therapy of HCC. Further, they support the possible use of curcumin, alone or in combination, in particular with cisplatin, in the treatment of this cancer.

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