

# Cytotoxicity of flutamide and 2-hydroxyflutamide and their effects on CYP1A2 mRNA in primary rat hepatocytes<sup>1</sup>

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**KEY WORDS** flutamide; 2-hydroxyflutamide; cytotoxicity; cytochrome P-450 CYP1A2

## ABSTRACT

**AIM:** To compare the cytotoxicity of flutamide and its active metabolite 2-hydroxyflutamide and their effects on cytochrome P-450 1A2 mRNA in primary rat hepatocytes. **METHODS:** After the isolation of hepatocytes and the primary incubation for 4 h, flutamide and 2-hydroxyflutamide were added respectively to the medium at the concentration of 10, 20, and 50 mg/L and incubated for 8 h. Cytotoxicity of hepatocytes was assessed by Trypan blue exclusion, lactate dehydrogenase (LDH) leakage, percentage of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) release, and reduced glutathione (GSH). The effect of flutamide and 2-flutamide on the CYP1A2 mRNA level was further analyzed by Northern blot. **RESULTS:** After incubation for 8 h, cell viability was observed by Trypan blue exclusion. The increase of ALT and AST activity and the decrease of glutathione content were also noted at 10, 20, and 50 mg/L of flutamide and 50 mg/L of 2-hydroxyflutamide as compared with normal rat hepatocytes. Induction of CYP1A2 mRNA were 2-, 5-, and 7.5-fold at 10, 20, and 50 mg/L of flutamide and 3.5-fold at 50 mg/L of 2-hydroxyflutamide. **CONCLUSION:** Cytotoxicity of flutamide and its effect on CYP1A2 mRNA were stronger than those of its active metabolite 2-hydroxyflutamide in primary rat hepatocytes.

## INTRODUCTION

Flutamide (3'-trifluoromethyl-4'-nitro-2-methyl-

propionylanilide) is a nonsteroidal antiandrogen devoid of other hormonal activity and is recognized world-wide as the most beneficial drug for the treatment of patients with advanced prostate cancer when used in combination with various luteinizing hormone-releasing factor (LHRH) agonists<sup>[1,2]</sup>. This antiandrogen is also used in combination with oral contraceptives for the treatment of hirsutism<sup>[6]</sup> and benign prostatic hyperplasia<sup>3,4</sup>.

The therapeutic activity of flutamide is compromised because of its potential liver toxicity. In 1091 patients consecutive treated with flutamide, the incidence of liver toxicity (defined as  $\geq 4$ -fold increase in serum transaminase activity) was 0.36%<sup>[5]</sup>. Flutamide-induced hepatitis is cholestatic and/or hepatocellular in type, and fulminant hepatitis has been observed. Some patients have documented blood eosinophilia, suggesting that an immune mechanism is possible<sup>[6]</sup>. In isolated rat hepatocytes, flutamide toxicity can be decreased by piperonyl butoxide (an inhibitor of cytochrome P450) and increased by  $\beta$ -naphthooflavone (an inducer of cytochrome P450 1A), indicating that cytochrome P-450 plays an important role in flutamide induced liver toxicity.

2-Hydroxyflutamide is the major active metabolite of flutamide *in vivo* and has a lower IC<sub>50</sub> than flutamide in specific binding experiments in mice, rats, and human androgen-sensitive tissues. Our studies compared the cytotoxicity of flutamide and 2-hydroxyflutamide and their effects on CYP1A2 mRNA in primary rat hepatocytes.

## MATERIALS AND METHODS

**Animals** Adult male Sprague-Dawley rats weighing between 200 - 250 g (Experiment Animal Center of Fudan University, Grade II, Certification No 01212) were housed at a temperature of 20 - 25 °C under a 12-h light/dark cycle with 50 % of relative humidity and kept in filtered and pathogen-free air.

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**Chemicals** Flutamide was provided by Hongqi Pharmaceutical Corporation (Shanghai, China). 2-Hydroxyflutamide was synthesized by Prof XIA Peng (Department of Pharmaceutic Chemistry, School of Pharmacy, Fudan University). These two chemicals were dissolved in ethanol. Materials for cultivating hepatocytes such as *D*-Hanks', Dulbecco's Modified Eagle's Medium (DMEM), *O*-phthalaldehyde (OPT), Triton X-100, and calf serum were obtained from Shanghai Shisheng Biology Corporation. Trypsin and collagenase were purchased from Sigma Chemical Corporation (St Louis, MO, USA). The radiolabeling kit and nylon or nitrocellulose membranes were obtained from Amersham (Aylesbury, UK) and the *Taq* DNA polymerase was purchased from Pharmacia (Uppsala, Sweden). [ $\alpha$ - $^{32}$ P]dCTP was obtained from ICN Bio-medicals, Inc (Costa Mesa, CA). All other chemicals were of high purity and purchased from commercial sources.

**Isolation and culture of hepatocytes** Male Sprague-Dawley rats were anesthetized by injection of sodium pentobarbital (50 mg/kg, ip). Hepatocytes were isolated using a two-step perfusion technique as described previously<sup>[7]</sup>. The portal vein was perfused with *D*-Hanks buffer at a flow rate of 2.5 mL/min for 8 min to remove blood. As soon as the liver became grayish brown in color, a second buffer solution containing collagenase (type IV, 0.03 %, pH 7.6, 37 °C) was perfused at the same rate for 10 min. Following mechanical dissociation, the cells were filtered through four layers of cotton gauze. The filtrate was centrifuged at 1000 × *g* for 2 min and the sediment was washed twice by *D*-Hanks buffer and resuspended in DMEM containing 20 % fetal calf serum and supplemented with streptomycin (0.1 g/L), benzylpenicillin (100 kU/L), dexamthasone (0.1 μmol/L), and insulin (0.1 μmol/L). After cultured for 4 h at 37 °C in 5 % CO<sub>2</sub> atmosphere, non-adherent hepatocytes were eliminated by removing the medium. Cell viability was assessed using 0.4 % Trypan blue exclusion and was always more than 90 % prior to each experiment. Isolations that yielded cells with less than 90 % viability were not included in this study.

**Cytotoxicity studies** Hepatocytes (5 × 10<sup>6</sup> cells/cm<sup>2</sup>) were seeded onto 24-well tissue culture plates. Final concentrations of flutamide or 2-hydroxyflutamide in medium were 10, 20, and 50 mg/L. Untreated hepatocytes were used as control. Cells alone or in

combination with flutamide or 2-hydroxyflutamide at each concentration were cultured for 8 h at 37 °C in 5 % CO<sub>2</sub> atmosphere.

At the end of the incubation period, the cells were loosened with 2 % trypsin, and washed three times with medium, each time decanting the medium after cold and low-speed centrifugation (4 °C, 500 × *g* for 1 - 2 min) before examining for viability and enzyme levels. Cell viability was assessed by Trypan blue exclusion and percentage of viable cells was calculated.

Culture supernatant 100 μL was removed from culture dishes following 8 h of exposure to the drugs and analyzed for LDH leakage into culture medium. LDH leakage was expressed in percentage of total LDH in cells at the beginning of the incubation.

Cytotoxicity was also expressed as the percentage of ALT or AST released by cells into the culture medium, and calculated by the following formula:

$$\text{ALT or AST release (\%)} = (A/B) \times 100 \%$$

Where A represents ALT or AST released into culture medium after cell incubation, B represents the total ALT or AST activity obtained by lysing the cells in culture medium containing 0.1 % Triton X-100 (v/v).

**Reduced glutathione (GSH)** After incubation with different concentrations of flutamide and 2-hydroxyflutamide, 5 × 10<sup>6</sup> hepatocytes were lysed with 0.1 % Triton X-100 in medium and centrifuged to isolate supernatant. The amount of GSH was determined by reaction with OPT 30 mg/L (pH 8.0) and measured in 1 × 10<sup>6</sup> cells incubated with various concentrations of flutamide and 2-hydroxyflutamide.

**Northern blot analysis** After incubation for 8 h at various concentrations of flutamide and 2-hydroxyflutamide, total hepatocyte RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD) and used for hybridization. Northern blot was performed after the denatured RNA (10 μg) was size-fractionated on a 1 % agarose gel containing formaldehyde. Hybridization proceeded at 42 °C overnight in a mixture containing 50 % formamide, 5 × Denhardt's solution, 5 × SSPE, salmon sperm DNA 0.1 μg/L, and <sup>32</sup>P-labeled cDNA probes. cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the intrinsic standard during Northern blot analysis. cDNA probe of rat CY1A2 was synthesized by Shanghai Sangon Co. The primer sequences were: sense primer, 5'-GGACCCTGG-GGCTTGCCCTTC-3'; antisense primer, 5'-AGCCTC-TTGTCTCACGTGCTC-3'. Blots were washed twice for 15 min with 2 × SSC, 0.1 % SDS at 65 °C, and then

twice for 15 min with  $0.2 \times$  SSC, 0.1 % SDS at 60 °C. Intensity of the hybridized bands was measured with a bioimage analyzer (BAS2000; Fujix, Tokyo, Japan) and normalized to the intensity of the hybridized band for the rat GAPDH probe. Membranes were exposed to Fuji X-ray film at -80 °C using an intensifying screen. The hybridized cDNA probe was stripped from the membranes between each hybridization by soaking the membranes in boiling water. Experiments were repeated at least twice and the same results were observed in all cases<sup>(8)</sup>.

**Scanning densitometry** Scanning densitometry was performed with a microcomputer imaging device, model MI (Imaging Research, St Catharines, Ontario, Canada). The area of each lane was integrated using MCID software (version 4.20, revision 1.0), with background subtraction.

**Statistical analysis** One-way analysis of variance (ANOVA) was used to assess the differences between treated groups. For each significant effect of treatment, the Newman-Keuls test was used for comparison of multiple group means. The criterion for statistical significant was  $P < 0.05$ .

## RESULTS

**Cytotoxicity of flutamide and 2-hydroxyflutamide** After incubation for 8 h, the percentage of viable cells assessed by Trypan blue exclusion decreased substantially in 10, 20, and 50 mg/L of flutamide and 50 mg/L of 2-hydroxyflutamide, respectively. The per-

centage of ALT, AST, and LDH leakage release of the hepatocytes exposure to flutamide was higher than that of 2-hydroxyflutamide at a given concentration of each corresponding group. Conversely, the GSH content of rat hepatocytes decreased substantially in the flutamide exposure (Tab 1).

**Effects on CYP1A2 mRNA** The effects of flutamide and 2-hydroxyflutamide on the CYP1A2 gene subfamily were assessed by mRNA levels. CYP1A2 mRNA increase 2-, 5-, and 7.5-fold following exposure to flutamide at 10, 20, and 50 mg/L, respectively, in hepatocytes cultured for 8 h. In contrast, cells treated with 2-hydroxyflutamide showed no increase in CYP1A2 mRNA at 10 and 20 mg/L, while 3.5-fold increase was noted at the high concentration (50 mg/L) treatment (Fig 1).

## DISCUSSION

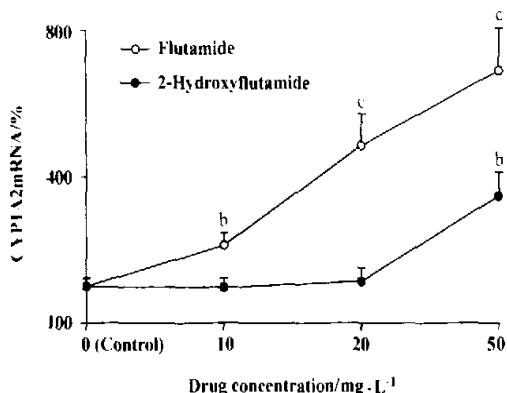
Since flutamide was marketed in February 1989, serious hepatic toxicity resulted from flutamide had been reported in the general medical literature. The major symptoms include anorexia, nausea, vomiting, fatigue, discolored urine, abdominal discomfort, and jaundice. To prevent the development of serious hepatic dysfunction, all patients receiving flutamide should be monitored clinically for signs and symptoms of hepatic injury<sup>(9)</sup>.

In the present study, isolated hepatocytes were used to probe the mechanism of liver toxicity due to flutamide. Isolated hepatocytes are a useful model for pharmaco-

**Tab 1. Cytotoxic effect of flutamide and 2-hydroxyflutamide in primary rat hepatocytes.  $n = 5$ .  $x \pm s$ . <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control.**

Group	Viable cells/%	LDH/%	ALT/%	AST/%	GSH/ $\mu$ g
Control	94 $\pm$ 4	100 $\pm$ 31	17.1 $\pm$ 1.3	22 $\pm$ 5	8.3 $\pm$ 0.8
Flutamide					
10 mg/L	82 $\pm$ 19 <sup>b</sup>	158 $\pm$ 35 <sup>b</sup>	29 $\pm$ 6 <sup>b</sup>	29 $\pm$ 8 <sup>b</sup>	6.36 $\pm$ 0.6
20	74 $\pm$ 20 <sup>c</sup>	557 $\pm$ 42 <sup>c</sup>	36 $\pm$ 11 <sup>c</sup>	34 $\pm$ 12 <sup>c</sup>	6.68 $\pm$ 0.14 <sup>b</sup>
50	58 $\pm$ 12 <sup>c</sup>	914 $\pm$ 48 <sup>c</sup>	40 $\pm$ 14 <sup>c</sup>	46 $\pm$ 19 <sup>c</sup>	5.0 $\pm$ 0.6 <sup>c</sup>
2-Hydroxyflutamide					
10 mg/L	88 $\pm$ 6	100 $\pm$ 51	8 $\pm$ 5	23 $\pm$ 7	8.5 $\pm$ 0.6
20	85 $\pm$ 8	121 $\pm$ 16	22 $\pm$ 6	25 $\pm$ 10	7.29 $\pm$ 0.09
50	76 $\pm$ 7 <sup>b</sup>	248 $\pm$ 62 <sup>b</sup>	33 $\pm$ 13 <sup>b</sup>	32 $\pm$ 8 <sup>c</sup>	6.17 $\pm$ 0.21 <sup>b</sup>

The LDH release in culture is expressed in percentage of control value, AST or ALT activity obtained by lysing the cells with culture medium containing 0.1 % Triton X-100 is defined as 100 %. GSH was measured in  $1 \times 10^6$  cells incubated with various concentrations of flutamide and 2-hydroxyflutamide.



**Fig 1.** Effect of flutamide and 2-hydroxyflutamide on CYP1A2 mRNA in primary hepatocyte culture. CYP1A2 mRNA was quantified by densitometry and expressed in percentage of control hepatocytes.  $n = 5$ .  $\bar{x} \pm s$ . <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control.

logical, toxicological, metabolic, and transport studies of xenobiotics since the development of techniques for high-yield isolation of rat hepatocytes<sup>[7]</sup>. For xenobiotic studies, freshly isolated hepatocytes and their short-term culture are currently being used. Liver injury would alter the molecular structure of cell membranes and results in permeability changes leading to hepatocyte necrosis<sup>[10]</sup>. Leakage of enzymes is considered to be a marker of cellular injury<sup>[11]</sup>. In our studies, flutamide 10, 20, and 50 mg/L and 2-hydroxyflutamide 50 mg/L resulted in an increase of ALT or AST and a decrease of GSH in primary hepatocyte cultures. These results show that flutamide can induce hepatotoxicity. In contrast, its major active metabolite, 2-hydroxyflutamide, has no apparent hepatotoxicity using these indications.

Previous results showed that CYP1A2 was involved in flutamide bioactivation<sup>[12]</sup>. The CYP1A and CYP3A inducers,  $\beta$ -naphthoflavone and dexamethasone, can increase LDH release. Female rats lacking cytochrome P450 3A2 exhibited lower covalent binding and lower LDH release<sup>[13]</sup>. Our studies indicated that CYP1A2 mRNA levels increase in parallel to hepatotoxicity caused by flutamide treatment. Because flutamide can be hydroxylated in the primary carbon atom and the aromatic ring or hydrolysed to form other products *in vitro*, free radicals formation also be proved<sup>[14]</sup>, the hepatotoxicity mechanism of flutamide remains further study in the future.

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**氟他胺和 2-羟基氟他胺对大鼠原代培养肝细胞毒性及 CYP1A2 mRNA 的影响<sup>1</sup>**

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**关键词** 氟他胺; 2-羟基氟他胺; 细胞毒性; 细胞色素 P-450 CYP1A2

**目的:** 比较氟他胺及其活性代谢产物 2-羟基氟他胺对原代培养大鼠肝细胞毒性及对 CYP1A2 mRNA 的影响。 **方法:** 分离大鼠肝细胞, 原代培养 4 h, 台盼蓝拒染法检测肝细胞活力。 氟他胺和 2-羟基氟他胺

在培养液中浓度分别为 10、20 和 50 mg/L。 肝细胞毒性检测包括台盼蓝拒染法, 乳酸脱氢酶(LDH)释放量, 谷丙转氨酶(AST)和谷草转氨酶(ALT)释放百分比, 谷胱甘肽(GSH)含量。 同时采用 Northern blot 方法进一步研究两药对 CYP1A2 mRNA 的影响。 **结果:** 药物作用 8 h 后, 氟他胺三个剂量组和 2-羟基氟他胺 50 mg/L 组出现肝细胞损伤, 表现为 ALT 和 AST 释放百分比增加, GSH 含量下降。 氟他胺三个剂量组使 CYP1A2 mRNA 水平分别升高 2、5 和 7.5 倍, 而 2-羟基氟他胺仅在 50 mg/L 时使 CYP1A2 mRNA 水平升高 3.5 倍。 **结论:** 氟他胺对原代培养大鼠肝细胞的毒性大于其活性代谢物 2-羟基氟他胺, 且明显增加 CYP1A2 mRNA 水平。

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