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"In vitro" and "ex vivo" effects of picotamide, a combined thromboxane A_2-synthase inhibitor and -receptor antagonist, on human platelets

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Summary. Picotamide (G 137), a new non prostanoid inhibitor of in vitro arachidonic acid induced platelet aggregation, has been further characterized in in vitro and ex vivo studies.

When whole blood was activated with collagen in the presence of picotamide 5×10^{-4} M, thromboxane B_2 production was decreased, and 6-keto-$PGF_1\alpha$ generation was significantly increased, suggesting a reorientation of platelet endoperoxide metabolism following blockade of thromboxane synthetase. Picotamide also inhibited platelet aggregation and clot retraction induced by the endoperoxide analogue U46619 in human platelets, indicating thromboxane A_2-receptor antagonism, possibly of competitive nature.

A single oral dose of picotamide 1 g in 24 healthy volunteers produced a significant inhibition of collagen, arachidonic acid and U46619-induced platelet aggregation. Serum levels of thromboxane B_2 were also reduced.

Chronic administration of picotamide 1.2 g/d to patients with vascular disease resulted in a prompt and persistent fall in their increased plasma levels of β-thromboglobulin.

The results indicate that picotamide is a combined thromboxane B_2-synthetase inhibitor and thromboxane A_2-receptor antagonist in human platelets, and that it may prove useful as an antithrombotic agent.

Key words: platelet inhibition, picotamide, TxB_2 inhibitor, antithrombotic drug, TxA_2 receptor antagonist, platelet endoperoxides, side effects

In response to various activating stimuli, blood platelets produce eicosanoid mediators, including thromboxane A_2 (TxA_2). It is the most potent vasoconstrictor known and it is also an agonist stimulating platelet aggregation and secretion [1]. Recognition of the role of TxA_2 as a physiological modulator and mediator in thrombotic, vasospastic and broncospastic conditions [2] has provided a strong rationale for developing TxA_2-blocking drugs. Two major classes of such compounds have recently become available: a) TxA_2-synthetase inhibitors, i.e. compounds that suppress the conversion of PGH_2 into TxA_2 [3]; and, b) TxA_2-receptor antagonists, that would block the activity of TxA_2 on its target cells [4].

In previous studies of the pharmacological actions of 4-OH-isophthalic acid derivatives, a number of inhibitors of platelet aggregation were identified [5]. Picotamide (G 137), a compound entirely devoid of antiinflammatory properties, is the most interesting compound in the series, as it had an evident and selective inhibitory activity on arachidonic acid induced aggregation of human platelets in vitro [6].

The inhibitory activity of picotamide on human platelets is further characterised here, and data have been obtained indicating that it possesses both TxA_2-synthetase and TxA_2-receptor antagonist activity. Studies in human volunteers and in patients with vascular disease suggest that the drug is an effective inhibitor of platelet activation. A preliminary report of the results has already been published as an abstract [7].

Materials and methods

Reagents were of the best grade available. The tartrate salt of picotamide (N, N bis (3-picolyl)-4-methoxy-isophtalamide, or Plactidil, Samil, Rome, Italy) was dissolved in sterile saline to give a 10 mM stock solution which was stored at −20 °C. Immediately before use, the stock solution was further diluted with saline to the desired concentration, and the pH was adjusted to 7.3 by adding 0.1 N NaOH. The control solution consisted of isotonic saline at the same pH. For the ex vivo studies, picotamide was supplied by the manufacturer in 250 or 300 mg capsules. ADP, epinephrine and collagen were purchased from Mascia Brunelli, Milan, Italy; arachidonic acid (sodium salt, >99% pure), from Sigma Chemicals Co., St. Louis, USA; batroxobin (reptilase reagent), from Boehringer Mannheim, West Germany; radioimmunoassay kits for β-thromboglobulin from the Radiochemical Centre (Amersham, England). The stable prostaglandin analogue, 9,11-dideoxy-11a, 9a-epoxymethano-prostaglandin F2α (U46619), a kind gift of Dr. J. Pike from Upjohn, Kalamazoo, USA, was dissolved in ethyl alcohol, stored at −20 °C and diluted in saline just prior to each series of tests.

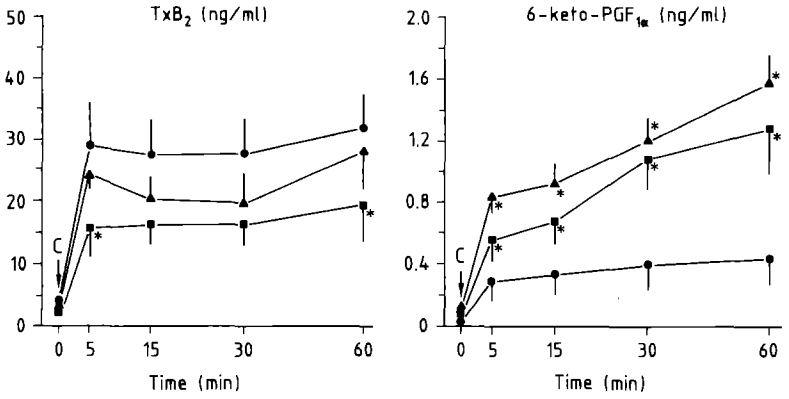

Fig. 1. TxB_2 and 6-keto-PGF_1 production in citrated whole blood from 5 normal subjects at various times after the addition of 1 μg/ml of collagen (C), in the presence of control solution (●—●), picotamide 5×10^{-5} M (▲—▲) and 5×10^{-4} M (■—■). * = $P < 0.01$

Healthy volunteers of both sexes, aged 21 to 42 y, were selected from laboratory staff, students and hospital personnel. They denied having taken any drug in the previous 10 days. *Patients* with peripheral arterial disease (Stage II or III of Fontaine) were considered for an open, uncontrolled long term study when a) they had not received therapy in the previous two months; b) they had had an increased plasma level (> 60 ng/ml) of β-thromboglobulin on at least two recent (within 2 months) occasions; and c) they had given informed consent to the trial. Patients were instructed to avoid the use of other drugs during the study and to maintain their usual condition of life.

Blood was collected under fasting conditions and, unless otherwise specified, 3.8% trisodium citrate 0.1 vol was added as anticoagulant.

Platelet aggregation studies were carried out as described elsewhere [8]. In brief, platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by sequential centrifugation of blood at room temperature, at 150 g for 15 min and at 2000 g for 20 min, respectively. Platelet aggregation curves were recorded at 37 °C using an ELVI-840 dual channel aggregometer (Logos, Milan, Italy). In order to avoid the individual variability of response at a low concentration of inducer, and to obtain maximal platelet stimulation in all individuals, unless otherwise specified, the aggregating agent was added at final concentrations that gave irreversible aggregation curves in normal subjects, namely 2 μM ADP, 10 μM adrenaline, 4 μg/ml collagen, 2.5 mM arachidonic acid and 0.5 μM U46619. Quantitative analysis of the aggregation curves was carried out by measuring: 1) the "lag" time, i.e. the time in seconds between the addition of the stimulus and the beginning of the aggregation curve; 2) the "slope" of the curve, i.e. the percent increase in light transmission observed during the first minute of aggregation; and 3) the "maximal amplitude" (M.A.), i.e. the maximal percentage deflection seen over 5 min.

Platelet malondialdehyde (MDA) generation was measured in washed platelets using the thiobarbituric acid method [9]. For the experiments that required analysis of MDA in PRP the method of Villa et al. [10] was employed.

Thomboxane B_2(TxB_2) and *6-keto-$PGF_1\alpha$* were measured by radioimmunoassay (RIA) using kits from New England Nuclear (Dreieich, West Germany). The generation of TxB_2 and 6-keto-$PGF_1\alpha$ in whole blood was measured by the method of Defreyn et al. [11]. In brief, picotamide or control solutions were incubated with citrated blood at 37 °C for 15 min. After a subaliquot had been withdrawn for the base line assay, 1 μg/ml collagen was added and further samples were taken at fixed intervals. Samples were immediately centrifuged at 4000 g for 2 min and the plasma stored at −20 °C (for no more than 10 days) for RIA. For the ex vivo studies, blood samples were anticoagulated with a mixture of indomethacin 0.1 mg · ml^{-1} and EDTA 0.033 M. Plasma for 6-keto-$PGF_1\alpha$ assay was then obtained by centrifugation at 2000 g for 20 min at 0 °C. To measure serum levels of TxB_2, parallel samples of blood were collected in glass tubes, incubated with 1 U · ml^{-1} of thrombin at 37 °C for 30 min, and then centrifuged at 2000 g for 20 min.

The *reptilase clot retraction test* was performed according to De Gaetano et al. [12]. PRP 0.5 ml from 3 normal volunteers was incubated with picotamide or control solution at 37 °C for 15 min, then 0.1 ml U46619 (0.5 μM final concentration) and 0.1 ml reptilase reagent were added in succession. The mixture was rapidly transferred to glass tubes and kept at 37 °C. Tubes were inspected every 10 min for 1 h and the degree of clot retraction was evaluated by relating the volume of serum to the total amount of the initial mixture.

The *bleeding time* was measured by a standardized template method [13].

Statistical evaluation of the results was performed by Wilcoxon's signed rank test and Student's *t*-test for paired samples. Results are expressed as mean with (SD).

Results

Arachidonic acid-induced platelet aggregation, TxB_2 generation and MDA production in PRP

The effect of picotamide on arachidonic acid-induced platelet aggregation, TxB_2 generation and MDA production was simultaneously assessed in PRP from 11 healthy volunteers. PRP 1 ml (2×10^8 platelets · μl^{-1}) was incubated for 15 min at 37 °C with increasing concentrations of picotamide or control solutions, and placed in an aggregometer cuvette. Arachidonic acid 2.5 mM (final conc) was added and platelet aggregation was recorded for 5 min. An aliquot (200 μl) was then withdrawn from the cuvette for the RIA of TxB_2 and the remainder was used to measure MDA [10]. The results are summarized in Table I.

Table 1. Platelet aggregation, MDA production and TxB_2 generation induced by arachidonic acid (2,5 mM) in PRP from 11 healthy volunteers in the presence of control solution or increasing concentrations of picotamide. In these experiments, MDA generation was measured according to Villa et al. [10] (*$P < 0.01$; $ $P < 0.05$)

	Control	Picotamide (G137)		
		5×10^{-6} M	5×10^{-5} M	5×10^{-4} M
Platelet aggregation				
– Lag time (s)	46.1 (19.2)	55.0 (20.5)	88.2 (86.9)	119 (124)*
– Slope (%)	42.5 (16.6)	35.2 (13.1)	30.2 (19.4)	18.7 (16.3)*
– M.A. (%)	81.9 (6.1)	76.9 (5.7)	69.3 (23.9)*	33.3 (26.1)*
Platelet MDA production ($nM/3 \times 10^8$ pl)	3.41 (1.16)	3.07 (1.13)	2.06 (0.95)*	1.65 (1.05)*
Platelet TxB_2 generation ($ng \cdot ml^{-1}$)	2.41 (1.76)	1.87 (1.04)$	0.62 (0.68)*	0.10 (0.06)*

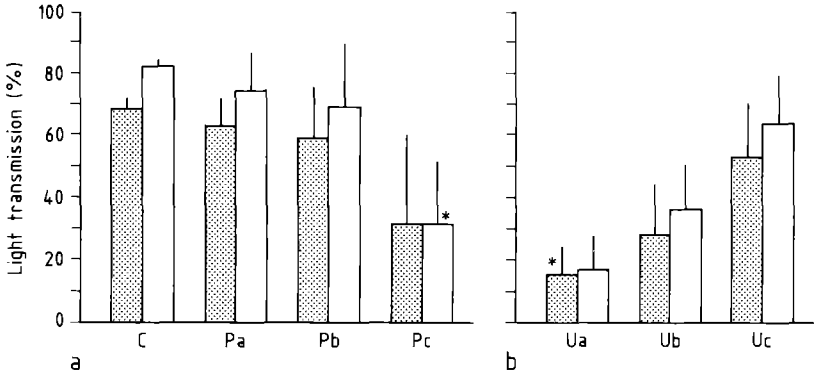

Fig. 2 a, b. Effect of picotamide on U46619-induced platelet aggregation. **a** PRP from 5 healthy volunteers was incubated at 37 °C for 15 minutes with increasing concentrations of picotamide ($Pa = 1 \times 10^{-6}$ M; $Pb = 1 \times 10^{-5}$; $Pc = 1 \times 10^{-4}$ M) or control solutions (C) and platelet aggregation was then induced by U46619 (0.5 μM final concentration); **b** PRP from healthy volunteers was incubated with picotamide (2.5×10^{-4} M final concentration) and platelet aggregation was then induced with increasing concentrations of U46619 (Ua = 0.5 μM; Ub = 0.75 μM; Uc = 1 μM) and compared to that observed when PRP was incubated with control solution (C) and stimulated with 0.5 μM U46619. Platelet aggregation is expressed as "slope" and "maximal amplitude" (mean and SD) of the aggregation curve □ Slope (%) ▨ M.A. (%)

Dose-dependent inhibition of arachidonic acid-induced platelet aggregation was observed in all the subjects tested and was found to be statistically significant in the presence of the two higher concentrations of picotamide. The drug prolonged the "lag" time as well as decreased the "slope" and the "maximal amplitude" of the aggregation curves. Inhibition of arachidonic acid-induced platelet aggregation was accompanied by a consistent dose-dependent reduction in MDA generation and a more pronounced decrease in TxB_2 synthesis. The $IC_{50\%}$, i.e. the concentration of drug required to achieve the half maximal effect, were 4×10^{-4} M for arachidonic acid-induced platelet aggregation (maximal amplitude), 2.8×10^{-5} M for TxB_2 generation and 3.4×10^{-4} M for MDA production.

TxB_2 and 6-keto-$PGF_1\alpha$ generation in whole blood

The effect of two concentrations of picotamide (5×10^{-5} and 5×10^{-4} M) on the generation of TxB_2 and 6-keto-$PGF_1\alpha$ was evaluated in citrated whole blood from 5 normal subjects (Fig. 1). In comparison with control solutions, picotamide decreased TxB_2 synthesis, and the effect was statistically significant with the highest concentration 5 and 60 min after the addition of collagen. The production of 6-keto-$PGF_1\alpha$, however, was significantly increased by both picotamide concentrations at all the times studied.

Platelet aggregation and clot retraction induced by U46619

Picotamide exerted a dose-dependent inhibitory effect on platelet aggregation induced by the prostaglandin endoperoxide analogue U46619 (Fig. 2a); the inhibition was significant at the highest concentration. The $IC_{50\%}$ was estimated to be 7.8×10^{-5} M. The effect could be overcome by increasing the endoperoxide analogue concentration (Fig. 2b), so that a significant difference from the control solution was not detected when platelets were stimulated with 1 μM U46619. The same inhibitory activity was also observed in PRP obtained from the same normal subjects 12 h after they had taken 0.8 g aspirin (data not shown).

The antagonism on TxA_2 receptors was confirmed by the observation of reduced U46619-induced clot retraction when PRP from 3 normal subjects was incubated with 2.5×10^{-4} M picotamide (Fig. 3).

Ex vivo studies

A single dose (1 g, four 250 mg capsules) was orally administered to 24 healthy volunteers of both sexes, and blood was collected before and after 2, 4, 6 and 10 h. As summarized in Table II, ADP-induced platelet aggregation was not affected by the drug, while significant inhibition of the platelet aggregation induced by epinephrine, collagen, arachidonic acid and U46619 was observed between the 4th and the 10th hours, when platelet MDA and TxB_2 synthesis were also significantly reduced. No significant change was detected in plasma 6-keto-$PGF_1\alpha$.

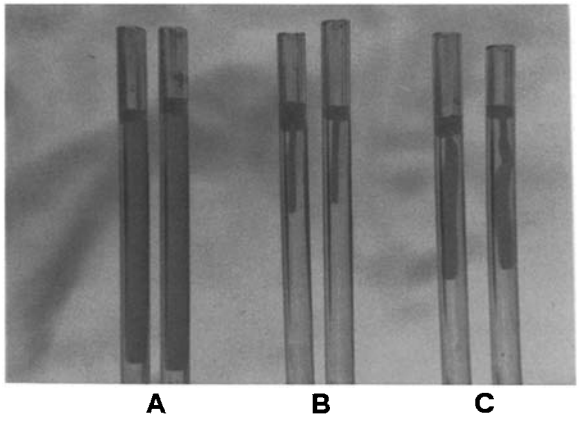

Fig. 3. Reptilase clot retraction observed in PRP stimulated with U46619. A = Reptilase; B = Reptilase + U46619 (1 μM) + control solution; C = Reptilase + U46619 (1 μM) + picotamide 2.5×10^{-4} M (Time of observation = 1 h)

Table 2. Platelet aggregation, MDA production, serum levels of thromboxane B_2, and plasma levels of 6-keto-$PGF_1\alpha$ after a single dose (1 g) of Picotamide in 24 healthy subjects. The data are expressed as mean (± SD, in parenthesis). Platelet aggregation is represented as "maximal amplitude" of the curves; equivalent changes were observed in the other quantitative parameters ($ = $P < 0.05$; * = $P < 0.01$)

	Before	2 h	4 h	6 h	10 h
Platelet aggregation by:					
ADP	79.5	80.7	82.6	76.8	80.0
(2 μM)	(9.7)	(10.0)	(6.5)	(9.3)	(12.1)
Epinephrine	72.6	66.2	62.3	58.3$	63.2
(10 μM)	(12.5)	(21.0)	(19.3)	(17.6)	(25.1)
Collagen	77.3	76.5	71.3	60.7$	68.6
(4 μg · ml^{-1})	(7.0)	(9.7)	(8.4)	(7.3)	(9.9)
Arachidonic	72.5	65.8	53.5$	51.4*	39.5*
acid (1 mM)	(12.8)	(18.3)	(14.0)	(15.3)	(17.2)
U-46619	77.9	72.9	70.8	49.5*	40.3*
(0.5 μM)	(6.4)	(7.9)	(8.1)	(23.3)	(25.4)
MDA	4.35	3.69	4.42	3.52$	3.68$
(nM/3 × 10^8 pl.)	(0.72)	(1.70)	(1.84)	(1.32)	(1.41)
TxB_2, serum	777	568	653	553$	488*
(ng · ml^{-1})	(214)	(387)	(403)	(351)	(323)
6-keto-$PGF_1\alpha$	38.5	36.7	33.5	44.3	38.6
(pg · ml^{-1})	(22.7)	(10.8)	(11.4)	(12.3)	(12.9)

In an open, uncontrolled study ten patients with peripheral arterial disease and increased plasma β-thromboglobulin received picotamide capsules 300 mg qid, for three months.

Before and during treatment (on Days 7, 30 and 90, before the morning dose), the following tests were performed: a) RIA for plasma β-thromboglobulin; b) platelet aggregation by ADP, collagen, epinephrine and arachidonic acid; c) MDA generation by washed platelets; and d) bleeding time.

A persistent decrease in plasma level β-thromboglobulin was rapidly produced by picotamide. Although platelet aggregation due to arachidonic acid and collagen was significantly lower than baseline values in those patients at certain times during the study, the differences were slight. No significant change was found in platelet aggregation induced by ADP or epinephrine (data not shown), in platelet MDA generation or in the bleeding time (Table 3). No side effects were reported either by normal subjects or patients during picotamide administration.

Discussion

The present investigation was undertaken to study the mechanism by which the non prostanoid compound picotamide produces in vitro inhibition of arachidonic acid-induced aggregation of human platelets [6], and to ascertain whether the drug was active and safe on oral administration to man. Since picotamide was found to be a rather selective inhibitor, in that it did not affect ADP-induced platelet aggregation and only slightly reduced "in vitro" collagen and epinephrine-induced aggregation [5, 6], we focused our attention on platelet prostaglandin metabolism. Platelet cyclooxygenase transforms arachidonic acid into prostaglandin endoperoxides, which aggregate platelets either directly or after conversion to thromboxane A_2. The marked inhibitory activity of picotamide on arachidonic acid-induced platelet aggregation, confirmed in this study (Table I), could be ascribed to an enzyme block (cyclooxygenase or thromboxane-synthetase) or to an antagonism at the thromboxane/endoperoxide receptor level. The interference of picotamide with the enzyme cyclooxygenase can be reasonably excluded because the drug did not suppress the synthesis of 6-keto-$PGF_1\alpha$ in whole blood but, on the contrary, increased it. These data are also in accordance with previous observations of a complete lack of antiinflammatory activity of picotamide [6].

The inhibition of arachidonic acid-induced platelet aggregation by picotamide was found to be accompanied by a marked decrease in platelet thromboxane B_2 production and a less pronounced, but significant reduction in MDA generation. These findings suggested a possible inhibitory activity on the enzyme thromboxane-synthase, since this enzyme, besides producting thromboxane A_2, simultaneously converts PGH_2 into 12-hydroxy-5-cis-8-trans-hepta-decatrienoic acid (HHT) and MDA [3]. Further evidence for the inhibitory activity of picotamide on thromboxane-synthetase was provided by the observation that the drug significantly increased the synthesis of 6-

Table 3. Effect of 3 month picotamide administration (300 mg qid) on platelet function tests in 10 patients with peripheral obstructive arterial disease ($ = $P < 0.05$; * = $P < 0.01$)

	Before	7th day	1st month	3rd month
β-thromboglobulin				
(ng · ml^{-1})	133 (65.5)	55.3 (46.6)*	68.5 (46.6)$	73.1 (54.7)
Platelet aggregation by AA (1 mM)				
– Lag time (s)	91.5 (44)	101.5 (76.5)	117.7 (81.6)	105.0 (91.0)
– M.A. (%)	80.2 (3.4)	64.9 (25)$	69.0 (22)	68.8 (21.4)
Platelet aggregation by collagen (4 μg/ml)				
– Lag time (s)	44.5 (10.1)	57.0 (9.4)$	58.8 (13.4)$	53.7 (17.0)
– M.A. (%)	84.0 (3.9)	79.8 (5.0)$	78.4 (4.8)$	80.0 (3.7)
Platelet MDA production				
(nM/3 × 10^8 pl)	3.98 (1.8)	3.35 (1.5)	3.45 (1.3)	2.76 (1.7)
Bleeding time (min)	2.58 (1.0)	3.44 (3.3)	3.06 (2.2)	3.15 (1.7)

keto-$PGF_1\alpha$ in whole blood. An impaired generation of thromboxane A_2 and MDA synthesis concomitant with an increase of prostacyclin synthesis has been reported to be a typical feature of patients with congenital thromboxane-synthetase deficiency [14] and in studies of selective thromboxane-synthetase inhibitors [11]. A reorientation of endoperoxide metabolism following thromboxane-synthetase block has been proposed to explain the observed rise in prostacyclin synthesis. Indeed, the inhibition of thromboxane A_2 synthesis by picotamide has been recently shown to be associated with a remarkable increase in platelet PGE_2 [15].

The rise of 6-keto-PGF_1 induced by picotamide in vitro was already apparent with drug concentrations lower than those required to give significant inhibition of TxB_2 synthesis. Similar observations have been reported by Gresele and coworkers [15] who proposed a possible direct stimulatory effect of picotamide on PGI_2 synthesis.

The inhibiton of thromboxane B_2 synthesis by picotamide was consistently accompanied by a decreased arachidonic acid-induced platelet aggregation in all the subjects studied, while it is known that selective inhibitors of thromboxane-synthase suppress arachidonic acid-induced platelet aggregation only in a small percentage of individuals, generally defined as "responders" [16, 17]. These observations suggested that additional mechanisms could be operative in picotamide-mediated inhibition of platelet aggregation. The ability of the drug to inhibit U46619-induced platelet aggregation and clot retraction strongly indicates that picotamide is also an antagonist of thromboxane/endoperoxide receptors in human platelets, possibly through a competitive mechanism of action.

A rise of intraplatelet levels of cyclic AMP (cAMP), due to the accumulation of antiaggregatory prostaglandins that stimulate adenylate cyclase, might also account for the diminished platelet response to U46619 [17, 18], but this mechanism is probably not prominent in the inhibitory effects of picotamide since it would counteract the aggregation induced by whatever stimulus, including ADP [19]. Picotamide by itself does not increase cAMP in unstimulated platelets [15], and the combined block of thromboxane synthetase and receptors using an association of the respective selective inhibitors has been reported to produce an increase in intraplatelet cAMP only in stimulated platelets [20].

The combination of thromboxane-synthetase inhibition and thromboxane A_2 receptor antagonism in a single compound, now described for other experimental drugs [21], makes picotamide an interesting antithrombotic drug. It has been proposed that the association of dazoxiben, a selective thromboxane A_2-synthetase inhibitor, with BM 13177, a selective receptor antagonist, could represent a step forward in antithrombotic strategy [20, 22] because the receptor antagonism would neutralize the activity of proaggregatory endoperoxides accumulated after thromboxane-synthetase block. Experimental in vivo studies in the animal [23] and in man [20] seem to support this suggestion. In an experimental model of endotoxic shock, picotamide proved to be as effective as a receptor antagonist (BM 13177) in preventing endotoxin-induced death in rabbits [22], while dazoxiben alone was not effective.

Our preliminary study on the oral administration of picotamide to healthy subjects and patients with vascular disease suggests that the drug is an effective and safe inhibitor of platelets in vivo. After a single dose administered to healthy subjects, the maximal inhibitory effect was observed 4–10 h later (Table 2) while plasma peak levels occur at 2–3 h (M. De Regis, personal communication). This delay could be explained assuming that the in vivo effect on platelet function is mediated by one or more active metabolites, and this hypothesis is further supported by the observation that the picotamide concentrations necessary to obtain significant effects in vitro (5×10^{-4} M) are probably not achievable in vivo, unless the drug is actively accumulated within the platelet, as recently suggested by Di Perri et al. [25]. The presence of active metabolites in blood after oral administration of picotamide is currently under investigation.

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