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SHORT COMMUNICATIONS

The metabolism of tamoxifen by human liver microsomes is not mediated by cytochrome P450IID6

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Tamoxifen, a non-steroidal oestrogen receptor antagonist, is widely used to treat both early and advanced female breast cancer [1]. It is currently undergoing trials for the prophylaxis of this disease in high risk women [2]. However, tamoxifen is only partially effective as an anti-cancer agent with average response rates of 30–35% [3]. The drug is mainly given at a fixed dose of 20 mg/day but plasma concentrations of tamoxifen and its metabolites vary widely between patients [3]. Thus, differences in the pharmacokinetics and metabolism of tamoxifen may contribute to the extent to which patients respond to the drug.

The drug undergoes extensive hepatic metabolism by N-demethylation (principal route), N-oxidation and 4-hydroxylation (Fig. 1) [3]. At steady state, tumour biopsy concentrations of *N*-demethyltamoxifen are about twice those of tamoxifen, whereas 4-hydroxytamoxifen concentrations are only about 2% of those of the parent compound [4]. However, 4-hydroxytamoxifen is 100 times more potent as an oestrogen antagonist than tamoxifen [5] and may contribute to the clinical response.

There is evidence that tamoxifen is metabolized by the cytochrome P450 enzyme system [6]. P450IID6 represents one form of this superfamily of enzymes. It exists in polymorphic forms and has been shown to hydroxylate about 25 drugs [7]. About 8% of Caucasian populations are phenotypically poor metabolizers (PMs*) of these drugs because of an inherited absence of hepatic P450IID6.

Like virtually all of the drugs metabolized by P450IID6 tamoxifen is a lipid-soluble base [7]. Examples of P450IID6-mediated pathways include a number of aromatic hydroxylation reactions but there is no clear example of N-dealkylation [7]. Thus, it is feasible that P450IID6 catalyses the formation of 4-hydroxytamoxifen. If this were the case, PM phenotypes would have an impaired ability to form 4-hydroxytamoxifen and as a result they may show markedly less therapeutic response to the drug compared to the rest of the population (EMs) who have normal levels of hepatic P450IID6. Accordingly, the aim of our study was to define the role of P450IID6 in the metabolism of tamoxifen by human liver microsomes, using specific probes developed for this isozyme. First, tamoxifen metabolism was compared in liver microsomes from two phenotypic EMs and one PM. The ability of quinidine, a potent and apparently specific inhibitor of P450IID6 activity [8], to impair tamoxifen metabolism was then tested.

Materials and Methods

Tamoxifen citrate (batch No. CHK63057), its metabolites and droloxifen *N*-oxide were supplied by Dr P. Jank, Klinge Pharma (Munich, Germany). Other chemicals, biochemicals and solvents were obtained commercially and were of the highest grade available. Samples of human liver were obtained from three renal transplant donors with the approval of the Hospital Ethics Committee and the knowledge of the local Coroner. Clinical details and the phenotypes of the donors have been published previously [8, 9]. One liver was from a phenotypic P450IID6 PM and the other two livers from EM subjects (EM1 and EM2). Liver microsomes were prepared as described previously [8]. Microsomal protein concentration was measured by the method of Lowry *et al.* [10] using bovine serum albumin (fraction V) as the standard. The incubation mixture comprised 0.2 mL microsomal suspension (equivalent to 1.5 mg microsomal protein), 2 μL ethanol/acetone (1:1 v/v) containing tamoxifen (36 μM), 0.2 mL of 1.15% (w/v) KCl (in some experiments containing quinidine) and 0.4 mL of an NADPH-generating system dissolved in 0.2 M potassium phosphate buffer (pH 7.4). The NADPH-generating system consisted of 4 μmol of G6P, 0.4 μmol of NADP, 0.4 U of G6PD and 2 μmol of $MgCl_2$. All incubations were carried out in triplicate at 37° for 60 min in a shaking waterbath. The reaction was stopped by the addition of 10 μL 0.15 M NaOH.

After the addition of droloxifen *N*-oxide as internal standard (100 ng) samples were extracted (vortex, 5 sec) twice with methyl *t*-butyl ether (1 × 2 mL, 1 × 1 mL). The ether layer was removed and evaporated to dryness under nitrogen at room temperature. Samples pending analysis were stored in the dark at 4° before being reconstituted with a mixture of mobile phase and water (2:1 v/v, 1 mL). An aliquot (100 μL) of a 1:20 dilution of the reconstituted sample was injected onto the chromatograph. HPLC was performed using a stainless steel column (250 mm, 4.6 mm i.d.) containing 5 μ Hyperchrome spherisorb ODS1 reversed phase packing material fitted with a guard column packed with Nucleosil C_{18} and heated to 40° by a waterbath, a photochemical reactor, a fluorescence detector (Model 820FP, Jasco) and an integrator (Model SP 4270, Spectra Physics). The photochemical reactor was made from 3.3 metres of Teflon tubing (1.6 mm o.d., 0.5 mm i.d.) crocheted into 5 mm loops and wound around a lattice fixed at a distance of 8 cm from a low pressure UV lamp (Osram HNS 10 W/U) [11]. The excitation and emission wavelengths of the fluorometer were set at 260 and 375 nm, respectively. The mobile phase comprised 10 mM KH_2PO_4 (adjusted to pH 3.7 with orthophosphoric acid)/methanol/acetonitrile (2:3:5 v/v). Chromatography was performed isocratically at a flow rate of 1 mL/min. The retention times of tamoxifen, *N*-demethyltamoxifen, tamoxifen *N*-oxide, 4-hydroxytamoxifen, *N*-demethyl-4-hydroxytamoxifen and droloxifen *N*-oxide were 36.7, 29.6, 21.8, 18.1, 15.4 and 11.9 min, respectively.

Tamoxifen was initially used in the form of its base, which at a substrate concentration of 36 μM was found to contain small but significant amounts of compounds with identical HPLC retention times to those of authentic metabolites. Since only small quantities of tamoxifen

* Abbreviations: PM, poor metabolizer; EM, extensive metabolizer.

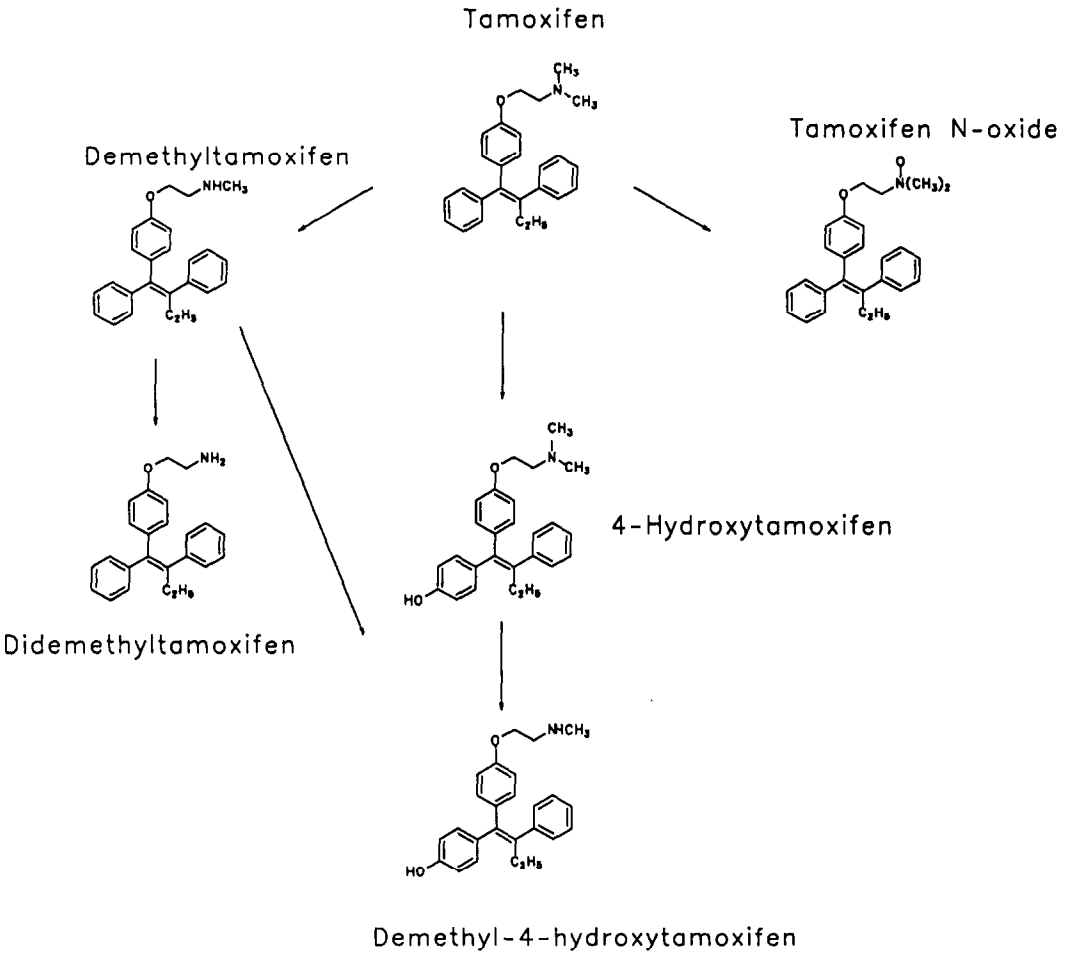

Fig. 1. The major routes of tamoxifen metabolism in humans.

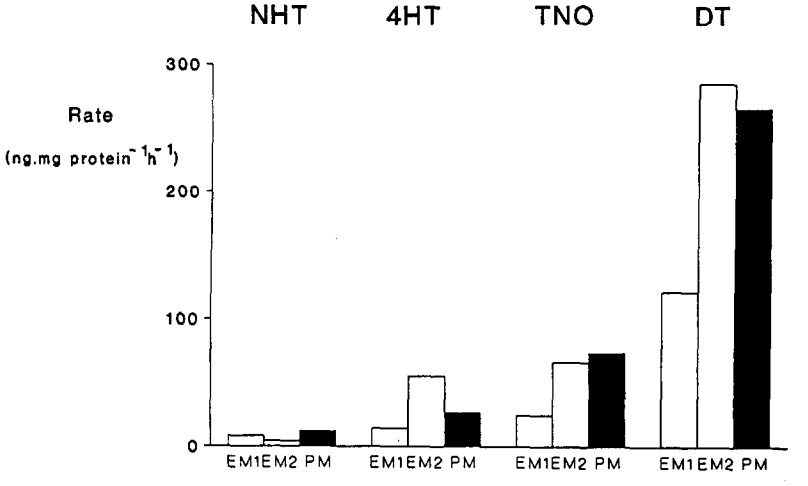

Fig. 2. Tamoxifen metabolism in liver microsomes from poor (PM) and extensive metabolizer (EM) subjects. Tamoxifen (36 μM) was incubated for 60 min with microsomes and an NADPH-generating system. Mean ± SD values (three incubations) for the rate of appearance of metabolites (ng/hr/mg protein) were: EM1 liver: 4-hydroxytamoxifen (4HT) 14 ± 8, tamoxifen *N*-oxide (TNO) 25 ± 11, *N*-demethyltamoxifen (DT) 121 ± 50, *N*-demethyl-4-hydroxytamoxifen (DHT) 8 ± 3; EM2 liver: 4HT 55 ± 28, TNO 66 ± 13, DT 286 ± 61, DHT 4 ± 1; PM liver: 4HT 27 ± 7, TNO 74 ± 17, DT 266 ± 68, D4HT 12 ± 6.

Table 1. The effect of quinidine on tamoxifen metabolism by liver microsomes from an extensive metabolizer (EM1)

Quinidine concentration (μM)	Rate of metabolite appearance (ng/mg protein/hr)			
	DT	TNO	4HT	DHT
Control	142	20	16	6
0.1	108	41	14	8
1.0	147	30	12	12
10.0	240	40	18	4
100.0	58	27	9	ND

ND, not detected.

The values represent the means of two results from separate experiments.

DT, *N*-demethyltamoxifen; TNO, tamoxifen *N*-oxide; 4HT, 4-hydroxytamoxifen; DHT, *N*-demethyl-4-hydroxytamoxifen.

metabolites are produced by human liver microsomal cytochrome P450, such contamination seriously confounded the interpretation of metabolic data. Attempts at purification of the base by recrystallization under an atmosphere of argon were unsuccessful but it was eventually found that batch No. CHK 63057 of the citrate salt of tamoxifen did not contain detectable quantities of the contaminants and this was used for further studies. A further difficulty was the instability of tamoxifen metabolites. Although samples could be stored in the dark at 4° for up to 6 hr in the dry state without significant loss of metabolites, it was considered necessary to carry out the analysis as rapidly as possible after incubation.

Results and Discussion

Microsomes from all three livers converted tamoxifen to *N*-demethyltamoxifen, 4-hydroxytamoxifen, tamoxifen *N*-oxide and *N*-demethyl-4-hydroxytamoxifen (Fig. 2). N-Demethylation was quantitatively the most important route. If P450IID6 catalyses tamoxifen metabolism, significant impairment of oxidation by the liver from the phenotypic PM would be expected. The findings did not support this hypothesis as the rates of appearance of all four tamoxifen metabolites in PM microsomes were similar to or higher than those in EM liver microsomes. Quinidine, when incubated with EM1 liver microsomes, had no effect on tamoxifen *N*-oxide appearance and was only a weak inhibitor of the other pathways of metabolism (Table 1).

These results indicate that P450IID6 does not play a significant role in the metabolism of tamoxifen. Although the drug has some properties in common with other known substrates of P450IID6, the relationship between chemical structure and metabolism has not been defined precisely for this P450 isoenzyme [12, 13].

Recently Islam, *et al.* [14] have generated a molecular template for substrates of P450IID6 using interactive molecular graphics. Tamoxifen fits poorly into this template, an observation which supports our experimental findings. Although PMs are unlikely to differ in their response to tamoxifen as a result of impaired metabolism, cytochrome P450-mediated metabolism may still be a major determinant of the action of this anticancer drug. Thus, it is important to characterize further the role of individual human cytochrome P450 isoenzymes in the metabolism of tamoxifen. Since this work was performed, the IIIA P450 sub-family has been shown to catalyse the N-demethylation of tamoxifen [15].

In conclusion, our findings suggest that, although tamoxifen metabolism is catalysed by human liver microsomal enzymes, its metabolism is not mediated by P450IID6.

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REFERENCES

1. British National Formulary, British Medical Association of Great Britain, 1990.
2. Powles TJ, Tillyer CR, Jones AL, Ashley SE, Treleaven J, Davey JB and McKenna JA, Prevention of breast cancer with tamoxifen—an update on the Royal Marsden Hospital pilot programme. *Eur J Cancer* **26**: 680–684, 1990.
3. Buckley MM-T and Goa KL, Tamoxifen—a reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic use. *Clin Pharmacokinet* **37**: 451–490, 1989.
4. Daniel PC, Gaskell J, Bishop H, Campbell C and Nicholson R, Determination of tamoxifen and biologically active metabolites in human breast tumour and plasma. *Eur J Cancer Clin Oncol* **17**: 1183–1189, 1981.
5. Jordan VC, Colms MM, Rowsby L and Prestwick G, A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* **75**: 305–316, 1977.
6. Metzler NM, Stang P and Sternson LA, Influence of tamoxifen and its *N*-desmethyl and 4-hydroxy metabolites on rat liver microsomal enzymes. *Biochem Pharmacol* **33**: 115–123, 1984.
7. Lennard MS, Genetic polymorphism of sparteine/debrisoquine oxidation: a reappraisal. *Pharmacol Toxicol* **67**: 273–283, 1990.
8. Otton SV, Crewe HK, Lennard MS, Tucker GT and Woods HF, Use of quinidine inhibition to define the role of the sparteine/debrisoquine cytochrome P450 in metoprolol oxidation by human liver microsomes. *J Pharmacol Exp Ther* **247**: 242–247, 1988.
9. Shaw L, Lennard MS, Tucker GT, Bax NDS and Woods HF, Irreversible binding and metabolism of propranolol by human liver microsomes—relationship to polymorphic oxidation. *Biochem Pharmacol* **36**: 2283–2288, 1987.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Jank P, Kern D, Huber HJ and Stanislaus F, Direct determination of droloxifene using HPLC with pre-column switching, post-column photochemical derivatisation and fluorescence detection. In: *Proceedings of the Third European Congress of Biopharmaceutics and Pharmacokinetics, Freiberg*, pp. 511–520, 1987.
12. Meyer UA, Gut J, Kronbach T, Skoda RC, Meier UT, Catin T and Dayer P, The molecular mechanisms of two common polymorphisms of drug oxidation—

evidence for functional changes in cytochrome P-450 isozymes catalysing bufuralol and mephenytoin oxidation. *Xenobiotica* **16**: 499–464, 1986.
13. Van Acker SA, Kaymans L, Donne-Opden Kelder GM, te Kopple JM and Vermeulen NPE, Mapping the active site of cytochrome P450IIDI. *Pharmaceut Weekblad* **12**(Suppl. B): B9, 1990.
14. Islam SA, Wolf CR, Lennard MS and Sternberg MJE, A three-dimensional molecular template for substrates of human cytochrome P450 involved in debrisoquine hydroxylation. *Carcinogenesis*, in press.
15. Jacolot F, Simon T, Dreano Y, Beaune P, Riche C and Berthou F, Identification of the cytochrome P450 IIIA family as the enzymes involved in the N-demethylation of tamoxifen in human liver microsomes. *Biochem Pharmacol* **41**: 1911–1919, 1991.

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Partial agonistic activity of two irreversible β-adrenergic receptor ligands, bromoacetylated derivatives of alprenolol and pindolol

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Irreversible antagonists are required for classical pharmacological determinations of agonist affinity and the assessment of non-linear receptor–effector coupling (formerly referred to as "spare receptors") [1]. Irreversible antagonists have also been used in studies of receptor metabolism [2]. The assessment of non-linear receptor–effector coupling is especially important in studies of β-adrenergic receptors since agonist potency and signal amplification may vary by several orders of magnitude among model systems [1].

Many irreversible probes for β-adrenergic receptors have been utilized but most of them have low affinity for β-adrenergic receptors and non-specific interactions with other cellular proteins [3, 4]. Bromoacetylated derivatives of alprenolol (BAAM*) and pindolol (BIM) were introduced as high-affinity, irreversible antagonists for the β-adrenergic receptor [4–6]. Because alprenolol and pindolol have considerable partial agonistic activity [7, 8], it is conceivable that BAAM and BIM may also be partial agonists. Such partial agonism might undermine the utility of BAAM and BIM as irreversible probes of β-adrenergic receptors.

The present study was designed to determine whether BAAM and BIM possess partial agonistic activity. For this purpose we have employed a recently developed biochemical assay in established cell lines using forskolin to amplify the stimulation of cAMP accumulation by partial agonists [7, 8].

Materials and Methods

Wild-type (strain 24.3.2) S49 lymphoma cells were maintained in suspension culture as described [7]. BC_3H_1 smooth muscle-like cells were grown as monolayers as described [8], and experiments were conducted after 4–6 days of confluence (≈10^6 cells/35 mm dish).

To determine cAMP accumulation, S49 cells were incubated at 37° for 5 min with the indicated drugs in the presence or absence of 1 μM forskolin, and intracellular cAMP accumulation was assayed as described previously [7]. A similar procedure was used with BC_3H_1 cells, as described previously [8]. The generated cAMP was measured with a competitive protein binding assay as described [7].

Bromoacetylated derivatives of alprenolol (BAAM: N^8-(bromoacetyl)-N^1-[3-[(*o*-allylphenyl)oxy]-2-hydroxypropyl]-(Z)-1, 8-diamino-*p*-menthane) and pindolol (BIM: N^8-(bromoacetyl)-N^1-[3-(4-indolyloxy)-2-hydropropyl]-(Z)-1, 8-diamino-*p*-menthane) were provided by Dr. Josef Pitha (Gerontology Research Centre, National Institute of Aging, The Francis Scott Key Medical Center, Baltimore, MD).

The maximal stimulation of cAMP accumulation and the $-\log EC_{50}$ were calculated by fitting the pooled experimental data to a sigmoid curve using the InPlot program (GraphPAD Software, San Diego, CA). Calculating the parameters from the pooled data does not allow standard error determination for the fitted parameters, but readers can estimate the confidence intervals from the raw data given in the figures.

Results and Discussion

Similar to alprenolol, pindolol, and many other weak partial agonists at β-adrenergic receptors [7, 8], BAAM and BIM did not cause detectable stimulation of cAMP accumulation in S49 lymphoma cells ([2] and data not shown). In the presence of 1 μM forskolin, however, BAAM and BIM stimulated cAMP accumulation (Fig. 1). The maximal enhancement of cAMP accumulation was similar to that previously reported for the respective parent compounds and was approximately 5% of that for the full agonist isoproterenol [7, 8]. The $-\log EC_{50}$ values for BAAM and BIM were 8.1 and 8.8, respectively (mean of five experiments each). These values are in good agreement with the apparent affinity of these drugs at β_2-adrenergic receptors in binding studies [2, 6]. Similarly, we have previously observed a good correlation between affinity and $-\log EC_{50}$ for stimulating cAMP accumulation in the presence of forskolin for a wide variety of other partial agonists at β-adrenergic receptors [8].

The partial agonistic activities of BAAM and BIM were not restricted to S49 lymphoma cells but were also seen in BC_3H_1 smooth muscle cells (Fig. 2). In this cell line we also found that the agonistic effects of BAAM and BIM on cAMP accumulation were blocked by the β-adrenergic receptor antagonist propranolol. The stimulation of cAMP accumulation by pindolol in S49 cells is also blocked by propranolol [7]. The facts that the potency for stimulating cAMP accumulation match well the affinity for β-adrenergic binding sites and that propranolol blocks cAMP

* Abbreviations: BAAM, bromoacetyl alprenolol menthane; and BIM, bromoacetyl indolyloxy methane.