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Quinolones as enhancers of camptothecin-induced cytotoxic and anti-topoisomerase I effects

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ABSTRACT

Camptothecins (CPTs) are topoisomerase I (topo I) inhibitor chemotherapeutic agents. Studies indicate that combination therapy is needed in most therapeutic protocols with camptothecins. Certain fluoroquionolones inhibit topoisomerase II activity in eukaryotic cells. We showed previously that the fluoroquionolone moxifloxacin inhibited purified human topoisomerase II, acted synergistically with etoposide and enhanced anti-proliferative effect in THP-1 and Jurkat cells. There is no information on flouroquionolone's activity on topoisomerase I. We examined the effect of moxifloxacin and ciprofloxacin alone or in combination with camptothecin on purified topoisomerase I activity and further analysed their combined activity on proliferation and apoptosis in HT-29 cells. Moxifloxacin and ciprofloxacin alone slightly inhibited purified topoisomerase I activity; however in combination with camptothecin it led to a 82% and 64% reduction in enzyme activity, respectively. Moreovwer, our studies indicate that incubation of HT-29 cells with a combination of moxifloxacin or ciprofloxacin with CPT increases cellular topoisomerase I inhibitory activity. In cell proliferation assays, addition of moxifloxacin to 1 nM camptothecin enhanced its cytotoxic activity by three-fold and was similar to that of 50 nM camptothecin alone (45 ± 2.1% inhibition). Ciprofloxacin enhanced cytotoxic activity to a lesser extent. Apoptosis studies showed up to 1.6-fold increase in annexin V positive cells when the fluoroquinolones were combined with camptothecin as compared to camptothecin alone. Analysis of the proangiogenic factors IL-8 and VEGF showed significant reduction in IL-8 production by moxifloxacin and ciprofloxacin up to 48% and in VEGF secretion from the cells. Further in vivo and clinical studies of camptothecins combined with the above fluoroquinolones are warranted.

1. Introduction

Mammalian DNA topoisomerases are the cellular targets of several anti-cancer drugs used today in clinical practice. These enzymes are classified as type I and type II topoisomerases, and members of each family are distinct in structure and function (reviewed in Ref. [1]). Camptothecin (CPT), is a selective inhibitor of topoisomerase I (topo I), an enzyme

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which takes part in replication and transcription of DNA. CPT is a potent anti-cancer agent and its derivatives toptecan and irinotecan (CPT-11) are used today for treatment of many types of solid tumors including lung, colorectal, ovarian, uterine and gastric carcinomas [2,3]. However, the response rate to CPT-11 administered alone is not always sufficient due to various factors and combination therapy is needed in most therapeutic protocols. Studies have shown that a combination of CPT-11 with gefitinib may be benefitial in the treatment of certain gastric cancers [4]. Similary, irinotecan, combined with fluorouracil and folinic acid was found to be effective in patients with advanced gastric cancer [5] and a combined treatment of irinotecan with low-dose cisplatinum was effective in patients with metastatic gastric or colonic cancer [6]. Combination chemotherapy is commonly used for treatment of many tumor types and is considered to provide numerous advantages over single drug regimens.

Quinolones are synthetic, potent, broad-spectrum antimicrobial agents currently used for community and hospital acquired infections. Fluoroquinolones exhibit bactericidal activity by inhibiting bacterial DNA gyrase and topoisomerase (reviewed by in Ref. [7]). Although most fluoroquinolones are highly specific for prokaryotic type II topoisomerases, several agents have been described that are also very active against eukaryotic topo II [8]. Thus, CP-115,953, a fluoroquinolone closely related to ciprofloxacin, was found to be a potent eukaryotic topo II poison [8,9]. Studies in yeast demonstrated that topo II is the primary physiological target for this quinolone [10]. Other studies have shown that nalidixic and oxolinic acids have 50% inhibitory concentrations (IC_{50}s) of 500 and 100 μg/ml, respectively, for topo II isolated from Hela cell nuclei in a decatenation activity assay [11]. A similar degree of inhibition was found against topo II isolated from calf thymus nuclei by Hussy et al. [12]. To the best of our knowledge no significant inhibitory effect on topo I activity by quinolones was observed. In one study [12], nalidixic acid and ofloxacin were shown to have no inhibitory activity against calf thymus nuclear topo I at concentrations of up to 1000 μg/ml. Norfloxacin and ciprofloxacin (CIP) exhibited limited inhibition in these tests, with IC_{50}s between 300 and 400 μg/ml [12].

Our group and others assessed the in vitro activity of certain quinolones against various tumor cell lines. CIP was found to inhibit tumor cell growth of bladder transitional cell carcinoma, colon cancer, and prostate cancer cell lines at concentrations achievable by oral administration of commonly used therapeutic dosages [13–15]. The above studies concentrated mainly on apoptosis and cell cycle progression, but have not studied the effects of the quinolones on topoisomerases in this setting. In a recent study we have shown that the fluoroquinolone moxifloxacin (MXF) slightly inhibited the activity of purified human topo II while in combination with the topo II inhibitor anti-cancer drug etoposide it lead to a 73% reduction in enzyme activity [16]. Moreover, our study showed that MXF enhanced the antiproliferative and apoptotic effects of etoposide towards THP-1 and Jurkat tumor cells and at the same time inhibited the enhanced release of pro-inflammatory cytokines (IL-8, IL-1 and TNF-α) from the cells, induced by the drug [16]. In the present study we investigated the in vitro effects of the two quinolones MXF and CIP alone and in combination with CPT on isolated calf thymus topo I activity and further studied the effect of the combination on cell proliferation and apoptosis in HT-29 colon cancer cell line. In addition, we investigated the effect of MXF and CIP on CPT-induced release of pro-angiogenic cytokines including IL-8 and VEGF in these cells.

2. Materials and methods

2.1. Drugs

Stock solutions of CPT (Sigma–Aldrich Corp., St. Louis, MO, USA) at 10 mM in dimethyl sulfoxide were stored in aliquots at −70 °C. Moxifloxacin HCL (89.6% activity) (MXF) and ciprofloxacin HCL (89.4% activity) (CIP) were a generous gift from Bayer AG, Wuppertal, Germany. Concentrations of quinolones in clinical setting and in vitro antimicrobial studies are commonly expressed as μg/ml. In the current study, where CPT concentrations are expressed on a molar basis (nM) we have used the molar equivalent of quinolones concentrations corresponding to drug concentrations ranging from 10 to 40 μg/ml.

2.2. Topo I assay

A previously described method was used with slight modifications [17]. Purified calf thymus topo I (0.5–4 units) (MBI Fermentas, Hanover, MD) or increasing concentrations of nuclear proteins were added to a topo I reaction mixture containing, at a final volume of 25 μl, 20 mM Tris–HCl, pH 8.1, 1 mM dithiothreitol, 20 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 30 μg/ml bovine serum albumin and 250–750 ng of pUC19 super coiled DNA plasmid (MBI Fermentas, Hanover, MD). Different concentrations of MXF (51–102 μM) (20–40 μg/ml), CIP (33.7–135 μM) (10–40 μg/ml) and CPT (0.1–1 μM) were added. After incubation at 37 °C for 10 min, the reaction was terminated by adding 5 μl of stopping buffer (final concentration, 1% SDS, 15% glycerol, 0.5% bromphenol blue and 50 mM EDTA, pH 8). The reaction products were analyzed by electrophoresis on 1% agarose gel using a Tris–borate/EDTA buffer (89 mM Tris–HCl, 89 mM boric acid, and 62 mM EDTA) at 1 V/cm, stained by ethidium bromide (1 μg/ml) and photographed using a short-wavelength UV lamp (ChemiImager 5500; Alpha Innotech, San Leandro, CA, USA). Densitometric analysis of the results were performed using the AlphaEasFC image processing and analysis software, and the percentage of topo I activity was calculated using the following equation: [1 − (sample/control) × 100] as previously described [17,19,20].

2.3. Preparation of nuclear extracts

Nuclear extracts from HT-29 colon cancer cells were prepared as described previously [17]. A mixture of protease inhibitors (final concentrations 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml antipain and 100 μg/ml phenylmethylsulfonyl fluoride) were added to the extraction buffers. Total protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). In experiments where the effect of quinolones on topo I activity in the cells was examined, HT-29 cells were incubated in the presence of 51 μM (20 μg/ml) MXF, 67.5 μM (20 μg/ml) CIP or

2.5 μM CPT for various periods of time as described in the experiments. Nuclear extracts were prepared as described above and 6 ng of nuclear protein were added to a topo I reaction mixture.

2.4. 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% CO_2. The cells were maintained in log phase by seeding twice a week at a density of 5×10^4 ml^{-1} and the experiments were performed 1 day after trypsinization. In order to increase constitutive VEGF expression of HT-29 cells, all the experiments for determination of VEGF and IL-8 activities were carried out in serum-deprived condition, by excluding FBS from standard culture medium.

2.5. 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 and various concentrations of MXF, CIP, CPT and their combination were added. The cells were incubated for 24–72 h at 37 °C in 5% CO_2 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 Corporation, Sunnyvale, CA).

2.6. Apoptosis assay

Apoptosis was measured by flow cytometry after concurrent staining with fluorescein-conjugated annexin V and propidium iodide (PI) (Annexin V-FITC kit, Bender MedSystems GmbH, Vienna, Austria) according to manufacturer instructions. In brief, following incubation of HT-29 cells with 5 nM CPT and various concentrations of MXF or CIP and their combination for 24 h, cells were collected, washed, stained with annexin V-PI and subjected to flow cytometric analysis on FACScan (Becton Dickinson, Franklin Lakes, NJ).

2.7. IL-8 and VEGF secretion

The concentration of IL-8 and VEGF in the medium of control and drug treated cells was measured using commercially available sandwich enzyme-linked immunoassay (ELISA) kit according to the manufactures' instructions (R&D Systems Inc., Minneapolis, MN). 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, CIP or CPT given as a single drug or in combination, in serum-free conditioned medium [18]. After 48 h cell-free supernatants were recovered, and the concentrations of IL-8 and VEGF were determined using ELISA (R&D Systems Inc.). The sensitivity of the assay for IL-8 is >10 pg/ml and for VEGF >31.2 pg/ml.

2.8. Statistical analysis

Statistical significance was determined by paired *t*-test (for MTT and for cytokine secretion) and ANOVA: two-factor without replication test (for the annexin-PI studies). A *p*-value of ≤0.05 was considered significant.

3. Results

3.1. MXF and CIP increase the inhibition of DNA relaxation activity of purified calf thymus topo I induced by CPT

The effect on DNA relaxation activity of eukaryotic topo I by various concentrations of MXF, CIP and CPT was investigated using purified calf thymus topo I added to a specific reaction mixture containing super coiled pUC 19 DNA as the substrate. The reaction products were analyzed by agarose gel electrophoresis and the percentage of topo I activity was calculated using densitometric analysis as previously described [16]. As shown in Fig. 1A, MXF at concentrations of 51 and 102 μM (20

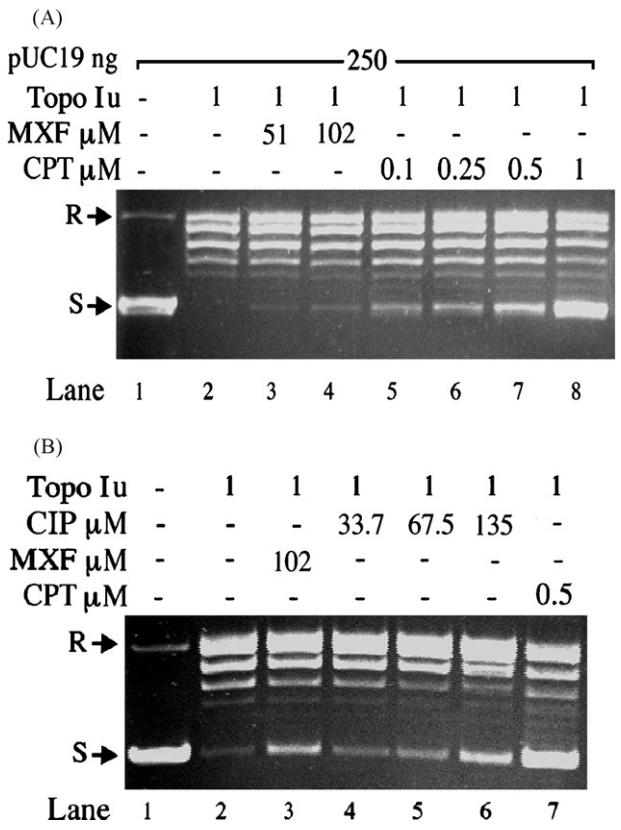

Fig. 1 – The effect of increasing doses of MXF, CIP and CPT on the activity of topo I. Representative agarose gel electrophoresis analysis of the topo I reaction products, obtained with increasing amounts of (A) MXF and CPT or (B) CIP. The pUC 19 supercoiled DNA plasmid and the relaxed forms are shown. R: relaxed DNA, S: supercoiled DNA, u: units.

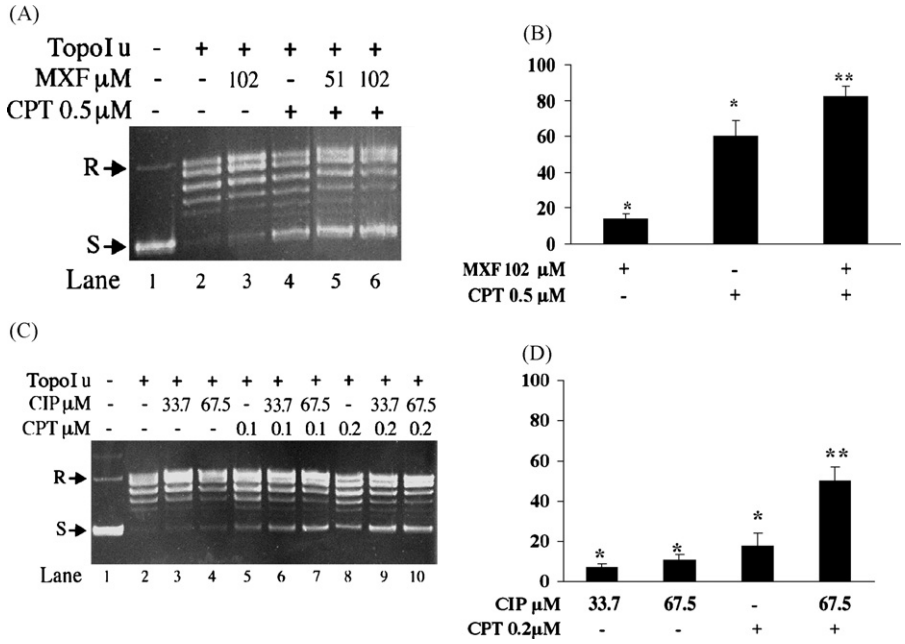

Fig. 2 – MXF and CIP increase the CPT-induced inhibition of purified calf thymus topo I DNA relaxation activity. Representative agarose gel electrophoresis analysis of the topo I reaction products, obtained with (A) MXF and CPT added separately or in combination (C) CIP and CPT added separately or in combination. Densitometric analysis was performed and the percentage of topo I inhibition by (B) MXF and CPT or (D) CIP and CPT was calculated. The results are the mean ± S.E. of three to four experiments. R: relaxed DNA, S: supercoiled DNA, u: units. $^{*}p < 0.05$ cells treated with drugs vs. control. $^{**}p < 0.05$ for cells treated by CPT + quinolones vs. CPT alone.

and 40 μg/ml) caused a 9–14% inhibition of topo I activity (lanes 3 and 4, respectively). CPT reduced topo I activity in a dose dependent manner (32%, 37%, 62% and 85% inhibition at CPT concentrations of 0.1, 0.25, 0.5 and 1 μM, respectively). Fig. 1B shows that CIP at concentrations of 33.7, 67.5 and 135 μM (10, 20 and 40 μg/ml) inhibited topo I activity in a dose dependent manner (18%, 30% and 68% inhibition, respectively) (lanes 4, 5 and 6, respectively).

To investigate the effect of a combined drug treatment on topo I activity 0.5 μM of CPT was used in combination with 51 or 102 μM (20 or 40 μg/ml) of MXF. Fig. 2A shows a representative experiment in which the additive effect of

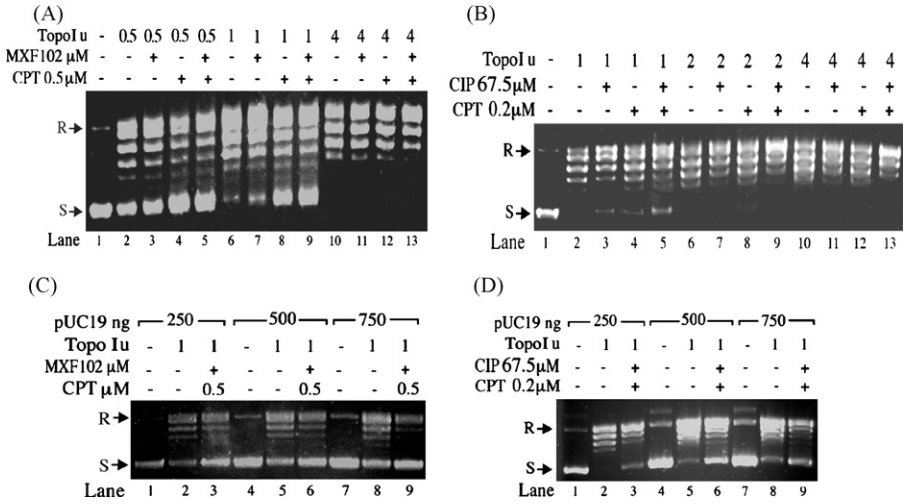

Fig. 3 – Representative agarose gel electrophoresis analysis of the topo I reaction products obtained with increasing amounts of topo I (0.5–4 units) in the presence of constant amount of pUC 19 DNA (250 ng) and (A) 0.5 μM CPT + 102 μM MXF or (B) 0.2 μM CPT and 67.5 μM CIP. (C and D) increasing amounts of pUC 19 DNA (250–750 ng) were added to topo I reaction mixture containing constant amount of topo I (1 unit) and 0.5 μM CPT + 102 μM MXF or 0.2 μM CPT + 67.5 μM CIP, respectively. R: relaxed DNA, S: supercoiled DNA, u: units.

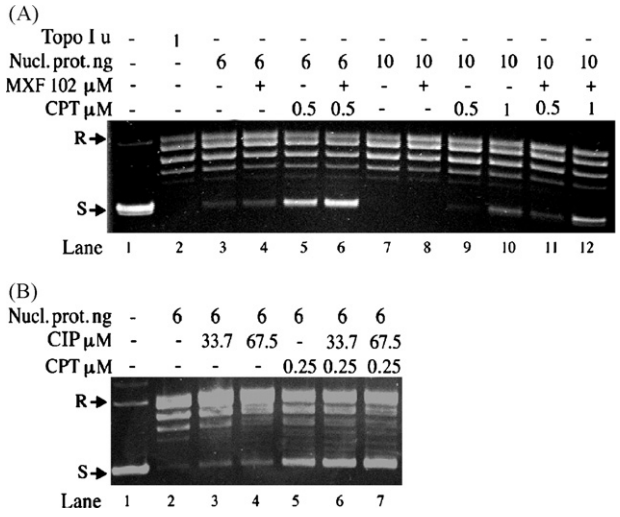

Fig. 4 – The effect of MXF, CIP and CPT on the activity of topo I from nuclear extract. Nuclear extract was prepared from HT-29 cells, and 6–10 ng nuclear (nucl) proteins (prot) were added to specific topo I reaction mixture in the presence of CPT (0.25–1 μM) and (A) MXF or (B) CIP. The reaction products were analyzed by agarose gel electrophoresis. R: relaxed DNA, S: supercoiled DNA, u: units.

MXF and CPT on topo I inhibition is shown. The addition of 102 μM (40 μg/ml) MXF or 0.5 μM CPT added separately to 1 unit topo I induced 14% and 62% inhibition in enzyme activity (lanes 3 and 4, respectively), while the addition of the combination of the two drugs induced a 82% inhibition (lane 6). A similar inhibitory effect was observed with CIP and CPT (Fig. 2C). The addition of 67.5 μM (20 μg/ml) CIP or 0.2 μM CPT added separately to 1 unit topo I induced 12% and 30% inhibition in enzyme activity (lanes 4 and 8, respectively), while the addition of the combination of the two drugs induced a 64% inhibition (lane 10). Densitometric analysis was performed and the percentage of topo I inhibition was calculated. Fig. 2B and D shows the mean results ± S.E. of four experiments. The figures show that the inhibition of topo I activity is significantly enhanced following the addition of a combination of MXF and CPT (Fig. 2B) or CIP and CPT (Fig. 2D) compared to the inhibitory effect observed with each drug added alone.

The observed increased inhibitory effect of CPT by MXF or CIP might be due to (1) a direct effect of the quinolones on the topo I protein, rendering it more susceptible to the action of CPT, or (2) MXF or CIP affect the DNA (e.g. intercalation) in a way that increases the CPT-induced stabilization of the DNA–enzyme cleavable complexes. To determine which of the possibilities do occur, we performed two classical biochemical competition based assays. Topo I activity was measured in the presence of constant amounts of DNA and MXF/CPT or CIP/CPT, and increasing amounts of topo I enzyme (Fig. 3A and B, respectively) or *vice versa*; topo I activity was measured in the presence of constant amounts of enzyme and MXF/CPT or CIP/CPT, and increasing amounts of DNA (Fig. 3C and D, respectively). The results show that only by increasing the amount of topo I enzyme it was possible to overcome the inhibitory effect of MXF/CPT or CIP/CPT (Fig. 3A and B compared to Fig. 3C and D), suggesting a possible, yet unclear, interaction of MXF and CIP with the topo I protein.

3.2. *Inhibition of topo I activity in nuclear extracts derived from HT-29 cells*

Nuclear protein lysates were prepared from HT-29 cells as described previously [17] and 6–10 ng nuclear proteins were added to specific topo I reaction mixture containing pUC 19 super coiled DNA (250 ng) in the presence of CPT (0.25–1 μM)

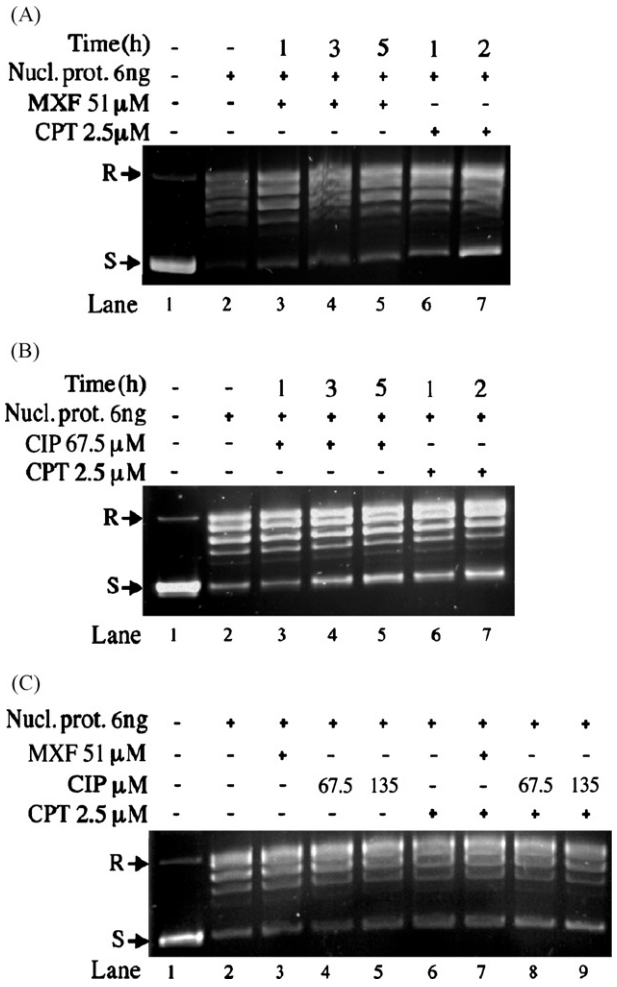

Fig. 5 – Inhibition of topo I DNA relaxation activity in MXF- and CIP-treated cells. HT-29 cells were incubated for the indicated periods of time with (A) 51 μM MXF or 2.5 μM CPT or (B) 67.5 μM CIP. Nuclear extracts were prepared and 6 ng nuclear (nucl) proteins (prot) were added to specific topo I reaction mixture. The reaction products were analyzed by agarose gel electrophoresis. (C) HT-29 cells were incubated for 2 h with the indicated concentrations of MXF or CIP, CPT was added to some of the cultures (as indicated) and the cells were incubated for additional 60 min. Nuclear extracts were prepared and 6 ng nuclear proteins were added to topo I reaction mixture and the data were analyzed as described for (A and B). R: relaxed DNA, S: supercoiled DNA, u: units.

and MXF or CIP. The reaction products were analyzed by agarose gel electrophoresis. Fig. 4A and B indicates that MXF and CIP act synergistically with CPT in inhibiting the activity of both purified and unpurified topo I (lane 6 compared to lanes 5 and 4 in Fig. 4A and lanes 6 and 7 compared to lanes 5, 4 and 3 in Fig. 4B).

3.3. *Inhibition of topo I DNA relaxation activity in MXF- and CIP-treated cells*

The results described above suggest that the quinolones MXF and CIP inhibit topo I activity when purified enzyme or nuclear extract proteins are used. To investigate the ability of these compounds to affect the activity of topo I within the cell, we first treated the cells for various intervals with 51 μM (20 μg/ml) MXF, 67.5 μM (20 μg/ml) CIP or 2.5 μM CPT. Nuclear extracts were prepared and 6 ng of nuclear proteins were added to a topo I reaction mixture. The results presented in Fig. 5A and B indicate that the inhibitory effects of the quinolones on topo I increased with the duration of cell exposure to the drugs. Incubation of the cells with 51 μM (20 μg/ml) MXF (Fig. 5A) or 67.5 μM (20 μg/ml) CIP (Fig. 5B) for 1–5 h resulted in 11–23% and 24–43% reduction of topo I activity, respectively.

A marked inhibition of topo I activity was observed in cells incubated with 2.5 μM CPT (23% and 56% inhibition upon incubation of 1 or 2 h, respectively). To investigate whether the quinolones enhance the inhibitory effect of CPT, HT-29 cells were incubated with the quinolones for 2 h, followed by CPT treatment for an additional hour (cells were exposed to the quinolones for 3 h and to CPT for 1 h). Fig. 5C shows that MXF (lanes 3 and 6 vs. lane 7) and CIP (lanes 4 and 6 vs. lanes 8 or 5 and lane 6 vs. lane 9) increase the inhibitory activity of topo I induced by CPT.

3.4. *Effect of MXF and CIP on the anti-proliferative activity of CPT*

The aforementioned results suggest that treatment of cells with quinolones enhanced the inhibition of cellular topo I by CPT. To examine the biological significance of these results we investigated the effect of the combination of quinolones with CPT on cell proliferation. In the first experiment we performed time dependent studies on the effect of various concentrations of CPT (1–50 nM) on cell proliferation. Fig. 6A indicates that the decrease in cell proliferation was dose- and time-dependent. Maximal inhibition (up to 45 ± 2.1%) was observed following incubation of the cells for 72 h with 50 nM CPT. The additional experiments combining quinolones with CPT were performed with cell incubation for72 h. Fig. 6B shows that MXF given alone at concentrations of 25.5 or 51 μM (10 or 20 μg/ml) inhibited cell proliferation only slightly (8 ± 0.2% and 14 ± 0.1%, respectively). In contrast, the combination of CPT with MXF induced a marked decrease in cell proliferation. Exposure of the cells to a combination of 1 nM CPT and 25.5 or 51 μM (10 or 20 μg/ml) MXF, resulted in a decrease up to 33 ± 1% and 44 ± 2%, respectively, compared to 14 ± 0.5% inhibition of proliferation induced by 1 nM CPT given alone ($p \leq 0.003$). The enhanced effect of the combination does not increase when CPT doses are elevated to 10 nM, indicating a more pronounced additivity or synergy when sub-inhibitory concentrations are combined. It

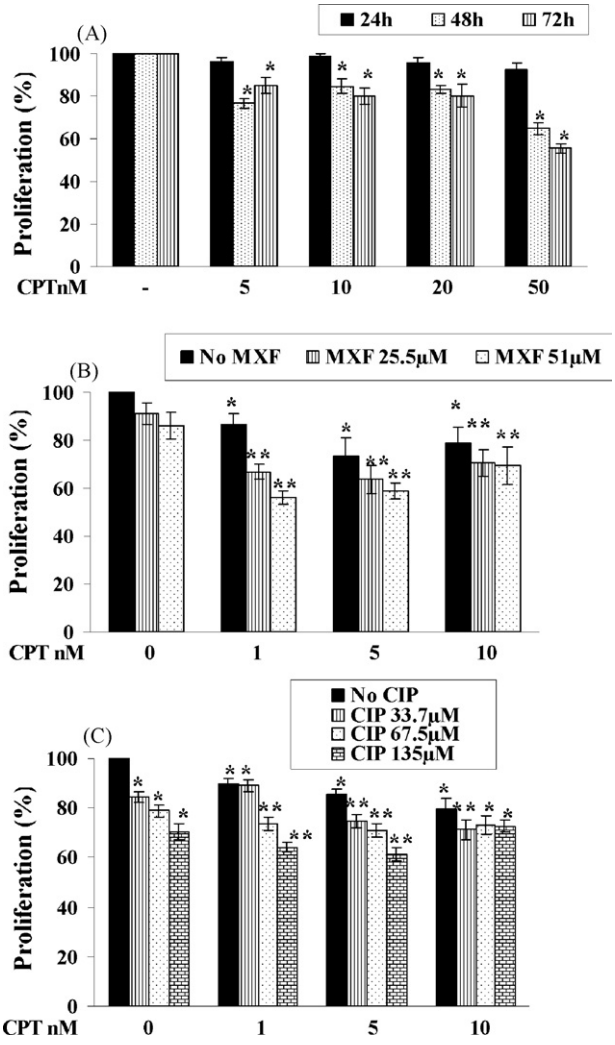

Fig. 6 – MXF and CIP enhance the anti-proliferative effect of CPT. (A) Time and dose dependent studies. (A) HT-29 cells were incubated for 24–72 h with increasing concentrations of CPT. Cell proliferation was determined by colorimetric MTT assay. Effect of (B) MXF and (C) CIP on cell proliferation following incubation of the cells for 72 h in the presence or absence of CPT and quinolones. Cell proliferation was determined as described above. Results are expressed as mean ± S.E. of four experiments performed in triplicate. *$p < 0.05$ cells treated with drugs vs. control. **$p < 0.05$ for cells treated by CPT + quinolones vs. CPT alone.

should be noted that the level of proliferation inhibition following exposure of the cells to a combination of low dose of the cytotoxic drug CPT (1 nM) and 51 μM (20 μg/ml) MXF (Fig. 6B) was similar to the level of inhibition obtained with a 50-fold higher dose of CPT (50 nM) alone (Fig. 6A: 44 ± 2% vs. 45 ± 2.1%, respectively, $p < 0.001$). Fig. 6C shows that CIP alone inhibited cell proliferation (15.5 ± 0.25%, 21 ± 0.5% and 30 ± 1% inhibition at (33.7, 67.5 and 135 μM, respectively) (10, 20 and 40 μg/ml) ($p < 0.001$), but it was less potent in enhancing the cytotoxic activity of CPT compared to MXF. The addition of 135 μM (40 μg/ml) CIP to 1 nM CPT induced a 36 ± 1% inhibition

in cell proliferation, a similar inhibitory effect observed with the addition of only 25.5 μM (10 μg/m) MXF to the same concentration of CPT (33 ± 1%).

3.5. *Effect of MXF and CIP on CPT-induced apoptosis*

To determine whether the observed decrease in cell number following exposure to quinolones and CPT is associated with increase in apoptosis, we exposed HT-29 cells for 24 h to 5 nM CPT, 25.5 and 51 μM (10 and 20 μg/ml) MXF or 67.5 and 135 μM (20 and 40 μg/ml) CIP alone or in combination. Following exposure, the cells were stained for annexin V and PI (Fig. 7). Cells staining positive for annexin only (Fig. 7A, lower right panel) represent early apoptosis, while cells staining positive for both annexin and PI represent cells in late apoptosis (upper right panel). Exposure to 51 μM (20 μg/ml) MXF or 135 μM

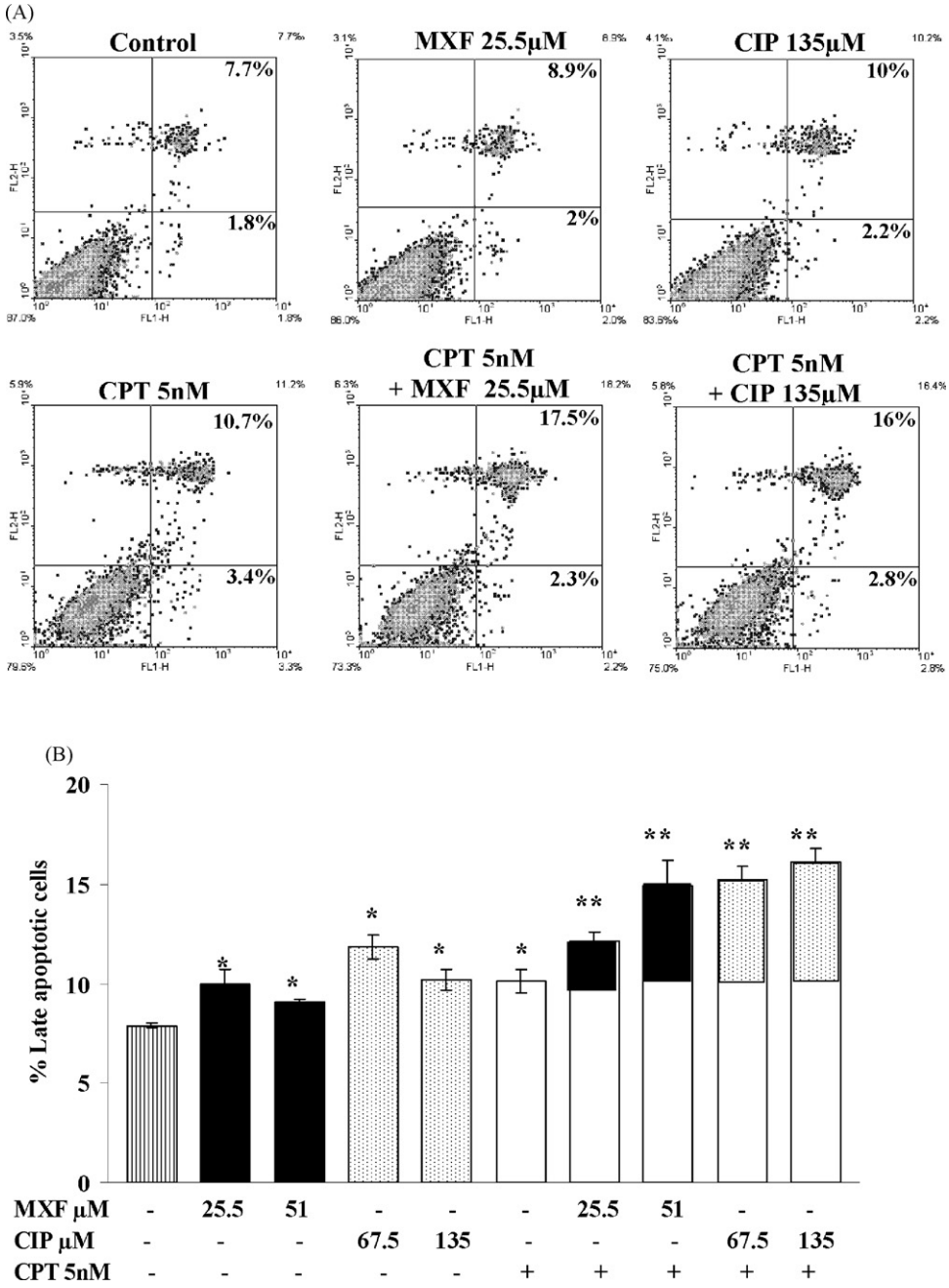

Fig. 7 – MXF and CIP enhance apoptosis induced by CPT. HT-29 cells were incubated for 24 h with CPT and MXF or CIP as indicated and flow cytometric analysis was performed by binding of annexin V and uptake of PI. A representative experiment is shown in A. Results (mean ± S.E.) of three independent experiments are shown in B. The percentage of annexin V-positive, PI-negative cells is indicated in the lower right quadrangle (early apoptosis) and of annexin V-positive, PI-positive cells in the upper right quadrangle (late apoptosis). The X-axis shows log annexin V fluorescence intensity and the Y-axis shows PI fluorescence intensity. $^{*}p < 0.05$ for cells treated with MXF, CIP or CPT given alone vs. control. $^{**}p < 0.05$ for cells treated with CPT + MXF or CPT + CIP vs. CPT alone.

(40 μg/ml) CIP alone slightly enhanced late apoptosis. A 1.5-fold increase in late apoptosis was observed after exposure to 5 nM CPT and a further 1.5- and 1.6-fold increase in late apoptosis was observed with the addition of 51 μM (20 μg/ml) MXF or 135 μM (40 μg/ml) CIP to CPT (Fig. 7A and B) ($p < 0.05$).

3.6. *Effect of MXF and CIP on the secretion of pro-angiogenic factors induced by CPT*

We investigated the effect of MXF and CIP on the secretion of IL-8 and VEGF by HT-29 cells. The concentrations of the pro-angiogenic cytokines were determined by ELISA, as previously described by us [16]. Fig. 8A and B shows that MXF at concentrations of 25.5 and 51 μM (10 and 20 μg/ml) and CIP at 135 μM (40 μg/ml) significantly decrease spontaneous release of IL-8 ($p < 0.01$). Incubation of the cells for 48 h with increasing concentrations of CPT (1–20 nM) resulted in a dose dependent increase in IL-8 release, (up to 1.85-fold, $p < 0.01$). MXF at concentration of 51 μM (20 μg/ml) significantly reduced the enhanced release of IL-8 induced by 20 nM CPT (48% reduction, $p < 0.01$). Similarly, CIP (135 μM) (40 μg/ml) led to 26% reduction of CPT-induced IL-8 release ($p < 0.01$). 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 25.5 and 50 μM (10 and 20 μg/ml) inhibited the spontaneous release by 17% and 33%, respectively (Fig. 8C). Neither CIP nor CPT affected spontaneous release of VEGF (data not shown).

4. Discussion

The central point of the present work is the finding that two fluoroquinolones, MXF and CIP, known to be highly specific for prokaryotic type II topoisomerase and are active against eukaryotic topo II, can also slightly inhibit eukaryotic topo I activity but significantly enhance the cytotoxic effects of CPT.

In a previous study we have shown that MXF alone slightly inhibited human topo II activity, but in combination with VP-16 led to a significant increase in the inhibitory effect of the anti-cancer drug on topo II activity and on the decrease of proliferation of THP-1 and Jurkat cells [16]. The present study investigated the effect of MXF and CIP in combination with the anti-topoisomerase I chemotherapeutic agent CPT on the activity of calf thymus topo I by measuring the relaxation of supercoiled pUC 19 DNA plasmid.

We found that MXF or CIP alone (at concentrations of 51–135 μM) (or 20 or 40 μg/ml) only slightly inhibited calf thymus topo I activity, but in combination with CPT led to a significant increase in the inhibitory effect of the anti-cancer drug on topo I activity. In addition, we found that the inhibitory effect of the combined drugs on topo I could be overcome only by increasing the amount of the enzyme protein in the reaction and not by adding higher concentrations of the DNA substrate. These observations imply that MXF and CIP enhance the inhibitory effects of CPT on topo I by affecting the enzyme protein in a way that renders it to become more susceptible to CPT. However, since fluroquinolones bind DNA [21] it is also possible that their binding to the DNA promote the binding of CPT to the enzyme–DNA complex.

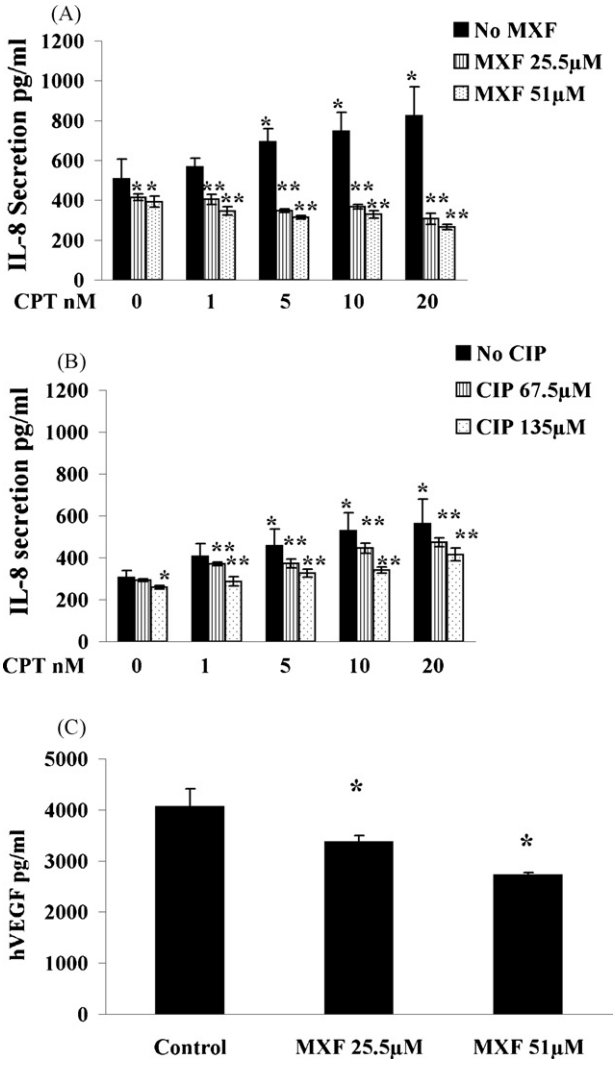

Fig. 8 – Effect of MXF and CIP on pro-angiogenic factors secretion. HT-29 cells were incubated for 48 h with increasing concentrations of CPT in the presence or absence of (A) MXF or (B) CIP and the concentrations of IL-8 (A and B) were measured by ELISA. The cells were incubated for 48 h in the absence or presence of the quionolones and the concentration of VEGF (C) was measured by ELISA. The values are the mean ± S.E. of six experiments performed in duplicates. $^{*}p < 0.01$ drug treated cells vs. control. $^{**}p < 0.05$ CPT + MXF or CPT + CIP vs. CPT treated cells.

The ability of the two quionolones to inhibit purified topo I activity in vitro and enhance the inhibitory effect of CPT does not necessarily indicate that topo I is indeed the target of these drugs within the cell. Therefore, we determined the effect of treating HT-29 cells with 51 μM (20 μg/ml) MXF or 67.5 μM (20 μg/ml) CIP alone or in combination with 2.5 μM CPT on the activity of cellular topo I. We found that both MXF and CIP enhanced significantly the inhibition of cellular topo I activity induced by CPT in a time dependent manner.

We have also defined the functional interaction of the drugs by investigating their effect on the cytotoxic activity

towards HT-29 cells. We found that MXF or CIP alone (at a concentration of 51 or 67.5 μM, respectively (20 μg/ml)) induced a slight anti-proliferative effect on HT-29 cells (up to 14 ± 0.1% and 21 ± 0.5% decrease in cell proliferation, respectively). In contrast, when the quinolones were added to CPT, a significant decrease in cell proliferation occurred. It is reasonable to suggest that the anti-proliferative effect of the investigated quinolones in combination with CPT is the result of their combined influence on the topoisomerases as was observed in the preceding experiments.

Camptothecin and its derivatives are currently used for the treatment of several types of solid tumors including lung, colorectal, ovarian, uterine and gastric carcinoma [2,3], however, the high cytotoxic adverse events of the drugs prompted the search for modulators of the therapeutic effect which would potentially allow the response to lower doses of these agents. Combined chemotherapy of irinotecan and other chemotherapeutic agents is recommended. Studies have shown that irinotecan and low-dose cisplatin were effective in patients with metastatic gastric, colonic and biliary tract cancers [6,22,23]. Response rates of up to 50% have been observed in patients with metastatic colon cancer following treatment with selected combinations of 5-FU, CPT-11 and oxaliplatin [24–26].

Fluoroquinolone antibiotics are considered non-toxic agents and can be readily administered orally. Recent reports documented that some fluoroquinolones show growth inhibition in a variety of human tumor cells such as human leukemic cells, osteoblast-like MG-63 human osteosarcoma cells, transitional cell carcinoma of the bladder and prostate cancer [15,27–31].

To the best of knowledge, the experiments reported here are the first to examine the interaction of MXF and CIP on the cytotoxic activity of CPT and are the first to describe their combined inhibitory effects on topoisomerase I. We were also able to substantiate the combined anti-proliferative effects by demonstrating a significant enhancement of apoptosis by this specific drug combination in the human tumor cell line HT-29.

An important observation is the fact that 1nM CPT combined with 51 μM (20 μg/ml) of MXF or 135 μM (40 μg/ml) CIP led to the same inhibition of cell proliferation as 50 nM CPT alone. This may imply a cytotoxic-drug ''sparing effect'' by the two quinolones. The translation of this phenomenon in the clinical setting is that instead of increasing the dose of the cytotoxic agent, along with its associated toxic side effect, one may use a lower dose of the cytotoxic agent and add the antimicrobial agents MXF or CIP with their excellent safety profile, and obtain the same anti-tumor effects with much less toxicity and adverse effects. It should also be noted, that the concentration of MXF and CIP cited above are attainable in certain tissues such as colon, bladder, prostate and lung cells following the commonly used dosages of MXF and CIP.

We show in the present study that treatment of HT-29 cells with CPT induced the release of the pro-inflammatory cytokine IL-8 and that MXF and CIP significantly inhibited the CPT-enhanced production of IL-8. The inhibitory effect of MXF on cytokine secretion from HT-29 cells also confirms our previous observations of MXF inhibition of the synthesis of pro-inflammatory cytokines in THP-1 cells and human peripheral blood monocytes stimulated with LPS-phorbol myristate acetate [32] or *Aspergillus fumigatus* [33]. Additionally, MXF inhibited NFκB and mitogen-activated protein kinase activation in THP-1 cells and in a human respiratory epithelial cell line [32,34]. Using a murine model of *Candida* pneumonia in immune suppressed animals, we found that MXF exerted a protective anti-inflammatory effect, resulting in a marked decrease in bronchopneumonia and enhanced survival. This protective efficacy was associated with a significant reduction in IL-8 and TNF-α in lung homogenates and an inhibition of NFκB nuclear mobilizatoin in alveolar macrophages and lung epithelial cells [35].

In summary, this study demonstrates an important role for MXF and CIP in enhancing the cytotoxic effects of CPT while, at the same time, decreasing CPT-induced pro-inflammatory cytokine secretion from cells, which may be harmful during chemotherapeutic treatment. Our results suggest that MXF and CIP may be a valuable new addition to chemotherapeutic armamentarium, simultaneously improving the cytotoxic activity, and reducing the side effects of CPT and similar agents. In addition a reduction in the total dose of the cytotoxic agent may be attained when administered in combination with these quinolones.

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