ABSTRACT:

The antiandrogenic drug, flutamide, is widely used in the treatment of carcinoma of the prostate. The present study examines the metabolism of flutamide by human liver microsomes and purified recombinant human cytochrome P450s (CYP), expressed as fusion proteins. These studies show the principal role of CYP1A2 in the metabolism of flutamide to 2-hydroxyflutamide. A minor metabolite, 4'-hydroxyflutamide, was underwritten by an unrestricted educational grant from Robert A. Welch Foundation (to R.W.E.) and DK 47657 (to M.J.M.) and by Grant I-1090 from the National Institutes of Health (NIH).

Materials and Methods

Chemicals. Unlabeled flutamide and hydroxyflutamide were obtained from Schering-Plough (Somerville, NJ). [3H]Flutamide (specific activity 13 Ci/mmol) and 2-[3H]hydroxyflutamide (specific activity 9 Ci/mmol) were tritiated by Moravek Biochemicals (Brea, CA). Acetanilide, phenacetin, estriol, paracetamol, imipramine, NADPH, DOPC, CHAPS, sodium isocitrate, isoctane, and 2-[3H]estradiol (specific activity 11 Ci/mmol) were purchased from Amersham. All reagents were of the highest purity available.

The non-steroidal antiandrogen, flutamide (3’-trifluoro-2-methyl-4’-nitro-2-methyl-propionylanilide) is a drug widely used in the treatment of prostate cancer (1, 2). It has been reported (3) that flutamide is rapidly converted in the liver to 2-hydroxyflutamide by first pass metabolism with subsequent hydrolysis to 3’-trifluoromethyl-4’-nitroaniline or glucuronidation of the hydroxylated aromatic ring and excretion in urine. It has been suggested that the biological activity of flutamide is associated with its metabolite, 2-hydroxyflutamide (4). Of concern is the report of hepatotoxicity associated with the clinical use of this drug (5), although it is described as “sporadic” induced liver injury with a very low incidence.

Berson et al. (6) and Fau et al. (7) have studied the metabolism of flutamide by isolated rat hepatocytes, human liver microsomes, and yeast microsomes expressing human liver cytochrome P450s. They concluded that “metabolic activation of flutamide in the human liver may be mediated mainly by cytochromes P450 3A4 and 1A2.” This conclusion was based, in part, on the ability to show covalent binding of radioactive flutamide (or its metabolites) to microsomal membrane fragments. Their studies also showed that “flutamide was not detectably reduced to its corresponding nitro anion free radical by the microsomal NADPH-cytochrome P450 reductase” excluding this pathway as a possible mechanism for formation of toxic reactive oxygen species (6).

The present study was undertaken to compare the metabolism of flutamide by microsomes prepared from human liver with metabolism using a variety of purified, recombinant human CYPs,1 expressed as enzymatically active, self-sufficient CYP fusion proteins. An HPLC method was developed to monitor the rates of metabolism of flutamide and the characteristics of the metabolites formed. Use of α-naphthoflavone, a potent inhibitor of CYP1A2 reactions (8), indicates that in vitro the major pathway of metabolism of flutamide is by CYP1A2. This is confirmed by studies using purified recombinant human CYP1A2. Of interest is the observation that no metabolism is seen with purified, recombinant CYP1A1. Purified, recombinant CYP3A4 catalyzes the formation of a minor polar metabolite, but only in the presence of an excess of added purified NADPH-P450 reductase. A role for drug-drug interactions is suggested from in vitro studies showing the inhibition of flutamide metabolism by phenacetin and imipramine. Conversely, flutamide inhibits the CYP1A2-catalyzed 2- and 4-hydroxylation of estradiol.

CV1 cells do not metabolize flutamide to 2-hydroxyflutamide. In assays performed using this cell line transfected with the cDNA for the androgen receptor, flutamide is a pure antagonist, and 2-hydroxyflutamide, while a more potent androgen receptor (AR) agonist, activates the AR at higher concentrations. Stable expression of CYP1A2 in these CV1 cells causes flutamide to exhibit agonistic properties at higher concentrations, a behavior not exhibited by cells stably transfected only with the expression vector encoding the AR. These findings raise the possibility that increased conversion of flutamide to 2-hydroxyflutamide or accumulation of 2-hydroxyflutamide in cells may contribute to the anomalous responses to flutamide that are observed in some advanced prostate cancers.

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The microsomes were diluted with 1.5 ml of TM buffer, to give the final CYP concentration of 0.5 nmol of P450/ml. The microsomes were incubated at 37°C and radiolabeled [3H]flutamide was added to a final concentration of 5 μM with 120,000 cpm/0.5 ml. The reaction was initiated by adding NADPH (1 mM, final concentration) and a regenerating system. 0.5-ml aliquots were taken at the times indicated and extracted with methylene chloride. Samples were analyzed by reversed-phase HPLC as described under Materials and Methods. The inset shows an HPLC chromatogram of flutamide and its metabolites.

**Fig. 1.** Time course of [3H]-flutamide metabolism using pooled human liver microsomes.

Flutamide metabolism by purified recombinant human CYPs, cytochrome bs, and inhibitors, incubations were carried out as described by Shet et al. (13). Briefly, an aliquot of the purified fusion protein containing a specific CYP was incubated at 37°C as a concentrated protein solution (ca. 75 μM) with an aliquot of concentrated cytochrome bs (ca. 200 μM), DOPC, and CHAPS. After 10 min, the concentrated mix was diluted with TM buffer followed by the addition of radioactive [3H]flutamide and the NADPH regenerating system. The final concentrations of CYP, cytochrome bs, DOPC, and CHAPS in the diluted reaction mixture were 0.5 μM, 0.5 μM, 312 μg/ml, and 250 μg/ml, respectively.

Estradiol 2- and 4-hydroxylase activities were assayed using the purified fusion protein containing human CYP1A2 (0.5 μM, final concentration) in the presence of added NADPH-P450 reductase diluted in TM buffer at 37°C. The ratio of CYP:reductase was 1:5 (nmol:nmol). [3H]Estradiol was added to give a final concentration of 100 μM followed by the addition of catalase (1000 units/ml) and 100 μM ascorbic acid. The reaction was started by the addition of the NADPH regenerating system. Samples were extracted with methylene chloride and analyzed using reversed-phase HPLC as described previously by Fisher et al. (14).

**Analysis of Flutamide Metabolites by Reverse-phase HPLC.** The methylene chloride layer of the quenched reaction mixture was removed and evaporated to dryness with a stream of nitrogen at 30°C and dissolved in 100 μl of methanol. The conversion of flutamide to its metabolites was analyzed using a computerized Waters reversed-phase HPLC system connected to an optical absorbance detector and a Radiometer Flo-1 radioactive detector. Separation of flutamide and its metabolites was accomplished using a C-18 uBondapak (3.9 × 300 mm) column and a gradient solvent system containing (in pump A) 60% (v/v) methanol and 40% (v/v) water and (in pump B) 100% (v/v) methanol. Initial conditions were established by equilibration with 100% contents of pump A. A linear mixing gradient, at a flow rate of 1 ml/min. was used to achieve at 40 min a mixture of 60% the contents of pump A and 40% the contents of pump B. The column was then flushed for 10 min with 100% methanol (pump B) followed by 100% contents of pump A to reestablish initial conditions. Using this HPLC method (fig. 1, inset)
The following retention times were obtained for radioactive flutamide and its metabolites: metabolite III, 7.2 min; metabolite II, 9.5 min; metabolite I (2-hydroxyflutamide), 12.5 min; and flutamide, 18.3 min. Metabolite I showed the same retention time as authentic 2-hydroxyflutamide.

**Other Methods.** CYP content was determined by the method of Omura and Sato (15) using a DW2a spectrophotometer. Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard (16).

**Cell Transfection.** One plate of CV1 cells was transfected with 1 µg of pSV2neo (17) and 10 µg of the empty expression vector pCMV5 (18) using the calcium phosphate precipitation method (19). A second plate of CV1 cells was transfected in parallel with 1 µg of pSV2neo and 10 137 m g of the same expression vector encoding the human CYP1A2. At confluence, the cells were split into new dishes and subjected to selection with the antibiotic G418 (Geneticin, GIBCO/BRL) at a final concentration of 400 µg/ml active ingredient. G418-resistant colonies appeared in both populations at a similar rate (approximately 100 colonies/10⁶ transfected cells). The plates containing the G418 resistant colonies were trypsinized, and the analyses were performed on the two resistant cell populations that were propagated as pools (designated pSV2neo/CMV 5 and pSV2neo/CYP1A2).

Transient transfections to assess the activation of the androgen receptor by the various ligands were performed as described previously (20). In brief, monolayers of the different cell populations (pSV2neo/CMV 5 and pSV2neo/ CYP1A2) were plated in 6-well plates (2 × 10⁵ cells per well). Twenty hours later, the cells were transfected with a calcium phosphate precipitate containing an expression plasmid encoding the human AR and the androgen-responsive reporter gene MMTV-luciferase. Twenty four hours after the addition of the precipitate, the medium was removed and replaced with fresh medium containing 5% charcoal-stripped serum and various concentrations of the ligands under study. For each 6-well plate, three wells were incubated with stripped serum alone and three were incubated with stripped serum containing the ligand to be assayed (yielding triplicate basal and stimulated values). Forty eight hours after the addition of the ligand, the cells were harvested, and the levels of luciferase activity were measured.

**Results**

**Metabolism by Human Liver Microsomes.** Incubation of radio-labelled flutamide with human liver microsomes revealed the NADPH-dependent metabolism of this drug to a single major product and two minor products. The initial rate of metabolism, using 5 µM flutamide, was about 0.5 ± 0.05 nmol/min/mg microsomal protein. The time course for metabolism is shown in fig. 1.

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illustrates the HPLC profile of metabolites generated after 10 min of incubation. HPLC analysis of authentic 2-hydroxyflutamide showed that it had a retention time the same as the major metabolite formed enzymatically (metabolite I of fig. 1). The structure of the two minor metabolites remains unknown. Similar studies using mouse liver microsomes revealed the same pattern of metabolites formed at approximately the same rate of metabolism (0.38 ± 0.04 nmol/min/mg microsomal protein).

The Effect of Inhibitors. It has been reported that flutamide is metabolized by CYPs of the 1A and 3A family (6, 7). To determine the relative contribution of these two pathways of metabolism using human liver microsomes, we measured the rate of conversion of flutamide to each metabolite. As shown in fig. 2, a low concentration of α-naphthoflavone nearly completely inhibits the metabolism of flutamide by human liver microsomes. An equivalent concentration of ketoconazole was less effective and only partially inhibited the reaction. Also shown in fig. 2, the addition of purified NADPH-P450 reductase to the microsomal suspension increased the rate of flutamide metabolism approximately 1.5-fold and also increased the rate of formation of metabolite III. Of interest is the observation that ketoconazole completely abolishes the formation of metabolite III. These studies indicate a major role for a CYP of the 1A family and a minor role of CYP3A4 in the metabolism of flutamide.

Metabolism of Flutamide by Purified Recombinant Human CYPs 1A1, 1A2, and 3A4. This laboratory has expressed a number of CYPs in E. coli, permitting the purification and characterization of their enzymatic properties. Greatest emphasis has been directed toward the study of recombinant fusion proteins containing the heme domain of a CYP linked to the flavin domain of rat NADPH-P450 reductase. All studies to date have shown that these fusion proteins have the same enzymatic properties (but with higher k_m activities) as purified CYPs reconstituted with purified NADPH-P450 reductase. In the present study, we have used purified fusion proteins containing human CYP1A1 (rF450[mHum1A1/mRatOR][L1]), human CYP1A2 (rF450[mHum1A2/mRatOR][L1]), or human CYP3A4 (rF450[mHum3A4/mRatOR][L1]). As shown in fig. 3A, flutamide is not metabolized by CYP1A1 and poorly metabolized by CYP3A4, even when the enzyme is preincubated with phospholipid (DOPC), detergent (CHAPS), and cytochrome b_5. In contrast, a significant rate of flutamide metabolism is obtained using CYP1A2. Of interest is the observation that the addition of a 5-fold excess of purified NADPH-P450 reductase to the reaction system significantly increases the rate of flutamide metabolism by both CYP1A2 and CYP3A4 fusion enzymes. This effect of added purified NADPH-P450 reductase on the enzymatic activities of recombinant fusion proteins has been reported elsewhere (13).

HPLC analysis of the products formed during enzymatic metabolism of flutamide by the purified, recombinant fusion proteins containing CYP1A2 or CYP3A4 showed (fig. 3B) that CYP1A2 forms only 2-hydroxyflutamide while CYP3A4 (in the presence of added NADPH-P450 reductase) generates a product with an HPLC retention time similar to that of metabolite III (cf. fig. 1 inset). An attempt to identify metabolite II as a product formed during the further metabolism of 2-hydroxyflutamide, using either CYP1A2 or CYP3A4, was not successful.

Inhibition of Flutamide Metabolism Using Purified CYPs. A series of experiments was carried out to determine the patterns of inhibition of flutamide metabolism by α-naphthoflavone or ketoconazole using purified CYPs 1A2 or 3A4. The conditions of incubation were similar to those described in fig. 3A, where the fusion protein containing CYP1A2 (in the absence of added purified NADPH-P450 reductase) or the fusion protein containing CYP3A4 (in the presence of purified NADPH-P450 reductase) was used. As shown in fig. 4, A and B, the metabolism of flutamide by CYP1A2 is very sensitive to inhibition by α-naphthoflavone, while the metabolism by CYP3A4 is unaffected by this agent. Conversely, ketoconazole is a more effective inhibitor of flutamide metabolism by CYP3A4 than CYP1A2, although both reactions are inhibited by this chemical.

When varying concentrations of flutamide were incubated with CYP1A2, it was observed (fig. 5A) that the half-maximal rate of metabolism occurred with about 6 ± 0.5 μM flutamide. At concentrations of flutamide greater than 40 μM, it was observed that the initial rate of flutamide metabolism was progressively inhibited as the concentration of flutamide was increased. Also of interest is the observation that 2-hydroxyflutamide is an effective inhibitor of flutamide metabolism (fig. 5B). At equimolar concentrations of 2-hydroxyflutamide and flutamide, one observes a 50% inhibition of the initial rate of metabolism of flutamide in vitro.

Inhibition of Flutamide Metabolism by Other Drugs. We examined the inhibitory effect of other substrates of CYP1A2 on the rate of metabolism of flutamide. As shown in fig. 6A, phenacetin, imipramine, and caffeine inhibit the metabolism of flutamide but with different inhibitory constants. Of interest is the result that 0.5 mM acetanilide and paracetamol are poor inhibitors of flutamide metabolism. The most effective inhibitory substrate tested was estradiol (K_i = ca. 50 μM). Conversely, flutamide is an effective inhibitor of the 2- and 4-hydroxylation of estradiol by CYP1A2 (fig. 6B). Clearly, a large number of drugs and other chemicals influence the metabolism of flutamide.

The Expression of CYP1A2 Alters the Properties of Flutamide Metabolism...
A series of samples containing 0.5 nmol/ml of CYP1A2 fusion protein in TM buffer was incubated with different concentrations (0–100 μM) of [3H]flutamide. For set B, purified CYP1A2 fusion protein (0.5 nmol/ml, final concentration) in TM buffer was incubated with increasing concentrations of 2-hydroxyflutamide for 10 min at 37°C followed by the addition of [3H]flutamide (25 μM, final concentration). NADPH (1 mM, final concentration) containing a regenerating system was added to start the reaction as described in fig. 1. The samples were analyzed as described. The uninhibited rate of flutamide metabolism was 1.4 ± 0.1 nmol/min/nmol.

In A, purified CYP1A2 fusion protein (0.5 nmol/ml, final concentration) was diluted in TM buffer and incubated for 10 min at 37°C with different concentrations of estradiol, phenacetin, imipramine, caffeine, and paracetamol. [3H]Flutamide was added to a final concentration of 25 μM, and the reaction was started by the addition of NADPH and the regenerating system. The samples were analyzed using reversed-phase HPLC as described previously. The uninhibited (100%) rate of flutamide metabolism using the purified fusion protein containing CYP1A2 was 1.4 ± 0.1 nmol/min/nmol P450. In B, the purified CYP fusion protein was incubated with NADPH-P450 reductase for 5 min at 37°C and diluted with TM buffer to give final concentrations of 0.5 μM (CYP) and 2.5 μM (NADPH-P450 reductase). The concentrations of flutamide are indicated in the figure. Flutamide was added to the reaction mixture and incubated for 10 min at 37°C before the addition of [3H]flutamide (25 μM, final concentration). NADPH containing the regenerating system was added to start the reaction. Samples were analyzed as described under Materials and Methods.

### Discussion

Metastatic prostatic cancer is a major health problem, and as no agents are available for the successful long-term treatment of this disease, androgen ablation therapy remains an important component of advanced prostatic cancers. Flutamide is a drug that has been used for many years with success. It is believed that this non-steroidal anti-androgen exercises its influence as a competitive antagonist of the androgen receptor via 2-hydroxyflutamide, the major metabolite of flutamide. As shown in table I, in cells transfected with the empty pCMV CMV CYPIA2 were transiently transfected with a cDNA encoding the human AR and the androgen-responsive reported plasmid MMTV luciferase. Twenty four hours following transfection, the cultures were treated with the ligands shown for 48 hr. At the end of the incubation, the cultures were harvested and assayed to measure the levels of luciferase activity. The concentrations of ligands used are indicated (nM for mibolerone [Mb], μM for the remaining ligands). Each of the experimental values is the average of single luciferase measurements of samples derived from three separate transfections (see Materials and Methods). The data presented are from a single representative experiment. Fold induction is the level of stimulated luciferase activity divided by the activity measured in cells incubated with no ligand (854 and 449 light units for cells transfected with pCMV 5 and pCMV CYPIA2, respectively). — indicates that the levels of luciferase were lower than those measured in the baseline (unstimulated) samples.

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### TABLE 1

**Activities of a model androgen-responsive reporter gene in CV1 cells stably transfected with a cDNA encoding CYP1A2**

Cell populations previously stably transfected with pSV2neo and pCMV 5 or pCMV CYPIA2 were transiently transfected with a cDNA encoding the human AR and the androgen-responsive reported plasmid MMTV luciferase.


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experiments. Our studies suggest that flutamide has a limit of solubility of about 50 µM. Also, Berson et al. (6) report the level of radioactivity associated with precipitated and extracted rat liver microsomes to be occurring at a rate of between 5 and 10 pmol/min/mg protein. Our results indicate a rate of flutamide metabolism to be at least 100 times faster than this rate of covalent binding.

A further complication in comparing results reported in this paper with the earlier report by Berson et al. (6), using yeast microsomes expressing specific human cytochrome CYPs, is the observation that they obtained 10–20 times greater binding of radioactive flutamide in the tumor cells themselves.

The present study shows the dominant role of CYP1A2 in the metabolism of flutamide to 2-hydroxyflutamide in vitro and suggests the possible influence of external agents (other drugs) on the effectiveness of flutamide as a therapeutic agent. CYP1A2 is a member of the family of P450s known to be readily induced by chemicals that interact with the Ah locus (21, 22). Planar, polycyclic aromatic hydrocarbons, such as benzo(a)pyrene or 3-methylcholanthrene, or pyrolysis products of amino acids formed during the barbecuing of meat and fish are recognized as inducers of P450s of the 1A family. Thus, the level of CYP1A2 in an individual may vary greatly depending on diet and personal habits, such as smoking. Further, the ability of other drugs, such as phenacetin, imipramine, or caffeine, to compete with flutamide for metabolism by CYP1A2 suggests a role for drug-drug interactions modifying the rate of formation of 2-hydroxyflutamide.

Also of interest is the observation that flutamide can influence the metabolism of estradiol for the 2- and 4-hydroxylation of this essential hormone. This result confirms the study by Zumoff et al. (23) who reported on the in vivo influence of flutamide on 2-hydroxyestradiol formation in patients with prostate cancer.

Although 2-hydroxyflutamide is a more potent antagonist of AR activation than is flutamide itself, in cotransfection assays 2-hydroxylation rate of the human AR is significantly higher than that of flutamide itself. While much attention has been focused on the role of mutations in the AR, in the progression to a state in which prostate tumors fail to respond to flutamide or exhibit the flutamide-withdrawal syndrome, the current studies suggest an alternative mechanism that might contribute to this behavior: the conversion to (or accumulation of) 2-hydroxyflutamide in the tumor cells themselves. Additional studies will be required to establish whether the expression of CYP1A2 can be detected in tumors that exhibit such a phenotype.

References