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# Effect of Substrate Size on Activities of Thiocarbamides with the Human Flavin-containing Monooxygenase 3

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# 사람 Flavin-containing Monooxygenase 3의 Thiocarbamide 화합물의 기질 크기에 따른 효소활성에 관한 연구

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## 요 약

FMOs (Flavin-containing monooxygenases, EC1.14.13.8)는 다양한 종류의 식품, 약물이나 기타 외래 유래물질 (xenobiotics)를 산화시키는 NADPH와 $O_2$ 의존성 약물대사효소이다. 현재까지 5종의 subfamily가 존재하는 것으로 보고 되어 있으며 그 중 잘 알려진 FMO3는 대표적인 subfamily로서 주로 간에 존재한다. 사람 FMO에 관한 연구는 최근들어 활성화되기 시작했으며 질소, 황이나 인 등을 포함하는 친핵성 (nucleophilic) 화합물이 대표적인 기질로 보고되어 있다. 본 연구에서는 thiocarbamide를 포함하고 있는 화합물에 대한 사람의 FMO3의 기질특이성을 알아보고자 하였다. 사람 FMO3를 baculovirus system을 이용하여 대량으로 발현시킨 후 그 microsomal FMO3를 분리하여 thiocholine assay를 시행하였다. 그 결과 methimazole, thiourea, and phenylthiourea는 낮은 $K_m$ (4-10 μM)값을 갖는 반면, 이보다 기질의 크기가 큰 1, 3-diphenylthiourea, 1, 3-bis (3, 4-dichlorophenyl)-2-thiourea, 1, 1-dibenzyl-3-phenyl-2-thiourea 에서는 효소활성이 나타나지 않았다. 이는 사람 FMO1과 비교하여 볼 때 큰 차이는 없었으며, 다른 pig, guinea pig, rat, rabbit에서 보다 받아들일 수 있는 기질의 크기가 더 제한적임을 알 수 있었다.

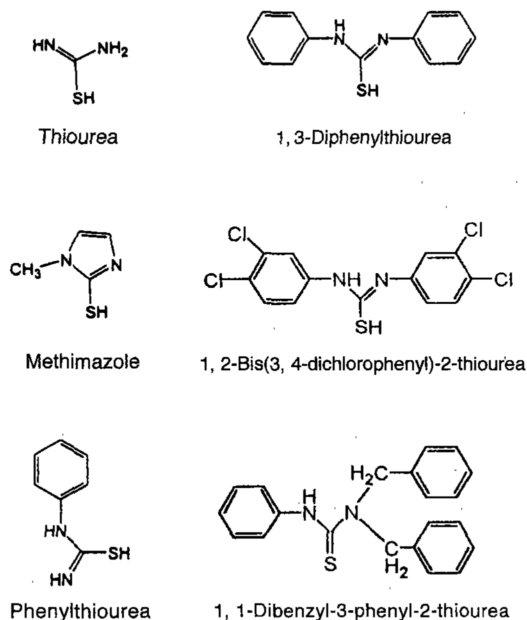
**Key words** : flavin-containing monooxygenase, thiocarbamides, baculovirus

## INTRODUCTION

The flavin-containing monooxygenase (FMO) consists of a family of enzymes involved in the metabolism of drugs and endogenous chemicals (Cashman JR, 1995; Ziegler DM, 1993). In humans, the flavin-containing monooxygenase form 3 (FMO3) (EC 1.14.13.8), is the predominant form of flavin-containing monooxygenase expressed in adults human liver (Lomri N *et al.*, 1992; Hines RN *et al.*, 1992). cDNA-expressed human FMO3 (Lomri N *et al.*, 1993; Cashman JR *et al.*, 1995) and human liver microsomal FMO3 (Cashman JR *et al.*, 1993) have been observed to N- and S-oxygenate nucleophilic nitrogen- and sulfur-containing drugs and chemicals, respectively. The physiological role of human FMO3 in hepatic metabolism is not clear, but it has been suggested that human FMO3 participates in the detoxication of nucleophilic heteroatom-

containing dietary, endogenous or xenobiotic chemicals to produce polar, oxygenated metabolites that are readily excreted (Ziegler DM, 1988).

There are 5 families of FMO found in mammals, FMO1 through FMO5. The mechanism of these enzymes differ form that of all other monooxygenases in that the oxygenatable substrate is not required for dioxygen activation. These flavo-proteins are apparently present within the cell in the 4a-hydroperoxyflavin form and any soft nucleophile that gains access to this potent monooxygenating agent (Ball S *et al.*, 1980) will be oxidized. How access to the enzyme-bound oxidant is controlled is still not clear, but the number and position of charged groups on the nucleophile are important (Taylor KL *et al.*, 1987). In addition, a report indicates that overall substrate size may be another important factor. The latter parameter appears largely responible for many (but not all) differences in substrate specificities of the flavoproteins isolated from rabbit lung and pig liver. Thiocarbamides with increasingly bulky substituents to the one and three positions are also apparently capable of serving as selective substrate probes for FMO isoforms in mamamlian microsomes (Guo *et al.*, 1992). The studies described in this report were undertatken to probe size restrictions for thiocarbamides and other nucleophilic xenobiotic substrates for human FMO3.




**Scheme 1.** Structures of thiocarbamides used in this work.

## MATERIALS AND METHODS

### 1. Chemicals

N-Benzylimidazole, acetylcholine chloride, and all thiocarbamides (Scheme 1) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Methimazole, $NADP^+$, glucose-6-phosphate, L-mesenteroides glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grades available commercially.

### 2. Synthesis

Thiocholine chloride was prepared by the methanolysis of acetylthiocholine chloride as follows. 200 mg of acetylcholine dissolved in 2 ml of dry, oxygen-free methanol saturated with HCl was incubated at room temperature overnight. The reaction was usually complete after only 5h but prolonged incubation had no detectable effect on yield of thiocholine. After incubation, methanol, methylacetate, and excess HCl were removed under vacuum or by purging with a stream of dry argon. Aqueous 10 mM solutions of thiocholine hydrochloride are stable for several months at 0~4°C. Phenylthiourea, 1, 3-diphenylthiourea, and larger thiocarbamides were recrystallized from ethanol and stored at 0~5°C. Fifty mM solutions of these thiocarbamides in ethanol were prepared daily. Methimazole and thiourea are more stable and aqueous solutions (0.1 M) of these substrates can be stored at 5~10°C for 2~3 weeks without any detectable changes.

### 3. Construction of recombinant of baculovirus

FMO3 recombinant baculovirus was constructed

by minor modifications of the procedure described by Dolphin *et al.* (1998). The insert was excised by incubation with XbaI and HindIII, then gel-purified and ligated into pFastBac1 (GibcoBRL, Rockville MD) to give FMO3/pFastBac1. FMO3 recombinant baculovirus was generated by transfection of *Spodoptera frugiperda* (Sf)9 cells with the corresponding recombinant bacmid DNA, obtained via site-specific transposition using the Bac-to-Bac Baculovirus Expression System (GibcoBRL, Rockville, MD).

#### 4. Baculovirus-mediated expression of FMO3

For expression, 500 ml of Sf9 cells, grown to a density of $1 \times 10^6$ cells/ml in a 2 liter spinner flask, were infected with virus at a multiplicity of infection of 7 and incubated by stirring at 100 rpm in a stirring platform at 27°C for 72 hours. Cells were pelleted, resuspended in 50 ml of lysis buffer, consisting of 50 mM Tris-HCl (pH 7.4), 0.154 M KCl, 0.2 mM phenylmethylsulfonyl fluoride. The cells were broken by two passes through a pre-cooled French press cell disrupter at 800 psi. The lysed cells were centrifuged at 3,000 g for 15 min at 4°C. The supernatant was saved and microsomal fraction was obtained by centrifugation of the resulting supernatant at 100,000 g for 1 hour at 4°C. Microsomal pellets were resuspended in 10 ml of 10 mM HEPES (pH 7.5) buffer containing 0.154 M KCl, 1 mM EDTA, and 20% (v/v) glycerol. The preparation was stored in aliquots at −80°C until use. Protein concentration was determined by the method of Bradford with Bio-Rad (Hercules, CA) reagents using bovine serum albumin as a standard.

#### 5. Activity measurements

The oxidation of thiocarbamides catalyzed by microsomes was determined by following substrate-dependent thiocholine oxidation as described (Guo *et al.*, 1992). The reaction was carried out in 10 ml Erlenmeyer flasks in a metabolic shaker at 37°C in 0.1 M phosphate (pH 7.4), 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 1.5 U glucose-6-phosphate dehydrogenase, 2 mM N-benzylimidazole and 150 μM thiocholine. After 3∼4 min temperature equilibration, the microsomes were added and 1min later the reaction was initiated by adding the compounds. Aliquots (0.4 ml), routinely withdrawn at 0, 3, 6, and 9 min were transferred to tubes containing 0.04 ml 3.0 M trichloroacetic acid. The concentration of thiocholine in the protein free supernatant fractions was determined as described earlier (Guo *et al.*, 1992).

## RESULTS AND DISCUSSION

To determine if the human FMO3 being expressed in the baculovirus vector system was full length, extracts were prepared from the insect cells carrying FMO3/Bacmid. This extract was analyzed by SDS-PAGE and western blotting with antisera prepared against FMO. The FMO3 could be seen on the stained gel, and the western blot showed band (∼60 kDa) indicating that the human FMO3 was being expressed in the baculovirus system.

Thiocarbamide-dependent oxidation of thiocholine catalyzed by microsomes isolated from Sf9 cells infected FMO3 baculovirus indicated that the method (Guo *et al.*, 1991) described for measuring the oxidation of thiourea to formamidine sulfenic acid can also be used to measure the oxidation of other thiocarbamide substrates for this enzyme. All are apparently oxidized to sulfenic acids at essentially the same maximal velocities, indicating that substrate-dependent oxidation of thiocholine appears to give a reliable estimation for the oxidation of these thiocarbamides to their sulfenic acids. In the absence of substrate there was no detectable loss of thiocholine for up to 12 minutes incubation with the complete reaction medium. Microsomes isolated from control Sf9 cells were also completely devoid of methimazole S-oxidase activity.

All of the compounds in Scheme 1. are structures of thiocarbamides used in this work.

While the $K_m$ for methimazole, thiourea and phenylthiourea are virtually the same (4∼10 μM), the larger 1, 3-diphenylthiourea, 1, 3-bis (3, 4-dicholo-

**Table 1.** $K_m$ and $V_{max}$ values for the oxidation of thiocarbamide compounds catalyzed by human FMO3 expressed in insect cells

| Substrate | $K_m$ | $V_{max}$ |
|---|---|---|
| | μM | nmol substrate oxidized/min/mg |
| Thiourea | 4.4 | 17.20±0.49 |
| Methimazole | 5.2 | 10.86±0.88 |
| Phenylthiourea | 10.9 | 14.10±0.47 |
| 1, 3-Diphenylthiourea | | ND |
| 1, 3-Bis (3, 4-dichlorophenyl) -2-thiourea | | ND |
| 1, 1-dibenzyl-3-phenyl -2-thiourea | | ND |

Activities were measured by following substrate-dependent thiocholine oxidation as described in the text. Also see Guo *et al.* (1992). Values are means±S.E. of triplicate measurements for each preparation. Kinetic constants were obtained from reciprocal plots of velocity of substrate-dependent thiocholine oxidation versus substrate concentration above and below the concentration required to give half maximal velocity.
ND, Activity not detected at the highest concentrations soluble in the reaction medium.

rophenyl)-2-thiourea, and 1, 1-dibenzyl-3-phenyl -2-thiourea are virtually excluded from the enzyme -bound 4α-hydoperoxyflavin in human FMO3 (Table 1).

Kinetic constants, calculate from rate of substrate dependent thiocholine oxidation catalyzed by microsomes, indicate that thiourea, methimazole and phenylthiourea were oxidized at the almost same (Fig. 1, Fig. 2). The measurements were quite reproducible and the rates determined with thiourea or methimazole were not significantly different. On the other hand, 1, 3-diphenylthiourea, 1, 3-bis (3, 4-dicholorophenyl)-2-thiourea, and 1, 1-dibenzyl-3-phenyl -2-thiourea dependent oxidation of thiocholine could not be detected in reactions catalyzed by human FMO3. 1, 3-diphenylthiourea is apparently completely excluded from the catalytic site, these amines drugs are probably approaching the upper size limits of xenobiotics accepted by human FMO3. The sulfur atom in the thiocarbamides used is relatively close to the bulky substituents on the nitrogens and differences in the van der Waals' surface area appear to reflect the effect of size on substrate acces-

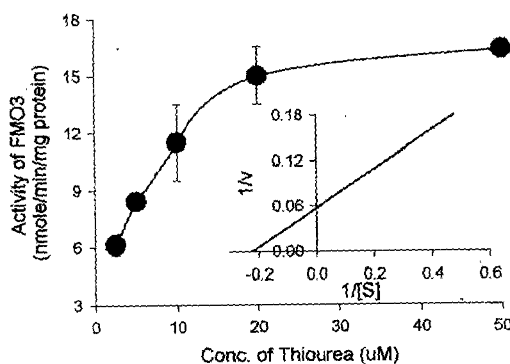



**Fig. 1.** The oxidation activity of thiourea catalyzed by human FMO3 expressed in the baculovirus system.

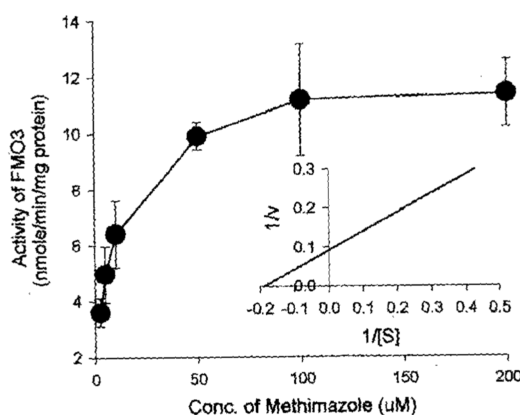



**Fig. 2.** The oxidation activity of methimazole catalyzed by human FMO3 expressed in the baculovirus system.

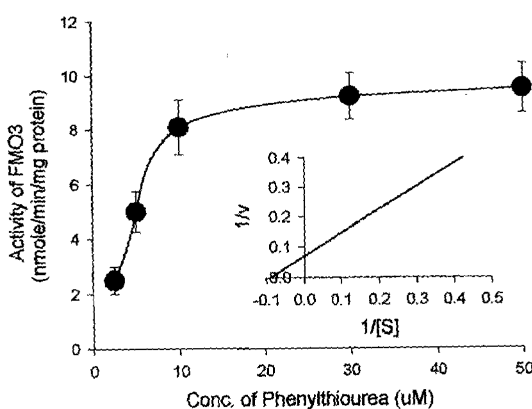



**Fig. 3.** The oxidation activity of phenylthiourea catalyzed by human FMO3 expressed in the baculovirus system.

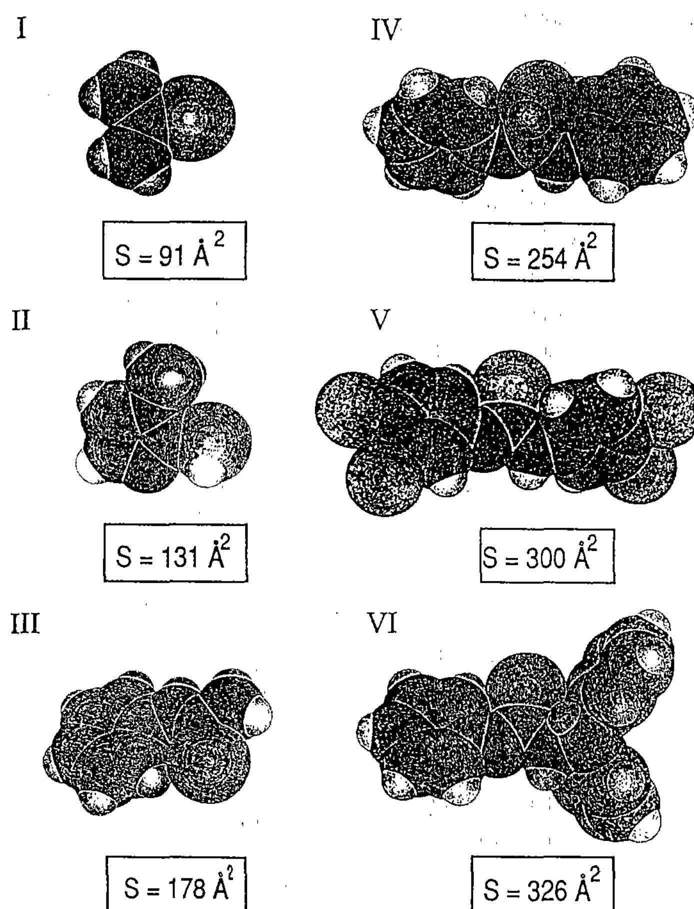



**Fig. 4.** Space-filling models of thiocarbamides at a minimum energy level determined by the PC model software program S is the van der Waals' surface area. Thiourea (I) and phenylthiourea (III) are in the ketone form, whereas methimazole (II), 1, 3-diphenylthiourea (IV), 1, 3-bis (3, 4-dichlorophenyl)-2-thiourea (V) and 1, 1-dibenzyl-3-phenyl-2-thiourea (VI) are in their imine forms.

sibility to the active site. The dimensions of the computerized space-filling models of methimazole and five thiocarbamides at minimum energies, determined by a software program (PC model), are shown in Fig. 4.

Without exception, microsomal preparations which do not show activity toward a thiocarbamide with intermediate size will not catalyze the oxidation of larger thiocarbamides while smaller analogs are readily accepted. The substrate specificity of this isoform in humans appears considerably more restricted than that of pig, guinea pig, rat or rabbit FMO3. The size limits for substrates accepted by human FMO3 appear similar to those observed for rabbit FMO3 (Guo *et al.*, 1992).

The studies reported in this paper indicate that size limitations may serve as an initial guide for predicting substrate activity in humans of a specific xenobiotic nucleophile bearing bulky substituents.

## CONCLUSION

Thiocarbamide compounds were examined for substrate activity with microsomes isolated from

(Sf)9 cells infected with human FMO3 recombinant baculovirus. While the $K_m$ for methimazole, thiourea and phenylthiourea are virtually the same (4~10 μM), the larger 1,3-diphenylthiourea, 1,3-bis(3,4-dicholorophenyl)-2-thiourea, and 1,1-dibenzyl-3-phenyl-2-thiourea are virtually excluded from the enzyme-bound 4α-hydoperoxyflavin in human FMO3. 1,3-diphenylthiourea is apparently completely excluded from the catalytic site, these amines drugs are probably approaching the upper size limits of xenobiotics accepted by human FMO3. The substrate specificity of this isoform in humans appears considerably more restricted than that of pig, guinea pig, rat or rabbit FMO3.

## ABSTRACT

The flavin-containing monooxygenases (FMOs) (EC1.14.13.8) are NADPH-dependent flavoenzymes that catalyze oxidation of soft nucleophilic heteroatom centers in a range of structurally diverse compounds, including foods, drugs, pesticides, and other xenobiotics. In humans, FMO3 is quantitatively a major human liver monooxygenase. In the present study, the baculovirus expression vector system was used to overexpress human FMO3 in insect cells for catalytic studies. Microsomes isolated from Spodoptera frugiperda (Sf)9 cells infected with human FMO3 recombinant baculovirus catalyzed the NADPH- and $O_2$-dependent oxidation of methimazole, thiourea, and phenylthiourea. However there was no detectable activity with 1,3-diphenylthiourea or larger thiocarbamides. Microsomes from control Sf9 cells were devoid of methimazole or thiourea S-oxygenase activity. 1,3-diphenylthiourea is apparently completely excluded from the catalytic site, these amines drugs are probably approaching the upper size limits of xenobiotics accepted by human FMO3. The substrate specificity of this isoform in humans appears considerably more restricted than that of pig, guinea pig, rat or rabbit FMO3.

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