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Oral nafazatrom in man: effect on inhaled antigen challenge

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1 The effect of oral nafazatrom (Bay g6575, 2 × 3 g) or placebo on inhaled antigen challenge was assessed in a double-blind study.

2 In four subjects antigen challenge resulted in an immediate fall of 93.2 ± 3.36% in airflow at 40% of vital capacity (Vp_{40}) and a 45.85 ± 4.95% reduction in forced partial expiratory volume at one second (FEV_1). Neither nafazatrom nor placebo had any effect on baseline lung function or that after challenge.

3 Leukotriene B_4 was generated by *ex vivo* stimulus of blood with ionophore A23187, and quantified by high performance liquid chromatography (h.p.l.c.)-radioimmunoassay. No inhibition of LTB_4 formation occurred *ex vivo* following oral nafazatrom, although addition of 10^{-5} M nafazatrom to blood *in vitro* significantly inhibited LTB_4 release.

4 Peak plasma nafazatrom levels during the study ranged from 3.3×10^{-7} M to 1.47×10^{-6} M which are below the concentration (10^{-5} M) at which significant 5-lipoxygenase inhibition occurs *in vitro*.

5 Oral nafazatrom is ineffective as a 5-lipoxygenase inhibitor in man, probably because of poor bioavailability after administration.

Keywords nafazatrom leukotriene B_4 5-lipoxygenase inhibitor

Introduction

Antigen-induced bronchoconstriction is believed to occur through the release of mediators, some of which are products of arachidonic acid metabolism. A number of eicosanoids are reported to be bronchoconstrictors *in vivo*, including prostaglandin D_2 (Fuller *et al.*, 1986) and the peptidoleukotrienes (Lewis & Austen, 1981; Dahlen *et al.*, 1980), however, as yet, there is little evidence linking the specific biosynthetic inhibition of these bronchoconstrictor eicosanoids with a reduction in all of the symptoms of allergen-induced bronchoconstriction.

Although steroid treatment is successfully used in the management of asthma, and has been shown to act as a phospholipase inhibitor (Blackwell & Flower, 1983), few would argue that the effects of these drugs are all mediated through inhibition of eicosanoid formation. Inhibition of the cyclooxygenase pathway has been reported to reduce the antigen-induced late reaction in man (Fairfax *et al.*, 1983; Shephard *et al.*, 1985), indicating some role for the prostanoids, perhaps as secondary mediators. Although there have been a number of studies on leukotriene biosynthesis inhibition *in vitro* (Taylor & Clarke, 1986), there is little information available on the effects of these drugs in man.

Nafazatrom (Bay g6575, 2,4-dihydro-5-methyl-2-[2-naphthyloxyethyl]-3H-pyrazol-3-one), which has been reported to possess antithrombotic and antimetastatic activity (Wong *et al.*, 1982; Marnett *et al.*, 1984; Seuter, 1979; Honn *et al.*, 1986), has recently proposed as an alternative therapeutic agent for asthma on the basis of its inhibitory effects on leukotriene biosynthesis (Busse *et al.*, 1982). At sub-micromolar concentrations, nafazatrom inhibited biosynthesis of leukotriene B_4 (LTB_4) from arachidonic acid

in vitro but had no effect on its formation from LTA_4 (McMillan *et al.*, 1986). Further, it has been reported that oral nafazatrom (3 g) inhibited *ex vivo* LTB_4 release from human isolated neutrophils (Strasser *et al.*, 1985).

We have compared the effect of oral nafazatrom (2 × 3 g) and a matched placebo on allergen induced bronchoconstriction in atopic volunteers. LTB_4, generated *ex vivo* by ionophore A23187 stimulus, was measured by high performance liquid chromatography-radioimmunoassay (h.p.l.c.-RIA) in order to determine whether there was any correlation between changes in *ex vivo* LT production and lung function.

Methods

In vitro *study*

Varying concentrations of nafazatrom (10^{-6}–10^{-4} M) were added to 10 ml samples of blood, from four subjects, which were challenged with 10 μl calcium ionophore A23187 (5 mM in dimethylsulphoxide [DMSO]). The mixture was incubated for 10 min at 37° C before the reaction was terminated by 10 ml of 0.002 M EDTA, 2.149 μM nordihydroguaritic acid (NDGA) and 112 mM NaCl at pH 7.2. Samples were centrifuged and then the concentration of LTB_4 in the plasma was measured using h.p.l.c. and radioimmunoassay as described below.

In vivo *study*

Five male and three female atopic subjects between 20 and 40 years of age took part in the study which had approval from the joint ethics committee of the Hammersmith and Queen Charlotte's Hospitals Special Health Authority and the Royal Postgraduate Medical School.

Lung function Measurements of airflow and volume were made using a rolling seal spirometer (P. K. Morgan) linked to a microcomputer (IBM-PC). The best of three vital capacity manoeuvres were used as the reference volume for the subsequent measurements. A forced partial expiratory manoeuvre was performed by the subject first inhaling to end tidal inspiration then forcibly exhaling into the spirometer to residual volume before inhaling to total lung capacity (Barnes *et al.*, 1981). Flow at 40% of the reference vital capacity (Vp_{40}) was then computed. Forced expiratory volume at 1 s (FEV_1) was measured by a dry wedge spirometer (Vitalograph).

LTB_4 and nafazatrom assays Blood was collected in vials containing heparin and approximately 16000 d min^{-1} of [^{3}H]-LTB_4 (Amersham international) as an internal standard. One minute after the collection, the blood was challenged with 10 μl of 5 mM calcium ionophore A23187 in DMSO and incubated at 37° C for 10 min. The incubation was terminated by the addition of 10 ml of 0.002 M EDTA, 2.149 μM NDGA and 112 mM NaCl at pH 7.2. After the sample had been centrifuged for 10 min at 10° C, the plasma was stored at −20° C prior to analysis. The plasma was diluted to approximately four times its volume with 0.04% acetic acid adjusted to pH 3.8 with ammonium hydroxide and then centrifuged. The supernatant was adjusted to pH 3.8 and injected onto a dual pump h.p.l.c. system (Waters Ass., Harrow, Middx.) which incorporates column switching from an MCH10 loading column (Varian) to a Hypersil ODS analytical column (Taylor *et al.*, 1986). LTB_4 was eluted at 1 ml min^{-1} in a linearly increasing methanol gradient (57%–96% methanol in 0.017% aqueous acetic acid) over 90 min. Fractions (2 ml) of h.p.l.c. eluant were collected; aliquots were taken for identification of eluting position by scintillation counting. Fractions containing [^{3}H]-LTB_4 were pooled as were background reference fractions on either side of the radiolabelled peak. The pooled fractions were dried under vacuum and resuspended in 300 μl of RIA buffer (Amersham LTB_4 RIA kit). A sample (100 μl) was removed for scintillation counting to determine LTB_4 recovery and the remaining sample was assayed by RIA. LTB_4 levels were determined by subtracting background immunoreactivity and correcting for recovery losses.

Plasma nafazatrom level analysis was determined by h.p.t.l.c. by the method of Ritter (1984) at Bayer AG, Wuppertal, FRG. Nafazatrom in the samples was stabilised with L-cysteine hydrochloride; samples were stored at −20° C until assayed.

Protocol

Screening Eight subjects attended the laboratory to assess their response to appropriate dose of inhaled antigen. Allergen extract (grass pollen and house dust mite (Pharmacia)) was reconstituted in saline and then ten fold dilutions were made. Prick tests were performed with the full range of concentrations of antigen. A ten fold dilution of the concentration which caused a 3 mm skin wheal was then used for the first dose of inhaled antigen. Following inhalation of control (saline) solution and basal Vp_{40} and FEV_1 measurements, the subjects inhaled five breaths

of the starting concentration of allergen and once again lung function was measured. After 10 min, a dose of antigen five times the original concentration was inhaled. The dose was increased until a 40% fall in Vp_{40} was achieved at any point during the 10 min after antigen inhalation. Readings of Vp_{40} and FEV_1 at 30 min intervals up to 6 h after the original antigen challenge were taken in order to detect a late response (a greater than 20% fall in Vp_{40} between 4 and 6 h after challenge).

Nafazatrom study Four subjects exhibiting both an early and late response took part in the double blind study. Following baseline measurements of lung function (Vp_{40} and FEV_1) an initial oral dose of 3 g of nafazatrom or placebo was given to the fasting volunteers. Lung function was measured for the next 3 h before a second dose (3 g) of the drug or placebo was administered, the subjects were then challenged with the inhaled dose of antigen which caused a $> 40\%$ fall in Vp_{40}. Lung function measurements were made at regular intervals for the next 6 h. Heparinized blood samples were taken before drug administration and then 1, 3, 4, 7 and 24 h after the first drug administration for *ex vivo* leukotriene B_4 production and drug level analysis.

Results

In vitro *study*

Stimulus of whole blood *in vitro* with calcium ionophore A23187 increased LTB_4 production from 220 ± 86 pg ml^{-1} (DMSO only) to 1883 ± 279 pg ml^{-1}. Nafazatrom produced a dose related inhibition of calcium ionophore A23187 stimulated LTB_4 production. The maximum observed inhibition (69.1%) occurred at a concentration of 10^{-4} M nafazatrom ($P < 0.02$) with no significant inhibition observed at 10^{-6} M (Table 1).

In vivo *study*

Of the eight atopic subjects studied seven had early responses and four had both early and late responses. Challenge of the four chosen atopic subjects (two male) with specific antigen resulted in an immediate mean fall of $93.2 \pm 3.36\%$ in Vp_{40} and $45.85 \pm 4.95\%$ in FEV_1 which returned to starting levels within 3 h. Between 4 and 6 h following challenge there was a further mean fall of $74.6 \pm 26.38\%$ and $24.6 \pm 6.27\%$ in Vp_{40} and FEV_1 respectively. Lung function responses for placebo or nafazatrom were almost identical both in individuals and the group as a whole, with no alteration in the early or the late phase changes (Figure 1). There was no evidence of inhibition of *ex vivo* LTB_4 production by nafazatrom following ionophore stimulus of blood (Table 2), although the results were more variable in individual subjects compared with lung function data. Plasma nafazatrom was detected in each of the four subjects with peak concentrations of $8.3 \pm 4.8 \times 10^{-7}$ M. There was substantial intersubject differences in the profiles of plasma drug levels achieved (Table 3). This may arise through intersubject variability in first-pass metabolism.

Table 1 Leukotriene B_4 generation *in vitro* following ionophore A23187-stimulation of blood in the presence of 10^{-4} to 10^{-6} M nafazatrom ($n = 4$, mean ± s.e. mean). Concentrations of 10^{-5} M (2.68 μg ml^{-1}) are required for significant inhibition of LTB_4 synthesis *in vitro*

Nafazatrom concentration (M)	*LTB_4 (pg ml^{-1})*
Blank	220 ± 86
0	1883 ± 279
10^{-6}	1749 ± 419
10^{-5}	693 ± 271
10^{-4}	281 ± 44

Discussion

Using ionophore-stimulated whole blood, coupled with a specific h.p.l.c.-radioimmunoassay, we have correlated the biological effects of nafazatrom, a 5-lipoxygenase inhibitor, on antigen challenge in man with a direct measurement of *ex vivo* LTB_4 production. The method is simple, and does not require separation of the components of blood prior to analysis; there is, therefore, no possibility of dissociation of cell and inhibitor which would lead to false negative results.

Nafazatrom at concentrations *in vitro* at 10^{-5} M and above causes significant inhibition of 5-lipoxygenase; at 10^{-6} M, no significant decrease in LTB_4 production was observed. In no subject did the peak nafazatrom concentration rise above 1.47 ± 10^{-6} M (395 ng ml^{-1}), and indeed in most cases, the levels were well below 10^{-6} M, and the failure to inhibit LTB_4 production *ex vivo* is therefore not surprising.

In a recent study in rats, McMillan and colleagues (McMillan *et al.*, 1986) reported on the effect of oral administration of several 5-lipoxygenase inhibitors on *ex vivo* generation of LTB_4 from ionophore-stimulated blood. Nafazatrom,

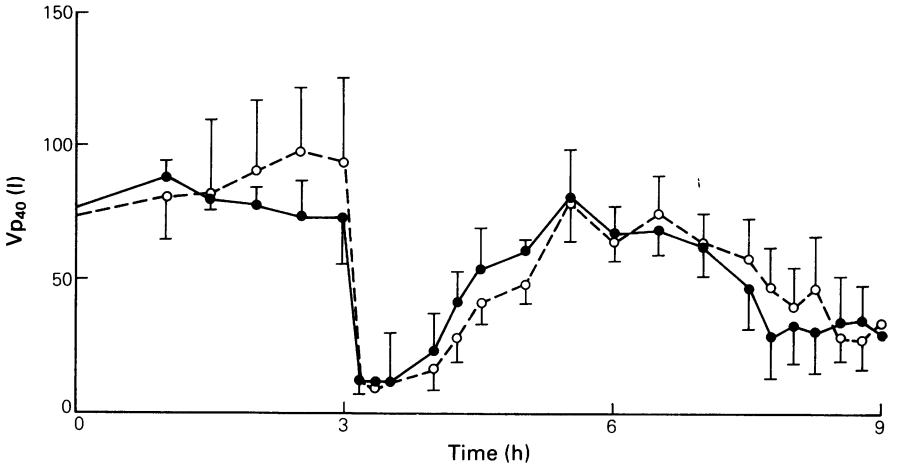

Figure 1 Flow at 40% of the reference vital capacity (Vp_{40}, l). Vp_{40} values (mean ± s.e. mean) of four subjects. Nafazatrom (solid line, •) or placebo (dashed line, ○) was taken orally at $t = 0$ h and $t = 3$ h. Antigen challenge was undertaken at $t = 3$ h.

Table 2 *Ex vivo* generation of LTB_4 following two 3 g oral doses of nafazatrom or placebo at $t = 0$ and $t = 3$ h [$n = 4$, mean ± s.e. mean]

Time (h)	*Placebo* LTB_4 ($\mu g\ ml^{-1}$)	*Nafazatrom* LTB_4 ($\mu g\ ml^{-1}$)
0	0.77 ± 0.28	1.63 ± 0.63
1	1.50 ± 1.15	3.97 ± 2.21
3	0.95 ± 0.35	3.34 ± 1.72
7	0.99 ± 0.30	2.64 ± 1.85
24	0.79 ± 0.19	1.97 ± 1.11

There is no evidence the inhibition of LTB_4 generation in the presence of nafazatrom. The apparent stimulation observed was not significant.

Table 3 Plasma nafazatrom measurements (ng ml^{-1}) in four subjects after oral doses of 3 g each at time 0 h and 3 h

Time (h)	*Nafazatrom concentrations (ng ml^{-1}) in each subject* 1	2	3	4
0	< 10	< 10	—	45
1	207	81	75	203
3	74	29	122	153
4	195	14	175	395
7	78	54	204	165
24	< 10	< 10	< 10	< 10

The limit of detection for the assay is 10 ng ml^{-1}. In no subject did the concentration of nafazatrom attain a level at which significant inhibition of LTB_4 synthesis occurred *in vitro* (10^{-5} M, 2.68 $\mu g\ ml^{-1}$).

although an effective 5-lipoxygenase inhibitor *in vitro*, at oral doses up to 100 mg kg^{-1} nafazatrom was inactive.

Strasser and colleagues (1985) measured LTB_4 formation in purified human neutrophils following ionophore stimulus and observed a 40% decrease in *ex vivo* LTB_4 generation 3 h after a single 3 g oral dose, although plasma nafazatrom levels were not determined, it was assumed they were in the range $1–4 \times 10^{-6}$ M. The differences in *ex vivo* LTB_4 generation between our study and that of Strasser *et al.* (1985) may have arisen as a result of purification of the neutrophils prior to ionophore-stimulation, or the less specific methods which were used for assaying LTB_4. In the same study, Strasser *et al.* (1985) also demonstrated *in vitro* that 10^{-6} M nafazatrom resulted in a 35% decrease in LTB_4 formation. This is in contrast with our data in whole blood (Table 1), and also that of McMillan and colleagues (1985) on human isolated leukocytes.

We conclude that a 6 g oral dose of nafazatrom has no effect on the early or late phases of bronchoconstriction, and that plasma concentrations are too low for *ex vivo* 5-lipoxygenase inhibition to be observed. This negative finding does not exclude the possibility that other 5-lipoxygenase inhibitors with effective plasma levels could be beneficial in the treatment of asthma.

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References

Blackwell, G. J. & Flower, R. J. (1983). Inhibition of phospholipase. *Br. med. Bull.*, **39**, 260–264.

Barnes, P. J., Gribben, H. R., Osmanliev, D. & Pride, N. B. (1981). Partial flow-volume curves to measure bronchodilator dose-response curves in normal humans. *J. applied. Physiol.*, **50**, 1193–1197.

Busse, W.-D., Mardin, M., Gruetzmann, R., Dunn, J. R., Theodorou, M., Sloane, B. F. & Honn, K. V. (1982). Nafazatrom (Bay g6575), an inhibitor of cellular lipoxygenase activity. *Fed. Proc.*, **41**, 1717.

Dahlen, S. E., Hedqvist, P., Hammarstrom, S. & Samuelson, B. (1980). Leukotrienes are potent constrictors of human bronchi. *Nature*, **288**, 484–486.

Deckmyn, H., Van Houtte, E., Verstraete, M. & Vermylen, J. (1983). Manipulation of the local thromboxane and prostacyclin balance *in vivo* by the antithrombitic compounds dazoxiben, acetylsalicylic acid and nafazatrom. *Biochem. Pharmac.*, **32**, 2757–2762.

Fairfax, A. J., Hanson, J. M. & Morley, J. (1983). The late reaction following bronchial provocation with house dust mite allergen. Dependence on arachidonic acid metabolism. *Clin. exp. Immunol.*, **52**, 393–398.

Fuller, R. W., Dixon, C. M. S., Dollery, C. T. & Barnes, P. J. (1986). Prostaglandin D_2 potentiates airway responsiveness to histamine and methacholine. *Am. Rev. resp. Dis.*, **133**, 252–254.

Honn, K. V. (1982). Control of tumor growth and metastasis with prostacyclin and thromboxane synthetase inhibitors: Evidence for a new antitumor and antimetastatic agent (BAY g6575). In *Interaction of platelet and tumor cells*, ed. Jamieson, G. A., pp. 295–331. New York: Alan R. Liss.

Lewis, R. A. & Austen, K. F. (1981). Mediation of local homeostasis and inflammation by leukotrienes and other mast cell-dependent compounds. *Nature* **293**, 103–108.

Marnett, L. J., Siedlik, P. H., Ochs, R. C., Pagels, W. R., Das, M., Honn, K. V., Warnock, R. H., Tainer, B. E. & Eling, T. E. (1984). Mechanism of the stimulation of prostaglandin H synthase and prostacyclin synthase by the antithrombotic and antimetastatic agent, nafazatrom. *Mol. Pharmac.*, **26**, 328–335.

McMillan, R. M., Masters, D. J. & Sterling, W. W. (1985). Modulation of leukotriene B_4 biosynthesis in human leukocytes. *Br. J. Pharmac.*, **84**, 44P.

McMillan, R. M., Millest, A. J., Proudman, K. E. & Taylor, K. B. (1986). Evaluation of lipoxygenase inhibitors *ex vivo*. *Br. J. Pharmac.*, **87**, 53P.

Ritter, W. (1984). Determination of nafazatrom in body fluids by high performance thin-layer chromatography with post-chromatographic derivatisation. *J. Chromatogr.*, **290**, 97–105.

Seuter, F. (1979). The antithrombotic agent of BAY g6575 (nafazatrom). *Arzneim.-Forsch. Drug Res.*, **29**, 54–59.

Shephard, E. G., Malan, L., MacFarlane, C. M., Mouton, W. & Joubert, J. R. (1985). Lung function and plasma levels of thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$ and β-thromboglobulin in antigen-induced asthma before and after indomethacin treatment. *Br. J. clin. Pharmac.*, **19**, 459–470.

Strasser, T., Fischer, S. & Weber, P. C. (1985). Inhibition of leukotriene B_4 formation in human neutrophils after oral nafazatrom (Bay g6575). *Biochem. Pharmac.*, **34**, 1891–1894.

Taylor, G. W., Chappell, C. G., Clarke, S. R., Heavey, D. J., Richmond, R., Turner, N. C., Watson, D. & Dollery, C. T. (1986). Mass spectrometric and immunological assays for the leukotrienes. In *The leukotrienes: their biological significance*, ed. Piper, P. J., pp. 67–90. New York: Raven Press.

Taylor, G. W. & Clarke, S. (1986). The leukotriene biosynthetic pathway: a target for pharmacological attack. *Trends Pharmac. Sci.*, **7**, 100–103.

Wong, P. Y.-K., Chao, P. H.-W. & McGiff, J. C. (1982). Nafazatrom (BAY g6575), an antithrombotic and antimetastatic agent, inhibits 15-hydroxyprostaglandin dehydrogenase. *J. Pharmac. exp. Ther.*, **223**, 757–760.

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