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STEREOCHEMICAL SELECTIVITY IN THE INDUCTION OF CYTOCHROME P450IVA1 (P452)-DEPENDENT FATTY ACID HYDROXYLATION AND PEROXISOME PROLIFERATION

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Abstract—Induction of hepatic microsomal cytochrome P450IVA1 and peroxisomal enzymes of the β-oxidation spiral were observed when male Long Evans hooded rats were administered optically pure enantiomeric forms and a racemic mixture of a clofibrate analogue [2-[4-(4-chlorophenyl)benzyloxy]-2-phenylacetic acid] at a dose level of 80 mg/kg for 3 days. The *R*(−)-enantiomer was found to be a more potent inducer of microsomal cytochrome P450IVA1 and its associated lauric acid 12-hydroxylase activity than its corresponding *S*(+)-antipode. This difference in potency was reflected by a eudismic ratio (*R*/*S* activity ratio) of approximately 3, whereas the racemic mixture exhibited a potency intermediary between the two isomers. An identical enantiomeric selectivity was observed for the phenomenon of peroxisome proliferation as judged by induction of cyanide-insensitive palmitoyl CoA oxidation and the bifunctional protein of the peroxisomal β-oxidation spiral. The highest potency was shown by the *R*(−)-isomer resulting in approximately a 3–6-fold increase over the control value. These increases were paralleled by an increase in total carnitine acetyl transferase activity with a eudismic ratio of approximately 4. In addition, immunochemical detection by Western blotting analysis for both the microsomal cytochrome P450IVA1 isozyme and the peroxisomal bifunctional protein was in agreement with the above modulation of catalytic activities. These results are therefore not inconsistent with the hypothesis that cytochrome P450IVA1 induction and peroxisome proliferation are intimately linked. Whether the observed stereochemical selectivity resides in xenobiotic recognition or disposition still remains to be determined.

The administration of clofibrate and various other hypolipidaemic agents including non-therapeutic compounds such as the phthalate ester plasticizers, results in several, characteristic hepatic changes in rodents. These induced hepatic responses include hepatomegaly, proliferation of smooth endoplasmic reticulum and induction of cytochrome P450IVA1-dependent fatty acid omega hydroxylase activity, peroxisome proliferation with associated changes in enzyme composition and alteration in mitochondrial number and structure with concomitant increases in certain enzyme levels [1–4]. These subcellular changes are toxicologically important as sustained proliferation of peroxisomes in rodents is frequently associated with the development of hepatocellular carcinomas and this has led to the suggestion that peroxisome proliferators constitute a novel class of non-genotoxic hepatocarcinogens [5–7]. In particular, the relationship between the xenobiotic-mediated induction of cytochrome P450IVA1 and peroxisome proliferation is at present still not clear. Various suggestions have been proposed including the presence of a common or related organelle biogenesis [8] or a cytosolic "receptor protein" [9]. The involvement of such a receptor in the induction phenomenon would, by its presence or absence, readily explain the well-documented tissue, sex and species differences in response to these agents, but the existence of this putative receptor still remains a matter of debate [10].

More recently, attempts have been made to explore any correlation between the induction of several enzymes related to both drug and lipid metabolism following treatment with structurally diverse peroxisome proliferators [4]. Results from such studies have led to the proposal that catalytically-competent cytochrome P450IVA1 is a necessary prerequisite for peroxisome proliferation by these xenobiotics, and the initial liver response is induction of microsomal cytochrome P450IVA1, subsequently resulting in increased cellular concentrations of long chain, dicarboxylic acids. As these latter metabolites are preferentially degraded by β-oxidation in the peroxisome (as opposed to the mitochondrion), it has been proposed that dicarboxylic acids then form the proximal stimulus for peroxisome proliferation in an attempt by the cell to maintain lipid homeostasis [4].

Based on preliminary studies at ICI Pharmaceuticals by Dr Brian Holloway, the present study examines the above hypothesis [4] further by comparing the relative abilities of optically-active enantiomers and the racemate of a clofibrate analogue to induce microsomal cytochrome P450IVA1 and/or peroxisome proliferation.

MATERIALS AND METHODS

Chemicals. The optically pure *R*(−)-isomer, S(+)-isomer and the racemic mixture of a clofibrate analogue [2-[4-(4-chlorophenyl)benzyloxy]-2-phenylacetic acid] (Fig. 1), were kindly provided by

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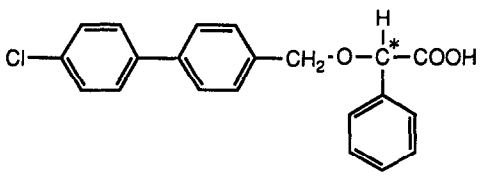

Fig. 1. Structure of 2-[4-(4-chlorophenyl)benzyloxy]-2-phenylacetic acid, showing the chiral centre.

ICI Pharmaceuticals (Macclesfield, U.K.) and synthesized by Dr M. E. Edge. Lauric acid, NADPH and prestained molecular weight markers (Stock No. SDS-7B) were purchased from the Sigma Chemical Co. (Poole, U.K.). [1-^{14}C]Lauric acid was supplied by the Radiochemical Centre (Amersham, U.K.).

Pure cytochrome P450IVA1 and the corresponding anti-serum were kind gifts from Drs M. N. Milton and Raj Sharma (University of Surrey, Guildford, U.K.). Anti-bifunctional peroxisomal enzyme serum was a kind gift from Dr D. Cinti (University of Connecticut Health Centre, CT, U.S.A.). Donkey anti-sheep enzyme label, donkey anti-rabbit enzyme label, pre-immune sheep and pre-immune rabbit sera were kindly supplied by Guildhay Antisera Ltd (Guildford, U.K.).

All other chemicals were obtained from commercial sources and were of the highest purity available.

Animals and drug pretreatment. Male Long Evans hooded rats (125–150 g body weight, University of Surrey Breeders) were pretreated by gavage once daily for 3 consecutive days with each enantiomer or the racemic mixture at a dose level of 80 mg/kg. Compounds were administered in gum tragacanth as vehicle and the control group received the same volume of vehicle only. All animals were killed at the start of the fourth day, i.e. 24 hr after the last dose.

The livers, following excision, were rinsed and perfused with 0.9% (w/v) sodium chloride to remove contaminating blood, blotted dry and weighed. All subsequent steps were performed at 4°. The livers were scissor-minced and homogenized in 0.25 M sucrose using a Potter-Elvehjem glass–Teflon homogenizer. The liver homogenate was adjusted to 25–33% (w/v) by the addition of 0.25 M sucrose. Microsomes were prepared by a standard centrifugation method and stored in 50 mM phosphate buffer, pH 7.25, containing 20% glycerol at −80° until required, without loss of original activity.

Enzyme assays. Total carbon monoxide-discernible cytochrome P450 was determined by the method of Omura and Sato [11] using a difference absorption coefficient (450–490 nm) of 91 $mM^{-1}\,cm^{-1}$ for the sodium dithionite–reduced CO adduct. Specific ELISA-based cytochrome P450IVA1 determination, potassium cyanide-insensitive palmitoyl CoA oxidation and carnitine acetyl transferase activities were determined as previously described [4].

Immunochemical quantitation of peroxisomal bifunctional protein was carried out by adaptation of the ELISA method used for quantifying cytochrome P450IVA1, with the use of donkey anti-rabbit horseradish peroxidase enzyme label instead of the corresponding donkey anti-sheep product [12].

The 11- and 12-hydroxy metabolites of lauric acid were separated by reverse-phase HPLC using a Micropak MCH-10 column (30 × 0.4 cm, Varian Associates Ltd, Walton-on-Thames, U.K.) using a linear gradient of water : methanol (45 : 55 containing 0.1% acetic acid) to 100% methanol over a 45 min period at a flow rate of 1.0 mL/min, as previously described [4]. The HPLC eluate containing radioactive metabolites, was passed through a Berthold LB503 radiodetector flow-cell (Lab-Impex, Twickenham, U.K.), interfaced with a Commodore PET (Series 4000), enabling quantitative analyses of lauric acid metabolism.

Western blotting. Qualitative evaluation of microsomal cytochrome P450IVA1 [4] and peroxisomal bifunctional protein [12] were carried out by minor modifications of the basic procedures of Towbin *et al.* [13] and Burnett [14], protein being determined by the Lowry method [15].

Statistical analysis. Statistical data evaluation was performed using the Student's *t*-test [16].

RESULTS

The effect of administration of the pure enantiomers and racemic mixture of the clofibrate analogue on the liver/body weight ratio (a measure of hepatomegaly), total carbon monoxide-discernible cytochrome P450 and specific cytochrome P450IVA1 isozyme levels are presented in Table 1.

Induction of specific cytochrome P450IVA1 was more accurately reflected in the enzyme-linked immunosorbent assay (Table 1). The cytochrome P450IVA1 isozyme level in the control group was approximately 5% of the total cytochrome P450 population, and induction by the isomers and racemate led to a 1.5–4-fold induction over this constitutive level. The highest fold increase in cytochrome P450IVA1 was observed after treatment the *R*(−)-enantiomer and corresponded to a eudismic ratio of approximately 3. Furthermore, the influence of treatment on cytochrome P450IVA1-dependent 11- and 12-hydroxylation of lauric acid, as determined by reverse phase HPLC analysis is shown in Fig. 2. The data shows preferential induction of the 12-hydroxylase activity with the highest fold increase of 4 effected by the *R*(−)-enantiomer and a 1.3-fold induction with the corresponding *S*(+)-antipode. Such a preferential induction of 12-hydroxylase activity has been previously demonstrated with other peroxisome proliferators [4], and reflects the regioselectivity of the cytochrome P450IVA1 isoenzyme for lauric acid hydroxylation [2].

Western blotting analysis of hepatic microsomes derived from the various treatment groups was performed using a sheep polyclonal antibody raised against purified rat hepatic cytochrome P450IVA1 (Fig. 3). In particular, Fig. 3 demonstrates that the anti-cytochrome P450IVA1 serum recognizes two proteins from both control and treatment groups. It appears that the lower of these two bands, which is preferentially induced, co-migrates with authentic

Table 1. Stereoselective induction of hepatomegaly and cytochrome P450/P450IVA1 content in male long Evans hooded rat

Treatment	Liver/body weight ratio (%)	Total Cytochrome P450 specific content (nmol/mg)	Cytochrome P450IVA1 quantitation	
			Specific cytochrome P450IVA1 (nmol/mg)	% Total
Control	5.64 ± 0.31	0.48 ± 0.06	0.028 ± 0.003	5.95 ± 0.88
R(−)-Isomer	6.67 ± 0.41*	0.57 ± 0.06*	0.107 ± 0.012‡	19.40 ± 3.27‡
S(+)-Isomer	5.83 ± 0.19	0.49 ± 0.07	0.037 ± 0.007†	7.44 ± 0.41†
Racemic mixture	5.97 ± 0.26	0.55 ± 0.09*	0.059 ± 0.006‡	10.88 ± 0.75‡

Values are mean ± SD of six animals (control) or three animals in the test groups. *P < 0.01, †P < 0.005, ‡P < 0.001.

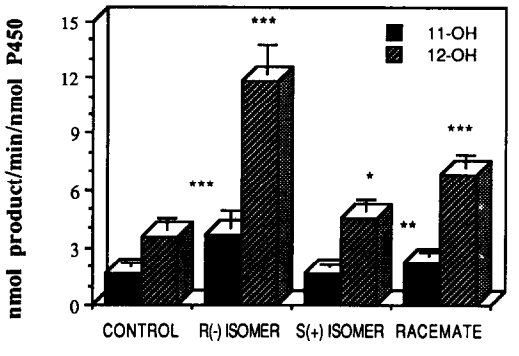

Fig. 2. Stereochemical induction of lauric acid hydroxylation by rat liver microsomes. Rats (six animals in control and three in the test groups) were pretreated with the clofibrate analogue isomers and racemate and laurate hydroxylase activities determined by reverse phase HPLC as described in Materials and Methods. *P < 0.1, **P < 0.01, ***P < 0.001.

hepatic cytochrome P450IVA1. Furthermore, following treatment (particularly with the *R*(−)-enantiomer), the levels of hepatic cytochrome P450IVA1 as determined by ELISA, in addition to the induction of the cytochrome P450-dependent 12-hydroxylation of lauric acid noted above, approximated more closely to the induction of the lower of the two bands. This data therefore lends further support to the conclusion that in the liver, the cytochrome P450IVA1 isozyme is represented by the lower molecular mass species, as previously discussed [17]. From the relative intensities of the protein bands shown in Fig. 3, the *R*(−)-isomer was again the more potent inducer of cytochrome P450IVA.

The effect of treatment on peroxisomal and mitochondrial lipid-metabolizing enzymes is presented in Table 2. The data presented are for peroxisomal cyanide-insensitive palmitoyl CoA oxidation (PCOA) and total (peroxisomal plus mitochondrial) carnitine acetyl transferase activity (CAT). The *R*(−)-enantiomer was more potent than the *S*(+)-isomer in inducing peroxisomal β-oxidation activity, the induction corresponding to a eudismic ratio of approximately 5. Similarly, carnitine acetyl transferase activity was also demonstrated to show stereoselectivity in the induction process. Although this latter enzyme, unlike palmitoyl CoA oxidase, is not a specific peroxisomal marker, much of the observed increase in enzyme activity may be ascribed to induction of the peroxisomal rather than mitochondrial enzyme activity [8]. A eudismic ratio of approximately the same magnitude as that for

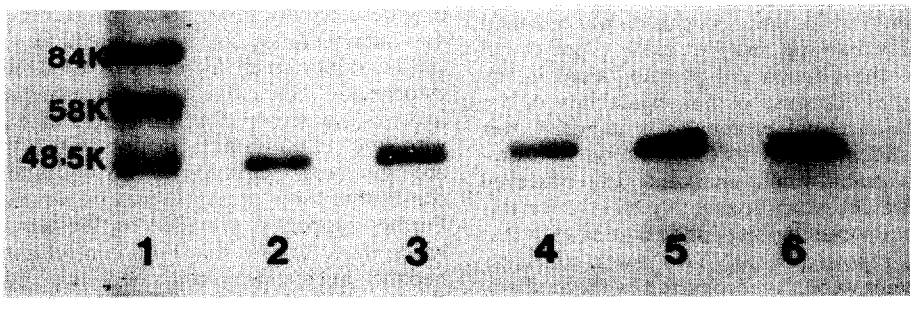

Fig. 3. Western blot analysis of cytochrome P450IVA1. Rats were pretreated with the *R*(−)-isomer, the *S*(+)-isomer or the racemate of the clofibrate analogue and Western blot analysis carried out as described in Materials and Methods. Ten micrograms of microsomal protein (from pooled microsomal fractions) were loaded on each track as follows. Track: 1, prestained molecular weight markers; 2, control microsomes; 3, racemate pretreatment; 4, *S*(+)-enantiomer pretreatment; 5, *R*(−)-enantiomer pretreatment; 6, authentic cytochrome P450IVA1 (0.5 pmol).

Table 2. Differential induction of palmitoyl CoA oxidation and carnitine acetyltransferase activity

Treatment	Palmitoyl CoA oxidation (nmol NADH/min/mg protein)	Carnitine acetyl transferase activity (nmol CoA/min/mg protein)
Control	4.42 ± 0.91	1.42 ± 0.44
R(−)-Isomer	11.67 ± 2.66‡	14.68 ± 4.90†
S(+)-Isomer	5.20 ± 0.62*	2.71 ± 0.79†
Racemic mixture	8.71 ± 1.42‡	6.01 ± 0.61‡
R/*S* Activity ratio	2.24	5.42

Values are mean ± SD of either six animals (control) or three animals in the test groups. *P < 0.1, †P < 0.01, ‡P < 0.001.

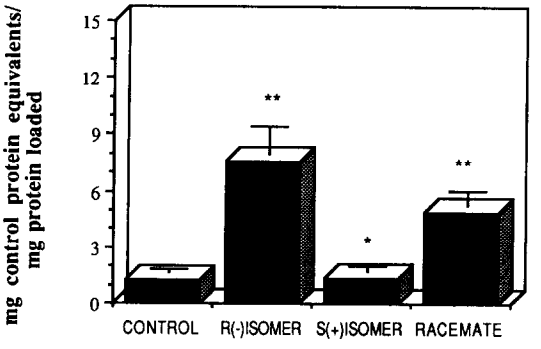

Fig. 4. Immunochemical detection of peroxisomal bifunctional protein. ELISA determinations were carried out as described in Materials and Methods from pooled homogenates (six in control group, three in test groups). One hundred nanograms of protein were loaded from each of the *R*(−)-isomer and racemate-treated groups and 500 ng from the *S*(+)-treatment group. Freeze-dried anti-bifunctional enzyme immune serum was reconstituted and diluted 1 : 200,000. Data are expressed as (units) mg control animal protein equivalents/mg protein and expressed as mean ± SD of 3–6 animals in each group. *P < 0.01, **P < 0.001.

KCN-insensitive palmitoyl CoA oxidation was observed (Table 2).

Results for the ELISA-based quantitation of the bifunctional protein of the peroxisomal β-oxidation spiral are presented in Fig. 4, and demonstrates that the *R*(−)-isomer was more potent in inducing this protein (*R*/*S* activity ratio of 5.4). Further evidence lending support to this latter conclusion was obtained from Western blotting analyses using a polyclonal antibody to the bifunctional protein raised in the rabbit (Fig. 5). Because of non-availability of the authentic bifunctional protein as a standard, this protein was identified by its co-migration with pre-stained molecular weight markers. The observed molecular weight of approximately 80 kDa for this peroxisome associated polypeptide agreed with that of previous reports [19, 20]. Two lower molecular weight proteins were also recognized by the antibody to the bifunctional protein, and are believed to be previously described proteolysis products [21].

DISCUSSION

It is well established that *in vivo* treatment with a number of structurally diverse hypolipidaemics to susceptible species such as the rat and mouse results in changes in liver biochemistry and morphology [4–6, 22, 23]. The results of the present study clearly demonstrates that there is a high degree of enantiomeric induction selectivity in lipid-metabolizing enzymes and that the *R*(−)-isomer of a clofibrate analogue appears to be the most potent of the enantiomeric forms administered. Our results also demonstrate the close relationship that exists between induction of the microsomal and peroxisomal lipid-metabolizing enzymes following challenge by these xenobiotics. The following induction potency was obvious in all the parameters investigated in our study: *R*(−)-isomer > racemate > *S*(+)-isomer. Moreover, the *R*(−)-enantiomer caused approximately double the inductive effects of the racemate suggesting that, at the dose levels administered and the duration of study, it is this enantiomeric form that mainly contributes to the observed changes in hepatic enzymes.

The induction of cytochrome P450IVA1 is mirrored by a concomitant parallel increase in lauric acid hydroxylase activity. Cytochrome P450IVA1 has previously been documented as the only hepatic isozyme of cytochrome P450 that exhibits a preferential regioselectivity for the 12-hydroxylation of lauric acid in the rat [4, 23]. Results obtained by reverse phase HPLC analysis (Fig. 2), further substantiated the phenomenon of enantioselectivity in the induction process and again the *R*(−)-isomer was much more potent in inducing lauric acid 12-hydroxylase activity. Very clearly, a eudismic ratio of approximately 3 is further evidence for enantioselectivity in this process. Hence, although the administration of these clofibrate analogues caused rather small increases in total microsomal cytochrome P450 content, the large increase in 12-hydroxylase activity for lauric acid as exemplified with the *R*(−)-isomer, demonstrates that there are significant changes occurring among some constitutive forms present in the rat liver microsomes. Further support is provided by Western blot data which demonstrates that both control and induced hepatic microsomes contained two cytochrome P450IVA1-related polypeptides that are recognized by the antibody (Fig. 3). These two polypeptides are of unequal intensity with the lower-molecular-mass band predominating in all the various treatment groups. The molecular weight of the lower band is consistent with that of authentic rat hepatic

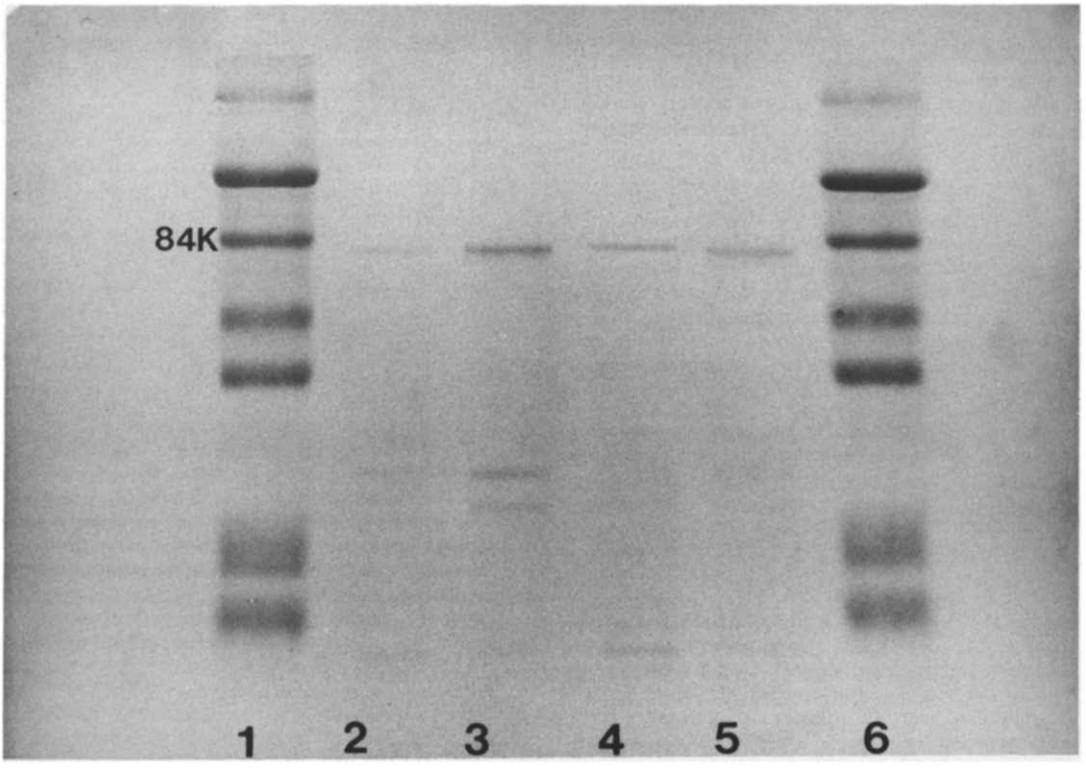

Fig. 5. Western blot analysis of the peroxisomal bifunctional protein. Rats were pretreated with the *R*(−)-isomer, the *S*(+)-isomer or the racemate of the clofibrate analogue and Western blot analyses carried out as described in Materials and Methods. Thirty micrograms of total homogenate protein was loaded on each track (pooled from 3–6 animals) as follows. Track: 1, prestained molecular weight standards; 2, control homogenate; 3, *R*(−)-enantiomer pretreatment; 4, *S*(+)-enantiomer pretreatment; 5, racemate pretreatment; 6, prestained molecular weight standards.

cytochrome P450IVA1 (51.5 kDa) and the higher band estimated to be 52 kDa. At present, the precise nature of the 52 kDa polypeptide is unknown, but may be a closely related member of the cytochrome P450IVA sub-family based on immunochemical reactivity [17, 24].

Many critical features of the biological action of xenobiotics arise from their interaction with endogenous chiral molecules such as receptors or enzymes and it is therefore not surprising that the biological actions of chiral, pharmacologically active molecules reside predominantly in one enantiomer [25–28]. For example, the 2-arylpropionic acids, an important group of non-steroidal anti-inflammatory drugs (the profen series), contain a chiral centre and *in vitro* studies on inhibition of prostaglandin synthesis demonstrates that activity resides almost exclusively in the *S*(+)-isomer with a eudismic ratio of 160 [29]. However, *in vitro*, this activity ratio is dramatically reduced to 1.3, due to the unidirectional metabolic inversion of the chiral centre from the inactive *R*(−)-isomer to the *S*(+)-antipode [30]. Although the metabolic disposition and potential metabolic chiral inversion of the enantiomeric clofibrate analogues described herein have not been investigated, it would appear unlikely that metabolic chiral inversion occurs in this case as may be deduced from the induction studies reported herein.

The stereospecificity of liver enzyme induction noted in our studies may be explained by differences in xenobiotic absorption, distribution or tissue uptake. Alternatively, enantiospecific selectivity may reside in the specific interaction of the inducer with a chiral intracellular macromolecule, which then initiates the induction process. This macromolecule is probably not a classical cytosolic protein receptor [9, 10], but may be associated with the 5′-flanking regulatory segments of the cytochrome P450IVA and/or peroxisomal fatty acid β-oxidation genes or the associated nuclear binding proteins, based on the fact that clofibrate induction of cytochrome P450IVA1 is by transcriptional activation of the corresponding gene [24].

In conclusion, we have been able to demonstrate that there is stereochemical specificity in the induction of microsomal cytochrome P450IVA1-dependent fatty acid hydroxylase activity. Our results also identify the *R*(−)-enantiomer as the eutomer and its corresponding *S*(+)-antipode as the distomer, with the racemic mixture intermediary in inducing this enzyme activity. Also an identical enantiomeric selectivity was observed for the phenomenon of peroxisome proliferation by these compounds. Thus,

taken collectively, the above findings are not inconsistent with the previously stated hypothesis [4] that cytochrome P450IVA1 induction and peroxisome proliferation are intimately linked. Whether the observed stereochemical selectivity resides in xenobiotic recognition or disposition still remains to be determined.

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