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IN VIVO AND *IN VITRO* EFFECTS OF NAFAZATROM (BAY G 6575), AN ANTITHROMBOTIC COMPOUND, ON ARACHIDONIC ACID METABOLISM IN PLATELETS AND VASCULAR TISSUE

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Abstract—Nafazatrom, given acutely to male volunteers, had no effect on platelet aggregation, associated thromboxane B_2 (TXB_2) formation or the evaluated hormonal, renal and cardiovascular parameters. Only slight increases in plasma levels of 6-keto-$PGF_{1\alpha}$ and in platelet counts were observed. However, a marked influence of nafazatrom on arachidonic acid metabolism in certain *in vitro* systems was found. Prostaglandin synthesis by rabbit kidney cortex microsomes was significantly enhanced, PGI_2 being stimulated the most. In normal human platelets arachidonic acid metabolism was not influenced significantly by nafazatrom which was taken up by the platelets in a dose-dependent manner. In contrast, in platelets with a high peroxide level probably due to depletion of reducing cofactors, 12-hydroperoxy-eicosatetraenoic acid was transformed to 12-hydroxy-eicosatetetraenoic acid by nafazatrom, while the formation of TXB_2 was stimulated. These findings suggest that nafazatrom may act as a reducing cofactor for the hydroperoxidase involved in the cyclooxygenase- and lipoxygenase-pathways of arachidonic acid metabolism.

Hydroperoxides like 12- and 15-HPETE* are known to influence the metabolic conversion of arachidonic acid in platelets [1, 2] and vascular tissue [3, 4]. Furthermore enhanced levels of hydroperoxides are claimed to be involved in the pathogenesis of athero-thrombotic processes [5].

Nafazatrom (Bay g 6575, Fig. 1) has been reported to possess considerable antithrombotic and thrombolytic activity in various animal models [6]. Moreover, an antimetastatic potential has been described [7]. The mechanism of action of this compound is presently unclear. Stimulation of prostacyclin biosynthesis, which has been reported from several studies *ex vivo* [8–10], was proposed as a possible mode of action. Subsequently nafazatrom was claimed to serve as a reducing cofactor for the hydroperoxidase component of fatty acid cyclooxygenase [10].

To evaluate the mode of action of nafazatrom further we studied in man the effects on platelet aggregation and TXB_2 formation *ex vivo* and on 6-keto-$PGF_{1\alpha}$ formation *in vivo*. The influence of nafazatrom on arachidonic acid metabolism *in vitro* was investigated in human platelets and in rabbit kidney cortical microsomes.

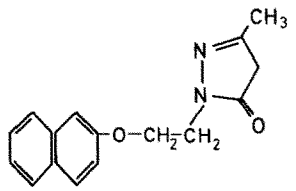

Fig. 1. Chemical formula of nafazatrom (Bay g 6575), 1-[2-(β-naphthyloxy)ethyl]-3-methyl-2-pyrazolin-5-one.

MATERIALS AND METHODS

In vivo *studies*

First study. A single dose of 2 g nafazatrom or placebo was administered orally after an overnight fast to six healthy males, aged 25–55 years, double blind, cross over with an 8-week washout period. Blood from an antecubital vein was drawn for hormone analyses and platelet function tests before drug ingestion, at 8 a.m. and 0.5, 1, 2, 3, 4 and 24 hr after ingestion of the drug. Urine samples were taken before (8 hr period) and 2, 4 and 6 hr after the drug. Parameters measured were: blood pressure and heart rate at 30 min intervals; complete blood count, platelet count (Coulter counter) and screening for liver and kidney function before and 24 hr after administration of the drug; platelet aggregation in platelet-rich plasma on stimulation with arachidonic acid, collagen and ADP by Born's method [11] and associated TXB_2 formation on stimulation with arachidonic acid and collagen by RIA [12] before and 1 and 3 hr after nafazatrom. In each plasma sample: renin by RIA (EDTA-plasma) [13], cate-

* Abbreviations: HPLC = high pressure liquid chromatography; RIA = radioimmunoassay; TLC = thin layer chromatography; PG = prostaglandin; TXB_2 = thromboxane B_2; 12-HHT = 12-hydroxy-heptadecatrienoic acid; 12-HETE = 12-hydroxy-eicosatetraenoic acid; 12-HPETE = 12-hydroperoxy-eicosatetraenoic acid; 15-HPETE = 15-hydroperoxy-eicosatetraenoic acid; THETEs = positional isomers of trihydroxy-eicosatetraenoic acids; HEPA = 10-hydroxy-11,12-epoxy-eicosatetraenoic acid.

cholamines (norepinephrine, epinephrine, dopamine) radioenzymatically (EGTA-plasma, glutathione) [14] and 6-keto-$PGF_{1\alpha}$ by RIA [15, 16]. In each urine sample: volume, Na^+ and K^+ and PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ by RIA [17, 18], aldosterone by RIA [19] and kallikrein by use of a chromogenic substrate (S-2266, Kabi, Stockholm).

Second study. Since the pharmacokinetics of nafazatrom and potentially active metabolites have not been resolved yet a second study was performed. Three doses of nafazatrom, 0.8 g each, were administered orally at 8 a.m. and 30 and 60 min later to eight healthy males, aged 23–27 years. Analyses performed were as follows. Platelet counts (by phase contrast microscopy) and plasma levels of 6-keto-$PGF_{1\alpha}$ [15, 16] were measured before and 90 min after ingestion of the first dose of nafazatrom. Platelet counts were repeated 24 hr later.

In vitro *studies*

Incubation of rabbit kidney cortex microsomes with [^{14}C]arachidonic acid in the presence of nafazatrom. Rabbit kidney cortex microsomes were prepared according to Blackwell *et al.* [20] and stored lyophilized at −70°. Preincubations were performed in 0.5 ml Tris–HCl (50 mM, pH 7.4, 1 mM EDTA) with 5 mg of lyophilized microsomes (42% protein, measured by the Lowry procedure [21]) and 0 (control), 50 μM, 250 μM and 1 mM nafazatrom for 5 min at 20°. Incubation was started by the addition of the microsomes to 4 μCi of [^{14}C]arachidonic acid in 20 μl ethanol, corresponding to a final concentration of 150 μM arachidonic acid which resulted in a maximum conversion to PGs. After 1 hr at 37° the incubate was acidified to pH 3.5 with concentrated HCOOH and PGs were extracted by a C18 reverse-phase cartridge (Sep-Pak®, Waters Assoc., Milford, U.S.A.) [22]. The extract was applied onto a TLC plate together with authentic standards and developed in ethyl acetate–trimethylpentane–acetic acid–H_2O (110:50:20:100, organic phase). Radioactive zones were located by radiochromatogram scanning, scraped off the plate and eluted with CH_3OH. Radioactivity was determined by liquid scintillation counting. The amount of PGs formed was expressed relative to the control experiment, which was taken as 100%. TXB_2 and PGE_2 were further separated by reverse-phase HPLC using a μ-Bondapak C18 column (Waters Assoc., Milford, U.S.A.) and H_2O–acetonitrile–acetic acid (740:260:2) as the solvent system.

Binding studies of [^{14}C]nafazatrom to human platelets. Human blood paltelets were prepared as described by Okuma and Uchino [23]. Contamination with leucocytes was excluded by light microscopy.

(a) In order to study the uptake of [^{14}C]nafazatrom, 200 μl of platelet suspension (corresponding to 6×10^8 platelets, resuspended in Tris-HCl, 25 mM, pH 7.4) was incubated with 0 (control), 25, 50, 100 and 250 μM [^{14}C]nafazatrom (1.7 μCi/mg) in 1.1 ml Tris–HCl (50 mM, pH 7.4) for 8 min at 37°. Thereafter platelets were washed three times with Tris–HCl and counted for radioactivity. The same incubations were performed in the presence of arachidonic acid (50 μM).

(b) To evaluate the kinetics of the uptake of nafazatrom, 6×10^8 platelets were incubated with 250 μM [^{14}C]nafazatrom for 1, 2, 5, 10 and 30 min, cooled to 0°, washed and then counted for radioactivity.

(c) For the evaluation of the intracellular distribution of nafazatrom, 6×10^8 platelets were preincubated with 250 μM [^{14}C]nafazatrom, washed and disrupted by freeze-thawing three times (liquid N_2, water bath 37°). The platelet homogenate was centrifuged at 100,000 *g* for 1 hr and the pellet and supernatant were counted for radioactivity.

Effects of nafazatrom on the uptake and conversion of [^{14}C]arachidonic acid by human platelets. (a) To investigate the effects of nafazatrom on the uptake of [^{14}C]arachidonic acid, 200 μl of platelet suspension (6×10^8 platelets) was incubated with 0 (control), 50, 100 and 250 μM unlabelled nafazatrom for 1 min at 20° and then with 0.5 μCi [^{14}C]arachidonic acid for 8 min at 37°. After washing, platelets were counted for radioactivity.

(b) To study the influence of nafazatrom on conversion of [^{14}C]arachidonic acid, 200 μl of platelet suspension (6×10^8 platelets, resuspended in Tris–HCl, 25 mM, pH 7.4) was preincubated with 0 (control), 250 μM nafazatrom and 2 mM aspirin (24) in 0.3 ml Tris–HCl (0.1 M, pH 7.4) for 5 min at 37°. In the same way, preincubations with suspensions of platelets lysed by freeze-thawing three times (liquid N_2, water bath 37°) or stored at −20° for 1–2 weeks were performed. These platelets usually exhibited additional formation of THETEs and HEPA upon incubation with arachidonic acid. Reactions were started by adding the platelets to 0.5 μCi [^{14}C]arachidonic acid in 10 μl ethanol, giving a final concentration of 20 μM. Incubations at 37° were stopped after 8 min by bringing the pH to 3.5 with 1 N HCl. Acetone (400 μl) was added and the mixture was extracted twice with 4 ml ethyl acetate. After TLC separation with two different solvent systems (system 1: ethyl acetate–isooctane–acetic acid–water, 110:50:20:100, organic phase; system 2: ligroin–ether–acetic acid, 40:60:1) radioactive products were visualized by radiochromatogram scanning. Radioactively labelled authentic standards served as reference compounds. TXB_2 peaks were scraped off the plates, eluted with CH_3OH and quantified by liquid scintillation counting. 12-HETE and 12-HPETE were also purified by straight-phase HPLC using a μ-Porasil column (Waters Assoc., Milford, U.S.A.) and hexane–2-propanol–acetic acid (989:10:1) as the solvent system [25].

Materials

Nafazatrom (Bay g 6575 and [^{14}C]nafazatrom (34.4 μCi/mg) were a gift from Bayer AG (Wuppertal, F.R.G.). Solutions of nafazatrom for *in vitro* studies were prepared as described in [7]. [^{14}C]arachidonic acid (56.5 mCi/mmole) and [^{3}H]prostaglandin standards were purchased from New England Nuclear (Dreieich, F.R.G.). Arachidonic acid and ADP were from Serva (Heidelberg, F.R.G.), collagen from Hormon-Chemie (Munich, F.R.G.), and silica TLC plates G 254 and all solvents used were from Merck (Darmstadt, F.R.G.).

RESULTS

In vivo *studies*

When nafazatrom was given in a single dose (2 g orally) to six healthy male volunteers no effects were seen on blood pressure, heart rate, the evaluated vasoactive hormonal systems and sodium and potassium excretion. *Ex vivo* platelet aggregation and associated TXB_2-formation were also not influenced by the drug. However, trends towards increased platelet counts 24 hr after nafazatrom (control 256 ± 38, nafazatrom 299 ± 44 × $10^3/\mu l$, mean ± S.D., $P < 0.05$) and towards increased levels of plasma 6-keto-$PGF_{1\alpha}$ 2 hr after the drug (control 9 ± 14, nafazatrom 13.2 ± 19 pg/ml, mean ± S.D., n.s.) were observed. Safety data (complete blood counts, liver and kidney function) were unchanged after nafazatrom.

In the second experiment with three oral doses of 0.8 g nafazatrom given in 30 min intervals, the tendency for increased platelet counts and plasma 6-keto-$PGF_{1\alpha}$ levels observed in the first experiment was verified. The results are shown in Table 1.

Table 1. Platelet counts and plasma 6-keto-$PGF_{1\alpha}$ levels before and after ingestion of 3 × 0.8 g nafazatrom in 30 min intervals

Time (hr)	−1	+1.5	+24
Platelet count ($\times 10^3/\mu l$)	204 ± 40	227 ± 44*	225 ± 36*
6-Keto-$PGF_{1\alpha}$ (pg/ml)	3.5 ± 2.1	6 ± 2.7†	n.d.

Mean ± S.D. significance: * $P < 0.02$; † $P < 0.05$ (paired *t*-test). n.d. = Not determined.

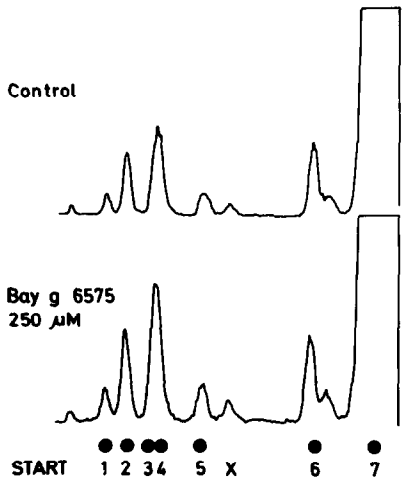

Fig. 2. Radiochromatograms of incubations of [^{14}C]arachidonic acid with rabbit kidney cortex microsomes. R_f values of reference compounds are indicated. 1 = 6-Keto-$PGF_{1\alpha}$, 2 = $PGF_{2\alpha}$, 3 = TXB_2, 4 = PGE_2, 5 = PGD_2, 6 = OH-fatty acids, 7 = arachidonic acid, × = unknown. Solvent system: ethyl acetate–tri-methylpentane–acetic acid–H_2O, 110:50:20:100 (organic phase).

In vitro *studies*

Influence of nafazatrom on PG formation by rabbit kidney cortex microsomes. Figure 2 shows the radiochromatograms of incubations of [^{14}C]arachidonic acid with rabbit kidney cortical microsomes. Total conversion of arachidonic acid to PGs was about 2% in the control experiment. In Table 2 stimulation of PG formation by various concentrations of nafazatrom is presented, showing a bell-shaped dependency for $PGF_{2\alpha}$, TXB_2/PGE_2 and PGD_2. The stimulation of conversion of [^{14}C]arachidonic acid was most pronounced for 6-keto-$PGF_{1\alpha}$ and PGD_2. TXB_2 and PGE_2 were further separated by reverse-phase HPLC. A significant stimuation of TXB_2, which represents only about 6% of the PGs formed, could not be established. Therefore, stimulation of the TXB_2/PGE_2 fraction was mostly due to increased PGE_2 formation.

Binding studies of [^{14}C]*nafazatrom to human platelets.* When washed human platelets were incubated with increasing concentrations of [^{14}C]nafazatrom, a dose-dependent binding curve was obtained ending in a plateau value above 100 µM. One to two per cent (in the presence of physiological concentrations of albumin 0.25–0.5%) of the total radioactivity was taken up by the platelets and the binding of nafazatrom was complete after 1 min of incubation. When incubations were performed in the presence of arachidonic acid, 3 to 5-fold higher amounts of [^{14}C]nafazatrom were bound by the platelets (Fig. 3). Platelets incubated with [^{14}C]nafazatrom were lysed by freeze-thawing and centrifuged at 100,000 *g*. The membrane pellet contained 76 ± 5.3% (S.D., $n = 3$ experiments) of the radioactivty, the rest was recovered in the supernatant.

Influence of nafazatrom on arachidonic acid uptake and metabolism by human platelets. Nafazatrom did not influence the uptake of [^{14}C]arachidonic acid by human platelets. Furthermore, formation of TXB_2, 12-HHT and 12-HETE was not modulated by 100 µM nafazatrom when platelets were incubated with 20 µM [^{14}C]arachidonic acid. At 250 µM nafa-

Table 2. Formation of PGs by rabbit kidney cortex microsomes incubated with [^{14}C]arachidonic acid

PGs	Nafazatrom (µM) 0	50	250	1000
6-Keto-$PGF_{1\alpha}$	100	140 ± 8‡	168 ± 9§	214 ± 14†
$PGF_{2\alpha}$	100	113 ± 4.5*	137 ± 5§	131 ± 11§
TXB_2/PGE_2	100	128 ± 11	147 ± 9†	114 ± 5.5†
PGD_2	100	145 ± 13	154 ± 12†	128 ± 7.5

Results are expressed as a percentage of the amount of prostaglandin formed in the absence of nafazatrom. Mean ± S.E.M., $n = 5$. Significance: * $P < 0.025$, † $P < 0.01$, ‡ $P < 0.005$, § $P < 0.0025$ (Student's *t*-test).

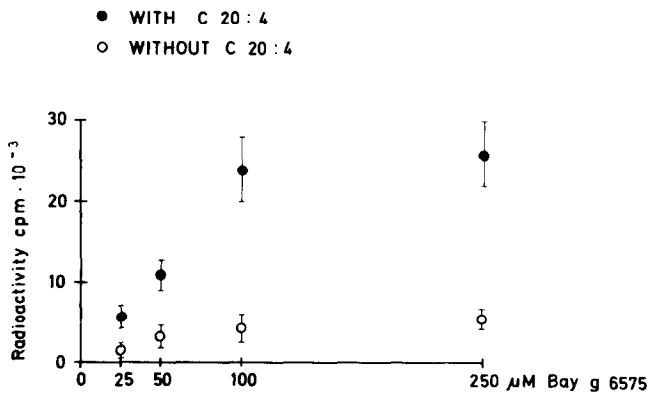

Fig. 3. Radioactivity bound by platelets when incubated with various concentrations of [^{14}C]nafazatrom in the presence and absence of arachidonic acid. Mean ± S.E.M., $n = 4$.

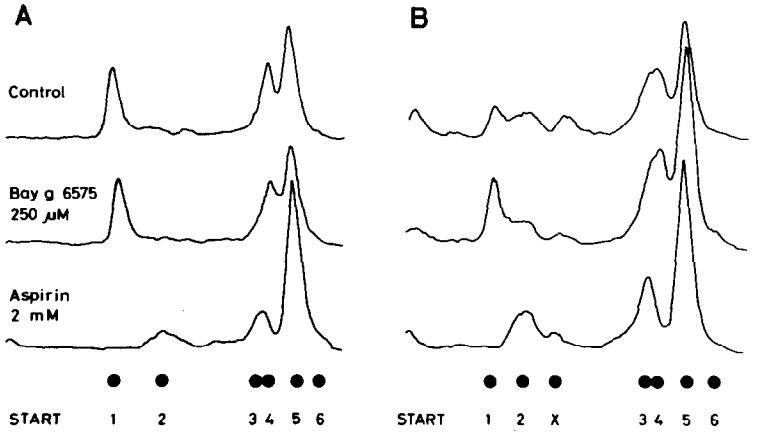

Fig. 4. Radiochromatograms of incubations of [^{14}C]arachidonic acid with human platelets. A = intact platelets, B = lysed platelets. 1 = TXB_2, 2 = THETEs, 3 = HEPA, 4 = 12-HHT, 5 = 12-HETE, 6 = arachidonic acid, × = unknown. Solvent system as in Fig. 2.

zatrom even a slight inhibition (n.s.) of TXB_2 formation was found. However, in platelets showing the additional formation of hydroxy acids tentatively identified as THETEs and HEPA [26], a significant stimulation of TXB_2 formation by nafazatrom was observed (Fig. 4, Table 3). In these platelets high levels of 12-HPETE were also observed. Nafazatrom stimulated the transformation of 12-HPETE to 12-HETE in these platelets to a similar extent as reduced glutathione (Fig. 5). The increase in 12-HETE and the decrease of 12-HPETE levels under the influence of nafazatrom were further confirmed by straight-phase HPLC using radioactively labelled authentic standards.

Table 3. TXB_2 formation by human platelets incubated with 20 µM [^{14}C]arachidonic acid

	Nafazatrom (µM)	
	0	250
Intact platelets	100	86 ± 18
Lysed platelets	100	182 ± 26*

Results are expressed as a percentage of the amount of TXB_2 formed in the absence of nafazatrom. Mean ± S.E.M., $n = 4$. Significance: * $P < 0.025$ (Student's t-test).

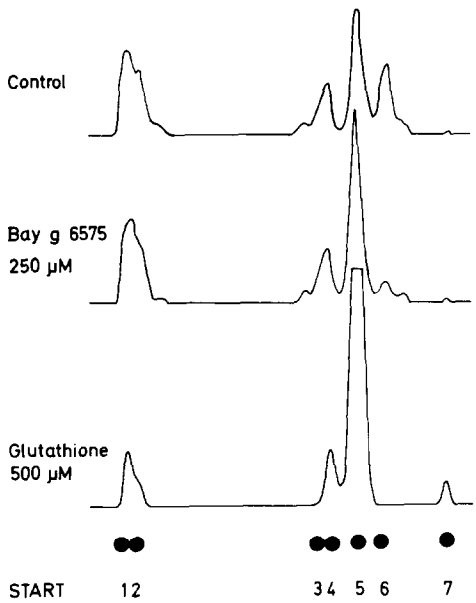

Fig. 5. Radiochromatograms of incubations of [^{14}C]arachidonic acid with lysed human platelets. 1 = TXB_2, 2 = THETEs, 3 = HEPA, 4 = 12-HHT, 5 = 12-HETE, 6 = 12-HPETE, 7 = arachidonic acid. Solvent system: ligroin–ether–acetic acid, 40:60:1.

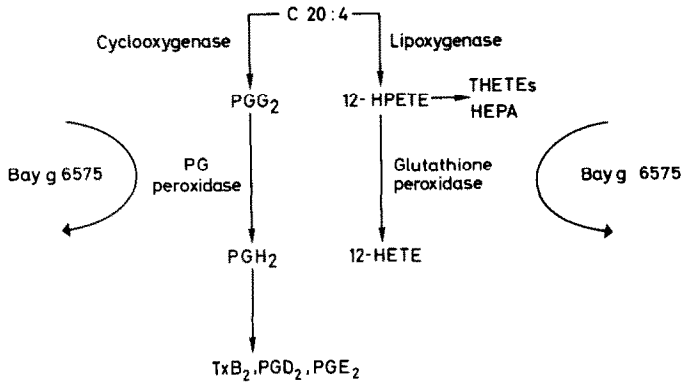

Fig. 6. Scheme of the proposed effect of nafazatrom (Bay g 6575) on arachidonic acid metabolism by human platelets.

DISCUSSION

A major goal of our *in vivo* studies, where nafazatrom was given acutely to normal volunteers, was the assessment of 6-keto-$PGF_{1\alpha}$ plasma levels. However, only slight increases were found, suggesting that the stimulatory effect of nafazatrom on PGI_2 formation may be small under physiological conditions. Furthermore, we could not observe any effects of nafazatrom on platelet aggregation *ex vivo* and associated TXB_2 formation. We also evaluated the effects of nafazatrom on cardiovascular and renal functions, since the drug is structurally related to muzolimine (Bay g 2821), a non-sulphonamide diuretic [27]. But no residual activity of the drug on these parameters was found. The slight increase in platelet counts after nafazatrom administration may be due to a prolonged half-life of platelets or to an increased *de novo* formation.

In contrast, our *in vitro* experiments exhibited a marked influence of nafazatrom on arachidonic acid metabolism. To study the effects of the drug on cyclooxygenation of arachidonic acid, we chose rabbit kidney cortical microsomes [20, 28], composed to a predominant part of vascular tissue and having about a 5-fold higher activity than human kidney cortical microsomes [29]. Incubations were performed without any cofactors and showed a dual influence of nafazatrom on PG formation. Stimulation at lower concentrations of nafazatrom may be attributed to enzyme preservation by the drug [30], whereas the inhibitory phase at 1 mM nafazatrom for $PGF_{2\alpha}$, E_2 and D_2 may be explained by the observation that small quantities of hydroperoxides are necessary to initiate the cyclooxygenase reaction [31]. This dual action on cyclooxygenase is a common feature of nafazatrom with many radical-scavenging reducing agents [32]. The finding that stimulation of 6-keto-$PGF_{1\alpha}$ did not show this dual behaviour but was highest at 1 mM nafazatrom might be explained by an additional protective effect of the drug on PGI_2-synthetase, which is most sensitive to radicals generated by hydroperoxides [3, 4, 33, 34].

Nafazatrom is a highly lipophilic compound and interaction with membranes where PG biosynthesis takes place can be assumed. Binding studies of [^{14}C]nafazatrom with human platelets indicated a rapid uptake of the drug in a dose-dependent manner. Between 100 and 250 μM, where maximal effects of nafazatrom on arachidonic acid conversion were observed, a saturation of binding had been achieved. The lipophilic character of the drug was confirmed by the finding that after lysis of the platelets and ultracentrifugation the major portion of [^{14}C]nafazatrom was bound to the membrane pellet. The enhanced uptake of [^{14}C]nafazatrom by platelets in the presence of arachidonic acid may be due to arachidonic acid-induced aggregation of the platelets, which, in turn, may increase the uptake of the drug.

In our *in vitro* studies on the metabolism of exogenous arachidonic acid by intact human platelets, we did not find any significant effects of nafazatrom on the formation of TXB_2, 12-HHT and 12-HETE [35]. However, in platelets with considerable levels of 12-HPETE [36] decomposing non-enzymatically to trihydroxy- and hydroxy-epoxy-fatty acids [26], a significant stimulation of TXB_2 biosynthesis and a decrease of the 12-HPETE levels were observed under the influence of nafazatrom. These findings may be explained according to the hypothetical scheme shown in Fig. 6. In platelets where the levels of reducing cofactors are lowered, PGG_2 and 12-HPETE may accumulate and deactivate the cyclooxygenase [1, 30]. Addition of nafazatrom provides reducing equivalents and thereby reactivates the peroxidases, which, in turn, lower the high peroxide tone. Thus cyclooxygenase activity is restored and 12-HPETE, which is an important regulator of arachidonic acid metabolism in platelets [1], is transformed enzymatically to 12-HETE. The reduction of 12-HPETE either in the presence of nafazatrom or reduced glutathione [37] suggests a similar mode of action for both compounds. In normal intact platelets sufficient amounts of reducing cofactors may be present and therefore nafazatrom does not influence arachidonic acid metabolism to a significant extent.

In conclusion, our data suggest that effects of nafazatrom on vascular and platelet cyclooxygenase and lipoxygenase pathways *in vivo* may be small under normal conditions and therefore difficult to

detect. Only in systems probably depleted of reducing cofactors like microsomes or platelets with a high peroxide level an effect of nafazatrom on arachidonic acid metabolism could be demonstrated. Relatively high nafazatrom concentrations were required to demonstrate these effects *in vitro* contrasting to the *in vivo* potency of the drug in animal models at very low doses [6]. Our findings may well support the hypothesis that nafazatrom is effective *in vivo* at local sites, where decreased levels of reducing cofactors are found and radical formation is increased, as has been claimed in athero-thrombotic and metastatic processes [5, 38].

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