Biochemical Pharmacology 79 (2010) 1100–1107

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Moxifloxacin increases anti-tumor and anti-angiogenic activity of irinotecan in human xenograft tumors

Debby Reuveni [a], Drora Halperin [a], Ina Fabian [a,*], Galia Tsarfaty [b], Nadir Askenasy [c], Itamar Shalit [d,e]

[a] Department of Cell and Developmental Biology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel
[b] Imaging Radiology, Sheba Medical Center, Ramat Gan, Israel
[c] Frankel Laboratory for Bone Marrow Transplantation, Department of Pediatric Hematology-Oncology, Schneider Children's Medical Center of Israel, Israel
[d] Schneider Children's Medical Center of Israel, Petach-Tikva, Israel
[e] Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

ARTICLE INFO

Article history:
Received 27 October 2009
Accepted 1 December 2009

Keywords:
Irinotecan
Moxifloxacin
Chemotherapy
Angiogenesis

ABSTRACT

Camptothecins (CPTs) are topoisomerase I inhibitors chemotherapeutic agents used in combination chemotherapy. We showed previously that combination of moxifloxacin (MXF) and CPT induced inhibitory effects on topoisomerase I activity, on proliferation of HT-29 cells in vitro and enhanced apoptosis, compared to CPT alone. Analysis of secretion of the pro-angiogenic factors IL-8 and VEGF showed significant reduction by MXF. Using a murine model of human colon carcinoma xenograft, we compared the effects of MXF/CPT in vitro to MXF/irinotecan combination in vivo. We show that the MXF/CPT inhibitory effects observed in vitro are reflected in the inhibition of the progressive growth of HT-29 cells implanted in SCID mice. Using caliper measurements, Doppler ultrasonography, image analyses and immunohistochemistry of nuclear proteins (Ki-67) and vascular endothelial cells (CD-31) we show that addition of MXF (45 mg/kg) to a relatively ineffective dose of irinotecan (20 mg/kg), results in a 50% and 30% decrease, respectively, in tumor size and a decrease in Ki-67 staining. Power Doppler Ultrasound showed a significant, pronounced decrease in the number of blood vessels, as did CD-31 staining, indicating decreased blood flow in tumors in mice treated with MXF alone or MXF/irinotecan compared to irinotecan. These results suggest that the combination of MXF/irinotecan may result in enhanced anti-neoplastic/anti-angiogenic activity.

1. Introduction

Camptothecins (CPTs) are topoisomerase I inhibitors and their derivatives are potent anti-cancer drugs. Irinotecan (CPT-11) is currently used in therapeutic regimens of several solid tumors and irinotecan-based combination protocols are employed as first- and second-line therapy in colorectal cancer [1,2]. Several clinical trials have shown the improved outcome of combination therapy compared to mono-therapy [3] and there is an ongoing search for less toxic combinations with improved activity.

Fluoroquinolones are potent, broad spectrum antimicrobial agents commonly used for the treatment of community and hospital-acquired infections and have a very good safety and tolerability profile. Certain members of this family (i.e. ciprofloxacin, moxifloxacin, trovafloxacin) were shown by us and other groups to exert anti-tumor activity in several cancer cell lines [4–7] and in an in vivo animal model [8]. Activity was shown mainly against colon cancer, bladder cancer and leukemia cell lines [5,7,9]. The anti-tumor activity was linked in several studies to the anti-topoisomerase II activity of the quinolones which was demonstrated in eukaryotic and tumor cells [7,10,11].

In a previous study we have shown that moxifloxacin (MXF) slightly inhibited the activity of purified human topoisomerase II, however, in combination with the anti-topoisomerase II chemotherapeutic agent VP-16 it acted synergistically leading to a 73% reduction in enzyme activity [7]. Furthermore, MXF significantly enhanced the anti-proliferative activity and apoptotic effects of VP-16 in THP-1 and Jurkat tumor derived cell lines and inhibited the enhanced release of IL-8, IL-1β and TNF-α exerted by VP-16 in THP-1 cells [7].

In a more recent study we have analyzed the effect of MXF and ciprofloxacin on topoisomerase I activity and their combination with the anti-topoisomerase I chemotherapeutic agent CPT [12]. The quinolones alone slightly inhibited purified topoisomerase I activity, however, an additive effect leading to 82% and 64% reduction in topoisomerase I activity was demonstrated when MXF and

* Corresponding author at: Department of Cell and Developmental Biology, Sackler School of Medicine, Tel.: Aviv University, P.O.B. 39040, Tel Aviv 69978, Israel. Tel.: +972 3 6409628; fax: +972 3 6407432.
E-mail address: inaf@post.tau.ac.il (I. Fabian).

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doi:10.1016/j.bcp.2009.12.001

ciprofloxacin, respectively, were added to CPT. We further analyzed the anti-proliferative apoptotic effects of CPT in combination with the quinolones in HT-29 cells. Addition of MXF to CPT enhanced its apoptotic (annexin V) and cytotoxic (MTT) activity. In addition, we analyzed the effect of the quinolones on the pro-angiogenic factors IL-8 and VEGF. CPT given alone induced an increase in IL-8 release from HT-29 cells while the addition of a combination of MXF and CPT resulted in a significant decrease of the enhanced release of IL-8 and reduction in spontaneous release of VEGF. Taken together our in vitro studies demonstrated a significant enhancement of CPT cytotoxic activity by MXF and ciprofloxacin in HT-29 cells associated with the enhancement of anti-topoisomerase I activity. Anti-angiogenic effects were also demonstrated by the quinolones following spontaneous or CPT induced release of pro-angiogenic cytokines.

In the present study we investigated the in vivo effect of combination therapy with irinotecan and MXF in a human colon cancer xenograft in SCID mice. Since we noticed in our in vitro studies both anti-proliferative and anti-angiogenic effects after adding MXF to CPT, we performed studies to elucidate both effects. Thus, in addition to caliper measurements of tumor growth we have performed a Power Doppler Ultrasound analysis of the tumors to assess blood flow and distribution of small vessels in the tumor. In addition, we performed immunohistochemistry staining on tumor sections for measurement of the nuclear protein Ki-67 as a marker for assessment of cancer cells proliferation, and of CD-31 for the demonstration and measurement of vascular endothelial cells in the tumor. Finally, we measured the levels of the pro-angiogenic chemokine IL-8 in tumor homogenates to further assess the potential effect of MXF on angiogenesis.

2. Materials and methods

2.1. Drugs

Irinotecan (Camptosar®) was supplied as irinotecan hydrochloride by Pharmacia & Upjohn, Kalamazoo, MI. MXF was a generous gift from Bayer AG, Wuppertal, Germany.

2.2. Generation of human colon cancer xenografts and evaluation of tumor growth

Female NOD.SCID mice, 5–6 weeks of age (NOD.CB17-Prkdcsid/J mice—Jackson Laboratories, Bar-Harbor, ME, USA), were housed and handled in a pathogen-free environment. Two sets of 42 mice each were implanted s.c. in their flanks with 5×10^6 HT-29, human colon carcinoma cells. Subsequently the mice were divided into 7 groups. Group 1: control group, mice were injected i.p. 3 times/day with saline. Group 2: mice received 3 times/day i.p. injection of 15 mg/kg MXF (the dose of MXF is based on our previous in vivo studies showing a protective anti-inflammatory effect of MXF in a murine model of *Candida albicans* pneumonia in immunosuppressed animals [13] and is considered an equi-human dose). Injections started on day 1 post implantation of HT-29 cells. Groups 3–5: mice received on days 7, 14 and 21 after HT-29 cell implantation an i.p. injection of 20, 35 or 50 mg/kg irinotecan, respectively [14]. Groups 6 and 7: mice received a daily injection of MXF as in group 2 and on days 7, 14 and 21 an injection of 20 or 35 mg/kg irinotecan, respectively, as in groups 3 and 4. Animal weights were monitored and tumor dimensions were measured in two perpendicular dimensions once every 3 days, using calipers, starting on day 1 and the tumor volumes were calculated according to the following formula: $V = (a^2 \times b)/2$, where 'a' is the width of the tumor (smaller diameter) and 'b' is the length (larger diameter) [15].

2.3. Power Doppler Ultrasound imaging of tumors

Power Doppler Ultrasound imaging of tumors was performed according to a previously published method [16,17]. Tumor-bearing mice (6 mice/group) were anesthetized (2.25 mg/kg ketamine, 0.15 mg/kg xylazine) to minimize echogenicity attributable to tissue motion. After the hair surrounding the tumor area was removed using a depilatory cream (Carter Product, New York, NY), the mice were placed on a heating pad to maintain body temperature and to minimize temperature-induced changes in blood flow. An imaging gel was placed on the tumor. Ultrasound measurements were carried out with a 15L8s, linear transducer (Acuson Sequoia 512 Siemens).

To localize tumor boundaries and basal blood flow, Power Doppler Ultrasound was performed. Imaging settings were standardized and unchanged throughout the experiment. No major near-field artifacts were encountered. Images were obtained by experienced sonographers who were not informed of the treatment status of the animals throughout the study.

For analysis, a computer based software (MICA) was used to quantify Power Doppler signals. Color pixels representing Power Doppler signals as well as gray pixels representing tumor area were separated from the background in Power Doppler images converted to JPEG format. Blood flow values were calculated as the ratio between gray pixels and color pixels.

All studies involving animal models were approved by the Van Andel Research Institute Institutional Animal Care and Use Committee (ultrasound assessments) or by the Committee of Animal Use and Care at Tel Aviv University (tumor growth assessments).

2.4. Antibodies

The anti-Ki-67 antibody was a rabbit polyclonal antibody (Lab Vision, Fremont, CA, USA) and the anti-CD-31 was a rat monoclonal antibody (mAb) (Abcam, Cambridge, MA, USA). The secondary antibody used for immunohistochemistry was biotin conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.5. Tumor homogenates

Tumors were removed, weight and lysed in Greenburger lysis buffer (300 mmol/L NaCl, 15 mmol/L Tris, 2 mmol/L $MgCl_2$, 2 mmol/L Triton X-100) (Sigma–Aldrich Corp., St Louis, MO, USA), pepstatin A, leupeptin and aprotinine (Roche, Mannheim, Germany, all 20 ng/mL) at pH 7.4, (150 μl buffer/100 mg tissue) as previously described by us [18]. Tumor lysates were obtained by homogenization with a tissue homogenizer (Polytron PT1200 model, PT 1200 CL, Kinematica AG Littau, Switzerland), then incubated for 30 min on ice and centrifuged twice (10 min, 14,000 × g). Homogenates were stored at −20 °C until use.

2.6. Immunohistochemistry staining of paraffin-embedded blocks

Tumors were fixed in 4% paraformaldehyde for 24 h washed with phosphate buffered saline (PBS) and dehydrated in increasing alcohol concentrations and embedded in paraffin. Deparaffinization in xylene and hydration in decreasing alcohol concentrations were performed prior to immunohistochemistry staining as previously described [19]. CD-31 staining was carried out using a Rat on Mouse HRP polymer kit (Biocare medical, Pike Lane, Concord, CA, USA) according to the manufacturer's instructions with slight modifications: Histo/Zime solution (Diagnostic Bio-Systems, Pleasanton, CA, USA) was used for antigen retrieval, and primary antibody against CD-31 (1:100) was used for staining.

Ki-67 staining was processed as described [19] and inactivation of avidin–biotin nonspecific binding was prevented by a blocking kit according to the manufacturer's protocol. The sections were further incubated at 4 °C overnight with the primary antibody against Ki-67 (1:300). The secondary antibody was biotin conjugated goat anti-rabbit IgG. Slides were then stained with ABC peroxidase system, developed with diaminobenzidine (DAB) (Vector Laboratories, Inc., Burlingame, CA, USA) and counter-stained with hematoxylin (Bio-Optica, Milano, Italy).

The slides were examined using an Olympus BX 52 light microscope and images were taken with Olympus DP50 digital camera system. Slides were scanned at low power (10× magnification) to identify areas of proliferation and highest vascularity. Thirty-two high power (20×) fields were then selected randomly within these areas (4 mice/group, 4 fields/mouse, two independent experiments). The slides were photographed and the blood vessels/cells were counted by two blinded observers and the mean ± SEM was calculated.

2.7. ELISA for IL-8 evaluation

Tumor lysates were obtained as described above. IL-8 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (R&D systems, Abingdon, UK human CXCL8/IL-8). The sensitivity of the assay is >8 pg/mL.

2.8. Statistical analysis

Comparison between animal groups treated with CPT + MXF vs animal groups treated with CPT alone was performed by Student's *t* test, analyzing tumor growth, body weight, blood flow and histochemical analysis. Results are considered significant for a *p*-value <0.05.

3. Results

3.1. Effect of combined treatment with MXF and increasing doses of irinotecan on the growth of human xenograft tumors in SCID mice

To determine the effect of the combined treatment of irinotecan and MXF on the in vivo growth of human tumor, SCID mice were inoculated s.c. with HT-29 colorectal carcinoma cells and divided into 7 groups as detailed in Section 2.

The pooled results of two experiments show that HT-29 cells injected s.c. in SCID mice grew quite rapidly, and tumor masses became detectable 7 days after xenotransplantation. Tumors in control animals showed a progressive enlargement in their dimensions and an exponential growth. A mean volume of 1665 ± 142 mm^3 was reached at day 23 when the animals were sacrificed (Fig. 1A and B). Treatment with 20 mg/kg irinotecan inhibited tumor growth as compared to control, starting on day 15 and reaching a significant reduction to 1219 ± 151 mm^3 (26.8 ± 3%) at day 23 ($p < 0.02$) (Fig. 1A and B). MXF by itself induced a decreased to 1149 ± 154 mm^3 (31 ± 2%) in tumor growth (on day 23) ($p < 0.025$) while treatment with a combination of irinotecan + MXF induced a significant additional regression of tumor volume up to 779 ± 112 mm^3 (53 ± 4%) decrease compared to control ($p < 0.05$) (Fig. 1A and B). A similar significant decrease in tumor volume, was observed upon treatment of mice with a combination of 35 mg/kg irinotecan + MXF to 754 ± 54 mm^3 (55 ± 3% decrease, $p < 0.005$) compared to a decrease to 1192 ± 131 mm^3 (28 ± 2%) in mice treated with 35 mg/kg irinotecan alone ($p < 0.025$), (Fig. 1A and B). It should be noted that treatment of mice with a combination of low dose irinotecan (20 mg/kg) and MXF induced a higher inhibition in tumor growth than a 2.5-fold higher concentration of irinotecan (50 mg/kg) given alone ($p < 0.018$) (Fig. 1B). As previously described by other

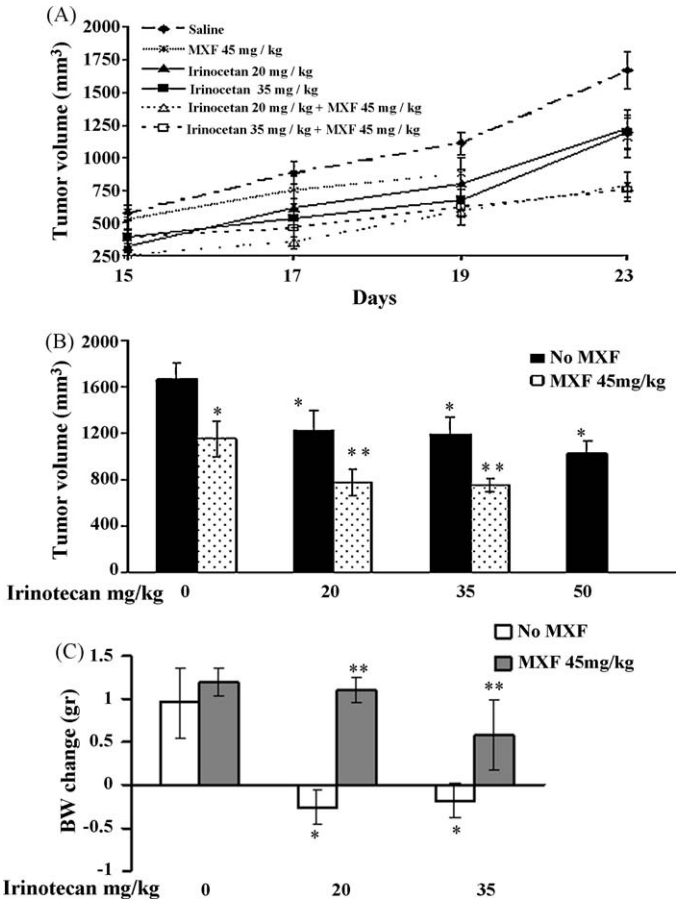

Fig. 1. The effect of combined treatment with MXF and increasing doses of irinotecan on growth of human xenograft tumors in SCID mice and on body weight change. SCID mice with human tumor xenografts of human HT-29 cells were divided into 7 groups. Group 1: control mice were injected with saline. Group 2: mice received 3 times/day i.p. injection of 15 mg/kg MXF. Groups 3–5: mice received on days 7, 14 and 21 after HT-29 cell implantation an i.p. injection of 20, 35 or 50 mg/kg irinotecan, respectively. Groups 6 and 7: mice received a daily injection of MXF as in group 2 and on days 7, 14 and 21 an injection of 20 or 35 mg/kg irinotecan, respectively. Tumor size was measured by caliper 3 times a week and the mean tumor volume of (A) mice treated with saline, MXF, irinotecan (20 and 35 mg/kg) given alone or in combination was plotted as a function of time after tumor cell implantation. (B) Tumor volume measured on day 23 in mice treated with increasing doses of irinotecan, up to 50 mg/kg. (C) Body weight (BW) changes were calculated on day 23 in mice treated with the indicated doses of irinotecan alone, MXF alone or the combination of irinotecan and MXF. Data are expressed as mean ± SE. *Significant difference compared to control; **significant difference between irinotecan + MXF vs irinotecan (n = 10 mice/group).

investigators [20,21] we observed severe diarrhea and corresponding loss of body weight in all the mice treated with irinotecan. In contrast we observed a 4.1 ± 0.06% and 5.7 ± 0.03% increase in body weight on day 23 in control and MXF treated mice, respectively (Fig. 1C). The addition of MXF to irinotecan treatment significantly prevented the reduction in body weight induced by irinotecan administered alone (Fig. 1C).

3.2. MXF enhances the inhibition of cellular proliferation in human colon xenografts induced by irinotecan

Ki-67 is a nuclear protein, associated with somatic cell proliferation and is used as an immunohistological marker for the assessment of cancer cell proliferation. Reduction in Ki-67 expression correlates with responsiveness to chemotherapy [22,23]. The number of Ki-67 positive cells/0.8 mm^2 was significantly higher in sections of untreated tumors (32 ± 2), (Fig. 2A) than in sections of animals treated with 20 mg/kg irinotecan (Fig. 2C)

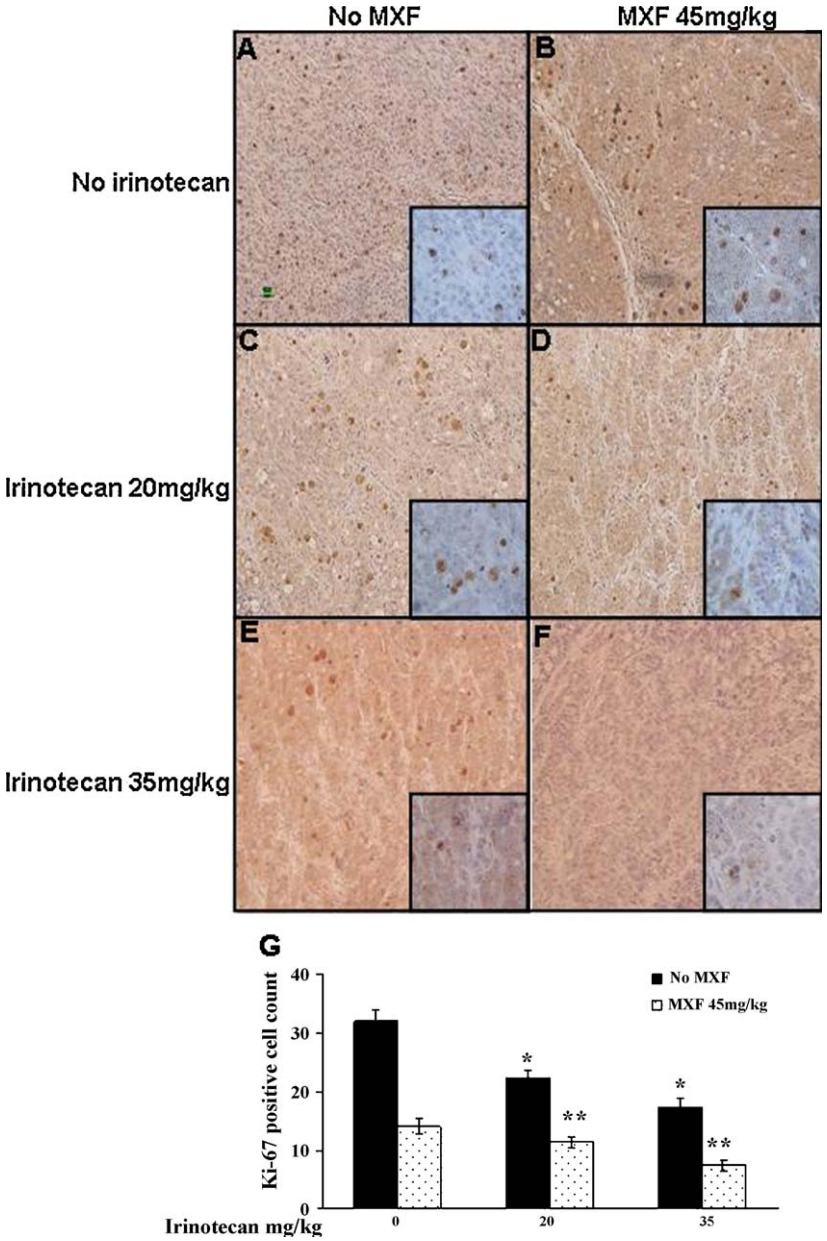

Fig. 2. The effect of irinotecan and MXF on expression of nuclear Ki-67 protein. Sections from tumors of (A) saline, (B) MXF, (C) 20 mg/kg irinotecan, (D) 20 mg/kg irinotecan + MXF, (E) 35 mg/kg irinotecan or (F) 35 mg/kg irinotecan + MXF treated mice, were stained with an antibody against Ki-67 then with DAB, and were counterstained with hematoxylin. The magnifications in the insert are 100× and 400×. The slides were examined using an Olympus BX 52 light microscope and images were taken with Olympus DP50 digital camera system. (G) The slides were photographed and the quantification of the number of positive nuclear Ki-67 was performed. Four different fields in each experimental group (4 mice/group, two independent experiments) were analyzed and the mean ± SEM were calculated. *Significant difference compared to control; **significant difference between irinotecan + MXF vs irinotecan (n = 8 mice/group).

or 35 mg/kg irinotecan (Fig. 2E) (22.3 ± 1.4 and 17.2 ± 1.7, respectively). A significant decrease of positive cells was observed upon treatment with MXF alone (14.1 ± 1.2) (Fig. 2B and G) or in animals treated with the combination of MXF and 20 mg/kg irinotecan (Fig. 2D and G) or 35 mg/kg irinotecan (Fig. 2F and G) (11.4 ± 0.9, 7.4 ± 0.9, respectively) ($p < 0.045$). The results indicate that MXF significantly enhanced the inhibitory effect of irinotecan on cellular proliferation in the HT-29 tumor xenografts.

3.3. MXF treatment reduces blood flow in xenograft tumors as measured by Power Doppler Ultrasound imaging

The effect of MXF on tumor blood flow was assessed in one set of experiments (6 mice/group) using Power Doppler Ultrasound imaging as previously described [16,17]. Power Doppler analysis performed on day 23 post implantation of HT-29 cells showed that the color area of the tumor (i.e. blood flow) decreased dramatically to 15% (1.7/11.2) ± 6% of that in the control group in mice treated with MXF alone ($p < 0.004$) (Fig. 3A and B). Irinotecan treated mice showed no change (20 mg/kg irinotecan) or a slight significant increase in blood flow (35 mg/kg irinotecan) (Fig. 3B). In the combined treatment groups (20 or 35 mg/kg irinotecan + MXF), the color area decreased to 19% (2.1/11.1) ± 2% and 32% (5.2/16.2) ± 7%, respectively, of the mean of the single irinotecan treatment groups ($p < 0.001$) (Fig. 3A and B). To evaluate the magnitude of the MXF effect, we used a computer based software (MICA) to quantify Power Doppler signals. The studies were performed on groups of 5–6 mice/group. Our results showed the marked significant inhibitory effect of MXF on tumor blood volume (Fig. 3A and B).

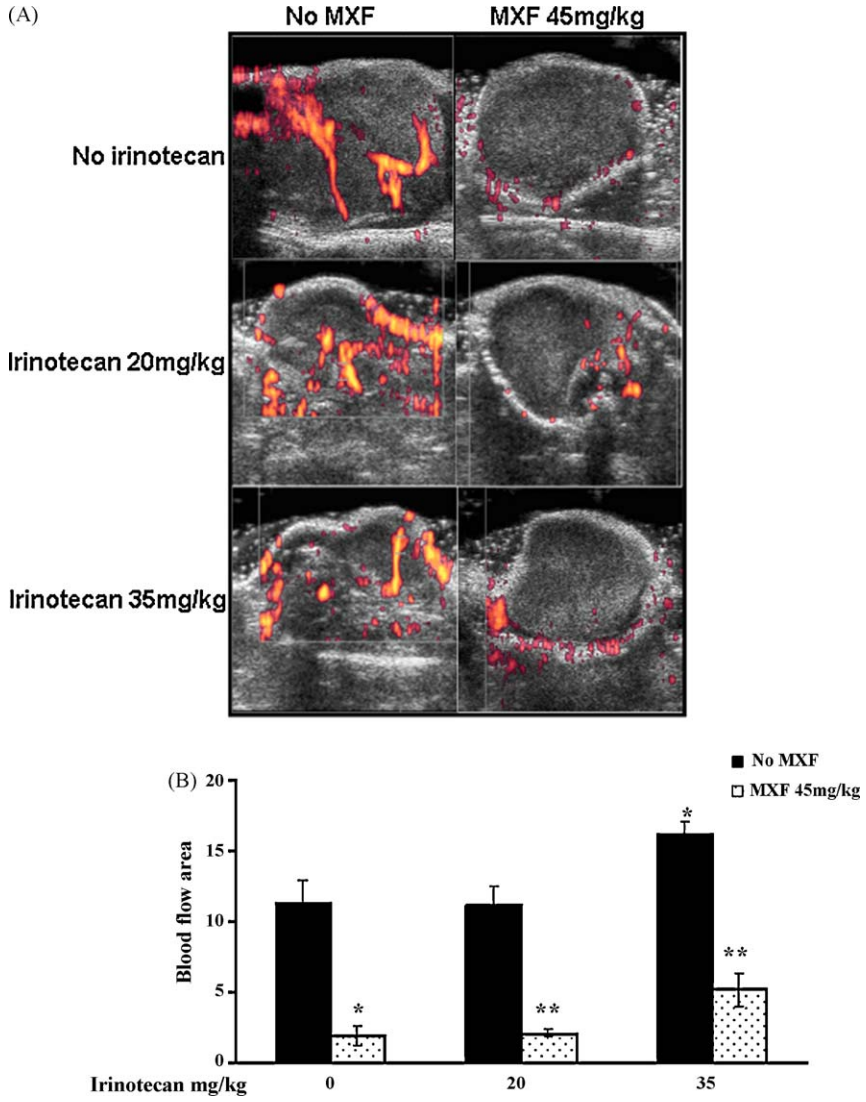

Fig. 3. MXF induces decrease in the blood flow in xenograft tumors, as measured by Power Doppler Ultrasound imaging. (A) Comparison of Doppler Ultrasound images taken from individual tumors of mice bearing a xenograft tumor that were injected with saline, MXF, or with 20 or 35 mg/kg irinotecan alone or in combination with MXF. Blood flow is depicted as red spots. (B) Quantification of Doppler Ultrasound analysis showed a decrease in blood flow following MXF treatment. *Significant difference compared to control; **significant difference between irinotecan + MXF compared to irinotecan treated mice (6 mice/group).

3.4. MXF treatment reduces the expression of platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD-31)

Platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD-31), a member of the Ig superfamily, is expressed strongly at endothelial cell-cell junctions, on platelets, and on most leukocytes. CD-31 has been postulated to play a role in vasculogenesis and angiogenesis [24]. Staining of sections with rat anti-mouse mAb detects vascular endothelial cells in tumors. Capillary density was slightly higher in sections of untreated tumors (Fig. 4A) than in sections treated with 20 mg/kg irinotecan (Fig. 4C) or 35 mg/kg irinotecan (Fig. 4E). A significant decrease in capillary density was observed upon treatment with MXF alone (Fig. 4B) or with the combination of irinotecan (20 or 35 mg/kg) and MXF (Fig. 4D and F) ($p < 0.05$).

3.5. MXF treatment reduces the enhanced IL-8 levels in tumor homogenates, induced by irinotecan

The pooled results of two experiments depicted in Fig. 5 show a marked and significant increase (1.8-fold) in the levels of the pro-angiogenic chemokine IL-8 in tumors of mice treated with 20 mg/kg irinotecan compared to control ($p < 0.05$). Treatment of mice with a combination of irinotecan and MXF prevented the enhanced IL-8 levels in tumors ($p < 0.05$).

4. Discussion

In the present in vivo study we demonstrated significant anti-proliferative and anti-angiogenic effects of MXF in a human colon carcinoma xenograft. In addition, MXF significantly enhanced the anti-proliferative effects of the chemotherapeutic agent irinotecan as demonstrated by tumor growth measurements and immunohistochemistry analysis of Ki-67 nuclear protein. An additional clinical effect was noted whereby MXF alone or in combination with irinotecan abolished or ameliorated the significant weight loss of animals treated with irinotecan alone. At the end of the treatment and assessment protocol, combination of MXF and irinotecan led to twice the reduction in tumor size, to a 68–81% decrease in tumor blood flow and to weight gain rather than weight loss compared to irinotecan single therapy. Moreover, MXF mono-therapy showed a significant effect on tumor growth and a

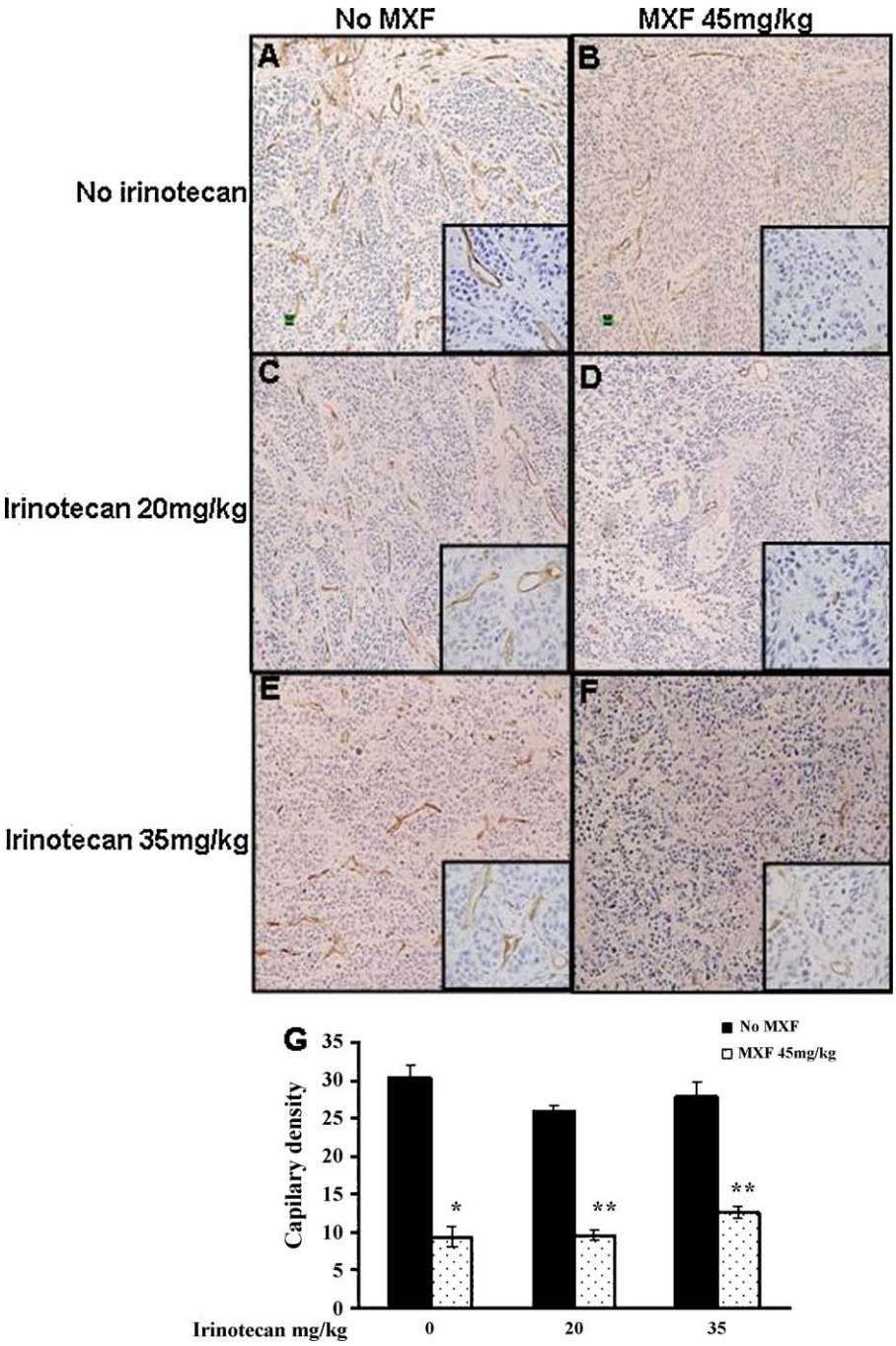

Fig. 4. The effect of irinotecan and MXF on expression of angiogenic markers in HT-29 tumors. Representative images of immunohistochemistry stained sections of mice treated with: (A) saline, (B) MXF, (C) 20 mg/kg irinotecan, (D) 20 mg/kg irinotecan + MXF, (E) 35 mg/kg irinotecan or (F) 35 mg/kg irinotecan + MXF treated mice. Sections from tumors were stained with an antibody against PECAM-1/CD-31 then with DAB and counterstained with hematoxylin, as indicated in Section 2. The magnifications in the insert are 100× and 400×. Quantification of the capillary density was performed as described in legend to Fig. 2. *Significant difference compared to control; **significant difference between irinotecan + MXF compared to irinotecan treated mice (n = 8 mice/group).

dramatic effect (85% reduction) on tumor blood flow as measured by Power Doppler.

Previous studies on the anti-proliferative effects of fluoroquinolones in cancer have concentrated mainly on their in vitro effects in several cancer cells lines. The fluoroquinolones ciprofloxacin, moxifloxacin and trovafloxacin were found to inhibit in vitro the growth of bladder transitional cell carcinoma, colon cancer, prostate cancer and the tumor derived cells lines THP-1 and Jurkat cells [5–9].

The effect was noted at drug concentrations achievable in relevant organs (i.e. bladder, prostate and colon) following usual dosage regimens of the drugs. A single in vivo study indicated an anti-tumor effect of trovafloxacin in neutropenic mice with lung infection injected with murine leukemia cells. The drug was effective in preventing metastasis of leukemic cells to various organs and in prolonging animal survival [8].

The underlying mechanism of activity of the fluoroquinolones on tumor cells was mainly attributed to the anti-topoisomerase II activity of the drugs. As antimicrobial agents these compounds inhibit the prokaryotic DNA-gyrase. However, it was found that the above agents also inhibit the eukaryotic analogue of DNA-gyrase—topoisomerase II [10,11]. Such inhibition was found by us in a

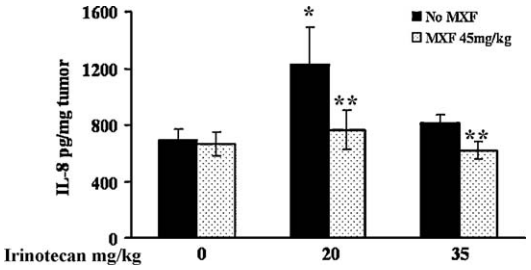

Fig. 5. Human IL-8 concentrations in tumor homogenates. Results expressed as mean ± SEM, $n = 9$ mice/group. *Significant difference compared to control; **significant difference between irinotecan + MXF compared to irinotecan treated mice.

previous study in isolated topoisomerase II as well as in tumor cells including HT-29 [7].

Several groups have synthesized quinolone analogues with more potent activity against topoisomerase II such as tricyclic quinolones [25], piperazinyl quinolones [26], and more complex compounds [27,28]. These agents have demonstrated anti-tumor activity in cell lines and few were shown to have anti-proliferative effects in animal models [10]. Nonetheless, these are experimental compounds in contrast to the widely used fluoroquinolones ciprofloxacin and MXF with their favorable safety profile.

Our current in vivo findings confirm a range of in vitro observations on the anti-tumor activity of MXF alone and in combination with the anti-topoisomerase I chemotherapeutic agent CPT. These in vitro studies demonstrated enhanced inhibition of purified topoisomerase I by the drug combination as well as increased cytotoxic effect and apoptosis in HT-29 cells [12]. Our current in vivo findings confirm the greater cytotoxic effect of the combination of MXF/irinotecan as demonstrated by the significant decrease in tumor dimension in mice treated by the two drugs compared to treatment with each drug alone. In addition, the Ki-67 nuclear protein staining further demonstrated a significant decreased in the number of positive cells in animals receiving the combined treatment compared to each drug alone, indicating that MXF effectively increases inhibition of cellular proliferation in the tumors, induced by irinotecan.

It should be noted that the effect of MXF in combination with a lower dose of irinotecan (20 mg/kg) was comparable and even slightly better than the effect of 50 mg/kg irinotecan given alone. This demonstrates a potential "cytotoxic drug sparing effect" conferred by MXF. This is desirable in order to reduce the toxic side effects of irinotecan. The clinical correlate of such "sparing effect" was in fact demonstrated in our study. Animals treated with irinotecan alone suffered from diarrhea and showed significant weight loss during the study period. In contrast, animals treated with MXF alone or with irinotecan in combination with MXF showed either no diarrhea and weight gain similar to controls or significant amelioration of signs and smaller weight gain than controls without any weight loss.

The most important finding in our study is the pronounced anti-angiogenic effect of MXF administered alone or in combination with irinotecan in the human colon cancer xenograft. We have elected to utilize the method of Power Doppler to elucidate and quantify the effect of the drug on tumor blood flow and monitor precisely the anti-angiogenic effects. Gee et al. [17] and Donnelly et al. [29] utilized this modality successfully to quantify tumor blood flow in murine xenograft models of melanoma, renal cell carcinoma and glioblastoma. The main advantage of this method was the ability to quantify precisely tumor blood flow, blood vessels distribution and evolution of blood flow and vessels during tumor growth phases. In addition, they showed strong correlation between the quantitative Power Doppler findings and complementary methods including measurements of tumor dimensions, confocal microscopy of vessels labeled in vivo by fluorochrome-conjugated lectin and immunohistological examinations. We have complemented our Power Doppler analysis by immunohistochemistry utilizing anti-CD-31 antibodies to detect vascular endothelial cells in tumor sections and found a strong correlation between these two distinct methods. Our findings showed a dramatic 85% reduction in blood flow in the tumor xenograft in animals treated with MXF alone compared to controls. Irinotecan treatment alone did not lead to reduced blood flow; however, the addition of MXF significantly reduced tumor blood flow (68–81% reduction). Such an effect was predictable to an extent based on our previous in vitro studies where we showed that MXF significantly reduced the secretion of the pro-angiogenic chemokine IL-8 as well as VEGF from HT-29 and other tumor cell lines [12]. It should be noted that in our in vitro study, irinotecan alone enhanced the secretion of IL-8 from HT-29 cells and MXF significantly decreased this enhancement. Similarly, in our present in vivo study, treatment of the tumor-bearing mice with 20 mk/kg irinotecan was associated with a significant enhancement of IL-8 secretion from the tumor, and addition of 45 mg/kg MXF significantly decreased this secretion (Fig. 5). This anti-angiogenic effect can potentially be of great clinical significance as a complementary anti-tumor effect during treatment with drug combination protocols. To the best of our knowledge this is the first report on such anti-angiogenic effects of MXF or other fluoroquinolones in an in vivo tumor model.

Several investigators have looked at the anti-angiogenic effects of irinotecan alone or in combination with other drugs. Bocci et al. [30] studied the effect of high dose irinotecan (100 mg/kg = maximal tolerated dose—MTD) administered every 7 days in comparison to metronomic daily treatment of irinotecan (total dose of 28% of MTD), or loading dose of 100 mg/kg followed by metronomic treatment in mice with HT-29 xenografts. Analysis of CD-31 showed a non-significant reduction in all the three groups while tumor growth was reduced significantly with all regimens. It should be noted that in our study we used a much lower dosage of irinotecan administered every 7 days (20 and 35 mg/kg) and have likewise not seen an effect of the drug alone on CD-31. The low dose may also explain the lack of effect of irinotecan on blood flow as measured quantitatively by Power Doppler Ultrasound.

An interesting study by Wildiers et al. [31] looked at the tumor uptake of irinotecan following a single 100 mg/kg i.p. dose in animals with HT-29 xenograft, pre-treated with anti-VEGF mAb. Despite significant reduction in tumor vascular density following anti-VEGF mAb, there was no reduction and even slight increase in irinotecan-uptake by the tumor. Thus, reduction in blood flow conferred by anti-angiogenic agents does not impair the availability and tumor concentration of the chemotherapeutic agent irinotecan.

Combinations of irinotecan with anti-angiogenic agents have been studied and utilized in advanced colorectal cancer and other types of malignancies [32–35]. Currently, the most common combination includes bevacizumab (Avastin®) and irinotecan-based combination regimens for the treatment of metastatic colorectal cancer. These regimens showed statistically significant improved outcomes compared to non-bevacizumab containing regimens [32,33,36]. Favorable results also exist for bevacizumab–irinotecan combination in the treatment of malignant gliomas [35].

Our present study suggests that the combination of MXF and irinotecan may offer similar advantages based on the pronounced anti-angiogenic effect of the MXF-containing treatment and the significant cytotoxic, tumor reducing effect of the combination. The long term clinical experience with MXF and its favorable safety profile, along with its pronounced effect on reducing clinical toxic effects (diarrhea and weight loss) of irinotecan should lead to further investigation of its clinical utility as an adjunct treatment for colon cancer and other malignancies.

Acknowledgments

This work constitutes a section of Debby Reuveni's PhD thesis at the Tel Aviv University, Israel.

We thank Dr. Gideon Halperin for assistance in statistical analyses, Mrs. Hasida Orenstein for assistance in the preparation of tumor sections for immunohistochemistry and Mrs. Dafna Shryber for Power Doppler Ultrasound assessments.

This study was supported in part by Tel Aviv University grants.

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