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Inhibition of Fibroproliferation by Pentoxifylline

ACTIVITY OF METABOLITE-1 AND LACK OF ROLE OF ADENOSINE RECEPTORS

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ABSTRACT. We have reported previously that pentoxifylline and adenosine decrease platelet-derived growth factor- (PDGF) stimulated fibroproliferation. To determine the role of adenosine receptors in the inhibition of fibroproliferation observed with pentoxifylline, we used a non-selective adenosine receptor antagonist, 8-phenyltheophylline, and specific A_1 and A_2 adenosine receptor antagonists. If the A_2 receptor, which is present on fibroblasts, mediates the inhibition of fibroproliferation which occurs with pentoxifylline, then pretreatment of fibroblasts with receptor antagonists prior to the addition of pentoxifylline should prevent the action of pentoxifylline. The results indicated that pretreatment of fibroblasts with 8-phenyltheophylline (100 μM) did not alter the inhibitory effect of pentoxifylline on PDGF-stimulated fibroproliferation. These results argue against a mechanism involving inhibition of adenosine reuptake as the mechanism for pentoxifylline's effect in this system. 8-Phenyltheophylline also did not alter the effect of pentoxifylline on baseline proliferation, suggesting that these effects of pentoxifylline are not mediated by adenosine receptors. Pentoxifylline is metabolized to several metabolites including 1-(5-hydroxyhexyl)-3,7-dimethylxanthine (metabolite-1). Metabolite-1 significantly reduced PDGF-stimulated fibroproliferation and was as effective as pentoxifylline. The combination of pentoxifylline and metabolite-1 had an additive effect. Metabolite-1 and pentoxifylline also reduced baseline proliferation. Preincubation of fibroblasts with 8-phenyltheophylline did not prevent the inhibitory action of metabolite-1 on PDGF-stimulated proliferation or on basal proliferation of fibroblasts, suggesting that the action of metabolite-1 on fibroproliferation was not mediated by adenosine receptors. Results using A_1 and A_2 adenosine receptor antagonists further suggest that the effect of pentoxifylline was not mediated by adenosine receptors. BIOCHEM PHARMACOL 52;4:597–602, 1996.

KEY WORDS. adenosine receptor; fibroproliferation; pentoxifylline; 8-phenyltheophylline; methylxanthines; metabolite-1

Fibroproliferation is a key event in liver fibrosis [1]. In a previous study, selected drugs were assessed for their ability to inhibit fibroproliferation and thus be potential candidates as new antifibrotic drugs [2]. During this assessment, several drugs were identified; among these were pentoxifylline, adenosine, and other methylxanthines. Though these drugs inhibited PDGF†-stimulated fibroproliferation, recent studies suggest that this is not due to a direct effect on the PDGF receptor [3]. Several of the drugs tested have the ability to act on the adenosine receptor [4] or block adenosine reuptake [5], thus potentiating the actions of adenosine. In this study, we investigated the role of adenosine receptors in the block of PDGF-stimulated proliferation which occurs with pentoxifylline. The effect of metabolite-1 of pentoxifylline was also assessed. Adenosine A_2 receptors are present on fibroblasts and may play a role in fibroproliferation [6]. If pentoxifylline is acting directly on the receptor or indirectly on the A_2 receptor inhibiting adenosine reuptake, then block of the receptor by an adenosine antagonist should prevent the actions of pentoxifylline. In this study, we used the adenosine receptor antagonist 8-phenyltheophylline [7] and specific A_1 and A_2 adenosine receptor antagonists [8, 9] to test whether the effect of pentoxifylline on fibroproliferation occurs via an adenosine receptor.

MATERIALS AND METHODS

Fibroproliferation

The fibroproliferative activity of PDGF was assessed by modification of the tritiated thymidine incorporation method of Dohlman *et al.* [10], using normal skin fibroblasts. Tritiated thymidine incorporation is a standard method for assessing fibroblast proliferation. Medium was removed by aspiration from flasks containing confluent

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† *Abbreviations:* PDGF, platelet-derived growth factor; DBE, Dulbecco's modified Eagle's medium; metabolite-1, 1-(5-hydroxyhexyl)-3,7-dimethylxanthine; CPSR2, control process serum replacement-2; CYP1A2, cytochrome P4501A2; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; and DMPX, 3,7-dimethyl-1-propargylxanthine.

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monolayers of human fibroblasts at passages 5–12. The cells were rinsed briefly with 10–20 mL of sterile saline to remove any remaining medium. Fibroblasts were removed from the flasks by the addition of 5 mL of trypsin–EDTA (Gibco, Ontario, Canada) for 30 sec with gentle rocking at room temperature followed by pouring off most of the trypsin solution and incubation at 37° for 3–5 min. Cells were resuspended in 10 mL DBE (Gibco), antibiotic/antimycotic, (Gibco) and 0.5% CPSR2 (Sigma Chemical Co., St. Louis, MO, U.S.A.). CPSR2 is a serum replacement that has low mitogenic activity. Aliquots (200 μL) of cell suspension (8×10^3 cells) were added to 0.32 cm^2 flat bottom wells of 96-well microtiter plates and incubated for 24 hr at 37° in 5% CO_2 in air. The medium was removed from each well by aspiration, replaced by 200 μL of DBE supplemented with factors, and incubated as above for a further 22 hr. PDGF A/B (R&D Systems Inc., Minneapolis, MN, U.S.A.) 8 ng/mL, was used to stimulate proliferation of fibroblasts in the presence or absence of pentoxifylline (Sigma) or metabolite-1 (gift from Hoechst–Roussel Canada Inc., Montreal, Canada). When 8-phenyltheophylline (Sigma) was used, it was incubated with fibroblasts for 2 min before the addition of other factors. When DPCPX (5 μM) and DMPX (10 μM) were used, they were incubated with fibroblasts for 2 and 30 min before the addition of other factors. [*methyl*-^{3}H]Thymidine, 0.5 μCi (Amersham, Ontario, Canada), was added to each well, and the incubation was continued for an additional 2 hr. To harvest the fibroblasts, the medium was removed by aspiration and replaced by 100 μL of trypsin–EDTA for a few minutes at 37°. This trypsinization was verified as sufficient to detach all fibroblasts. The loose fibroblasts were then aspirated onto glass fiber filters using a Brandel Cell Harvester (Xymotech Biosystems, Ontario, Canada) and washed 8–10 times with phosphate-buffered saline; the radioactivity was determined by liquid scintillation. All samples were tested in quadruplicate. The [^{3}H]-thymidine uptake assay has been verified as a good measure of proliferation of fibroblasts, in response to stimuli or inhibitors, by manual cell counts and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [2, 11].

Statistical Analysis

An unpaired Student's *t*-test was used to compare two variables, and ANOVA and the Student–Newman–Keuls test were used when more than two variables were compared [12].

RESULTS

The results in Fig. 1 show the concentration-related effects of metabolite-1 on PDGF-driven fibroproliferation. The fibroblasts were stimulated to proliferate with PDGF (8 ng/mL). The effect of increasing concentrations of metabolite-1 on PDGF-driven fibroproliferation was tested. It is evident that metabolite-1 in concentrations ranging from 225 μM to 2.25 mM (0.066 to 0.66 mg/mL) significantly reduced PDGF-driven fibroproliferation (Fig. 1).

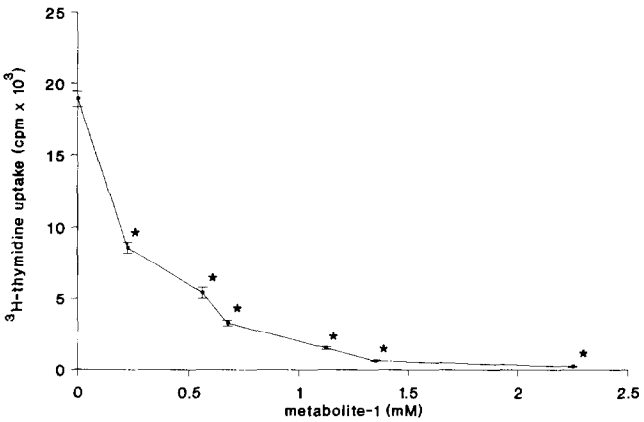

FIG. 1. Effect of metabolite-1 on PDGF-stimulated proliferation. Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts and expressed in counts per minute (cpm). Metabolite-1 was assessed at 225 μM, 560 μM, 680 μM, 1.125 mM, 1.36 mM, and 2.25 mM (0.066, 0.165, 0.2, 0.33, 0.4, and 0.66 mg/mL, respectively). PDGF was tested at 8 ng/mL. Results are expressed as means ± SEM. Each treatment was done on wells in quadruplicate, and experiments were repeated. Key: (*) $P < 0.05$ compared with fibroblasts stimulated with PDGF in the absence of metabolite-1.

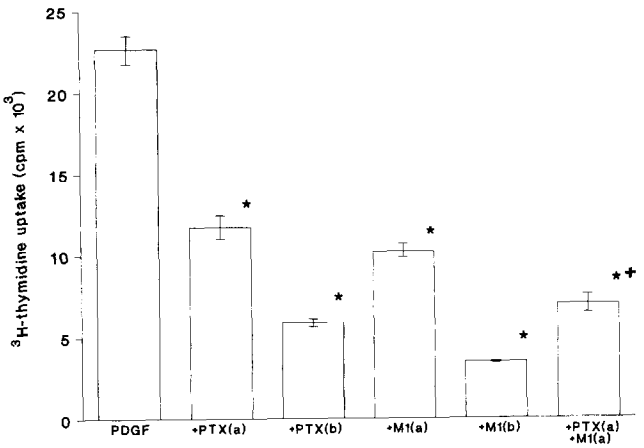

FIG. 2. Relative effects of metabolite-1 (M1) and pentoxifylline (PTX) on PDGF-stimulated proliferation. Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts and expressed in counts per minute (cpm). PTX was assessed at two concentrations [PTX(a) = 240 μM (0.066 mg/mL), PTX(b) = 720 μM (0.2 mg/mL)], and M1 was assessed at two concentrations [M1(a) = 225 μM (0.066 mg/mL), M1(b) = 675 μM (0.2 mg/mL)]; PDGF was tested at 8 ng/mL. The effect of combining PTX (240 μM) and M1 (225 μM) on PDGF-stimulated fibroproliferation was also assessed. Results are expressed as means ± SEM. Each treatment was done on wells in quadruplicate, and experiments were repeated. Key: (*) $P < 0.05$ compared with fibroblasts stimulated with PDGF in the absence of PTX or M1, and (+) $P < 0.05$ compared with fibroblasts stimulated with PDGF in the presence of either PTX (240 μM) or M1 (225 μM).

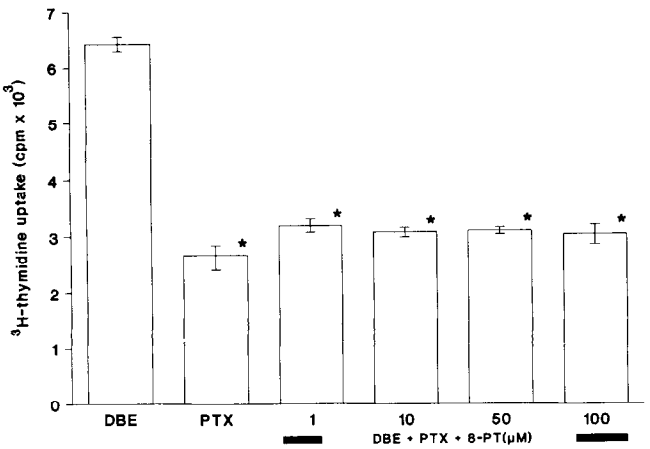

FIG. 3. Effect of 8-phenyltheophylline (8-PT) on the inhibition of baseline proliferation by pentoxifylline (PTX). Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts and expressed in counts per minute (cpm). PTX was assessed at 240 μM, and increasing concentrations of 8-PT were used as indicated on the figure. Baseline fibroproliferation was assessed on fibroblast cultures in DBE plus 0.5% CPSR2. Results are expressed as means ± SEM. Each treatment was done on wells in quadruplicate, and experiments were repeated. Key: (*) $P < 0.05$ compared with DBE.

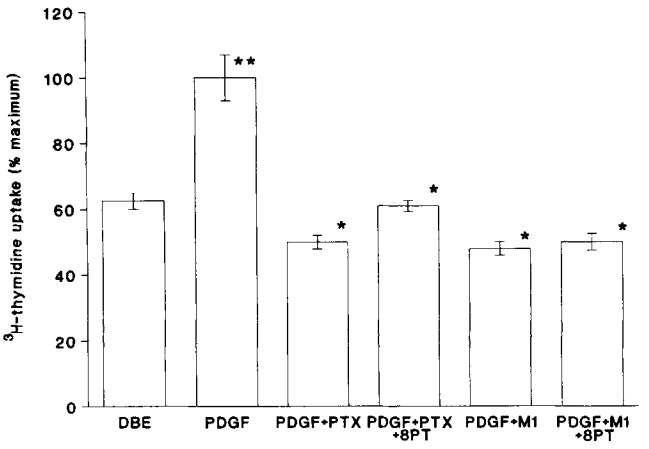

FIG. 5. Effect of 8-phenyltheophylline (8-PT) on the inhibitory actions of pentoxifylline (PTX) and M1 on PDGF-stimulated fibroproliferation. Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts in counts per minute. Concentrations tested: PTX, 240 μM; M1, 225 μM; PDGF, 8 ng/mL; and 8-PT, 100 μM. Results are expressed as means ± SEM (% maximum stimulation). Baseline proliferation (in DBE + 0.5% CPSR2) was 7620 ± 290 cpm. Each treatment was done on wells in quadruplicate, and experiments were repeated. Key: (*) $P < 0.05$ compared with fibroblasts stimulated with PDGF alone in the absence of PTX or M1; and (**) $P < 0.05$ compared with baseline (DBE + 0.5% CPSR2).

The relative effects of pentoxifylline and metabolite-1 on PDGF-driven fibroproliferation are illustrated in Fig. 2. Proliferation of fibroblasts was stimulated with PDGF (8 ng/mL), and the inhibitory effects of pentoxifylline (240 and 720 μM) and metabolite-1 (225 and 675 μM) were tested. The results clearly indicate that pentoxifylline and metabolite-1 both significantly reduced PDGF-driven fibroproliferation and indicate that there was no difference between the effects of pentoxifylline and metabolite-1, suggesting that the parent compound and its metabolite-1 had similar effects on fibroproliferation. The results further in-

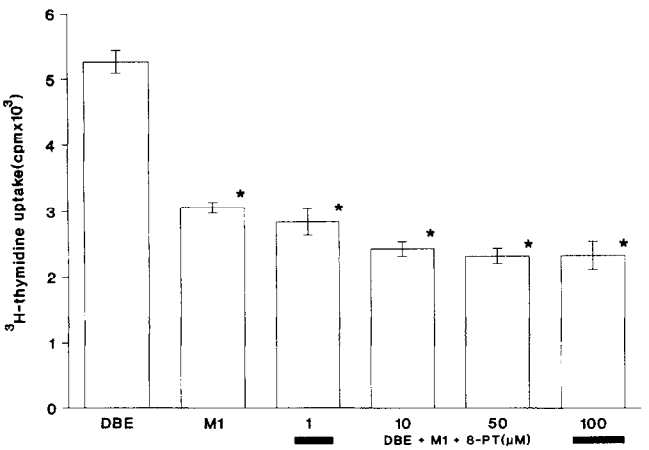

FIG. 4. Effect of 8-phenyltheophylline (8-PT) on the inhibitory actions of metabolite-1 (M1) on baseline proliferation. Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts and expressed in counts per minute (cpm). M1 was assessed at 225 μM, and 8-PT was added in increasing concentrations as indicated on the figure. Results are expressed as means ± SEM. Each treatment was done on wells in quadruplicate, and experiments were repeated. Key: (*) $P < 0.05$ compared with DBE.

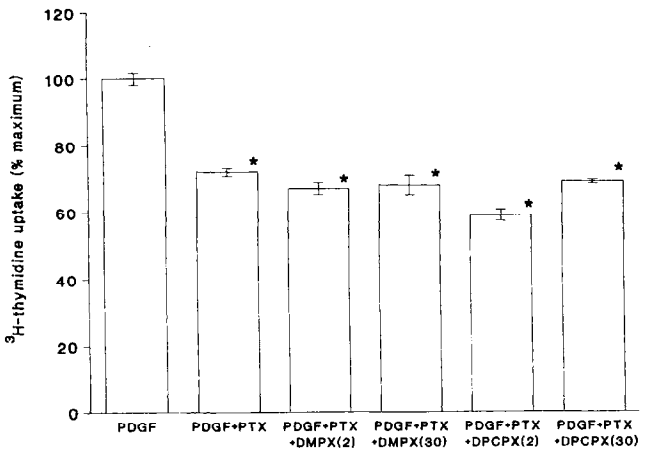

FIG. 6. Effects of DPCPX and DMPX on the inhibitory actions of pentoxifylline (PTX) on PDGF-stimulated fibroproliferation. Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts in counts per minute. PTX (240 μM), PDGF (8 ng/mL), DPCPX (5 μM) and DMPX (10 μM) were assessed. DPCPX and DMPX were preincubated with fibroblast cultures for 2 and 30 min prior to addition of PDGF and PTX. Results are expressed as means ± SEM (% maximum stimulation). Each treatment was done on wells in quadruplicate, and experiments were repeated. Key: (*) $P < 0.05$ compared with fibroblasts stimulated with PDGF alone in the absence of PTX, DPCPX, and DMPX.

dicate that the combination of pentoxifylline and metabolite-1 significantly reduced PDGF-stimulated fibroproliferation and appeared to be additive.

To test the hypothesis that pentoxifylline inhibits fibroproliferation via adenosine receptors, a non-selective adenosine receptor antagonist, 8-phenyltheophylline, was used. 8-Phenyltheophylline was tested at 1, 10, 50, and 100 μM concentrations. 8-Phenyltheophylline did not alter the effect of pentoxifylline on baseline proliferation, suggesting that these effects of pentoxifylline were not mediated by adenosine receptors (Fig. 3). In this figure, baseline proliferation was stimulated by DBE containing CPSR2, and pentoxifylline (240 μM) reduced this by greater than 50%. Addition of 8-phenyltheophylline (1–100 μM) did not prevent the inhibitory effects of pentoxifylline on baseline proliferation.

The effect of increasing concentrations of 8-phenyltheophylline on metabolite-1 inhibition of baseline proliferation is shown in Fig. 4. Metabolite-1 inhibited baseline proliferation, and the four concentrations of 8-phenyltheophylline tested did not alter or prevent the inhibition of proliferation due to the M-1 metabolite.

The inhibitory effect of pentoxifylline on PDGF-stimulated fibroproliferation was not altered by 8-phenyltheophylline (Fig. 5). PDGF (8 ng/mL) significantly stimulated proliferation of fibroblasts, and addition of pentoxifylline (240 μM) to an incubation mixture containing PDGF (8 ng/mL) significantly reduced proliferation of fibroblasts to near baseline levels. Addition of 8-phenyltheophylline (100 μM) did not prevent the depression of fibroproliferation that occurred with pentoxifylline. We also investigated whether 8-phenyltheophylline had any influence on metabolite-1 inhibition of PDGF-stimulated proliferation. Metabolite-1 inhibited PDGF-stimulated proliferation, and the results demonstrated no effect of 8-phenyltheophylline on metabolite-1 inhibition of PDGF-stimulated proliferation.

Preincubation of fibroblasts with DPCPX and DMPX, specific A_1 and A_2 adenosine receptor antagonists, for 2 or 30 min did not prevent the inhibitory effect of pentoxifylline on PDGF-stimulated fibroproliferation (Fig. 6).

DISCUSSION

The results from this study clearly establish that pentoxifylline and its metabolite-1 inhibit PDGF-stimulated fibroproliferation and suggest that this effect is not mediated by adenosine receptors. The results further establish that the inhibition by pentoxifylline and metabolite-1 is of a similar magnitude, thus suggesting that the actions of pentoxifylline may not be related directly to pentoxifylline blood levels, but are, in fact, related to the blood level resulting from the summation of pentoxifylline and metabolite-1. The combination of pentoxifylline and metabolite-1 had an apparent additive effect.

It has been established that pentoxifylline is metabolized to seven metabolites in mammals [13–16]. The first five metabolites are dimethylxanthines with substitutions at the N-1 position, including two carboxylic acid metabolites 4 and 5. Metabolites 6 and 7 are 3-methylxanthines having been demethylated at the 7 position from pentoxifylline and metabolite-1, respectively [17]. The major metabolites of pentoxifylline are metabolites 1 and 5 [18], and metabolite-1 has been shown to have pharmacological properties similar to those of pentoxifylline in peripheral vascular disease [19, 20]. For this reason, the actions of metabolite-1 on fibroproliferation were assessed in our assay procedure.

Results from this study suggest that metabolite-1 is as effective as pentoxifylline in inhibiting fibroproliferation stimulated by PDGF. Pentoxifylline (240 and 720 μM) reduced PDGF-stimulated proliferation 51 and 74%, respectively. Metabolite-1 (225 and 675 μM) reduced PDGF-stimulated proliferation 56 and 85%, respectively. The effects of pentoxifylline and metabolite-1, at corresponding concentrations, were not significantly different. These results suggest that the parent compound pentoxifylline and its metabolite-1 are equally potent in inhibiting PDGF-stimulated proliferation, thus suggesting that the biological activity of pentoxifylline will be underestimated if one does not take into account the production of an active metabolite-1 with equal biological activity in inhibiting fibroproliferation.

Pentoxifylline metabolism and the levels of metabolite-1 are altered by ciprofloxacin [21], smoking [22], and liver disease [23]. Co-administration of ciprofloxacin with pentoxifylline increases plasma levels of pentoxifylline and metabolite-1 [21]. Ciprofloxacin inhibits cytochrome P4501A2 [24, 25]. The interaction between ciprofloxacin and pentoxifylline likely occurs via the cytochrome P4501A2 system, which N-demethylates pentoxifylline and metabolite-1 to metabolites 6 and 7, respectively [25–27]. Smoking reduces pentoxifylline and metabolite-1 plasma concentrations [22] compared to non-smokers, likely via an effect on cytochrome P4501A2 [28]. Factors that modify CYP1A2 would be expected to alter levels of metabolite-1. Pentoxifylline and metabolite-1 plasma levels are elevated markedly in cirrhotic patients [23], and CYP1A2 activity is decreased preferentially in patients with cirrhosis [29]; thus, the elevation in metabolite-1 observed in patients with cirrhosis [23] likely relates to an inhibition of CYP1A2 activity in these patients. These reports together with our results on the equipotent effect of pentoxifylline and metabolite-1 on fibroproliferation suggest that the antifibroproliferative effect of pentoxifylline in patients with cirrhosis may be greater than expected due to the additional biological activity derived from metabolite-1 and to the inhibitory effect of cirrhosis on CYP1A2, thus elevating blood levels of both pentoxifylline and metabolite-1. These implications are of great importance because the target population of patients for new anti-fibroproliferative drugs such as pentoxifylline consists of those patients with liver disease who may demonstrate this altered pentoxifylline metabolism.

Previous results had indicated that pentoxifylline, other methylxanthines, and adenosine inhibit PDGF-stimulated fibroproliferation [2]. In this study, we used 8-phenyltheophylline as an adenosine receptor antagonist at concentrations that are sufficiently high to block adenosine receptors. The preincubation of cells with 8-phenyltheophylline prior to addition of PDGF and pentoxifylline did not alter the inhibitory effect of pentoxifylline on PDGF-stimulated fibroproliferation. Similarly, the results obtained when 8-phenyltheophylline was preincubated with cells prior to the addition of PDGF and metabolite-1 indicated that 8-phenyltheophylline did not block the inhibitory effect of metabolite-1 on PDGF-stimulated fibroproliferation. Using the specific A_1 and A_2 receptor blockers DPCPX [8] and DMPX [9], as well as a longer preincubation time, the results tend to eliminate the possibility that a subset of adenosine receptors is involved in the action of pentoxifylline on PDGF-stimulated proliferation. Therefore, although adenosine A_2 receptors are present on fibroblasts [6] and hepatic Kupffer cells [30], these results clearly indicate that pentoxifylline and its metabolite-1 inhibit PDGF fibroproliferation by a mechanism other than a direct or indirect effect on adenosine receptors. Pentoxifylline, adenosine, theophylline, and trapidil have all been reported to inhibit PDGF-stimulated fibroproliferation [2]; thus, an alternative mechanism may relate to an elevation of cyclic AMP via an effect on phosphodiesterase activity [31].

In conclusion, our results suggest that (i) both pentoxifylline and metabolite-1 have similar biological activity in inhibiting fibroproliferation, and (ii) it is unlikely that this activity is due to an effect at the adenosine receptor. The reduced CYP1A2 activity reported to occur in liver disease patients may allow reduction in the dose of pentoxifylline required to achieve blood levels that are sufficiently high to block fibroproliferation [2].

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