ANTICANCER RESEARCH *25*: 2119-2128 (2005)

Sabarubicin- (MEN 10755) and Paclitaxel Show Different Kinetics in Nuclear Factor-KappaB (NF-kB) Activation: Effect of Parthenolide on their Cytotoxicity

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Abstract. *Background: Several antitumor drugs have been described to induce nuclear factor kappaB (NF-Î B), but results about its role in regulating apoptotic cell death are quite controversial. In this paper, we studied NF-Î B induced by the two anticancer agents Sabarubicin (MEN 10755) and paclitaxel (Taxol) and the effects of its pharmacological inhibition. Materials and Methods: In the human colon cancer cell line HCT-116, we investigated NF-Î B activation induced by the two anticancer agents using electrophoretic mobility shift assay (EMSA), while drug-induced cytotoxicity was measured by trypan blue staining. Apoptosis was analyzed using a cell death detection enzyme-linked immunosorbent assay (ELISA) kit, flow cytometry and caspase-3 activation assay. Results: The combination with the NF-Î B inhibitor parthenolide increased Sabarubicin- but not paclitaxel-induced cell death. EMSA experiments demonstrated that the two antitumor drugs induced NF-Î B complexes with different kinetics but similar subunit composition. Moreover, Sabarubicin elicited NF-Î B activation definitely earlier than DNA fragmentation, whereas with paclitaxel the kinetics of the two phenomena were similar.*

Nuclear factor kappaB (NF-Î B) is a transcriptional factor which can be activated by a wide range of immunological and inflammatory stimuli. It is formed by two subunits, usually p65/p50 or p50/p50, kept inactive in the cytoplasm by the association with the inhibitory protein of NF-Î B, IkBα. Upon appropriate stimulation, IkBα is phosphorylated by the upstream IkB kinase (IKK), and then polyubiquitinated and degraded by proteases, causing the release and translocation of NF-Î B into the nucleus (1, 2).

NF-Î B nuclear translocation can also be induced by different classes of antitumor drugs. Topoisomerase poisons (camptothecin, etoposide, anthracyclines), DNA-intercalating agents (mitoxantrone) or antimicrotubule drugs such as paclitaxel (Taxol) have been described as potent inducers of NF-Î B activation (3-7). Much evidence suggests that NF-Î B activation by topoisomerase poisons is induced by DNA-strand breaks through a pathway leading from the nucleus to cytoplasmic IKK, which probably involves signaling proteins such as the ataxia-teleangiectasia mutated kinase ATM , ATM-Rad3-related (ATR) and DNA-dependent protein kinases (8-10). However, in the case of microtubule targeting drugs, such as paclitaxel, it is less clear what events induce IkB degradation, whether nuclear signals are involved and how NF-Î B is connected to the apoptotic pathway (11). Some authors demonstrated that paclitaxel depends on protein kinase C (PKC) conventional isotypes for NF-Î B activation and that the c-Jun N-terminal protein kinase (JNK) pathway is induced by paclitaxel and involved in NF-Î B-mediated gene expression (12, 13). Regarding paclitaxel, it has been observed that NF-Î B inhibition can both increase the rate of cytotoxic cell death or decrease drug-mediated apoptosis (6, 11, 14). When anthracyclines or camptothecins are employed, an anti-apoptotic function of NF-Î B has been described, even if it has been reported that, in endothelial cells, NF-Î B inhibition reduces doxorubicin (DXR)-induced cell death (3, 15-19). Therefore, it is clearly emerging how NF-Î B transcriptional activity is strongly influenced by the type of stimulus and also by the cellular histotype, since different genetic patterns and experimental models could affect NF-Î B activation (20, 21).

The aim of this study was to investigate, in the human colon cancer cell line HCT-116 which has wild-type p53, the effect on NF-Î B induction of two anticancer agents, characterized by completely different mechanisms of action, *i.e.* the new disaccharide anthracycline Sabarubicin (MEN

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Key Words: NF-kappaB activation, Sabarubicin, paclitaxel, apoptotic DNA fragmentation, parthenolide, colon cancer cells.

10755) and the microtubule stabilizing drug paclitaxel (22, 23). Human colon cancer cell lines, in most of which NF-Î B can be strongly induced by antitumor drugs, are well described as being poorly sensitive to anthracycline cytotoxic effects both *in vitro* and *in vivo* models (24, 25). We evaluated whether the sensitivity of HCT-116 cells to Sabarubicin or paclitaxel could be increased by NF-Î B inhibition, attained using the sesquiterpene lactone parthenolide, which inhibits NF-Î B by targeting the IkB kinase complex (26). Our data indicated a different effect of the NF-Î B-inhibitor when combined with these two drugs, since blocking the early activation of Sabarubicin-induced NF-Î B seemed to ameliorate anthracycline cytotoxic activity. On the contrary, inhibition of the late NF-Î B induced by paclitaxel did not increase antitumoral-mediated cell death. Furthermore, the NF-Î B activation cascade induced by Sabarubicin preceded DNA apoptotic fragmentation, while in the case of paclitaxel the signaling for NF-Î B activation was subsequent to the induction of DNA apoptotic damage.

Materials and Methods

Cell line and culture conditions. The human colon cancer cell line HCT-116, which is wild-type for p53, was purchased from ATCC. The cells were routinely propagated in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine (Gibco BRL, Invitrogen, Milan, Italy) and maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Chemicals and antibodies. Sabarubicin was synthesized as already described (22). Paclitaxel was kindly provided by Bristol-Myers Squibb. Stock solutions were prepared in distilled water and stored at –20˚C. Parthenolide (Sigma, St. Louis, MO, USA) was dissolved in DMSO and stored at –20˚C. [γ-^{32}P]-ATP (3,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Antibodies to NF-Î B subunits p50 (sc-114 X), p65 (sc-109 X), p52 (sc-298 X), c-rel (sc-6955 X) and RelB (sc-226 X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tumor necrosis factor-α (TNF-α) was purchased from Alexis Biochemicals (Lausen, Switzerland) and used at the concentration of 20 ng/ml. Propidium iodide (PI) was purchased from Sigma and dissolved in hypotonic buffer (0.1% Na citrate) at the concentration of 50 μg/ml.

Determination of cell death. Cytotoxic cell death was measured by plating 2x10^5 cells in 24-well plates with 1 ml medium containing 10% FBS and incubating overnight. Next day, the cells were incubated with different concentrations of antitumoral drugs; after 24 h, the cells were harvested by trypsinization, stained by trypan blue dye and the percentage of dead cells measured by counting with a hemocytometer.

DNA fragmentation assay. 10^4 cells were plated in 96-well plates, incubated for 4, 14, or 24 h with different concentrations of antitumoral drugs and then assayed for the formation of cytoplasmic histone-associated-DNA-fragments using the cell death detection enzyme-linked immunosorbent assay (ELISA) plus kit (Roche, Indianapolis, IN, USA). Briefly, after pharmacological treatment, the cells were lysed in 200 μl lysis buffer for 30 min, centrifuged and 20 μl of the lysate transferred to streptavidin-coated microplate wells together with 80 μl of anti-histone-biotin- and anti-DNA-peroxidase-conjugated antibodies. After 2 h at room temperature under gently shaking, the solution was removed by tapping and 100 μl 2,2'-Azino-di-[3-ethyl-benz-thiazo-line sulfonate(6)] (ABTS) were added and the wells incubated for 20 min until color development was sufficient for photometric analysis. Absorbance was measured at 405 nm against ABTS solution as a blank using a Victor microplate reader (Wallac, Turku, Finland). The results are expressed as DNA fragmentation index, which is the ratio between the absorbance of the sample and that of the untreated control .

Flow cytometric analysis. Apoptosis in HCT-116 cells was detected by flow cytometry using PI staining, as previously described (27). Briefly, trypsinized adherent and floating cells were collected, washed twice with cold PBS and fixed with 70% ethanol. After fixation, they were washed with PBS and resuspended at a density of about 10^{6}cells/ml in 50 μg/ml PI containing 1.25% RNAsi at 0.5 mg/ml and 0.1% Nonidet P40 (NP40), and incubated at room temperature for 30 min in the dark before acquisition using a FACSort flow cytometer (Becton Dickinson, Mountain View, CA, USA). Apoptosis was measured using CellQuest and Cellfit analysis software (Macintosh, Facstation, Becton Dickinson).

Nuclear extracts preparation. Following treatments, the cells were washed quickly with ice-cold PBS, harvested by brief trypsinization and incubated with 0.5 ml buffer A [10 mM HEPES (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA;1 mM dithiothreitol (DTT); 0.75 mM spermidine; 1 mM phenylmethylsulfonyl fluoride (PMSF); aprotinin, leupeptin and pepstatin (2 μg/ml each)] for 10 min on ice. After centrifugation, the pellets were resuspended with 0.5 ml buffer B (buffer A containing 0.5% NP-40) and further incubated for 10 min on ice. Lysates were spun to isolate nuclear pellets and the proteins extracted with 50 μl of buffer C [20 mM HEPES (pH 7.9); 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF; and aprotinin, leupeptin and pepstatin (2 μg/ml each)] for 1 h at 4˚C with rotation. Protein concentration was calculated with the Bio-Rad Protein assay (Munchen, Germany).

Electrophoretic mobility shift assay (EMSA). The double-stranded NF-Î B oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') was purchased from Promega (Madison, WI, USA) and 5' end-labelled with [γ-^{32}P]-ATP and T4 polynucleotide kinase for 1 h at 37˚C. Binding reactions were performed using 3-5 μg of nuclear extracts. Proteins were pre-incubated for 10 min at room temperature with binding buffer [10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 0.05% NP-40, 10% glycerol and 1 μg dI-dC]. Labelled NF-Î B oligo (10,000 cpm) was then added and the reaction allowed to proceed for a further 20 min at room temperature. For supershift experiments, the antibody to each subunit was incubated with nuclear extracts in binding buffer for 30 min at room temperature before addition of labelled oligo. Control reactions included a cold-specific NF-Î B competitor and a cold-unrelated competitor. NF-Î B complexes were resolved on 4% acrylamide gels, dried and exposed to autoradiography.

Fluorogenic substrate assay for caspase-3 activity. HCT-116 cells were seeded in 96-well plates at 3x10^4 cells/well. After 18-20 h, the cells were pre-incubated for 20 min with 20 μM parthenolide and then Sabarubicin or paclitaxel were added for a further 24 h. At the end of the incubation time, the cells were lysed for 5 min in 20 μl lysis

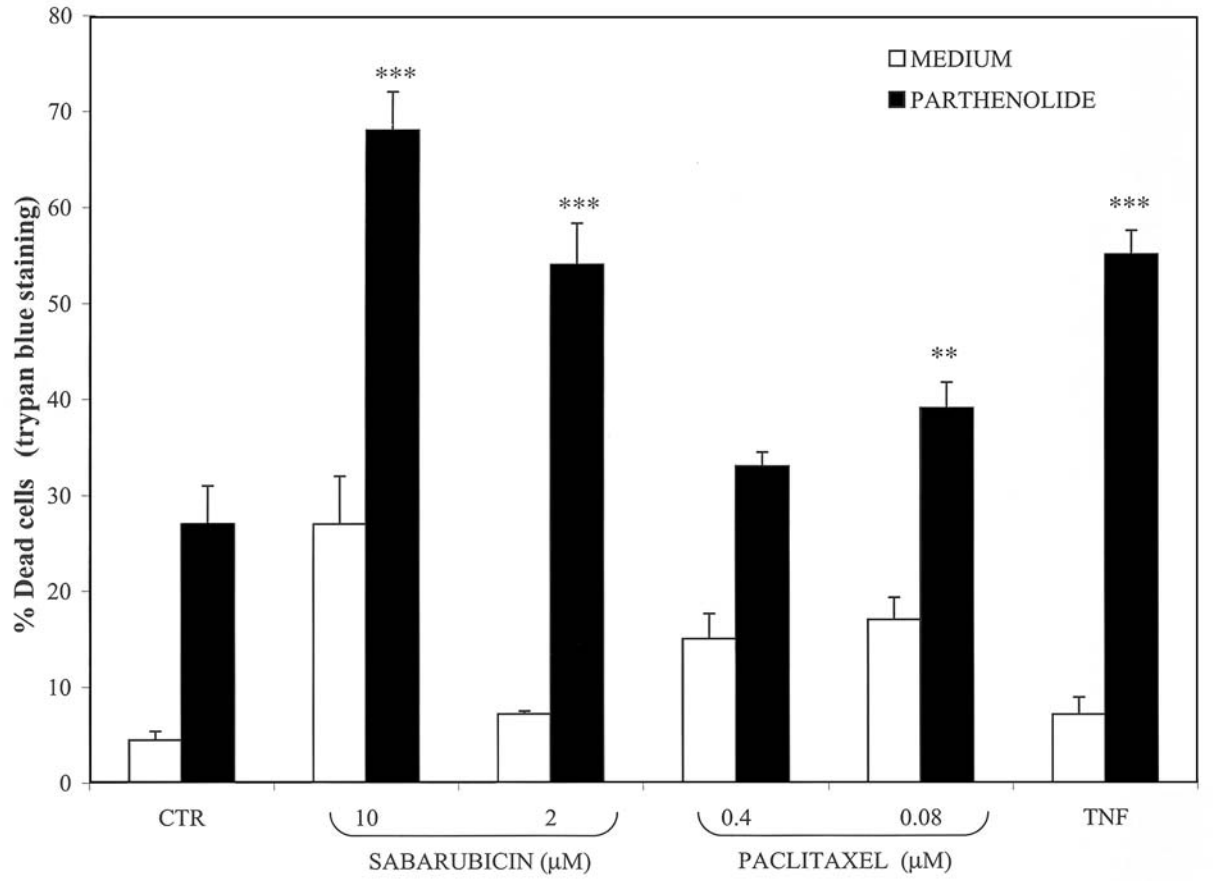

Figure 1. *Determination of cell death induced by Sabarubicin or paclitaxel in the presence of parthenolide. HCT-116 cells were exposed to 10 or 2 μM Sabarubicin, 0.4 or 0.08 μM paclitaxel or 20 ng/ml* TNF-α *for 24 h after a pre-treatment of 30 min with 20 μM parthenolide. The percentage of dead cells was measured by trypan blue staining. Data represent the mean±SE from four independent experiments. (**) $p<0.01$, (***) $p<0.001$, significantly different from cells treated with drug alone.*

buffer (1% Triton, 130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaF, 100 mM PMSF). Caspase-3-like activity was measured by incubation of the cell lysate with 25 μM of the fluorogenic substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin (Ac-DEVD-amc) (Alexis Corporation, Lausen, Switzerland) in a 200 μl cell-free system buffer, comprising 20 mM Hepes, 10% glycerol, 2 mM DTT. After 2 h at 37˚C, the release of fluorescence was measured using a Victor microplate reader (Wallac, Turku, Finland).

Statistics. All results were expressed as the mean±SE of data obtained from three to four separate experiments. The data were entered into Instat 2.03 GraphPad software to perform Tukey's test. Differences between groups with $p<0.05$ were considered significant.

Results

Parthenolide increases Sabarubicin- but not paclitaxel-induced cell death. In order to evaluate the effect of NF-Î B inhibition on antitumor cytotoxicity, HCT-116 cells were incubated for 24 h in the presence of parthenolide plus Sabarubicin or paclitaxel at concentrations which showed to be equitoxic into a 96-h cytotoxicity assay (10 and 2 μM for Sabarubicin and 0.4 and 0.08 μM for paclitaxel). Parthenolide was used at 20 μM, which is the concentration corresponding to the IC_{30} extrapolated from dose-response cytotoxicities at 24 h (data not shown). Dead cells were measured by trypan blue staining. As shown in Figure 1, the percentage of dead cells induced by 10 and 2 μM Sabarubicin was from three- to ten-fold, respectively, increased by the combination with parthenolide, while the effect of the association between this NF-Î B inhibitor and paclitaxel was weaker and only in one case significantly different from single-drug treatment. As control, the effect of parthenolide on cell mortality induced by TNF-α was measured, since many tumor cell lines become resistant to the death effect of TNF-α which strongly stimulates the NF-Î B survival cascade. We found that the inhibition of NF-Î B nuclear translocation can restore TNF-α cytotoxicity and this effect is quite beyond the cytotoxic effect of parthenolide alone (Figure 1).

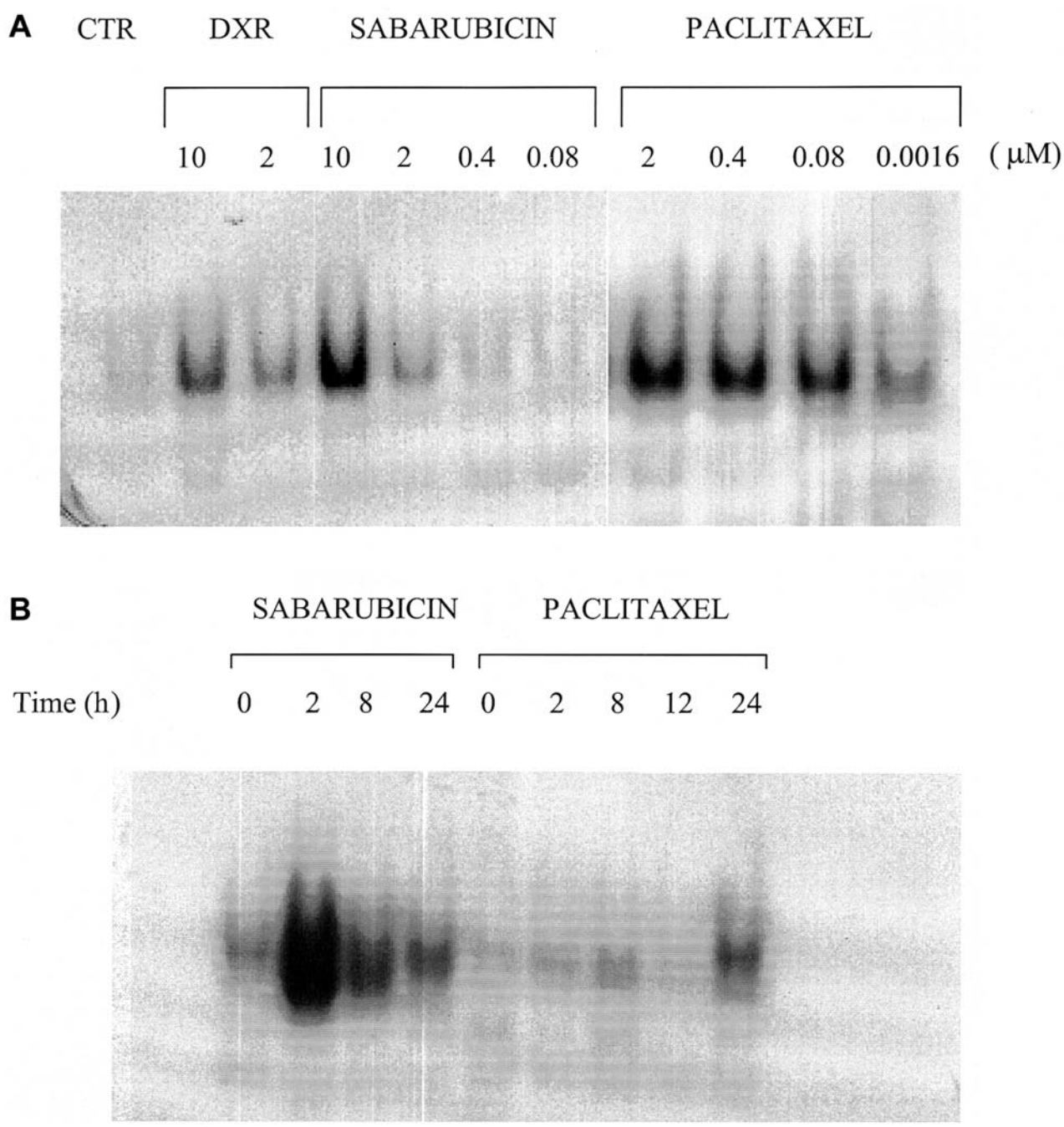

Figure 2. *Electrophoretic mobility shift assay for NF-Î B nuclear translocation. (A) Dose-response of NF-Î B activation. HCT-116 cells were treated with increasing concentrations of DXR (2 h), Sabarubicin (2 h), or paclitaxel (24 h). Nuclear extracts (4 μg of proteins) were assayed for NF-Î B-DNA binding using ^{32}P-labelled NF-Î B consensus oligonucleotide. (B) Kinetics of NF-Î B activation following treatment with Sabarubicin or paclitaxel at the concentrations of 10 μM and 0.08 mM, respectively.*

Sabarubicin or paclitaxel treatment induces NF-Î B activation and parthenolide inhibits it. We performed EMSA on nuclear extracts from HCT-116 cells using a Î B sequence from *k* light chain enhancer in order to compare the effect of Sabarubicin and paclitaxel on NF-Î B induction. As shown in Figure 2A, Sabarubicin stimulated DNA binding of NF-Î B after 2 h of incubation at the concentrations of 10 and 2 μM. This effect was very similar to that observed with DXR, even though 10 μM Sabarubicin seems to more strongly induce NF-Î B. On the contrary, dose-response experiments with paclitaxel

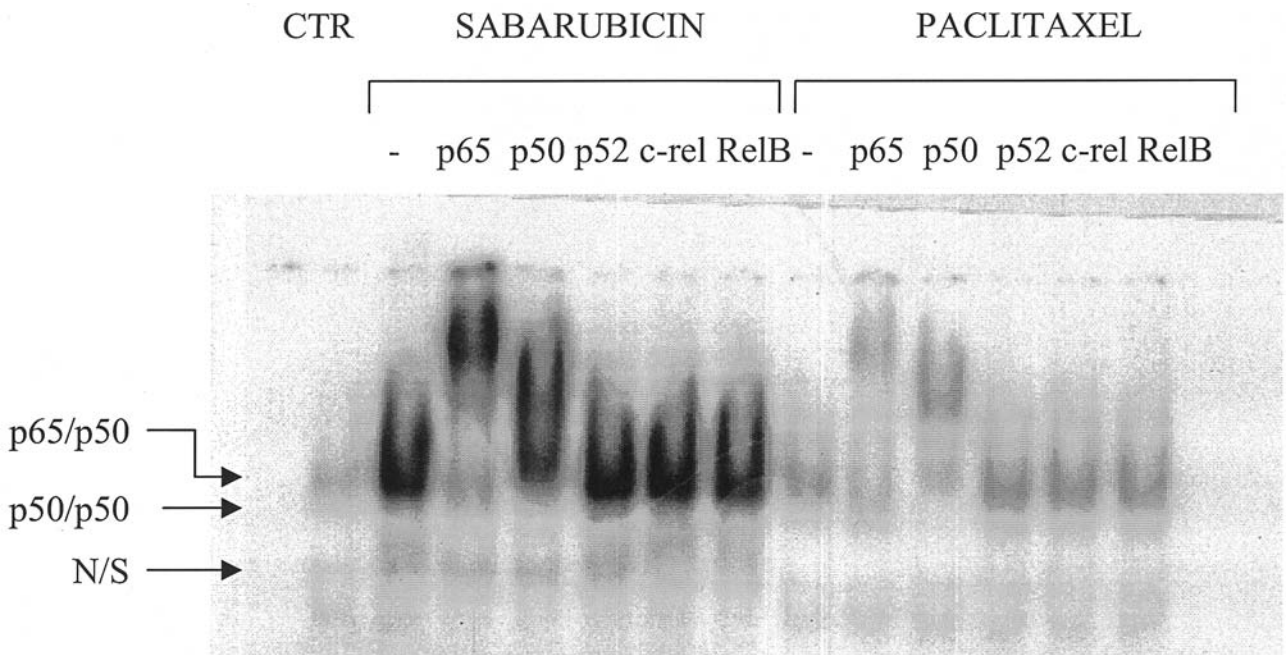

Figure 3. *Supershift assay of Sabarubicin- or paclitaxel-induced NF-Î B subunits. HCT-116 cells were treated with Sabarubicin or paclitaxel at 10 or 0.08 μM for 2 or 24 h of stimulation, respectively. Nuclear extracts (4 μg of proteins) were pre-incubated for 30 min at room temperature with 2 ml of the specific antibodies directed against p50, p65, p52, c-rel, or RelB subunits before addition of the ^{32}P-labelled NF-Î B consensus oligonucleotide. N/S: non-specific binding.*

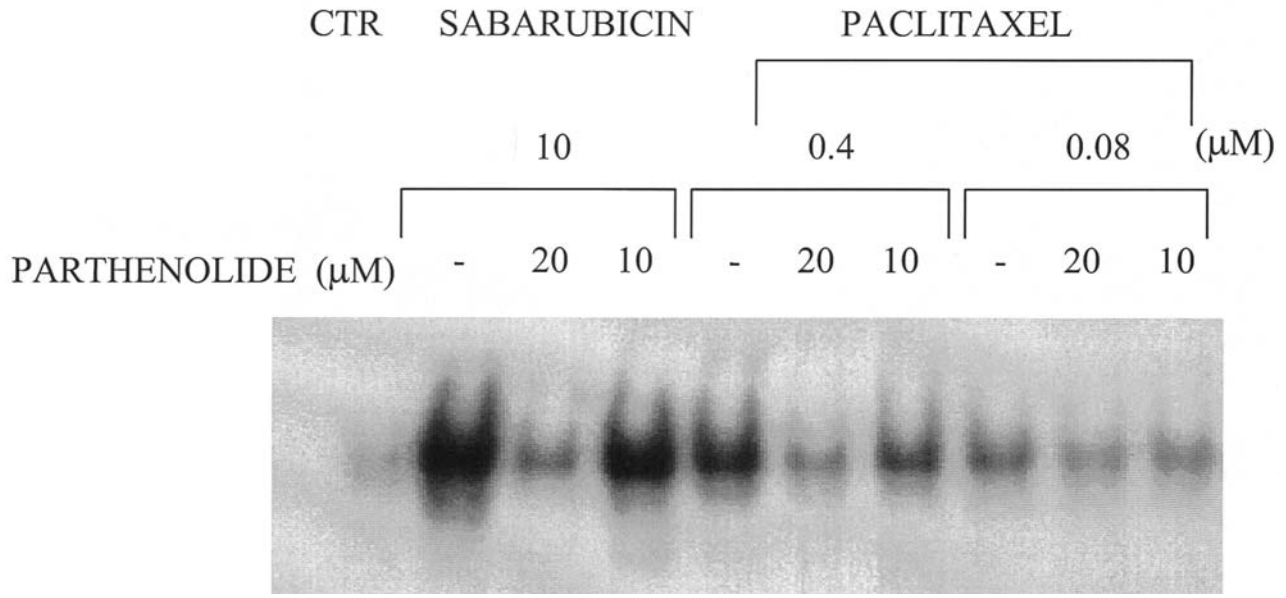

Figure 4. *Effect of parthenolide on NF-Î B activation induced by Sabarubicin or paclitaxel. HCT-116 cells were pre-treated with 20 or 10 μM parthenolide for 30 min and then exposed to 10 μM Sabarubicin or 0.4 and 0.08 μM paclitaxel for 2 or 24 h, respectively. Nuclear extracts (4 μg of proteins) were analyzed by EMSA.*

showed that short-time exposure (2-8 h) did not increase NF-Î B binding even at high concentrations (30-10 μM, data not shown). Paclitaxel treatment induced the appearance of significant NF-Î B DNA binding only after 24 h of incubation, thus suggesting that the kinetics of NF-Î B induction were definitely different between the two compounds (Figure 2B).

Supershifting experiments performed with antibodies directed toward NF-Î B proteins demonstrated that the

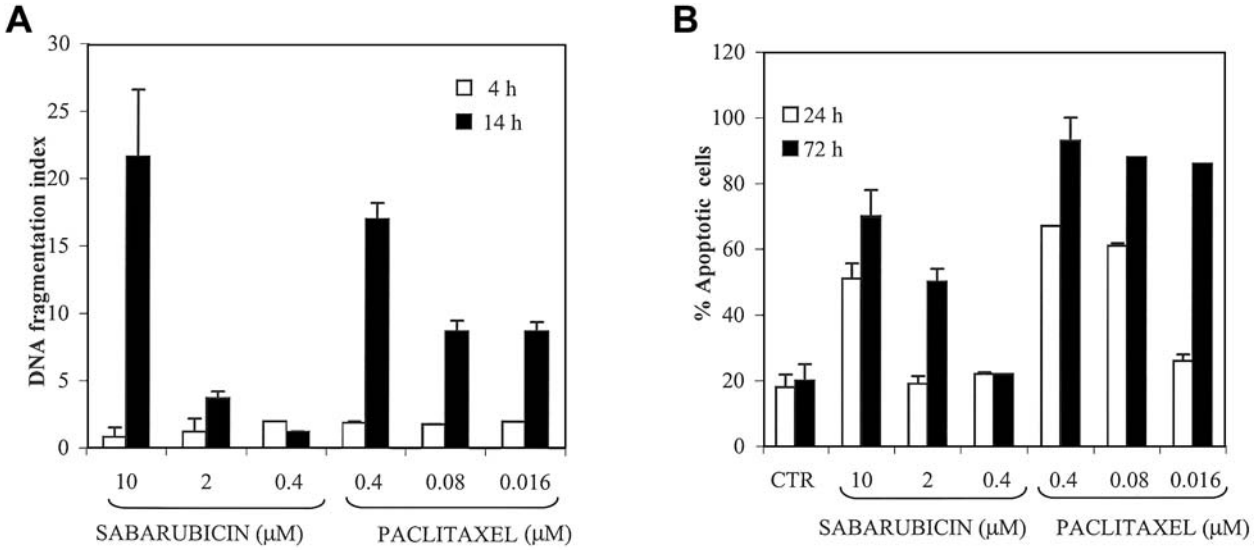

Figure 5. *(A) Determination of histone-associated DNA fragments in the cytoplasm of drug-treated HCT-116 cells. After incubation with equitoxic concentrations of Sabarubicin or paclitaxel at 4, 14 and 24 h, cells were lysed in 200* μl *lysis buffer for 30 min, centrifuged and 20* μl *of the lysate transferred to streptavidin-coated microplate wells together with 80* μl *of anti-histone-biotin- and anti-DNA-peroxidase-coniugated antibodies. The results are expressed as DNA fragmentation index, that is the ratio between the absorbance of the sample and that of untreated control. (B) Evaluation of apoptotic cells by flow cytometric analysis following Sabarubicin or paclitaxel treatment. HCT-116 cells were exposed to equitoxic concentrations of Sabarubicin or paclitaxel for 24 or 72 h, stained with 50* μg/ml *PI , and then acquired with a Facsort instrument. Samples were analyzed with Cellfit software and apoptotic cells measured as the hypodiploid sub-G1 peak. Data represent the mean ± SE of three independent experiments.*

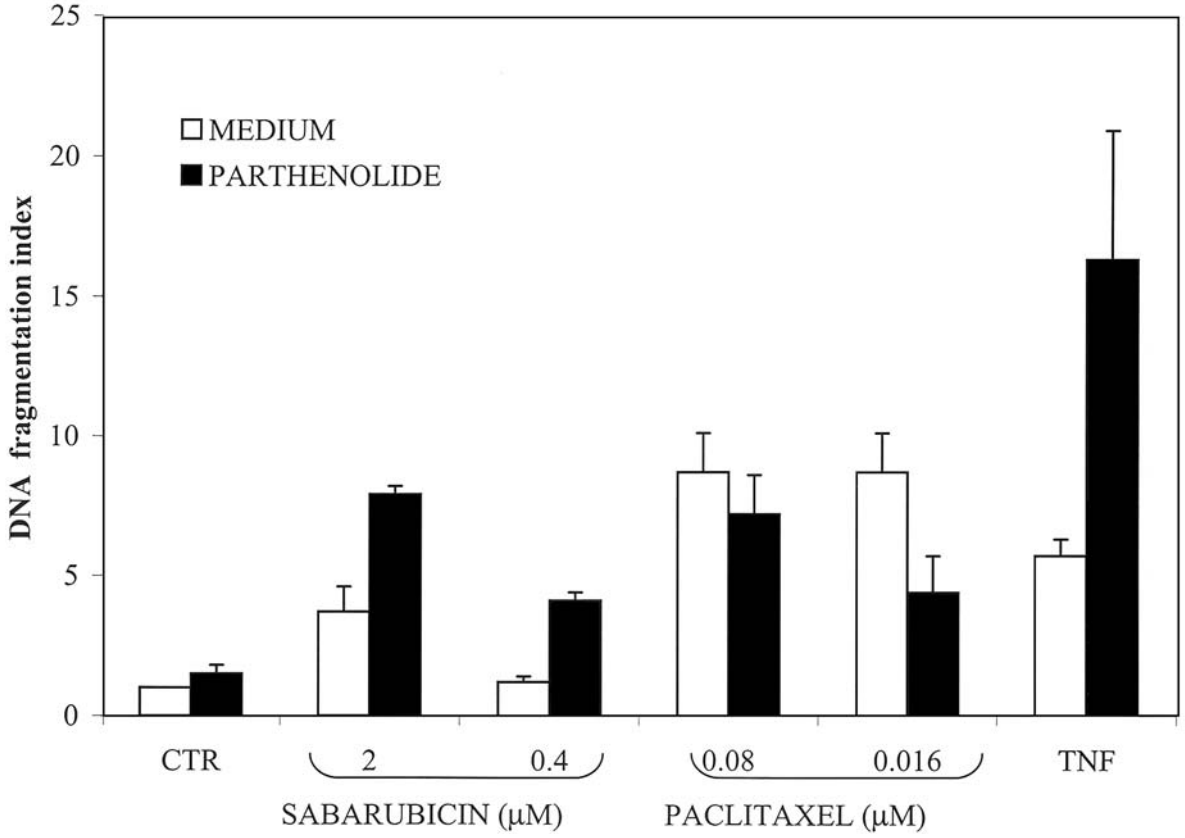

Figure 6. *Effect of parthenolide on cytoplasmic histone-associated DNA fragments induced by Sabarubicin or paclitaxel. HCT-116 cells were pre-treated for 30 min with 20 μM parthenolide and then incubated with 2 or 0.4 μM Sabarubicin, 0.08 and 0.016 μM paclitaxel or 20 ng/ml TNF-α for 14 h. Data represent the mean±SE of three independent experiments.*

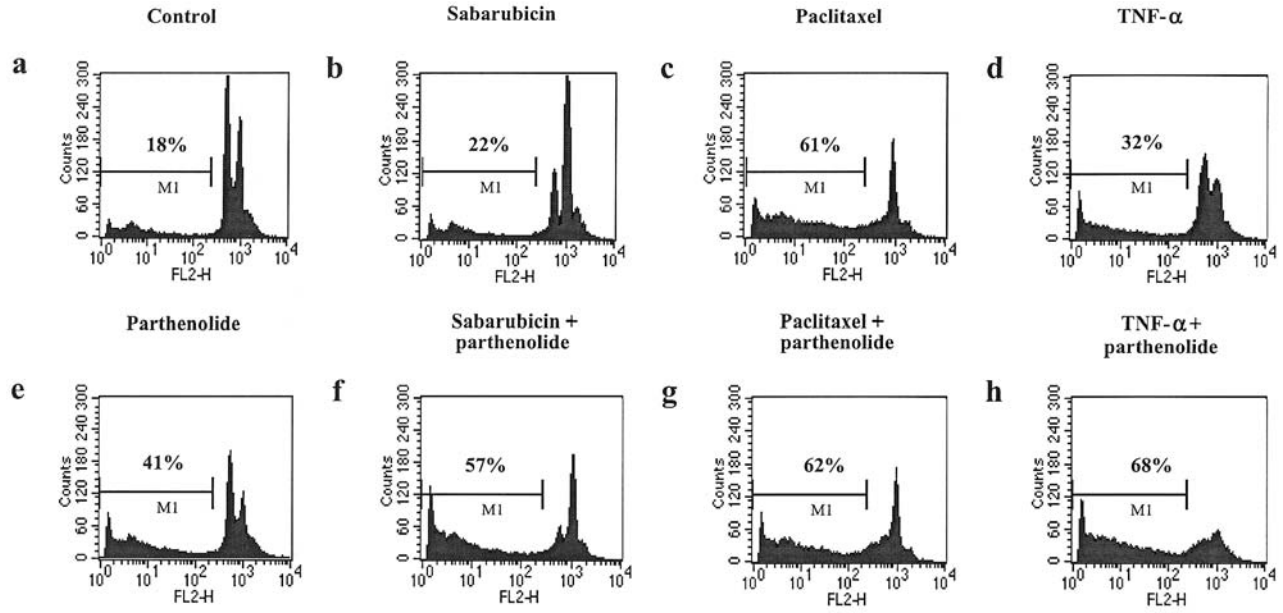

Figure 7. *Flow cytometric determination of Sabarubicin- or paclitaxel-induced apoptosis in the presence of parthenolide. HCT-116 cells were pre-incubated with parthenolide and then exposed to Sabarubicin or paclitaxel or TNF-α for 24 h. (a) control, (b) 2 μM Sabarubicin, (c) 0.08 μM paclitaxel, (d) 20 ng/ml TNF-α, (e) 20 μM parthenolide, (f) 2 μM Sabarubicin + 20 μM parthenolide, (g) 0.08 μM paclitaxel + 20 μM parthenolide, (h) 20 ng/ml TNF-α + 20 μM parthenolide. Apoptotic cells were measured by CellQuest software. This experiment is representative of three similar ones.*

subunit composition of NF-Î B induced by Sabarubicin or paclitaxel was similar. In our experimental conditions, autoradiographies showed two adjacent NF-Î B bands: a dense upper band, probably corresponding to p65/p50 complexes, and a fainter and faster migrating band, probably p50 homodimers. p65 antibodies displaced the upper band, while p50 antibodies supershifted the lower band and part of the upper band both for Sabarubicin- or paclitaxel-induced NF-Î B (Figure 3). p52, c-rel and rel-B antibodies did not affect the migration of drug -induced NF-Î B.

The effect of parthenolide on Sabarubicin- or paclitaxel-induced NF-Î B activation was evaluated after 2 or 24 h of incubation, respectively. Twenty μM parthenolide suppressed 50% of NF-Î B DNA binding induced by 10 μM Sabarubicin or by 0.4 and 0.08 μM paclitaxel, as evaluated by densitometric analysis (Figure 4).

Sabarubicin- or paclitaxel- induced apoptotic DNA fragmentation is correlated to NF-Î B induction and is affected differently by NF-Î B inhibition. To investigate whether the induction of oligonucleosomal DNA fragmentation could be correlated to NF-Î B activation, we measured cytoplasmic histone-associated-DNA-fragments after incubation with Sabarubicin or paclitaxel alone or in combination with parthenolide. Figure 5A shows the different behavior of these two antitumoral drugs at their equitoxic concentrations: Sabarubicin induced significant oligonucleosomal fragmentation at 10 μM after 14 h,

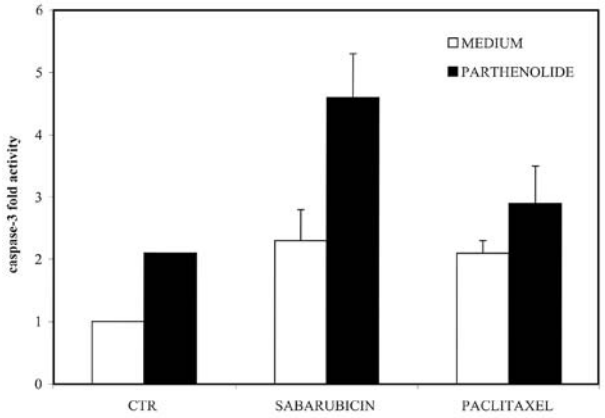

Figure 8. *Effect of parthenolide on caspase-3 activity induced by Sabarubicin or paclitaxel. HCT-116 cells were pre-treated for 30 min with 20 μM parthenolide and then incubated with 2 μM Sabarubicin or 0.08 μM paclitaxel for 24 h. Data represent the mean±SE of three independent experiments.*

but the effect was definitely lower at 2 and 0.4 μM (DNA fragmentation index: 3.7 and 1.2, respectively). On the contrary, paclitaxel also at 0.08 and 0.016 μM showed a higher DNA fragmentation index (DNA fragmentation index: 8.7). These data are in agreement with cytofluorimetric studies, which confirm that, in this cell line, equitoxic concentrations of the two agents resulted in a different degree of apoptosis:

after both 24 and 72 h of exposure, paclitaxel activated a strong apoptotic response at all tested concentrations, whereas Sabarubicin induced the apoptotic process only at 10 μM or after longer incubation times, suggesting that HCT-116 cells are more resistant to anthracycline-induced apoptosis (Figure 5B).

In the presence of 20 μM parthenolide, DNA fragmentation at 14 h was increased by the association between the NF-Î B inhibitor and Sabarubicin, but unaffected when the combined cytotoxic agent was paclitaxel (Figure 6). TNF-α-induced DNA fragmentation was increased by parthenolide, as reported in the literature (28). Also, cytofluorimetric PI staining confirmed that the presence of parthenolide improved Sabarubicin-mediated apoptotic cell death in an additive manner (Figure 7).

In order to further support data obtained by DNA fragmentation measurements, we analyzed the effect of the combination between parthenolide and Sabarubicin or paclitaxel on caspase-3 activity after 24 h of drug treatment. Figure 8 shows that the association of parthenolide with Sabarubicin significantly doubled caspase-3 activation, compared to Sabarubicin alone, while the effect of the NF-Î B inhibitor on paclitaxel-induced caspase-3 was lower.

Discussion

In this study, we compared the effect of the NF-Î B inhibitor parthenolide on the cytotoxicity of the new anthracycline Sabarubicin and of the well-known anti-microtubule agent paclitaxel. We investigated whether NF-Î B induction could be: i) responsible for a reduced drug sensitivity; ii) related to apoptotic DNA fragmentation. For this purpose, we used the human colon cancer cell line HCT-116: in these cells the sensitivity to anthracyclines is lower when compared to other cell lines, contrary to paclitaxel sensitivity, and NF-Î B can be strongly activated by antitumoral drugs (24, 29). Furthermore, since it has been demonstrated that mutated p53 changes the normal transcriptional cross-talk with NF-Î B, we chose to use a p53 wild-type cell line (30).

Pharmacological inhibition of NF-Î B was obtained using parthenolide, a sesquiterpene lactone that is a specific inhibitor of IKK, functioning similarly to an IkBα super-repressor (26). Although some studies indicated that parthenolide can affect other cellular targets, the effect on the NF-Î B pathway seems to be prevalent (31). Following the association of parthenolide and Sabarubicin, we observed a marked improvement of cytotoxic cell death, suggesting a survival role for Sabarubicin-induced NF-Î B (Figure 1). On the other hand, we found that the association between the NF-Î B inhibitor and paclitaxel did not affect drug-induced cell death, so we hypothesize that NF-Î B triggered by paclitaxel might have a different function than Sabarubicin (6).

Previous studies in HCT-116 cells demonstrated that NF-Î B inhibition through IkBα super-repressor does not affect the response to cytotoxic drugs (29, 32), which is in contrast with our data. However, it has been supposed that the molecular inhibition of NF-Î B through stable transfection could select cell clones with alternative survival pathways and that this model would not be appropriate to investigate the effects of the NF-Î B blockade (24). Moreover, in cancer cells of different histotypes, NF-Î B induction could be less important as a survival mechanism.

We also demonstrated that the kinetics of NF-Î B induction by the two drugs is definitely different: Sabarubicin strongly induced NF-Î B DNA binding in EMSA assay at 10 and 2 μM after 2 h of treatment while, after paclitaxel challenging, NF-Î B became evident only after 24 h (Figures 2A and 2B). The evaluation of NF-Î B dimer composition in supershifting showed that p65/p50 and p50/p50 were the predominant dimers for both Sabarubicin- and paclitaxel- induced NF-Î B at 2 and 24 h of stimulation, respectively (Figure 3).

Thus, from the above results, we can conclude that, under our experimental conditions, the two antitumoral drugs induce NF-Î B complexes which seem to affect cytotoxic cell death differently, and show different kinetics but similar subunits. In order to better characterize the NF-Î B-related differences between the two drugs, we analyzed the appearance of apoptotic DNA damage following drug treatment and the possible connection with NF-Î B activation. Oligonucleosomal DNA fragmentation is an early apoptotic marker which signals the activation of endogenous Ca^{2+}- and Mg^{2+}-dependent endonucleases: the enrichment of mono- and oligonucleosomes in the cytoplasm of apoptotic cells occurs several hours before plasma membrane breakdown (33). In our experimental system, both drugs induced a maximum of oligonucleosomal fragmentation after 14 h of drug treatment (Figure 5A). Comparing these results with the NF-Î B induction kinetics, we concluded that, for Sabarubicin-incubated cells, NF-Î B induction appears definitely earlier than DNA fragmentation, probably as a consequence of topoisomerase II-mediated DNA strand breaks whereas, for paclitaxel-treated cells, the kinetics of the phenomena are superimposed. Moreover, cytofluorimetric studies with longer incubation times demonstrated that the degree of apoptosis induced by paclitaxel was higher than that induced by Sabarubicin, especially at 24 h when paclitaxel-induced NF-Î B was substantially activated, as though the appearance of NF-Î B following paclitaxel treatment does not have anti-apoptotic effects as in cells grown in the presence of Sabarubicin (Figure 5B). Since, in HCT-116 cells, paclitaxel is not able to induce p53, p21 and bax up-regulation, it is possible that NF-Î B, deprived of important transcriptional competitors, could modulate a broader panel of genes, not only anti-apoptotic ones (34). Interestingly, when NF-Î B

was pharmacologically-inhibited, as demonstrated by EMSA experiments (Figure 4), this event affected Sabarubicin- but not paclitaxel-induced DNA fragmentation (Figure 6). Apoptosis measurements by cytofluorimetry and caspase-3 experiments confirmed these results of the combination between the NF-Î B inhibitor and the two anticancer agents (see Figures 7 and 8). The lesser improvement of cell death observed with elevated concentrations of Sabarubicin (see Figure 1) could be explained considering that, at high concentration, other transcriptional regulators might be induced, which could either compete with NF-Î B activity or be part of a NF-Î B-independent survival route.

In summary, our findings suggest that, in HCT-116 cells, Sabarubicin induces an early pro-survival NF-Î B, probably activated by early DNA strand breaks and whose inhibition enhances anthracycline killing. Conversely, paclitaxel activates first cytoplasmic pathways, including mitogen-activated protein kinases (MAPKs) and other signals probably mediated by alterations of microtubule dynamics, and targets the nucleus only as secondary issue (35); thus, we suppose that paclitaxel-induced NF-Î B could depend not only on cytoplasmic, but also on nuclear signaling and that the loss of its survival function could be related to its late appearance. In conclusion, many different factors can affect the role of NF-Î B as a modulator of the apoptotic process and these factors include the type of inducing stimuli, the cell line used and the kinetics of activation. Therefore, these aspects must be considered when planning new antitumoral therapeutic strategies employing NF-Î B inhibitors.

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Received August 9, 2004
Revised January 24, 2005
Accepted February 9, 2005