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INDUCING EFFECT OF OXFENDAZOLE ON CYTOCHROME P450IA2 IN RABBIT LIVER

CONSEQUENCES ON CYTOCHROME P450 DEPENDENT MONOOXYGENASES

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Abstract—Male New Zealand rabbits were dosed with either 0.9, 4.5 or 22.5 mg/kg/day of oxfendazole by gastric intubation for 10 days. Oxfendazole administered at the therapeutic dose (4.5 mg/kg) and at the highest dose (22.5 mg/kg) increased 1.54- and 2.36-fold the total liver microsomal cytochrome P450 and more particularly the isoenzyme P450IA2 (95 and 184% increases) as demonstrated by western blotting. Increases in ethoxyresorufin O-deethylation and hydroxylations of benzopyrene and acetanilide occurred in livers of the same animals without any change in N-demethylation of aminopyrine, benzphetamine or erythromycin. Because of the unchanged level of mRNA specific to cytochrome P450IA2, as shown by northern blot analysis of poly mRNA, an enzyme stabilization rather than a transcriptional activation of IA2 genes should be involved in the P450IA2 regulation mechanisms. Oxfendazole bound strongly to cytochrome P450, giving rise to a type II spectrum, and inhibited non-competitively the ethoxyresorufin *O*-deethylase and acetanilide hydroxylase activities, this confirmed that oxfendazole interacts only with the P450IA family. On the basis of a comparison of the enzymatic activities induced by various imidazole drugs, it was concluded that oxfendazole, like omeprazole and albendazole, behaved as a 3-methylcholanthrene-type inducer. These three benzimidazoles did not all belong to the same category of cytochrome P450 inducers as the antifungal drugs miconazole, clotrimazole and ketoconazole.

Oxfendazole ([5-(phenylsulfinyl)-1*H*-benzimidazol-2-yl] carbamic acid methyl ether) (Fig. 1) is a thio-substituted benzimidazole derivative which is extensively used in veterinary medicine as a potent antinematodal drug against gastrointestinal and pulmonary nematodes in ruminants, horses and pigs. Its mode of anthelmintic action would consist of the inhibition of both glucose uptake in the parasitic worm and tubulin polymerization [1]. The pharmacokinetics of this drug are characterized by a biological half-life of elimination around 30 hr in ruminant species [2]. Since divided administration produced sustained high plasma levels responsible for enhanced potency, this drug is currently administered as slow release devices in breeding animals.

Many imidazole-containing drugs such as miconazole, ketoconazole, clotrimazole and 1-benzylimidazole have been described as inducing hepatic drug metabolizing enzymes in male rats [3, 4]. More particularly, miconazole and ketoconazole displayed biphasic effects on the mixed-function oxidase activities with specific binding to cytochrome P450 resulting in a type II spectra and inhibition towards the *p*-nitroanisole *O*-demethylase activity of the induced microsomes. On the other hand, albendazole, a closely related anthelmintic benzimidazole, has been described as increasing cytochrome P448 and benzo[*a*]pyrene hydroxylase, 7-ethoxycoumarin or 7-ethoxyresorufin *O*-deethylase activities in the liver of rats receiving 10.6 mg/kg per day for 10 consecutive days [5].

Because of the repeated use of oxfendazole in breeding animals, as well as the biological persistence of this drug and the inductive properties of a structurally related benzimidazole, it was decided to study the possible interaction of oxfendazole with liver mixed-function oxidases and the male rabbit was used as a model. The levels of the main cytochrome P450 isoenzymes and specific mRNA were determined by immunoblotting and northern blotting analysis giving a tentative explanation of the mode of inducing action of oxfendazole. The *in vitro* interaction of oxfendazole was also determined by investigating the optical difference spectrophotometry of microsomal fractions and the inhibition towards cytochrome P450 dependent monooxygenases.

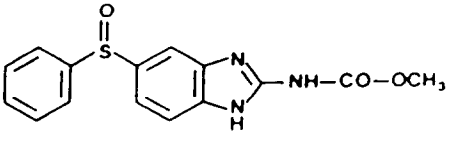

Fig. 1. Chemical structure of oxfendazole.

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MATERIALS AND METHODS

Chemicals. Oxfendazole was a gift from Rhône-Merieux Laboratory (Toulouse, France); 7-ethoxyresorufin, NADPH and erythromycin were purchased from Boehringer-Mannheim (Meylan, France); resorufin from Aldrich-Chimie (Strasbourg, France); different IgGs, benzopyrene and aminopyrine from Sigma (La Verpillière, France); and acetanilide and acetaminophen from Serva (Heidelberg, Germany). Anti-rabbit-P450 IA1, IA2, IIB1, IIIA IgG, rabbit liver cytochrome P450 IA2 and cDNA probes were kindly provided by Dr P. Maurel (INSERM U.128, Montpellier, France).

Treatment of animals. Sixteen male adult New Zealand white rabbits (2.0 ± 0.1 kg) were used; they were housed individually and allowed free access to food (UFAC, Rental Languedoc, Toulouse, France) and water. They were randomly divided into four groups of four animals: one control group and three oxfendazole-treated groups receiving either 0.9, 4.5 (therapeutic dose) or 22.5 mg/kg [respectively 8, 40, 200 mg dissolved in 10 mL of 5% (w/v) arabic gum water solution]. Controls received similar volumes of the alone vehicle. Treatments were performed each day by gastric administration for 10 days. Rabbits were killed 24 hr after the last administration; livers were immediately perfused *in situ* with 0.5 mM ethylene glycol-bis (β-amino ethyl Ether) *N,N,N',N'*-tetraacetic acid at the rate of 20 mL/min, removed and weighed.

Preparation of microsomes and RNA. Hepatic microsomes were prepared by differential centrifugation as described previously [6] and stored at −80° until used. Total RNA was prepared from the whole liver by the guanidine-thiocyanate procedure [7]. The yield of RNA redissolved in 300 μL of Tris–EDTA buffer averaged 1.53 mg/g.

Determination of protein and cytochrome P450 content. Protein concentration was assayed by the method of Lowry *et al.* [8] using bovine serum albumin as standard. Total cytochrome P450 content was spectrophotometrically determined according to the method of Omura and Sato [9].

Mixed-function oxidase assays. The N-demethylation of aminopyrine, benzphetamine and erythromycin was estimated by the formaldehyde formation detected by the method of Nash modified by Cochin and Axelrod [10].

The resorufin-producing activity of 7-ethoxyresorufin *O*-deethylase was determined fluorimetrically by a modification of the method of Lake [11]. The incubation mixture contained 1 mg of microsomal protein and 100 μL of 8.5 mM NADPH in a final volume of 850 μL in 12.5 mM Tris–HCl buffer (pH 8.4). After a 5 min preincubation in a shaking water bath (37°), the reaction was started by the addition of 50 μL of 8.5 μM ethoxyresorufin and stopped by 500 μL of 5% (w/v) zinc sulfate and 500 μL of saturated barium hydroxide. After 5 min of centrifugation (13,000 rpm), 500 μL of the deproteinized supernatant were removed and added with 1 mL of 0.5 M glycine–NaOH buffer (pH 8.5). Fluorescence was measured on a Perkin-Elmer MPF3 spectrofluorimeter with excitation and emission set to 535 and 582 nm, respectively. A 4.5 μM solution of resorufin was used to establish a standard curve between 0 and 0.04 μM.

Concerning the activity of acetanilide hydroxylase, the concentrations of acetanilide and acetaminophen in microsomal hepatic fractions were determined by means of a modification of a high performance liquid chromatography method, previously described [12]. Samples (0.1 mL) were added to 1.0 mL ethyl acetate in a 2.0 mL polypropylene tube. After shaking and centrifugation, the organic phases were removed and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μL of elution solvent and injected into the column (250 × 4.6 mm reverse phase partisil ODS 3 Whatman) of a chromatograph (Model 201, Waters) equipped with an ultraviolet detector (detection wavelength: 254 nm). The mobile phase acetic acid–2% methanol (70:30; v/v) was pumped at a rate of 1.5 mL/min. The retention times were 3.5 and 8.0 min for acetaminophen and acetanilid, respectively.

Determination and quantification of cytochrome P450 isoenzymes. Solubilized microsomes were electrophoresed in 9% polyacrylamide gel in the presence of SDS according to the method of Laemmli [13]. The separated microsomal proteins were electrophoretically transferred to nitrocellulose membranes as described elsewhere [14, 15]. The membranes were first incubated with anti P450 IA1, IA2, or IIIA7 IgG produced in sheep or goat, then with rabbit anti-sheep or anti-goat IgG and finally with peroxidase-labelled sheep anti-rabbit IgG. Specific content of each form of cytochrome P450 was quantified by densitometry by using a Shimadzu CS,930 scanner.

Northern blots. Electrophoresis on 1.2% agarose gel of RNA (10 μg) prepared from whole liver, transfer to nylon membrane (Zetabind, Cuno Inc., Meriden, NC), prehybridation and hybridation with appropriate radiolabelled probes (kit from Amersham, les Ulis, France): 5'pIA2, 3'pIA1, pIIIA7, or pGAPDH were carried out as previously described [14]. Membranes were autoradiographed for 48 hr at −80°. In control experiments, membranes were dehybridized and rehybridized with radiolabelled pGAPDH to ascertain that identical amounts of mRNA had been analysed for each sample.

Optical difference spectrophotometry. Difference spectra were recorded on a Kontron Uvikon 860 double beam spectrophotometer using quartz cuvettes. A baseline was realised with 1.5 mL of a microsomal suspension (1 mg protein/mL) in 0.1 M potassium phosphate buffer (pH 7.4) in sample and reference cuvettes. Oxfendazole (1 mM) dissolved in DMSO was added in aliquots of 0.5 μL up to a volume of 2.5 μL to the test cuvette and an equal volume of DMSO was added to the reference cuvette. Spectra were recorded between 350 and 500 nm and the absorbance peak minus trough values were used to construct a double reciprocal plot of ΔO.D. versus oxfendazole

Table 1. Cytochrome P450 and mixed-function oxidase activities in rabbit liver microsomes after oral administration of vehicle (controls), 0.9, 4.5 and 22.5 mg/kg/day of oxfendazole for 10 days

Enzymes	Controls	Oxfendazole (mg/kg/day)		
		0.9	4.5	22.5
Cytochrome P450				
Total P450	0.881 ± 0.186	0.971 ± 0.162	1.356 ± 0.225*	2.084 ± 0.480*
P450IA2 isoenzyme	0.604 ± 0.121	0.512 ± 0.103	1.180 ± 0.185*	1.580 ± 0.342*
N-Demethylation				
Aminopyrine	1.60 ± 0.17	1.51 ± 0.25	1.37 ± 0.17	1.36 ± 0.12
Benzphetamine	3.35 ± 0.57	3.22 ± 0.44	2.94 ± 0.45	3.38 ± 0.36
Erythromycin	0.52 ± 0.22	0.60 ± 0.14	0.53 ± 0.11	0.53 ± 0.14
O-Deethylation				
Ethoxyresorufin	10.88 ± 1.37	12.20 ± 2.96	15.36 ± 0.93*	20.92 ± 3.40*
Hydroxylation				
Acetanilide	4.19 ± 0.32	4.64 ± 0.30	5.05 ± 0.35*	5.08 ± 0.24*

Values are means ± SD of eight liver microsomal fractions. They are expressed as nmol/mg for cytochrome concentrations, and as nmol/min/mg proteins for mixed-function oxidase activities.
* Significantly different ($P < 0.05$) from control.

concentration, to determine the dissociation constant (K_s).

In vitro *interaction of oxfendazole with P450 dependent monooxygenases*. Benzphetamine and erythromycin *N*-demethylase, ethoxyresorufin *O*-deethylase or acetanilide hydroxylase activities were determined in the absence or in the presence of increasing oxfendazole concentrations (0–117 μM). Different oxfendazole concentrations (0, 35, 117 μM) were assayed with increasing ethoxyresorufin concentrations (0.1–0.5 μM). The results were reported as Lineweaver–Burk plots and the inhibitory constant (K_i) was determined.

Statistical analysis. The results were reported as the mean ± SD for four animals and two liver lobes per animal (N = 8). The statistical significance between treatments was achieved with analysis of variance followed by Dunnett's test. Differences were considered to be significant at $P < 0.05$ level.

RESULTS

Effect of oxfendazole on hepatic mixed-function oxidases

Whatever dose of oxfendazole was used, there was no significant change in either body weight (2.32 ± 0.19 kg), liver weight (96.9 ± 13.6 g) or concentration of microsomal proteins (17.6 ± 0.5 mg/g) as compared with untreated control animals. When oxfendazole was administered at a dose of 0.9 mg/kg/day, the total liver microsomal cytochrome P450 remained unchanged (Table 1) whereas the administration of 4.5 or 22.5 mg/kg oxfendazole for 10 days was associated with significant cytochrome P450 increases: 54 and 136% of control, respectively. While aminopyrine, benzphetamine and erythromycin *N*-demethylase activities were not influenced by the different treatments, ethoxyresorufin *O*-deethylase and acetanilide hydroxylase activities though unchanged by the lower dose (0.9 mg/kg/day), significantly increased (41.5 or 92.3% for the ethoxyresorufin and 20.5 or 21.3% for the acetanilide) in liver of animals receiving 4.5 or 22.5 mg/kg/day doses, respectively.

Effect of oxfendazole on accumulation of cytochrome P450 isoenzymes and specific mRNA

Western blot analysis of cytochromes P450IA1, IIB4 and IIIA7 isoenzymes revealed no significant change in the level of these forms in the liver of treated rabbit, by comparison to control animals, which received the vehicle only. In contrast, significant increases (95.4 or 184.1%) in P450IA2 isoenzyme occurred in the microsomal fractions of animals receiving 4.5 or 22.5 mg/kg/day of oxfendazole (Table 1, Fig. 2). Northern blot analysis of poly mRNA prepared from untreated or oxfendazole-treated animals, using 3′pIA1 and 5′pIA2 cDNA probes did not reveal any significant change in the level of mRNA specific to the two cytochromes P450IA1 or P450IA2 (Fig. 3).

In vitro *interaction of oxfendazole with cytochrome P450*

The addition of oxfendazole to microsomal suspensions gave similar difference spectra for control and oxfendazole pre-treated rabbits (Fig. 4). The spectrum was characterized by trough, isobestic and peak wavelength at 391, 406 and 422 nm, respectively. This indicated a type II spectrum related to the interaction of the imidazole group of the drug with oxidized cytochrome P450 [16]. The dissociation constant of the complex ($K_s = 1.63 \pm 0.20$ μM) was evaluated from the double reciprocal plot of O.D. change versus oxfendazole concentration (Fig. 5).

In vitro *interaction of oxfendazole with cytochrome P450 dependent monooxygenases*

As shown in Fig. 6, oxfendazole dose-dependently inhibited both ethoxyresorufin *O*-deethylase and acetanilide hydroxylase activities whatever the origin of the microsomal fractions was. Under these conditions, the concentration of oxfendazole which provoked a 50% inhibition of these two oxidases (I_{50}) was around 30 μM. In contrast,

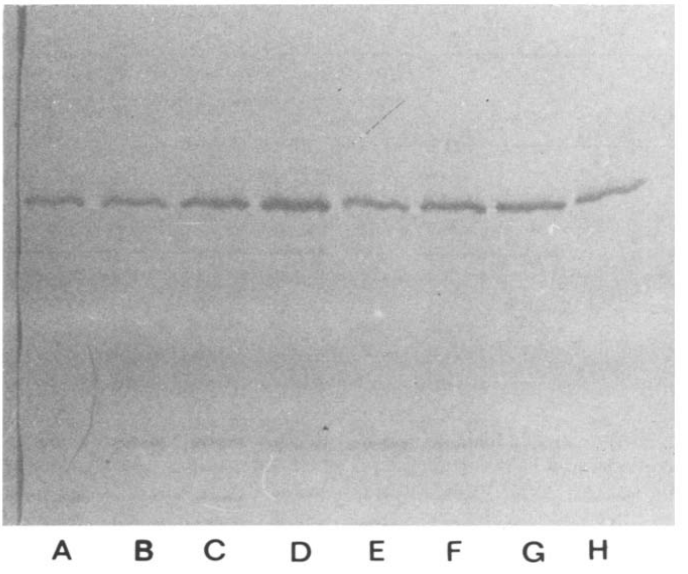

Fig. 2. Western blot analysis of liver microsomes from control (B, E), 4.5 mg/kg/day (C, F) or 22.5 mg/kg/day (D, G) oxfendazole-treated rabbits. Lanes A and H correspond to 2 and 4 pmoles of purified rabbit P450IA2 isoenzyme, respectively. Microsomal proteins (5 μg) were submitted to electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies prepared against rabbit liver cytochrome P450IA2.

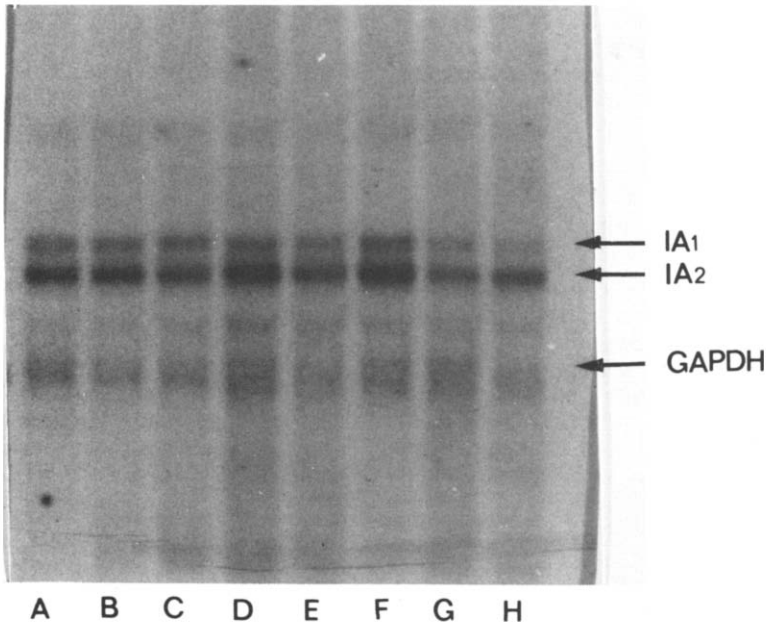

Fig. 3. Effect of oxfendazole on the level of P450IA1 and P450IA2 mRNA from liver of control (A, B, C), 4.5 mg/kg/day (D, E, F) or 22.5 mg/kg/day (G, H) treated rabbits. RNA (10 μg) was submitted to electrophoresis on a 1.2% agarose gel, transferred to nylon filter and probed with radiolabelled [^{32}P]3′pIA1 and [^{32}P]5′pIA2 (10^7 cpm). Nick translated pGAPDH was used to ascertain that identical amounts of mRNA were loaded on the gel. The filter was autoradiographed for 16 hr at −80°.

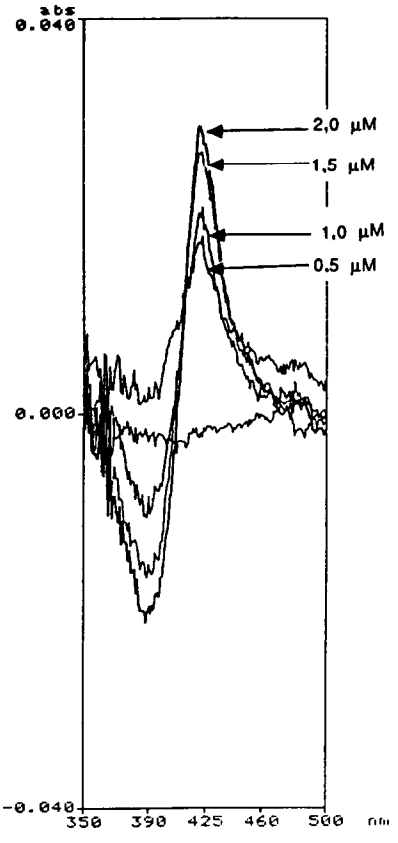

Fig. 4. Type II spectra of rabbit liver microsomes in presence of 0, 0.5, 1.0, 1.5 and 2.0 μL of 1 mM oxfendazole solution in DMSO.

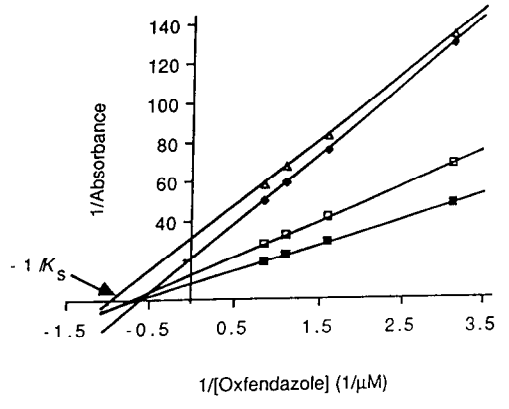

Fig. 5. Double reciprocal plot of absorbance change between 391 and 422 nm vs oxfendazole concentration for microsomes from control (△), 0.9 mg/kg/day (◆), 4.5 mg/kg/day (□) and 22.5 mg/kg/day (■) treated rabbits. The corresponding K_s values were respectively: 1.44 ± 0.80, 1.77 ± 0.30, 1.51 ± 0.20 and 1.82 ± 0.33 μM. Each curve is the mean of four determinations.

oxfendazole did not affect the N-demethylation of benzphetamine or erythromycin. The analysis of the Lineweaver–Burk plot of ethoxyresorufin *O*-deethylase inhibition by oxfendazole revealed that the inhibition was non-competitive (Fig. 7). This inhibition was characterized by a K_m value (ethoxyresorufin) of 0.32 ± 0.04 μM and a K_i value (oxfendazole) of 51 μM.

DISCUSSION

Observation of animals in the course of oxfendazole treatment and at autopsy revealed that the anthelminitic drug did not provoke any toxicity in rabbits receiving up to 5-fold the therapeutic dose, each day for 10 days.

The data presented in this paper showed that after subchronic dosing of male rabbits with oxfendazole at 0.9 mg/kg, there was no effect on either hepatic cytochrome P450 level or P450-dependent monooxygenases. In contrast, the daily administration of 4.5 or 22.5 mg/kg resulted in significant increases in total cytochrome P450 and more particularly the P450IA2 isoenzyme. From our results, the P450IA2 corresponded in control animals to about 60% of the total hepatic cytochrome P450; this would mean that the IA2 isoenzyme is a major form in rabbit liver, as generally observed [17]. The inducing effect of oxfendazole on P450IA2 isoform is in good agreement with the corresponding enhancements of both ethoxyresorufin *O*-deethylase and acetanilide hydroxylase activities, since the cytochrome P450IA family has been described as being involved in the metabolism of ethoxyresorufin [18] and acetanilide [19]. Furthermore, oxfendazole was devoid of inducing properties, even at the highest dose level, towards P450IIB and P450IIIA isoenzymes as demonstrated by western blotting analysis; this is also supported by the unchanged N-demethylation of aminopyrine, benzphetamine or erythromycin. Indeed, these two last compounds are now well established as specific marker substrates for cytochromes P450IIB4 and P450IIIA7, respectively [20].

Such a specific pattern of hepatic mixed-function oxidase induction by oxfendazole may be related to that obtained with the closely-related anthelmintic albendazole, and omeprazole, a benzimidazole used in the treatment of gastric ulcers. These drugs increased both cytochrome P450IA1 and IA2 dependent monooxygenases without any effect on benzphetamine N-demethylation or other cytochrome P450 in rats receiving a daily oral administration of 10.6 mg/kg for 10 days [5] or in human hepatocytes [21]. Because the two anthelmintics are thio-substituted benzimidazoles, one could suppose the existence of a common active binding site on cytochrome P450IA2 which accepts both sulfur (albendazole) or sulfoxide (oxfendazole, albendazole sulfoxide) regardless of the aliphatic (albendazole) or aromatic (oxfendazole) nature of the 5-thio-substituted group [22]. In relation to their different chemical structures, the inducing properties of these drugs appear quite different from those of imidazole-containing

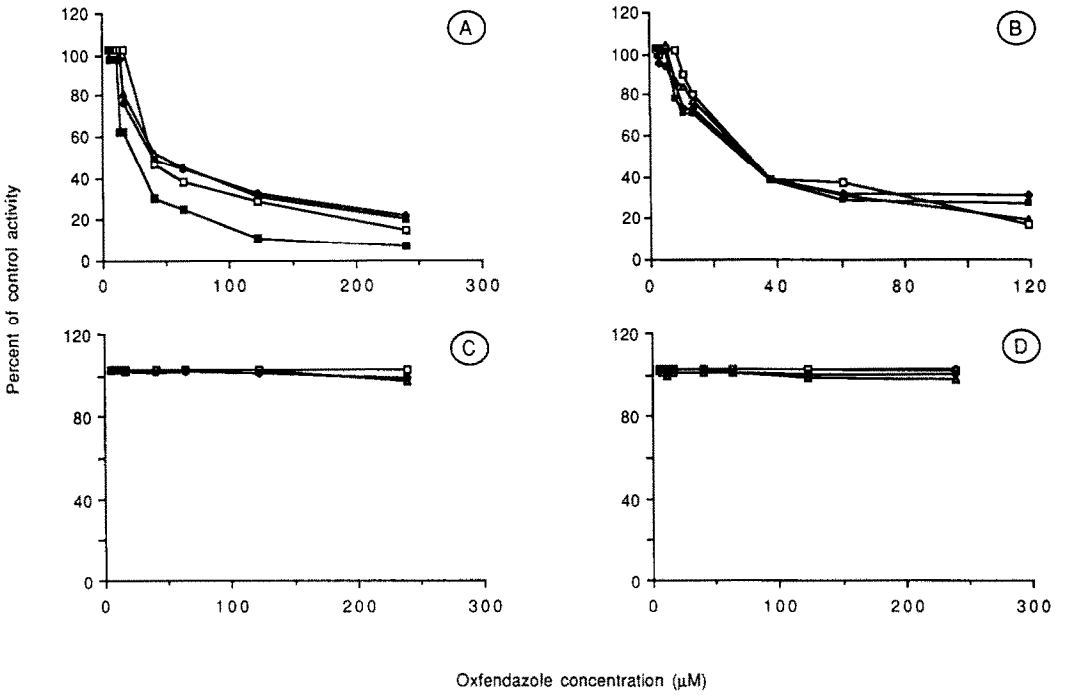

Fig. 6. Dose–response curves for the inhibition of ethoxyresorufin *O*-deethylase (A), acetanilide hydroxylase (B), benzphetamine (C) and erythromycin (D) *N*-demethylase activities by oxfendazole. The inhibitory effect of the drug was assayed on liver microsomes from control (△), 0.9 mg/kg/day (◆), 4.5 mg/kg/day (□) and 22.5 mg/kg/day (■) treated rabbits. The control activities were respectively 10.83 (A), 4.20 (B), 3.5 (C) and 0.59 (D) nmol/min/mg proteins.

antifungals: clotrimazole, miconazole and ketoconazole. These agents have been previously described as inducers of the rat hepatic microsomal mixed-function oxidases, displaying selectivity towards the P450IIB (phenobarbital inducible) and P450IIIA (PCN inductible) families of liver cytochrome P450 proteins [23].

The *in vitro* inhibition of both ethoxyresorufin O-deethylation and acetanilide hydroxylation but not of N-demethylation of benzphetamine or erythromycin confirms the substrate selectivity of oxfendazole for the cytochrome P450IA family. Despite the fact that no evident metabolic complex occurs between oxfendazole and cytochrome P450 (unpublished complementary assay), the drug was shown to elicit a type II difference spectrum with microsomal preparations from either untreated or oxfendazole-treated rabbit livers. The low K_s value obtained (1.63 μM) clearly indicates a high affinity interaction associated with the formation of ferrihaemochrome with cytochrome P450 [15]. This interaction would influence the normal activity of ethoxyresorufin O-deethylation as demonstrated *in vitro* by the non-competitive inhibition of this activity by oxfendazole. The nature of this inhibition would suggest that oxfendazole binds to the cytochrome P450IA2 in a region distinct from the ethoxyresorufin binding site [24]. This region is most probably the haem prosthetic group which is the target of many inhibitors and critical for monooxygenase activity [25].

The results of the northern blotting analysis of mRNA clearly indicate that the induction of P450IA2 by oxfendazole in rabbit liver is not due to an accumulation of mRNA specific to P450IA2. In consequence, the mechanism of induction should not be related to transcriptional activation of IA2 gene expression but could rather involve a differential regulation by enzyme stabilization. Such a mode of action could be related to that of polychlorinated and polybrominated biphenyls which are described as potent inducers of cytochrome P450IA2, and are unusual in that they induce more P450IA2 than P450IA1 [26, 27]. Several of these inducers would form stable complexes with P450IA2 [28, 29] that might also stabilize the enzyme against degradation, prolonging its half-life and increasing its specific content in the endoplasmic reticulum.

The discovery of the inducing properties of the benzimidazole sulfoxide derivative oxfendazole on hepatic cytochrome P450 is of interest. Indeed, it could be related to the recent description of a similar potency of albendazole sulfoxide which was described as an inducer of cytochrome P448 and ethoxyresorufin O-deethylation in cultures of human hepatoma Hep G2 cells whereas albendazole (the sulfur parent drug) fails to increase these enzyme activities [30]. Finally, the present study demonstrates the incidence of a repetitive administration of therapeutic doses of oxfendazole on the liver mixed-function oxidases by increasing the stability of cytochrome P450IA2. In cases of such an induction in breeding animal species, this could

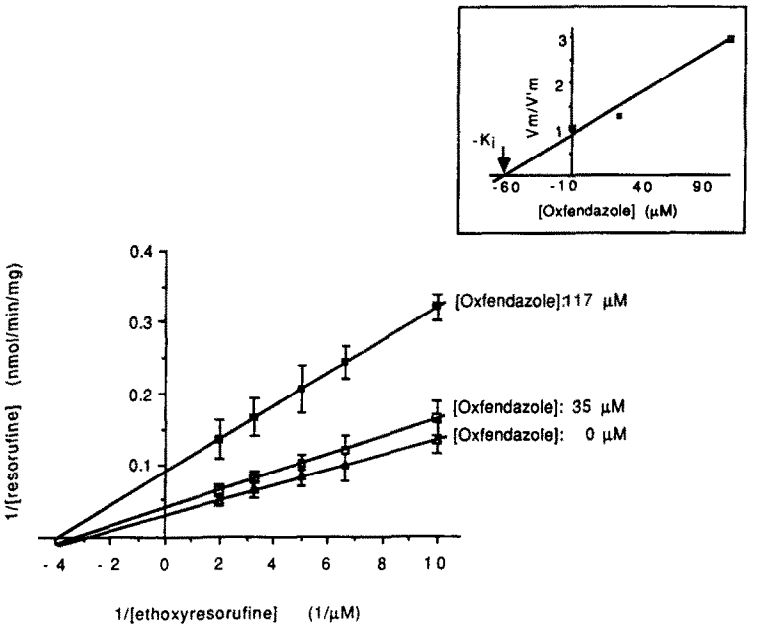

Fig. 7. Inhibitory effect of oxfendazole on the rate of O-deethylation of ethoxyresorufin. This activity was measured in the absence (△) or the presence of 35 μM (□) or 117 μM (■) oxfendazole. The figure at the bottom shows double reciprocal plots of the rate of ethoxyresorufin O-deethylation vs concentrations of ethoxyresorufin. The figure at the top shows a plot of oxfendazole vs the slopes of the lines from the lower figure.

change the capacity of liver for handling endogenous substrates, drugs and other xenobiotics with possible consequences in the drug dosage adjustment or in veterinary drug retention in the animal's body.

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