THE AROMATASE INACTIVATOR 4-HYDROXYANDROSTENEDIONE (4-OH-A) INHIBITS TAMOXIFEN METABOLISM BY RAT HEPATIC CYTOCHROME P-450 3A: POTENTIAL FOR DRUG-DRUG INTERACTION OF TAMOXIFEN AND 4-OH-A IN COMBINED ANTI-BREAST CANCER THERAPY

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ABSTRACT:
Tamoxifen (tam), an anti-breast cancer agent, is metabolized into tam-N-oxide by the hepatic flavin-containing monooxygenase and into N-desmethyl- and 4-hydroxy-tam by cytochrome P-450s (CYPs). Additionally, tam is metabolically activated by hepatic CYP3A, forming a reactive intermediate that binds covalently to proteins. Tam and 4-hydroxyandrostenedione (4-OH-A) are currently used to treat breast cancer, and it has been contemplated that 4-OH-A be given concurrently with tam to contravene potential tumor resistance to tam. Because alterations in tam metabolism may influence its therapeutic efficacy, the effect of 4-OH-A on tam metabolism was examined. Incubation of tam with liver microsomes from phenobarbital-treated rats, in the presence of 4-OH-A (10–100 μM), resulted in marked inhibition of tam-N-demethylation and tam covalent binding and in decreased tam-N-oxide accumulation; however, there was no inhibition of the formation of 4-hydroxy-tam and of 3,4-dihydroxytamoxifen. These findings indicate that 4-OH-A inhibits CYP3A, but not P-450(s) that catalyze tam 4-hydroxylation. The diminished tam-N-oxide accumulation could be due to decreased N-oxide formation and/or due to increased N-oxide reduction. Incubation of tam-N-oxide with liver microsomes containing heat-inactivated flavin-containing monooxygenase demonstrated that 4-OH-A increases the accumulation of tam, possibly by diminishing its P-450-mediated metabolism. Kinetic studies indicate that 4-OH-A is a competitive inhibitor of CYP3A, but not a time-dependent inactivator. Consequently, the concurrent treatment of tam and 4-OH-A may result in increased tam half-life and thus could potentiate the therapeutic efficacy of tam and diminish the potential side effects of tam by inhibiting its covalent binding to proteins and possibly to DNA.

Tamoxifen (tam)1, a triphenylethylene, is currently the endocrine therapeutic agent of choice for all stages of breast cancer (Jordan, 1993; Chlebowski et al., 1993). Recent large scale clinical trials in the U. S. in women considered at risk of breast cancer demonstrated that tam reduced the incidence of breast cancer in 45% of the subjects (Fisher et al., 1998). However, the observations that tam treatment increases the incidence of endometrial cancer in breast cancer patients (Killacky et al., 1985; Fornander et al., 1989), causes hepatocarcinoma in animals (Fendel and Zinniski, 1992; Williams et al., 1993), and elicits a high frequency of p53 mutations (Vancutsem et al., 1994) are of concern.

Tam undergoes extensive metabolism yielding numerous metabolites, the major ones being the N-desmethyl-, 4-hydroxy- and N-oxide tam-derivatives (Fig. 1; Reunitz et al., 1984; McCague and Seago, 1986; Mani et al., 1993a). Also, smaller amounts of α-hydroxy-tam, α-hydroxy-tam-N-oxide, α-hydroxy-N-desmethyl-tam, 4-hydroxy-tam-N-oxide, 3,4-dihydroxy-tam, 3′,4′-dihydroxy-tam, and tam-1,2-epoxide have been observed (Reunitz et al., 1984; Poon et al., 1993; Lim et al., 1994; Phillips et al., 1994). In vitro studies demonstrated that N-desmethyl-tam and 4-hydroxy-tam (4-OH-tam) are formed by rat and human hepatic cytochrome P-450s (CYPs; Jacolot et al., 1991; Mani et al., 1993a) and that tam-N-oxidation is catalyzed primarily by the flavin-containing monooxygenase (FMO; Mani et al., 1993b). Although N-demethylation is catalyzed by CYP3A, 4-hydroxylation in rat liver is supported by a hitherto unidentified constitutive CYP. Chicken and chick embryo livers effectively catalyze tam-4-hydroxylation and 2,3,7,8-tetrachlorodibenzo-p-dioxin and β-naphthoflavone treatment of chick embryos markedly increased 4-hydroxylation through the induction of the P-450 2,3,7,8-tetrachlorodibenzo-p-di-

1 Abbreviations used are: tam, tamoxifen [Z (1-(4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-butene); FMO, flavin-containing monooxygenase; CYP, cytochrome P-450; PB, phenobarbital; 4-OH-A, 4-hydroxyandrostenedione; 4-OH-tam, 4-hydroxytamoxifen; [3H]SAM, [3H-methyl]-N-adenosyl-L-methionine; TLC, thin-layer chromatography; DTT, dithiothreitol; PB-microsomes, liver microsomes from PB-treated rats.
oxin isoform that catalyzes that reaction (Kupfer et al., 1994). Recently, it was demonstrated that 4-hydroxylation of tam in humans is primarily catalyzed by the hepatic CYP2D6 (Dehal and Kupfer, 1997; Crewe et al., 1997).

Additionally, it was established that tam undergoes metabolic activation, yielding a reactive intermediate that binds covalently to proteins, and that this reaction is catalyzed by CYP3A (Mani and Kupfer, 1991; Mani et al., 1994; Kupfer, 1996). Moreover, metabolically activated tam binds to DNA (Han and Liehr, 1992; White et al., 1992; Randerath et al., 1994; Kupfer, 1996). Recently, we demonstrated that 4-OH-tam and the subsequently produced catechol 3,4-di-OH-tam are the major route of metabolism leading to covalent binding to proteins (Dehal and Kupfer, 1996). However, it is not known whether the same reactive intermediate binds to proteins and DNA.

The therapeutic function of tam is thought to involve its inhibition of the estrogen-dependent tumor growth by acting as an antiestrogen at the estrogen receptor site (Fig. 2; Jordan, 1984). Surprisingly, a significant number of tumors lacking the estrogen receptor respond to tam therapy; however, the mechanism of this tam action is not understood. A second mode of breast cancer therapy that aims to reduce estrogen production by using inhibitors of the P-450 aromatase has also been conceived (Schwarzel et al., 1973; Brodie et al., 1977) and is now used (Coombes et al., 1984). A prototype of such an aromatase inhibitor is 4-hydroxyandrostenedione (4-OH-A), which acts as a potent and specific mechanism-based inhibitor of aromatase (Fig. 2), apparently without interfering with biosynthetic and metabolic pathways of other adrenal steroids (Brodie and Longcope, 1980; Brodie et al., 1981). Because the therapeutic function of tam may involve the action of its metabolites, particularly of the potent antiestrogen 4-OH-tam (Jordan et al., 1977; Borgna et al., 1981), and because of the possibility of initiation of combined or sequential application of tam and 4-OH-A in the treatment of breast cancer, it was deemed of interest to determine whether 4-OH-A affects tam metabolism. Additionally, it appeared that such a study would provide information on the potential for the metabolic drug-drug interaction between 4-OH-A and tam. The current investigation was undertaken with these considerations in mind.

Materials and Methods

NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, EDTA, catechol-O-methyltransferase and S-adenosyl-L-methionine iodide salt were purchased from Sigma Chemical Co. (St. Louis, MO). 4-OH-A was synthesized as described previously (Brodie et al., 1977). Dithiothreitol (DTT) was purchased from Calbiochem Corp. (La Jolla, CA). [14C-ring-labeled]tam citrate (21.1 mCi/mmol) is currently available only through custom synthesis. [3H-N-methyl]tam (85.6 Ci/mmol) and [3H-methyl]-S-adenosyl-L-methionine ([3H]SAM, 15 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). Phenobarbital (PB) sodium salt was obtained from Mallinckrodt, Inc. (St. Louis, MO). Ultima-Gold biodegradable scintillation fluid was purchased from Packard (Downers Grove, IL). Normal phase thin-layer chromatography (TLC) plates, containing fluorescent indicator and predesorbent strip, were purchased from Whatman, Inc. (Clifton, NJ). All other chemicals were of reagent grade quality and were used without further purification.

Animals and Treatment. Male Sprague-Dawley CD rats (90–100 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA) and were housed under controlled temperature (22°C) and light (12-h light/dark cycle; lights off at 7:00 PM). PB treatment (37.5 mg/kg i.p. in 0.2 ml of deionized water, twice daily) was for 4 days. Liver microsomes from PB-
treated rats (PB-microsomes) were prepared 12 h after the last dose. Control rats received the same injection regimen of the deionized water only.

**Preparation of Microsomes.** Microsomes were prepared by homogenization of the livers in 0.25 M sucrose (5 ml/g liver) and by differential centrifugation, as previously described (Kupfer and Dehal, 1966) and, unless stated otherwise, represent a pool of four to eight livers. The resulting microsomal pellet was resuspended in 1.15% aqueous KCl solution with a Potter-Elvehjem Teflon plunger/glass homogenizer and centrifuged at 105,000g for 1 h. The supernatant was discarded and the microsomal pellet was covered with −2.0 ml of KCl solution and stored at −70°C until use.

**Incubations and Tam Metabolism.** Incubations of the above microsomal pellets were carried out essentially as previously described (Kupfer and Dehal, 1966; Mani et al., 1994; Dehal and Kupfer, 1996). Briefly, the KCl solution covering the microsomal pellet was thawed and discarded and the pellet was suspended in fresh KCl solution using a Potter-Elvehjem homogenizer. Protein concentration was determined as previously described (Kupfer and Dehal, 1966). Incubations were conducted in 20-ml glass scintillation vials (open to air atmosphere) containing the following constituents: 0.6 ml of sodium phosphate buffer (pH 7.4, 60 mmol) containing EDTA (0.1 mmol); 0.1 ml of MgCl₂ (10 mmol); microsomal suspension (1.0 mg protein); and [14C]tam (200,000 dpm, 100 nmol, except when indicated otherwise) in 10 μl ethanol, in the presence or of in the absence of 4-OH-A. Deionized water was added to a final volume of 0.9 ml. After preincubation at 37°C for 2 min, the reaction was initiated by the addition of NADPH-regenerating system ([NADPH, 0.5 mmol; glucose-6-phosphate, 10 mmol; glucose-6-phosphate dehydrogenase, 2 IU] in 0.1 ml of sodium phosphate buffer (10 mmol, pH 7.4)) and incubated with shaking at 37°C for 60 min (unless stated otherwise). The enzymecatalyzed reaction was terminated by addition of 10 ml of ethanol.

The above aqueous ethanolic mixture was filtered through a 2.4-cm Whatman GF/C glass microfiber filter (Whatman, Ltd., Maidstone, Kent, England) in a filter holder (Schleicher and Schuell, Inc., Keene, NH) attached to a vacuum filter flask. The filter containing trapped protein precipitate was washed sequentially with ethanol (20 ml) and methanol (10 ml) followed by various other organic solvents to elute the loosely bound tam metabolites, as previously described (Mani and Kupfer, 1991; Mani et al., 1993; Dehal and Kupfer, 1996). To elute the proteins, the filter was placed in a 20-ml scintillation vial containing 2 ml of 2% aqueous SDS solution and incubated at 37°C for 2 h. The solution was transferred into a 12 × 75 mm glass culture tube. The vial containing the filter paper was rinsed with 1 ml of fresh 2% aqueous SDS solution. The combined SDS solution was processed as previously described (Mani and Kupfer, 1991; Dehal and Kupfer, 1996). An aliquot of the SDS solution was analyzed for radioactivity by scintillation spectrometry and the rest was used for protein determination. The covalent binding of activated tam metabolite(s) is expressed as nanomoles of tam equivalent bound per mg of protein in the SDS solution.

**Analysis of Tam Metabolites.** The combined ethanolic and methanolic filtrate (approximately 30 ml) from the above incubation was evaporated to dryness under a stream of nitrogen at ambient temperature. The residue was taken up in 2.0 ml of ethanol and the radioactivity of an aliquot (10 μl) in duplicate was determined in a Packard Tri-Carb 460 CD liquid scintillation spectrometer, using an automatic quench correction curve previously generated with a series of quenched [14C] and 3H standards. Routinely, 10 to 20% of the sample was used for chromatographic separation and quantification of metabolites on TLC and the rest was used for the subsequent isolation and identification of the metabolites. Chromatographic separation was performed on Whatman silica gel TLC plates and developed in CHCl₃:CH₃OH:NH₄OH (80:20:0.5; v/v/v), slightly modified from the system described previously (Reunitz et al., 1984). Radioabeled metabolites on TLC corresponding to authentic compounds (in adjacent lanes) were quantified with System 2000 Imaging Scanner (Bioscan, Inc., Washington, DC).

**Examination of Time-Dependent Inhibition by 4-OH-A.** To examine the possibility that 4-OH-A elicits a time-dependent inhibition of tam metabolism, 4-OH-A was preincubated with liver microsomes at 37°C for 30 min in the presence or absence of NADPH. Tam was added and incubations were conducted for 60 min as described above under Incubations and Tam Metabolism, and the rate of formation of the tam metabolites was determined as above.

**Catechol Formation.** Incubations were conducted in 20-ml glass scintillation vials open to air atmosphere with minor modifications (Dehal and Kupfer, 1996) of a previously described procedure for quantifying catechol estrogens as radiolabeled monomethyl ether (Hoffman et al., 1980; Kupfer et al., 1990). Microsomes (1.0 mg protein in 1.15% aqueous KCl) were added to sodium phosphate buffer (60 mmol, pH 7.4, 0.6 ml) containing EDTA (0.1 mmol), MgCl₂ (10 mmol, 0.1 ml), radioinactive tam or 4-OH-tam (25 or 100 μmol in 10 μl ethanol), DTT (50 nmol in 10 μl H₂O), [3H]SAM (1 μCi; 200 nmol in 12 μl H₂O), and water to a final volume of 0.9 ml. After a preincubation at 37°C for 2 min, the reaction was started by the addition of NADPH-regenerating system in a final volume of 1.0 ml. Values represent a mean of quadruplicate measurements. *p ≤ .01; **p ≤ .001, B, lineeweaver-Burk plot of the inhibition of covalent binding of [14C]tam by 4-OH-A to liver microsomal proteins from PB-treated male rats. [14C]Tam (100 nmol/200,000 dpm) and 4-OH-A were incubated with liver microsomes (1.0 mg protein) at 37°C for 60 min in the presence of NADPH-regenerating system in a final volume of 1.0 ml. Values represent a mean of duplicate measurements.

**FIG. 3.** A, inhibition by 4-OH-A of covalent binding of [14C]tam to liver microsomal proteins from PB-treated male rats.

A. Inhibition by 4-OH-A of covalent binding of [14C]tam to liver microsomal proteins from PB-treated male rats.
Results demonstrated a marked inhibition of tam covalent binding by ortholog of CYP3A4, which is the major P-450 in human liver. The model for human liver because CYP3A1, induced by PB, is an enzyme that catalyzes the metabolism of tam. To determine whether 4-OH-A, a mechanism-based aromatase inhibitor, exhibits an effect on the rate and extent of tam covalent binding to microsomal protein, incubations were conducted with [14C]tam and PB-microsomes in the presence of NADPH-regenerating system in a final volume of 1.0 ml. Values represent a mean ± S.D. of triplicate measurements. *p ≤ .05; **p ≤ .02; ***p ≤ .01; ****p ≤ .003.

Fig. 4. A, effect of 4-OH-A on metabolism of tam by liver microsomes from PB-treated male rats.

4-OH-A (Fig. 3A), suggesting a decrease in tam transformation into a reactive intermediate and/or interference with binding to the acceptor protein. A double reciprocal plot of the initial reaction rates of covalent binding versus tam concentration (Fig. 3B) suggested that the inhibition by 4-OH-A was competitive and that the apparent Ki was 21 μM. Furthermore, 4-OH-A inhibited the N-demethylation of [14C]tam by PB-microsomes (Fig. 4A), indicating that CYP3A is inhibited by 4-OH-A (previous studies demonstrated that N-demethylation of tam is catalyzed primarily by CYP3A (Jacolot et al., 1991; Mani et al., 1993a, 1994). The inhibition of N-demethylation of tam by 4-OH-A appeared to be competitive (Fig. 4B), the Ki being approximately 20 μM. Tam-N-oxide accumulation was also diminished by 4-OH-A (Fig. 4A); the decrease was statistically significant at 25, 50, and 100 μM. In contrast, 4-OH-tam accumulation was not inhibited. The slight increase (not statistically significant) in 4-OH-tam formation was probably due to inhibition of tam N-demethylation, thus providing a larger concentration of unaltered tam.

In incubations of 4-OH-tam, there was a small decrease in catechol formation by 4-OH-A. However, there was a small increase by 4-OH-A of tam-catechol (3,4-di-OH-tam) accumulation derived from tam incubation, but that increase was statistically significant only at 100 μM 4-OH-A (Fig 5). The elevation of the tam-catechol could be either due to increased concentration of 4-OH-tam, the precursor of catechol formation, and/or inhibition of the consumption of the catechol in the covalent binding reaction. Interestingly, there was a small inhibition of tam-catechol accumulation derived from 4-OH-tam; this effect was probably due to inhibition by 4-OH-A of the CYP3A-mediated 3-hydroxylation of 4-OH-tam. Previously, we demonstrated that CYP3A catalyzes effectively the ortho-hydroxylation of a variety of phenols (Stresser and Kupfer, 1997; S.S.D and D.K., in preparation).

To determine whether 4-OH-A, a mechanism-based aromatase in-
hibitor, is also an inactivator of the P-450s that catalyze tam metabolism, 4-OH-A was preincubated for 30 min with PB-microsomes in the presence of NADPH, followed by incubation of tam. The preincubation of 4-OH-A did not enhance the inhibition of tam metabolism that is normally observed by coincubation of tam with 4-OH-A (not shown), indicating that 4-OH-A is not a mechanism-based inactivator of the P-450s catalyzing tam metabolism.

To ascertain whether 4-OH-A stimulates the reduction of the N-oxide back to tam by P-450s, we performed experiments in which 4-OH-A was added to rat liver microsomes in the absence of the ambiguity from the simultaneous FMO-catalyzed N-oxidation, FMO was inactivated by heating the hepatic microsomes at 50°C for 90 s before incubation with [14C]tam or [14C]tam-N-oxide. The heat treatment essentially eliminated the FMO activity as seen from the diminished tam-N-Oxide. Values represent a mean ± S.D. of triplicate measurements. * Also, liver microsomes were preheated at 50°C for 90 s (to inactivate FMO). A, [14C]tam-N-oxide and 4-OH-A (50 μM) were incubated with liver microsomes (1.0 mg protein) at 37°C for 60 min in the presence of NADPH-regenerating system, in a final volume of 1.0 ml. B, [14C]tam was incubated as above in (A), however, without 4-OH-A. T-NO, N-desmethyl-tam; T-NO= tam-N-Oxide. Values represent a mean ± S.D. of triplicate measurements. * Liver microsomes were exposed to 50°C for 90 s. ** indicates the presence of NADPH, as seen from the diminished tam-N-Oxide accumulation of tam by reduction of the N-oxide back to tam and/or by P-450-mediated reduction of tam-N-oxide. In addition, tam and 4-hydroxy-tam were a gift from ICI Pharmaceuti-

**Fig. 6. Effect of 4-OH-A on tam-N-oxide or tam metabolism by liver microsomes from PB-treated male rats.**

When so indicated, liver microsomes were preheated at 50°C for 90 s to inactivate FMO. A, [14C]tam-N-oxide and 4-OH-A (50 μM) were incubated with liver microsomes (1.0 mg protein) at 37°C for 60 min in the presence of NADPH-regenerating system, in a final volume of 1.0 ml. B, [14C]tam was incubated as above in (A), however, without 4-OH-A. T-NO, N-desmethyl-tam; T-NO= tam-N-Oxide. Values represent a mean ± S.D. of triplicate measurements. * Liver microsomes were exposed to 50°C for 90 s. ** indicates the presence of NADPH, as seen from the diminished tam-N-Oxide accumulation of tam by reduction of the N-oxide back to tam and/or by P-450-mediated reduction of tam-N-oxide. In addition, tam and 4-hydroxy-tam were a gift from ICI Pharmaceuti-

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