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The Effect of Indobufen on the Activities of Selected Rat Liver Phase I and Phase II Drug Metabolizing Enzymes, Peroxisomal β-oxidation and Hepatic Glutathione Status

H. THOMAS†, M. STROLIN BENEDETTI*, P. DOSTERT* AND F. OESCH

*Institute of Toxicology, University of Mainz, Obere Zahlbacher Str. 67, D-6500 Mainz, Germany, *Pharmacia-Farmitalia Carlo Erba, Via Carlo Imbonati 24, I-20159 Milan, Italy, and †Ciba-Geigy Limited, Toxicology Services, R-1066.4.32, Postfach, CH-4002 Basel, Switzerland*

Abstract—Oral administration of indobufen to male rats for three days at daily doses of 5, 10 and 20 mg kg^{-1} resulted in no changes in liver total glutathione, cytosolic glutathione *S*-transferases or microsomal epoxide hydrolase. Reduced glutathione appeared slightly diminished to about 84% of control at the highest dose level. Microsomal cytochrome P450-dependent ethoxyresorufin *O*-de-ethylase and pentoxyresorufin de-alkylase activities were decreased to 64% (not significantly) and 67% of control at the lowest dose level. 6α- and 7α-Hydroxytestosterone activities were decreased to 67 and 68% of control at the highest dose level. Cyanide-insensitive peroxisomal fatty acid β-oxidation was increased to 223, 261 and 232% of control at doses of 5, 10, and 20 mg kg^{-1}, respectively. The results obtained in this study are indicative of the action of indobufen as a weak peroxisome proliferator in male rat liver, and suggest a slight but toxicologically insignificant inhibitory action of this drug on microsomal cytochrome P450-dependent enzyme activities.

Indobufen, (±2)-[*p*-(1-oxo-2-isoindolinyl)]phenyl butyric acid, is a potent inhibitor of cyclo-oxygenase and platelet aggregation with antithrombotic activity in animals and in man (Bergamaschi et al 1979, 1984; Vittoria et al 1981; Di Minno & Silver 1983). In clinical studies, indobufen proved to be 2–5 times more potent than acetyl salicylic acid as an inhibitor of adenosine diphosphate- and collagen-induced platelet aggregation (Pogliani et al 1977). In contrast to acetyl salicylic acid, however, the inhibition produced by indobufen is reversible (Fuccella et al 1979; Vinazzer & Fuccella 1980; Pogliani et al 1981).

Pharmacokinetic studies in young healthy subjects revealed that indobufen is rapidly and almost completely absorbed after oral administration and is excreted mainly in urine with an elimination $t_{\frac{1}{2}}$ of 7–8 h. In urine, about 75% of the dose was recovered within 48 h of administration as total indobufen (unchanged drug plus glucuronide) (Fuccella et al 1979), and no changes in drug disposition kinetics were found following repeated treatment (Tamassia et al 1979). The cytochrome P450 system does not appear to be involved in the metabolism of indobufen in man. Compared with man, indobufen metabolism and disposition in the rat is very different: the major route of excretion in the rat is the bile (Strolin Benedetti et al 1990; Grubb et al 1991) and the major urinary metabolites are the 5-hydroxy derivative and its sulphate (Tonani et al 1980; Grubb et al 1991, 1993). This study was intended to characterize biochemically the effects of indobufen on selected oxidative systems, such as peroxisomal β-oxidation and cytochrome P450-dependent drug-metabolizing enzyme activities. Also investigated was the glutathione status in liver of male CD(SD)BR rats following oral treatment at indobufen dose levels of 5, 10 and 20 mg kg^{-1} day^{-1} for three consecutive days, as this might have changed following phase I metabolic reactions of indobufen in the rat. The daily doses were selected according to those used in long-term safety evaluation studies in the rat, as the aim of this work was mainly to collect biochemical information which might contribute to the global interpretation of the safety evaluation studies in the rat.

Correspondence: M. Strolin Benedetti, Pharmacia-Farmitalia Carlo Erba, Via C. Imbonati 24, I-20159 Milan, Italy.

Materials and Methods

Chemicals

[^{3}H]2-Phenyloxirane (11·7 GBq $mmol^{-1}$) and [^{3}H]*trans*-2,3-diphenyloxirane (0·41 GBq $mmol^{-1}$) were synthesized as described by Oesch et al (1971, 1980), respectively. Dithiothreitol, coenzyme A (CoA), flavine adenine dinucleotide (FAD), 1-chloro-2,4-dinitrobenzene, bovine serum albumin, 3-(*N*-morpholino)propane-sulphonic acid (MOPS), glutathione: reduced form (GSH), palmitoyl-CoA, and steroid standards were purchased from Sigma (Deisenhofen, Germany). Oxidized glutathione (GSSG), β-nicotinamide adenine dinucleotide: reduced form (NADH), β-nicotinamide adenine dinucleotide (NAD) (free acid, grade II), β-nicotinamide adenine dinucleotide phosphate: reduced form (NADPH), 7-ethoxyresorufin, 7-pentoxyresorufin and glutathione reductase were obtained from Boehringer (Mannheim, Germany). Resorufin was a product of Pierce (Rockford, IL, USA). Indobufen (batch no. RR 16NO24) of 99·12% purity was provided by Farmitalia Carlo Erba (Milan, Italy). All other chemicals were of analytical grade or the purest grade commercially available.

Experimental animals

Male CD(SD)BR rats, initially weighing 200–220 g, were obtained from Charles River Wiga GmbH (Sulzfeld, Germany) and randomly assigned to groups of four. The

animals were housed in standard Macrolon cages on softwood bedding at 50% relative humidity and 22 ± 1°C with a light/dark cycle of 12 h. All animals had free access to a standardized diet (Altromin, Lage/Lippe, Germany) and tap water. The animals were allowed to adjust to the environmental conditions six days before treatment with indobufen.

Indobufen treatment

Indobufen was administered orally (gavage) as a homogeneous suspension in 0·5% carmellose to allow for final concentrations of 5, 10 and 20 mg kg^{-1} body weight in a final volume of 0·5 mL/100 g body weight. Animals of the control group received an equivalent volume of 0·5% carmellose. Treatment was performed for three consecutive days at 1030 h, and the animals were killed on the fourth day at 0830 h.

Subcellular fractionation

All animals were killed under CO_2 anaesthesia by exsanguination; livers were removed and weighed, and a defined part of each liver was immediately frozen in liquid nitrogen and stored at −80°C for the determination of total and reduced hepatic glutathione. The residual part of each liver was subsequently homogenized in ice-cold 10 mM Tris/HCl buffer, pH 7·4, containing 250 mM sucrose, with an Ultra-Turrax homogenizer to give a 25% (w/v) homogenate. The homogenate was centrifuged for 10 min at 600 *g*, 4°C, and the supernatant, after removal of an aliquot for the determination of the cyanide-insensitive peroxisomal β-oxidation activity and protein content, was centrifuged for 15 min at 10 000 *g*, 4°C. The resulting supernatant was spun for 60 min at 100 000 *g* and 4°C. The supernatant (cytosol) was divided into portions and immediately frozen in liquid nitrogen. The microsomal pellet was resuspended in a volume of homogenization buffer equivalent to the initial liver weight, divided into portions, frozen in liquid nitrogen, and stored at −80°C until assayed.

Enzyme assays

Cyanide-insensitive peroxisomal β-oxidation was measured in the 600 *g* supernatant as outlined by Bieri et al (1984) by recording the FAD/NAD-dependent oxidation of palmitoyl-CoA at 340 nm in the presence of 2 mM KCN. Cytosolic epoxide hydrolase activity was determined in the cytosolic liver fraction with [^{3}H]*trans*-2,3-diphenyloxirane as substrate using the method of Schladt et al (1986), and the activity of soluble glutathione *S*-transferases (GST) was determined from the same enzyme source at 25°C with 1-chloro-2,4-dinitrobenzene as substrate using the spectrophotometric method of Habig et al (1974).

Microsomal epoxide hydrolase with broad substrate specificity for xenobiotic epoxides, including benzo(a)pyrene 4,5-oxide activity, was evaluated with [^{3}H]2-phenyloxirane as substrate as described by Oesch et al (1971) under the conditions given by Oesch (1974). Microsomal 7-ethoxyresorufin *O*-de-ethylase and 7-pentoxyresorufin *O*-de-alkylase activities were determined fluorimetrically by monitoring the fluorescence of resorufin at excitation and emission wavelengths of 522 and 586 nm, respectively (Burke & Mayer 1974, 1976), and corresponding standard curves were obtained with appropriate concentrations of resorufin.

Regio- and stereoselective testosterone hydroxylation as an assay method to evaluate the activities of individual cytochrome P450 isoenzymes was performed according to van der Hoeven (1984).

Determination of reduced and total glutathione

GSH in the liver was determined as described by Sedlak & Lindsay (1968) from acid liver extracts prepared according to Akerboom & Sies (1981). To 0·3–0·5 g frozen liver tissue, 4 vol ice-cold 1 M $HClO_4$, 2 mM ethylenediaminetetraacetic acid (EDTA), were added, followed by homogenization. The acid extracts were centrifuged at 5000 *g* for 5 min, and suitable aliquots (5–15 μL) of the acidic tissue extracts were used for the reduction of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB).

Total glutathione in liver was determined from the same perchloric acid liver extracts following neutralization of a 200 μL aliquot with 80 μL 2 M KOH, 0·3 M MOPS. Ten microlitres of the neutralized extract was used in a kinetic assay, in which catalytic amounts of GSH or GSSG and glutathione reductase bring about the continuous reduction of DTNB by NADPH (Akerboom & Sies 1981).

Protein

Protein was determined according to Lowry et al (1951) using bovine serum albumin as a standard.

Statistical analysis

Data are presented as means ± standard deviations from four animals per experimental group. Two-sided Student's *t*-test of differences between two sample means was used to assess the significance of differences between groups (Lorenz 1988).

Results

Animals

No signs of toxicity were observed in rats in any of the groups (5, 10, and 20 mg kg^{-1}) throughout the treatment period, and no macroscopic changes were detectable in any of the rats of the control and the treatment groups at death. Absolute liver weights were unaffected by indobufen treatment (Table 1).

Liver protein

In livers of untreated rats, the 600 *g* supernatant had a protein content of 19·9 ± 2·4 mg mL^{-1} (100%); the protein contents of the corresponding liver fractions were reduced in indobufen-treated animals to 11·3 ± 1·3 (57%), 9·5 ± 0·7 (48%), and 12·9 ± 1·1 mg mL^{-1} (65%) at the 5, 10, and 20 mg kg^{-1} dose levels, respectively. Conversely, microsomal fractions had slightly increased protein ranging from 134% to 127% of control in the low and high dose groups, respectively. No treatment-related changes were observed for the protein of the cytosolic liver fractions from any of the indobufen treatment groups (Table 1).

Enzyme activities

Among the investigated peroxisomal and metabolizing enzyme activities, cyanide-insensitive peroxisomal fatty acid β-oxidation was slightly to moderately increased in

Table 1. The effect of indobufen treatment on absolute liver weights, protein contents of subcellular fractions, activities of cyanide-insensitive peroxisomal fatty acid β-oxidation, cytosolic glutathione *S*-transferases, cytosolic and microsomal epoxide hydrolases, 7-ethoxyresorufin *O*-de-ethylase and 7-pentoxyresorufin *O*-de-alkylase activities and total and reduced glutathione.

Dose	0 (Control)	5	10	20
Absolute liver weight (g)	9·1 ± 0·8	9·7 ± 1·5	9·4 ± 2·4	9·0 ± 0·6
Microsomal protein (mg mL^{-1})	7·7 ± 0·4	10·3 ± 1·2**	10·1 ± 2·4	9·8 ± 0·8**
Cytosolic protein (mg mL^{-1})	16·7 ± 3·5	17·5 ± 1·2	17·5 ± 1·7	16·3 ± 1·2
Peroxisomal β-oxidation (nmol min^{-1} mg^{-1})	3·1 ± 0·3	6·9 ± 1·6**	8.1 ± 1·0***	7·2 ± 1·0***
Cytosolic epoxide hydrolase (pmol min^{-1} mg^{-1})	36·1 ± 6·4	51·2 ± 10·4	54·7 ± 27·6	43·2 ± 12·4
Microsomal epoxide hydrolase (nmol min^{-1} mg^{-1})	11·5 ± 2·5	13·7 ± 1·8	12·9 ± 3·2	12·4 ± 0·9
Cytosolic glutathione *S*-transferases (μmol min^{-1} mg^{-1})	1·4 ± 0·2	1·6 ± 0·1	1·5 ± 0·3	1·6 ± 0·1
7-Ethoxyresorufin *O*-de-ethylase (pmol min^{-1} mg^{-1})	20·4 ± 7·3	13·0 ± 1·3	18·5 ± 4·7	21·2 ± 2·8
7-Pentoxyresorufin *O*-de-alkylase (pmol min^{-1} mg^{-1})	5·7 ± 1·1	3·8 ± 0·5*	5·7 ± 1·4	6·8 ± 2·7
Total glutathione (μmol g^{-1})	5·2 ± 1·1	5·0 ± 0·7	5·0 ± 1·5	5·1 ± 0·6
Reduced glutathione (μmol g^{-1})	4·9 ± 0·6	4·1 ± 0·7	4·1 ± 0·7	4·1 ± 0·1*

Values given are means ± standard deviation from four animals per experimental group. Asterisks indicate results significantly different (two-sided Student's *t*-test) from control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

all treatment groups. These increases amounted to 223, 261 and 232% of the corresponding control activity at the 5, 10 and 20 mg kg^{-1} dose level, respectively. Similarly, cytosolic epoxidehydrolase displayed a tendency to slightly elevate activities in all treatment groups which were highest (152% of control) in cytosol of animals that had received 10 mg kg^{-1} indobufen. However, none of these changes reached statistical significance (Table 1). No treatment-related alterations were found for cytosolic glutathione *S*-transferase or microsomal benzopyrene oxide activities at any of the investigated dose levels (Table 1). Ethoxyresorufin *O*-de-ethylase and pentoxyresorufin *O*-de-alkylase activities were determined to assess the effect of indobufen-treatment on the activities of microsomal cytochromes P450 of the gene families CYP1A (ethoxyresorufin *O*-de-ethylase) and CYP2B (pentoxyresorufin *O*-de-alkylase), respectively (Table 1) and were found to be reduced to 64% (not significantly) and 67% of control at the lowest dose level. An increase in the daily dose of indobufen to 10 and 20 mg kg^{-1} resulted in concomitantly increased activities to 91 and 100%, and 104% and 119% of untreated controls, respectively.

The metabolites of testosterone generated by the investigated liver microsomes are presented in Table 2. They are arranged according to the catalytic activity of the cytochromes CYP2C11 (2α- and 16α-hydroxylation), CYP3A1/A2, (2β-, 6β- and 15β-hydroxylation), CYP2B1 (16β-hydroxylation and 17-oxidation to androstenedione), and CYP2A1 (6α- and 7α-hydroxylation). However, the only reliable indicators of the catalytic activity of CYP2A1, CYP2B1, and CYP2C11 are testosterone 7α-, 16β- and 2α-hydroxylation, respectively. The results in Table 2 indicate that the total testosterone hydroxylation was slightly reduced by indobufen treatment in an apparently dose-dependent manner to 73% of control at the highest dose level. This change, however, did not reach the level of statistical significance. Except for the 6α-, 6β-. and 7α-hydroxylation which appeared marginally, but not statistically significantly, elevated in microsomes of the 5 mg kg^{-1} group, all testosterone oxidation pathways were reduced in all indobufen treatment groups. The most prominent dose-dependent suppression (67% and 68% of control) was noted for the rates of testosterone 6α- and 7α-hydroxylation which are a reliable index for CYP2A1.

Total and reduced glutathione in liver

As shown in Table 1, total liver glutathione contents remained essentially unaffected by indobufen treatment at all dose levels. GSH appeared slightly diminished by about 16% in all indobufen-treated animals. However, these changes reached statistical significance only at the highest dose.

Table 2. The effect of indobufen on microsomal cytochrome P450-dependent testosterone hydroxylation.

Testosterone metabolite	Indobufen dose (mg kg^{-1} day^{-1})			
	0 (Control)	5	10	20
2α-OH	2096 ± 673	1575 ± 514	1369 ± 453	1528 ± 881
16α-OH	2177 ± 662	1719 ± 540	1493 ± 527	1635 ± 907
2β-OH	257 ± 76	236 ± 64	209 ± 76	189 ± 35
6β-OH	1218 ± 454	1250 ± 361	1156 ± 444	992 ± 181
15β-OH	53 ± 8	51 ± 11	48 ± 17	47 ± 4
16β-OH	85 ± 38	74 ± 17	66 ± 49	76 ± 8
Androstenedione	1521 ± 422	1313 ± 429	1022 ± 237	967 ± 393
6α-OH	61 ± 8	69 ± 14	53 ± 14	41 ± 3**
7α-OH	798 ± 146	850 ± 91	694 ± 188	542 ± 78*
Total activity	8265 ± 2209	7137 ± 2013	6108 ± 1897	6016 ± 2440

The specific activity is given in pmol min^{-1} (mg microsomal protein)$^{-1}$. Values given are means ± standard deviation from four animals per experimental group. Asterisks indicate results significantly different (two-sided Student's *t*-test) from control: *$P < 0.05$, **$P < 0.01$.

Discussion

The liver is the major metabolizing organ for a number of xenobiotics including environmental chemicals and drugs. The adaptive responses most frequently encountered in rat liver include a reversible hepatomegaly and the reversible induction of a variety of cytosolic, microsomal, and peroxisomal enzyme activities (Conney 1967, 1986; Hawkins et al 1987). On the protein and enzyme activity level, inductive effects are expressed within a few days, and the potency of the inducing agent may be readily delineated from the extent of alteration at a defined dose by comparison with the effects of well-characterized model compounds.

Indobufen, at single and daily doses of 5, 10 and 20 mg kg^{-1} to male CD(SD)BR rats for three consecutive days, did not exert any significant effect on the absolute liver weights. However, reduced protein in the liver 600 *g* supernatant and slightly elevated protein in the microsomal fractions argue for an effect of indobufen on the liver (Table 1). From the enzyme parameters known to respond rapidly to a chemical challenge, the activities of the microsomal cytochrome P450-dependent mono-oxygenase system rank among the most sensitive (Okey 1990; Testa & Jenner 1981; Murray & Reidy 1990). The ethoxyresorufin *O*-deethylase and pentoxyresorufin de-alkylase activities did not reflect any notable adverse action of indobufen on these mono-oxygenases at the 10 and 20 mg kg^{-1} doses (Table 1). The slightly reduced activities recorded at the 5 mg kg^{-1} dose may indicate a weak inhibitory interaction of the drug with constitutively expressed cytochrome P450 isoenzymes which catalyse, although to a limited extent, the de-alkylation of alkoxyresorufins (Burke et al 1985).

The alterations noted in the regio- and stereoselective hydroxylation of testosterone by liver microsomes from indobufen-treated rats were marginal (Table 2), and with the exception of the 6α- and 7α-hydroxylation which were found reduced to about 67–68% of control at the highest target dose, none of them reached the level of statistical significance. Also, these changes appear too small to be regarded of any toxicological significance as far as the oxidative metabolism of endogenous substrates and foreign compounds is concerned. Testosterone 6α- and 7α-hydroxylation are diagnostic for cytochrome CYP2A1 which is constitutively expressed in rats of both sexes. However, testosterone 7α-hydroxylation, the major pathway reported for cytochrome P4502A1, is catalysed by microsomes from mature female rats at two or three times the rate catalysed by microsomes from male rats (Sonderfan et al 1987; Dutton et al 1987). This isoenzyme has been reported to be inducible by a factor of 2 by phenobarbitone and by 2·5 by 3-methylcholanthrene, and was found to be relatively refractory to treatment with pregnenolone-16α-carbonitrile. Other inducers such as troleandomycin and spironolactone were shown to even reduce CYP2A1-related testosterone 7α-hydroxylation to about 80% of control (Sonderfan et al 1987). This earlier observation compares with 32–33% reduction of 7α-hydroxylation by the highest tested dose of indobufen (Table 2), which excludes a phenobarbitone- or 3-methylcholanthrene-like action of indobufen. On the other hand, essentially unchanged or even slightly reduced testosterone 2β-, 6β- and 15β-hydroxylation rates exclude any substantial inductive potency of indobufen similar to that of pregnenolone-16α-carbonitrile (Sonderfan et al 1987) (Table 2).

No alterations following indobufen treatment were recorded for the microsomal epoxide hydrolase with broad substrate specificity and cytosolic GST activities. By contrast, cytosolic epoxide hydrolase appeared slightly increased (although not significantly) 1·2- to 1·5-fold at all dose levels (Table 1). For comparison, dietary administration of fenofibrate, a potent peroxisome proliferator to male Fischer 344 rats at mean daily doses of 8, 40 and 230 mg kg^{-1} for four days, caused a concomitant 2·8-fold induction of both cytosolic epoxide hydrolase and peroxisomal fatty acid β-oxidation, respectively, at the lowest dose level (Oesch et al 1988). Similarly, indobufen-mediated cytosolic induction was accompanied by a slight induction of peroxisomal β-oxidation at all investigated dose levels. Maximal induction to 261% of control was recorded at 10 mg kg^{-1} (Table 1).

At the same time, a marginal 16% decrease in the hepatic content of GSH was observed (Table 1).

The changes in cytosolic epoxide hydrolase and peroxisomal fatty acid β-oxidation activity are good indicators for the action of indobufen as a weak peroxisome proliferator in the rat liver (Hawkins et al 1987). This view is supported by the established alteration in the hepatic content of GSH, which is essentially in agreement with the previously reported increase in the biliary excretion of GSSG when lauric acid, a substrate for peroxisomal fatty acid β-oxidation, was infused into the livers of rats treated with the peroxisome proliferator nafenopin (Lake & Russel 1989).

However, when compared with a series of known peroxisome proliferators, the potency of indobufen is regarded similar to, or even lower than that of the weak peroxisome proliferator acetylsalicylic acid. The latter was shown to cause a dose-dependent induction of peroxisomal β-oxidation from 1·6-fold at 0·2% (w/w) in the diet (corresponding to 160 mg kg^{-1} day^{-1}) to 4·7-fold at 1% (w/w) dietary concentration (corresponding to 800 mg kg^{-1} day^{-1}) following treatment for seven consecutive days, while the indobufen-related increase in this enzyme activity reached a plateau of 2·6-fold induction even at the 10 mg kg^{-1} dose. This means that the maximal inducibility of this enzyme activity by indobufen is considerably less than that by aspirin (Schladt et al 1987).

In conclusion, indobufen was identified in this investigation as a weak peroxisome proliferator in the male rat liver. However, peroxisome proliferation in general has been characterized as a reversible and rodent-specific adaptive response so far, which may not be of toxicological significance to man (Hawkins et al 1987; Stott 1988; Grasso & Sharratt 1991).

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