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A novel drug interaction between the quinolone antibiotic ciprofloxacin and a chiral metabolite of pentoxifylline☆

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ABSTRACT

Pentoxifylline (PTX), a methylxanthine derivative, is metabolized to seven compounds in vivo, with metabolites 1 and 5 possessing biologic activity. Metabolite-1 is a chiral molecule and its S-enantiomer is selectively formed during PTX metabolism in vivo. We have developed a reproducible method of synthesizing a racemic mixture of the chiral metabolite-1 (M-1) of PTX. In this study, we examined the kinetics of racemic M-1 in mice compared to PTX. An interaction between PTX and the quinolone antibiotic ciprofloxacin has been demonstrated. A goal of this study was to determine if a similar interaction occurs between ciprofloxacin and M-1 in vivo. M-1 and PTX had similar absorption and elimination rates. M-1 was rapidly converted to PTX, while very little PTX was converted to M-1 in vivo. The peak concentration of biologically active drug (PTX + M-1) was 36% higher when M-1 was administered compared to PTX. Combination of ciprofloxacin and PTX significantly increased serum concentrations of both PTX and M-1 (2-fold) compared to controls. The combination of M-1 and ciprofloxacin significantly increased serum concentration of M-1 (3-fold) and PTX (2-fold). The ciprofloxacin/M-1 combination produced a significantly higher sera concentration of bioactive drug compared to all other groups suggesting that this combination may enhance the anti-fibrogenic effect.

1. Introduction

Pentoxifylline (PTX; 1-(5-oxyohexyl)-3,7-dimethylxanthine) is a methylxanthine derivative and non-specific phosphodiesterase (PDE) inhibitor [1,2]. It is widely prescribed for the treatment of certain vascular disorders such as intermittent claudication due to its ability to improve blood flow through narrow vessels [2,3]. In addition to its effects on the microcirculation, PTX is known to have diverse anti-inflammatory actions, including in vitro and in vivo inhibition of T_H1 cytokine synthesis (e.g. TNF-α, IL-12, IL-2 and IFN-γ) by various immune cells [4–11]. In addition to its effects on the immune system, results from our lab. and others have shown that PTX is a potent anti-fibrogenic compound. PTX inhibits proliferation and collagen synthesis by cultured fibroblasts, myofibroblasts, hepatic stellate cells, renal myofibroblasts, intestinal smooth muscle cells and vascular smooth muscle cells in the presence of various fibrogenic stimuli [12–18]. PTX also attenuated fibrosis in animal models of glomerulonephritis [19], Peyronie's disease [20] and hepatic fibrosis [13,21–23]. The combined anti-inflammatory and anti-fibrotic effects of this drug, along with its safety and affordability, make PTX an attractive candidate for treatment of chronic inflammatory and fibrotic diseases.

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PTX is metabolized to at least seven molecules in mammals with metabolites 1 and 5 being the major products formed [24]. Metabolite-1 (M-1; 1-(5-hydroxyhexyl)-3,7-dimethylxanthine) is formed from PTX in vivo by the reduction of a single carboxyl group to a corresponding hydroxyl group, forming a chiral center in the molecule (Fig. 1). The S-enantiomer of M-1 (M-1S) is selectively formed during PTX metabolism in vivo and is thought to have biological properties similar to PTX although few studies have examined this [1,25,26]. We have been investigating the in vitro effects of racemic M-1 (R/S) and have shown that it is as effective as PTX in inhibition of PDGF-stimulated human skin fibroblast proliferation [27] and is more potent than PTX in inhibiting collagen synthesis by PDGF-stimulated porcine hepatic myofibroblasts [13,28]. These results indicate that M-1 has potential as an anti-fibrogenic agent.

A pharmacologic interaction between PTX and the antibiotic ciprofloxacin in humans was reported to produce elevated serum levels of both PTX and M-1 in vivo with increased formation of the transient M-1R enantiomer [29]. We have shown that this interaction can be reproduced in mice [30], and that elevated serum drug levels are not due to down-regulation of the CYP1A2 gene, but rather to inhibition of the catalytic activity of CYP1A2 and an induction of CYP2E1, liver enzymes involved in PTX metabolism [31]. Since both PTX and M-1 are biologically active compounds, elevated levels of these drugs in mice in vivo would likely improve the therapeutic effects of PTX administration. A direct interaction between M-1 and ciprofloxacin has not been examined. The goals of the present study were to compare the kinetics of racemic M-1 and PTX in mice, since racemic M-1 had not previously been administered in vivo, and to determine if a pharmacologic interaction between ciprofloxacin and the novel M-1 occurs in mice.

2. Methods

2.1. Animals

Male CD1 mice weighing approximately 25 g were obtained from Charles River Laboratories and maintained in the Carlton

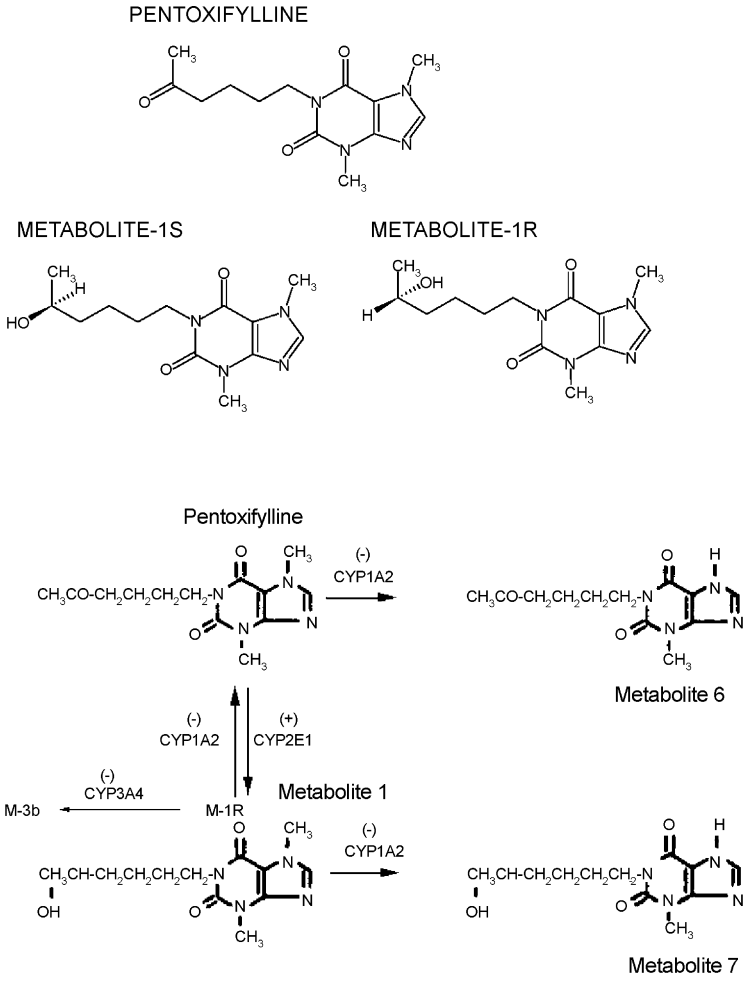

Fig. 1 – Structures of pentoxifylline and its major metabolite (M-1). M-1 is formed from the reduction of a carboxyl group (C═O) on the PTX molecule (arrow) to a corresponding hydroxyl group (–OH), forming a chiral center in the metabolite. The different spatial orientations of the hydroxyl group around the chiral carbon atom of M-1 give rise to the two enantiomers of the molecule (M-1S and M-1R). The S-enantiomer is the major metabolite formed in vivo during PTX metabolism. Also shown are the metabolic pathways of pentoxifylline that we propose are affected by ciprofloxacin. The effect is shown as (+) or (−) above the CYP enzyme.

Animal Care Facility at Dalhousie University. Animals were housed six per cage with a 12 h light:12 h dark cycle and ad libitum access to water and standard chow. Animals were given 1 week to acclimatize to the facility before experimentation began. The experimental protocol was approved by the University Committee on the use of Laboratory Animals at Dalhousie University.

2.2. Chemical synthesis of metabolite-1 from pentoxifylline

Metabolite-1 is a chiral molecule derived from PTX by the reduction of a single ketone functional group to a hydroxyl group (Fig. 1). A racemic mixture of metabolite-1 was synthesized from PTX (Sigma; Oakville, ON, Canada) in a chemical reduction reaction carried out in the Department of Chemistry at Dalhousie University with the assistance of Dr. Jim Pincock. PTX (10 g) was dissolved in HPLC-grade methanol (500 ml) and sodium borohydride ($NaBH_4$; 1.8 g) was slowly added as the reducing agent. The reaction proceeded for 1 h at room temperature with constant stirring and the methanol was evaporated using a rotary evaporator. The crude product was dissolved in 150 ml of distilled water (dH_2O) and was extracted four times using dichloromethane (4 ml × 100 ml CH_2Cl_2). A powdered drying agent (Na_2SO_4) was added to the organic phase to absorb water and the solution was gravity filtered. The remaining organic solvent (CH_2Cl_2) was evaporated leaving a powdered compound (M-1) behind. This product was recrystallized from 2-propanol overnight at 4 °C, dried by suction filtration the following day, and was allowed to air dry for several days before use. A yield greater than 80% was consistently achieved with this method. The product was identified as M-1 by proton nuclear magnetic resonance spectroscopy (^1H NMR). The synthesis method employed was a non-selective chemical reduction reaction producing a racemic mixture (1:1) of M-1 enantiomers [32].

2.3. Kinetics of M-1 and PTX in vivo

Mice were injected with heparin (1000 units/kg i.p.; Leo Pharma Inc., Thornhill, ON, Canada) to prevent blood from clotting during the procedure. After 40 min, animals received an injection of either PTX or M-1 (100 mg/kg i.p.). Blood samples (~50 μl) were collected at 5, 10, 15, 25, 40, 60 and 120 min post-injection from a small puncture at the tip of the tail. Blood was centrifuged and serum was collected for HPLC analysis.

2.4. Interaction between M-1 and ciprofloxacin in vivo

Combination of ciprofloxacin and PTX in vivo is reported to elevate serum levels of PTX in humans with increased formation of the rare R-enantiomer of M-1, which is not generally produced during PTX metabolism [29]. A direct interaction between ciprofloxacin and racemic M-1 has never been investigated. In order to assess the interaction of ciprofloxacin with PTX and the novel M-1 in mice, animals were first divided into two groups of 15. One group received daily injections of the antibiotic ciprofloxacin (25 mg/kg i.p.; Bayer) for 9 days while the control group received daily injections of sterile 0.9% saline solution. On the ninth day, five animals from each group received an injection of PTX (100 mg/kg i.p.), five animals received an injection of M-1 (100 mg/kg i.p.), and the remaining animals received an equivalent volume of saline (~200 μl). Table 1 gives a summary of the treatment groups. Animals were sacrificed at 30 min post-injection and blood was collected by cardiac puncture for serum analysis by HPLC. This timepoint was chosen based on the kinetics data (Fig. 2) since it is a point beyond the peak absorption of both PTX and M-1 by the i.p. route.

Table 1 – Treatment groups in ciprofloxacin experiment

DAY 1–9	DAY 9	N (mice)
Saline (i.p.)	Saline (i.p.)	5
Saline (i.p.)	PTX (100 mg/kg i.p.)	5
Saline (i.p.)	M-1 (100 mg/kg i.p.)	5
Cipro® (25 mg/kg i.p.)	Saline (i.p.)	5
Cipro® (25 mg/kg i.p.)	PTX (100 mg/kg i.p.)	5
Cipro® (25 mg/kg i.p.)	M-1 (100 mg/kg i.p.)	5

2.5. High performance liquid chromatography (HPLC)

Serum drug levels were measured using reverse-phase HPLC [33,34] by comparing samples of unknown drug concentration to standard curves prepared using a range of known concentrations of PTX and M-1 (final concentration 1–15 μg/ml) referenced to phenacetin, the internal standard. Phenacetin was used because it has a retention time similar to PTX and can be visualized on the same HPLC chromatogram. Samples and standards were prepared for HPLC analysis by extraction through 1 ml C-18 Solid Phase Extraction cartridges (JT Baker) into glass test tubes and were dried by nitrogen evaporation. Dried samples could be stored for extended

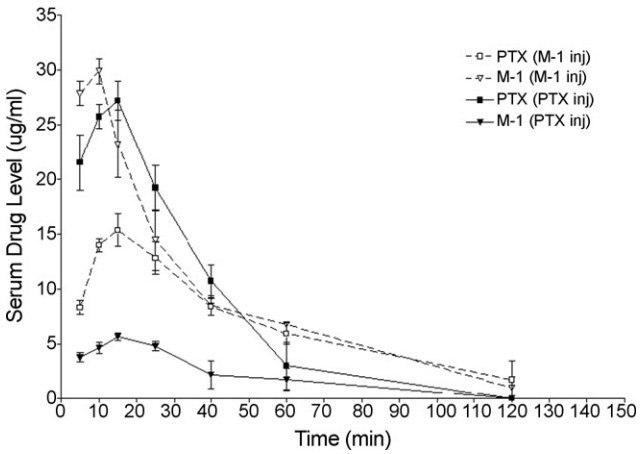

Fig. 2 – Kinetics of M-1 and PTX in vivo. Animals were injected with M-1 (dashed lines) or PTX (solid lines) (100 mg/kg i.p.) and serum samples were analyzed by HPLC at 5, 10, 15, 25, 40, 60 and 120 min. Interconversion of PTX and M-1 occurs in vivo thus both drugs are detected by HPLC analysis following injection of either drug alone. Rate of elimination between the 20 and 40 min timepoints was approximately 0.75 μg/ml/min for both PTX and M-1. Each point is mean drug concentration (μg/ml) ± S.E.M. for four separate animals.

periods at 4 °C until HPLC analysis. Dried samples were dissolved in HPLC mobile phase (100 μl; 75% mH_2O: 25% acetonitrile) and 10 μl was injected into the HPLC injection port using a glass syringe. The HPLC instrument used was Beckman System Gold consisting of an Analogue Interface Module 406 coupled to a UV detector Module 166 (set at 273 nm) and Programmable Solvent Module 126. The stationary phase used was a C-18 column (250 mm length, i.d. 4.6 mm; Econosource) and the flow rate was 1 ml/min with a run-time of 15 min. HPLC printouts were obtained consisting of a chromatograph and relevant data for each sample, including retention times, peak height and peak area. The ratio of the area under the drug peak to the area under the phenacetin peak (peak $area_{drug}$/peak $area_{phen}$) was used to construct a standard curve, with drug concentration (μg/ml) on the *x*-axis and peak area ratio (peak $area_{drug}$/peak $area_{phen}$) on the *y*-axis. Serum drug levels from treated animals were determined by calculating peak $area_{drug}$/peak $area_{phen}$ for each sample and comparing to the standard curves ($x = y - b/m$).

3. Results

3.1. Kinetics of M-1 and PTX

Kinetics of M-1 and PTX in vivo are shown in Fig. 2. HPLC analysis of serum drug levels following M-1 (dashed lines) and PTX (solid lines) injection indicated that both drugs were detectable in serum following injection of either drug alone. This was expected since the conversion between PTX and M-1 is a reversible reaction that takes place in the blood. M-1 had reached a peak by 10 min post-injection while PTX peaked at 15 min. The rate of elimination for both M-1 (following M-1 injection) and PTX (following PTX injection) was 0.75 μg/ml/min between the 20 and 40 min timepoints. Serum half-lives of PTX and M-1 were 15 and 20 min, respectively. Levels of PTX and M-1 in serum following M-1 injection reached near equilibrium by 25 min, whereas only a small amount of PTX was converted to M-1 following PTX injection (Fig. 2). This resulted in 36% higher peak combined drug levels (M-1 + PTX) following M-1 injection compared to PTX (Fig. 3). Since both drugs are active in vivo, the combined levels of PTX and the M-1 will determine the overall biological effects of drug administration. While no drugs were detected in serum 120-minutes after PTX injection, detectable amounts of both M-1 and PTX remained following injection of the metabolite.

3.2. Interaction between M-1 and ciprofloxacin in vivo

To investigate the possibility of an interaction between ciprofloxacin and M-1 in vivo, mice were pre-treated with ciprofloxacin or saline for 9 days before receiving an injection of M-1 or PTX (100 mg/kg i.p.). Ciprofloxacin pre-treatment increased serum drug levels following injection of either PTX or M-1 (Fig. 4). Serum M-1 levels were increased 3-fold ($p < 0.05$; Fig. 5) compared to serum M-1 levels in mice not pre-treated with ciprofloxacin. A statistically significant 2-fold increase in serum drug levels was achieved in all other groups due to ciprofloxacin pre-treatment ($p < 0.05$). Since the therapeutic effects of PTX or M-1 depend on the total amount of both active compounds in the body, results were graphed as total drug (PTX + M-1) in the serum at 30 min post-injection (Fig. 6). Total drug levels following M-1 injection alone were significantly higher than following PTX injection alone (23.6 ± 1.7 μg/ml versus 15 ± 1.0 μg/ml; $p < 0.05$). Ciprofloxacin pre-treatment significantly elevated total drug levels following PTX or M-1 injection ($p < 0.05$). The combination of ciprofloxacin and M-1 produced a significantly higher total drug level (57 ± 3.3 μg/ml) compared to PTX with ciprofloxacin pre-treatment (28 ± 4.6; $p < 0.05$).

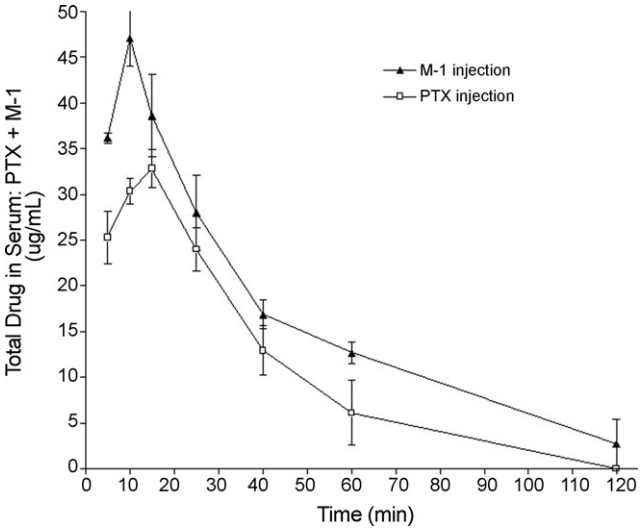

Fig. 3 – Kinetics of M-1 and PTX in vivo graphed as total drug (PTX + M-1) in serum. Combined levels of PTX and M-1 give an estimate of relative bioactivity in vivo. Each point is mean drug concentration (μg/ml) ± S.E.M. for four separate animals.

4. Discussion

We have previously demonstrated that racemic M-1 inhibits in vitro proliferation and collagen synthesis in fibroblasts stimulated with PDGF, implicating M-1 as a potential anti-fibrogenic compound [13,27]; however, its effects in vivo were previously unexplored. Injection of M-1 (32 and 100 mg/kg i.p.) into 25 g mice revealed no signs of toxicity. Signs of toxicity would include dizziness (rotating head movements), lethargy and hypersalivation (excessive licking movements). No signs of toxicity were observed in CD1 mice with these doses of drug.

Examination of M-1 kinetics compared to PTX revealed that both drugs reached peak serum levels 10–15 min after i.p. injection with similar α-elimination rates of 0.75 μg/ml/min. The serum half-lives of M-1 and PTX were 15 and 20 min, respectively. Differences in the metabolic profiles of the two compounds were observed by analyzing the conversion of M-1 to PTX in vivo and vice versa. Following injection of PTX, very little M-1 was detected in the serum. Following M-1 injection, the levels of M-1 and PTX in serum reached equilibrium by 40 min, indicating that PTX is a major metabolite formed from

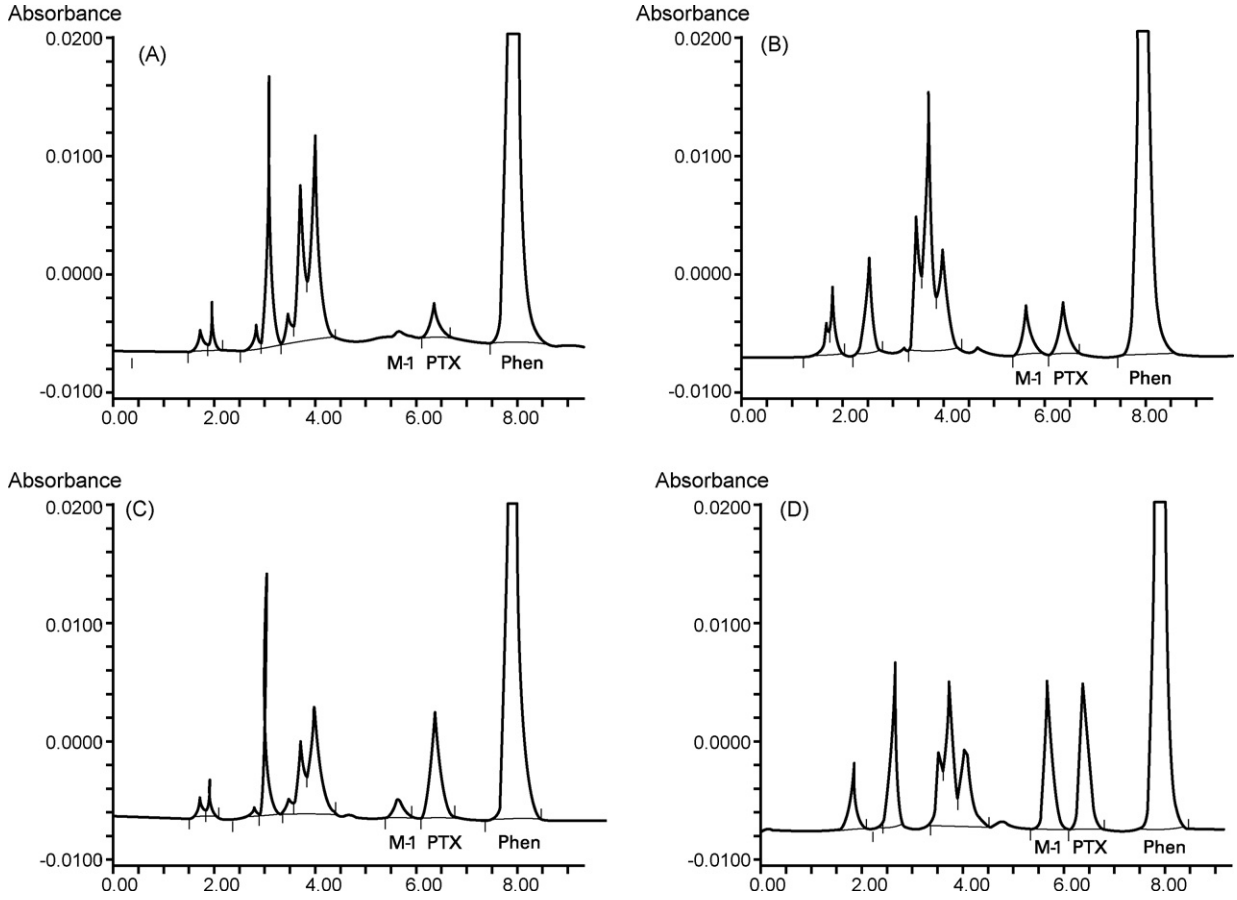

Fig. 4 – Representative HPLC chromatographs demonstrating the effect of ciprofloxacin pre-treatment on serum levels of PTX and M-1. This figure shows the visual difference in HPLC peak area for each drug between treatment groups. Animals were treated with ciprofloxacin (25 mg/kg) or saline for 9 days before receiving an injection of PTX or M-1 (100 mg/kg i.p.). Serum samples were collected 30 min after PTX or M-1 injection. (A) PTX injection (saline pre-treatment). (B) M-1 injection (saline pre-treatment). (C) PTX injection (ciprofloxacin pre-treatment). (D) M-1 injection (ciprofloxacin pre-treatment). Phenacetin (30 μg/ml) was used as the internal standard for all samples so that serum drug levels could be calculated from a standard curve.

racemic M-1 in vivo. A recent study demonstrated that reversible interconversion of PTX and M-1 takes place rapidly in human erythrocytes (CYP450-independent mechanism) with the conversion of M-1 to PTX being favored 4-fold over the conversion of PTX to M-1 [35], consistent with our findings in mice. The rate of conversion of M-1S to PTX was 4-fold higher than conversion of M-1R in this study, and the authors indicate that liver enzymes likely participate in conversion of M-1R to PTX in vivo. In support of this, conversion of M-1R back to PTX has been reported in human liver microsomes and is thought to occur partially via CYP1A2 and also via an unidentified CYP450-independent mechanism, which could include cytosolic oxidases [36,37].

Since both PTX and racemic M-1 are active in vivo, the higher total drug levels (M-1 + PTX) achieved in serum following M-1 injection would likely result in enhanced therapeutic effectiveness of this compound. The total drug levels achieved in mouse serum following M-1 and PTX injection were 171 and 125 μM, respectively, concentrations at which significant anti-fibrogenic effects have been reported in vitro [13,27]. In a previous study [13], M-1 was more potent as an anti-fibrogenic drug (IC50 value 35 μM) compared to PTX (IC50 value 161 μM) for the inhibition of collagen synthesis in hepatic stellate cells. The higher levels of M-1 achieved in this study by injecting the metabolite directly therefore could result in enhanced anti-fibrogenic effects in vivo.

An in vivo drug interaction between the quinolone antibiotic ciprofloxacin and PTX has been demonstrated in humans that resulted in elevated plasma levels of PTX and M-1 [29]. Our group has reproduced this interaction in mice and demonstrated that pre-treatment of CD1 mice with ciprofloxacin (25 mg/kg) resulted in a 2-fold increase in serum concentrations of PTX and M-1 compared to controls [12,30]. Increased serum drug levels were due to inhibition of CYP1A2 catalytic activity and induction of CYP2E1 [12], consistent with ciprofloxacin as a competitive inhibitor of the CYP1A2 enzyme [38,39].

A goal of the present study was to determine if a similar interaction exists between ciprofloxacin and M-1. Consistent with our previous published results, a 2-fold increase in serum drug levels was measured 30 min after PTX injection (100 mg/kg i.p.) in the ciprofloxacin pre-treated group [12]. In the

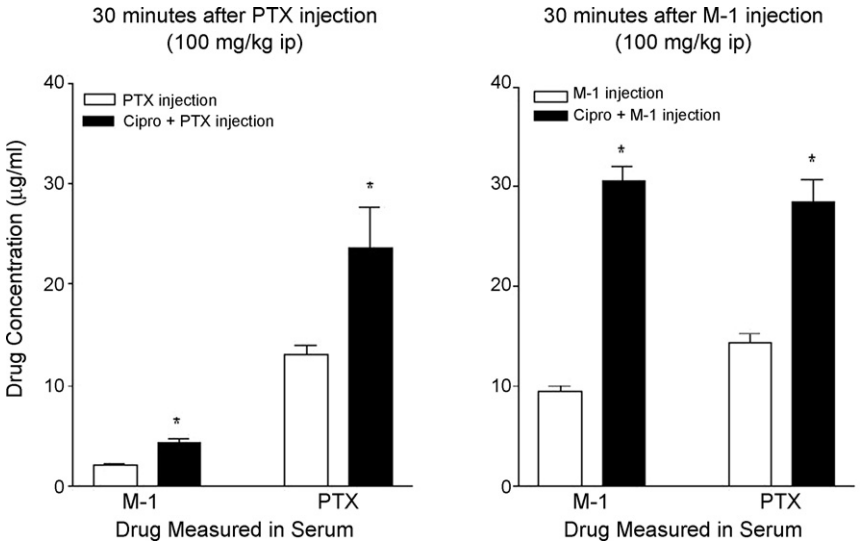

Fig. 5 – Effect of ciprofloxacin pre-treatment on serum levels of PTX and M-1 30 min after injection of either drug. Animals were treated with ciprofloxacin (25 mg/kg) or saline for 9 days before receiving an injection of M-1 or PTX (100 mg/kg i.p.). Interconversion of PTX and M-1 takes place in vivo thus both compounds are detected in serum after injection of either drug alone. Each bar represents mean drug concentration (μg/ml) ± S.E.M. for five animals. Figure is representative of two separate experiments yielding similar results. (*) Significant difference from same group without ciprofloxacin pre-treatment; $p < 0.05$.

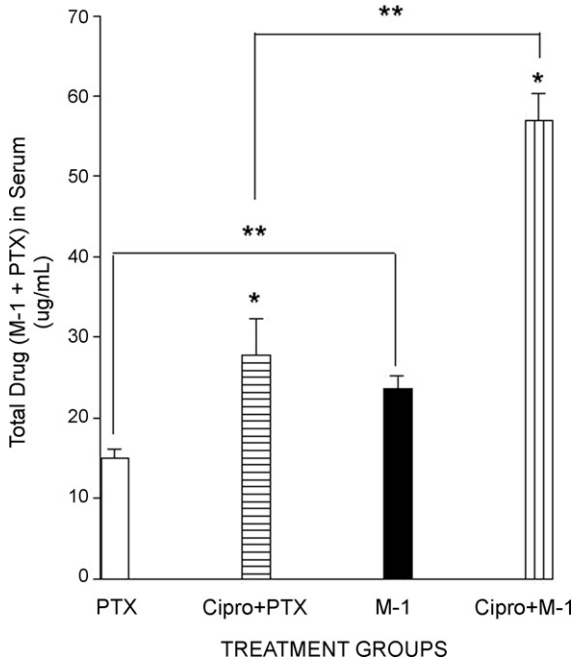

Fig. 6 – Effect of ciprofloxacin pre-treatment on total drug (PTX + M-1) levels in serum 30 min after M-1 or PTX injection. Mice were treated with ciprofloxacin (25 mg/kg) of saline for 9 days before receiving an injection of PTX or M-1 (100 mg/kg i.p.). Results are expressed as mean drug concentration (μg/ml) ± S.E.M. for five animals. Figure is representative of two separate experiments yielding similar results. (*) Significant difference from same group without ciprofloxacin pre-treatment; $p < 0.05$. (**) Significant difference between groups indicated; $p < 0.05$.

ciprofloxacin/M-1 group, there was a significant 2-fold increase in PTX serum levels and a 3-fold increase in serum M-1 levels seen at 30 min post-injection. The total drug levels (PTX + M-1) achieved in serum following M-1 injection were higher than the total drug levels achieved following PTX injection. The interaction between ciprofloxacin and M-1 significantly elevated total serum drug levels 2-fold greater in the ciprofloxacin/M-1 group (57 ± 3.3 mg/ml which is equivalent to 240 μM) compared to all other groups (Fig. 6). The drug concentration achieved (240 μM) is a concentration that has previously been shown to significantly inhibit proliferation [40] and collagen synthesis [13] in hepatic stellate cells in vitro, key parameters in fibrosis. This suggests that the drug combination of M-1 and ciprofloxacin has tremendous potential as an anti-fibrotic therapy.

The 3-fold increase in M-1 levels in the ciprofloxacin/M-1 group suggests that an additional pathway of M-1 metabolism was blocked since a 2-fold increase was seen in all other groups. CYP1A2 is important for the *N*-demethylation of PTX and M-1 to M-6 and M-7, respectively [12]. Also, CYP1A2 plays a partial role in the conversion of M-1R back to PTX [37]. CYP3A4 mediates the conversion of M-1R to M-3b (*trans*-4,5-diol of M-1), a metabolite of M-1R formed in vivo [41]. Ciprofloxacin is a competitive inhibitor of CYP3A4 [42] and likely contributes to the dramatic elevation in M-1 levels seen with the ciprofloxacin/M-1 combination since pathways to three of its metabolites (M-6, M-3b and PTX) are likely inhibited by ciprofloxacin. Inhibition of CYP1A2 and CYP3A4 by ciprofloxacin is competitive in nature and therefore pre-treatment with ciprofloxacin is likely not necessary to achieve increased serum levels of PTX and M-1 solely by its action on CYP1A2 and CYP3A4 but recent evidence suggests that pre-treatment with ciprofloxacin induced CYP2E1 [12], which would result in greater

conversion of pentoxifylline to metabolite-1. These combined effects would explain the 3-fold elevation in blood levels of M-1 when it is administered following ciprofloxacin.

The combination of M-1 and ciprofloxacin should be examined in animal models of disease, specifically diseases involving hyperproliferation, inflammation and fibrosis. Since ciprofloxacin is commonly prescribed to patients with Crohn's disease to prevent or treat bacterial overgrowth [43,44], the combination of ciprofloxacin with M-1 in these patients warrants further investigation as an aid to preventing stricture formation.

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