SIZE LIMITS OF THIOCARBAMIDES ACCEPTED AS SUBSTRATES BY HUMAN FLAVIN-CONTAINING MONOOXYGENASE 1

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ABSTRACT:

Microsomes isolated from Spodoptera frugiperda (Sf)9 cells infected with human flavin-containing monoxygenase (FMO1) recombinant baculovirus catalyzed the NADPH- and O2-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 oxidation of methimazole, thiourea, and phenylthiourea. However, there was no detectable effect on the oxidation of 10 µM methimazole (Km = 5 µM) but 1.0 mM N,N-dimethylaniline or chlorpromazine inhibited the oxidation of 1.0 mM methimazole 50 and 70%, respectively. Although products were not isolated, the pronounced inhibition of methimazole S-oxygenation suggests that these amines are alternate substrates for human FMO1. Because 1,3-diphenylthiourea is apparently completely excluded from the catalytic site, tricyclic amine drugs are probably approaching the upper size limits of xenobiotics accepted by human FMO1. The substrate specificity of this isoform in humans appears considerably more restricted than that of pig or guinea pig FMO1. Differences in the size of nucleophiles accepted must be considered in attempting to extrapolate the extensive structure-activity studies available for pig FMO1 to this FMO isoform in humans.

The quantitative description of structural elements controlling substrate specificities of enzymes catalyzing metabolism of xenobiotics is a major objective of predictive toxicology and drug metabolism. Because the metabolism of virtually all xenobiotics is catalyzed by more than one enzyme or at least by multiple isoforms whose expression can vary with species, tissue, and development, such a comprehensive analysis is extremely difficult. However, the broad specificities of some drug metabolizing enzymes suggest that selective binding to the catalytic site is not essential for catalysis, and isoform specificity depends on other more readily defined parameters. For instance, numerous structure activity studies with purified and microsomal flavin-containing monoxygenases (FMOs) suggest that the overall size and shape of a nucleophilic xenobiotic is a major factor responsible for differences in the specificities of FMO isoforms [cf. review by Poulsen and Ziegler (1995) for references to original literature supporting this conclusion]. To what extent this will be true for all mammalian FMO isoforms is not known, but because rate constants for the oxidation of sulfur compounds by the enzyme-bound and protein-free 4a-hydroperoxylavlin are essentially the same (Jones, 1985), this assumption is probably valid for xenobiotics bearing nucleophilic sulfur or selenium functional groups. On the other hand, although enzyme protein has a large effect on the oxidation of amines, substrate size was still a major factor responsible for the differences in substrate specificities of pig FMO1 and rabbit FMO2 for a homologous series of tricyclic xenobiotics bearing the same N,N-dimethyl functional group (Nagata et al., 1990). Thio carbamides with increasingly bulky substituents on the one and three positions are also apparently capable of serving as selective substrate probes for FMO isoforms in mammalian microsomes (Guo et al., 1992). The studies described in this report were undertaken to probe size restrictions for thiocarbamides and, by extension, other nucleophilic xenobiotic substrates for human FMO1.

Materials and Methods

N-Benzylimidazole, acetylcholine chloride, and all thiocarbamides were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methimazole, NADP+, glucose 6-phosphate, L-mesenteroides glucose 6-phosphate dehydro-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km</th>
<th>Vmax</th>
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<tbody>
<tr>
<td>Methimazole</td>
<td>5</td>
<td>18.09 ± 0.46</td>
</tr>
<tr>
<td>Thiourea</td>
<td>4</td>
<td>21.12 ± 0.75</td>
</tr>
<tr>
<td>Phenylthiourea</td>
<td>3</td>
<td>20.16 ± 0.14</td>
</tr>
<tr>
<td>1,3-Diphenylthiourea</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND, activity not detected at the highest concentrations soluble in the reaction medium.
Microsomal thiocarbamide S-oxygenase activities were measured by following substrate-dependent thiocarboline oxidation as described (Guo et al., 1992). The reaction medium contained 0.1 M phosphate (pH 7.4), 0.25 mM NADP<sup>+</sup>, 2.5 mM glucose 6-phosphate, 1.5 U glucose 6-phosphate dehydrogenase, 2 mM N-benzylimidazole, and 150 µM thiocline. Aliquots withdrawn at 3-min intervals were deproteinized and analyzed for thiocholine exactly as described previously (Guo et al., 1992). Substrate activities of trimethylamine, N,N-dimethylaniline, and chlorpromazine were estimated by their inhibition of 10 mM methimazole S-oxygenation.

**Results and Discussion**

Initial studies carried out with commercial human Supersomes and microsomes isolated in this laboratory from Sf9 cells-infected FMO1 baculovirus indicated that both preparations catalyzed methimazole-dependent oxidation of thiocholine. In the absence of methimazole, there was no detectable loss of thiocholine for up to 12-min incubation with the complete reaction medium. Microsomes isolated from control Sf9 cells were also completely devoid of methimazole S-oxygenase activity. In addition, neither 1.0 mM trimethadylamine nor methimazole at the highest concentration soluble in the assay medium had any detectable effect on the methimazole S-oxygenase activity of microsomes bearing expressed human FMO1. In contrast, N,N-dimethylaniline and chlorpromazine at 1.0 mM inhibited the oxidation of 10 µM methimazole 50 and 70%, respectively. Although oxidation products were not isolated, the pronounced inhibition of methimazole oxidation suggests that these amines can serve as alternate substrates for human FMO1. This interpretation is consistent with earlier studies of Lemone et al. (1990), who reported that human kidney, but not human liver microsomes catalyzed the N-oxidation of imipramine. The enzymatic basis for this difference was subsequently shown to be due to the expression of different FMO isoforms in these tissues in adults (Dolphin et al., 1996; Lang et al., 1998). Although nucleophiles not much bigger than methimazole are readily accepted by essentially all FMO isoforms, in humans, only FMO1 catalyzes the oxidation of the much larger tricyclic drugs. Rettie has proposed that the N-oxidation of imipramine or orphenadrine can serve as marker activities for human FMO1 (A. Rettie). However, this leaves unanswered the question whether this isoform, like pig FMO1, can catalyze the oxidation of nucleophiles bearing even more bulky substituents.

This question is addressed by the data summarized in Table 1, which indicate that the tricyclic drugs approach the upper size of compounds that can enter the active site of human FMO1. Although the <i>K</i><sub>m</sub> values for methimazole, thiourea, and phenylthiourea are virtually the same (3–5 µM), the larger 1,3-diphenylthiourea is virtually excluded from the enzyme-bound 4a-hydroperoxyflavin in human FMO1. These data suggest that pig FMO1, which accepts thiocarbamides much larger than 1,3-diphenylthiourea (Guo et al., 1992), would be a poor model for human FMO1. The size limits for substrates accepted by human FMO1 appear more similar to those observed for rat or rabbit FMO1.

The observation that the <i>K</i><sub>m</sub> and <i>V</i><sub>max</sub> values for methimazole and thiocarbamides that can access the enzyme-bound oxidant of human FMO1 are essentially the same suggest that the tight binding enzyme-substrate complexes are not required for catalysis, and size restrictions measured with thiocarbamides can be used to predict activity with other xenobiotic nucleophiles.

For instance, all of the compounds in Fig. 1, except for 1,3-
diphenylthiourea, are substrates for human FMO1. Although they differ considerably in structure, the space filling models, Fig. 2, reveal some common features. First, in the projections shown, the width of the smaller substrates (thiourea, phenylthiourea, methimazole, and \( \text{N,N}-\text{dimethylaniline} \)) is no more than 4.2 Å, and other substituents on the heteroatoms are limited to methyl groups or hydrogens. This suggests that these substrates could readily contact the 4a-hydroperoxy flavin located at the bottom of a substrate channel (or cleft) at least 5 Å in diameter. The depth of the substrate site cannot be estimated with any precision, but it appears to accommodate the side chains of imipramine, orphenadrine, and chlorpromazine. Although phenothiazine is \( S \)-oxygenated by pig FMO1 (Nagata et al., 1990), activity with human FMO1 could not be detected at the highest concentrations soluble in the assay medium. The sulfur atom in phenothiazine, like that of 1,3-diphenylthiourea, is apparently excluded from the catalytic site by bulky groups adjacent to the sulfur atom. On the other hand, the side chain tertiary amine group of chlorpromazine is accepted by human FMO1 as are those of the

![Fig. 2. Space filling models of the compounds in Fig. 1.](image)
structurally similar imipramine and orphenadrine. From the dimensions in Fig. 2, it appears that the hydrophenoxylflavin in human FMO1 lies no more than 5 Å below the surface of amino acid side chains that control access of nucleophiles into the substrate site.

Although activity of ranitidine with human FMO1 was not measured directly, the dimensions of the molecule shown in Fig. 2 suggest that human FMO1 can catalyze N-oxygenation but not S-oxygenation of ranitidine. The substituents on sulfur in ranitidine like those adjacent to sulfur in 1,3-diphenylthiourea would prevent contact of sulfur in these compounds with the enzyme-bound 4a-hydroperoxy flavin. The dimensions of the side chain bearing the N,N-dimethyl group suggest that ranitidine should be a substrate not only for human FMO1 but also for FMO3 or any other isoform that accepts methimazole as a substrate.

Although size restrictions limiting access of nucleophiles into the catalytic sites of FMO isoforms can serve as a guide for predicting substrate activity, considerable caution must be exercised. Except for relatively rigid molecules such as sulindac sulfide (Light et al., 1982), most xenobiotics are flexible molecules that can assume different shapes in solution, and it is quite possible that only a very minor form would be responsible for substrate activity.

Despite these obvious limitations, the studies reported in this article indicate that size limitations may serve as an initial guide for predicting substrate activity in humans of a specific xenobiotic nucelophile bearing bulky substituents. The data also show that human FMO1 only catalyzes the oxidation of xenobiotics considerably smaller than those accepted by pig or guinea pig FMO1.

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