# Flavin-Containing Monooxygenase Isoform Specificity for the N-Oxidation of Tamoxifen Determined by Product Measurement and NADPH Oxidation

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**ABSTRACT: The $K_m$ value for tamoxifen is 1.2 mM for mouse FMO1 (human FMO1 is not expressed in adults) and 1.4 mM for human FMO3, with no detectable activity being expressed toward tamoxifen by FMO5 from either mouse or human. These data are derived from experiments using $^3$H-tamoxifen as substrate in which the product, tamoxifen *N*-oxide, was measured directly. It was not possible to derive meaningful data from the measurement of NADPH consumption because *Escherichia coli* preparations, in the presence of tamoxifen, regardless of whether the *E. coli* was expressing an FMO isoform, consumed large amounts of NADPH without the appearance of tamoxifen *N*-oxide or other discernable product.** © 2000 John Wiley & Sons, Inc. J Biochem Toxicol 14: 118–120, 2000

KEYWORDS: Flavin-Containing Monooxygenase; FMO; FMO Isoforms; Tamoxifen; Human; Mouse.

## INTRODUCTION

Tamoxifen is a widely used anti-estrogenic drug for the treatment of breast cancer, which may also have a role as a breast cancer chemopreventive agent [1]). However, epidemiological studies have demonstrated that tamoxifen is associated with a small increase in the incidence of endometrial cancers. Although the mechanism of carcinogenic activity is not fully understood, tamoxifen metabolites, which bind covalently to protein [2–4] and to DNA [5–8] have been identified. Most recently, nearly 50% of women treated with tamoxifen were demonstrated to have tamoxifen-DNA adducts in endometrial tissue, which established the potential genotoxicity of this drug [9].

Four major metabolites of tamoxifen are the result of oxidative metabolism by cytochrome P450 (CYP) and flavin containing monooxygenases (FMO). These metabolites include *N*-desmethyl-tamoxifen, 4-OH-tamoxifen, tamoxifen-*N*-oxide, and 3,4 dihydroxytamoxifen. Metabolism studies using expression systems have demonstrated that *CYP3A* is primarily responsible for the formation of *N*-desmethyl-tamoxifen [10–11] and that *CYP2D6* is solely responsible for the production of 4-OH-tamoxifen (the putative active anticancer tamoxifen metabolite) [12]. Studies using heat inhibition and antibodies to NADPH-P450 reductase showed that FMOs were primarily responsible for the production of tamoxifen-*N*-oxide [13], although the specific isoforms involved in its formation were not identified. The purpose of the present study was to determine, using mouse and human isoforms of FMO, which isoforms had the greatest involvement in the production of tamoxifen-*N*-oxide.

## MATERIALS AND METHODS

### Tamoxifen Incubations

*Escherichia coli* JM109 cells transformed with pJL-2 (pJL) and human FMO isoforms 3 (pJL-FMO3h) and 5 (pJL-FMO5h) were provided by R.M. Philpot (NIEHS, Research Triangle Park). The isolation and characterization of mouse FMO isoforms 1 (pJL-FMO1m) and 5 (pJL-FMO5m) were previously described by Cherrington et al. [14]. Briefly, single colonies were added to 5 mL of LB-ampicillin (50 μg/mL; LB-Amp) for overnight growth at 37°C. These cultures were added to 500

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**TABLE 1.** NADPH Consumption of *E. coli* Preparations in the Presence of Tamoxifen

| *E. coli Preparation* | *NADPH Consumption (nmole/min/mg protein)* |
|---|---|
| Empty | |
| + tamoxifen | 16.4 |
| − tamoxifen | 0.6 |
| Expressing FMO1 | |
| + tamoxifen | 14.7 |
| − tamoxifen | 1.8 |

**TABLE 2.** Tamoxifen Michaelis Constants for FMO Isoforms Expressed in E. Coli

| | *FMO1* | *FMO3* | *FMO5* |
|---|---|---|---|
| Mouse | 1.2 mM | Inactive[b] | Inactive[b] |
| Human | [a] | 1.43 mM | Inactive[b] |

[a]FMO1 is a fetal isoform in humans and is not expressed in adult human liver.
[b]No activity at substrate concentrations up to 1.5 mM

mL LB-Amp and grown at 37°C for 3 hours until an $OD_{600}$ reading of 0.5 was obtained. After the cultures were cooled to room temperature, isopropyl-thio-β-D-galactoside was added to a final concentration of 1 mM, and the cultures grown overnight at 30°C with shaking at 150 rpm. Subcellular fractions were then prepared as described by Lawton and Philpot [15]. Protein content of the microsomal fraction was determined using the BCA protein kit.

NADPH oxidations were performed essentially as described previously [16] using tamoxifen as substrate. Briefly, sample and reference cuvettes contained a 1 mL reaction mixture containing 0.1 M Tricine/KOH, (pH 8.4), 0.1 mM EDTA, 0.06 mM DTT, 0.1 mM NADPH, 50–200 μg microsomal protein, and 100 nmole tamoxifen (sample cuvette only). The incubation mixture was measured at 37°C for 2 minutes using a Shimadzu double beam spectrophotometer.

Tamoxifen metabolism was also monitored using [$^3$H] tamoxifen. One mL incubations were conducted in 13 × 100 mm test tubes containing 100 μg microsomal protein, 0.1 M Tricine/KOH (pH 8.4), 0.1 mM EDTA, 0.1 mM NADPH, and [$^3$H ]tamoxifen (80,000 dpm and 25–200 nmole) in 5 μl ethanol. Reactions were initiated by adding NADPH, and incubations were conducted for 1 hour at 37°C. The reactions were terminated by the addition of 1 mL methanol, and the incubate evaporated at room temperature under a stream of nitrogen. The residue was brought up in 100 μl methanol and applied to Whatman silica gel plates (LK5DF), which were developed in chloroform:methanol:ammonium hydroxide (80:20:0.5) as described by Mani et al. [10]. Lanes on the plates were scraped in 1 cm sections and counted by liquid scintillation. The only radioactive peak on the plate other than tamoxifen was identified as tamoxifen *N*-oxide by cochromatography with synthetic radioinert tamoxifen *N*-oxide.

## RESULTS AND DISCUSSION

Spectrophotometric determinations of NADPH oxidation activity have been used extensively in our laboratory to determine FMO activity toward a variety of substrates. Previous studies examining the stoichiometry of substrate addition, NADPH oxidation, and $O_2$ consumption have validated this quantitation method for a variety of FMO substrates [17,18]. Use of this method in the case of tamoxifen incubations with *E. coli*-expressed FMOs, however, resulted in high rates of NADPH oxidation that were independent of whether an FMO isoform was present in the incubation (Table 1). Closer analysis of the products of the reaction, using radiolabeled tamoxifen, revealed that in the absence of FMO, no product was formed. Our speculation is that *E. coli* proteins are involved in futile cycling of NADPH in the presence of tamoxifen.

In order to determine the rate of *N*-oxide formation, radiolabeled tamoxifen was incubated with each FMO isoform. Product formation was determined by counting radioactive spots on thin-layer plates. The only metabolite observed in any of these assays was tamoxifen-*N*-oxide. The rate of *N*-oxide formation, at the same concentration of tamoxifen used in the NADPH oxidation assays, was 4.5 nmole/min/mg protein; approximately 30% of the NADPH oxidation rate. $K_m$ values for tamoxifen using mouse and human isoforms 1, 3, and 5 are shown in Table 2. The $K_m$ for tamoxifen was 1.2 mM for mouse FMO1 and 1.4 mM for human FMO3. No detectable activities toward tamoxifen were determined for FMO5 from either species. Since FMO1 is not expressed in adult human tissues, the majority of tamoxifen *N*-oxide formed in humans is likely to be the result of FMO3 expression.

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