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Effects of pentoxifylline and its metabolites on platelet aggregation in whole blood from healthy humans

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Abstract

It is known that pentoxifylline inhibits platelet aggregation *in vitro*, but the effects from pentoxifylline and its main metabolites: 3,7-dimetyl-1(5′hydroxyhexyl)xanthine (R-M1 and S-M1), 3,7-dimetyl -1(4-carboxybutyl)xanthine (M4), 3,7-dimetyl -1(3-carboxypropyl)xanthine (M5), on platelet aggregation in whole blood *in vitro* and *in vivo* have not been studied. We found that pentoxifylline, *rac*-M1, R-M1, S-M1 and M4 significantly inhibit ADP induced platelet aggregation in whole blood *in vitro* in a concentration-dependent manner, R-M1 being the most potent followed by *rac*-M1, S-M1, pentoxifylline, and M4. In this series of experiments the effects on aggregation induced ATP-release were less pronounced and were only significant after treatment with pentoxifylline, *rac*-M1 and R-M1, but the potency order appears to be the same. Since the metabolites are not available for use in humans, and also since each substance would be extensively metabolised *in vivo*, we made an attempt to estimate the relative contribution of each substance to the total effect of pentoxifylline *in vivo*. Previously published concentrations of pentoxifylline and these metabolites in humans, after administration of pentoxifylline, were used in combination with the potency ratios from this study. The findings from these calculations were that the main effect *in vivo* comes from S-M1 followed by pentoxifylline, the other metabolites contribute less than 10% each. In conclusion: in the following potency order R-M1, *rac*-M1, pentoxifylline, S-M1 and M4 all have significant effects on platelet aggregation in whole blood *in vitro*. However, it appears that the main effects *in vivo* are caused by S-M1 and pentoxifylline.

Keywords: Pentoxifylline; Metabolite effect; Platelet aggregation in whole blood

1. Introduction

Pentoxifylline 3,7-dimetyl-1(5′-oxo-hexyl)xanthine is a haemorheologic drug used in the treatment of peripheral vascular disease and other conditions with insufficient regional microcirculation (Moher et al., 2000; Samlaska and Winfield, 1994; Ward and Clissold, 1987). Pentoxifylline acts primarily by increasing erythrocyte flexibility, by reducing blood viscosity, and by decreasing the potential for platelet aggregation and thrombus formation. In addition, pentoxifylline inhibits platelet aggregation in patients with peripheral vascular disorders (Angelkort, 1979), and also prevents shunt thrombosis formation in dialysis patients (Radmilović et al., 1987). Pentoxifylline is metabolised in humans to at least seven metabolites (Hinze et al., 1972). The major metabolites in humans are the hydroxy metabolite 3,7-dimetyl-1-(5′hydroxyhexyl)xanthine (M1), and the two carboxylic acid metabolites 3,7-dimetyl -1-(4-carboxybutyl)xanthine (M4) and 3,7-dimetyl -1(3-carboxypropyl)xanthine (M5). After oral administration of pentoxifylline to healthy volunteers the areas under the plasma concentration curves (AUCs) of M5 and racemic-M1 (*rac*-M1) exceeded that of pentoxifylline while that of M4 was lower (Beermann et al., 1985; Bryce and Burrows, 1980; Nicklasson et al., 2002; Smith et al., 1986). Using chiral separation we found that S-M1 is the major (>96%) and R-M1 the minor metabolite (Nicklasson et al., 2002). Metabolism of

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pentoxifylline to M1 takes place both in the liver (Lillibridge et al., 1996) and in the erythrocytes (Ings et al., 1982; Nicklasson et al., 2002) and is reversible (Hinze et al., 1972; Lillibridge et al., 1996; Nicklasson et al., 2002).

Antiplatelet effects of pentoxifylline only were studied by De la Cruz et al. (1993). They found pentoxifylline to be more potent in whole blood than in platelet rich plasma. A comparison of the haemorheologic effects of pentoxifylline, *rac*-M1, and six other metabolites *in vitro* showed pronounced effects of the two former species and M5 on erythrocyte filterability and platelet aggregation (Ambrus et al., 1995). We have previously investigated the effect of pentoxifylline and its metabolites on another of its modes of action, namely retinal blood flow, in a randomised, placebo controlled, four-period cross over study in healthy volunteers (Magnusson et al., 2005). Pentoxifylline was administered as intravenous infusions alone or after pre-treatment of the subjects with ciprofloxacin or rifampicin, and a placebo infusion was used as negative control. Retinal blood flow was measured by scanning laser Doppler flowmetry in a selected area of the central temporal retina. The pre-treatments with rifampicin (an inducer of several enzymes of the cytochrome P450 system) and ciprofloxacin (an inhibitor of CYP1A2) were used in order to create different blood concentration ratios between pentoxifylline and its metabolites within the same subject. This enabled a comparison of blood flow effects of pentoxifylline and its metabolites *in vivo* by means of a linear multiple-regression model. Pentoxifylline, R-M1 and M5 significantly increased retinal blood flow while S-M1 and M4 appeared to counteract this effect. Thus, although the mechanisms of action differ, it is interesting to note that the findings on filterability *in vitro* and blood flow *in vivo* were in general agreement as regards the activity of the metabolites. However, the influence of the enantiomers of M1 was not investigated in the *in vitro* study on platelet aggregation.

The aim of this study was to investigate the relative potencies of pentoxifylline and metabolite M1, M4 and M5 to inhibit platelet aggregation in whole blood, and in particular to clarify contributions of the two enantiomers of M1, to this effect.

2. Material and methods

2.1. Materials

Pentoxifylline and *rac*-M1, M4 and M5 were kindly supplied by Aventis Pharma (Stockholm, Sweden). The R enantiomer of M1 was a gift from Cell Therapeutics (Seattle, WA, USA). ATP standards and luciferin luciferase reagents from Chrono-Lume-reagents, Trio-lab (Göteborg, Sverige) were used and ADP from Sigma (Stockholm, Sverige).

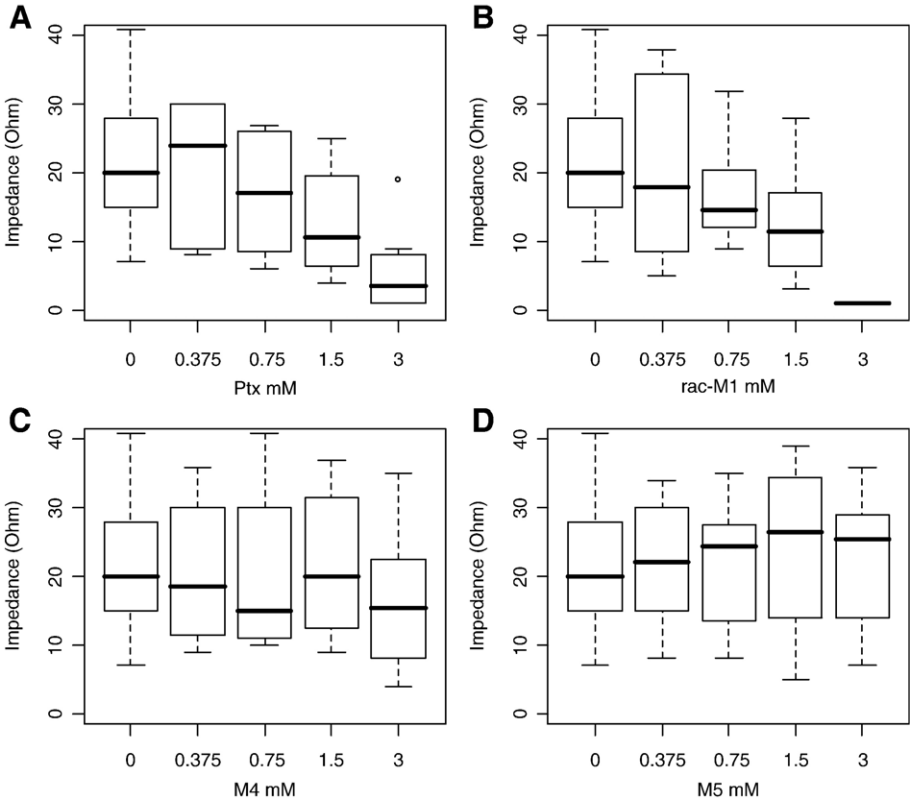

Fig. 1. Box plots of ADP induced platelet aggregation in whole blood *in vitro*, measured as impedance, after incubation with a series of concentrations of: (A) pentoxifylline, (B) *rac*-M1, (C) M4, and (D) M5. The lower, middle and upper lines of each box are the first, second and third quartiles, respectively. The bars extend from the lowest to the highest non-outlier observation of the data set. Outliers are defined as values outside 1.5 times the box width from either end of the box, and indicated by open dots.

2.2. Platelet aggregation

Measurement of platelet aggregation were done in whole blood from 8 healthy volunteers (2 males and 6 females) by impedance technology (electrical resistance between two electrodes immersed in whole blood), using whole blood lumi-aggregometer, (Chrono log modell 560 Ca, Chrono Log Corp, Havertown, PA, USA) (Podczasy et al., 1996; Vucenik and Podczasy, 1998).

The subjects were informed about the study and gave oral informed consent prior to blood sampling. The subjects had not been taking any drugs 24 h prior to blood donation and had not been taking drugs containing acetylsalicylic acid within one week prior to blood donation. The study was performed according to the declaration of Helsinki and approved by the Ethics committee of Lund University.

Blood was sampled at two occasions from each subject by venepuncture and collected into vacutainer tubes containing 1:10 (volume:volume) 0.129 mol/L citrate. All tests were completed within four hours after the blood was drawn. At one occasion ($n=8$) pentoxifylline, *rac*-M1, M4 and M5 were investigated and on another occasion ($n=8$) *rac*-M1 and R-M1.

Platelet aggregation was studied in whole blood diluted in a 1:1 ratio with 0.9% saline (450 μL each). The samples were placed in plastic cuvettes containing a magnetic stir bar and were incubated in 37 °C for at least 5 min prior to testing and stirred at 1200 rpm.

The test procedure was started by adding 0.008 mg luciferin luciferase to the sample; the samples were incubated in 37 °C for 1 min prior to addition of the test substances. Pentoxifylline, *rac*-M1, R-M1, M4, M5 or saline (vehicle control) was added to the sample and incubated in 37 °C for 1 min prior to addition of ADP (10 μM final concentration), aggregation was monitored for 6 min. Pentoxifylline and its metabolites were diluted in saline 0.9%, 60 μL of the stock solutions were added to the samples, giving the final concentrations 0.375 mM, 0.75 mM, 1.5 mM or 3 mM. Saline 0.9% was used as control. The effects of S-M1 were calculated from a comparison between R-M1 and *rac*-M1 since we did not have access to pure S-M1.

In the same specimens ATP-release from the platelets was observed from the luciferin luciferase reaction. In these observations the ATP-release was calculated based on an ATP standard (2 nmol).

2.3. Statistics

For statistical analysis the mixed procedure in SAS (version 8.2; SAS Institute, Cary, NC, USA) was used. In the analysis of ADP induced platelet aggregation and ATP-release we used logarithmically transformed observations as dependent variables. Concentrations of the studied substances were used in the mixed model as fixed effects whereas subjects were entered as random effects. Least square

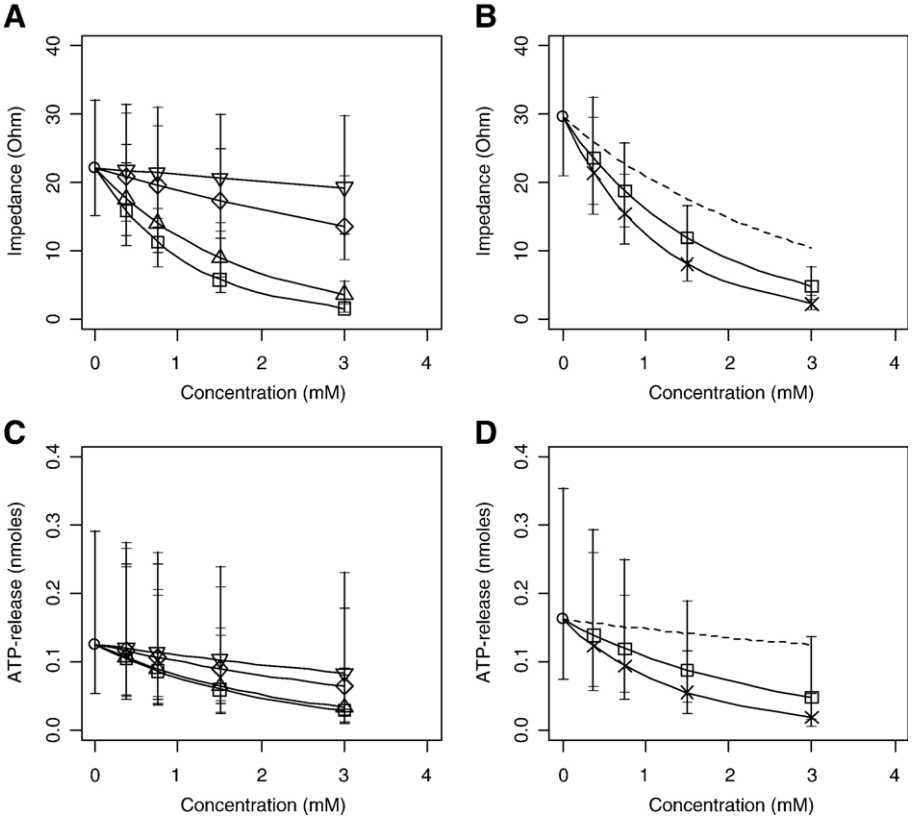

Fig. 2. (A, B) The estimated least square mean impedance values (ohm) and their 95% confidence limits from the mixed model after incubation with 2A pentoxifylline r, *rac*-M1 o, M4 ¯, and M5 s and in B (rac-M1 o, R-M1 ×, and S-M1 dotted line). (C, D) Estimated least square mean values and their 95% confidence limits from the mixed model for ATP-release (nmol) after incubation with pentoxifylline r, *rac*-M1 o, M4 ¯, and M5 s and in D (*rac*-M1 s, R-M1 ×, and S-M1 dotted line.

Table 1
The remaining aggregation in whole blood after addition of a series of concentrations of each investigated substance (pentoxifylline, *rac*-M1, M4 and M5), expressed as fractional effect at 1 mM, mean and 95% confidence interval (CI)

	Fractional effect at 1 mM estiate	Fractional effect at 1 mM 95% CI	*P*-value
Pentoxifylline	0.54	0.49–0.61	<0.0001
rac-M1	0.40	0.36–0.45	<0.0001
M4	0.85	0.76–0.95	0.0049
M5	0.95	0.85–1.07	0.42
	Ratio estimate	95% CI	*P*-value
Pentoxifylline/*rac*-M1	1.3474	1.1727–1.5482	<0.0001
Pentoxifylline/M4	0.64	0.56–0.74	<0.0001
Pentoxifylline/M5	0.57	0.50–0.66	<0.0001
rac-M1/M4	0.48	0.41–0.55	<0.0001
rac-M1/M5	0.42	0.37–0.49	<0.0001
M4/M5	0.89	0.77–1.02	0.094

Ratios of the fractional effects at 1 mM for platelet aggregation in whole blood, mean and 95% CI. *P*-values against the null hypothesis of unity are given. *P*-values against the null hypothesis of unity (i.e. saline) are given.

Table 3
The remaining ATP-release in whole blood after addition of a series of concentrations of each investigated substance (pentoxifylline, *rac*-M1, M4 and M5), expressed as fractional effect at 1 mM, mean and 95% confidence interval (CI)

	Fractional effect at 1 mM estimate	Fractional effect at 1 mM 95% CI	*P*-value
Pentoxifylline	0.64	0.48–0.85	0.0020
rac-M1	0.61	0.45–0.81	0.0011
M4	0.80	0.60–1.06	0.12
M5	0.87	0.66–1.16	0.34
	Ratio estimate	95% CI	*P*-value
Pentoxifylline/*rac*-M1	1.05	0.74–1.49	0.78
Pentoxifylline/M4	0.80	0.57–1.12	0.19
Pentoxifylline/M5	0.73	0.52–1.03	0.072
rac-M1/M4	0.76	0.53–1.08	0.12
rac-M1/M5	0.70	0.49–0.99	0.043
M4/M5	0.92	0.65–1.29	0.62

Ratios of the fractional effects at 1 mM for ATP-release in whole blood, mean and 95% CI. *P*-values against the null hypothesis of unity are given. *P*-values against the null hypothesis of unity (i.e. saline) are given.

estimates and 95% confidence limits after back transformation are given. Statistical significance was accepted at $P<0.05$ (two tailed).

3. Results

Pentoxifylline, *rac*-M1 and M4 inhibited platelet aggregation, measured as impedance, in a concentration-dependent manner (Fig. 1). Concentration–effect relationships were also found for *rac*-M1 and R-M1 with a more pronounced effect for R-M1. In all experiments we could se that the effects on aggregation were mirrored by changes in ATP-release. Although the observations from the different series of experiments showed large variability we could successfully model the effect in mixed model analyses.

The predicted impedance values from the mixed model are shown in Fig. 2A (pentoxifylline, rac-M1, M4, and M5) and in Fig. 2B (*rac*-M1, R-M1, and S-M1). Aggregation was reduced in a concentration-dependent manner for *rac*-M1, R-M1, S-M1, pentoxifylline and M4. The intercept and slopes for the impedance–concentration curves were used for calculation of fractional effect at 1 mM and are shown in Table 1 (pentoxifylline, *rac*-M1, M4 and M5) and Table 2 (*rac*-M1, R-M1, and S-M1). The most potent inhibitor of aggregation was *rac*-M1 followed by pentoxifylline and M4. However, when the R-enantiomer of M1 was compared with *rac*-M1 we found that this enantiomer inhibited aggregation 23% more efficiently than *rac*-M1 and 41% more than S-M1.

Simultaneously with aggregation, secretion of dense granule ATP was measured in the same sample. Predicted values from the mixed model for ATP-release are shown in Fig. 2C (pentoxifylline, *rac*-M1, M4 and M5) and 2D (*rac*-M1, R-M1, and S-M1). Pentoxifylline, *rac*-M1 and R-M1 significantly decreased ATP-release, Tables 3 and 4.

Table 2
The remaining aggregation in whole blood after addition of a series of concentrations of *rac*-M1, R-M1, S-M1, expressed as fractional effect at 1 mM, mean and 95% confidence interval (CI)

	Fractional effect at 1 mM estimate	Fractional effect at 1 mM 95% CI	*P*-value
rac-M1	0.54	0.46–0.64	<0.0001
R-M1	0.42	0.35–0.50	<0.0001
S-M1	0.71	0.51–0.98	0.036
	Ratio estimate	95% CI	*P*-value
R-M1/*rac*-M1	0.77	0.63–0.94	0.011
S-M1/*rac*-M1	1.30	1.07–1.60	0.011
R-M1/S-M1	0.59	0.40–0.88	0.011

Ratios of the fractional effects at 1 mM for platelet aggregation in whole blood, mean and 95% CI. *P*-values against the null hypothesis of unity are given. *P*-values against the null hypothesis of unity (i.e. saline) are given.

Table 4
The remaining ATP-release in whole blood after addition of a series of concentrations of *rac*-M1, R-M1, S-M1, expressed as fractional effect at 1 mM, mean and 95% confidence interval (CI)

	Fractional effect at 1 mM estimate	Fractional effect at 1 mM 95% CI	*P*-value
rac-M1	0.67	0.45–0.98	0.038
R-M1	0.48	0.33–0.71	0.0004
S-M1	0.92	0.45–1.87	0.81
	Ratio estimate	95% CI	*P*-value
R-M1/*rac*-M1	0.73	0.47–1.12	0.14
S-M1/*rac*-M1	1.38	1.12–2.11	0.14
R-M1/S-M1	0.53	0.22–1.24	0.14

Ratios of the fractional effects at 1 mM for ATP-release in whole blood, mean and 95% CI. *P*-values against the null hypothesis of unity are given. *P*-values against the null hypothesis of unity (i.e. saline) are given.

4. Discussion

The aim of this study was to investigate the relative potencies of pentoxifylline and metabolite M1, M4 and M5 to inhibit platelet aggregation in whole blood, and in particular to clarify contributions of the two enantiomers of M1, that are formed to very different extents *in vivo*, to this effect.

We found that pentoxifylline, *rac*-M1, R-M1, S-M1 and M4 significantly inhibit platelet aggregation in a concentration-dependent manner, R-M1 being the most potent followed by *rac*-M1, S-M1, pentoxifylline, and M4. The effects on ATP-release were less pronounced and were only significant after treatment with pentoxifylline, *rac*-M1 and R-M1, and the potency order appears to be the same, but these measurements exhibit more scatter and thus less explained variability. Thus, in the following we focus on the effect on platelet aggregation.

Previously Ambrus et al. (1995) studied the effects of pentoxifylline, *rac*-M1, M4 and M5 on aggregation in platelet rich plasma using approximately similar concentration to ours. They found that *rac*-M1, pentoxifylline and M5 but not M4 inhibited aggregation and that *rac*-M1 was more potent than pentoxifylline. Our results are in agreement with the findings of Ambrus and co-workers regarding the most potent substances, but not regarding M4 and M5. A direct comparison between studies is always difficult. Further, the methodologies differ: Ambrus et al. (1995) used aggregation in platelet rich plasma, and De la Cruz et al. (1993) used both platelet rich plasma and whole blood, whereas we studied platelet aggregation in whole blood. Our method should be more relevant for *in vivo* situations since we studied aggregation in whole blood instead of aggregation in platelet rich plasma. Aggregation in whole blood is preferable, both since it evaluates the platelets in a physiologic milieu in the presence of red and white blood cells, which are known to modulate platelet function, and also since aggregation in platelet rich plasma require centrifugation that causes injury to the platelets and loss of giant thrombocytes (Dyskiewicz-Korpanty et al., 2005). In addition, Ambrus et al. (1995) could not distinguish between the enantiomers of M1, which, as we now show, differ significantly in their potencies.

In vivo there will always be a mixture of the parent compound and its metabolites after administration of pentoxifylline, which allows pharmacological interactions between the different species. An aspect that has not been investigated *in vitro*, where only one specimen is added at the time, with the obvious exception of the racemate. Even if there is an *in vitro* metabolism in red blood cells, the extent of this phenomenon should be negligible taken into account the abundance of the species added and the short duration of the experiment (Nicklasson et al., 2002). In addition, the relative plasma concentrations of pentoxifylline and the metabolites will be very different from those in the *in vitro* experiments, so that activities are compared for different regions of the underlying concentration–effect relationships.

In an attempt to estimate the relative contribution of each substance to the total effect of pentoxifylline on platelet aggregation *in vivo* we use our *in vitro* data from this study together with our previously published concentrations of pentoxifylline and its metabolites in humans after administration of pentoxifylline (Nicklasson et al., 2002). In that study we found that the relative mean concentrations (calculated from ratios of AUCs of the metabolites over the AUC of pentoxifylline) were 0.058, 2.91, 0.28, and 2.22 for R-M1, S-M1, M4, and M5, respectively. If we combine these results with the potencies found in this study, we then conclude that the main effect on platelet aggregation *in vivo* should actually be brought about by S-M1 and pentoxifylline, and that the remaining metabolites would contribute by less than 10% each. This approximation holds true also if we take the inter-subject variability of the metabolism of pentoxifylline into account. Thus, even if R-M1 is twice as potent as S-M1 *in vitro* the low concentration achieved after administration of pentoxifylline results in only a small contribution to the total effect on platelet aggregation *in vivo*.

In conclusion, we found that in the following potency order R-M1, *rac*-M1, pentoxifylline, S-M1 and M4 all have significant effects on platelet aggregation in whole blood *in vitro*. When combining our findings in this study with previously known concentration data in humans after administration of pentoxifylline it appears that the main effects *in vivo* are caused by S-M1 and pentoxifylline. However, further studies are needed in order to confirm this.

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