

Moxifloxacin enhances etoposide-induced cytotoxic, apoptotic and anti-topoisomerase II effects in a human colon carcinoma cell line

DEBBY REUVENI¹, DRORA HALPERIN¹, ITAMAR SHALIT², ESTHER PRIEL³ and INA FABIAN¹

¹Department of Cell and Developmental Biology, Sackler School of Medicine, Tel-Aviv University, Tel Aviv;

²Schneider Children's Medical Center of Israel, Petach-Tikva, and Sackler School of Medicine, Tel-Aviv University,

Tel-Aviv; ³The Shraga Segal Department of Microbiology and Immunology, BGU Cancer Research Center,

Faculty of Health Sciences, Ben-Gurion University, Beer-Sheva, Israel

Received February 18, 2010; Accepted April 12, 2010

DOI: 10.3892/ijo_00000695

Abstract. Etoposide (VP-16) is a topoisomerase-II (topo II) inhibitor chemotherapeutic agent. Studies have shown that a combination of VP-16 with other drugs demonstrates better clinical responses. The aim of this study was to investigate the effects of moxifloxacin (MXF) and VP-16 on cellular topo II activity in drug-treated cells and evaluate the influence of MXF on the mode of action of VP-16, on proliferation and apoptosis of HT-29 cells. Decatenation assay, band depletion and Western blot analysis, cytotoxic assay (MTT), flow cytometric studies (cell cycle and survivin expression), apoptosis (DAPI-sulforhodamine staining and caspase 3 activity) and IL-8 and VEGF secretion were determined. MXF or VP-16 slightly affected cellular topo II activity in nuclear extracts derived from drug-treated cells while the combination enhanced inhibitory activity and the reduction in band depletion of topo II. VP-16 induced cell cycle arrest at G₂/M and the appearance of the subG₁ peak which was increased by the addition of MXF. Apoptosis studies (DAPI staining and caspase 3 activity) showed a marked increase in the presence of MXF and VP-16 compared to VP-16 alone. VP-16 induced the release of IL-8, and addition of MXF reduced enhanced release and the spontaneous release of VEGF from the cells. In conclusion, the results suggest that the enhancement in the reduction of topo II activity by the combined MXF/VP-16 treatments was probably due to the increase in the level of the DNA-enzyme cleavable complexes formed by both drugs. The unique combination of MXF/VP-16 may have clinical benefits and a cytotoxic drug 'sparing effect' and should be further studied *in vivo*.

Introduction

Colorectal carcinoma is the most common gastrointestinal neoplasm and the second cause of death from cancer in the Western world (1). Despite recent advances in neoadjuvant therapeutic modalities, treatment success is limited in advanced stages of colorectal carcinoma. Additional modalities are needed to cope with this challenge, and biological response modifiers may represent such novel pharmacological options (2).

Etoposide (VP-16) is one of the most widely used anticancer drugs. It belongs to the family of DNA-topoisomerase II (topo II) inhibitors. Topoisomerases form transient covalent adducts (cleavage complexes) with DNA. Etoposides stabilize the transient 'cleavable complexes' formed between topo II and DNA in the process of untangling the DNA helix and thus introduce double-strand breaks (DSBs) in the DNA (3,4). Unrepaired DNA DSBs cause cell death by apoptotic process. VP-16 is used today as the front-line therapy for a variety of human malignancies, including leukemias, lymphomas and several solid tumors (5,6). Previous studies have shown that VP-16 is ineffective as a single agent in colorectal carcinoma (7), but a combination of VP-16 and mitomycin C demonstrated activity in advanced colorectal cancer (1) and in upper-gastrointestinal tumors (8), metastatic breast cancers (9) and metastatic carcinomas of unknown primary (10). Clinical studies in colorectal carcinoma using VP-16 in combination with other agents, mainly cisplatin, showed partial clinical responses (11,12).

It was recently shown that the fluoroquinolone ciprofloxacin, a commonly used broad-spectrum antibiotic with an excellent safety profile, induced apoptosis and inhibited proliferation of human colorectal carcinoma cells (13) and colon cancer cell lines (14) and induced the growth inhibition and apoptosis of osteosarcoma cells (15). Fluoroquinolones are highly effective antibiotics with a broad antibacterial spectrum (16). At high concentrations, some fluoroquinolones exhibit genotoxic effects in eukaryotic systems as a result of topoisomerase II inhibition (17). Quinolone-induced inactivation of topo II α at high concentrations was proposed to involve the direct binding of quinolones to DNA, and their mode of inhibition was shown to be distinct from the inhibitory

Correspondence to: Dr Ina Fabian, Department of Cell and Developmental Biology, Sackler School of Medicine, Tel-Aviv University, Tel Aviv 69978, Israel
E-mail: inaf@post.tau.ac.il

Key words: moxifloxacin, topoisomerase II, etoposide, colon carcinoma, chemotherapy, angiogenesis

mechanism of agents such as VP-16 (18). We showed previously that the fluoroquinolone moxifloxacin (MXF) inhibited purified human topoisomerase II, acted synergistically with VP-16 at the purified enzyme level and enhanced its anti-proliferative effects in THP-1 and Jurkat cells (19).

In the present study we further investigated the effects of MXF alone and in combination with VP-16 on the cellular topo II activity. In order to investigate the mechanism by which MXF affects VP-16 cytotoxicity, we determined the formation of the cellular covalent topo-DNA complexes in drug-treated cells (MXF and VP16 administered alone or in combination). Moreover, we expanded our studies to investigate the effects of MXF and VP-16 on various parameters of cell proliferation, cell cycle progression, apoptosis and signal transduction pathways in the HT-29 human colon adenocarcinoma cell line. In addition, we investigated the effect of MXF on VP-16-induced release of pro-angiogenic cytokines including IL-8 and VEGF in these cells.

Materials and methods

Cell culture. The human colon adenocarcinoma cell line, HT-29, was obtained from the American Type Culture Collection (ATCC, USA) and cultured in DMEM/F-12 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C (complete medium) in a humidified incubator with 5% CO₂. The cells were maintained in log phase by seeding twice a week at a density of 5x10⁴/ml, and the experiments were performed 1 day after trypsinization.

Preparation of nuclear extracts. Nuclear extracts from HT-29 colon cancer cells were prepared as described previously (20). HT-29 cells were washed twice with cold PBS, scrubbed and collected into tubes. The cells were resuspended in hypotonic buffer A [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1 mM EDTA and a mixture of protease inhibitors: 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 2 µg/ml antipain and 100 µg/ml PMSF-phenylmethylsulfonyl fluoride] and incubated in ice for 15 min. Cell lysis was performed by passing the solution 15 times through a 21G needle followed by centrifugation at 13,000 rpm at 4°C for 10 min. The supernatant was collected as the cytosolic fraction, and the pellet containing intact nuclei was resuspended with buffer B [10 mM Tris-HCl (pH 7.4), 10 mM NaCl and 1.5 mM MgCl₂ and protease inhibitors], and 1 M (final concentration) of NaCl was added for a 30-min incubation on ice. The samples were centrifuged (13,000 rpm, 4°C, 20 min), and the supernatant was collected as the nuclear extract. Total protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA).

To determine the effect of MXF on topo II activity in the cells, HT-29 cells were incubated in the presence of 40 µg/ml MXF or 35-50 µg/ml VP-16 for 3 and 1h, respectively, and the decatenation assay was performed.

Decatenation assay. The method was previously described (21). The assay is based on decatenation of kinetoplast DNA (kDNA), and since this assay is specific for topo II enzyme,

it can be carried out with crude nuclear extracts. kDNA is a large network of plasmids originating from the trypanosome *Crithidia fasciculata*. Due to its structure, when kDNA is analyzed by gel electrophoresis, it penetrates only slightly into the agarose gel. Upon decatenation by topo II mini circles monomers of DNA are formed. Nuclear proteins (1.2 µg) were added to a specific topo II reaction mixture containing a final volume of 25 µl: 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA, 25 µg/ml BSA, 1 mM ATP and 80 ng kDNA (TopoGEN, Port Orange, FL, USA).

The reaction products were analyzed by electrophoresis on 1% agarose gel using a Tris-borate/EDTA buffer (89 mM boric acid and 62 mM EDTA) at 1 V/cm, stained with ethidium bromide (1 µg/ml) and photographed using a short-wave-length UV lamp (ChemImager 5500; Alpha Innotech, San Leandro, CA), as previously described by us (19).

Band depletion and Western blot analysis. Topoisomerases form transient covalent adducts (cleavage complexes) with DNA in intact cells during the DNA relaxation process. Treatment of cells with etoposide stabilizes these cDNA-enzyme cleavage complexes. As a consequence, when samples from the drug-treated cells are rapidly denatured and analyzed on SDS polyacrylamide gel electrophoresis, only a few topoisomerase molecules are free to migrate to their actual molecular weight size. The covalent-topoisomerase-DNA complexes are large in size and exhibit a lower mobility after denaturation. Therefore, Western blot analysis using anti-topoisomerase antibody demonstrates a significant decrease in the band of topoisomerase; this is the basis for the band depletion assay. The band depletion assay was conducted as described by Kaufmann and Svingen (22). Briefly, HT-29 cells were seeded at a concentration of 1x10⁶/ml and incubated with 40 µg/ml MXF alone, 8-24 µg/ml VP-16 alone (for 3 and 1 h, respectively) or in combination of the two drugs. The cells were lysed in denaturing agent [62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA and 2% SDS] and sonicated (40 bursts, 2 sec each). The lysates were mixed with 4X SDS loading buffer [200 mM Tris-HCl (pH 6.8), 8% SDS, 0.4% bromophenol blue, 40% glycerol, 20% β-mercaptoethanol] and separated on 10% SDS-PAGE gels for Western analysis.

After electrophoresis and electrophoretic transfer of proteins to nitrocellulose membranes (Schleicher and Shuell, Dassel, Germany), the membranes were blocked with 6% non-fat milk in PBS (pH 7.4) for 1 h. Membranes were then rinsed three times in PBST and incubated overnight at 4°C with goat polyclonal anti-topo II-α (1:2500) (Santa Cruz Biotechnology, Santa Cruz CA, USA). Actin levels were assessed as loading control. The blots were then incubated with a secondary antibody, horseradish peroxidase-linked anti-goat IgG (Santa Cruz Biotechnology) for 1 h at room temperature, washed three times in PBST and incubated in enhanced chemiluminescence reagent (ECL, Amersham Pharmacia Biotech). Relative density values of topo II-α were determined by densitometric analysis followed by photographing the specific bands (Kodak XLS-1 film).

Cytotoxicity assay. HT-29 cells, cultured as described above, were seeded on 96-well plates at a concentration of

5×10^4 cells/0.1 ml/well in triplicates. Various concentrations of MXF, VP-16 and their combination were added. The cells were incubated for 48-72 h at 37°C in a 5% CO₂ atmosphere. For the last 3 h of incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (5 mg/ml) in phosphate-buffered saline (PBS) was added to each well. The cells were incubated at 37°C for 3 h, and 0.04 M HCl was added to dissolve the formazan crystals. The absorbance was then measured at 560 nm with a spectrophotometer (ELISA Reader Molecular Devices Corp., Sunnyvale, CA).

Flow cytometric cell analysis (cell cycle and survivin expression). Cell cycle distribution was analyzed by flow cytometry as described previously (23). Briefly, cells were incubated for 48 h with 0.1-5 µg/ml of VP-16 in the presence or absence of MXF. Following incubation, cells were trypsinized, washed with PBS fixed with 70% cold ethanol and stained with propidium iodide (PI). The stained cells were analyzed using the FACScan laser flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Intracellular levels of survivin were determined using an anti-human survivin-fluorescein monoclonal antibody, according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN). Briefly, HT-29 cells were serum starved for 24 h (24) then pre-incubated with 20 µg/ml MXF for 24 h after which time 0.1-9 µg/ml VP-16 was added for an additional 24 h. Cells were collected and fixed in 4% cold paraformaldehyde for 10 min. Cells were washed twice in PBS and resuspended in SAP buffer [0.1% (w/v) saponin, 0.05% (w/v) NaN₃ in Hank's balanced salt solution (HBSS)]. Following centrifugation at 200 x g for 7 min, the supernatant was decanted, ensuring that ~200 µl of the SAP buffer remained in the tubes. The pellet was gently resuspended, and 10 µl of the conjugated antibody was added to each sample. After 45 min of incubation in the dark, the cells were washed twice in SAP buffer and resuspended in PBS for final flow cytometric analysis. Raw flow cytometric data were analyzed by WinMDI software.

DAPI-sulforhodamine staining. Cells were evaluated for apoptosis by studying their morphology after staining with 4',6-diamidino-2-phenylindole (DAPI)-sulforhodamine, which highlights cellular DNA. Cells were incubated for 48 h with the indicated concentrations of VP-16 and MXF as described above. Approximately 30,000 freshly harvested cells were distributed on glass slides and fixed by immersion in 4% paraformaldehyde for 10 min. Upon drying, the slides were treated with PBS and stained with 10 µl of Vectashield mounting medium with DAPI (1.5 µg/ml) (Vector Laboratories, Burlingame CA). The cells were examined by fluorescence microscopy. The morphological criteria of apoptosis included: a) cytoplasmic and nuclear shrinkage; b) chromatin condensation; and c) cytoplasmic blebbing with maintenance of the integrity of the cell membrane (25).

Fluorogenic assay for caspase 3 activity. Caspase 3 was measured as previously described by us (26). HT-29 cells were incubated for 48 h with 5 and 10 µg/ml VP-16 or 10-20 µg/ml MXF, or combination of both drugs. Following incubation, the cells were collected, washed, resuspended in 50 mM

HEPES, (pH 7.4), 0.1% CHAPS, 5 mM DTT and 0.1 mM EDTA, incubated for 15 min on ice and lysed by three successive freeze-thaw cycles in dry ice/37°C. Cell lysates were centrifuged at 14,000 rpm for 15 min, and the supernatants were stored at -70°C. The protein concentration of each sample was estimated using the Bradford Bio-Rad protein assay. For caspase 3 activity a total of 25 µg protein was incubated with 30 µM ac-DEVD-AMC (BioMol Research Laboratories, Plymouth Meeting, PA) at 37°C for 60 min in the dark. The release of 7-amino-4-methylcoumarine was monitored by a spectrofluorometer using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. In some experiments, a caspase 3 inhibitor (Z-DEVD-FMK, Calbiochem, Nottingham, UK) was added directly to the medium 30 min prior to the addition of 20 µg/ml VP-16.

IL-8 and VEGF secretion. In order to increase constitutive VEGF and IL-8 secretion of HT-29 cells, all of the experiments were carried out in a serum-deprived condition by excluding FBS from the standard culture medium (27). The supernatants of the cells grown in serum-deprived medium served for the determination of VEGF and IL-8. VP-16 and MXF were added at the start of the experiments. The concentration of IL-8 and VEGF in the medium of control and drug-treated cells was measured using a commercially available sandwich enzyme-linked immunoassay (ELISA) kit according to the manufacturer's instructions (R&D Systems Inc.). Briefly, cells were placed in 24-well culture plates at a concentration of 1×10^6 cells/ml and treated with different concentrations of MXF or VP-16 administered as a single drug or in combination, in serum-free conditioned medium. After 48 h, the cell-free supernatants were recovered, and the concentrations of IL-8 and VEGF were determined using ELISA. The sensitivity of the assay for IL-8 is >10 pg/ml and for VEGF >31.2 pg/ml.

Statistical analysis. Statistical significance was determined by the paired t-test. A p-value of <0.05 was considered significant.

Results

Inhibition of topo II activity in MXF- and VP-16-treated cells. Our previous results suggest that the addition of MXF to VP-16 enhances inhibition of decatenation activity of topo II when purified topo I (28) or nuclear extract derived topo I (data not shown) are used. To investigate the ability of these compounds to affect the activity of topo II within the cell, we first treated the cells for 3 h with 40 µg/ml MXF, or for 1 h with 35-50 µg/ml VP-16. Nuclear extracts were prepared, and 1.2 µg of nuclear proteins was added to a topo II reaction mixture. Incubation of the cells with 35 or 50 µg/ml VP-16 alone, resulted in 40±8% and 45±5% reduction in the decatenation activity of topo II, respectively (Fig. 1A, lanes 4 and 6 compared to lane 2 and Fig. 1B). The addition of 40 µg/ml MXF alone only slightly affected topo II activity (16±2% inhibition) (Fig. 1, lane 3 compared to lane 2). In contrast, up to 76±17% and 70±21% inhibition of topo II activity was observed in cells incubated with 35 or 50 µg/ml VP-16,

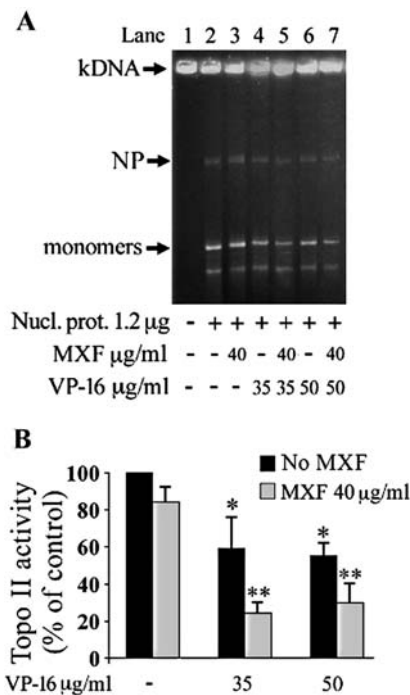


Figure 1. Inhibition of decatenation activity of topo II in MXF- and VP-16-treated cells. (A) kDNA only (lane 1). HT-29 cells were incubated for 3 h with 40 μ g/ml MXF only (lane 3) and with 35 or 50 μ g/ml VP-16 only (for 1 h) (lanes 4 and 6). To some of the cultures, MXF was added for 3 h, and for the last hour various concentrations of VP-16 were added (lanes 5 and 7). Nuclear extracts were prepared, 1.2 μ g nuclear proteins was added to the topo II reaction mixture, and decatenation of topo II activity was determined. The reaction products were analyzed by agarose gel electrophoresis. NP, nuclear protein. (B) Results (mean \pm SE) of three independent experiments are shown. Relative density values were determined by densitometric analysis followed by photographing the specific bands. * p <0.05, cells treated with drugs vs. control; ** p <0.05, cells treated with VP-16+MXF vs. VP-16 alone.

respectively, in combination with 40 μ g/ml MXF (lane 5 compared to lane 4 and lane 7 compared to lane 6 and Fig. 1B).

Effect of MXF and VP-16 on topo II cleavage activity. To determine the effect of MXF and VP-16 on the amount of drug-stabilized cleavable complex formation, the band depletion assay (as described in Materials and methods) was used. Band depletion assays allow detection of topo II complexes with DNA. The topo II enzymes that are covalently bound to DNA (cleavable complexes) are prohibited from entering the acrylamide gel resulting in a band depletion, whereas topo II enzymes not bound to DNA readily enter the gel. A slight band depletion was observed following exposure of the cells to MXF alone. VP-16 is a potent anti-topo II drug and at high concentrations causes a high reduction in the topo II band which effaces the effect of MXF. Therefore, lower doses of VP-16 were used for the band depletion assay. Exposure to 8 μ g/ml VP-16 alone resulted in 27 \pm 1% (p <0.01) depletion of the topo II band, whereas in cells exposed to a combination of the two drugs, a 45 \pm 7% reduction was observed (p <0.03), suggesting an increase in cleavable complex formation (Fig. 2A and B). Exposure of the cells to 15 μ g/ml VP-16 resulted in 48 \pm 8% depletion of topo II (p <0.04) whereas no further depletion of topo II was observed

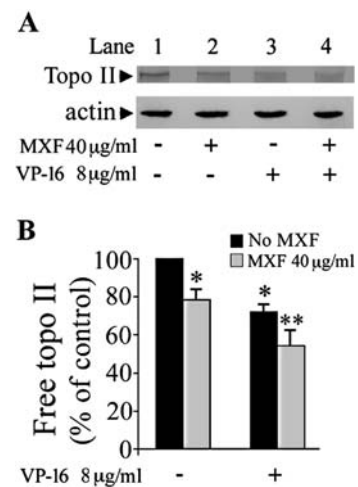


Figure 2. Band depletion assay of topo II in cells incubated with (A) buffer (lane 1), 40 μ g/ml MXF (lane 2), 8 μ g/ml VP-16 (lane 3) or the combination of MXF and topo II (lane 4). (B) Densitometry analysis relative to untreated cells (mean \pm SE) (n =4). Topo II was depleted in the presence of the indicated concentrations of drugs, suggesting the formation of cleavable complexes. * p <0.05, cells treated with drugs vs. control; ** p <0.05, cells treated with VP-16+MXF vs. VP-16 alone.

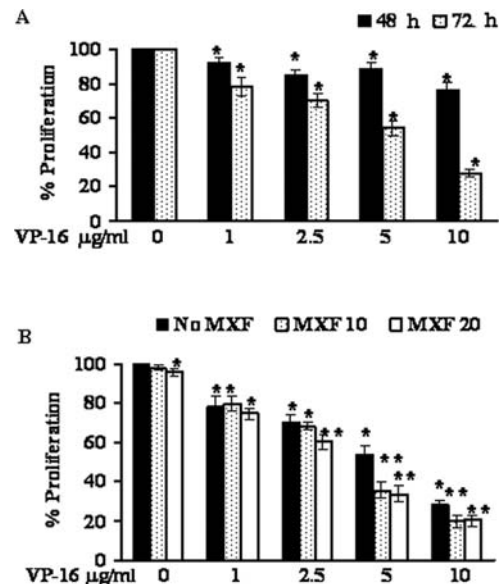


Figure 3. MXF enhances the anti-proliferative effect of VP-16. Time- and dose-dependent studies. (A) HT-29 cells were incubated for 48 or 72 h with increasing concentrations of VP-16. (B) Cells were incubated for 72 h in the presence of VP-16 and MXF. Cell proliferation was determined by the colorimetric MTT assay. Results are expressed as mean \pm SE of five experiments performed in triplicate. * p <0.05, cells treated with drugs vs. control; ** p <0.05, cells treated with VP-16+MXF vs. VP-16 alone.

when the cells were exposed to the combination of the two drugs (data not shown).

Effect of MXF on the anti-proliferative activity of VP-16. The afore-mentioned results suggest that treatment of cells with MXF enhanced the inhibition of cellular topo II by VP-16. To examine the biological significance of these results, we

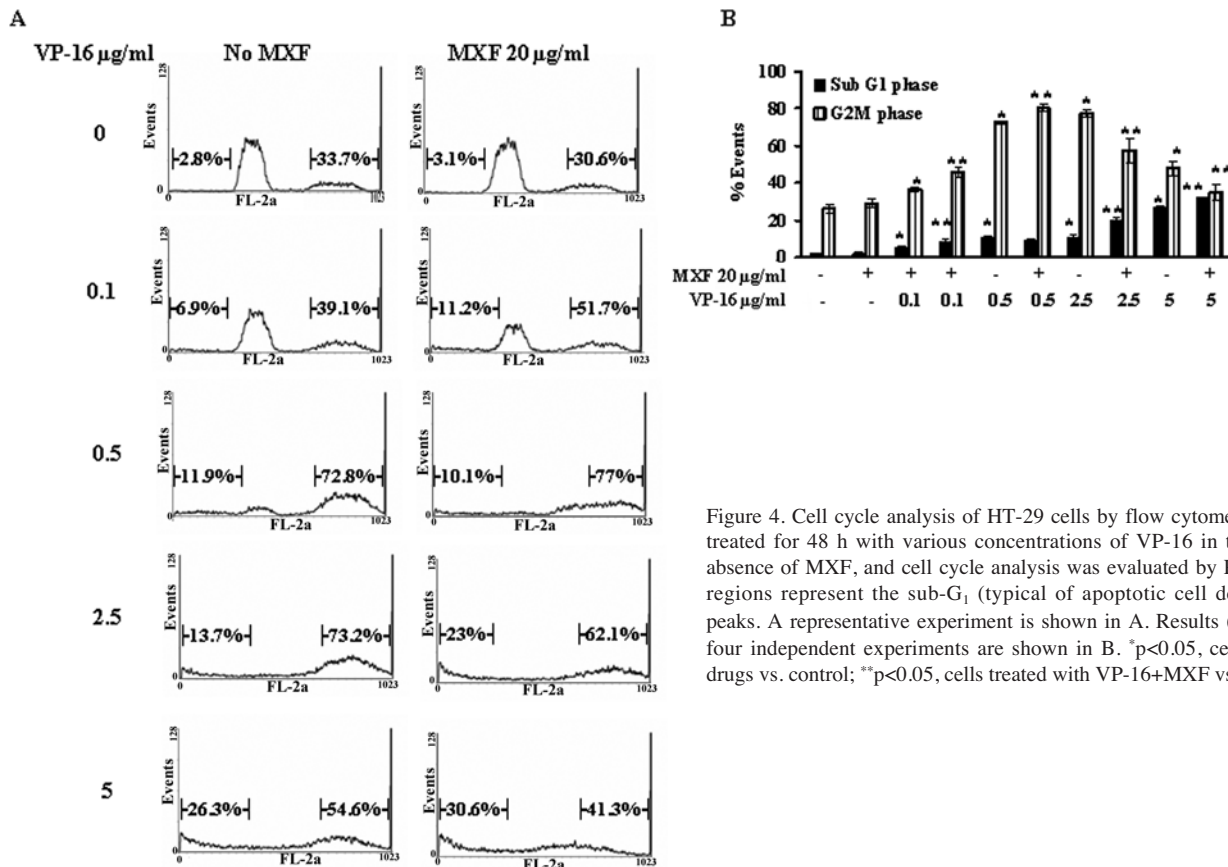


Figure 4. Cell cycle analysis of HT-29 cells by flow cytometry. Cells were treated for 48 h with various concentrations of VP-16 in the presence or absence of MXF, and cell cycle analysis was evaluated by PI staining. The regions represent the sub-G₁ (typical of apoptotic cell death) and G₂M peaks. A representative experiment is shown in A. Results (mean \pm SE) of four independent experiments are shown in B. * $p < 0.05$, cells treated with drugs vs. control; ** $p < 0.05$, cells treated with VP-16+MXF vs. VP-16 alone.

investigated the effect of the combination of MXF with VP-16 on cell proliferation. In the first experiment we performed time-dependent studies on the effect of various concentrations of VP-16 (1-10 μ g/ml) on cell proliferation. Fig. 4 indicates that the decrease in cell proliferation was dose- and time-dependent (Fig. 3A). Maximal inhibition (up to $72 \pm 7\%$) was observed following incubation of the cells for 72 h with 10 μ g/ml VP-16. The additional experiments combining MXF with VP-16 were performed with cell incubation for 72 h. Fig. 3B shows that MXF given alone at concentrations of 20 μ g/ml inhibited cell proliferation only slightly ($11 \pm 0.02\%$). In contrast, the combination of VP-16 with MXF induced a marked decrease in cell proliferation. Exposure of the cells to a combination of 5 μ g/ml VP-16 and 20 μ g/ml MXF, resulted in a decrease up to $66 \pm 6\%$ compared to $46 \pm 4\%$ inhibition of proliferation induced by 5 μ g/ml VP-16 administered alone ($p < 0.001$) and was similar to the inhibitory effect induced by 10 μ g/ml VP-16 administered alone ($72 \pm 7\%$ inhibition).

Cell cycle arrest. We evaluated whether VP-16 and MXF affect the cell cycle profile. The cells were treated with various concentrations of VP-16 in the presence or absence of MXF for 48 h and were subjected to flow cytometric analysis after DNA staining. Histograms of flow cytometric data are shown in Fig. 4A. VP-16 induced a dose-dependent cell cycle arrest at G₂M (up to a concentration of 2.5 μ g/ml), and the appearance of a sub-G₁ peak (Fig. 4A and B), which is typical of apoptotic cells. The cell fraction in the sub-G₁ peak increased in the presence of increasing concentrations of

VP-16, and reached a maximum of 26.3% in the presence of 5 μ g/ml VP-16 (Fig. 4A). Fig. 4A and B indicate that MXF administered alone at a concentration of 20 μ g/ml did not affect the cell cycle. In contrast, a 4-fold increase in the fraction of sub-G₁ cells was observed in the presence of MXF and a very low dose of VP-16 (0.1 μ g/ml) and was similar to the effect of 0.5 μ g/ml VP-16 given alone, indicating that MXF markedly enhances the cytotoxic activity of VP-16. MXF enhanced G₂M arrest induced by 0.1-0.5 μ g/ml VP-16 while at higher concentrations a decrease in G₂M was observed in the presence of VP-16 and MXF as compared to VP-16 administered alone (Fig. 4A and B).

Effect of MXF and VP-16 on survivin expression. Survivin is a recently discovered member of the inhibitors of apoptosis (IAP) family that plays a dual role in suppressing apoptosis and regulating cell division (29). Survivin is regulated in a highly cell cycle-dependent manner, with a marked increase in the G₂M phase (30).

We investigated the effect of MXF and VP-16 on survivin expression within the cells by flow cytometry, using a specific antibody, as described in Materials and methods. HT-29 cells were pre-incubated with 20 μ g/ml MXF for 24 h, and then VP-16 at different concentrations (0.1-9 μ g/ml) was added for an additional 24 h. In agreement with other studies that demonstrated that VP-16 upregulates survivin expression (31), we showed that exposure of HT-29 cells to various concentrations of VP-16 resulted in a 5- to 20-fold increase in survivin expression (Fig. 5A and B). MXF administered alone did not affect survivin expression. The

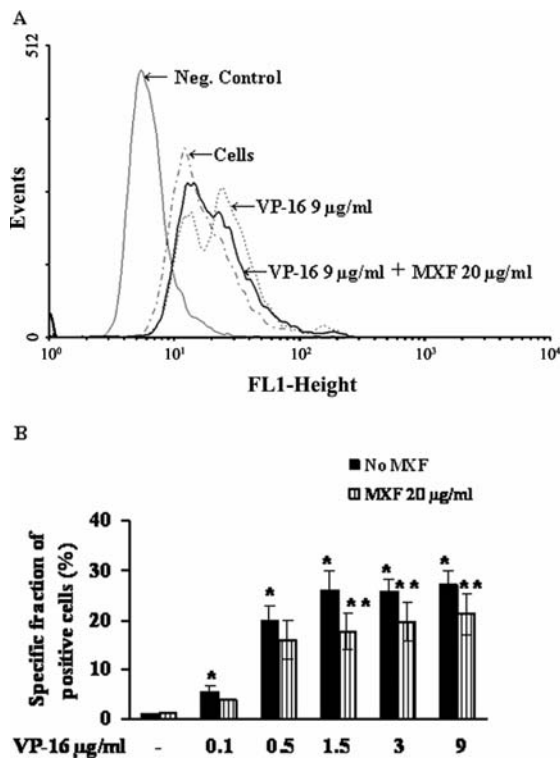


Figure 5. Flow cytometric studies of the expression of survivin in cells treated with VP-16 and MXF. HT-29 cells were serum starved for 24 h, 20 $\mu\text{g/ml}$ MXF was added, and cells were incubated for an additional 24 h, after which different concentrations of VP-16 were added to some of the cultures for an additional 24 h. Cells were collected and stained with anti-human survivin-fluorescein monoclonal antibody. A representative experiment is shown in A. Results (mean \pm SE) of four independent experiments are shown in B. The specific fraction of positive cells (%) was calculated as follows: percentage of stained drug-treated cells - percentage of stained untreated cells. * $p < 0.05$, cells treated with drugs vs. control, ** $p < 0.05$, cells treated with VP-16+MXF vs. VP-16 alone.

addition of 20 $\mu\text{g/ml}$ MXF to 1.5-9 $\mu\text{g/ml}$ VP-16 inhibited survivin expression by 32-22%, respectively (Fig. 5B).

Effect of MXF and VP-16 on morphological changes in HT-29 cells. Fluorescence microscopy of DAPI-stained nuclei was employed to visualize chromatin condensation. Incubation of cells for 48 h with VP-16 alone resulted in numerous cells that had smaller nuclei with chromatin condensation and increased fluorescence intensity (Fig. 6A). The addition of MXF to VP-16 resulted in an increased concentration of cells with condensed and apoptotic nuclei compared to the effect noted with VP-16 alone (Fig. 6A). After treatment with 5 or 10 $\mu\text{g/ml}$ VP-16, the apoptotic rate was increased from $1 \pm 0.5\%$ in control cultures to $15.5 \pm 0.6\%$ and $19.7 \pm 2.2\%$, respectively. A further significant increase in the apoptotic rate was observed upon incubation of the cells with 5 or 10 $\mu\text{g/ml}$ VP-16 and 20 $\mu\text{g/ml}$ MXF (up to $22.2 \pm 1.7\%$ and $26 \pm 2.6\%$, respectively; $p < 0.05$) (Fig. 6B).

Caspase 3 activation. DEVD-AMC is a specific substrate for caspase 3 which mimics the PARP cleavage site. To test caspase 3 activation, cell lysates from HT-29 cells that were treated with VP-16 in the presence or absence of 10-20 $\mu\text{g/ml}$ MXF for 48 h were incubated with the substrate, and the

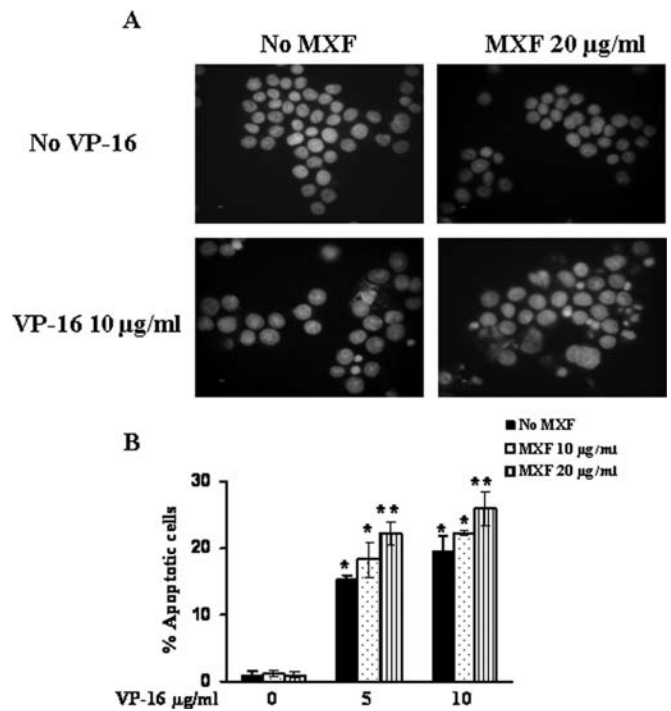


Figure 6. DAPI staining of an HT-29 cell monolayer after treatment with VP-16 and MXF. HT-29 cells were treated for 48 h with the indicated concentrations of VP-16 in the absence or presence of MXF, and DAPI staining was performed.

increase in fluorescence due to enzymatic cleavage of the peptides was measured with a fluorometer. As shown in Fig. 7, treatment with 5 or 10 $\mu\text{g/ml}$ VP-16 significantly increased caspase 3 activation ($p < 0.02$ and < 0.03 , respectively). The addition of 20 $\mu\text{g/ml}$ MXF further enhanced this increase by 1.8-fold in the presence of 5 $\mu\text{g/ml}$ VP-16 ($p < 0.03$) and by 1.7-fold in the presence of 10 $\mu\text{g/ml}$ VP-16 ($p < 0.04$). To confirm that the caspase activity observed was specific to caspase 3, we used a caspase 3-specific inhibitor, Z-DEVD-FMK. Treatment of cells with the inhibitor prior to the addition of 20 $\mu\text{g/ml}$ VP-16 reduced the degree of caspase 3 activation (insert in Fig. 7).

Effect of MXF on the secretion of pro-angiogenic factors induced by VP-16. We investigated the effect of MXF on the secretion of IL-8 and VEGF by HT-29 cells. Fig. 8A shows that MXF at concentrations of 10 and 20 $\mu\text{g/ml}$ significantly decreased spontaneous release of IL-8 ($p < 0.05$). Incubation of the cells for 48 h with 5 or 10 $\mu\text{g/ml}$ VP-16 alone resulted in a dose-dependent increase in IL-8 release. Addition of MXF at a concentration of 10 or 20 $\mu\text{g/ml}$ significantly reduced the enhanced release of IL-8 induced by 5 or 10 $\mu\text{g/ml}$ VP-16. A 25% reduction in the enhanced increase induced by 10 $\mu\text{g/ml}$ VP-16 was observed in the presence of 20 $\mu\text{g/ml}$ MXF ($p < 0.05$).

The spontaneous release of VEGF from HT-29 cells grown in serum-free medium for 48 h was 4059 ± 354 pg/ml. MXF at concentrations of 10 and 20 $\mu\text{g/ml}$ inhibited the spontaneous release by 17 and 33%, respectively (Fig. 8B). VP-16 did not affect VEGF release from the cells.

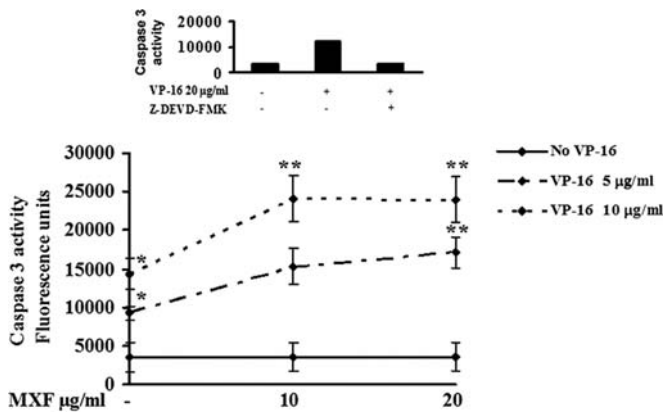


Figure 7. MXF enhances caspase 3 activation induced by VP-16. Fluorogenic studies. HT-29 cells were incubated with the indicated concentrations of VP-16 and MXF for 48 h, and lysates were prepared. Caspase 3 activity was measured using DEVD-AMC as the substrate. The data represent the mean \pm SE of three experiments. Effect of caspase 3 inhibitor is shown in the insert. Z-DEVD-FMK (caspase 3 inhibitor) was added 30 min prior to exposure to 20 μ g/ml VP-16. * p <0.05; ** p <0.012, VP-16+MXF- vs. VP-16-treated cells.

Discussion

The study presented here expands our previous observations on the effect of MXF added alone or in combination with the cytotoxic agent VP-16 on purified topo II activity and proliferation of THP-1 and Jurkat cell lines (19). In the present study we elected to use the more clinically relevant HT-29 human colon adenocarcinoma cell line, and we analyzed, in-depth, the intracellular effects of the two drugs on topoisomerase II and performed broad spectrum experiments on proliferation, apoptosis, the cell cycle, signal transduction and pro-angiogenic cytokine expression in this cell line. In our previous study (19) we investigated the effect of the drugs on the activity of purified human topo II enzyme by measuring the relaxation of the supercoiled pUC19 DNA plasmid. We demonstrated that MXF alone slightly inhibited the activity of human topo II, while the combination with VP-16 led to a marked reduction in enzyme activity. The ability of MXF to inhibit purified topo II activity *in vitro* and to enhance the inhibitory effect of VP-16 does not necessarily indicate that topo II is indeed the target of the drugs within the cell. Therefore, we determined the effect of treating HT-29 cells with 40 μ g/ml MXF alone or in combination with 35-50 μ g/ml VP-16 on the activity of cellular topo II. We found that MXF significantly enhanced the inhibition of cellular topo II activity induced by VP-16. To investigate the mode of action by which the combined treatment MXF/VP-16 enhanced the reduction in topo II activity in drug-treated cells, we examined the formation of DNA-enzyme covalent adducts (cleavage complexes). During the process of DNA relaxation by topo II, the enzyme forms transient covalent bonds with DNA. When cells are treated with VP-16, the drug stabilizes these cleavage complexes, prevents religation processes and thus introduces DNA DSBs which enter the cells into apoptosis. Using the band depletion assay for the determination of the DNA-topo II cleavage complexes we showed that MXF by itself slightly increased the formation of DNA-topo II adducts.

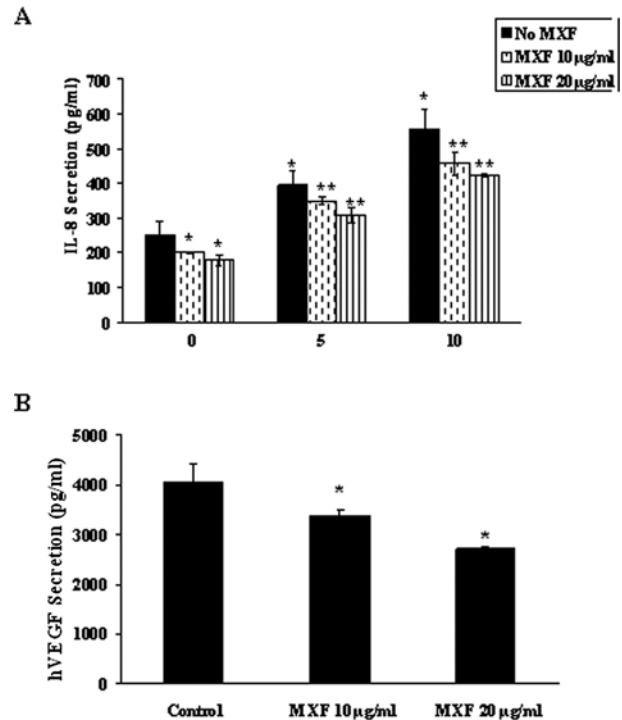


Figure 8. Effect of MXF on pro-angiogenic factor secretion. HT-29 cells were incubated for 48 h with increasing concentrations of VP-16 in the presence or absence of MXF, and the concentrations of IL-8 (A) were measured by ELISA. The cells were incubated for 48 h in the absence or presence of MXF, and the concentration of VEGF (B) was measured by ELISA. The values are the mean \pm SE of four experiments performed in duplicate. * p <0.05, drug-treated cells vs control; ** p <0.05, VP-16+MXF- vs. VP-16-treated cells.

However, when MXF was added with VP-16 it enhanced the accumulation of DNA-topo II adducts compared to VP-16 alone. This enhancement might be due to the ability of MXF to increase (although slightly) DNA-enzyme adducts. This enhancement might be due to additive effects of both drugs or alternatively to changes in topo II conformation by MXF which enhanced the cleavage complexes of topo II-DNA in the presence of VP-16. However, the accumulation of the topo II-DNA cleavage complex will enhance the cytotoxicity of the combined VP-16/MXF treatment.

Therefore, we also defined the functional interaction of the drugs by investigating their effect on the cytotoxic activity towards HT-29 cells. We found that MXF alone at a concentration of 20 μ g/ml induced a slight anti-proliferative effect on HT-29 cells (up to $11 \pm 0.02\%$ in cell proliferation). In contrast, when the quinolone was added to VP-16, a significant decrease in cell proliferation occurred. It is reasonable to suggest that the anti-proliferative effect of MXF in combination with VP-16 is the result of their combined influence on the topoisomerase as was observed in the previous experiments.

We confirmed previous studies indicating that VP-16 induces cell cycle arrest at G₂M (31) (at drug concentrations of 0.1-0.5 μ g/ml), but also showed that MXF added to higher concentrations of VP-16 (2.5 and 5 μ g/ml) induced a decrease in G₂M, which was accompanied by a marked increase in sub-G₁. Our results are in line with those of Jahnke *et al* (31) who

demonstrated that VP-16 at a concentration of 1 $\mu\text{g/ml}$ induced cell cycle arrest at G₂M in K562 cells while at higher concentrations (20 $\mu\text{g/ml}$) no cell cycle arrest was observed.

In our study we also observed that VP-16 induced an increase in survivin expression and that in the presence of MXF there was a decrease in elevated expression of survivin. Survivin is generally undetectable in differentiated normal tissues. Several reports have demonstrated survivin expression in a variety of human tumor types including colon cancers (32-35). Similar to our findings, survivin posttranslational upregulation was found in cancer cells exposed to diverse anti-neoplastic agents, such as adriamycin and paclitaxel (35) suggesting that this change in survivin expression could be a possible general effect of these drugs. Survivin is regulated in a highly cell cycle-dependent manner, with a marked increase in the G₂M phase (30,31). The decrease in survivin expression, observed by us, following exposure of cells to a combination of VP-16 and MXF can be explained by the decrease in G₂M arrested cells and an increase in the sub-G₁ fraction induced by the combination.

Finally, the increase in apoptosis observed upon co-incubation of HT-29 cells with VP-16 and MXF was preceded by an increase in caspase 3 activity, a critical component of the cellular response to genomic instability probably generated by the drug combination.

A possible explanation for this additive effect is the differential mechanisms of action against topoisomerase II conferred by the two drugs. Bromberg *et al* (36) found that VP-16 acts by inhibiting the ability of topoisomerase II to ligate cleaved DNA molecules, whereas quinolones have little effect on ligation but stimulate the forward rate of topoisomerase II-mediated DNA cleavage. These two distinct mechanisms may work in concert and lead to the observed additive/synergistic cytotoxic effects of VP-16 and MXF.

We showed in the present study that treatment of HT-29 cells with VP-16 induced the release of the pro-inflammatory, pro-angiogenic cytokine IL-8 and that MXF significantly inhibited the VP-16 enhanced production of IL-8. These observations confirm our previous studies that demonstrated significant inhibition by MXF of the synthesis of pro-inflammatory cytokines in THP-1 cells and in human peripheral blood monocytes stimulated with LPS-phorbol myristate acetate (37) or *Aspergillus fumigatus* (38). The combined effect of MXF on inhibition of VP-16-induced IL-8 and of spontaneous secretion of VEGF from HT-29 cells may be an important aspect of MXF activity. These two pro-angiogenic factors have been associated with enhanced tumor progression and with greater tendency and extent of tumor metastases, and their inhibition was shown to favorably affect both parameters (39,40).

In summary, this study demonstrated an important role for MXF in enhancing the cytotoxic effects of VP-16 in a human colon carcinoma cell line while, at the same time, decreasing VP-16-induced pro-inflammatory, pro-angiogenic cytokine secretion from cells, that may be harmful during chemotherapy. Our results suggest that MXF might be a valuable new addition to the therapeutic armamentarium, simultaneously improving the cytotoxic activity and reducing the side effects of VP-16 and similar agents. In addition, a reduction in the total dose of the cytotoxic agent may be

attained when administered in combination with this quinolone.

Acknowledgements

This study was supported by a research grant from Tel Aviv University and by The Cancer Biology Research Center of Tel Aviv University.

References

1. Seminara P, Pastore C, Iacone C, *et al*: Mitomycin C and etoposide in advanced colorectal carcinoma - A clinical in vitro experience that focuses on the problem of schedule dependence in combination therapy. *Chemotherapy* 53: 218-225, 2007.
2. Tanaka T, Halicka HD, Traganos F, Seiter K and Darzynkiewicz Z: Induction of ATM activation, histone H2AX phosphorylation and apoptosis by etoposide. *Cell Cycle* 6: 371-376, 2007.
3. Baldwin EL and Osheroff N: Etoposide, topoisomerase II and cancer. *Curr Med Chem Anticancer Agents* 5: 363-372, 2005.
4. Fan JR, Peng A, Chen HC, Lo SC, Huang TH and Li TK: Cellular processing pathways contribute to the activation of etoposide-induced DNA damage responses. *DNA Repair* 7: 452-463, 2008.
5. Nakasone H, Izutsu K, Wakita S, Yamaguchi H, Muramatsu-Kida M and Usuki K: Autologous stem cell transplantation with PCR-negative graft would be associated with a favorable outcome in core-binding factor acute myeloid leukemia. *Biol Blood Marrow Transplant* 14: 1262-1269, 2008.
6. You B, Tranchand B, Girard P, *et al*: Etoposide pharmacokinetics and survival in patients with small cell lung cancer: a multicentre study. *Lung Cancer* 62: 261-272, 1998.
7. Hande KR: Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur J Cancer* 10: 1514-1521, 1998.
8. Braybrooke JP, O'Byrne KJ, Saunders MP, *et al*: A phase II study of mitomycin C and oral etoposide for advanced adenocarcinoma of the upper gastrointestinal tract. *Ann Oncol* 8: 294-296, 1997.
9. Menichetti ET, Silva RR, Tummarello D, Miseria S, Torre U and Celerino R: Etoposide and mitomycin C in pretreated metastatic breast cancer. *Tumori* 75: 473-474, 1989.
10. Voog E, Merrouche Y, Trillet-Lenoir V, *et al*: Multicentric phase II study of cisplatin and etoposide in patients with metastatic carcinoma of unknown primary. *Am J Clin Oncol* 23: 614-616, 2000.
11. Planting AS, van der Burg ME, van der Bent MJ, de Boer-Dennert M, State G and Verweij J: Phase II study of a short course of weekly high dose cisplatin combined with long-term oral etoposide in metastatic colorectal cancer. *Br J Cancer* 73: 1265-1267, 1996.
12. Posner M, Slapak CA, Browne MJ, *et al*: A phase I-II trial of continuous-infusion cisplatin, continuous-infusion 5-fluorouracil, and VP-16 in colorectal carcinoma. *Am J Clin Oncol* 13: 455-458, 1990.
13. Herold C, Ocker M, Ganslmayer M, Gerauer H, Hahn EG and Schuppan D: Ciprofloxacin induces apoptosis and inhibits proliferation of human colorectal carcinoma cells. *Br J Cancer* 86: 443-448, 2002.
14. Shalit I, Nasrallah N, Bar-On S and Rabau M: In-vitro effect of ciprofloxacin and ofloxacin on murine and human colon carcinoma cell lines. *Drugs* 49: 296-297, 1995.
15. Duewelhenke N, Krut O and Eysel P: Influence on mitochondria and cytotoxicity of different antibiotics administered in high concentrations on primary human osteoblasts and cell lines. *Antimicrob Agents Chemother* 51:54-63, 2006.
16. Bakshi RP, Galande S and Muniyappa K: Functional and regulatory characteristics of eukaryotic type II DNA topoisomerase. *Crit Rev Biochem Mol Biol* 36: 1-37, 2001.
17. Robinson MJ, Martin BA, Gootz TD, *et al*: Effects of quinolone derivatives on eukaryotic topoisomerase-II. A novel mechanism for enhancement of enzyme-mediated DNA cleavage. *J Biol Chem* 266: 14585-14592, 1991.
18. Bromberg KD, Burgin AB and Osheroff N: Quinolone action against human topoisomerase II alpha: stimulation of enzyme-mediated double-stranded DNA cleavage. *Biochemistry* 42: 3393-3398, 2003.

19. Fabian I, Reuveni D, Levitov A, Halperin D, Priel E and Shalit I: Moxifloxacin enhances the antiproliferative and apoptotic effects of etoposide but inhibits its proinflammatory effects in THP-1 and Jurkat cells. *Br J Cancer* 95: 1038-1046, 2006.
20. Bendetz-Nezer S, Gazit A and Priel E: DNA topoisomerase I as one of the cellular targets of certain tyrphostin derivatives. *Mol Pharmacol* 66: 627-634, 2004.
21. Har-Vardi I, Mali R, Breietman M, *et al*: DNA topoisomerases I and II in human mature sperm cells: characterization and unique properties. *Hum Reprod* 22: 2183-2189, 2007.
22. Kaufmann SC and Svingen PA: Immunoblot analysis and band depletion assays. In: *Methods in Molecular Biology. Protocols in DNA topology and topoisomerase, Part I; DNA Topology and Enzymes*. Vol. 94. Mary-Ann Bjornsti and Neil Osheroff (eds). Humana Press Inc., Totowa, NJ, 253-268, 1999.
23. Riccardi C and Nicoletti I: Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 1: 1458-1461, 2006.
24. Jung YD, Nakano K, Liu W, Gallick GE and Ellis LM: Extracellular signal-regulated kinase activation is required for up-regulation of vascular endothelial growth factor by serum starvation in human colon carcinoma cells. *Cancer Res* 59: 4804-4807, 1999.
25. Cohen JJ: Apoptosis. *Immunol Today* 14: 126-130, 1993.
26. Gross-Fischer T and Fabian I: Protection by ascorbic acid from denaturation and release of cytochrome c, alteration of mitochondrial membrane potential and activation of multiple caspases induced by H₂O₂, in human leukemia cells. *Biochem Pharmacol* 63: 1325-1335, 2002.
27. Blau H, Klein K, Shalit I, Halperin D and Fabian I: Moxifloxacin but not azithromycin selectively inhibits IL-8, IL-6, ERK 1/2, JNK and NFκB activation in a cystic fibrosis epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* 292: L343-L352, 2007.
28. Reuveni D, Halperin D, Shalit I, Priel E and Fabian I: Quinolones as enhancers of camptothecin-induced cytotoxic and anti-topoisomerase I effects. *Biochem Pharmacol* 75: 1272-1281, 2008.
29. Altieri DC and Marchisio C: Survivin apoptosis: an interloper between cell death and cell proliferation in cancer. *Lab Invest* 79: 1327-1333, 1999.
30. Li F and Altieri DC: The cancer anti-apoptosis mouse survivin gene: characterization of locus and transcriptional requirements of basal and cell cycle-dependent expression. *Cancer Res* 59: 3143-3151, 1999.
31. Jahnke U, Higginbottom K, Newland AC, Cotter FE and Allen PD: Cell death in leukemia: Passenger protein regulation by topoisomerase inhibitors. *Biochem Biophys Res Commun* 361: 928-933, 2007.
32. Ambrosini G, Adida C and Altieri DC: A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 3: 917-921, 1997.
33. Gianani R, Jarboe E, Orlicky D, *et al*: Expression of survivin in normal, hyperplastic, and neoplastic colonic mucosa. *Hum Pathol* 32: 119-125, 2001.
34. Grossman D, McNiff JM, Li F and Altieri DC: Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. *J Invest Dermatol* 113: 1076-1081, 1999.
35. Wall NR, O'Connor DS, Plescia J, Pommier Y and Altieri DC: Suppression of survivin phosphorylation on Thr(34) by flavopiridol enhances tumor cell apoptosis. *Cancer Res* 63: 230-235, 2003.
36. Bromberg KD, Burgin AB and Osheroff N: A two-drug model for etoposide action against human topoisomerase II alpha. *J Biol Chem* 278: 7406-7412, 2003.
37. Weiss T, Shalit I, Blau H, *et al*: Anti-inflammatory effects of moxifloxacin on activated human monocytic cells: inhibition of NFκB and MAPK activation and synthesis of proinflammatory cytokines. *Anti Microb Agents Chem* 48: 1974-1982, 2004.
38. Shalit I, Halperin D, Haite D, *et al*: Anti-inflammatory effects of moxifloxacin on IL-8, IL-1β, and TNF-α secretion and NFκB and MAP-kinase activation in human monocytes stimulated with *Aspergillus fumigatus*. *J Antimicrob Chemother* 57: 230-235, 2006.
39. Rolny C, Capparuccia L, Casazza A, *et al*: The tumor suppressor semaphorin 3B triggers a prometastatic program mediated by interleukin 8 and the tumor microenvironment. *J Exp Med* 205: 1155-1171, 2008.
40. Amin DN, Bielenberg DR, Lifshits E, Heymach JV and Klagsbrun M: Targeting EGFR activity in blood vessels is sufficient to inhibit tumor growth and is accompanied by an increase in VEGFR-2 dependence in tumor endothelial cells. *Microvasc Res* 76: 15-22, 2008.