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NF-κB transcription factor induces drug resistance through MDR1 expression in cancer cells

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The ubiquitous NF-κB transcription factor has been reported to inhibit apoptosis and to induce drug resistance in cancer cells. Drug resistance is the major reason for cancer therapy failure and neoplastic cells often develop multiple mechanisms of drug resistance during tumor progression. We observed that NF-κB or P-glycoprotein inhibition in the HCT15 colon cancer cells led to increased apoptotic cell death in response to daunomycin treatment. Interestingly, NF-κB inhibition through transfection of a plasmid coding for a mutated IκB-α inhibitor increased daunomycin cell uptake. Indeed, the inhibition of NF-κB reduced *mdr1* mRNA and P-glycoprotein expression in HCT15 cells. We identified a consensus NF-κB binding site in the first intron of the human *mdr1* gene and demonstrated that NF-κB complexes could bind with this intronic site. Moreover, NF-κB transactivates an *mdr1* promoter luciferase construct. Our data thus demonstrate a role for NF-κB in the regulation of the *mdr1* gene expression in cancer cells and in drug resistance.
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Introduction

Numerous mechanisms of cancer cells resistance to chemotherapeutic agents are known (Goldie, 1992), such as decreased drug transport, increased drug or metabolite inactivation, reduced drug activation, increased DNA repair, target gene amplification or failure to undergo apoptosis. Reduced cellular drug uptake can be the consequence of P-glycoprotein (P-gp) overexpression. This 180 kDa protein belongs to the ATP-binding cassette (ABC) superfamily of membrane transporter proteins and is encoded by the *mdr1* gene (Ling *et al*., 1983; Gros *et al*., 1986; Biedler, 1994; Bosch and Croop, 1996). P-gp is expressed in some tissues such as kidney tubules, colon, pancreas and adrenal gland, and tumors derived from these tissues are often resistant to chemotherapeutic drugs (Goldstein *et al*., 1989). Furthermore, *mdr1* expression is also increased in many relapsing cancers.

P-gp might also confer resistance to TNF-α- and FasL-induced cell death through inhibition of downstream Caspase 3 and 8 activation. Two hypotheses could explain this effect: increased intracellular pH or P-gp-mediated inhibition of death-inducing-signaling complex (DISC) formation (Robinson *et al.,* 1997; Smyth *et al.,* 1998; Johnstone *et al.,* 1999). Moreover, TNF-α activates a sphingomyelinase that induces apoptosis through the generation of ceramides from sphingomyelin (Kolesnick, 1992; Hannun, 1994; Kolesnick and Golde, 1994). P-gp-dependent sphingomyelin excretion may inhibit TNF-α-induced ceramide production and subsequent apoptosis (Bezombes *et al*., 1998).

NF-κB is a family of ubiquitous transcription factors. In most cells, the NF-κB dimers are retained by an inhibitor (IκB) in the cytoplasm of nonstimulated cells. Following different stimuli, such as cytokines or DNA-damaging agents including chemotherapeutic drugs, IκB is phosphorylated by the IKK kinase complex, polyubiquitinated and degraded (Karin, 1999). Then, the NF-κB nuclear localization signal (NLS) is freed allowing the nuclear translocation of the transcription factor and the induction of its target genes. These target genes code for proinflammatory molecules as well as pro or antiapoptotic proteins (Pahl, 1999).

NF-κB has been shown to play an antiapoptotic role in cancer cells. In B lymphocytes, Hodgkin disease and some breast cancer cells, NF-κB activity is constitutive and protects against apoptosis (Wu *et al*., 1996; Bargou *et al.,* 1997; Sovak *et al.,* 1997). Indeed, NF-κB inhibition in these cells often induces cell death. Numerous apoptotic signals such as TNF-α, ionizing radiations and chemotherapeutic drugs have been shown to induce NF-κB (Pahl, 1999). Experimental data clearly demonstrated that the expression of an unresponsive mutated IκB-α inhibitor sequestered NF-κB in the cytoplasm and increased apoptosis following treatment by cytotoxic agents (Beg and Baltimore, 1996; Van Antwerp *et al*., 1996; Wang *et al*., 1996). In addition, gene inactivation experiments confirmed that the NF-κB activity inhibits TNF-α-induced liver apoptosis during embryonal development (Beg *et al*., 1995; Li

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et al., 1999). Conversely, in a few experimental conditions, NF-κB can play a proapoptotic role, for instance, after treatment with some chemotherapeutic drugs (Barkett and Gilmore, 1999). In addition, Ryan *et al*. showed that p53 induction induces apoptosis through NF-κB activation (Ryan *et al*., 2000).

The putative promoter region of *mdr1* does not have a TATA box, but has a consensus CAAT box and two GC box-like sequences (Ueda *et al*., 1987). Transcription of the *mdr1* is controlled by numerous transcription factors, such as SP1, NF-Y, YB1, MEF1 (MDR1 promoter-enhancing factor1), p53 and NF-R1 (Ogura *et al*., 1992; Madden *et al*., 1993; Sundseth *et al*., 1997; Ohga *et al*., 1998; Hu *et al*., 2000; Ogretmen and Safa, 2000; Sampath *et al*., 2001). Ogretmen *et al*. demonstrated that a protein complex consisting of NF-κB /p65 and c-Fos transcription factors interacts with the CAAT promoter region in MCF7 cells and negatively regulates the human *mdr1* promoter activity (Ogretmen and Safa, 1999). It has also been reported that an insulin-induced *mdr1* expression is mediated by NF-κB in rat hepatoma cells (Zhou and Kuo, 1997) and that NF-κB can protect kidney proximal tubule cells from cadmium and oxidative stress by increasing P-gp expression (Thevenod *et al.,* 2000). Recent papers reported that NF-κB was involved in TNF-α-induced *mdr1* expression in hepatocytes, in 2-acetylaminofluorene-induced MDR expression in liver cells and in constitutive MDR expression in drug-resistant cells (Ros *et al*., 2001; Um *et al*., 2001; Kuo *et al*., 2002).

In the present study, we report that the inhibition of NF-κB activity sensitizes resistant colon cancer cells to daunomycin through a decreased *mdr1* expression. This effect is cell- and signal-specific and depends on the level of NF-κB inhibition. Our results provide a novel link between NF-κB and resistance to chemotherapy through the regulation of human *mdr1* gene expression.

Results

Stable inhibition of NF-κB activity sensitizes HCT15 resistant cells to daunomycin and TNF-α cytotoxic effect

The HCT15 human colon cancer cells were stably transfected with a plasmid encoding a mutated, non-inducible HA-tagged IkB-α inhibitor (HCT15-MT cells) or the empty pcDNA3 vector (HCT15-H cells). Immunoblots performed with whole-cell extracts from HCT15-H cells or four HCT15-MT clones confirmed the expression of the mutated IkB-α inhibitor at various levels (Figure 1a).

We confirmed that the NF-κB activity was efficiently inhibited in the HCT15-MT clones. HCT15-H and HCT15-79-MT cells were treated with daunomycin or TNF-α, and nuclear NF-κB DNA-binding activity was evaluated by gel shift analysis performed with a palindromic κB probe. We could not detect any specific NF-κB activity in unstimulated or stimulated HCT15-79-MT cells, while such an activity was present in stimulated HCT15-H cells (Figure 1b). This inhibition was also confirmed by transient transfection of κB-dependent reporter plasmid. As compared to HCT15-H cells, basal and TNF-α-induced NF-κB transcriptional activity was completely abolished in HCT15-79-MT cells (data not shown).

Control HCT15-H cells and the four HCT15-MT clones were treated for 48 h with increasing concentra-

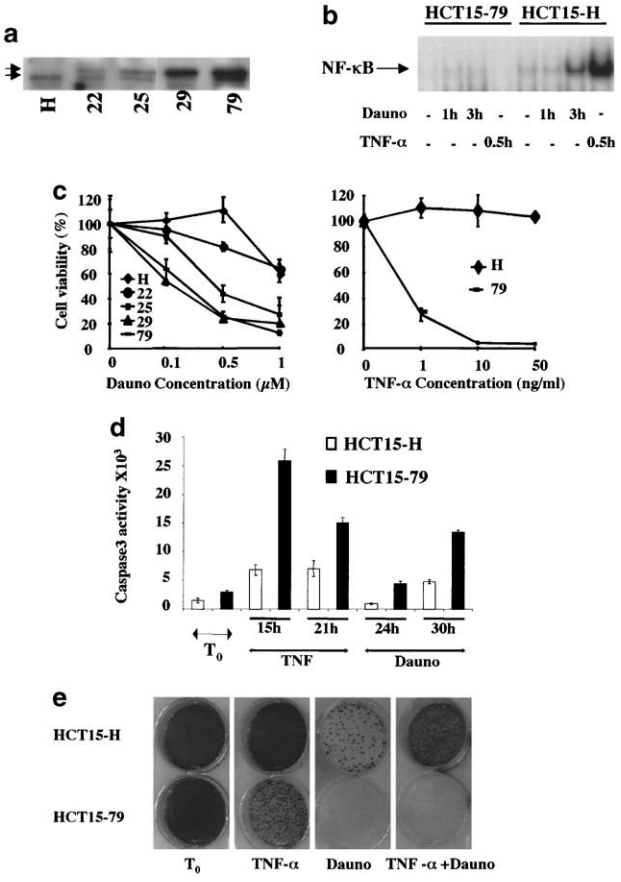

Figure 1 Inhibition of NF-κB sensitizes HCT15 cells to apoptotic cell death. (**a**) Expression of the IκB-α NF-κB inhibitor was compared in HCT15 cells stably transfected with an empty vector (HCT15-H) or with a vector coding for a mutated IκB-α protein (clones HCT15-22, 25, 29 and 79-MT cells). Whole-cell extracts were analyzed by Western blotting with a specific anti-IκB-α antibody. The upper arrow indicates the tagged mutated IκB-α protein and the lower indicates the endogenous wild-type IκB-α protein. (**b**) HCT15-H and HCT15-79-MT cells were left untreated or were stimulated with daunomycin (1 μM) or TNF-α (10 ng/ml) for the indicated times. Nuclear extracts were analyzed for DNA binding to a palindromic κB site. The arrow indicates the specific NF-κB complex. (**c**) HCT15-H and the four MT clones were left untreated or incubated with increasing concentrations of TNF-α or Dauno for 48 h. Cell viability was estimated with the WST-1 test. Values represent the means of three independent measures s.d. (**d**) Caspase 3 activity was measured in HCT15-H and HCT15-79-MT cells left untreated or incubated with increasing concentrations of TNF-α (10 ng/ml) or Dauno (1 μM) for the indicated times. Values represent the means of three independent measures ±s.d. (**e**) Clonogenic survival assay of HCT15-H and HCT15-79-MT cells treated for 48 h with TNF-α (10 ng/ml), Dauno (0.5 μM) or both (TNF-α (5 ng/ml), Dauno (0.25 μM)). The figure shows the colonies after staining with crystal violet

tions of daunomycin (Dauno) or TNF-α (Figure 1c). Survival curves showed that NF-κB inhibition (HCT-15-MT cells) increased daunomycin and TNF-α cytotoxic effect. Cellular viability of these different clones showed that the level of expression of the inhibitor is correlated with the daunomycin and TNF-α cytotoxic effect (Figure 1c and data not shown). Finally, treatment of HCT15-H and HCT15-79-MT cells with vinblastine or camptothecin under the same experimental conditions showed a significant but less important, increased cytotoxicity in MT cells (data not shown). However, NF-κB inhibition does not increase H_2O_2, C6 ceramide or sodium nitroprussiate cytotoxic effect in the same cell lines (data not shown).

To determine whether NF-κB inhibition sensitizes HCT15 cells to apoptosis, caspase 3 activity was assessed 15 and 21 h following TNF-α (10 ng/ml) or 24 and 30 h following daunomycin (1 μM) treatment (Figure 1d). Caspase 3 activity was higher in HCT15-79-MT cells than in HCT15-H cells. The same results were observed by FACS analysis of annexin V/propidium iodide staining of HCT15-H and HCT15-79-MT cells treated with TNF-α, indicating a higher rate of apoptosis in HCT15-79-MT than in HCT15-H cells (data not shown).

To investigate the consequence of NF-κB inhibition in clonogenic survival, HCT15-H and HCT15-79-MT cells were treated with daunomycin (0.1, 0.5, 1 and 2 μM), TNF-α (1, 10 and 50 ng/ml) or both of them for 48 h, incubated with fresh medium for 12 days and the colonies (>50 cells) were counted. Under these conditions, MT cells were less resistant than HCT15-H control cells to daunomycin or TNF-α (Figure 1e).

Inhibition of NF-κB reduces daunomycin uptake

In order to evaluate whether a modulation of P-gp activity explained the increased cytotoxic effect after NF-κB inhibition, HCT15-H and HCT15-79-MT cells were treated with daunomycin (0.5 or 1 μM) for 6, 24 or 32 h (Figure 2 and data not shown). FACS measurement of daunomycin fluorescence was performed and showed a higher accumulation of daunomycin in HCT15-79-MT cells than in HCT15 or HCT15-H control cells (Figure 2a). A similar conclusion was reached after observation of daunomycin uptake by fluorescent microscopy (data not shown).

To determine whether daunomycin or TNF-α cytotoxic effect was enhanced by a decrease in P-gp functional activity, we investigated the effect of P-gp pharmacological modulators in the presence or absence of the cytotoxic agents. HCT15 cells were incubated for 48 h with cyclosporine A (3 or 6 μg/ml) or verapamil (2 or 10 μM) and increasing concentrations of daunomycin. Both cyclosporine and verapamil sensitized HCT15 cells to Daunomycin cytotoxic effect (Figure 2b and data not shown), but did not have any effect on TNF-α-induced mortality (data not shown).

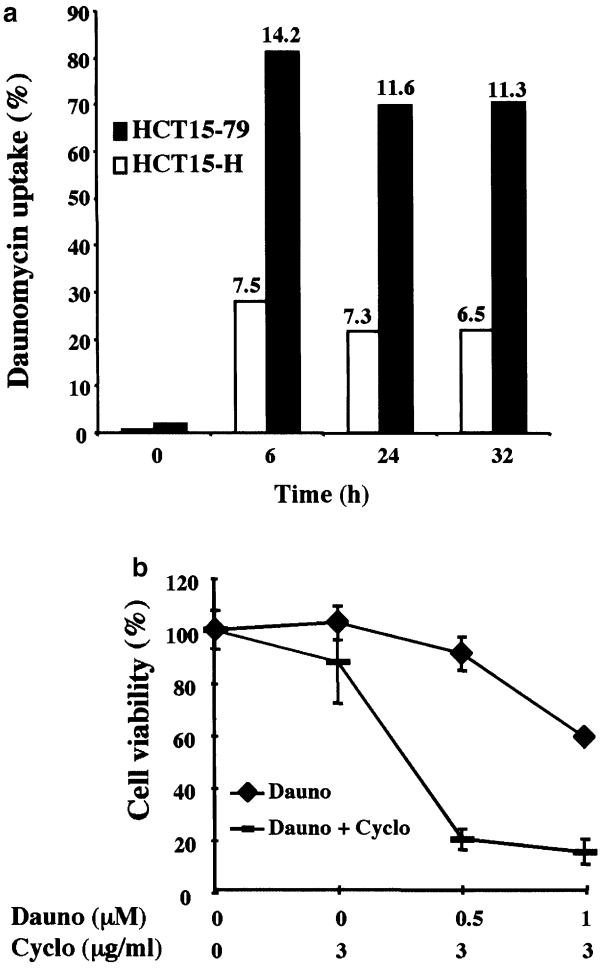

Figure 2 NF-κB inhibition decreases MDR1 functional activity. (**a**) HCT15-H and HCT15-79 cells were treated with Dauno (0.5 μM) as indicated and the percentage of fluorescent cells was measured by FACS. The mean values of fluorescence intensity are indicated on the top of the columns. The experiment was repeated with various daunomycin concentrations and generated reproducible data. The figure shows a representative experiment. (**b**) HCT15 cells were left untreated or incubated with daunomycin alone or in combination with cyclosporine (Cyclo) for 48 h. Cell viability was estimated with the WST-1 test. Each value represents the mean of three independent measures ±s.d

Inhibition of basal NF-κB activity downregulates endogenous mdr1 gene and P-gp protein expression

The expression of the *mdr1* mRNA and the P-gp protein were compared in control and transfected cell lines. Immunoblots performed with crude membrane fraction from cells treated or not with daunomycin or TNF-α showed that the expression of P-gp (180 kDa) is higher in HCT15-H cells, compared to HCT15-79-MT cells (Figure 3a and data not shown). To assess whether NF-κB regulated *mdr1* mRNA expression, total RNA was isolated from HCT15-H and HCT15-79-MT treated or not (24 or 32 h) with daunomycin (1 μm) or TNF-α (1 ng/ml) and *mdr1* expression was determined by quantitative real-time RT-PCR (Figure 3b and data not shown). We observed that the *mdr1* RNA expression

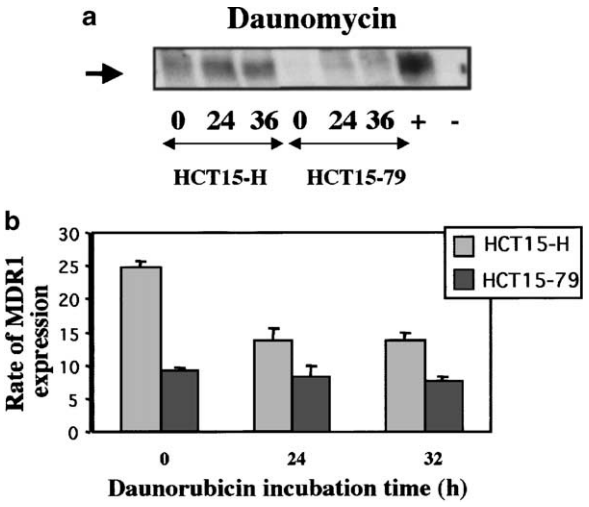

Figure 3 NF-κB inhibition in HCT 15 cells decreases MDR1 expression. (**a**) Immunoblots performed with the crude membrane fraction from HCT15-H and HCT15-79-MT cells treated or not with Dauno (1 μM) and revealed with an anti-P-gp antibody. The positive (+) and negative (−) controls were performed with the crude membrane fraction from CEM-VLB1OO resistant cells or CEM non resistant cells, respectively. The arrow indicates the specific P-gp band at 180 kDa. (**b**) *mdr1* mRNA expression in HCT15-H and HCT15-79 cells left untreated or incubated with daunomycin for the indicated times (1 μM). Total cellular RNAs were analyzed by real-time RT-PCR for *mdr1* and human Beta2-microglobulin mRNAs. The values indicate quantifications *of mdr1* transcripts normalized to Beta2-microglobulin mRNA expression

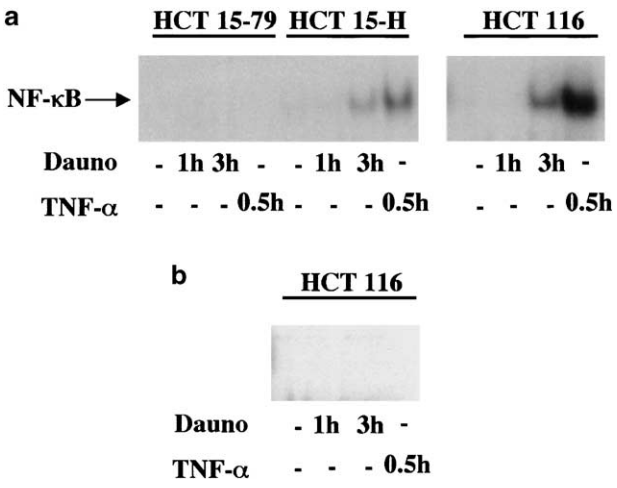

Figure 4 NF-κB binds a site in the first intron of the human *mdr1* gene. (**a**) HCT15-H, HCT15-79 and HCT116 cells were treated with TNF-α (10 ng/ml) or daunomycin (1 μM) for the indicated times and nuclear extracts were analyzed for DNA-binding to a probe containing the κB site from the first intron of the *mdr1* gene. (**b**) HCT116 cells were treated with TNF-α (10 ng/ml) or daunomycin (1 μM) for the indicated times and nuclear extracts were analyzed for DNA-binding to a probe containing the mutated κB site from the first intron of the *mdr1* gene

level was higher in HCT15-H cells than in HCT15-79-MT cells.

NF-κB binds the mdr1 promoter through an intronic site

We examined the *mdr1* gene sequence and identified a putative NF-κB binding site (CCTTTCGGGG) in the first intron of the *mdr1* gene promoter. EMSA performed with nuclear extracts from untreated daunomycin- or TNF-α-treated HCT116 or HCT15-H cells and oligonucleotide probes derived from this site showed a specific NF-κB binding, while we could not detect any binding with nuclear extracts from HCT15-79-MT cells (Figure 4a). Moreover, we could not detect any binding to a similar but mutated probe (Figure 4b). Competition experiments performed with unmodified and mutated unlabelled probes confirmed the specificity of the observed bands (data not shown).

NF-κB transactivates an mdr1 reporter plasmid

We also explored whether NF-κB can transactivate through the *mdr1* promoter. HCT15 cells were transfected with a luciferase reporter gene driven by a large fragment from the *mdr1* promoter (p225-LUC) and with expression vectors for NF-κB proteins. We observed that, while p50 homodimers did not have any significant effect, RelA, p50/RelA, p50/RelB and p50/c-Rel induced a dose-dependent activation of the human *mdr1*

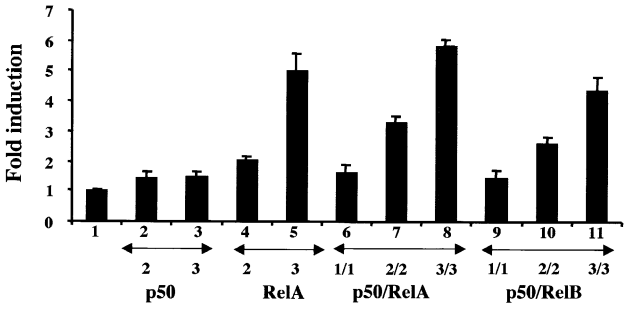

Figure 5 HCT15 cells were transfected with the *mdr1* promoter alone or in combination with increasing amounts of p50, RelA, p50/RelA or p50/RelB. The number indicates the amounts of transfected DNA (in μg). Luciferase activities in cell extracts were measured 24 h after transfection, normalized to β-galactosidase activities in the same extracts and reported as fold inductions over the activity observed after transfection of the reporter plasmid alone. Each value represents the mean of at least three independent measures (±s.d.)

promoter reporter plasmid in HCT15 cells (Figure 5 and data not shown).

Discussion

The role of NF-κB in the control of apoptosis has been extensively studied these last years. Although in most experimental settings NF-κB inhibits apoptosis through the regulation of antiapoptotic genes, a few reports also demonstrated that NF-κB could be required for apoptosis in some circumstances. Among these large number of studies, several indicated that NF-κB inhibition could increase cellular response to cytotoxic

drugs (Wang *et al*., 1996, 1999); however, this activity was probably dependent upon the cell lines, the compounds and the extent of NF-κB inhibition (Kasibhatla *et al*., 1998; Bentires-Alj *et al*., 1999). In the present paper, we confirm that NF-κB activity could reduce the cytotoxic effect of anticancer drug but this activity was again cell- and signal specific.

Once activated, NF-κB controls the expression of numerous genes and many of them regulate the apoptotic pathways. Therefore, the resistant phenotype of cancer cells associated with constitutive or induced NF-κB activity has been associated with NF-κB-dependent expression of proteins inhibiting the various proapoptotic pathways (Barkett and Gilmore, 1999; Bours *et al*., 2000). Our present data as well as a recent publication (Kuo *et al*., 2002) indicate that NF-κB also acts upstream by controlling drug efflux through P-gp expression in cancer cells while previous reports had demonstrated the NF-κB-dependent regulation of P-gp expression in renal tubules or liver which physiologically express the protein (Thevenod *et al.,* 2000; Ros *et al*., 2001). Therefore, it is likely that increased P-gp expression participates in NF-κB-related cancer cell resistance to treatment.

The exact mechanisms explaining the regulation of human P-gp expression by NF-κB remain controversial. Indeed, in the present paper, a sequence study of the promoter fragment we used identified a single consensus NF-κB-binding site in the first intron but the mutation of this site did not significantly modify the response of our reporter plasmid to overexpressed exogenous NF-κB proteins, indicating that other, less well conserved, sites are probably bound by these proteins (data not shown). Indeed, we identified in the promoter several sites presenting a single mismatch with the canonical consensus κB site. Very recently, Kuo *et al*. identified another NF-κB-binding site at position −6902 in the human *mdr1* promoter (Kuo *et al.,* 2002) but this site is not part of our promoter construct. Finally, it was reported that crosscoupled p65 and c-Fos repress the *mdr1* promoter in MCF7 cells (Ogretmen and Safa, 1999). Interestingly, our data indicate the functional and therapeutical consequences of P-gp regulation by NF-κB as NF-κB inhibitors facilitate daunomycin uptake.

Constitutive NF-κB activity has been associated with advanced breast cancers, lung cancers, Hodgkin diseases, multiple myelomas and leukemias (Rayet and Gelinas, 1999). It would therefore be most interesting to determine whether NF-κB activity in these cancers is associated with P-gp expression.

Our observations could have an important impact on the understanding of anti-MDR treatments and open new perspectives. CyclosporinA (CsA) is used in combination with chemotherapy to treat advanced and resistant multiple myelomas that usually express high levels of P-gp. It has been demonstrated that CsA directly inhibits the P-gp protein, but this drug can also block NF-κB activation in a signal-specific way (Schmidt *et al.,* 1990; Marienfeld *et al.,* 1997; Meyer *et al.,* 1997; Lee *et al*., 1999). Therefore, the role of CsA in the decrease of P-gp expression through the inhibition of NF-κB should be determined.

Other anticancer treatments are potential NF-κB inhibitors. Corticosteroids have been demonstrated to inhibit NF-κB activity, probably through a direct association with some NF-κB subunits (Ray and Prefontaine, 1994; Caldenhoven *et al*., 1995; Scheinman *et al*., 1995). Glucocorticoids are an important part of the therapeutic schemes in acute lymphoblastic leukemias, chronic lymphocytic leukemias, Hodgkin diseases, multiple myelomas and non-Hodgkin lymphomas. Interestingly, constitutive NF-κB activation, chromosomal translocation involving NF-κB subunits encoding genes and mutations of the IκB-α inhibitor were associated with most of these pathologies (Rayet and Gelinas, 1999). Moreover, progressive increase in P-gp expression is a hallmark of advanced multiple myelomas and has important repercussions on the response to treatment (Sonneveld *et al*., 1997). Therefore, it would be important to determine whether a link between the mechanisms of NF-κB activation, the response to corticosteroids and chemotherapy and the P-gp expression could be established. If such a link exists, NF-κB could be seen as an important target for the design of novel therapeutic agents acting both on the inhibition of apoptosis and on drug cellular clearance through P-gp expression.

Materials and methods

Chemicals

Daunomycin hydrochloride – CERUBIDIN® (DAUNO), (Rhône-Poulenc Rorer, Brussels, Belgium) was dissolved in distilled water (10 mM) and stored at −80°C. The human recombinant TNF-α (specific activity $>1.0\times10^8$ U/mg) and Cyclosporin (Sandimmun®) were purchased from Roche (Mannheim, Germany) and Novartis Pharma (Brussels, Belgium), respectively.

Cell culture

HCT15 human colon carcinoma cells (ATCC CCL225) were grown in RPMI 1640 medium supplemented with 1% L-glutamine (200 mM), 20% (v/v) fetal bovine serum (FBS, Life Technologies, Grand Island, NY, USA), penicillin (100 IU/ml) and streptomycin (100 μg/ml). HCT116 cells were grown in McCoy's medium supplemented with 1% L-glutamine (200 mM), 10% (v/v) FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). HCT15 cells were stably transfected with an IκB-α expression vector mutated at serines 32 and 36 (HCT15-MT; clones: HCT15-22, HCT15-25, HCT15-29, HCT15-79), or the empty vector pcDNA3 (HCT5-H). HCT15-H and HCT15-MT cells were cultivated in the same medium as untransfected cells supplemented with geneticin (500 μg/ml of G418 active concentration, Life Technologies).

Sequence analysis

Analysis of the *mdr1* promoter sequence was performed with the Ben (Belgian Embnet Node) software using the mapping program and Tf sites database.

Plasmids

The expression vector for pRSV-LacZ was kindly provided by Dr Winkler (Laboratoire d'Oncologie Moléculaire, Liége, Belgium). The mammalian PMT_2T expression vectors for p50, RelA and RelB were previously described (Bours *et al*., 1992). The p NF-κB -Luc reporter plasmid contains five κB sites from the human immunodeficiency virus-1 long terminal repeat cloned upstream of a luciferase (LUC) reporter gene.

The p225 plasmid contains a 976 bp polymerase chain reaction (PCR)-generated fragment *of mdr1* genomic sequence upstream of the initiating ATG. This fragment includes exon 1a, exon 1b, intron 1 and 430 bp upstream of the transcription intiation site (Ueda *et al*., 1987). This fragment was amplified using the oligonucleotides: 5′-ATATAAGCTTCTG-CAGGGGCTTTCCTGTG-3′ and 5′-TATAAAGCTTCTG-CAGAAAAATTTCTCCTAGCC-3′, and subcloned into *Hind*III sites of the pGL3-Basic vector (Promega, Madison, WI, USA) upstream of a luciferase (LUC) reporter gene. The *mdr1* reporter plasmids were controlled by sequencing.

Cellular extracts

Whole-cell extracts were prepared by cell lysis in SDS 1 % buffer. Nuclear protein extraction for EMSA was previously described (Dejardin *et al*., 1995). Briefly, the pelleted nuclei were resuspended in nuclear buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.63 M NaCl, 25% glycerol, and Protease inhibitor kit (Roche)), incubated for 20 min at 4°C and centrifuged for 30 min at 14 000 *g*. Protein amounts were quantified with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, II, USA).

The crude membrane protein extracts were prepared as described (Gala *et al*., 1995). Briefly, cells were washed with PBS, resuspended in lysis buffer (1.5 ml/30×10^6 cells; 0.01 M Tris-HCl, pH 7.4, protease inhibitors (Protease inhibitor kit, Roche)), freezed at −20°C, thawed and homogenized in a motor-driven Teflon glass homogenizer. After centrifugation for 10 min at 4 000 *g*, the supernatant was centrifuged again for 60 min at 35 000 *g* and the crude membrane fraction resuspended in the lysis buffer.

Immunoblot analysis

Proteins from the crude membrane fraction or whole-cell extracts were separated in reducing SDS–polyacrylamide gels and transferred to polyvinyl difluoride membranes (PVDF, Millipore). After overnight blocking at 4°C with TBS-T buffer plus 5% dry milk, the membranes were incubated for 1 h with the c-219 monoclonal anti-MDRl antibody (Calbiochem, Darmstadt, Germany), the monoclonal anti-βactin antibody (Sigma, Bornem, Belgium) or the polyclonal anti-IκB-α antibody (Upstate Biotechnology, Lake Placid, USA).

The reaction was revealed with the enhanced chemiluminescence detection method (ECL kit, Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays (EMSA) and supershifting analyses were performed as previously described (Bonizzi *et al*., 1996). The sequence of the κB probe from the *mdr1* promoter was 5′-TTGGCTGCAGGGGCTTTCCTG TGCGC-3′. The mutated κB probe from the *mdr1* promoter was: 5′-TTGGCTGCACTCGCTTTCCTGTGCGC-3′.

Reverse transcription and real-time quantitative RT-PCR

The total cellular RNA was prepared with the TriPure™ isolation reagent (Roche, Mannheim, Germany) according to the manufacturer's recommendation. In total 200 ng RNA was reverse transcribed for 1 h at 37°C with 100 pmol of random primers and 200 U of the MMLV-reverse transcriptase according to the manufacturer's recommendations (Life Science Biotech, GIBCO-BRL).

A real-time quantitative PCR analysis was performed with a PE Applied Biosystems 7700 Sequence Detector.

Primers and probe sequences for the *mdr1* gene were chosen as follows:

MDR1 Forward 5′-GGTTTATAGTAGGATTTA-CACGTGGTTG-3′
MDR1 Reverse 5′-AAGATAGTATCTTTGCCCAGA-CAGC-3′
MDR1 Probe 5′FAM CTAACCCTTGTGATTTTGG-CCATCAGTCC Tamra 3′

The calibration curve was performed with a plasmid containing the whole *mdr1* cDNA (a generous gift of Professor JP Marie, Hôtel Dieu Hospital, Paris, France).

The mRNA was quantified by a scale of mRNA (from 250 to 0.0025 ng) using the VIC Taqman kit for human Beta2-microglobulin (Applied Biosystems). Both assays used Taqman Universal PCR Master Mix containing UracylN-Glycosylase.

Transient transfections and luciferase assays

For DNA transfection, cells were plated at a density of 6.5×10^5 cells per 35 mm-diameter six-well culture dishes. After 16 h, the cells were transfected by the Fugene™ 6 transfection reagent as recommended by the manufacturer (Roche, Mannheim, Germany). To control transfection efficiency, 0.2 μg of the pRSV-LacZ expression plasmid was cotransfected in every well. Cells were harvested 24 h after transfection, and luciferase and β-galactosidase activities were measured using the β-Gal Reporter Gene assay chemiluminescent (Roche) and the Luciferase Reporter Gene assay (Roche) kits, respectively. Luciferase activities were normalized to the β-galactosidase activities measured in the same lysates.

Cytotoxicity assay

Stably transfected cells or parental cells were seeded at the concentration of 7×10^3 cells per well, in flat-bottom microplates. After 24 h, cells were cultivated with the appropriate cytotoxic agent for the indicated time or left untreated. Cell viability was measured by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial deshydrogenases in viable cells (Roche, Mannheim, Germany).

Long-term clonogenic survival assay

HCT15 cells (5×10^4) were seeded in six-well plates and treated at the indicated concentrations for 48 h. These cells were then washed twice in PBS and left in fresh medium for 12 days. The cells were fixed with paraformaldehyde 3% and the number of colonies were counted after staining with crystal violet (0.3%).

Caspase 3 activation

Caspase 3 activity was measured after cellular lysis (NP40 1%, Tris pH 6 20 mM, NaCl 137 mM, glycerol 10%, PMSF 1 mm,

Na orthovanadate 1 mM and protease inhibitors). Extracted proteins (50 μg) were then incubated for 4 h at 37°C in 150 μl of the buffer in the presence of the Ac-DEVD-AMC substrate, as recommended by the manufacturer (Calbiochem, Darmstadt, Germany). The fluorescence levels of the cleaved product were determined using a VictorTM 1420 multilabel counter (Wallac, Sweden). Excitation occurred at 355 nm and emission at 460 nm.

Uptake analysis

Cells were washed twice in phosphate-buffered saline (Life Technologies), fixed in paraformaldehyde (4%) and analyzed by fluorescence microscopy (Nikon Eclipse E800, Japan).

For uptake quantification, cells were washed twice in phosphate-buffered saline (Life Technologies), harvested after trypsin treatment, washed again and the daunomycin fluorescence was measured using an FACStar Plus (Becton Dickinson, Erembodegem, Belgium).

Abbreviations

NF-κB, nuclear factor-κB; IκB, inhibitor of κB; MDR, multidrug resistance; P-gp, P-glycoprotein.

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