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INHIBITION OF LEUKOTRIENE B_4 FORMATION IN HUMAN NEUTROPHILS AFTER ORAL NAFAZATROM (BAY G 6575)

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Abstract—Three grams of nafazatrom (Bay g 6575), given orally to healthy male volunteers in a single dose, significantly reduce the formation of leukotriene B_4 in polymorphonuclear leukocytes. LTB_4 synthesis fell from 57.1 ± 17.0 ng/10^7 PMNL, mean ± S.D., in control to 34.3 ± 14.4 ng/10^7 PMNL 3 hr after nafazatrom ($2P < 0.001$). *In vitro*, nafazatrom inhibited LTB_4 formation in human PMNL in a dose dependent manner. At 1 μM nafazatrom LTB_4 formation was reduced to 65% of the control value. Nafazatrom had no effect on the excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2, the major urinary metabolites of endogenously synthesized PGI_2 and TXA_2, respectively. Serum levels of TXB_2 in clotted whole blood also remained unchanged.

The inhibitory effect of nafazatrom on leukotriene biosynthesis in human PMNL suggests a therapeutic potential of this drug in processes like allergy and chronic inflammation, where leukotrienes play a pathogenetic role.

Nafazatrom (Bay g 6575) has been shown to possess antithrombotic and antimetastatic properties in several animal models [1, 2]. Stimulation of prostacyclin biosynthesis has been discussed as one possible mode of action [3] and indeed, stimulation of prostacyclin formation has been detected in various *in vitro* systems [4–6]. Since hydroperoxides reduce the synthesis of PGI_2,† a reduction of hydroperoxy levels by nafazatrom may contribute to that increase of PGI_2-synthesis *in vitro*. At variance to the *in vitro* results, no relevant changes of plasma 6-keto-$PGF_{1\alpha}$ concentrations were found and no effects on platelet aggregation and TXB_2 formation were detected *ex vivo* after oral nafazatrom in normal healthy volunteers [5]. One reason for these discrepancies may be that in healthy volunteers no significant levels of hydroperoxides exist *in vivo*. Another explanation might be that possible effects of nafazatrom on vascular and platelet cyclooxygenase are small under normal in vivo conditions and therefore difficult to detect.

Nafazatrom may act also on the lipoxygenase pathway. This possibility has already been addressed in recent *in vitro* studies, where nafazatrom was shown to reduce the 5-lipoxygenation of exogenous arachidonic acid in a tumor cell line [7]. Leukotrienes, derived from cellular arachidonic acid, are biologically important mediators of allergy and inflammation and may also contribute to atherothrombotic processes [8, 9, 10]. LTB_4, synthesized via the 5-lipoxygenase pathway in human neutrophils, exerts a variety of biological effects including chemokinesis, chemotaxis, stimulation of superoxide anion production and release of lysosomal enzymes [8, 9]. Nafazatrom may influence the 5-lipoxygenase activity through its well documented potency as a reducing cofactor [6] and by its action as a reactive scavenger of free radicals [11]. Therefore, we studied the effect of nafazatrom on LTB_4 formation in human polymorphonuclear leukocytes *ex vivo* and *in vitro*. In addition, we reevaluated possible effects of nafazatrom on PGI_2 and TXA_2 production *in vivo* by measuring their major urinary metabolites.

MATERIALS AND METHODS

Materials. Nafazatrom (2,4-dihydro-5-methyl-2-[2-(naphtyloxy)]ethyl-3H-pyrazol-3-one) was synthesized by Bayer AG, F.R.G. and provided as granules. Percoll was from Pharmacia, F.R.G.; bovine serum albumin (essentially fatty acid free), prostaglandin B_2 and ionophore A 23187 were from Sigma, F.R.G.; Hank's balanced salt solution and phosphate buffered saline were purchased from Serva, F.R.G.; SEP-PAK C 18 cartridges were from Waters, F.R.G. and the HPLC-column Nucleosil 5 C 18 from Machery u. Nagel, F.R.G. [^{3}H]-LTB_4 and [^{3}H]-5-HETE were purchased from NEN, U.S.A. Authentic LTB_4 was a gift from Dr. J. Pike, Upjohn Company, Kalamazoo, U.S.A. The TXB_2 antiserum was a gift from Dr. L. Levine, Brandeis University, Waltham, MA.

Study protocol. Nafazatrom (3 g) was given orally to seven healthy, male volunteers, aged 26 to 37 years, weighing between 68 and 90 kg. Subjects with abnormalities of prestudy laboratory data (including complete blood count, clinical chemistry, liver and kidney parameters) were excluded from the protocol. Blood for preparing PMNL and serum for TXB_2 analysis was taken from fasting volunteers before and 3 hr after ingestion of nafazatrom. Urine was collected −12 to 0 before, 0 to 6 and 6 to 12 hr after drug administration and safety data were repeated 24 hr after drug ingestion.

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† Abbreviations: HPLC, high performance liquid chromatography; GCMS, gas chromatography–mass spectrometry; PGI_2, prostaglandin I_2; TXA_2,B_2, thromboxane A_2, B_2; LT, leukotriene; PMNL, polymorphonuclear leukocytes; PGB_2, prostaglandin B_2; 5-HPETE, 5-hydroperoxy-eicosatetraenoic acid; 5-HETE, 5-hydroxy-eicosatetraenoic acid.

Cell preparation. Heparinized blood (50 ml, 10 U/ml) was collected by venipuncture and the platelet rich plasma was removed. After dilution with 10% platelet poor plasma blood was layered on a Percoll gradient and centrifuged according to Hjorth *et al.* [12]. The fraction containing PMNL was removed and washed in phosphate buffered saline (Ca^{2+}-free). After hypotonic lysis of the remaining erythrocytes PMNL were washed twice with phosphate buffer containing 15 mg% bovine serum albumin to remove contaminating platelets as described [13]. PMNL were resuspended in phosphate buffered saline (10^7 PMNL/300 μl saline). This preparation leads to 98% pure PMNL with a viability of 98% as judged by Trypan blue exclusion. The leukocyte to platelet ratio was always greater than 90:1.

Incubation conditions. 10^7 PMNL were preincubated at 37° for 10 min. Then, ionophore A 23187 (10 μM), dissolved in Hank's balanced salt solution was added to give a final volume of 1 ml (final Ca^{2+} concentration 0.8 mM). After 10 min, the incubation was stopped by adding 1.5 vol ice-cold ethanol.

For *in vitro* studies, PMNL were preincubated with nafazatrom in concentrations of 10^{-7} to 3×10^{-5} M for 5 min and stimulated with ionophore A 23187 as described above. A stock solution of nafazatrom (10^{-2} M) in Me_2SO was prepared and diluted with phosphate buffer prior to preincubation.

Extraction, purification and analysis of LTB_4. After precipitation of proteins, the samples were centrifuged and extracted according to [14] using SEP-PAK C 18 cartridges. Samples were analyzed by HPLC using a Nucleosil 5 C 18 column with MeOH:H_2O:acetic acid (70:30:0.01 v/v, pH adjusted to 5.7 with NH_4OH) as mobile phase (flow rate 1 ml/min). Leukotrienes were monitored using a UV spectrometer (Kontron, F.R.G.) set at 280 nm, monohydroxy fatty acids were monitored at 235 nm. Tritiated standards of LTB_4 and 5-HETE were measured using a radioactivity monitor (Berthold, F.R.G.). LTB_4 was calculated using PGB_2 as internal standard added before extraction of the samples.

Determination of urinary 2,3-dinor-TXB_2, 2,3-dinor-6-keto-$PGF_{1\alpha}$ and of serum TXB_2. Products were analyzed as described previously [15]. Briefly, 2,3-dinor-TXB_2 was measured by radioimmunoassay using [^{3}H]-TXB_2 as tracer and a TXB_2-antiserum, which showed a 50–60% cross reaction with authentic TXB_2. After extraction of the urine with SEP-PAK C 18 cartridges 2,3-dinor-TXB_2 and TXB_2 were separated on reverse-phase HPLC. Urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ was quantitated by GC-MS [16] according to a modified method of Falardeau [17] using deuterated 2,3-dinor-6-keto-$PGF_{1\alpha}$ as internal standard. For determination of serum TXB_2 a previously described radioimmunoassay method was used [18].

RESULTS

Synthesis of LTB_4 in human PMNL after nafazatrom

After ingestion of a 3 g dose of nafazatrom, the rate of LTB_4 production in human PMNL isolated 3 hr after dosing was decreased (Fig. 1). This decrease was observed in all volunteers as shown in

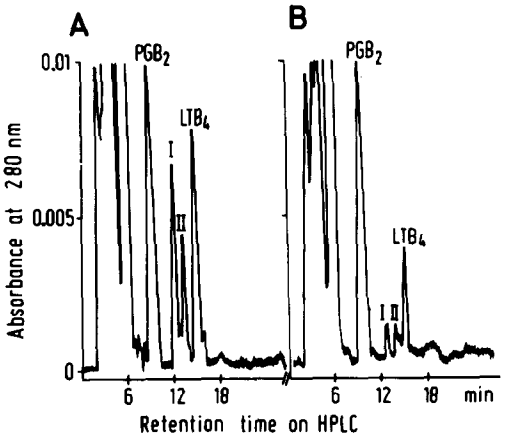

Fig. 1. Two representative HPLC-profiles of leukotriene B_4 isolated from stimulated PMNL before (A) and 3 hr after (B) ingestion of a 3 g dose of nafazatrom. 100 ng PGB_2 were added to the samples as internal standard. Peaks I and II are 5, 12-dihydroxy-6-trans-stereoisomers of LTB_4.

Fig. 2. Synthesis of LTB_4 was 57.1 ± 17.0 ng/10^7 PMNL before and 34.3 ± 14.4 ng/10^7 PMNL after nafazatrom (mean ± S.D., N = 7, $2P < 0.001$). The identity of LTB_4 was confirmed by comparison with authentic standards by HPLC and GC-MS (data not shown). No 5-HETE was detected in control PMNL and in PMNL obtained 3 hr after nafazatrom. Nafazatrom added *in vitro* to human PMNL inhibited formation of LTB_4 in a dose dependent manner. Table 1 shows, that the reduction of LTB_4 formation in human PMNL after *in vitro* pretreatment with 10^{-6} M nafazatrom for 5 min was about 35%. From these data an IC_{50} of approx. 3×10^{-6} M can be calculated.

Excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ and of 2,3-dinor-TXB_2 in urine; TXB_2 formation in serum

The excretion rates of 2,3-dinor-6-keto-$PGF_{1\alpha}$ and of 2,3-dinor-TXB_2 in urine were unaltered 0–6 hr and 6–12 hr after nafazatrom as compared to the control period (−12 to 0 hr) (Table 2). TXB_2 formation in clotted whole blood also was not influenced by nafazatrom (Table 2).

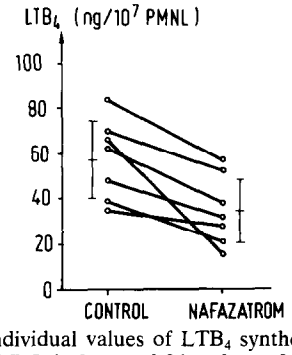

Fig. 2. Individual values of LTB_4 synthesis in stimulated human PMNL before and 3 hr after a 3 g dose of nafazatrom. Mean ± S.D. is also indicated ($2P < 0.001$).

Table 1. Inhibition of LTB_4 formation in human PMNL by different concentrations of Nafazatrom *in vitro*

Control	Nafazatrom 10^{-7} M	10^{-6} M	10^{-5} M	3×10^{-5}
100	98.3 ± 12.7	65.2 ± 6.1	26.3 ± 19.2	n.d.

Cells were preincubated with the drug for 5 min and stimulated with A 23187 (10 μM) for 10 min. Values are given in per cent of LTB_4 formed at control in 10^7 PMNL without the drug (Mean ± S.D., N = 3).
n.d. = not detectable

DISCUSSION

The aim of the present study was to investigate the effects of nafazatrom on arachidonic acid metabolism in human PMNL, which synthesize LTB_4 as the major arachidonic acid metabolite when stimulated with the ionophore A 23187. The demonstration of reduced *ex vivo* synthesis of LTB_4 after oral nafazatrom adds a new aspect in the discussion of the possible mode of action of nafazatrom *in vivo*. To our knowledge, this is the first report of a pharmacological effect of nafazatrom which is demonstrable *ex vivo* in man. Although our data do not allow a firm conclusion regarding the mechanism by which nafazatrom reduces the synthesis of LTB_4, several modes of action seem to be possible:

A reduction of hydroperoxy fatty acids has been described after nafazatrom using exogenous arachidonic acid as substrate [5]. By this mode of action, nafazatrom could reduce the concentration of 5-HPETE and consecutively the formation of LTA_4. However, under physiological conditions, endogenous cellular arachidonic acid serves as substrate for the lipoxygenase after release from phospholipids. In the present experiments, we determined the formation of LTB_4 synthesized from cellular arachidonic acid. In this setting, only very small concentrations of 5-HETE are detectable 10 min after stimulation of PMNL. As has recently been demonstrated in human PMNL, 5-HETE reaches a maximum 2 to 4 min after stimulation and is rapidly reesterified thereafter [19]. This prevents an accurate measurement of the concentration of 5-HETE formed during the incubation period. In addition, without a simultaneous measurement of 5-HPETE, our data do not allow a conclusion regarding an increased shift from 5-HPETE to 5-HETE induced by nafazatrom. But this could be one of the mechanisms whereby nafazatrom reduces substrate availability for LTA_4-synthetase and thereby formation of LTB_4.

Nafazatrom has also been found to increase the synthesis of 15-HETE, which is described as an inhibitor of the 5-lipoxygenase. An increased concentration of 15-HETE could, therefore, inhibit the formation of LTB_4 [20]. However, very little or no 15-HETE is synthesized in highly purified preparations of PMNL without exogenous arachidonic acid added as substrate. We could not detect 15-HETE in our PMNL preparation. Therefore, such a mode of action seems unlikely to be operative in our experiments.

The *in vitro* dose response curve showing an IC_{50} of approximately 3×10^{-6} M nafazatrom corresponds to results obtained previously in human PMNL (20). The plasma concentrations of nafazatrom after a single oral 3 g dose range from 4×10^{-6} to 10^{-6} M, 1–3 hr after dosing (E. Philipp, personal communication). Nafazatron is a highly lipophilic compound and is rapidly taken up by cellular membranes. As in platelets [5] about 65–80% of [^{14}C]nafazatrom are recovered in the 100,000 *g* membrane pellet of human PMNL (unpublished results). It is therefore likely that nafazatrom once bound to PMNL, remains within the PMNL membranes during the percoll preparation.

Enhanced levels of hydroperoxides may be involved in the pathogenesis of atherothrombotic processes by reducing the synthesis of PGI_2 [21, 23]. The possible beneficial effects of nafazatrom have therefore been related to a reduction of hydroperoxy fatty acids, leading to an increased synthesis of PGI_2 [22]. Since we could not detect changes in the excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$, such a mechanism in the control of PGI_2 formation may be of minor importance in healthy volunteers. We could also not detect any changes in the excretion of 2,3-dinor-TXB_2 and of TXB_2 formation *ex vivo* in whole clotted blood, which is in accordance with previous studies, in which no effects of nafazatrom on platelet TXB_2 formation and platelet aggregation have been found [5]. In addition, nafazatrom did not influence the synthesis of 12-HETE in intact washed platelets *in vitro* [5].

Taken together, it seems therefore possible, that the inhibitory effect of nafazatrom on the 5-lipoxygenase pathway is rather specific, may be a direct one and does not extend to other lipoxygenases or to the cyclooxygenase pathway. The possible involvement of leukotrienes in processes like chronic inflammation and allergy is presently under intensive investigation. Available data already indicate that increased leukotriene synthesis may play a role in certain diseases including psoriasis [24], Crohn's disease [25] and asthma [26]. Since we have demonstrated a significant inhibitory effect of nafazatrom on the biosynthesis of biologically active LTB_4 in healthy volunteers, it may be interesting to explore the usefulness of this drug in disorders, where increased formation of LTB_4 is demonstrable.

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Table 2. 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2 in urine and TXB_2 in serum before and after a 3 g dose of Nafazatrom

Volunteer	2,3-dinor-6-keto-$PGF_{1\alpha}$			2,3-dinor-TXB_2			TXB_2	
	Control −12–0 hr	0–6 hr after nafazatrom	6–12 hr after nafazatrom	Control −12–0 hr	0–6 hr after nafazatrom	6–12 hr after nafazatrom	Control	3 hr after nafazatrom
1	81	48	62	880	738	2102	115	140
2	84	102	60	3663	3925	2334	238	233
3	68	77	58	1048	727	1337	255	184
4	65	73	46	1440	1202	1414	191	223
5	93	56	119	233	n.d.	282	185	143
6	83	85	61	236	n.d.	448	172	189
7	137	128	113	464	509	507	282	237
Mean ± S.D.	87.3 ± 22.2	81.3 ± 25.2	74.1 ± 7.0	1137.7 ± 1110.2	1420.2 ± 1272.6	1203.4 ± 760.9	205.9 ± 52.3	192.7 ± 37.5

Results are given in ng/g creatinine (2,3 dinor-6-keto-$PGF_{1\alpha}$ and 2,3 dinor-TXB_2) in urine and ng/ml serum (TXB_2). Mean ± S.D., N = 7.
n.d. = not determined

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