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Metabolism of Flutamide in Diet Control Fischer 344 and Brown Norway × F 344 Rats, and Its Hydroxylation and Conjugation by Human CYP450s and UDP-Glucuronosyltransferases

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ABSTRACT

In our current report, flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl] propanamide) treated diet control and non limited-fed Fischer 344 (F344) and Brown Norway (BN) × F344 rats showed that diet control reduces spontaneous and flutamide-induced hyperplasia[(1)]. In this continued study, we found that serum concentrations of active metabolite of flutamide, 2-hydroxyflutamide (OH-flu), were 181 ± 26.6 ng/mL and 68 ± 8.0 ng/mL ($p<0.05$), in non-limited fed and diet control F344 rats. In BN × F344 rats, the serum concentrations of 2-OH-flutamide were 232 ± 57 ng/mL and 52 ± 6 ng/mL ($p<0.05$) in non-limited fed and diet control animals. In diet control groups, liver microsomes from flutamide-treated F344 rats showed high 7-ethoxyresorufin O-deethylase (EROD) activity, while 7-Benzoxyresorufin O-dealkylase (BROD) activity was not affected significantly. Both rat and human liver microsomes showed flutamide oxidation activity. Human liver microsomes showed 10 times higher activity than rat liver microsomes (0.673 ± 0.04 vs 0.063 ± 0.008 nmol OH-flu/min/mg protein). Microsomes from human tissues such as colon, colon cancer, kidney, bladder, pancreas, prostate, prostate cancer, or ovarian cancer, showed no or non-detectable activity for flutamide hydroxylation. CYP450 1A1, 1A2, 1B1 and 2C19 from human lymphoblastoid cell lines, oxidized flutamide to OH-flu *in vitro* with activities from 0.118 ± 0.005 to 0.275 ± 0.010 nmol/min/mg protein. Microsomes isolated from human kidney, colon, and liver showed UDP-glucuronosyltransferase (UGT) activity for glucuronidation of OH-flu. Human kidney showed the highest activity. Several human recombinant UGTs (1A1, 1A4, 1A6, 1A7, 1A9, and 1A10) also showed glucuronidation activity for OH-flu. It was found that UGT1A6 was more active than other human UGTs.

Key words: flutamide, diet restricted, non limited-fed, hydroxylation, glucuronidation, CYP450, UDP-glucuronosyltransferase.

INTRODUCTION

Although testicular cancer is a relatively rare disease in men, it usually occurs at an adult early age and thus constitutes one of the most serious forms of cancer in young men[(2-4)]. Epidemiological evidence suggests that a significant number of human testicular cancer cases may have an environmental component in their etiology, including a dietary factor[(3)]. Testicular tumor formation is relatively common in several strains of rats. Over 80% of male Fischer 344 rats exhibit multiple interstitial cell adenomas by 24 months of age[(5,6)]. In rodents, pathological data have shown that lymphatic and endocrine tissues, including testes, are the most sensitive to degenerative diseases, including cancer[(6)]. Long-term diet control reduces the incidence of multiple adenoma, and altered the testicular biochemical function[(7)]. Diet control also has been shown to influence liver enzyme activity[(8)]. It has been reported that Leydig cells are the primary sites for P450 activity in testis[(9)]. Human testes, which are less susceptible to interstitial cell tumorigenesis, do not express the same spectrum of cytochrome P450 isozymes as rat testis[(10)].

Flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide) is a non-steroidal antiandrogen used for the treatment of prostate carcinoma[(11-13)]. This drug inhibits feedback inhibition of testerone on the hypothalamus[(14,15)]. Long-term administration of flutamide to rodents resulted in an elevation of LH and testosterone[(1,16,17)] which induces Leydig cell hyperplasia and interstitial adenomas[(1)].

It has been reported that flutamide is metabolized rapidly and extensively in rats and men[(18,19)]. In high doses, it inhibits feedback inhibition of testosterone at the hypothalamic-testicular-pituitary axis stimulating in elevated LH levels with increased Leydig cell hyperplasia in rats[(20-22)]. The major metabolite that has been found with pharmacological activity equal to or greater than flutamide is hydroxyflutamide (OH-flu)[(19,23-25)]. OH-flu binds to androgen receptors and inhibits the binding of testosterone and dihydrotesterone[(25-28)]. Studies also show that flutamide reduces androgen synthesis through restoration of ovulation in young women[(29)]. Flutamide affects Leydig cells and macrophage numbers in adult rats through the interference of LH[(7)]. Flutamide is toxic to rat hepatocytes as a result of the cytochrome P450 3A- and 1A-mediated formation of electrophilic metabolites[(30)].

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Diet control has been shown to delay the onset and reduce the severity of degenerative diseases, including cancer[31,32]. In rodents, pathological data has shown that testes are the most sensitive to degenerative disease[6]. It has been shown that the incidence of testicular interstitial cell tumors in rats is correlated with caloric intake[6]. Long-term diet controls reduce the incidence of multiple adenoma to 25% in 2 year-old Fischer 344 rats. Diet control also appears to have altered the testicular biochemical function. For example, serum testosterone levels were decreased and serum estradiol levels increased in restricted male rats[33]. Thus, it is probable that diet control alters the expression and regulation of several enzymes within the Leydig cells. To support this, it has been demonstrated that diet control caused marked alterations in hepatic cytochrome P450-dependent monooxygenase activities in the Fischer 344 rats[9]. Diet control caused a 50% reduction in cytochrome 2C11-dependent testosterone-16α-hydroxylase activity, when compared to non-limited fed controls. It has also been established that Leydig cells express relatively high level of cytochrome P450 2A1 that is active in the metabolic activation of carcinogens such as DMBA.

In a previous study, we have shown that reduction of caloric intake (i.e. diet control) significantly reduces the severity of interstitial hyperplasia in flutamide-treated F344 and Brown Norway × F344 cross rats[1]. Diet control also alters steroid and arachidonic acid metabolism in rodent testes, and these changes are functionally related to a reduced incidence of spontaneous and flutamide-induced testicular tumors.

In this study, the non-limited fed or diet control rats were treated with flutamide for 90 days. Pharmacokinetics studies of the active metabolite, OH-flu, in the serum were reported. The flutamide oxidation activity of rat liver microsomes, CYP 450 isozymes from human lymphoblastoid cell lines, and human tissue microsomes were also determined. Conjugation of 2-hydroxyflutamide, active compound of flutamide, by UGT isoforms, and human liver, kidney, and colon microsomes were also investigated.

MATERIALS AND METHODS

I. *Animals*

Twenty-four non-limited fed male Fischer rats, 24 caloric restricted male Fischer 344 rats, 24 non-limited fed male Brown Norway × Fischer 344 (BN × F344) hybrid rats and 24 caloric restricted BN × F344 hybrid rats. The animals were maintained at the National Center for Toxicological Research (NCTR), FDA under standard conditions. At 8 months of age, the animals were divided into control and treatment groups (12 animals each). The treated animals received by gavarge administration of 250 mg/kg of flutamide. Dosing was continued for 90 days when the animals were put in euthanasia.

II. *Chemicals*

Flutamide was purchased from Schering-Plough Research Institute (Kenilworth, NJ). 2-hydroxyflutamide, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $MgCl_2$ were purchased from Sigma Chemical Co. UDP-glucuronosyltransferase 1A1, 1A4, 1A6, 1A9 were purchased from Gentest Co. (Woburn, MA), 1A7 and 1A10 were purchased from PanVera Co. (Madison, WI).

III. *Human Samples*

The human liver samples used in this study were provided by Dr. A. Li, Washington University, St. Louis, MO. Other human tissue microsomes were gifts from Dr. G. Y. Tang and Mr. R. Wiese at NCTR, and Ms. S. Nowell at Veteran's Hospital in Little Rock, AR. Human tissue microsomes were prepared by a modified method as described previously[34]. The homogenization buffer contained 250 mM sucrose containing 25 mM potassium chloride, 1 mM DL-dithiothreitol, 0.5 mM EDTA, 10 mM HEPES, 20% glycerol and 10 mg/50 mL PMSF, pH 7.4. Protein concentrations were measured by Lowry's method[35].

IV. *Lymphoblastoid Cell Cultures*

Lymphoblastoid cell lines expressing individual isoforms of either human cytochrome P450 (except CYP1B1, CYP2C8, CYP2C19 and CYP4A11 were obtained directly from Gentest Corp.) were grown in suspension culture in 2 liter Wheaton cell-stir flasks at 35˚C and 16% O_2/4.5% CO_2 humidified atmosphere. The culture medium obtained from the Gentest Co. consisted of an RPMI medium containing histidinol in place of histidine and was supplemented with 10% horse serum. The cells were grown to a density of 1.2×10^6 per mL and harvested by centrifugation and the cell pellets frozen in liquid nitrogen.

To prepare lymphoblastoid cell microsomes, cell pellets from approximately 10^9 cells were homogenized while still frozen in 10 mL of cold homogenization buffer using a Teflon-glass homogenizer. The homogenate was centrifuged at 4,000 × g for 3 minutes. The supernatant was then centrifuged at 100,000 × g for 10 minutes to obtain microsomal pellets. The pellets were resuspended in potassium phosphate buffer to a protein concentration of 10 mg/mL. Aliquots were frozen at –80˚C for further analysis. Microsomes from cells expressing UDP-glucuronosyltransferases were activated with Brii 58.

V. *Microsome Preparation*

Two grams of rat liver or testis, were prepared at 4˚C then mixed with 5 mL homogenization buffer containing 0.25 M sucrose, 25 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 10 mM HEPES, and 20% glycerol, pH 7.4. After the tissues had been homogenized at 4˚C, the homogenates were centrifuged at 7, 000 × g for 20 min at 4˚C. After centrifugation, the supernatants were ultracentrifuged at 100,000 × g for 30 min at 4˚C. After this process, the pellets were sus-

pended with 0.01M tris-acetate buffer containing 4% glycerol and 0.15 M KCl, pH 7.4, and then ultracentrifuged again at 100,000 × g for 20 min at 4˚C. The pellets were collected and suspended with 1.5 mL buffer containing of 0.05 M tris-acetate buffer and 20% glycerol, pH 7.4, and stored at –80˚C.

VI. *Oxidation of Flutamide by Microsomal Solution*

The microsomes were mixed with 2.76 µg flutamide and 100 µL cofactor (10 mM glucose-6-phosphate/$MgCl_2$ + 2.4 mM NADP and 4 units glucose-6-phosphate dehydrogenase) and incubated at 37˚C for 45 min. The mixtures were then prepared for HPLC analysis.

VII. *HPLC Analysis*

Plasma samples (250 μL) or microsomal mixtures were mixed with 250 μL acetonitrile in eppendorff tubes and vortexed for 15 seconds. The mixtures were then centrifuged at 2,000 × rpm or 5 min at 4˚C. The supernatants were transferred to micro auto sampler tubes and 200 μL of the samples was injected for HPLC analysis[36]. The samples were analyzed by running through a Waters Novapak C18 column (15 cm × 3.9 mm) and monitoring at 300 nm by using a Waters LC Module 1 system. The mobile phase was an isocratic solution of 50% of acetonitrile/acetic acid/water (5:1:94, v/v/v) and 50% acetonitrile. The flow rate was 0.5 mL/min.

VII. *UDP-Glucuronosyltransferase Activity Determination and Matrix-Assisted Laser Desorption/Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF/MS)*

Microsomes prepared from human or rat tissues, or UGT isoforms from human lymphoblastoid cells, were incubated with flutamide as described in the previous study[37]. HPLC analysis for glucuronidation of OH-flu followed a method from previous study[38] except the samples were not radiolabeled, and the products were monitored at 265 nm. The products were collected and analyzed for their mass by using MALDI-TOF/MS. Mass spectrometric determination for glucuronidation of OH-flu followed the method described by Chang *et al*.[39] except that matrix was not used in this study.

RESULTS AND DISCUSSION

Testicular tumor formation is relatively common in several strains of rats, including Fischer 344 rat. Diet control has been demonstrated to reduce the incidence of spontaneous testicular tumors of the Fischer 344 strain. It has been well established that diet control increases serum corticosterone levels[40]. We have shown that diet control for as little as 4 weeks also results in elevated corticosterone levels showing a peak coincidence with feeding[33]. Most striking differences are seen in the ratio of serum estradiol to serum testosterone, where the estradiol to testosterone ratio is significantly greater at all circadian time-points. It was previously shown that subchronic treatment with flutamide potentiates Leydig cell hyperplasia in F344 rats, while diet control reduces both spontaneous and flutamide-induced hyperplasia[1]. A pharmacokinetic study showed that serum concentrations of the active metabolite of flutamide, 2-hydroxyflutamide (OH-flu), were 232 ± 57 ng/mlL and 52 ± 6 ng/mL ($p<0.05$) in non limited- fed and diet control BN × F344 hybrid rats, respectively. In non-limited fed and diet control of F344 rats, the serum OH-flu levels were 181 ± 26 ng/mL and 68 ± 8 ng/mL ($p<0.05$), respectively (Table 1). Corresponding LH values were 0.22 ± 0.03 and 0.14 ± 0.02 ($p<0.05$) in non-limited fed and diet control F344 rats respectively[1]. The results revealed that diet control altered flutamide/hydroxyflutamide pharmacokinetics and by altering pituitary/LH response[1] in rats.

In BN × F344 rats, 7-benzoxyresorufin O-dealkylase (BROD) activity of liver tissues increased in flutamide fed, caloric restricted groups. There were no significant differences of 7-ethoxyresorufin O-deethylase (EROD) activity in liver tissues. In the testis tissues of BN × F344 rats, non-limited fed groups (treated with or without flutamide) showed higher BROD and EROD activities than caloric restricted groups (Table 2). In Fischer 344 rats, no differences of BROD and EROD activities were observed in testis tissues. The caloric restricted control group showed the highest liver BROD activity, and the caloric restricted flutamide-treated group showed the highest liver EROD activity (Table 2).

Flutamide is oxidized to its active metabolite 2-hydroxyflutamide (OH-flu) by using rat liver microsomes from different treatments. No significant differences of specific activity (ca. 0.054 ± 0.006 ~ 0.071 ± 0.008 nmol/min/mg protein) were found between groups. From a pharmacokinetic study (Table 1), we concluded that diet control decreased the production of flutamide active metabolite, OH-flu, in both BN × F344 and F344 rats. LH was also elevated in non-limited treated rats[1]. Flutamide 2-hydroxylation was catalyzed predominantly by human CYP1A1 (0.118 ± 0.005 nmol/min/mg protein), CYP1A2 (0.275 ± 0.010 nmol/min/mg protein), CYP1B1 (0.204 ± 0.003 nmol/min/mg protein), and CYP2C19 (0.158 ± 0.001 nmol/min/mg protein) (also see Table 3). No activity could be detected in microsomes from other cell lines. Flutamide is used as an androgen antagonist in the treatment of prostate cancer, but requires conversion to 2-hydroxyflutamide in order to exhibit antiandrogenic activity[25,41,42]. It has been reported that the metabolism of flutamide by human liver microsomes and isolated rat hepato-

Table 1. 2-hydroxyflutamide (OH-flutamide) level found in the serums from flutamide-treated BN × F344 and Fischer 344 rats in diet control or non-limited fed groups

Animal (flutamide treated) (n= 6)	OH-flu (ng/mL) found in serum
BN × F344 (non-limited fed)	232 ± 57[a]
BN × F344 (diet control)	52 ± 6
Fischer 344 (non-limited fed)	181 ± 26[b]
Fischer 344 (diet control)	68 ± 8

Values represent mean ± standard deviation.
[a,b] Significant difference from diet control groups ($p<0.05$).

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Table 2. Analysis of 7-benzoxyresorufin O-dealkylase (BROD) and 7-ethoxyresorufin O-deethylase activities from liver and testis microsomes of BN × F344 and Fischer 344 rats. (NL: non-limited fed; DC: diet control)

Animal (n= 3)	BROD* (Liver)	BROD* (Testes)	EROD* (Liver)	EROD* (Testes)
BN x F344 (DC control)	15.2 ± 1.5 ^{b**}	0.6 ± 0.1^{c}	56.1 ± 3.9^{b}	0.6 ± 0.1^{c}
BN x F344 (DC flutamide treated)	23.5 ± 2.0^{a}	0.3 ± 0.1^{c}	55.0 ± 11.9^{b}	0.5 ± 0.2^{c}
Fischer 344 (DC control)	29.8 ± 1.8^{a}	6.3 ± 1.3^{a,b}	49.7 ± 8.9^{b}	5.9 ± 0.9^{b}
Fischer 344 (DC flutamide treated)	19.6 ± 1.9^{b}	7.1 ± 1.6^{a,b}	98.3 ± 11.4^{a}	9.9 ± 2.2^{a}
BN x F344 (NL control)	6.7 ± 2.0^{c}	11.0 ± 2.5^{a}	54.3 ± 11.2^{b}	10.4 ± 1.1^{a}
BN x F344 (NL flutamide treated)	12.2 ± 1.1^{b}	9.3 ± 0.5^{a}	53.9 ± 7.6^{b}	13.5 ± 1.1^{a}
Fischer 344 (NL control)	13 ± 3.6^{b}	8.0 ± 0.2^{a,b}	25.7 ± 4.3^{c}	10.8 ± 2.7^{a}
Fischer 344 (NL flutamide treated)	19.5 ± 3.0^{a,b}	5.6 ± 0.6^{b}	37.2 ± 7.6^{c}	7.1 ± 1.2^{b}

* pmol/min/mg protein.

** Values represent mean ± standard deviation. Statistical comparisons within strains were performed by GLM-LSD Analysis. Similar letters are not significantly different, P< 0.05.

Table 3. Oxidation of flutamide by CYP450 isoforms from human lymphoblastoid cell lines (N=3)

CYP450 isoform	OH-flutamide produced (nmol/min /mg protein)
1A1	0.118 ± 0.005
1A2	0.275 ± 0.010
1B1	0.204 ± 0.003
2A6	–
2B6	–
2C8	–
2C19	0.158 ± 0.001
2E1	–
2D6	–
3A4	–
4A11	–
Human liver	0.637 ± 0.04

Values represent mean ± standard deviation.

cytes may be mainly mediated by CYP1A2 and 3A4[30,43]. Our study showed that CYP1A2 had the highest flutamide 2-hydroxylation metabolism rate than CYP1A1, CYP1B1 and CYP2C19 (Table 3). Shet *et al*.[44] also reported that CYP1A2 played a major role in the metabolism of flutamide 2-hydroxylation. Interestingly, CYP1B1 is also highly expressed in the human prostate[45]. Since we have demonstrated that CYP1B1 catalyzes flutamide 2-hydroxylation, it is likely that biologically active 2-hydroxyflutamide will be synthesized within the target tissue. Such an effect would be expected to enhance the efficacy of flutamide. Although induction studies in rodents suggested that CYP3A isoform converted flutamide to toxic metabolites, we found no evidence of such effects using CYP3A4. CYP2C19 was reported to catalyze 4-hydroxylation of tolbutamide and (*R*)-mephenytoin[46]. The same group also reported that human liver contained CYP2C19. These results support that CYP2C19 was one isoform that catalyzed hydroxylation of flutamide in our study.

Hydroxylation of flutamide was mainly catalyzed by human liver microsomes (0.637 ± 0.04 nmol/min/mg protein) in our study. This result was supported by the evidence that CYP1A1, 1A2, 1B1, and 2C19 were also expressed in human liver[44], and catalyzed hydroxylation of flutamide (Table 3). Microsomes prepared from human colon, colon cancer, kidney, bladder, pancreas, prostate, prostate cancer, or ovarian cancer tissues had none or undetectable effects on hydroxylation of flutamide (data not shown).

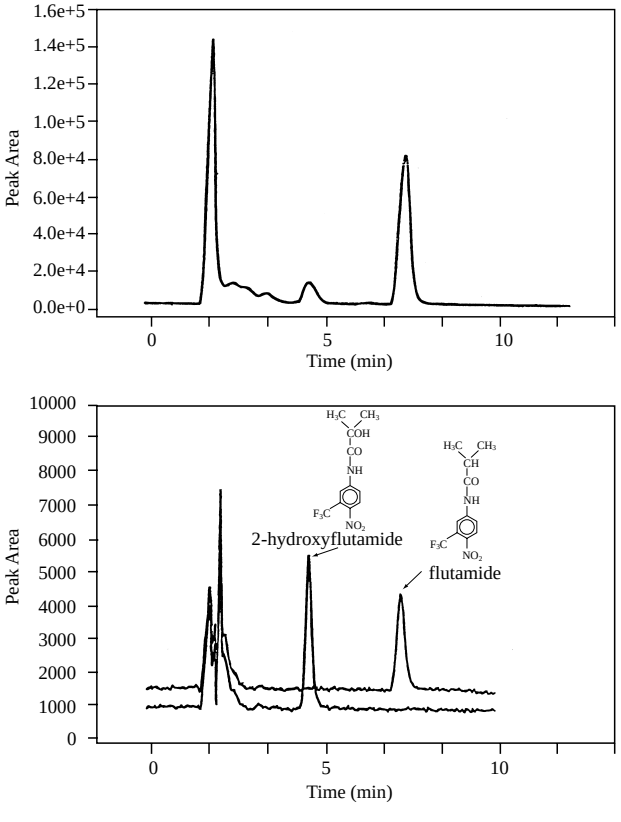

Figure 1. HPLC profile of 2-hydroxyflutamide, the active compound of flutamide was observed in the serum samples of non-limited fed F344 and BN × F344 rats (top). Standards showed that the retention time were 4.60 min and 7.27 min for 2-hydroxyflutamide and flutamide respectively (bottom).

Human kidney, liver, colon, and human recombinant UGTs catalyzed conjugation of 2-hydroxyflutamide by glucuronic acid. MALDI-TOF/MS results revealed that the fractions collected from a retention time of 8.65 minutes in a conjugation study for hydroxyflutamide by using human kidney microsomes (Figure 2) showed a mass of 469 Da which was consistent with the mass of glucuronic acid conjugated hydroxyflutamide (data not shown). These results were used to identify conjugated hydroxyflutamide from other studies. In a comparative study, human kidney microsomes showed relatively higher activity than liver and colon microsomes. The activity decreased in the order of human kidney > human

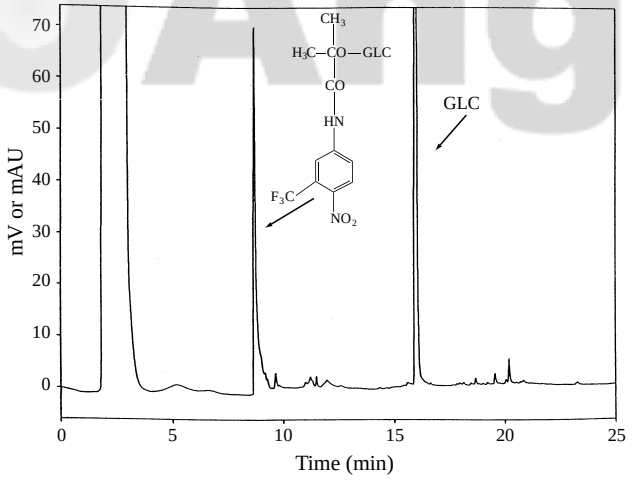

Figure 2. HPLC profile of glucuronidation of 2-hydroxyflutamide by human kidney microsomes. Metabolite eluted from retention time of 8.65 min was identified as the compound with mass of 469 Da by MALDI-TOF/MS. (GLC: glucuronic acid)

Table 4. Conjugation of OH-flutamide by human tissue microsomes and UGT isoforms (1A family)

UGT preparation	UGT activity
Human kidney microsomes	+++++
Human liver microsomes	++++
Human colon microsomes	+++
1A1	++
1A4	++
1A6	++
1A7	+
1A9	+
1A10	+
control	--

liver > human colon. UGT isoforms 1A1, 1A4 and 1A6 showed higher activities than 1A7, 1A9, and 1A10 (Table 4).

Interestingly, UGT 1A1, 1A3/1A4, 1A6, 1A8/1A9, and 1A10 were expressed in human kidney or kidney 293 cells[47-51]. UGT 1A1, 1A6, 1A9, and 1A10 were expressed in colon or Caco-2 cells[49,52]. UGT 1A1, 1A3, 1A4, 1A6, 1A7 and 1A9 were expressed in human liver[50,53-55]. Apparently, UGT 1A6 that had been found expressed in human kidney, colon and liver, showed higher activity than other UGT isoforms, and could be the most important human UGT for glucuronidation of 2-hydroxyflutamide. However, other human tissue microsomes such as from prostate or testis were not available or sufficient for finding other possible UGT effects in these tissues. In summary, diet control treatment decreased the opportunity for testicular tumor occurrence in rat models. It also eliminated the appearance of active compound 2-hydroxyflutamide in rats. Human *in vitro* studies showed that liver might be the main site for modification of flutamide to hydroxyflutamide. This conclusion is supported by the facts that human liver microsomes and human CYP 1A2 had highly activities for flutamide hydroxylation. It is also concluded that kidney is the main site for conjugation of hydroxyflutamide for elimination, and UGT 1A6 might be the major UGT for conjugation.

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食量控制對Flutamide在大白鼠體內代謝的影響，並人體細胞色素及尿甘二磷酸葡萄糖醛酸轉化酵素對其羥基化及接合之研究

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摘 要

使用flutamide餵食大白鼠（Fscher 344, Brown Norway × F344），並加上食量控制。結果顯示食量控制可減少由flutamide引起的細胞增生。在不限食量的F344大白鼠實驗中，血液內flutamide代謝產物（2-hydroxyflutamide）的含量爲181 ± 26.6 ng/mL。而在食量控制的大白鼠中，只有68 ± 8.0 ng/mL。在Brown Norway × F344大白鼠中，也有同樣的效應（各爲232 ± 57及52 ± 6.0 ng/mL）。在食量控制組中，肝細胞微體具較高之EROD活性。BROD則無明顯差異，大白鼠及人體肝細胞微體都有羥基化flutamide爲2-hydroxyflutamide的功能。人體肝細胞微體之功能約爲大白鼠肝細胞色素的十倍（0.673 ± 0.04對0.063 ± 0.008 nmol/min/mg蛋白質）。其他人體細胞色素則不具此效應。人體淋巴細胞株培養的CYP1A1, 1A2, 2B1及2C19亦有同樣的功能。人體的腎、肝及結腸細胞微體有接合2-hydroxyflutamide的功能，腎細胞微體比肝及結腸細胞體的功能強。以人體尿甘二磷酸葡萄糖醛酸轉化酵素群（UGT 1A1, 1A4, 1A6, 1A7, 1A9, 1A10）作2-hydroxyflutamide的接合研究，結果顯示UGT1A6的效果最好。

關鍵詞： flutamide, 2-hydroxyflutamide，食量控制，不限食量，羥基化，接合作用，細胞色素，細胞微體，尿甘二磷酸葡萄糖醛酸轉化酵素