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INHIBITORY EFFECTS OF NILUTAMIDE, A NEW ANDROGEN RECEPTOR ANTAGONIST, ON MOUSE AND HUMAN LIVER CYTOCHROME P-450

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Abstract—The effects of nilutamide were studied first with human liver microsomes. At concentrations expected in the human liver (110 μM), nilutamide inhibited hexobarbital hydroxylase, benzphetamine *N*-demethylase, benzo(*a*)pyrene hydroxylase and 7-ethoxycoumarin *O*-deethylase activities by 85, 40, 35 and 25%, respectively. There was no *in vitro* inhibition of NADPH–cytochrome *c* reductase activity, no *in vitro* loss of CO-binding cytochrome P-450, and no spectral evidence for the *in vitro* formation of a possible cytochrome P-450Fe(II)-nitroso metabolite complex. Other studies were performed with mouse liver microsomes. Nilutamide (550 μM) did not significantly increase the consumption of NADPH by aerobic microsomes, and did not modify the kinetics for the reduction of cytochrome P-450 by NADPH–cytochrome P-450 reductase in an anaerobic system. Nilutamide (22 μM) produced either a type I or a type II binding spectrum. Kinetics for the inhibition of hexobarbital hydroxylase were consistent with competitive inhibition. A last series of experiments was performed after administration of nilutamide in mice. Thirty minutes after administration of doses (15 or 30 $\mu mol.kg^{-1}$ i.p.) similar to those used in humans, the hexobarbital sleeping time was increased by 40 and 60%, respectively. There was no evidence, however, for the irreversible inactivation of microsomal enzymes since CO-binding cytochrome P-450 and monooxygenase activities remained unchanged in liver microsomes from mice killed 1 or 6 hr after administration of nilutamide (30 $\mu mol.kg^{-1}$ i.p.). These results show that nilutamide inhibits hepatic cytochrome P-450 activity, and suggest that inhibition may actually occur after therapeutic doses of nilutamide in humans.

Nilutamide (Fig. 1) is a new non-steroidal anti-androgen derivative behaving as a competitive antagonist of the androgen receptor [1]. The drug has been marketed in France since November 1987. Nilutamide is proposed in the treatment of metastatic prostatic carcinoma, in association with orchiectomy. Four randomized double-blind multicenter studies have shown that nilutamide combined with orchiectomy is more effective than orchiectomy alone on bone pain and several other criteria [1].

After oral administration of nilutamide in humans, absorption is essentially complete, the half-life is about 2 days, and urinary excretion is predominant, mainly in the form of metabolites [1]. The elimination half-life is 7 hr in rats. Nilutamide is extensively metabolized in the liver, undergoing mainly reduction of the nitro group to the hydroxylamine and the amine. Nilutamide itself, or the amine, may also undergo oxidation at several sites: one of the methyl groups is oxidized to the primary alcohol; oxidation of the benzene ring leads to the *p*-aminophenol derivative, while oxidation of the NH group of the hydantoin moiety leads to the *N*-hydroxyl metabolite [1].

Premarketing studies have shown that administration of nilutamide increased the hexobarbital sleeping time, and the anticoagulant effect of warfarin in rats [1]. To determine the mechanism for these effects and their possible clinical implications, we have studied the effects of nilutamide on hepatic cytochrome P-450 in mice and in humans.

MATERIALS AND METHODS

Chemicals. Nilutamide, 5,5-dimethyl-3-(4-nitro-3-trifluoromethylphenyl)-2,4-imidazolidinedione, was kindly given by Cassenne Laboratories (Paris, France). [2-^{14}C]Hexobarbital (9 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Its radiochemical purity was assessed by thin-layer chromatography and found to be 99%.

Human liver microsomes. The overall approach was similar to that used in previous studies on the effects of troleandomycin [2], erythromycin [3] and methoxsalen [4] on human liver cytochrome P-450. This study was approved by the Comité Consultatif

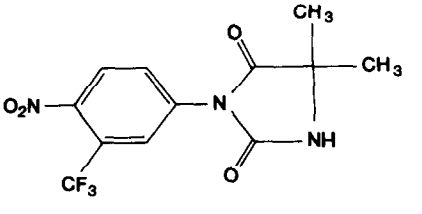

Fig. 1. Chemical structure of nilutamide.

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d'Ethique Médicale du centre Hospitalier et Universitaire Bichat-Beaujon. Informed consent was obtained from patients. A liver specimen was removed by surgical biopsy, or during a hepatic tumor resection, in 6 patients undergoing elective abdominal surgery for various reasons (digestive carcinomas, gall-bladder stones, hepatic tumors), and in whom a histologic examination of the liver was medically required. We excluded alcoholic patients or those who had taken drugs known to induce microsomal enzymes [5] during the two weeks preceding surgery; patients taking other drugs were not excluded since drugs had to be given for premedication or anesthesia, anyway. Patients were premedicated with alimemazine, atropine, and hydroxyzine. They were anesthetized with droperidol, enflurane, fentanyl, thiopental sodium, and pancuronium bromide.

Part of the liver was placed in Bouin's fluid, and sent to the pathologist. Another fragment of the surgical liver biopsy, or parts of the removed normal liver during tumor resection, were stored at −20° until the conclusion of the pathologist was available, a few days later. Only those livers with a normal histology were used in this study.

The liver fragment was then thawed, blotted dry, weighed and homogenized in 3 vol. of ice-cold, 0.15 M KCl, 0.01 M sodium–potassium phosphate buffer pH 7.4. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 60 min. Microsomal pellets were stored at −20° until analyzed, 1–3 days later (usually, 1 day).

Mouse liver microsomes. Male Crl: CD-1(ICR)BR Swiss mice were purchased from Charles River (Saint-Aubin-lès-Elbeuf, France). Animals were fed on a normal diet (M25 biscuits, Extra Labo, Piètrement, Provins, France) given *ad libitum*. Mice were killed by cervical dislocation. Hepatic microsomes were prepared as described above.

Some mice were killed 1, 4 or 6 hr after administration of nilutamide (15–300 μmol . kg^{-1} i.p.) in 0.1–0.25 ml of corn oil. Control mice received corn oil.

Microsomal enzymes and monooxygenase activities. Microsomal protein concentration was determined by the method of Lowry *et al.* [6]. Microsomal cytochrome P-450 was measured as described by Omura and Sato [7]. NADPH–cytochrome *c* reductase activity was determined as described by Mazel [8].

Monooxygenase activities were assayed by incubating various substrates (0.25 mM) with hepatic microsomes and the following NADPH-generating system: NADP (0.4 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (2 enzyme U/ml) and $MgCl_2$ (6 mM) in 0.05 M sodium–potassium phosphate buffer, pH 7.4. The incubation was carried out at 37° for 10 min. Benzo(*a*)pyrene hydroxylase activity was determined by the technique of Kuntzman *et al.* [9]. [2-^{14}C]Hexobarbital hydroxylase activity was measured as described by Kupfer and Rosenfeld [10]. The activity of benzphetamine *N*-demethylase was measured as reported by Mazel [8]. 7-Ethoxycoumarin *O*-deethylase activity was measured as reported by Greenlee and Poland [11].

Binding spectra. Because nilutamide absorbs light at 263 nm, a technique using four cuvettes was used to look for the presence of a binding spectrum of nilutamide with cytochrome P-450Fe(III). On each side were placed two cuvettes: one containing buffer, the other containing the microsomal suspension (2 mg of microsomal protein per ml). After recording the base-line, nilutamide at a final concentration of 22 μM, was added in 2 μl of ethanol, both to the microsomal suspension on the sample side and to the buffer solution on the reference side, whereas a same volume of ethanol was added to the two other cuvettes. The difference spectrum was recorded, with base-line subtraction, from 350 to 480 nm on an SLM. Aminco DW-2C spectrophotometer.

In vitro *destruction of cytochrome P-450.* A possible *in vitro* destruction of cytochrome P-450 was investigated as previously reported [4]. Human microsomes from 125 mg of liver were incubated in 1 ml of 0.06 M KCl, 0.025 M sodium-potassium phosphate buffer, pH 7.4, containing EDTA (1.5 mM), nilutamide (550 μM), and the NADPH-generating system described above. Microsomal protein in the assay was 4.6 ± 0.6 mg . ml^{-1}. In some flasks, nilutamide was omitted. Half of the flasks were kept in ice throughout, and served as zero-time samples. The other flasks were incubated at 37° for 20 min, and then placed on ice again. After adding 1 ml of buffer, the microsomal suspension was divided between two cuvettes, and cytochrome P-450 was then determined as the CO-difference spectrum of dithionite-reduced microsomes.

Cytochrome P-450Fe(II)–metabolite (nitroso) complexes. The possible *in vitro* formation of a cytochrome P-450Fe(II)–metabolite complex during incubation of nilutamide with liver microsomes was investigated as previously described [12]. Cuvettes divided into 2 compartments and maintained at 37° were used. A microsomal suspension containing NADPH (1 mM) and EDTA (1.5 mM) was placed in one compartment, while buffer was placed in the other compartment. After recording the baseline, nilutamide (550 μM) was added to the microsomal suspension on the sample side and to the buffer solution on the reference side. Successive spectra were recorded, for 20 min, from 420 to 490 nm.

The possible *in vivo* formation of a cytochrome P-450Fe(II)–metabolite complex after administration of nilutamide in mice was investigated as reported previously [2]. Uncomplexed cytochrome P-450 was measured as the CO-difference spectrum of dithionite-reduced microsomes. Total cytochrome P-450 was similarly measured, but after first adding 50 μM potassium ferricyanide to the microsomes, to disrupt a possible cytochrome P-450Fe(II)–nitroso metabolite complex. Complexed cytochrome P-450 was also directly looked for, by recording the absorbance around 455 nm after adding 50 μM of potassium ferricyanide to the reference cuvette only.

Reduction of cytochrome P-450 by NADPH–cytochrome P-450 reductase. Hepatic microsomes from 1 g of mouse liver were resuspended in 20 ml of 1.5 mM EDTA, 0.15 M KCl, 0.05 M sodium–potassium phosphate buffer, pH 7.4. Microsomes were studied in the presence or absence of 550 μM nilutamide. Cuvettes, maintained at 4°, contained

hepatic microsomes, glucose (13.3 mM), glucose oxidase (1200 units/ml) and catalase (11,000 units/ml) to remove traces of oxygen that had not been removed by bubbling carbon monoxide for 3 min. The initial, near linear reduction rate of cytochrome P-450 (during the first 7–20 sec), and the amount of cytochrome P-450 eventually reduced (after 5–9 min) were measured by recording at 450 nm the kinetics for the appearance of the cytochrome P-450Fe(II)–CO complex, after addition of NADPH (0.3 mM) to the sample cuvette.

Utilization of NADPH. Hepatic microsomes from 1 g of mouse liver were resuspended in 16 ml of 1.5 mM EDTA, 0.15 M KCl, 0.05 M sodium–potassium phosphate buffer, pH 7.4. The microsomal suspension was divided between 2 cuvettes, warmed at 37°. NADPH (0.05 mM) was added in the sample cuvette, and its absorption at 340 nm was recorded with time. The same experiment was repeated after addition of nilutamide (550 μM) in both cuvettes.

RESULTS

Human liver microsomes

Monooxygenase activities. Activities were studied in the presence of three concentrations of nilutamide (Fig. 2): (i) a first concentration (22 μM) corresponding to the plasma concentration observed in humans after therapeutic doses of nilutamide [1]; (ii) a second concentration (110 μM) representing the concentration expected in human liver, from the liver/plasma concentration ratio observed in several animal species [1], and (iii) a 5-times higher concentration (550 μM). Half-inhibition of hexobarbital hydroxylase, benzphetamine *N*-demethylase, benzo(*a*)pyrene hydroxylase and 7-ethoxycoumarin *O*-deethylase activities was observed with concentrations of about 20, 20, 180 and 550 μM, respectively, of nilutamide (Fig. 2).

CO-binding cytochrome P-450, complexed cytochrome P-450 and NADPH–cytochrome c reductase

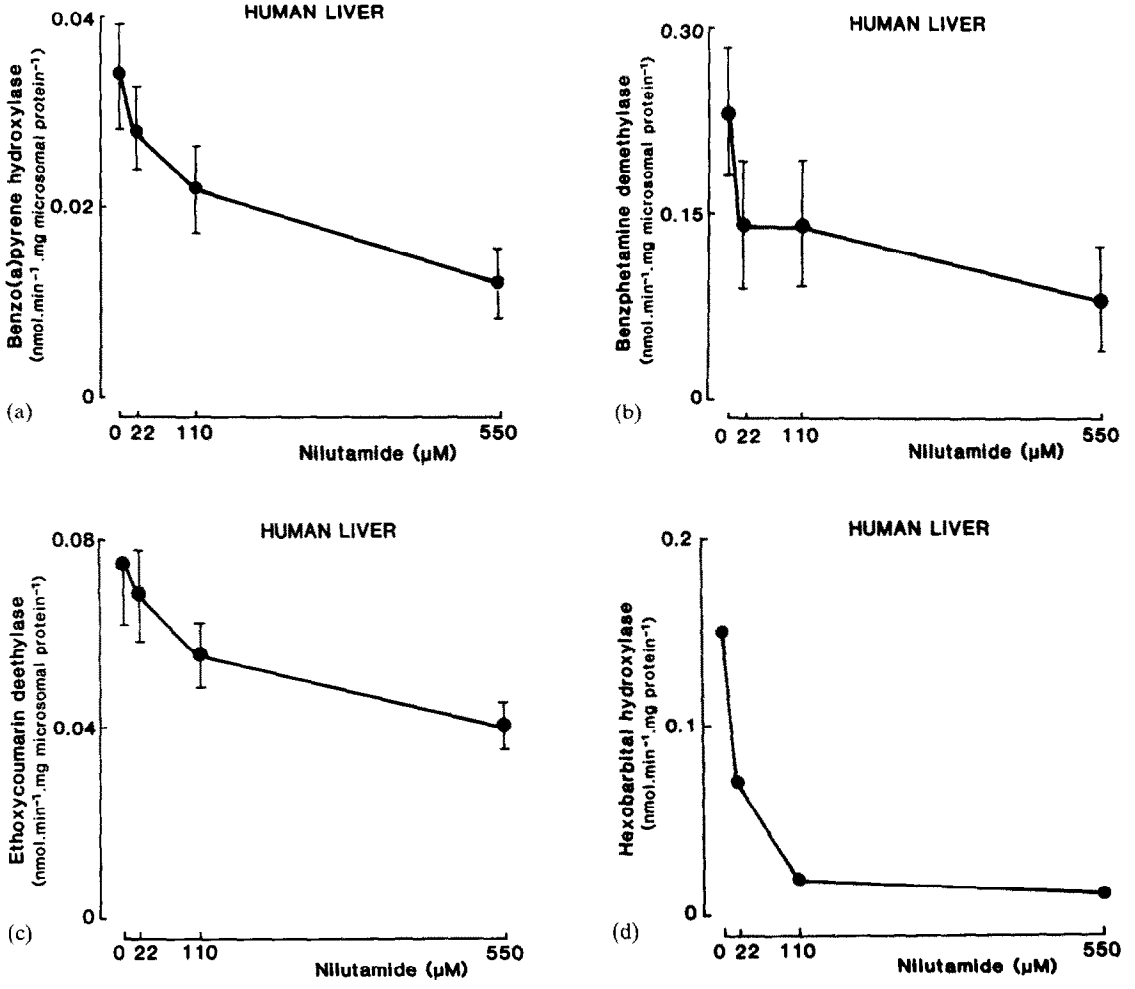

Fig. 2. *In vitro* effects of nilutamide on monooxygenase activities in human liver microsomes. Monooxygenase activities were measured by incubating various substrates (0.25 mM) with human liver microsomes and a NADPH-generating system at 37° for 10 min, in the presence of various concentrations of nilutamide. Results are means ± SEM for 4–5 human livers, except for hexobarbital hydroxylase activities which are the means for 2 human livers only.

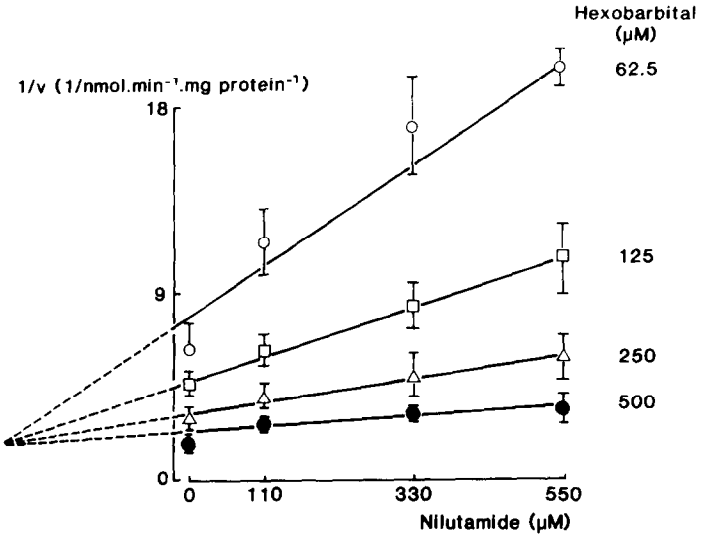

Fig. 3. Dixon plot for the inhibition of hexobarbital hydroxylase by nilutamide in mouse liver microsomes. Results are means ± SEM for 4 experiments.

activity. Cytochrome P-450 content, determined by CO-binding to dithionite-reduced microsomes was not modified when human liver microsomes were incubated at 37° for 20 min with 1.5 mM EDTA, nilutamide (550 μM) and a NADPH-generating system (not shown). We could find no spectral evidence for the *in vitro* formation of a cytochrome P-450Fe(II)–nitroso metabolite complex upon incubation of human liver microsomes with nilutamide (0.5 mM) and NADPH (1 mM). NADPH–cytochrome *c* reductase activity was not modified during incubation with various concentrations of nilutamide, up to 550 μM (not shown).

Mouse liver microsomes

Since we had only limited amounts of human liver microsomes, other mechanistic studies were performed with mouse liver microsomes.

Binding spectra. Nilutamide (22 μM) produced either a type I or a type II binding spectrum with mouse liver microsomes. Surprisingly, either one of these two spectra was observed, even in immediately consecutive experiments performed with the same batch of microsomes, under apparently identical conditions. No binding spectrum was observed with higher concentrations of nilutamide (110 or 550 μM).

Inhibition kinetics. Kinetics for the inhibition of hexobarbital hydroxylase activity by nilutamide were grossly consistent with competitive inhibition, both on a Lineweaver–Burk plot (not shown) and on a Dixon plot (Fig. 3). Kinetics for the inhibition of benzphetamine *N*-demethylase by nilutamide were

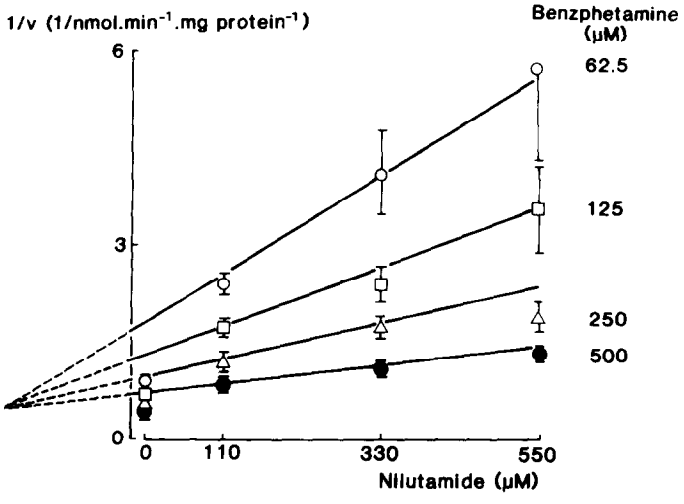

Fig. 4. Dixon plot for the inhibition of benzphetamine *N*-demethylase by nilutamide in mouse liver microsomes. Results are means ± SEM for 3 experiments. Lines have been drawn to fit points obtained at high concentrations of nilutamide.

Table 1. Effects of nilutamide on the utilization of NADPH, and the reduction of cytochrome P-450 by NADPH–cytochrome P-450 reductase in mouse liver microsomes

	Utilization of NADPH in an aerobic system at 37°	Reduction of cytochrome P-450 in an anaerobic system	
		Initial (linear) reduction rate during the first 7–20 sec	Amount eventually reduced after 5–9 min
		(nmol.mg protein^{-1}.min^{-1})	
Without nilutamide	3.5 ± 0.1	0.11 ± 0.02	0.33 ± 0.03
With nilutamide (550 μM)	3.8 ± 0.1	0.10 ± 0.02	0.32 ± 0.03

The utilization of NADPH (initial concentration: 0.05 mM) was recorded at 340 nm in an aerobic suspension of mouse liver microsomes at 37°. Kinetics for the reduction of cytochrome P-450 by NADPH–cytochrome P-450 reductase were determined at 4° as the appearance of the cytochrome P-450Fe(II)–CO complex after addition of NADPH (0.3 mM) to an anaerobic suspension of mouse liver microsomes in the presence of carbon monoxide (see Materials and Methods). Results are means ± SEM for 6 experiments.

Table 2. Effect of a low dose of nilutamide (30 μmol.kg^{-1} i.p.) on the hexobarbital sleeping time, cytochrome P-450 levels, and monooxygenase activities in mice

	Hexobarbital sleeping time (min)	Cytochrome P-450 (nmol.mg protein^{-1})	Hexobarbital hydroxylase (nmol.mg protein^{-1}.min^{-1})	Benzphetamine *N*-demethylase (nmol.mg protein^{-1}.min^{-1})
Control (1 hr)	36 ± 3	0.67 ± 0.04	0.69 ± 0.05	1.6 ± 0.1
Nilutamide (1 hr)	56 ± 4*	0.62 ± 0.04	0.70 ± 0.07	1.6 ± 0.1
Control (6 hr)	28 ± 2	0.86 ± 0.04	0.60 ± 0.04	1.1 ± 0.1
Nilutamide (6 hr)	50 ± 5*	0.75 ± 0.04	0.58 ± 0.04	1.2 ± 0.1

Mice were killed 1 or 6 hr after administration of nilutamide, and hepatic microsomes were prepared. Cytochrome P-450 was measured as the CO-difference spectrum of dithionite-reduced microsomes. Monooxygenase activities were measured in the presence of hexobarbital (0.25 mM) or benzphetamine (0.25 mM) with a NADPH-generating system. Other mice received hexobarbital (100 mg.kg^{-1} i.p.) 30 min or 6 hr after administration of nilutamide. Results are means ± SEM for 5–10 mice.

* Significantly different from control mice, $P < 0.01$.

more complex, being non-linear both on a Lineweaver–Burk plot (not shown) and on a Dixon plot (Fig. 4).

Reduction of cytochrome P-450 by NADPH–cytochrome P-450 reductase. Addition of nilutamide (550 μM) did not modify the initial, near-linear, reduction rate of cytochrome P-450 by NADPH–cytochrome P-450 reductase measured under anaerobic conditions at 4°, nor did it modify the amount of cytochrome P-450 eventually reduced (Table 1). Addition of nilutamide (550 μM) did not significantly increase the utilization of NADPH by aerobic mouse liver microsomes at 37° (Table 1).

Nilutamide administration. The dose of nilutamide given to humans is 15 to 30 μmol.kg^{-1}. Administration of 15 μmol.kg^{-1} i.p. of nilutamide, 30 min before hexobarbital, significantly increased the hexobarbital sleeping time by 40%, from 36 ± 2 min in

Table 3. Hepatic microsomal enzymes after administration of a high dose of nilutamide (0.3 mmol.kg^{-1} i.p.) in mice

	Microsomal protein	NADPH–cytochrome *c* reductase	Uncomplexed	Complexed	Total
	(mg.g liver^{-1})	(nmol.mg protein^{-1}.min^{-1})	Cytochrome P-450 (nmol.mg protein^{-1})		
Control (4 hr)	35 ± 1	38 ± 1	0.79 ± 0.02	0	0.78 ± 0.03
Nilutamide (4 hr)	37 ± 1	34 ± 1	0.74 ± 0.06	0	0.77 ± 0.06

Mice were killed 4 hr after administration of nilutamide, and hepatic microsomes were prepared. Uncomplexed cytochrome P-450 was measured as the CO-difference spectrum of dithionite-reduced microsomes. Total cytochrome P-450 was similarly measured, but after first adding 50 μM potassium ferricyanide to the microsomes to disrupt a possible cytochrome P-450Fe(II)–metabolite complex. Complexed cytochrome P-450 was looked for by recording the absorption around 455 nm after addition of 50 μM potassium ferricyanide to the reference cuvette only. Results are means ± SEM for 5 mice.

control mice, to 50 ± 4 min in nilutamide-treated mice (mean ± SEM for 10 mice). Thirty minutes and 6 hr after administration of 30 μmol.kg^{-1} i.p. of nilutamide, the hexobarbital sleeping time was increased by 60–80% (Table 2), but was unchanged at 24 hr, being 40 ± 4 min in control mice, and 41 ± 3 min in nilutamide treated mice (mean ± SEM for 10 mice). Cytochrome P-450 content, hexobarbital hydroxylase activity and benzphetamine *N*-demethylase activity were unchanged in microsomes from mice killed 1 or 6 hr after administration of nilutamide, 30 μmol.kg^{-1} i.p. (Table 2). Microsomal protein, NADPH–cytochrome *c* reductase activity and cytochrome P-450 content were not changed 4 hr after administration of a much higher dose of nilutamide, 0.3 mmol.kg^{-1} i.p. (Table 3) and there was no evidence for the *in vivo* formation of a cytochrome P-450Fe(II)–nitroso complex (Table 3).

DISCUSSION

Our results show that nilutamide inhibits monooxygenase activities in mice and humans (Figs 2–4). Although nilutamide is metabolized in part by reduction of the nitro group, it did not significantly increase the utilization of NADPH by aerobic suspensions of mouse liver microsomes (Table 1), suggesting that decreased availability of reducing equivalents is not the mechanism for inhibition. Nilutamide did not modify the NADPH–cytochrome *c* reductase activity of human liver microsomes, and did not interfere with the reduction of mouse liver cytochrome P-450 by NADPH–cytochrome P-450 reductase (Table 1), showing that inhibition did not involve the reductase, or its interaction with cytochrome P-450, and therefore probably affected cytochrome P-450 itself.

There was no evidence for an irreversible inactivation of cytochrome P-450 either *in vitro* or *in vivo* (Tables 2 and 3), suggesting reversible inhibition. Indeed, whereas the hexobarbital sleeping time was prolonged at early times after administration of nilutamide, in contrast, the hexobarbital hydroxylase activity remained unchanged in liver microsomes from mice killed at such times (Table 2). This suggests that the several dilutions involved in the preparation of hepatic microsomes and their incubation in the hexobarbital hydroxylase assay had markedly decreased the concentrations of nilutamide initially present in the liver, and had thereby reversed the inhibition.

Our results suggest that reversible inhibition was due to the interaction of nilutamide with the active site of cytochrome P-450. Indeed, nilutamide (22 μM) gave either a type II or a type I binding spectrum with mouse liver microsomes (Results), suggesting that nilutamide may either bind as a sixth, weak, ligand to the iron(III) of cytochrome P-450, or bind to the apoprotein at the active site. Hypothetically, bi-dentate ligation (to both the iron and the protein) might also occur; this might result in a rather strong interaction, which might explain that nilutamide is a rather potent inhibitor of cytochrome P-450 in mice and in humans. Consistent with an interaction at the active site of cytochrome P-450, the kinetics for the inhibition of hexobarbital hydroxylase activity were those of a competitive inhibition (Fig. 3). Similarly, nilutamide has been shown to inhibit competitively rat testicular 17α-hydroxylase and 17, 20-lyase activities [13]. The kinetics for mouse liver benzphetamine *N*-demethylase, however, were more complex (Fig. 4), probably reflecting the involvement of different cytochrome P-450 isoenzymes.

Our results also suggest that therapeutic doses of nilutamide will probably inhibit hepatic drug metabolism in humans. In mice (Table 2, Results), prolongation of the hexobarbital sleeping time occurred at doses of nilutamide (15 or 30 μmol.kg^{-1} i.p.) identical to the doses used in humans. In studies performed with human liver microsomes (Fig. 2), the concentration of 110 μM of nilutamide had been selected as one mimicking the concentration expected in human liver. This was calculated from the known plasma concentration of nilutamide in humans (22 μM), and the fact that hepatic concentrations are about 5-fold higher than plasma concentrations in several animal species [1]. This concentration of nilutamide (110 μM) inhibited hexobarbital hydroxylase, benzphetamine *N*-demethylase, benzo(*a*)pyrene hydroxylase and 7-ethoxycoumarin *O*-deethylase activities by 85, 40, 35 and 25%, respectively (Fig. 2).

The human cytochrome P-450 isoenzymes which metabolize these various substrates are still incompletely delineated. It appears that 7-ethoxycoumarin deethylase may be mediated by a number of different human isoenzymes [14]. In contrast, benzphetamine *N*-demethylase may be mainly mediated by human cytochrome P-450$_5$ [14], a form closely related or identical [14] to cytochromes P-450$_p$ [15] and P-450$_{NF}$ [16], which belong to the glucocorticoid-inducible P-450 III family [17]. Benzo(*a*)pyrene hydroxylase may be mediated by both the arylhydrocarbon-inducible cytochrome P-450 I family [9, 17] and also by human cytochrome P-450$_8$ [14], which is closely related [14, 18] to cytochrome P-450 mephenytoin [18]. To our knowledge, the isoenzyme(s) mediating hexobarbital hydroxylase activity have not been identified. The chemical structure of hexobarbital, however, is very close to that of mephobarbital, which is metabolized by cytochrome P-450 mephenytoin [19, 20]. It is tempting to speculate that hexobarbital, like mephobarbital, may be metabolized by this same isoenzyme. Taken together, these observations may indicate that nilutamide inhibits several human cytochrome P-450 isoenzymes, possibly including glucocorticoid-inducible isoenzymes and cytochrome P-450 mephenytoin. Further studies are required, however, to assess these hypotheses.

In summary, we conclude that nilutamide inhibits mouse and human liver cytochrome P-450, probably through reversible interaction with the active site of cytochrome P-450. We suggest that therapeutic doses of nilutamide may actually inhibit monooxygenase activities in humans.

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