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KETOCONAZOLE INHIBITS THE BIOSYNTHESIS OF LEUKOTRIENES *IN VITRO* AND *IN VIVO*

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Abstract—Ketoconazole inhibits *in vitro* (IC_{50}: 2.6×10^{-5} M) the formation of 5-HETE and LTB_4 by isolated, carrageenin-elicited rat peritoneal PMN leukocytes, challenged with the Ca^{2+}-ionophore A23187 in the presence of [^{14}C]-arachidonic acid ([^{14}C]-AA). The relative potency of various compounds tested in this respect is NDGA > nafazatrom > phenidone > ketoconazole > BW 755C. In contrast to the other compounds studies, ketoconazole *in vitro*, up to 1×10^{-4} M, has no effect on the fatty acid cyclo-oxygenase or the 12-lipoxygenase-mediated metabolism of [^{14}C]-AA by isolated human platelets; however, it stimulates the 15-lipoxygenase activity in phenylhydrazin-induced rabbit reticulocytes.

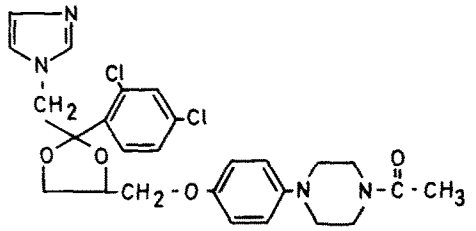
After oral administration (10–40 mg/kg, −2 hr), ketoconazole inhibits in a dose-dependent way, the leukotriene-mediated anaphylactic bronchoconstriction in guinea pigs. This study demonstrates that ketoconazole is a comparatively specific and orally active inhibitor of the 5-lipoxygenase activity bearing on the production of leukotrienes derived from arachidonic acid.

Leukotrienes, which are formed from arachidonic acid, are presumed to be important mediators in allergic and anaphylactic reactions and in inflammation [1]. It is now well established that the activity of the allergic mediator slow-reacting substance of anaphylaxis (SRS-A) is almost entirely attributable to the leukotrienes C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4) [1, 2]. Their powerful biological activities, especially on the human airways, indicate potential functions in asthma, anaphylaxis and allergy. Furthermore, another metabolite, leukotriene B_4 (LTB_4), is a potent chemokinetic and chemotactic agent towards leukocytes [3–5]. It has been proposed as an important mediator in inflammatory reactions [3, 6].

The peptido-leukotrienes LTC_4, D_4 and E_4 share a common intermediate LTA_4 with LTB_4. LTA_4 is biosynthesized from arachidonic acid through the subsequent actions of a 5-lipoxygenase, which transforms arachidonic acid to 5-HPETE, and a dehydrase, which converts 5-HPETE to LTA_4 [1]. Therefore, a specific inhibitor of the 5-lipoxygenase enzyme, the first step in the formation of the leukotrienes, could be useful as a tool for investigating the regulation mechanism of leukotriene biosynthesis, and also as a drug for treatment of disease states associated with hypersensitivity reactions and inflammation.

In the present study we investigated the effect of ketoconazole, an antimycotic drug (Fig. 1) on the metabolism of arachidonic acid to the leukotrienes *in vitro* and *in vivo* and compared its activity with that of known inhibitors of the lipoxygenase enzymes.

Ketoconazole appears to be a selective inhibitor of the leukotriene biosynthesis, with activity *in vivo* after oral administration.



Ketoconazole M.W.: 531

Fig. 1. Chemical structure of ketoconazole. (For review of its activities see references 48–50.)

MATERIALS AND METHODS

PMN incubations

Peritoneal polymorphonuclear leukocytes (PMN) were elicited in rats (Wistar, male, 200–250 g) by the I.P. injection of carrageenin (0.5 mg). After 3 hr, the cells were harvested by the injection of 10 ml Hank's balanced salt solution containing 10 U/ml heparin. The cells were isolated from the peritoneal washing fluid by centrifugation for 10 min at 200 *g*. The cell pellet was resuspended in 17 mM Tris–HCl buffer (pH 7.5) containing ammonium chloride to lyse contaminating erythrocytes, followed by centrifugation for 5 min at 200 *g*. The cell pellet was washed twice by resuspension in Tris–HCl buffer (50 mM, pH 7.5) with NaCl (100 mM) and centrifugation. The cells were finally resuspended in

Tris–HCl buffer (50 mM, pH 7.5 at 37°) with NaCl (100 mM) at a cell density of 6.25×10^6 cells/ml. The PMN suspension (800 μl) was pre-incubated for 10 min at 37° with 2 μl drug solution or solvent. Incubation was started by addition of [^{14}C]-arachidonic acid (3.7 μM; 54.5 mCi/mmol), calcium ionophore A23187 (1 μM) and $CaCl_2$ (2 mM) unless stated otherwise. The final volume of the incubate was 1 ml. After a 5 min incubation period, the reaction was stopped by addition of 2 ml acetone and acidification of the reaction mixture with formic acid (10% in H_2O) to pH 3.5. Radioactive reaction products were extracted twice in chloroform (2 ml) by Vortex mixing.

The combined chloroform fractions were dried under nitrogen; the residue was dissolved in chloroform : methanol (2/1) and applied on pre-coated silica gel plates. The plates were developed in hexane–diethyl ether–acetic acid (40:60:1).

Radioactive products were located by autoradiography and quantitated by optical density measurements with a Quantimet 900 (Cambridge Instruments).

Assay of 5-lipoxygenase (cell-free)

Rat peritoneal PMN were elicited and harvested as described above (PMN incubations). After the lysis of erythrocytes and washing of the PMN, the cells were finally resuspended in 50 mM phosphate buffer (pH 7.4 at 25°) containing 1 mM EDTA and 0.1% gelatine. The cell suspension was sonicated three times during 20 sec at 20 kHz and the 10,000 *g* supernatant fraction was prepared. Under the standard conditions for 5-lipoxygenase activity the fraction of the supernatant, obtained from 5×10^6 cells, and diluted to 800 μl with buffer was pre-incubated with suprofen (10 μM) and with the drug solution or solvent at 37° for 10 min. The incubation was started by addition of [^{14}C]-arachidonic acid (3.7 μM; 54.5 mCi/mmol), $CaCl_2$ (2 mM) and ATP (1 mM). The final volume of the incubate was 1 ml. After a 5 min incubation period the reaction was stopped by the addition of 2 vol. acetone and acidification to pH 3.5 with formic acid (10% in H_2O). Reaction products were extracted, separated by t.l.c. and quantitated as described above. The activity of the 5-lipoxygenase was expressed as the sum of 5-HETE, LTB_4 and all *trans*-5,12-diHETE.

Reticulocyte incubations

Reticulocytosis was provoked in male rabbits (New Zealand white, 2.0–2.5 kg) by the subcutaneous injection of phenylhydrazine daily for 7 days [7]. Blood was withdrawn on heparin (20 U/ml) by cardiac puncture. The blood was diluted with 1 volume of ice-cold 154 mM NaCl, containing 0.2% bovine serum albumin and centrifuged at 250 *g* for 10 min. The cell pellet was washed three times and finally resuspended in Hank's balanced salt solution. Specific staining with new methylene blue and subsequent microscopic counting showed ≥ 95% of the red cell population to consist of reticulocytes. Reticulocytes (5×10^7/ml) were preincubated with 2 μl drug solution or solvent for 10 min at 37°. The incubations were started by the addition of the substrate [^{14}C]-arachidonic acid (3.7 μM; 54.5 mCi/mmol) and the mixture was incubated for 10 min at 37°. The reaction was stopped by the addition of acetone and acidification to pH 3.0. Reaction products were extracted, separated by t.l.c. and quantitated as described above.

Platelet incubations

Washed, human platelets [8] were pre-incubated for 10 min at 37° with solvent or drug solution and then incubated for another 5 min with [^{14}C]-arachidonic acid in a final volume of 1 ml. The reaction was stopped by the addition of 2 ml ethyl acetate and acidification to pH 3.5. Reaction products were extracted, separated on t.l.c. and quantified as previously described [8].

Guaiacol peroxidation

Guaiacol (methoxyphenol 50 μM) peroxidation by horse radish peroxidase (0.62 μg/ml) in Tris 50 mM pH 7.4, in the presence of solvent (D.M.S.O. 1% v/v) or drugs, was initiated by the addition of H_2O_2 (100 μM) and quantified by measuring the optical density (o.d., Coleman Spectrophotometer) at 436 nm, in 2 ml samples at 3 min of reaction.

If required, the o.d. values obtained in the presence of drugs were corrected for those obtained in a similar system without guaiacol, before the calculation of the percentage inhibition produced by the drug.

Anaphylactic bronchoconstriction

Male guinea-pigs (350–400 g) were sensitized by the intraperitoneal injection of 1 mg ovalbumin and 1 ml of Bacto Bordetella pertussis antigen (1:10 dilution, Difco). Fourteen to 21 days after the sensitization, the animals were anaesthetized with pentobarbital (30 mg/kg i.p.). The right jugular vein and the trachea were cannulated for the administration of drugs and monitoring of the ventilation.

Succinylcholine (0.5 mg/kg, i.v.) and propranolol (3 mg/kg, i.p.) were administered. The animal was ventilated (Stefan Reanimator) at 50 strokes/min, a flow of 3 ml/min and an inflation pressure limit of 30 mbar. Tidal volume was measured by a thorax strain gauge plethysmograph (Janssen Scientific Instruments). After a 10 min stabilization period, following drugs were administered intravenously: pyrilamine (2 mg/kg), methysergide (0.1 mg/kg), propranolol (1 mg/kg), atropine (0.5 mg/kg) and indomethacin (10 mg/kg). Five minutes after the premedication, the animals were challenged with ovalbumin (0.3 mg/kg, i.v.). Tidal volume and inspiratory pressure are monitored up to 10′ after the challenge. Reduction of tidal volume was calculated as previously described [9].

h.p.l.c. analysis

h.p.l.c. analyses were carried out on a Varian 5000 liquid chromatograph. For the h.p.l.c. identification of the monohydroxy metabolites of arachidonic acid a Nucleosil C_{18} column (250 × 4.6 mm, Alltech associates) was utilized and elution was performed with methanol/water/acetic acid (78:22:0.01, by volume) at a flow of 1 ml/min. The effluent was monitored at 235 nm using a variable wavelength UV50 detector. Fractions of 1 ml were collected and

the radioactivity was determined by liquid scintillation counting. For the h.p.l.c. analysis of the more polar compounds, co-chromatographing with LTB_4 on the t.l.c.-plate, a Lichrosorb RP-18 cartridge (250 × 4 mmn, Merck) was used. The solvent system consisted of tetrahydrofuran/methanol/0.1% EDTA/acetic acid (25/30/45/0.1, by volume) which was brought to pH 5.5 with ammoniumhydroxide at a flow rate of 0.9 ml/min [10]. The u.v.-absorbance was monitored at 270 nm and the radioactivity in 0.5 ml fractions was determined.

Materials

Radioactive [^{14}C]-arachidonic acid (56.5 mCi/mmol) was purchased from New England Nuclear. The calcium ionophore A23187 was obtained from Calbiochem. Behring. Tris(hydroxymethyl)aminomethane (Tris), ovalbumin, carrageenin, guaiacol and horseradish peroxidase, were obtained from Sigma (London, U.K.), Phenylhydrazine was from Janssen Chimica*. For in-vitro studies, ketoconazole, suprofen (Janssen, Pharmaceutical), nordihydroguaretic acid (NDGA, Fluka AG*), nafazatrom (Bayer, Leverhusen, FRG), BW 755C (Wellcome Research Labs., Beckenham, U.K.), phenidone (Aldrich, Gillingham, U.K.) were dissolved in dimethylsufloxide. For in vivo studies, ketoconazole was dissolved in 20% PEG 400. FPL 55712 (a generous gift from Fison, Loughborough, U.K.) was solubilized with a minimal amount of tartaric acid and diluted with physiological saline under strict pH control and diethylcarbamazine (Mercken Cyanamid*) was dissolved in saline. Thin layer chromatography was performed on precoated silica plates $60F_{254}$ (Merck, Darmstadt, F.R.G.). All solvents and reagents were of analytical grade (Merck).

RESULTS

Identification of the metabolites formed by PMN and reticulocytes

Rat PMN. Upon stimulation of the rat PMN's with [^{14}C]-arachidonic acid and the calcium ionophore A23187 in the presence of the cyclo-oxygenase inhibitor suprofen (10^{-5} M F.C.) [11], three major metabolites of arachidonic acid were detected on the t.l.c. chromatogram, apart from the radioactivity found on the start and the unconverted arachidonic acid.

Those metabolites co-chromatographed with LTB_4, 5-HETE and 15-HETE. The different fractions were eluted from the plate and subjected to h.p.l.c. analysis. The LTB_4 fraction was further analysed with u.v. spectrometry.

h.p.l.c. analysis of the LTB_4 fraction revealed that three metabolites were present in the fraction. The major peak co-chromatographed with authentic LTB_4, while the two others had lower retention times (14.5 and 16 min vs 19 min for LTB_4). However, u.v. analysis of the three fractions revealed that they all three showed the typical pattern of u.v. absorption of conjugated trienes. The LTB_4 fraction showed a major absorption peak at 270 nm and two minor peaks at 260 and 280 nm, whereas both other fractions showed a major peak at 268 nm and two minor peaks at 258 and 280 nm. These results suggest that both minor metabolites are the two Δ^6-*trans*-isomers of LTB_4 (5S, 12S- and 5S, 12R-dihydroxy-6, 8,10,14-EEEZ-eicosatetraenoic acid), which are formed by non-enzymatic hydrolysis of LTA_4 [12, 13]. As the standards of both 6-*trans*-isomers were not available, the results could not be further confirmed. However, Powell [13], in a similar h.p.l.c.-system, reported retention times comparable to ours for the three isomers. This observation supports the contention that the two minor metabolites are the 6-*trans*-isomers of LTB_4. The relative abundance of the isomers were: 67% LTB_4, 21% isomer 1 (5S, 12R) and 11% isomer 2 (5S. 12S).

The h.p.l.c. analysis of the 5-HETE fraction showed only 1 peak which co-eluted with authentic 5-HETE. The 15-HETE fraction of the PMN consisted of two metabolites of arachidonic acid, which co-eluted on HPLC with authentic 12-HETE and 15-HETE, respectively. The ratio of 12-HETE over 15-HETE was 2/1. This fraction will be referred to as 12/15-HETE.

Rabbit reticulocytes. The rabbit reticulocytes converted the [^{14}C]-arachidonic acid to one major metabolite, which co-chromatographed on t.l.c. with 15-HETE.

h.p.l.c. analysis demonstrated the presence of 12-HETE, apart from 15-HETE. The relative proportion of 12-HETE was less than 10% of the amount of 15-HETE, as also reported earlier [7]. The 12-HETE might be derived from contaminating platelets. However, it has been suggested that this metabolite is a product of the reticulocyte 15-lipoxygenase [7].

Arachidonic acid metabolism in rat PMN leukocytes

PMN leukocytes, stimulated with the calcium ionophore A23187 and [^{14}C]-arachidonic acid, transform the arachidonic acid via the cyclo-oxygenase as well as via the lipoxygenase pathway. Addition of the cyclo-oxygenase inhibitor suprofen (10 μM) to the incubation mixture inhibited the transformation of arachidonic acid via the cyclo-oxygenase pathway, as evidenced by the diminished radioactivity in the start spot, where the prostanoids remain together with the phospholipids and by the disappearance of the HHT peak (R_f 0.46). Concomitantly, the formation of the 5-lipoxygenase-derived products 5-HETE and LTB_4 and isomers was increased (5-HETE: 120 ± 6% of control; LTB_4: 129 ± 8% of control). The total amount of unconverted arachidonic acid was also slightly increased.

In subsequent experiments, suprofen (10 μM) was always present in the incubation mixture. Leukotriene formation by PMN leukocytes has been shown to depend on the presence of extracellular calcium and calcium ionophore [14–16]. Under our assay conditions, hardly any lipoxygenase products could be detected in the absence of extracellular calcium. The addition of calcium to the medium, dependent on concentration (0.25–2 mM), increased the formation of the 5-lipoxygenase metabolites (LTB_4 and isomers, 5-HETE). Higher calcium concentrations (up to 10 mM) did not further influence the metabolisation of arachidonic acid. Similarly, in the absence of the calcium ionophore A23187, the arachidonic acid was not metabolized. The formation

Table 1. Inhibition of the oxygenation reactions in the several in-vitro systems by the different drugs

	IC_{50} (μM)							
	Cell-free 5-lipoxygenase	PMN		Platelets + suprofen	Platelets		Reticulocytes	Guaiacol peroxidation
		LTB_4	5-HETE	12-HETE	C.O.	12-HETE	15-HETE	
Ketoconazole	28	30	26	n.i.	n.i.	n.i.	↑	↑
BW 755C	35	33	36	63	21	> 100	1.2	3.2
Phenidone	n.d.	14.5	17	n.i.	> 100	↑	2.6	4.4
Nafazatrom	7.9	9.5	10	> 50	> 100	↑	9.1	10.9
NDGA	0.08	1.3	1.4	8.5	15	26	1.0	3.5

IC_{50}-values were computed from separate determinations: at least four for the PMN, reticulocytes and the guaiacol peroxidation and three for the incubations with platelets.
n.i., not inhibited.
↑, stimulated.
n.d., not determined.

of the 5-lipoxygenase products was stimulated by the calcium ionophore in a concentration-dependent manner from 0.1 μM up to 1 μM and was not further influenced by the higher concentrations of the ionophore (up to 10 μM). Therefore, in subsequent experiments, calcium at 2 mM and the calcium ionophore A23187 at 1 μM were always added to the incubation mixture together with the substrate [^{14}C]-arachidonic acid.

Under these controlled conditions, 20.6 ± 0.3% (mean ± S.E.M., $n = 21$) of the total amount of [^{14}C]-arachidonic acid was converted to LTB_4 and its isomers, and 18.0 ± 0.3% ($n = 21$) was recovered as 5-HETE. Only a small amount of the arachidonic acid was transformed into 15- and 12-HETE (3.0 ± 0.2%, $n = 21$).

Ketoconazole concentration-dependently inhibited the formation of the 5-lipoxygenase metabolites (Fig. 1). Ketoconazole was compared to well-known inhibitors of the lipoxygenases such as BW 755C, NDGA, nafazatrom and phenidone (Fig. 1 and Table 1). Ketoconazole was slightly more potent than BW 755C in inhibition the 5-lipoxygenase but less potent than phenidone (2×), nafazatrom (2.5×) and NDGA (20×). None of the inhibitors showed any preferential inhibition of one 5-lipoxygenase metabolite over the other, indicating that the inhibition occurred at the first step of the arachidonic acid metabolism, i.e. the formation of 5-HPETE.

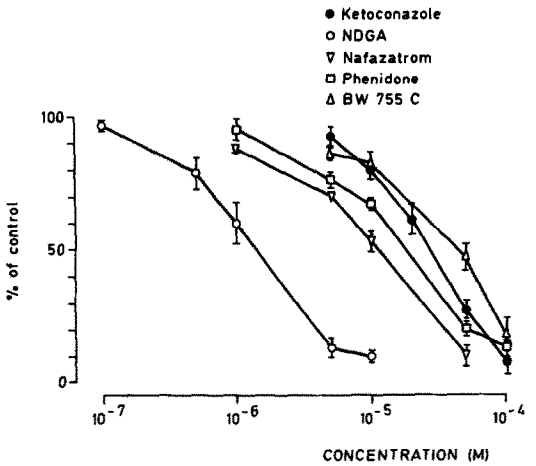


Fig. 2. Inhibition of the formation of 5-HETE in calcium ionophore stimulated rat peritoneal PMN leukocytes. Results are expressed as a percentage of the control incubation. Each point represents the mean ± S.E.M. of at least four separate determinations. ●: Ketoconazole; ○: NDGA; ▽: Nafazatrom; □: Phenidone; △: BW 755C.

Concomitant with the inhibition of the 5-lipoxygenase, ketoconazole stimulated the formation of 12/15-HETE in the incubation mixture (Fig. 3). Nafazatrom also induced a moderate increase (2×) of the 12/15-HETE fraction, whereas the other products hardly changed the transformation of arachidonic acid into 12/15-HETE.

However, if the PMN were incubated in the absence of suprofen, the increase of the 12/15-HETE fraction induced by ketoconazole was much less pronounced.

Finally, the effects of metyrapone, a cytochrome P-450 inhibitor, were investigated. Metyrapone also inhibited concentration-dependently the formation of the metabolites of the 5-lipoxygenase (IC_{50}: 2×10^{-4} M).

The assay of 5-lipoxygenase in a cell-free system

In order to confirm that ketoconazole directly inhibits the 5-lipoxygenase and does not work via an indirect mechanism (e.g. influence of calcium mobilization) incubations were carried out in the cell-free 10,000 *g* supernatant of homogenates of PMN. As shown in Table 1, all drugs inhibited the 5-lipoxygenase enzyme in the same concentration range as they did in intact cells except for NDGA, which was approximately 10 times more active in the cell-free supernatant, probably reflecting the poor penetration of NDGA through the membrane.

The metabolism of arachidonic acid in human platelets

When the cyclo-oxygenase of the platelets is inhibited by suprofen, 84 ± 1% (mean ± S.E.M., $n = 10$) of the [^{14}C]-arachidonic acid is converted to 12-HETE via the 12-lipoxygenase and 6.3 ± 0.8 is recovered as unconverted arachidonic acid.

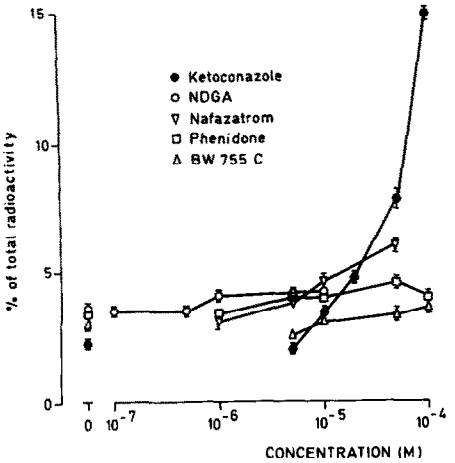


Fig. 3. Formation of 12- and 15-HETE in calcium ionophore stimulated rat peritoneal PMN leukocytes. Results are expressed as a percentage of the total radioactivity recovered from the t.l.c. plate. Each point represents the mean ± S.E.M. of at least four separate determinations. ●: Ketoconazole; ○: NDGA; ▽: Nafazatrom; □: Phenidone; △: BW 755C.

NDGA and BW 755C concentration-dependently inhibited the formation of 12-HETE (Fig. 4, Table 1), whereas phenidone had only a minor effect (20% inhibition at 5.10^{-5} M). Nafazatrom and ketoconazole had no effect on the 12-lipoxygenase.

When the platelet cyclo-oxygenase was not inhibited by suprofen, the metabolisation pattern was more complicated. The relative amounts of radioactivity associated with the different metabolites in the control incubations were (mean ± S.E.M., $n = 16$); 30.5 ± 0.7% (TXB_2), 34.7 ± 0.4% (HHT), 26.3 ± 0.5% (12-HETE) and 3.8 ± 0.5% (arachidonic acid). Only very small amounts of $PGF_{2\alpha}$, PGE_2 and PGD_2 were recovered (0.7 ± 0.1%, 1.0 ± 0.1% and 1.0 ± 0.1%, respectively).

In this system, NDGA inhibited both the cyclo-oxygenase and 12-lipoxygenase enzymes (Table 1, Fig. 4) with an IC_{50}-value of 1.5 and 2.6×10^{-5} M, respectively. BW 755C concentration-dependently inhibited the cyclo-oxygenase (IC_{50} 2.1×10^{-5} M) and inhibited at the highest concentration tested (10^{-4} M), the 12-lipoxygenase by 25%. However, at lower concentrations (5×10^{-5} to 5×10^{-6} M), BW 755C stimulated the formation of 12-HETE. Phenidone and nafazatrom only slightly inhibited the cyclo-oxygenase (30 and 20%, respectively at 5×10^{-5} M), but concomitantly stimulated the production of 12-HETE.

Ketoconazole had no effect on the cyclo-oxygenase nor on the 12-lipoxygenase activity in the platelets. However, ketoconazole inhibited the thromboxane synthase (IC_{50}: 4×10^{-5} M), as evidenced by the inhibition of the TXB_2 production and the concomitant increase of PGE_2, PGD_2 and $PGF_{2\alpha}$, the non-enzymatic breakdown products of the endoperoxide PGH_2.

The metabolism of arachidonic acid by rabbit reticulocytes

The radiochromatogram of the incubation of reticulocytes with [^{14}C]-arachidonic acid showed one major metabolite, which co-chromatographed with authentic 15-HETE and unconverted [^{14}C]-arachidonic acid. The metabolite was identified by HPLC as a mixture of 15- and 12-HETE (10:1).

Ketoconazole stimulated the formation of those 15-lipoxygenase products. At the highest concentration of ketoconazole (1×10^{-4} M), several other oxygenated products, which were not identified, appeared on the chromatogram (R_f-values: 15-HETE: 0.46; X_1: 0.14; X_2: 0.20; X_3: 0.39). The other lipoxygenase-inhibitors NDGA, BW 755C, phenidone and nafazatrom all inhibited concentration-dependently the formation of the 15- and 12-HETE. Metyrapone inhibited the 15-lipoxygenase at 5×10^{-3} M, but lower concentrations (1×10^{-3} to 5×10^{-5} M) had no significant effect.

Guaiacol peroxidation

The four antioxidants NDGA, phenidone, BW 755C and nafazatrom inhibited concentration-dependently the peroxidation of guaiacol by horse-radish peroxidase (Fig. 5 and Table 1). However, ketoconazole slightly stimulated this peroxidation in a concentration-dependent manner.

Antigen-induced bronchoconstriction in guinea pigs

Antigen challenge of the guinea pigs, pretreated with antagonists of histamine, serotonin, acetylcholine and β-receptors and with an inhibitor of the cyclo-oxygenase, resulted in bronchoconstriction, as evidence by the reduction of the tidal volume. In the control animals, the bronchoconstriction gradually increased and reached its maximum at ± 4 min.

Ketoconazole, given orally 2 hr before the challenge, reduced in a dose-dependent way this bronchoconstriction (Fig. 6).

In order to validate the leukotriene-mediated nature of the bronchoconstriction in the model two drugs were also tested: FPL 55172, an established antagonist of SRS-A [17], and diethylcarbamazine, a drug which inhibits the formation of SRS-A at the level of transformation of 5-HPETE to LTA_4 [18, 19].

FPL 55712 (10 mg/kg, given intravenously 2 min before the ovalbumin challenge) inhibited the bronchoconstriction by 85 ± 6.3% ($n = 4$). Intravenous administration of diethylcarbamazine (10 mg/kg bolus injection and 10 mg/kg as an infusion over 2 min) before the ovalbumin challenge also reduced the anaphylactic bronchoconstriction (58 ± 1.6% reduction, $n = 4$).

DISCUSSION

The present results demonstrate that ketoconazole, a substituted imidazole derivative, is a comparatively selective inhibitor of the 5-lipoxygenase enzyme, since it inhibits the production of 5-HETE and LTB_4 in rat peritoneal PMN leukocytes, but has no inhibitory effect on the 15- and 12-lipoxygenase nor on the cyclo-oxygenase of several cell systems *in vitro*.

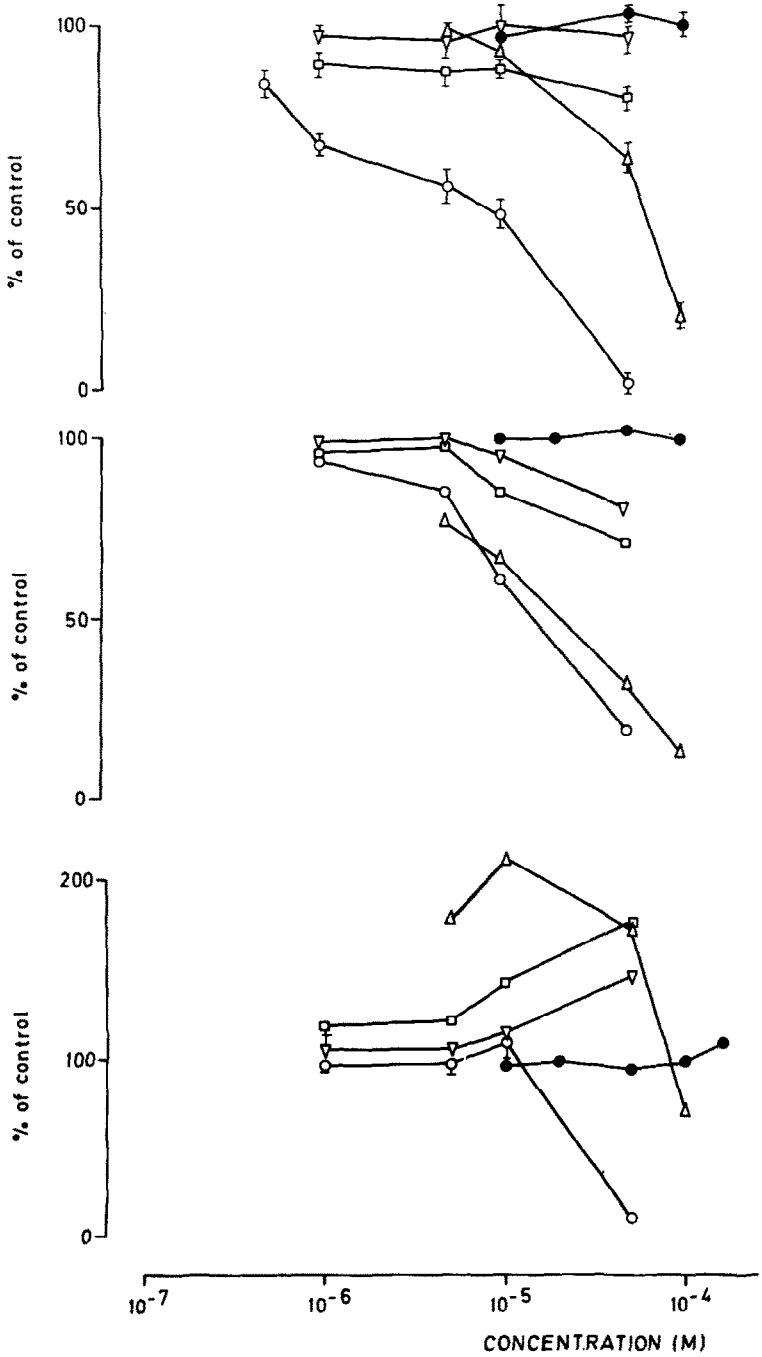


Fig. 4. The metabolisation of exogenous [^{14}C]-arachidonic acid by washed human platelets. Upper panel: the formation of 12-HETE by platelets pretreated with an inhibitor of the cyclo-oxygenase enzyme, suprofen. Middle panel: the inhibition of the cyclo-oxygenase enzyme in untreated platelets. Lower panel: the formation of 12-HETE by untreated platelets. Results are expressed as a percentage of the control incubations. Each point represents the mean of three separate determinations. ●: Ketoconazole; ○: NDGA; ▽: Nafazatrom; □: Phenidone; △: BW 755C.

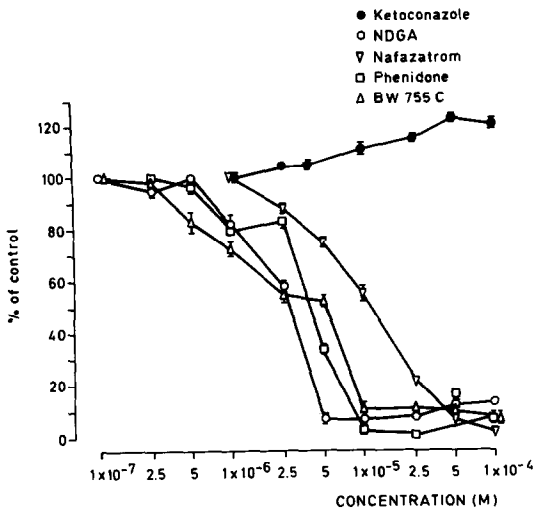


Fig. 5. Peroxidation of guaiacol by horseradish peroxidase. Results are expressed as a percentage of the control incubation. Each point represents the mean ± S.E.M. of at least four separate determinations. ●: Ketoconazole; ○: NDGA; ▽: Nafazatrom; □: Phenidone; △: BW 755C.

Ketoconazole inhibits in a concentration dependent manner the formation of the 5-lipoxygenase products in the rat PMN leukocytes *in vitro*. Its activity was compared with other known inhibitors of the lipoxygenase enzymes such as NDGA, BW 755C, nafazatrom and phenidone, which all inhibited the 5-lipoxygenase; the IC_{50}-values, found in our model, are in good agreement with previous published values in peritoneal PMN [15, 20–24]. Ketoconazole appears to be slightly more active than BW 755C, and 2–3 times less active than phenidone and nafazatrom. However, NDGA was the most potent inhibitor of the 5-lipoxygenase enzyme. For all compounds, the inhibition takes place at the level of the 5-lipoxygenase enzyme, as evidenced by the simultaneous inhibition of the formation of LTB_4 and 5-HETE. Ketoconazole also inhibits the 5-lipoxygenase reaction in a cell-free 10,000 *g* supernatant of homogenates of PMN leukocytes. This experiment excludes an indirect mechanism of the inhibitory activity, e.g. effect on the mobilization of calcium or on an interaction with the ionophore A23187 as has been shown for benoxaprofen [25, 26], and clearly demonstrate the direct effect of ketoconazole on the 5-lipoxygenase enzyme.

The inhibition of the 5-lipoxygenase by ketoconazole cannot be related to its radical scavenging and antioxidative properties, as has been suggested for the other tested inhibitors [27]. Indeed, ketoconazole stimulates the peroxidation of guaiacol in the concentration range where it inhibits the PMN-lipoxygenase, whereas the other tested compounds all inhibit the guaiacol peroxidation.

The interaction of ketoconazole with cytochrome P-450 dependent enzymes has been suggested as the molecular basis for its therapeutic effects [28]. Indeed, ketoconazole inhibits the cytochrome P-450 dependent enzymes of the ergosterol synthesis in yeast [28] and the cytochrome P-450 mediated testosterone production in man and animals [29, 30]. Recently, the formation of epoxides and mono- as well as dihydroxy-derivatives of arachidonic acid by microsomal cytochrome P-450 enzymes has been demonstrated [31–34]. Metyrapone, a documented inhibitor of cytochrome P-450 dependent oxidative reactions [35, 36], also inhibits the formation of the 5-lipoxgenase products by rat PMN leukocytes in a concentration dependent manner. Therefore, it is tempting to consider the formation of 5-HPETE by the 5-lipoxygenase and the subsequent transformation into leukotrienes in the rat PMN leukocytes as a cytochrome P-450 linked process. However, the enzymes involved in the formation of LTB_4 appear to be cytosolic [37], which is highly unusual for a cytochrome P-450 of higher animals. Furthermore, the 5-lipoxygenase and the LTA_4-hydrolase have recently been partially purified [38, 39], and no evidence for a cytochrome P-450 nature was provided. Therefore, the inhibition of the 5-lipoxygenase by

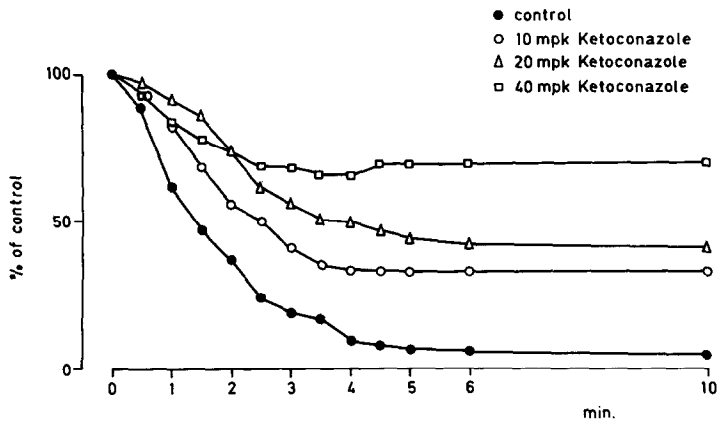


Fig. 6. Effects of ketoconazole on antigen-induced bronchoconstriction in guinea pigs. The animals were pretreated with ketoconazole orally. Two hours later, the animals were challenged with ovalbumin and the tidal volume was measured. Results are expressed as a percentage of the tidal volume before the challenge. Each point represents the mean of at least six separate observations. ●: Control; ○: 10 mg/kg ketoconazole; △: 20 mg/kg ketoconazole; □: 40 mg/kg ketoconazole.

ketoconazole must rely on an as yet unidentified mechanism.

Ketoconazole increases several times the formation of both hydroxy-derivatives 12- and 15-HETE, which are formed by the PMN leukocytes themselves and contaminating macrophages [40–42]. The other inhibitors of the 5-lipoxygenase hardly affect the 12/15-HETE production, except for nafazatrom and metyrapone which also increase the formation of both hydroxy-derivatives, although to a lesser extent. This increased formation of 12- and 15-HETE might be explained by a redirection of the substrate to the other lipoxygenases as the 5-lipoxygenase is inhibited. This concept is supported by the observation that the increase of 12/15-HETE is much less pronounced when no inhibitor of the cyclo-oxygenase is present in the incubation mixture, allowing a redirection of the metabolism of arachidonic acid to the cyclo-oxygenase pathway. However, ketoconazole directly stimulates the formation of 15-lipoxygenase products in the rabbit reticulocytes. Such a direct stimulation of the 15-lipoxygenase might partly contribute to the increased 15-HETE production and explain the slightly decreased ratio for 12-HETE/15-HETE.

It has been shown that 15-HETE selectively inhibits 5-HETE-formation in PMN leukocytes [43]. Therefore, the inhibition of the 5-lipoxygenase enzyme by ketoconazole, in theory, could also be explained by the increased production of 15-HETE. However, the maximal concentration of 15-HETE can only be estimated to be about 0.5 μM. Even taking into account the release of endogenous arachidonic acid, such a concentration is too low to produce a complete inhibition, the IC_{50}-value being 6 μM [43]. Therefore, the increased formation of 15-HETE can only contribute for a very small part to the inhibitory effect of ketoconazole on the 5-lipoxygenase enzyme. The inhibition, therefore, must be explained by another mechanism.

In contrast to the other lipoxygenase inhibitors, ketoconazole stimulates the 15-lipoxygenase of reticulocytes. The relative potencies of BW 755C, phenidone, nafazatrom and NDGA to inhibit the formation of 15-HETE in rabbit reticulocytes correlated well with those for inhibition of guaiacol peroxidation; on the other hand, the stimulation of the peroxidation by ketoconazole in one system matched that in the other one. The mechanism of this stimulatory activity remains unclear.

Ketoconazole has no effect on the cyclo-oxygenase nor on the 12-lipoxygenase in human platelets, in contrast to the other inhibitors, which all inhibit the cyclo-oxygenase of the platelets to some degree. However, ketoconazole inhibits the platelet thromboxane synthase, as evidenced by the reduced formation of TXB_2 and the concomitant increase of PGE_2, PGD_2 and $PGF_{2\alpha}$, the non-enzymatic breakdown products of the endoperoxide H_2, confirming previous experiments [44]. This inhibition of the TXB_2 formation can be rationalized through an interaction of ketoconazole with the cytochrome P-450. Indeed, the thromboxane synthase has been shown to be a cytochrome P-450 dependent enzyme [45].

Judged on the scarcity of reports, there seem to exist few selective lipoxygenase inhibitors with activity *in vivo* after oral administration. Our results indicate that ketoconazole is such an orally active inhibitor of the lipoxygenase *in vivo*. Indeed, ketoconazole reduced the antigen-induced bronchoconstriction in actively sensitized guinea pigs. Under the experimental conditions, the bronchoconstriction is primarily mediated by SRS-A [46, 47]. Indeed, the administration prior to the ovalbumin challenge of the receptor antagonists of histamine (pyrilamine), and of serotonin (methysergide) and the cyclo-oxygenase inhibitor (indomethacin), exclude the possible involvement of these mediators in the remaining ovalbumin-induced bronchoconstriction. Anderson *et al.* [46]. demonstrated that SRS-A appeared in the plasma during the anaphylactic shock and that the inhibition of the bronchoconstriction by the intravenous administration of the SRS-A synthesis inhibitor phenidone coincided with a dose-dependent reduction of the plasma SRS-A levels. Furthermore, in our experiments the antigen-induced bronchoconstriction was completely inhibited by the intravenous administration, prior to the challenge, of FPL 55 712, an established antagonist of SRS-A [17], and by the i.v. injection of diethylcarbamazine, a drug which inhibits the biosynthesis of SRS-A [18, 19]. Our experiments confirm previous published results [46, 47] supporting the SRS-A-dependent nature of the antigen-induced bronchoconstriction.

The inhibitory effect of ketoconazole on this type of bronchoconstriction demonstrates its ability to reduce the production of leukotrienes *in vivo* after oral administration.

In conclusion, our results demonstrate that ketoconazole is a comparatively selective inhibitor of the leukotriene biosynthesis *in vitro* as well as *in vivo* after oral administration.

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