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Oxidative Metabolism of Lansoprazole by Human Liver Cytochromes P450

LYDIANE PICHARD, ROZANGELA CURI-PEDROSA,[1,2] CLAUDE BONFILS, EVELYNE JACQZ-AIGRAIN, JACQUES DOMERGUE, HENRI JOYEUX, JOSÉ COSME, F. PETER GUENGERICH, and PATRICK MAUREL

INSERM U-128, CNRS, 34033 Montpellier, France (L.P., R.C.-P., C.B., P.M.), Unite de Pharmacologie Clinique, Hopital Robert Debré, 75019 Paris, France (E.J.-A.), Service de Chirurgie Digestive C, Hopital Saint Eloi, 34059 Montpellier, France (J.D.), Chirurgie Viscérale et Digestive, Institut Curie, 75231 Paris, France (H.J.), INSERM U-75, Hopital Necker, 75730 Paris, France (J.C.), and Department of Biochemistry, Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232–0146 (F.P.G.)

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SUMMARY

The aim of this work was to identify the form(s) of human cytochrome P450 (P450) involved in the hepatic biotransformation of lansoprazole to its two main metabolites, i.e., the sulfone and the hydroxy derivative. In liver microsomes, the production of the sulfone of lansoprazole correlated with the level of P450 3A4, cyclosporin oxidase, and the production of the hydroxy derivative, as well as of omeprazole sulfone. The production of hydroxylansoprazole moderately correlated with the level of P450 3A4, cyclosporin oxidase, and (*S*)-mephenytoin 4′-hydroxylase. The production of the sulfone and of the hydroxy derivative of lansoprazole was significantly inhibited by anti-P450 3A4 antibodies, by cyclosporin and ketoconazole, and by tolbutamide. Anti-P450 2C8 and 2C3 antibodies moderately inhibited the biotransformation of lansoprazole, whereas they completely inhibited (*S*)-mephenytoin 4′-hydroxylase activity under the same conditions. In primary cultures of human hepatocytes, the biotransformation of lansoprazole and the oxidation of cyclosporin were strongly increased by rifampicin and phenobarbital, whereas (*S*)-mephenytoin 4′-hydroxylation was not. β-Naphthoflavone did not induce the formation of the sulfones but stimulated the production of hydroxylansoprazole. Among several forms of cDNA-expressed human P450s, 3A4 generated significant amounts of the sulfones of lansoprazole and omeprazole and 2C18 was active for the production of hydroxylansoprazole but inactive in the 4′-hydroxylation of (*S*)-mephenytoin. We conclude that P450 3A4 is the major enzyme involved in the production of the sulfone of lansoprazole and that this P450, as well as P450 2C18 and/or another 2C-related form, could contribute to the production of hydroxylansoprazole.

LAN is a benzimidazole derivative that produces a potent inhibitory effect on the ATPase-dependent proton pump of the parietal cells of the stomach (1). This drug is being increasingly used in the treatment of severe esophagitis, the Zollinger-Ellison syndrome, and refractory peptic ulcer disease. In humans and laboratory animals, LAN has been shown to be extensively metabolized in the liver (2). In humans, the main metabolites found in the serum are LANS and OH-LAN, with the amount of LANS being greater than that of OH-LAN. These metabolites appear to be devoid of pharmacological effect. Because this drug is prescribed generally for treatment of long duration, it is of interest to identify the enzyme systems involved in its metabolism, to predict and/or avoid possible drug interactions and side effects.

P450s from families CYP1 to CYP4 are monoxygenases involved in the oxidative metabolism of many drugs, environmental pollutants, procarcinogens, and other xenobiotics (3). They are present in several organs, including the liver, intestine, kidney, skin, lung, and placenta, where they play a major role in the metabolic activation and elimination of xenobiotics. A recent study on the metabolism of omeprazole, a proton pump inhibitor structurally similar to LAN (4), implicated P450 3A4 and one or several 2C form(s) in the production of the sulfone and the hydroxy derivative, respectively (5). Andersson *et al.* (6) and Sohn *et al.* (7) observed that the oxidative metabolism of omeprazole correlated with (*S*)-mephenytoin 4′-hydroxylation in Caucasian and Chinese subjects. (*S*)-Mephenytoin is a prototypic drug in pharmacogenetics; its 4′-hydroxylation is polymorphic, with approximately 2–5% of Caucasian subjects being poor metabolizers (3). Analysis of this defect at

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[2] Present address: Department of Biochemistry, Federal University of Santa Caterina, CCB Campus, Trinidade/Florianopolis, Brazil.

ABBREVIATIONS: LAN, lansoprazole; CYP, cytochrome P450 gene; P450, cytochrome P450 enzyme; LANS, lansoprazole sulfone; OH-LAN, hydroxylansoprazole; HPLC, high performance liquid chromatography.

the molecular level revealed that (*S*)-mephenytoin is hydroxylated by one or several forms of P450 of the 2C subfamily, which comprises at least seven genes in humans (8). There was considerable controversy in the identification of this P450. Recently, two groups concluded, using different experimental approaches, that 2C19 is the (*S*)-mephenytoin 4′-hydroxylase in humans (9, 10).

Because no data are presently available on the metabolism of LAN in human liver microsomes, the aim of this work was to identify the form(s) of P450 involved in the biotransformation of this drug. Using human liver microsomes, primary cultures of human hepatocytes, and several cDNA-expressed P450s, we demonstrate that P450 3A4 is the major form involved in the production of LANS in humans, whereas one or several forms of the CYP2C subfamily could partly contribute to the production of OH-LAN. A preliminary report of this work has been published (11).

Materials and Methods

Chemicals. LAN and [^{14}C]LAN (specific activity, 51.85 mCi/mmol) were provided by Laboratoires Houdé (Paris, France). LAN metabolites, including LANS, OH-LAN, the hydroxysulfone, the sulfide, and the hydroxysulfide, were kindly provided by Drs. B. Flouvat and B. Delhotal Landes (CHU Ambroise Paré, Paris, France).

Human liver samples. The use of human liver samples for scientific purposes was approved by the French National Ethics Committee (date of last review and approval, November 1992). Human liver specimens were obtained from organ donors (livers not suitable for transplantation due to high levels of transaminases or steatosis) and from patients undergoing hepatic lobectomies for medical reasons completely unrelated to our research program. The use of a code number to identify hepatocyte cultures from different individuals (FH for donors, FT, or HTL, followed by numbers for the patients) ensures full protection of the privacy of the patients. The clinical characteristics of some donors and patients have been given in previous papers, as follows: FT1 and FT4 (12), FT8, 18190, and 61289 (13), HTL2 and HTL4 (14), HTL13 (15), HTL27 (16), HTL37, FT21, and FH6 (17), and FH7 (18). The clinical characteristics of other donors and patients are presented in Table 1.

Liver microsomes. Microsomes were prepared either from liver samples or from hepatocyte cultures by differential centrifugation and were stored as described previously (15). Protein concentration was determined by the bicinchoninic acid method, according to the protocol provided by the reagent manufacturer (Pierce Chemical Co., Rockford, IL). Bovine serum albumin (Pierce Chemical Co) was used as the standard.

TABLE 1

Clinical characteristics of donors or patients from whom liver specimens were obtained

Patient identification	Age	Gender[a]	Origin of specimen	Diagnosis
	years			
FH3	19	F	Whole liver	Brain injury from traffic accident
FH5	47	F	Whole liver	Respiratory failure
FT5	45	F	Left lobe	Metastasis from colon cancer
FT6	60	M	Right lobe	Metastasis from colon cancer
FT22	70	M	Left lobe	Hepatocarcinoma in normal liver
FT25	73	M	Left lobe	Hepatocarcinoma in cirrhotic liver
FT28	65	M	Left lobe	Metastasis from colon cancer
FT29	62	F	Right lobe	Metastasis from colon cancer
FT30	50	F	Left lobe	Biliary cyst
FT31	24	F	Right lobe	Adenoma
FT43	61	F	Right lobe	Metastasis from colon cancer
FT63	54	F	Right lobe	Metastasis from colon cancer
HTL84	70	F	Right lobe	Metastasis from rectum cancer
HTL86	66	M		Metastasis from rectum cancer

[a] F, female; M, male.

Assays with human liver microsomes. LAN oxidase activity was evaluated by HPLC as described (19). Microsomes (final protein concentration, 1 mg/ml) were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, in the presence of LAN (concentration range, 1–100 μM) and [^{14}C]LAN (0.5 μCi) and, after 3 min at 37°, the reaction was initiated by the addition of 1 mM NADPH. The final volume was 500 μl; LAN was dissolved in dimethylsulfoxide, the final volume of which never exceeded 1%. The reaction was stopped with 30% (by volume) acetonitrile, and the suspension was centrifuged at 10,000 × *g* for 5 min. One hundred microliters of the supernatant were injected onto a 4- × 250-mm, Nucleosil 100A, 5-μm, C_{18}, stainless steel column (Macherey-Nagel, Düren, Germany), which was protected by a guard column (4 × 30 mm) of the same material. The mobile phase consisted of a mixture of 10 mM potassium phosphate, 5 mM phosphoric acid, 10 mM triethylamine, 26 mM acetic acid, and 36% (by volume) acetonitrile. The column was eluted at a rate of 1 ml/min at room temperature. The radiocativity of the effluent from the column was analyzed with a LB506-CI radioactivity monitor (Berthold, Wildbad, Germany), after mixing with liquid scintillation cocktail (Quickszint Flow 302; Zinsser, Maidenhead, England) in a 1:3 volume ratio. Under these conditions, the elution times were as follows: OH-LAN, 3.14 min; LAN hydroxysulfone, 4.18 min; LAN hydroxysulfide, 5.24 min; LAN, 6.38 min; LANS, 8.42 min; and LAN sulfide, 16.44 min. The radiocativity peaks were integrated with an Epson computer (Epson-France, Levallois-Perret, France) and converted to molar concentrations. The rate of production of the metabolites was calculated from the linear part of the plots of metabolite concentration versus time. A relative uncertainty of ±15% was estimated from triplicate measurements on several different preparations of microsomes or of primary cultures of hepatocytes.

(*S*)-Mephenytoin 4′-hydroxylase, cyclosporin oxidase, and the production of omeprazole sulfone were determined by HPLC as described previously (8, 12, 13, 20).

Correlation analysis of data. The data characterizing LANS, OH-LAN, and omeprazole sulfone production, cyclosporin oxidase, (*S*)-mephenytoin 4′-hydroxylase, and the levels of P450 3A4 and 1A2 for each preparation of liver microsomes were compared by simple linear regression analysis for each pair of data, with the Macintosh StatView program.

Immunoinhibition of monoxygenase activities in hepatic microsomes. Anti-rabbit P450 1A1, 1A2, 2C3, and 3A6 antibodies (16, 17) and anti-baboon P450 2A antibody (21), cross-reacting with the human orthologous forms, and anti-human 2C8 antibody (8) were used in this work. The assay conditions were the same as those described above, except that microsomes were first incubated for 20 min at room temperature with the various anti-P450 antibodies (0–10 mg of IgG/ml). The substrate was then added to the reaction medium and the reaction was initiated by addition of NADPH.

Immunoquantitation of P450s. P450 1A2, 2C8–18, 2D6, and 3A4 were quantitated by immunoblotting using specific antibodies as described (15). Purified antigen (1 pmol) was loaded on each gel as a standard.

Primary cultures of human hepatocytes. Primary cultures of human hepatocytes were prepared from lobectomies and cultured as described in previous papers (15, 17). For the treatment of cells, inducers were diluted in dimethylsulfoxide and added to the culture medium at final concentrations of 50 μM for rifampicin and β-naphthoflavone, 1 nM for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and 2 mM for phenobarbital. In all cases the concentration of dimethylsulfoxide was 0.1%, and control cultures received only dimethylsulfoxide at the same concentration. Treatment with the inducers lasted for 96 hr and was renewed every 24 hr as the culture medium was changed.

Assays with primary cultures of hepatocytes. After 96 hr the culture medium was renewed, in the absence of the inducer or in the presence of 20 or 50 μM LAN and 0.5–1 μCi of the radiolabeled molecule.

The cells were then incubated under standard culture conditions for 0, 1, 2, 3, 6, or 24 hr. At the indicated time, 250-μl aliquots of the culture medium were collected and mixed with the HPLC solvent, and a 100-μl aliquot was analyzed by HPLC as described above. The amounts of parent molecule and of metabolites in the extracellular medium were calculated from the HPLC data and the total radioactivity recovered from the cells.

Assays with cDNA-expressed human P450s. Microsomes from a human lymphoblastoid cell line expressing human P450 1A1, 1A2, 2A6, 2B6, and 3A4 (Genetest Corp., Woburn, MA) and from transformed yeast (22) expressing P450 2C8, 2C9, and 2C18 were used in this work. In addition to the assays performed with LAN, these preparations were tested for several monoxygenase activities, including tienilic acid oxidase, cyclosporin oxidase, production of omeprazole sulfone, and (*S*)-mephenytoin 4′-hydroxylase. These activities are reported in the legend to Table 4. The experimental conditions were those described previously, except that the incubation time was 3 hr. Parent drug and metabolites were analyzed by HPLC as indicated above.

Results

HPLC analysis of LAN metabolites. The parent molecule and metabolites were quantitated by HPLC. Elution profiles are presented in Fig. 1. Some metabolites of LAN eluted before 3 min. These most likely represent spontaneous degradation products, because their formation was neither NADPH nor microsome dependent and their amount varied from one batch of radiolabeled drug to another and increased upon aging; in addition, they generally represented no more than 10% of the total amount of metabolites.

In good quantitative agreement with pharmacokinetic studies in humans (1, 2, 19), the main metabolites of LAN generated with human liver microsomes or hepatocytes in culture were LANS and OH-LAN; other metabolites, including the hydroxysulfone, the sulfite, and hydroxysulfite, accounted for <10% of total metabolites each.

Biotransformation of LAN in human liver microsomes. The biotransformation of LAN in 23 different preparations of human liver microsomes was investigated. All microsome preparations generated measurable amounts of metabolites when incubated in the presence of NADPH. From the decrease of the concentration of the unchanged drug with time, the V_{max} and K_m for the biotransformation of LAN were determined to be 1005 pmol/mg/min and 102 ± 25 μM, respectively, with sample 61289. With other microsome samples, K_m (LAN) ranged from 45 to 195 μM. Lineweaver-Burk plots did not exhibit biphasic behavior in the concentration range from 1 to 100 μM. Higher concentrations were not tested, because the maximum *in vivo* serum level of the drug is 1–5 μM (19). The rates of production of LANS and OH-LAN determined in human liver microsomes are presented in Fig. 2. A large interindividual variability in the capacity of liver microsomes to oxidize LAN was seen. In most of the preparations, LANS was produced in greater amounts than OH-LAN, in agreement with pharmacokinetic data (1, 2, 19). In one preparation (FT30), the two metabolites were produced in similar amounts; in two preparations (FH5 and HTL2), OH-LAN was produced in greater amounts than LANS. Among the preparations of microsomes used in these experiments, some have been characterized, by immunoblot analysis (data not shown), as originating from subjects that are deficient in P450 2D6. These include preparations FT6, HTL2, HTL4, HTL37, and FH5. Preparations FT6, HTL37, and FH5 did not exhibit significantly lower activity than the average. In particular, preparation HTL37 was one of the most active. This suggests that P450 2D6 is not implicated to a significant extent in the biotransformation of LAN in human liver microsomes. On the other hand, both HTL2 and HTL4 exhibited low activity, but this was shown to correlate also with low levels of P450 3A4 in these preparations (data not shown).

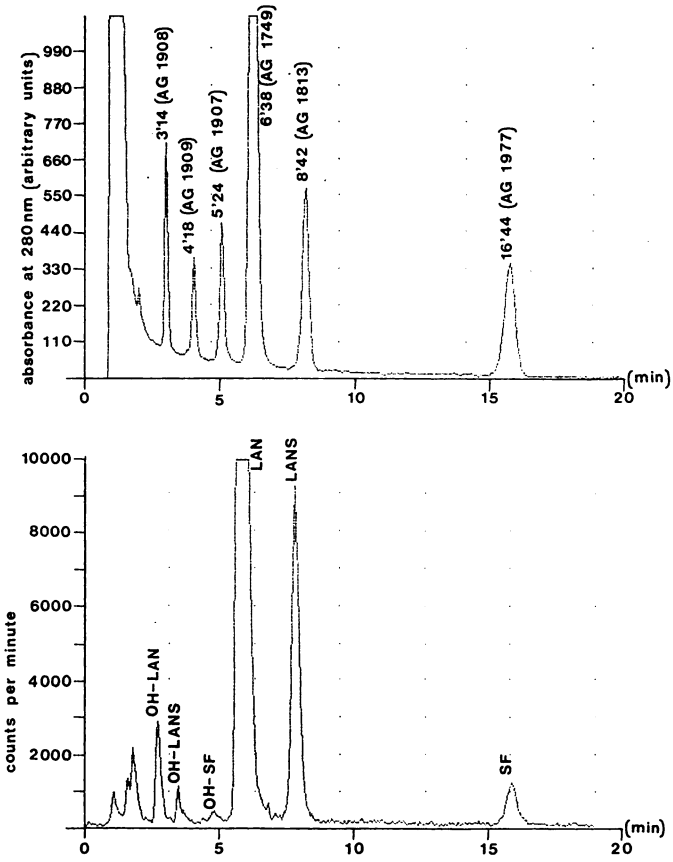

Fig. 1. HPLC analysis of LAN and metabolites. *Upper*, elution of authentic metabolites of LAN and authentic LAN, monitored by absorbance recording at 280 nm, is shown. *AG 1749*, LAN; *AG 1908*, OH-LAN; *AG 1909*, LAN hydroxysulfone (*OH-LANS*); *AG 1907*, LAN hydroxysulfite (*OH-SF*); *AG 1813*, LANS; *AG 1977*, LAN sulfite (*SF*). *Numbers above the peaks* refer to retention times. *Lower*, human liver 61289 microsomes (1 mg/ml) were incubated in a 0.1 M potassium phosphate buffer, pH 7.4, with 10 μM LAN in the presence of 0.5 μCi of the tritiated molecule, and the reaction was initiated at 37° by addition of 1 mM NADPH. After 30 min, the reaction was quenched by addition of acetonitrile and the suspension was centrifuged at 10,000 × *g* for 5 min. One hundred microliters of supernatant were loaded onto the HPLC column. The radioactivity of the effluent was recorded.

Correlations. The microsomes from our bank have been characterized with respect to their levels of several forms of P450 and some P450-specific monoxygenase activities. A series of simple linear regression plots were accordingly generated from the data, including the production of LANS and OH-LAN, the levels of P450 1A2 and 3A4 (determined by immunoblot), and monoxygenase activities including cyclosporin oxidase (P450 3A subfamily), (*S*)-mephenytoin 4′-hydroxylase (P450 2C subfamily), and the production of omeprazole sulfone. The results of such analyses are reported in Table 2, where the correlation coefficient (*r*) as well as the *p* value are quoted for each pair of data. The production of LANS correlated with the

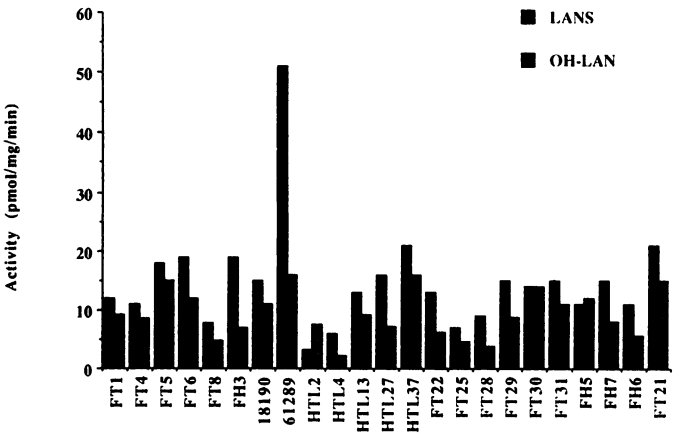

Fig. 2. Interindividual variability of the metabolism of LAN in human liver microsomes. Human liver microsomes were incubated in a 0.1 M potassium phosphate buffer, pH 7.4, with 10 μM LAN in the presence of 0.5 μCi of the radiolabeled molecule, and the reaction was initiated at 37° by addition of 1 mM NADPH. After 30 min, the parent molecule and metabolites were analyzed by HPLC. Liver microsomes FT6, HTL2, HTL4, HTL37, and FH5 were deficient in P450 2D6, as revealed by immunoblot. Liver microsomes FT4 and FT22 were inactive in (*S*)-mephenytoin 4′-hydroxylation.

level of P450 3A4 ($p = 0.0003$), cyclosporin oxidase activity ($p < 0.0001$), the formation of OH-LAN ($p = 0.0008$), and the formation of the sulfone of omeprazole ($p = 0.0001$). The production of OH-LAN exhibited a significant correlation with the production of omeprazole sulfone ($p = 0.0001$) and a weak correlation with the P450 3A4 level ($p = 0.011$), cyclosporin oxidase activity ($p = 0.022$), and (*S*)-mephenytoin 4′-hydroxylase activity ($p = 0.021$). Interestingly, sample HTL2, which exhibited a low LAN oxidase activity, was also the preparation with the highest (*S*)-mephenytoin 4′-hydroxylase activity of the bank; in contrast, samples FT4 and FT22 were inactive (Fig. 2). No correlation was observed between the production of LANS or omeprazole sulfone and the P450 1A2 level or (*S*)-mephenytoin 4′-hydroxylase activity; similarly, the production of OH-LAN did not correlate with the level of P450 1A2. On the other hand, cyclosporin oxidase correlated with the P450 3A4 level ($p = 0.0013$), as expected, and with the formation of omeprazole sulfone ($p = 0.0002$), and the formation of omeprazole sulfone correlated with the level of P450 3A4 ($p = 0.0013$).

Immunoinhibiton experiments. Several preparations of human liver microsomes exhibiting high activity, i.e., 61289, FT21, FT5, and FH7, were selected for immunoinhibition experiments. The results reported in Fig. 3 are for preparation 61289, but similar results were obtained with the other three preparations. Anti-P450 3A antibodies inhibited the production of LANS and OH-LAN by >60%, under conditions where cyclosporin oxidase activity was inhibited by >50% (see the legend to Fig. 3). These antibodies also inhibited the formation of other minor metabolites of LAN, including the hydroxysulfone and hydroxysulfide, by 50–75% (data not shown). Anti-P450 2C3 and 2C8 antibodies exerted a moderate inhibitory effect (30%) on the formation of LAN metabolites. However, in control experiments, these antibodies totally inhibited (*S*)-mephenytoin 4′-hydroxylase activity, although they did not affect cyclosporin oxidase activity significantly under the same conditions (see the legend to Fig. 3). Anti-P450 1A1 and 1A2 antibodies inhibited the production of OH-LAN in human liver microsomes by approximately 50% but did not inhibit the production of LANS. Interestingly, the production of the sulfite derivative of LAN was increased under those conditions where the metabolism of the drug was decreased, that is, in the presence of anti-P450 3A and 1A antibodies (data not shown). Finally, anti-P450 2A6 antibodies had no significant effects. These results suggest a major involvement of P450 3A and a partial involvement of P450 2C and P450 1A in the metabolism of LAN in human liver microsomes.

Inhibition by specific P450 substrates and/or inhibitors. To further characterize the form(s) of P450 involved in the biotransformation of LAN, we investigated the effect of well characterized specific substrates or inhibitors on these enzymes. For this purpose, a series of microsome preparations, including 61289, FT21, FT5, and FH7, were incubated with LAN, at concentrations lower than the K_m (10 and 20 μM), in the absence or presence of the putative inhibitory molecules. The following compounds were tested: propranolol (10 and 50 μM) as inhibitor of P450 1A2 (23), coumarin (10 μM) as substrate of P4502A6 (21), sulfaphenazole (10 and 50 μM), (*S*)-mephenytoin (50 and 100 μM), and tolbutamide (50 and 200 μM) as inhibitor and substrates, respectively, of the P450 2C subfamily (24, 25), quinidine (2 and 20 μM) as inhibitor of P450 2D6 (26), diethyldithiocarbamate (50 and 200 μM) as inhibitor of P450 2A6 and 2E1 (27, 28), and ketoconazole (5 and 50 μM) and cyclosporin A (10 and 50 μM) as inhibitor and substrate, respectively, of P450 3A4 (12, 13). Only cyclosporin A, ketoconazole, and tolbutamide exhibited significant and reproducible inhibitory effects on the biotransformation of LAN, as reported in Fig. 4. Cyclosporin A and ketoconazole inhibited the for-

TABLE 2

Correlations (r and p values) between the rate of production of LAN metabolites, other monoxygenase activities, and P450 1A2 and 3A levels in human liver microsomes

The *p* values are given in parentheses.

	Correlation *r* values					
	OH-LAN[a]	OMS[a,b]	CSA[c]	MP[c]	P450 3A[c]	P450 1A2[c]
LANS	0.650 (0.0008)	0.869 (0.0001)	0.946 (<0.0001)	0.128 (0.649)	0.809 (0.0003)	−0.364 (0.183)
OH-LAN		0.713 (0.0001)	0.583 (0.022)	0.588 (0.021)	0.633 (0.011)	−0.370 (0.174
OMS			0.823 (0.0002)	0.368 (0.177)	0.681 (0.0013)	−0.167 (0.494)
CSA				0.066 (0.816)	0.777 (0.0007)	−0.349 (0.202)
MP					0.044 (0.876)	−0.167 (0.551)
3A						0.220 (0.364)

[a] $n = 23$.
[b] OMS, production of omeprazole sulfone; CSA, cyclosporin A oxidase; MP, (*S*)-mephenytoin hydroxylase.
[c] $n = 15$.

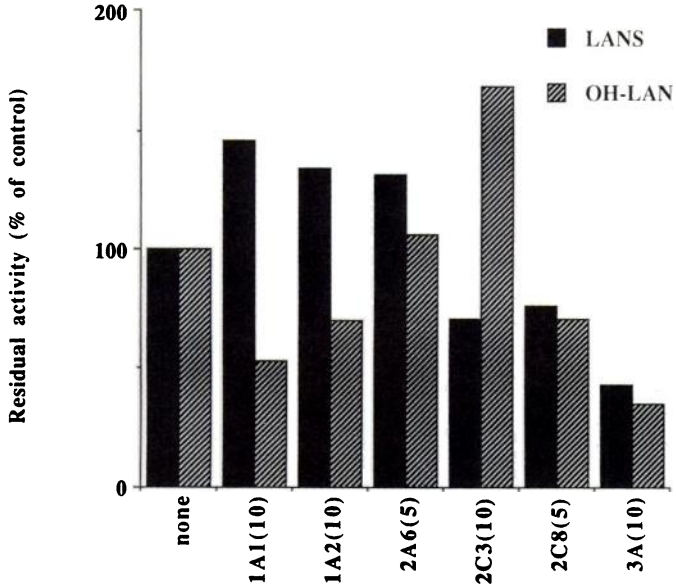

Fig. 3. Immunoinhibition of microsomal metabolism of LAN by specific anti-P450 antibodies. Human liver 61289 microsomes were incubated in a 0.1 M potassium phosphate buffer, pH 7.4, in the absence (control) (*none*) or in the presence of 5 or 10 mg/ml anti-P450 antibodies, for 20 min at room temperature. Then, 10 μM LAN and 0.5 μCi of the radiolabeled molecule were added to the suspension; the reaction was initiated at 37° by addition of 1 mM NADPH. Relative amounts of LANS and OH-LAN are presented as percentage of uninhibited activity. Unihibited activities were as follows: LANS, 50 pmol/min/mg; OH-LAN, 20 pmol/min/mg. Similar results were obtained with preparations FT21, FT5, and FH7. To test the inhibitory effects of the antibodies used in these experiments, their influences on (*S*)-mephenytoin 4′-hydroxylase and cyclosporin oxidase activities were determined in the same liver microsome preparations. (*S*)-Mephenytoin 4′-hydroxylase activity was inhibited by 100% with 5 mg/ml anti-P450 2C3 and anti-P450 2C8, whereas cyclosporin oxidase activity was inhibited by 50% with 10 mg/ml anti-P450 3A4 antibodies.

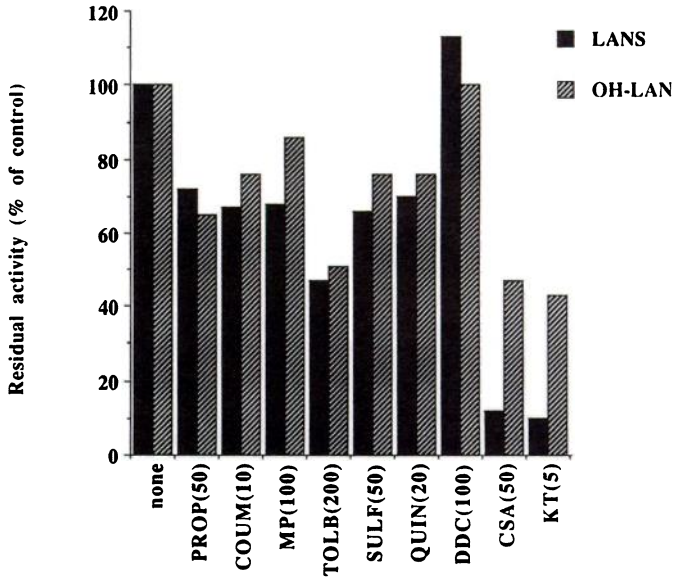

Fig. 4. Inhibition of microsomal metabolism of LAN by form-specific P450 substrates or inhibitors. Human liver 61289 microsomes were incubated in a 0.1 M potassium phosphate buffer, pH 7.4, in the absence (control) (*none*) or in the presence of P450 form-specific substrates or inhibitors (5–500 μM), with 10 μM LAN or omeprazole and 0.5 μCi of the radiolabeled molecule. The reaction was initiated at 37° by addition of 1 mM NADPH. Relative amounts of LANS and OH-LAN are presented as percentage of uninhibited activity. For unihibited activities, see the legend to Fig. 3. Substrates and inhibitors were as follows: *PROP(50)*, 50 μM propranolol; *COUM(10)*, 10 μM coumarin; *MP(100)*, 200 μM (*S*)-mephenytoin; *TOLB(200)*, 200 μM tolbutamide; *SULF(50)*, 50 μM sulfaphenazole; *QUIN(20)*, 20 μM quinidine; *DDC(100)*, 100 μM diethyldithiocarbamate; *CSA(50)*, 50 μM cyclosporin A; *KT(5)*, 5 μM ketoconazole. Similar results were obtained with preparations FT21, FT5, and FH7.

mation of LANS to a greater extent than the formation of OH-LAN, whereas the inhibitory effects of tolbutamide were similar for both metabolites and sulfaphenazole had no significant effect. These results are in agreement with the data presented above and provide additional evidence in favor of the involvement of P450 3A in the metabolism of LAN.

Biotransformation of LAN in human hepatocytes in primary culture. Several P450 forms, including P450 1A1, 1A2, and 3A (12, 15–17), have been shown to be inducible in human hepatocytes in primary culture by β-naphthoflavone (P450 1A1 and 1A2) and rifampicin or phenobarbital (P450 3A). We therefore evaluated the effect of such inducers on the biotransformation of LAN. For this purpose, three different cultures, HTL84, FT58, and FT63, were maintained for 96 hr either in the absence or in the presence of β-naphthoflavone, rifampicin, or phenobarbital. The biotransformation of LAN was then determined directly in the culture dishes. The levels of P450 1A1, 1A2, 2C, and 3A4 were determined, in parallel, by immunoblot analysis of the microsomes prepared from these cultures (data not shown). Cyclosporin oxidase and (*S*)-mephenytoin 4′-hydroxylase activities and the production of omeprazole sulfone were also measured in these cultures or microsomes. The results are reported in Fig. 5 and Table 3. The biotransformation of LAN was strongly increased in cultures treated with rifampicin and phenobarbital, in comparison with untreated cultures. The production of omeprazole sulfone was also strongly increased by these treatments in the same cultures (see the legend to Fig. 5). In cultures exposed to β-naphthoflavone, only the production of OH-LAN was increased. The production of LANS and omeprazole sulfone was correlated with the accumulation of P450 3A4 and cyclosporin oxidase activity in these cultures. Although this activity was induced in rifampicin- and phenobarbital-treated cells, as expected (12, 17), (*S*)-mephenytoin 4′-hydroxylase activity was not affected (Table 3). In addition, immunoblot analysis, using anti-P450 2C8 antibodies, of microsomes prepared from the induced cultures did not reveal any increase in the accumulation of P450 2C-related proteins (data not shown). Interestingly, LAN and omeprazole, which were recently shown to be mixed inducers of CYP1A and CYP3A families in human hepatocytes (29), increased their own biotransformation in these cultures. However, the extent of this increase was low, in comparison with that observed with rifampicin; this is in agreement with the finding that these compounds are only moderate inducers of P450 3A, compared with rifampicin (29).

Biotransformation of LAN by cDNA-expressed human P450s. We next investigated the ability of several cDNA-expressed human P450s to biotransform LAN. For this purpose, microsomes from a human lymphoblastoid cell line (Genetest Corp.) expressing P450 1A1, 1A2, 2A6, 2B6, and 3A4 and from yeast expressing P450 2C8, 2C9, and 2C18 were assayed under the experimental conditions previously used to characterize these preparations with prototypic substrates (see the legend to Table 4). The results are reported in Table 4, in which data concerning the production of omeprazole sulfone are also presented. P450 1A1, 1A2, 2A6, and 2B6 were inactive. The P450

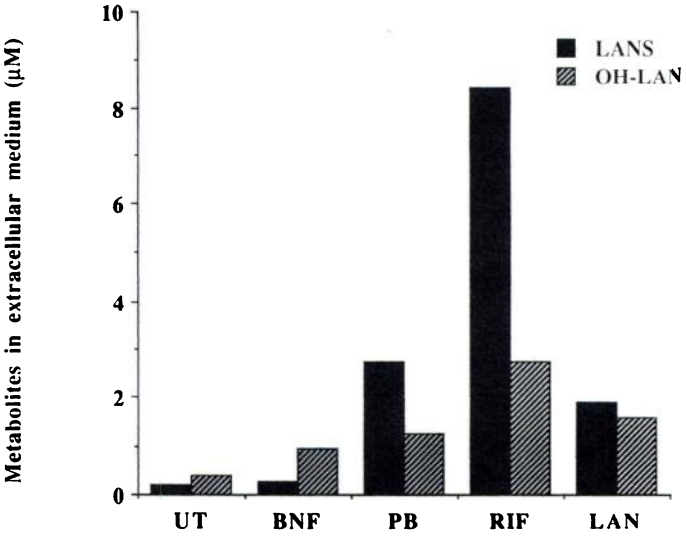

Fig. 5. Oxidative metabolism of LAN in human hepatocytes in primary culture. Human hepatocytes from patient FT63 were maintained in culture for 96 hr in the absence (*UT*) or in the presence of various inducers of P450, including 50 μM rifampicin (*RIF*), 50 μM β-naphthoflavone (*BNF*), or 2 mM phenobarbital (*PB*), as well as in the presence of 50 μM LAN. The medium was then renewed in the absence of the inducer but in the presence of 25 μM LAN and 1 μCi of the radiolabeled molecule. After 3 hr of incubation, an aliquot of extracellular medium was analyzed by HPLC and the amounts of LANS and OH-LAN released in the extracellular medium were determined. The amount of omeprazole sulfone released in the extracellular medium after 3 hr of incubation was also determined in this culture under conditions similar to those used for LAN; the levels were <0.2 μM in untreated cells and cells treated with β-naphthoflavone and 3 μM, 5 μM, and 1.5 μM in cells treated with phenobarbital, rifampicin, and omeprazole, respectively. Similar results were obtained with cultures HTL84 and FT58.

TABLE 3

Cyclosporin oxidase and (S)-mephenytoin 4′-hydroxylase activities in microsomes prepared from untreated and induced human hepatocytes in culture

Cultures HTL86 and FT63 were maintained for 96 hr in the absence or presence of 50 μM β-naphthoflavone (BNF), 50 μM rifampicin (RIF), 1 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), or 2 mM phenobarbital (PB). Microsomes were prepared from these cultures and assayed by HPLC for cyclosporin oxidase and (*S*)-mephenytoin 4′-hydroxylase activities.

Inducer	Cyclosporin oxidase		(S)-Mephenytoin	
	HTL86	FT63	HTL86	FT63
	pmol/mg/min		*pmol/mg/min*	
None	37 ± 4	12 ± 2	20 ± 2	25 ± 3
BNF	33 ± 3	9 ± 2	29 ± 3	17 ± 2
TCDD	48 ± 5		24 ± 3	
RIF	467 ± 47	156 ± 16	24 ± 3	20 ± 3
PB	185 ± 20	132 ± 15	11 ± 2	12 ± 2

2C forms generated measurable amounts of OH-LAN only, with P450 2C18 being 40 times more active than P450 2C8 and 2C9. However, this preparation of 2C18 was inactive in hydroxylating (*S*)-mephenytoin. On the other hand, P450 3A4 produced significant amounts of LANS and omeprazole sulfone, but not of OH-LAN. Interestingly, the turnover numbers for the production of LANS and omeprazole sulfone are very similar. In addition, the turnover numbers quoted in Table 4 are in good agreement with turnover numbers calculated from the microsomal activities presented in Fig. 2 (assuming that the P450 3A4 or 2C concentration is in the range of 100–200 pmol/mg in human liver microsomes). These results are in agreement with the observations described above and support

TABLE 4

LAN and omeprazole oxidase activities of several human forms of P450 expressed from cDNA in yeast or in a cell line

Microsomes from yeast (2C8, 2C9, and 2C18) or from a human lymphoblastoid cell line (3A4) were resuspended in a 0.1 M phosphate buffer, pH 7.4, at 2 mg/ml, in the presence of 10 μM LAN or omeprazole and of the NADPH-regenerating system. The incubation lasted for 3 hr at 37°. The reaction was quenched and metabolites were analyzed as indicated in Materials and Methods. Relative uncertainty was estimated at ±15%. Other forms of P450 (P450 1A1, 1A2, 2A6, and 2B6) were tested but they were inactive. Monoxygenase activities of these microsomes tested under the same experimental conditions were as follows: with tienilic acid (200 μM), undetectable, 1.6 pmol/pmol/min, and 0.5 pmol/pmol/min with 2C8, 2C9, and 2C18, respectively; with cyclosporin A (10 μM), 9.1 pmol/pmol/hr with P450 3A4.

Expressed P450	Metabolites		
	LANS	OH-LAN	Omeprazole sulfone
	pmol/pmol of P450/hr		
2C8	UD[a]	0.08	UD
2C9	UD	0.1	UD
2C18	UD	4.0	UD
3A4	4.9	UD	6.6

[a] UD, undetectable.

a major involvement of P450 3A4 in the biotransformation of LAN and a possible involvement of 2C18, or of another 2C form, in the production of OH-LAN.

Discussion

The results presented here show that P450 3A4 (and/or other forms of this family) is the major enzyme involved in the production of LANS, the main circulating metabolite of LAN. This conclusion is based on the following arguments: 1) in a bank of human liver microsomes, the production of LANS was significantly correlated with the level of P450 3A4 determined by immunoblotting, with cyclosporin oxidase activity, and with the formation of omeprazole sulfone; 2) the production of LANS was specifically and extensively inhibited by anti-P450 3A4 antibodies, by ketoconazole (a specific inhibitor), and by cyclosporin A (a specific substrate of P450 3A4); 3) in human hepatocytes in primary culture, the biotransformation of LAN was strongly accelerated by pretreatment of the cells with specific inducers of P450 3A4; and 4) cDNA-expressed P450 3A4 generated significant amounts of LANS.

The CYP3A subfamily in humans is known to have at least four different genes, including *CYP3A3*, *CYP3A4*, *CYP3A5*, and *CYP3A7* (30–34). These genes encode proteins that exhibit 83–98% similarity in terms of their primary sequence. It is therefore likely that the anti-P450 antibodies used in this work would cross-react with all forms of the subfamily. A contribution of P450 3A7, the human fetal P450 3A-related form, to the oxidative metabolism of LAN can be excluded on the basis of the finding that this form, which can be clearly resolved from the major P450 3A proteins expressed in adult human liver microsome preparations by electrofocusing (35), was not detected in the microsomes used here, prepared either from liver tissue or from human hepatocytes in culture.[3] On the other hand, it is now acknowledged that *CYP3A3* is an allelic variant of *CYP3A4*, which is the main form expressed in adult liver. Indeed, we demonstrated that *CYP3A4* mRNA and protein were specifically induced by rifampicin and phenobarbital in human hepatocytes in culture (29, 34). Aoyama *et al.* (32) have

[3] C. Bonfils, unpublished observations.

shown from heterologous expression of cDNA that P450 3A4 is the cyclosporin oxidase, although P450 3A5 could also partially account for this activity. However, P450 3A5 is known to be polymorphically expressed in only 10–30% of Caucasian subjects (32, 34). It is therefore likely that P450 3A4 is the major enzyme system involved in the oxidative metabolism of LAN. However, we cannot presently exclude a contribution of P450 3A5 to this activity.

Our finding that LANS is specifically produced by P450 3A4 is in good agreement with previous reports from this group (11) and another laboratory (5), in which it was concluded that P450 3A4 was the major form responsible for the production of the sulfone of omeprazole, another proton pump inhibitor of similar chemical structure. Indeed, we observed in this work that the production of LANS and that of omeprazole sulfone were strongly correlated in both human liver microsomes and human hepatocytes in culture and that cDNA-expressed P450 3A4 generated significant amounts of omeprazole sulfone. We recently showed that LAN and omeprazole are mixed inducers of *CYP1A* and *CYP3A* in human hepatocytes in primary culture (29). The present work suggests, therefore, that these drugs could enhance their own metabolism. However, because both compounds were shown to be moderate inducers of *CYP3A* (with respect to rifampicin, for example), this effect may not be clinically significant. To our knowledge, no such observation has yet been reported.

The finding that both LANS and omeprazole sulfone are specifically produced by P450 3A4 indicates that these benzimidazole derivatives represent another class of substrates for this P450 family. Interestingly, another benzimidazole derivative, fenbendazole (36), was recently shown to be oxidized to the sulfone by P450 3A in rats. The list of molecules that have been shown to be metabolized through P450 3A4 (or other human P450 3A-related enzymes) includes nifedipine, quinidine, diltiazem, erythromycin, cyclosporin A, midazolam, triazolam, ethynylestradiol, tamoxifen, dapsone, steroid hormones (6β-hydroxylation), cortisol (6β-hydroxylation), FK506, rapamicin, lovastatine, imipramine (*N*-demethylation), vindesine, vinblastine, lidocaine, estradiol, amiodarone, terfenadine, and zolpidem (34). A number of drugs have been reported to inhibit or induce this P450 in human liver microsomes and hepatocytes in culture (16, 17, 21, 34). These include, for example, ketoconazole, clotrimazole, nicardipine, isradipine, verapamil, ergotamine, and bromocryptine as inhibitors, with K_i values in the range of 0.1–10 μM, and rifampicin, phenytoin, phenobarbital, dexamethasone, and prednisone as inducers. Association of LAN or omeprazole with one (or several) of these molecules could accordingly result in drug interactions. Although a report suggests drug interactions involving omeprazole to be rare or not clinically significant (37), such interactions could arise as the use of these drugs continues to grow. Recent studies in which omeprazole was found to inhibit the metabolism of nifedipine (38) and cyclosporin A (39) *in vivo* and the metabolism of midazolam *in vitro* (40) seem to support our conclusions in this respect.

In contrast to LANS, several forms of P450 could be involved in the production of OH-LAN in human liver, including P450 3A4, P450 1A forms, and P450 2C forms. The first is P450 3A4. The rate of production of OH-LAN correlated significantly with the rates of both LANS and omeprazole sulfone production, as well as with the level of P450 3A4. In addition, immunoinhibition by anti-P450 3A antibodies was significant and the production of OH-LAN was induced by rifampicin and phenobarbital in hepatocytes in culture. On the other hand, cyclosporin and ketoconazole inhibited the formation of OH-LAN, although to a smaller extent than LANS, suggesting that P450 3A4 could be only partly involved. However, cDNA-expressed P450 3A4 did not produce detectable amounts of this metabolite. The reason for this inconsistency is not clear. One possibility is that the cDNA-expressed P450 3A4 is not active for this reaction in the microsomes from lymphoblastoid cells. Indeed, it is possible that some monoxygenase reactions require the presence of essential components that could be absent from the host cells. The second is P450 1A forms. Anti-P450 1A1 and 1A2 significantly inhibited the production of OH-LAN but not of LANS. Moreover, the production of OH-LAN, but not of LANS, was slightly increased in hepatocytes that had been pretreated with β-naphthoflavone, a *CYP1A1* and *CYP1A2* inducer in these cultures (15, 29). However, there was no correlation between the metabolism of LAN and the level of P450 1A2, and both P450 1A1 and 1A2 were inactive when expressed from cDNA in a heterologous system. This last discrepancy could result from a too limited expression of the active proteins in the lymphoblastoid system or from the considerations discussed above for cDNA-expressed 3A4. Another explanation is that our anti-1A antibodies cross-react with an enzyme that is responsible for the production of OH-LAN and whose amount or activity is increased in β-naphathoflave-treated human hepatocytes. These conflicting data do not allow us to definitely conclude that P450 1A forms are significantly involved in the biotransformation of LAN. The third is P450 2C forms. A P450 2C form has been suspected to be partly involved in the production of OH-LAN, on the basis of a moderate correlation with (*S*)-mephenytoin 4′-hydroxylase, immunoinhibition by anti-P450 2C8 and anti-2C3 antibodies, inhibition by tolbutamide, and significant production of this metabolite by cDNA-expressed P450 2C18.

Andersson *et al.* (5, 6) and Sohn *et al.* (7) concluded from *in vivo* studies in humans and from *in vitro* correlation and inhibition data that the hydroxy derivative of omeprazole was produced by a P450 form of the 2C subfamily responsible for (*S*)-mephenytoin hydroxylation. In humans, this subfamily is complex, with at least seven genes being present in the genome (8). There was considerable controversy regarding the identity of the P450 responsible for (*S*)-mephenytoin 4′-hydroxylation and, because this P450 had not been fully characterized by the time those data were published, those authors incorrectly suspected that P450 2C9 and/or P450 2C18 were involved. Recent results from Wrighton *et al.* (9) and Goldstein *et al.* (10), however, demonstrated that the (*S*)-mephenytoin 4′-hydroxylase in humans is P450 2C19. It is therefore likely that P450 2C19 is responsible for the formation of the hydroxy derivative of omeprazole. On the other hand, it has been suggested that 2C9 is the form responsible for the metabolism of tolbutamide in humans (10). Our results on the production of OH-LAN, however, do not fully support the implication of either 2C9 or 2C19. First, cDNA-expressed 2C18 produced much larger amounts of OH-LAN than did 2C8 and 2C9 under the same conditions; in addition, this 2C18 preparation was inactive for the production of the hydroxy derivative of omeprazole (data not shown) and the metabolism of (*S*)-mephenytoin. Second, sulfaphenazole, a potent and specific inhibitor of tolbutamide

hydroxylase in human liver microsomes, did not affect the metabolism of LAN. Third, whereas anti-2C3 and -2C8 antibodies completely inhibited (*S*)-mephenytoin 4′-hydroxylase and tolbutamide hydroxylase activities in human liver microsomes, they only moderately inhibited (by 30%) the biotransformation of LAN in the same samples of microsomes under the same conditions. Fourth, two preparations of human liver microsomes from our bank that were inactive for (*S*)-mephenytoin 4′-hydroxylation (FT4 and FT22) exhibited "normal" LAN hydroxylase activity. We suggest, therefore, that, if LAN is metabolized by the enzymes responsible for (*S*)-mephenytoin or tolbutamide hydroxylation, then their contribution is not major. It is worth emphasizing here that, in contrast to sulfonation, hydroxylation of LAN and omeprazole does not occur at the same position. Whereas omeprazole is hydroxylated at the 5-methyl carbon of the pyridinyl group, LAN is hydroxylated at carbon 5 of the benzimidazole moiety. It is therefore not surprising that hydroxylation of these drugs could be catalyzed by different enzymes. In conclusion, we have shown in this report that P450 3A4 is the major form involved in the production of LANS (the main metabolite of LAN found in human serum) and that this P450, as well as P450 2C18 (and/or another 2C-related form), could be partly involved in the production of OH-LAN.

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Send reprint requests to: Patrick Maurel, INSERM U-128, CNRS, Route de Mende, BP 5051, Montpellier 34033, France.