Biochemical Pharmacology, Vol. 39, No. 8, pp. 1327–1333, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

METABOLISM OF FLUPERLAPINE BY CYTOCHROME P450-DEPENDENT AND FLAVIN-DEPENDENT MONOOXYGENASES IN CONTINUOUS CULTURES OF RAT AND HUMAN CELLS

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(*Received* 2 *August* 1989; *accepted* 11 *November* 1989)

Abstract—The metabolism of fluperlapine, a neuroleptic dibenzazepine derivative with a *N*-methyl-piperazinyl substituent, was investigated in continuous cultures of rat and human cells which express various cytochrome P450-dependent monooxygenase activities. The differentiated rat hepatoma cells H4IIEC3/G$^-$ and their variants 2sFou and FGC-5 metabolized fluperlapine predominantly by N-oxygenation and only to a minor degree by N-demethylation or glucuronidation of primary phenolic products. Total fluperlapine metabolism in dedifferentiated rat hepatoma cells H5 and partially differentiated human hepatoma cells HepG2 was much smaller than in the differentiated rat hepatoma lines. This was primarily attributable to their low capacity for N-oxygenation. Human lung adenocarcinoma lines NCI-H322 and NCI-H358 formed only trace amounts of fluperlapine *N*-oxide. Pretreatment of 2sFou cells with benz(*a*)anthracene, phenobarbital or dexamethasone markedly increased the formation of N-demethylated and glucuronidated products but did not affect the rate of *N*-oxide formation. Guanethidine and cysteamine, inhibitors of flavin-dependent monooxygenase activity, reduced fluperlapine N-oxidation more strongly than aldrin epoxidation, a marker for cytochrome P450 activity. In contrast, *n*-octylamine inhibited aldrin epoxidation but was without effect on fluperlapine N-oxygenation. The results suggest that certain cells in continuous culture are capable of expressing flavin-dependent monooxygenase(s) in addition to cytochrome P450-containing monooxygenases. Such cells may offer useful systems for studying the oxidative metabolism of a broad spectrum of xenobiotics and analysing the importance of the two oxygenation reactions for the biological effects of their substrates.

During the last decades isolated hepatocytes have extensively been used to study the metabolism of drugs and environmental chemicals [1]. Since they retain the *in vivo* ability for metabolizing xenobiotics, at least for some time, they are highly suitable for identifying pathways of drug metabolism under well defined conditions. However, their general applicability to studies on metabolism and biological effects of drugs is severely restricted because they possess only a limited lifespan in culture [2] and show a considerable variability from preparation to preparation. Continuous hepatic cell lines would be attractive alternatives to hepatocyte cultures provided they are metabolically competent.

Recently certain cell lines derived from Reuber rat hepatoma [3] have been shown to maintain numerous liver specific functions including those of xenobiotic metabolism [4–7]. In particular, these cell lines were found to express cytochrome P450 forms which are typically contained in liver. A number of observations suggests that HepG2, an epithelial cell line, derived from a human hepatoblastoma [8, 9], also contains various liver specific functions including drug-metabolizing enzymes [10–14].

The present investigation had as a first aim to test whether these rat and human hepatoma cell lines are capable of mimicking the metabolism of drugs in hepatocyte cultures. As a model compound we chose the neuroleptic dibenzazepine derivative, fluperlapine, with a *N*-methyl-piperazinyl substituent. Fluperlapine has previously been analysed for its complex metabolism in hepatocytes derived from rat [15] and man [16]. It is metabolized via aromatic ring hydroxylation with subsequent conjugation reactions including glucuronidation and sulfation. The compound is also N-demethylated and N-oxygenated at the 4′-piperazinyl nitrogen (Fig. 1).

The second aim of the study was to more closely analyse the nature of the enzyme(s) involved in the oxidative metabolism of fluperlapine. The test compound presents an interesting case because its oxidation reactions are most likely mediated by different types of oxygenases, i.e. cytochrome P450-containing monooxygenase (P450‡) and flavin-containing monooxygenase (FMO). Thus ring hydroxylation and N-demethylation are typical actions of P450s, whereas the N-oxidation of tertiary amines with alpha-hydrogens, such as the one in fluperlapine, is preferentially carried out by FMO [17]. Other structurally related tricyclic antidepressant drugs like imipramine or chlorpromazine have been shown to be N-oxygenated by FMO of pig liver [18].

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‡ Abbreviations: P450, cytochrome P450-containing monooxygenase; FMO, flavin-containing monooxygenase; DEX, dexamethasone; BA, benz(*a*)anthracene; PB, phenobarbital; AHH, aryl hydrocarbon hydroxylase; AE, aldrin epoxidase.

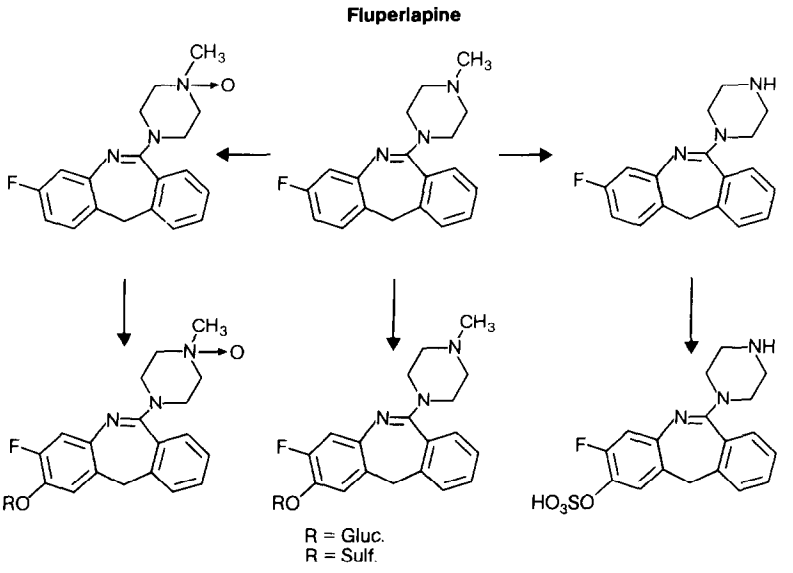

Fig. 1. Proposed pathway of biotransformation of fluperlapine [15, 16].

The possible involvement of P450s or FMO in fluperlapine oxygenation was studied in variants of the Reuber rat hepatoma cells which differ in their status of differentiation and expression of P450. The activity of major P450 forms was modulated by exposure of cells to various P450 inducers. In addition, we examined two human lung adenocarcinoma lines which resemble alveolar type II cells and Clara cells [19, 20] for their metabolism of fluperlapine as examples for extrahepatic cells. Finally, preliminary attempts were made, to distinguish P450 and FMO activities by using differential inhibitors of the two reactions.

MATERIALS AND METHODS

Chemicals. [^{14}C]Fluperlapine {3-fluoro-6-(4′-methyl-1′-piperazinyl)-11H-[6-^{14}C]dibenz[b,e]azepine} was synthesized by Dr R Voges of the Synthetic Tracer Laboratories of Sandoz Ltd, Basle. The specific activity was 30.5 μCi/mg and the purity was greater than 95%. Unlabeled fluperlapine, its *N*-oxide and N-demethylated product were obtained from the Preclinical Research Department of Sandoz Ltd, Basle. Guanethidine was generously supplied by Ciba-Geigy Ltd, Basle. All other chemicals were commercially available.

Cells. The differentiated hepatoma lines H4IIEC3/G$^-$ (H4IIE), FGC-5 and 2sFou and the dedifferentiated line hepatoma H5 were generously supplied by Dr M. Weiss, Institute Pasteur, Paris, France. HepG2 were obtained from Dr B. Knowles, Wistar Institute, Philadelphia, PA, U.S.A.; NCI-H358 and NCI-H322 from Dr H. Schuller, Vet. College, Knoxville, TN, U.S.A.

Culture and treatment of cells. Reuber hepatoma cells were grown in NCTC 135:HAM'S F12 (1:1) medium containing 5% fetal calf serum; HepG2 cells in MEM medium containing 10% fetal calf serum and 1 mM L-glutamine; NCI-H322 and NCI-H358 cells in DMEM medium containing 10% fetal calf serum and 2 mM L-glutamine. All media contained 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were seeded at a density of 0.05–1.0 × 10^6/21 cm^2 plate or 2–4 × 10^5/55 cm^2 plate. They were exposed to the various chemicals when they approached confluency, i.e. after 5–8 days of growth. For induction of P450 activity cells were treated with dexamethasone (DEX) (5 μM), benz(*a*)anthracene (BA) (22 μM) or phenobarbital (PB) (1.6 mM). The medium was then exchanged and cells were exposed to fluperlapine (39 μM) added in dimethyl sulfoxide. The final solvent concentration was 0.1%.

Enzyme assays. Aryl hydrocarbon hydroxylase (AHH) activity was determined according to Wiebel *et al.* [21] and aldrin epoxidase (AE) activity according to Wolff *et al.* [22]. Cellular protein was determined by the method of Lowry *et al.* [23] using bovine serum albumin as the standard.

HPLC analysis. Metabolites were separated by HPLC using a Merck Lichrosorb RP 18 column (8 × 250 mm). The material was eluted at ambient temperature with a flow rate of 4 mL/min. The mobile phase consisted of 10 mM ammonium carbonate (solvent A) and acetonitrile (solvent B). The proportion of solvent B was 0% up to 5 min and was increased linearly to reach 7% at 15 min, 25% at 75 min and 80% at 95 min. The eluent was monitored for UV absorbance at 250 nm and for radioactivity.

Radioactivity measurements. The total radioactivity of the sample solutions was measured in duplicate by liquid scintillation counting using standard procedures. Quench correction was performed by external standard ratio method. The radioactivity of the HPLC column effluent was determined by continuous flow scintillation spectroscopy.

RESULTS

Fluperlapine metabolism

When 2sFou cells were incubated with 39 μM fluperlapine for 24 hr, 75–88% of the administered radioactivity was found in the medium. Another 15–19% of the radioactivity was extractable from the

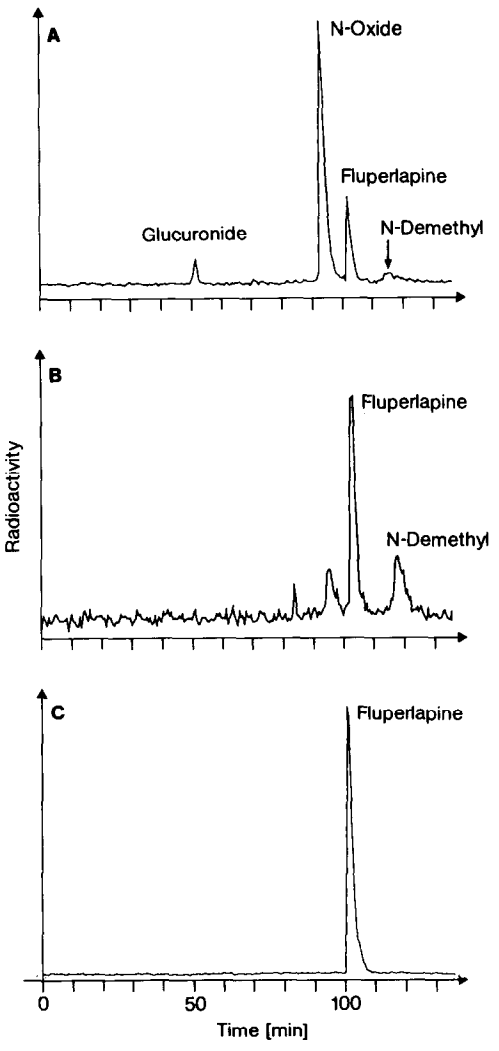

Fig. 2. HPLC separation of fluperlapine and its metabolites formed by H4IIE cells. H4IIE cells were incubated with fluperlapine (39 μM) for 24 hr. (A) Growth medium from 55 cm^2 plates containing approximately 3×10^6 cells; (B) extract of cells; (C) growth medium from plates without cells. Cells were pretreated with DEX for 30 hr. AE activity was 14 pmol/min/mg protein.

cells with methanol and less than 0.2% of the radioactivity remained associated with the cells.

The metabolism of fluperlapine was analysed by HPLC. An example for the separation of metabolites found in the growth medium of H4IIE cells after 24 hr of exposure is shown in Fig. 2A. Chromatography of unlabeled reference compounds allowed the assignment of the peak with a retention time of 101 min to unchanged drug and of the two peaks with retention times of 92 and 113 min to N-oxidized fluperlapine and N-demethylated fluperlapine, respectively. No final structural assignment was made for the more polar metabolite eluting at 52 min. However, incubation of the radioactive material in this peak with β-glucuronidase increased its retention time to 90 min indicating the formation of a less polar product, possibly a phenol, from a glucuronide. In addition, chromatography of a known sample of rat bile and comparison of the retention times supports the assignment of the peak eluting at 52 min to the fluperlapine-2-glucuronide. Relative amounts of the different metabolites found in the growth medium of H4IIE cells are given in Table 1 together with those formed by other cell lines. The major metabolite formed by H4IIE cells was fluperlapine-*N*-oxide representing 70% of the total radioactivity in the medium. *N*-Demethyl-fluperlapine and fluperlapine-glucuronide comprised 2.1 and 4.3%, respectively, of the radioactivity. Other metabolites accounted for less than 0.5%. About 25% of the drug remained unchanged under these conditions. The relative distribution of the metabolites retained in H4IIE cells differed somewhat from that in the growth medium. Methanol extracts of the cell fraction contained predominantly the N-demethylated fluperlapine and less of the N-oxygenated product (Fig. 2B). The more polar metabolite could not be detected in the methanol extracts. Fluperlapine was stable when kept in the growth medium in the absence of cells (Fig. 2C).

The total metabolism of fluperlapine considerably differed between the various cell lines examined (Table 1). The highest rates of metabolism were observed in the differentiated rat hepatoma lines 2sFou, H4IIE and FGC-5. Metabolic rates were intermediate in the dedifferentiated rat hepatoma line H5 and the human hepatoma line HepG2 and were barely detectable in human pulmonary cell lines NCI-H358 and NCI-H322.

The various cell lines also differed in their metabolite pattern. For example, HepG2 cells formed more *N*-demethyl fluperlapine and conjugated products than fluperlapine-*N*-oxide in contrast to the predominant formation of the *N*-oxide in the differentiated rat hepatoma cells, 2sFou and H4IIE. Similarly, the dedifferentiated H5 hepatoma cells produced sizeable amounts of the N-demethylated product but little *N*-oxide as compared to the differentiated hepatoma lines. In NCI-H322 and NCI-H358 cells only trace amounts of *N*-oxides were detectable.

The primary N-oxidation product of fluperlapine apparently did not undergo further biotransformation (Table 2). Thus prolonged exposure of 2sFou cells to fluperlapine for up to 72 hr did not change the yield of fluperlapine-*N*-oxide. Similarly, when growth media containing the fluperlapine-*N*-oxide from cultures exposed to fluperlapine for 24 hr was transferred to previously unexposed cultures, the *N*-oxide was nearly quantitatively recovered after 24 hr. To assess a possible reduction of the *N*-oxide within the cells, 2sFou cells were isolated and made permeable by freeze-thawing. Formation of radiolabeled *N*-oxide from radioactive fluperlapine by the cells was measured in the presence or absence of unlabeled *N*-oxide. The appearance of radioactive *N*-oxide was not dependent on the presence or absence of unlabeled *N*-oxide suggesting that the *N*-oxide is not reduced to a significant extent under the present conditions.

P450 activities

2sFou cells contained appreciable amounts of both

Table 1. Fluperlapine metabolism in various cell lines and its dependency on the status of P450 induction*

Cell line	Treatment	Formation of fluperlapine metabolites†				
		N-Oxide	*N*-Demethyl	Glucuronide	Others	Total metabolism
H4IIE	DEX + BA [30 hr]	69.5	2.1	4.3	0.5	75.9
2sFou	None	88.1	0.5	1.1	0.5	89.0
	DEX [72 hr]	85.4	4.2	3.2	2.2	95.0
	PB [72 hr]	93.8	2.9	1.5	1.0	99.2
	BA [24 hr]	86.8	1.8	2.5	0.5	91.1
	DEX + BA [30 hr]	92.7	0.7	3.5	—	96.9
FGC-5	None	46.3	0.5	0.5	0.5	46.3
H5	BA [24 hr]	5.3	3.7	0.5	0.9	9.9
HepG2	None	3.5	4.3	2.0	1.0	10.8
NCI-H358	None	1.3	0.5	—	—	1.8
NCI-H322	None	1.2	—	—	—	1.2

* Cells were treated with various P450-inducers as described in Materials and Methods before fresh medium containing 39 μM fluperlapine was added. For constitutive and induced P450 activities see Table 3.

† Values represent the per cent of initial amount of substrate in the growth medium. They give the mean of determinations from duplicate plates ranging by $\leq$15%.

Table 2. Metabolites of fluperlapine incubated for various time periods with 2sFou cells*

Time of incubation	*N*-Oxide	*N*-Demethyl	Fluperlapine
24 hr	89.2	0.7	10.1
48 hr	97.7	1.0	0.1
72 hr	97.0	0.2	0.1
2 × 24 hr	98.8	0.1	0.2

* Values give the mean of determinations from duplicate plates which varied by less than 6%. They represent the per cent of initial substrate concentration (39 μM). 2sFou cells were pretreated with DEX for 30 hr as described in Materials and Methods.

AHH and AE activities (Table 3). Pretreatment with DEX or PB caused an increase in both AHH and AE, whereas BA induced only AHH activity. The level of constitutive or DEX-induced AE activity did not significantly change during the test period. Constitutive and DEX-induced AHH activity doubled during the test period. In contrast, AHH activities induced by PB or BA markedly decreased in the absence of the inducer.

Effect of P450 inducers on fluperlapine metabolism

Pretreatment of 2sFou cells with DEX, PB or BA resulted in an increased formation of *N*-demethyl-fluperlapine and polar products (Table 1). The major metabolite fluperlapine-*N*-oxide, however, was not significantly affected by the different inducers. This was also true under conditions, where only 10–15% fluperlapine-*N*-oxide were formed, i.e. when 2sFou cells were exposed to a high concentration of fluperlapine (96 μM) for only 2 hr (data not shown). The rate of fluperlapine-*N*-oxide formation was approximately 500 pmol/min/mg protein under these conditions.

Differential inhibition of monooxygenase activities

In order to obtain some information on the nature of the N-oxidation of fluperlapine in differentiated hepatoma cells, we examined the effect of various compounds which differentially inhibit FMO or P450. Exposure of 2sFou cells to guanethidine or cysteamine, both known substrates of FMO [24, 25], inhibited fluperlapine-*N*-oxide formation by 33 and 72%, respectively (Fig. 3). Although P450, as determined by AE activity, was also affected by the two compounds, the degree of inhibition was considerably lower than for *N*-oxide formation of fluperlapine. In contrast to the inhibition by guanethidine or cysteamine, no reduction of fluperlapine-*N*-oxide was found when *n*-octylamine was added at concentrations inhibiting aldrin epoxidase activity. Guanethidine, cysteamine or *n*-octylamine, at the concentrations used, did not significantly affect the vitality of the cells as determined by the uptake of neutral red at the end of the expose period (data not shown).

DISCUSSION

The present results have shown that the metabolism of fluperlapine in certain continuous cultures is qualitatively similar to that in primary cultures of rat [15] and human hepatocytes [16] and in rat and

Table 3. Constitutive and induced P450 activities of the test cell lines*

Cell line	Treatment†	Monooxygenase activity (pmol/min/mg protein) AHH Treated‡	AHH 24 hr after treatment§	AE Treated‡	AE 24 hr after treatment§
2sFou	None	1.37 ± 0.10	2.96 ± 0.17	12.91 ± 2.11	15.36 ± 0.20
	DEX [72 hr]	4.59 ± 1.08	8.29 ± 0.88	25.91 ± 3.04	19.69 ± 1.60
	PB [72 hr]	12.60 ± 0.93	5.33 ± 0.20	61.32 ± 1.94	53.14 ± 3.89
	BA [24 hr]	136.40 ± 1.48	17.67 ± 3.09	13.23 ± 1.95	14.78 ± 1.34
FGC-5	None	0.41 ± 0.01	0.70 ± 0.01	22.63 ± 1.51	29.91 ± 0.03
H5	BA [24 hr]	54.03 ± 0.13	15.87 ± 0.90	0.61 ± 0.11	ND
HepG2	None	0.128 ± 0.023	ND	<0.1	ND
NCI-322	None	0.143 ± 0.000	ND	<0.1	ND
NCI-358	None	0.031 ± 0.004	ND	<0.1	ND

* Values represent mean ± range of determinations from duplicate plates.
† Cells were exposed to DEX, PB or BA for the times indicated in brackets as described in Materials and Methods.
‡ Enzyme activities after exposure to DEX, PB or BA.
§ Enzyme activities after additional 24 hr in the absence of DEX, PB or BA.
ND, not determined.

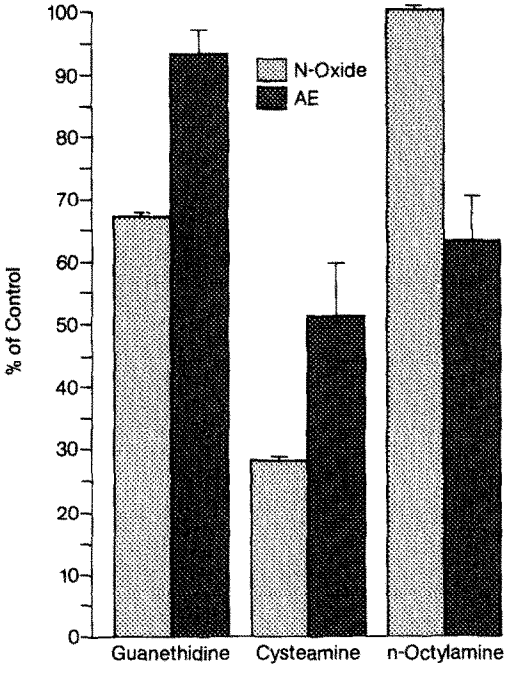

Fig. 3. Effect of guanethidine, cysteamine and *n*-octylamine on fluperlapine-*N*-oxide formation and aldrin epoxidation in 2sFou cells. 2sFou cells grown on 21 cm^2 plates using 3 mL growth medium were exposed to fluperlapine (39 μM) or aldrin (20 μM) in the absence or presence of guanethidine (2 mM), cysteamine (2 mM) or *n*-octylamine (0.1 mM) for 16 hr. Fluperlapine-*N*-oxide formation was measured by HPLC as described in Materials and Methods. For determination of aldrin epoxidation, 0.2-mL aliquots of the growth medium were extracted with 1 mL hexane. Amounts of dieldrin in the hexane phase were measured as described by Wolff *et al.* [22]. Values represent the percent of fluperlapine-*N*-oxide formation and AE activity in the absence of the inhibitors. These activities amounted to 500 and 6.1 pmol/min/mg protein, respectively. Mean and range were determined from duplicate plates for fluperlapine-*N*-oxide formation and from triplicate plates for AE.

man *in vivo* (unpublished data). The cells metabolized fluperlapine by N-oxygenation, N-demethylation and ring hydroxylation. In all systems *N*-oxides were the main products. For example between 45 and 60% of the substrate was found to be N-oxygenated in rat and human hepatocytes; more than 60% of the radioactivity in the plasma of rat after application of [^{14}C]fluperlapine consisted of *N*-oxide [15].

Although the present results do not prove, they strongly suggest that the N-oxygenation and N-demethylation of fluperlapine in hepatoma cells are mediated by different types of enzymes, i.e. flavin-dependent or cytochrome P450-dependent monooxygenases, respectively. A first line of evidence comes from the differential effects of P450 inducers on the two oxygenase reactions. Pretreatment of 2sFou cells with inducers of major P450 forms did not increase the rate of N-oxygenation under conditions where the formation of N-demethylated and ring hydroxylated products was increased and the marker activities for P450, AHH and AE, were markedly increased. As shown by Ziegler and co-workers, hepatic FMO is neither inducible by PB [26] nor 3-methylcholanthrene [27]. FMO activity might even be suppressed by treatment of rats with PB or a polycyclic aromatic hydrocarbon type inducer [28].

There was also no correlation between the constitutive activities of P450 and the rate of fluperlapine N-oxygenation. For example, aldrin epoxidation which is mediated by the majority of hepatic P450 forms [29] was higher in FGC-5 cells than in 2sFou cells, but the opposite was true for the rates of fluperlapine *N*-oxide formation in these cell lines. Likewise, the levels of AHH activities did not correlate with the rates of *N*-oxide formation in the various cell lines.

A second argument for the involvement of FMO, but not P450, in N-oxygenation of fluperlapine comes from the different effects of guanethidine, cysteamine, and *n*-octylamine on *N*-oxide formation and

aldrin epoxidation in 2sFou cells. Guanethidine *N*-oxide formation has been used as indicator for FMO activity [24]. Cysteamine is a physiological substrate for FMO [25]. Although guanethidine and cysteamine were also inhibitory to aldrin epoxidase activity, they suppressed N-oxygenation of fluperlapine more strongly. So far, no substance has been found which specifically blocks FMO activities without affecting P450 mediated oxidations [25]. The effects of *n*-octylamine on the test parameters were opposite to those of guanethidine or cysteamine. Fluperlapine-*N*-oxide formation was not altered, while AE activity decreased by 40% in the presence of *n*-octylamine. This is consistent with the observation that *n*-octylamine is a potent inhibitor of cytochrome P450 containing monooxygenase(s) [30], but has little or even small stimulatory effects on FMO in liver microsomes of rat [31, 32].

The N-oxygenation of fluperlapine appears to be a differentiated function of liver cells. The activity was relatively high in all of the differentiated rat hepatoma lines examined and minor in the dedifferentiated rat hepatoma line H5. The low degree of N-oxygenation in the human hepatoma line HepG2 is difficult to interpret. Human liver has been shown to contain FMO activity [28, 33]. Human hepatocytes in culture are capable of N-oxygenation of fluperlapine [16]. Although HepG2 cells have retained a number of differentiated functions of liver [8, 9], their status of differentiation is not clear in respect to the expression of hepatic xenobiotic metabolizing enzymes. The HepG2 cells used in this study possessed only trace activities of hepatic P450, i.e. of aldrin epoxidase. Thus FMO activity may be one of the functions which were lost during the partial dedifferentiation of HepG2 cells. The same applies in principle to the human pulmonary lines NCI-H322 and NCI-H358 which only marginally metabolized fluperlapine. The two cell lines resemble in their metabolism of benzo(*a*)pyrene human lung *in vivo* [34, 35]. Pulmonary forms of FMO of mouse, rabbit, rat, guinea-pig and hamster have been shown to differ from the major forms of FMO present in the liver [18, 31, 36–38]. They may accept fluperlapine as a substrate less readily than hepatic FMO forms. Imipramine and chlorpromazine two structurally related tricyclic antidepressants are readily N-oxygenated by FMO of pig liver but not of rabbit lung [18].

In conclusion, certain cells in continuous culture such as rat hepatoma cells 2sFou and H4IIE appear to express both P450-dependent and flavin-dependent monooxygenases. A panel of these cell lines differing in the expression of the two monooxygenases may be highly useful to analyse the oxidative metabolism of xenobiotics and monitor their biological effects on the cellular level.

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