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Advances in Brief

Tamoxifen Metabolic Activation: Comparison of DNA Adducts Formed by Microsomal and Chemical Activation of Tamoxifen and 4-Hydroxytamoxifen with DNA Adducts Formed *in Vivo*[1]

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Abstract

One of our laboratories recently showed by ^{32}P-postlabeling that administration of tamoxifen to mice induces two groups of hepatic DNA adducts comprising two major spots, nos. 3 and 5, respectively. 4-Hydroxytamoxifen and α-hydroxytamoxifen appear to be the proximate metabolites of groups I and II adducts, respectively. The relative significance of these two adduct groups for tamoxifen carcinogenicity remains to be established. To determine the activation mechanism(s) of tamoxifen and 4-hydroxytamoxifen, *in vivo* adducts were compared by ^{32}P-postlabeling with adducts generated by microsomal or chemical activation *in vitro*. Microsomal activation of 4-hydroxytamoxifen and tamoxifen, respectively, in the presence of DNA and cumene hydroperoxide, induced two adducts, which mapped similarly to the corresponding *in vivo* adduct spots 3 and 5. Chemical oxidation of 4-hydroxytamoxifen with silver(II) oxide, followed by incubation of the product(s) with DNA, elicited the formation of a major spot (Q1), while tamoxifen itself did not react. Rechromatographic analyses revealed that *in vitro* fractions 3 and Q1 (from 4-hydroxytamoxifen) matched the major *in vivo* group I adduct fraction 3, consistent with the hypothesis that 4-hydroxytamoxifen is a precursor for adduct fraction 3 *in vivo*. The *in vitro* adduct fraction 5 (from tamoxifen) was identical to that formed *in vivo*, indicating that the metabolic pathway for the formation of group II adducts did not involve 4-hydroxytamoxifen. In conclusion, the results support a model where primary metabolites of tamoxifen undergo secondary metabolism to form DNA adducts, which are detected *in vivo* after treatment with tamoxifen or 4-hydroxytamoxifen.

Introduction

The antiestrogen TAM,[3] an important drug against breast cancer, is currently being tested as a chemopreventive agent (1, 2). The fact that TAM causes hepatic carcinomas in rats (reviewed in Ref. 3) and increased incidences of highly malignant endometrial cancers in women undergoing TAM therapy (4, 5) has raised concerns regarding its choice as a cancer prophylactic (3). TAM induces complex DNA-adduct profiles in liver and extrahepatic tissues of several rodent species, suggesting that genotoxic mechanisms significantly contribute to its hepatocarcinogenicity (Refs. 6–11; reviewed in Ref. 3). TAM requires metabolic activation to exert its genotoxic effects (3, 10–12). Metabolic studies on TAM using rodent and human liver microsomes have identified several metabolites including 4-OH-TAM, *N*-desmethyl-TAM, and TAM-*N*-oxide (13). In addition, α-hydroxytamoxifen has been detected in a rat liver microsomal system (14) and in hepatocytes (15) upon treatment with TAM, and metabolite E is present in the plasma of TAM-treated breast cancer patients (16). Recent studies have demonstrated that microsomal activation of either TAM itself or its metabolites, 4-OH-TAM and metabolite E, produces up to eight DNA adducts (10–12). However, the relationship between the adducts formed *in vitro* and the DNA modifications formed *in vivo* is poorly understood.

One of our laboratories showed that exposure of mice to TAM elicits formation of two distinct groups (I and II) of hepatic DNA adducts and that 4-OH-TAM, the hormonally active form of TAM (17), is the proximate metabolite involved exclusively in group I adduct formation (9). Furthermore, co-exposure to PCP markedly enhances group I adduct levels *in vivo* (8, 9). Relative amounts of the different groups of hepatic adducts in mice strongly depend on the route of administration (8, 9). To clarify the mechanisms of metabolic activation of TAM to groups I and II DNA-adduct precursors, we have used ^{32}P-postlabeling to compare the major *in vivo* TAM and 4-OH-TAM adducts with adducts generated *in vitro* by microsomal or chemical activation of these compounds.

Materials and Methods

Chemicals. Sources of TAM, 4-OH-TAM, PCP, and other chemicals have been reported previously (9), as have materials for ^{32}P-postlabeling (8, 9, 18).

Animal Treatment. Female ICR mice were treated i.p. with a combination of 4-OH-TAM (120 μmol/kg) and PCP (75 μmol/kg) or with TAM (120 μmol/kg) only, once daily for 4 days (9). Controls received TO or PCP dissolved in TO. Tissues were taken 24 h after the last dose and stored at −80°C until DNA isolation.

Microsomal Activation. Microsomes were prepared from the livers of female rats pretreated with phenobarbital (12). The procedure for microsomal drug activation in the presence of the cofactor CuOOH and DNA has been reported (10–12).

Chemical Activation. The method for chemical activation of TAM or 4-OH-TAM by silver(II) oxide to DNA-binding product(s) was identical to that used for silver(II) oxidation of DES to DES-quinone, followed by incubation of the oxidation product with DNA (19, 20), except that DES was replaced with TAM or 4-OH-TAM.

DNA Analysis. Liver DNA was isolated by a modified solvent extraction procedure and quantified spectrophotometrically (9). Adducts from 10 μg of DNA were analyzed by dinucleotide/monophosphate ^{32}P-postlabeling (18), as modified for TAM (8, 9).

Re- and Cochromatography. Major *in vivo* and *in vitro* DNA adducts were compared by isolation from TLC sheets and re- and cochromatography as detailed previously (9).

Results

Treatment of mice with 4-OH-TAM combined with PCP [to generate enhanced levels of group I adducts (9) for further analysis] led to the formation of group I DNA adducts (nos. 1–4) exclusively, the

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[3] The abbreviations used are: TAM, tamoxifen; 4-OH-TAM, 4-hydroxytamoxifen; metabolite E, (*Z*)-1,2-diphenyl-1-(4-hydroxyphenyl)but-1-ene; PCP, pentachlorophenol; CuOOH, cumene hydroperoxide; DES, diethylstilbestrol; TO, trioctanoin; RAL, relative adduct labeling.

major adduct fraction by far being no. 3 (Fig. 1*B*). These adducts were absent in animals treated only with vehicle (Fig. 1*A*) or PCP (8). TAM itself i.p. (9), rather than 4-OH-TAM, gave rise to both group I (nos. 1–4) and group II (nos. 5, 6, and 8–12) adducts (Fig. 1*C*).

Microsomal activation of 4-OH-TAM in the presence of CuOOH as cofactor and DNA produced one major adduct fraction, indicated as spot 3 in Fig. 1*F*, which migrated similarly to the *in vivo* fraction 3 (Fig. 1, *B* and *C*). Adduct fractions 3 and X in Fig. 1*F* appeared incompletely resolved but could be further separated by rechromatography in 0.6 M lithium chloride, 0.37 M Tris-HCl, 6.38 M urea (pH 8.0), with R_fs of 0.47 (spot 3) and 0.67 (spot X) (not shown). The RAL $\times$ 10^8 values of adduct fractions 3, X, and Y (Fig. 1*F*) were 516.6, 79.1, and 24.7, respectively, *i.e.*, fraction 3 amounting to 83% of total labeling. Chemical activation of 4-OH-TAM with silver(II) oxide and DNA produced one major (Q1) and one minor (Q2) adduct fraction (Fig. 1*E*), with RAL $\times$ 10^8 values of 200.0 and 7.5, respectively. Chemical activation of TAM itself did not result in any detectable adducts (Fig. 1*D*). Re- and cochromatography experiments (Fig. 2) revealed the principal *in vivo* group I adduct fraction 3 (Fig. 1, *B* and *C*) to be identical to adduct fraction Q1 (Fig. 1*E*) generated by chemical activation of 4-OH-TAM. In fact, the individual subcomponents of these adduct fractions were also indistinguishable chromatographically (Fig. 2, *C-E, Lanes b, c,* and *e*). Adduct fraction 3 produced by CuOOH-dependent microsomal activation of 4-OH-TAM (Fig. 2, *A-E, Lanes a*) matched the major subcomponent of fraction 3 induced *in vivo* (Fig. 2, *A-E, Lanes c* and *d*) and fraction Q1 (Fig. 2, *A-E, Lanes b*). Thus, when chromatography was performed in 0.37 M Tris-HCl, 0.37 M boric acid, 7.5 mM EDTA, 0.97 M sodium chloride, 6 M urea, pH 8.0 (Fig. 2*D*), *Lane a* appeared to contain one major spot that matched the fast-moving component of *Lane c*. On the other hand, with 0.15 M sodium bicarbonate, 1.8 M urea (pH 8.3) as the solvent (Fig. 2*E*), *Lane a* matched the slow-moving component of *Lane c*. A few minor additional products were formed by CuOOH-dependent microsomal activation of 4-OH-TAM (Fig. 2, *D* and *E*).

CuOOH-dependent microsomal activation of TAM itself produced major adduct fraction 5, which consisted of two partially resolved spots (combined RAL $\times$ 10^8 = 9.8) and two minor additional slow-moving spots (Fig. 1*G*). Re- and cochromatographic analysis showed *in vitro* fraction 5 to be identical to the corresponding *in vivo* group II TAM fraction 5, each of which comprised two subcomponents (Fig. 3, *A* and *B*, *Lanes a, c,* and *d*). These results and the chromatographic identity of the subcomponents were confirmed upon chromatography in the additional solvent systems used for adduct 3 analysis listed in the legend of Fig. 2 (data not shown). Adduct fraction 3 produced *in vitro* by microsomal activation of 4-OH-TAM with CuOOH (Fig. 1*F*), on the other hand, did not match the *in vivo* TAM adduct fraction 5 (compare Fig. 3, *A* and *B*, *Lanes b, c,* and *e*). TAM *in vivo* group II adduct 12 (Fig. 3, *C* and *D*, *Lanes c*) was distinct from adducts Y (*Lane a*) and Q2 (*Lanes b* and *d*) induced *in vitro* by microsomal and chemical activation, respectively, of 4-OH-TAM.

Discussion

The present study has examined mechanisms of formation of the major groups I and II DNA adducts induced by TAM *in vivo*. Our findings demonstrate that microsomal activation of the TAM metabolite, 4-OH-TAM, in the presence of CuOOH produced adduct fraction 3 (Fig. 1*F*), which was identical (Fig. 2) to the corresponding *in vivo* fraction (Fig. 1*B*). These results suggested strongly that *in vivo* metabolic activation of 4-OH-TAM is involved in the formation of the group I adduct fraction and that cytochrome P-450 (acting as a peroxidase; Refs. 10, 21, and 22) may play a role in the production of the ultimate DNA-binding metabolite of 4-OH-TAM. Furthermore, the fact that chemical

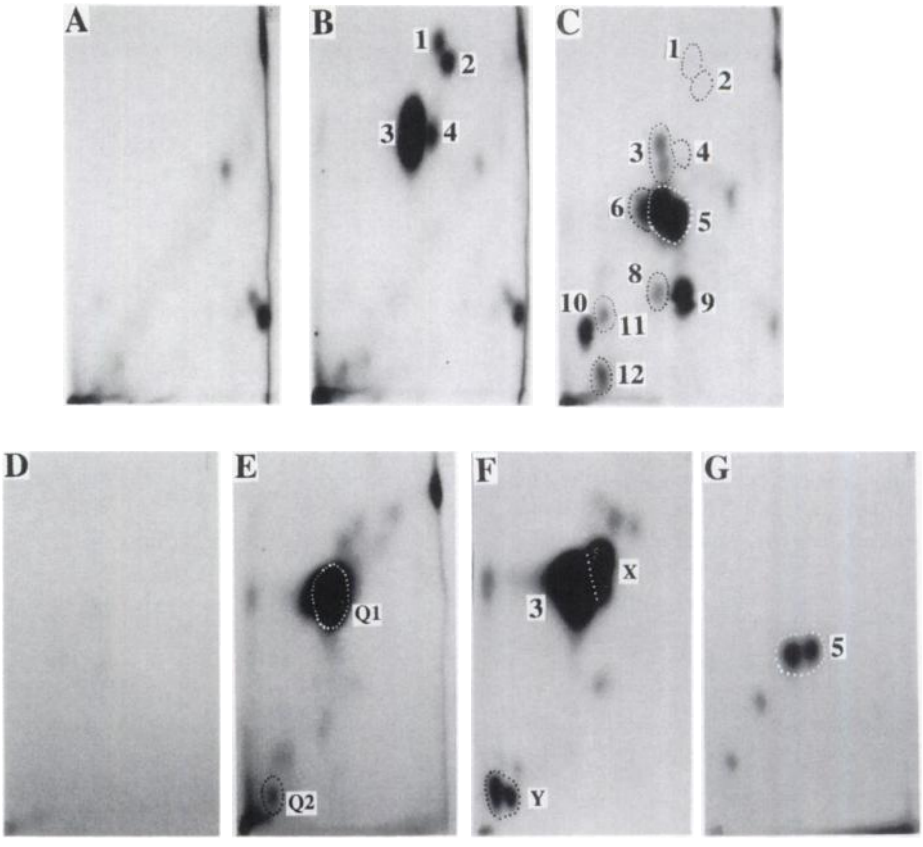

Fig. 1. Representative autoradiograms of TLC maps of ^{32}P-postlabeled DNA digests displaying TAM or 4-OH-TAM adducts obtained under various conditions. *A, B,* and *C,* adducts from liver DNA of individual mice exposed i.p. to vehicle (TO), PCP + 4-OH-TAM, and TAM, respectively; *D* and *E,* adducts from calf thymus DNA incubated with silver(II) oxide-reaction products of TAM (*D*) or 4-OH-TAM (*E*); *F* and *G,* adducts from microsomal activation of 4-OH-TAM (*F*) or TAM (*G*) in the presence of CuOOH. DuPont Cronex-4 X-ray film was used with intensifying screens at 23°C for 16 h (*A-C*) or 120 min (*D* and *E*) and at −80°C for 150 min (*F* and *G*). Detection of circled spots 1, 2, and 4 in *C* required exposure for up to 16 h at −80°C.

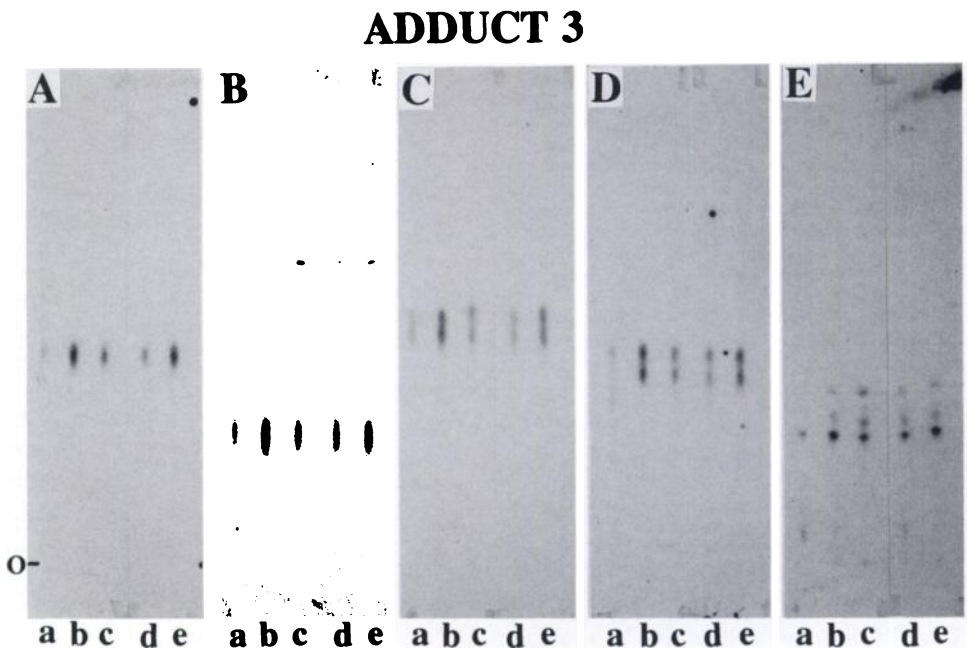

Fig. 2. Re- and cochromatographic comparison of 4-OH-TAM-induced DNA adducts. Isolated adducts (100 cpm) in 2–3 μl water were applied to origins of PEI-cellulose TLC sheets and resolved by one-dimensional chromatography to 14 cm above origin (*O*). Each lane corresponded to: (*a*) *in vitro* (microsomal) adduct fraction 3 (Fig. 1*F*); (*b*) (chemically synthesized) adduct fraction Q1 (Fig. 1*E*); (*c*) *in vivo* adduct fraction 3 (Fig. 1*B*); (*d*) mixture *a* and *c* (50 cpm each); and (*e*) mixture *b* and *c* (50 cpm each). The solvents were: 2.48 M lithium formate, 4.13 M urea, pH 3.35 (*A*); 0.42 M sodium hydrogen phosphate, 4.2 M urea, pH 6.4 (*B*); 0.6 M lithium chloride, 0.37 M Tris-HCl, 6.38 M urea, pH 8.0 (*C*); 0.37 M Tris-HCl, 0.37 M boric acid, 7.5 mM EDTA, 0.97 M sodium chloride, 6 M urea, pH 8.0 (*D*); and 0.15 M sodium bicarbonate, 1.8 M urea, pH 8.3 (*E*). Film exposure was at −80°C for 64 h using Kodak XAR-5 film with intensifying screens.

activation of 4-OH-TAM in the presence of silver(II) oxide produced major adduct fraction Q1 (Fig. 1*E*), which was also indistinguishable (Fig. 2) from adduct fraction 3 formed *in vivo* (Fig. 1*B*), specifically suggested that a 4-OH-TAM quinone methide derivative (Fig. 4) was the precursor of the major group I adducts *in vivo*.

Although the reaction product formed from 4-OH-TAM (an allylphenol; Fig. 4) by silver(II) oxide oxidation has not yet been identified, the following lines of evidence further support the notion of a quinonoid species as the ultimate electrophile: (*a*) silver(II) oxide oxidation of 4-OH-TAM, but not TAM (which lacks a phenolic hydroxyl group), yielded the adduct (Fig. 1*E*); (*b*) vinyl quinone methides are readily formed by silver oxide oxidation of allylphenols (23); (*c*) one of our laboratories has provided evidence that the TAM metabolite E, an allylphenol like 4-OH-TAM, is converted to a DNA-reactive quinone methide by silver oxide activation (11); (*d*) silver oxide treatment of DES, *i.e.*, an allylphenol structurally resembling 4-OH-TAM, yields DES quinone, an ultimate activated metabolite of DES *in vivo* (19, 20), presumably by an analogous mechanism.

As the formation of 4-OH-TAM, a major microsomal metabolite of TAM, is catalyzed by P450 *in vitro* in the presence of NADPH (13), a plausible mechanism (Fig. 4) of group I adduct formation *in vivo* is initial P450/NADPH-dependent conversion of TAM to 4-OH-TAM, followed by P450/peroxidase-mediated oxidation to 4-OH-TAM quinone methide as the ultimate electrophile. Potter *et al.* (24) previously hypothesized on chemical grounds that 4-OH-TAM quinone methide

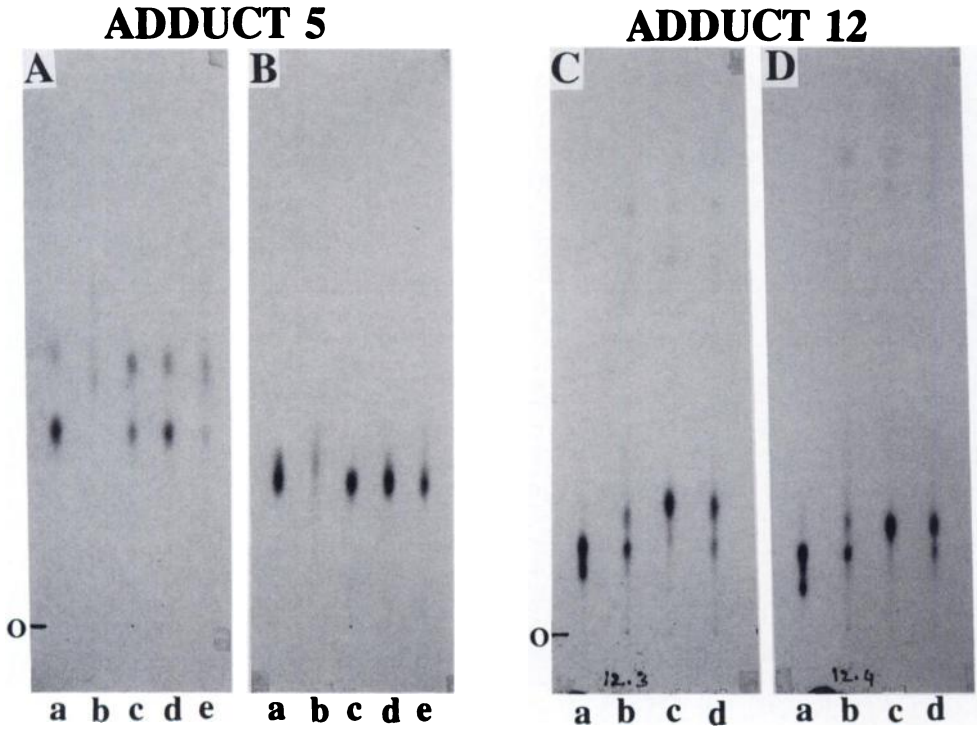

Fig. 3. Comparison of the major *in vivo* TAM group II adduct fractions 5 and 12 with different adduct fractions generated *in vitro* from TAM or 4-OH-TAM. Adduct fractions 5 (100 cpm) or 12 (250 cpm) were applied to the PEI-cellulose sheets. Adduct 5 comparisons: (*Lanes a*) *in vitro* adduct fraction 5 (Fig. 1*G*); (*b*) *in vitro* (microsomal) adduct fraction 3 (Fig. 1*F*); (*c*) *in vivo* adduct fraction 5 (Fig. 1*C*); (*d*) mixture *a* and *c* (50 cpm each); and (*e*) mixture *b* and *c* (50 cpm each). Adduct 12 comparisons: (*Lanes a*) *in vitro* adduct fraction Y (Fig. 1*F*); (*b*) fraction Q2 (Fig. 1*E*); (*c*) *in vivo* adduct fraction 12 (Fig. 1*C*); and (*d*) mixture *b* and *c* (125 cpm each). The solvents used for *A*, *B*, *C*, and *D* were the same as the corresponding ones in Fig. 2. Film exposure was at −80°C for 64 h using Kodak XAR-5 film with intensifying screens.

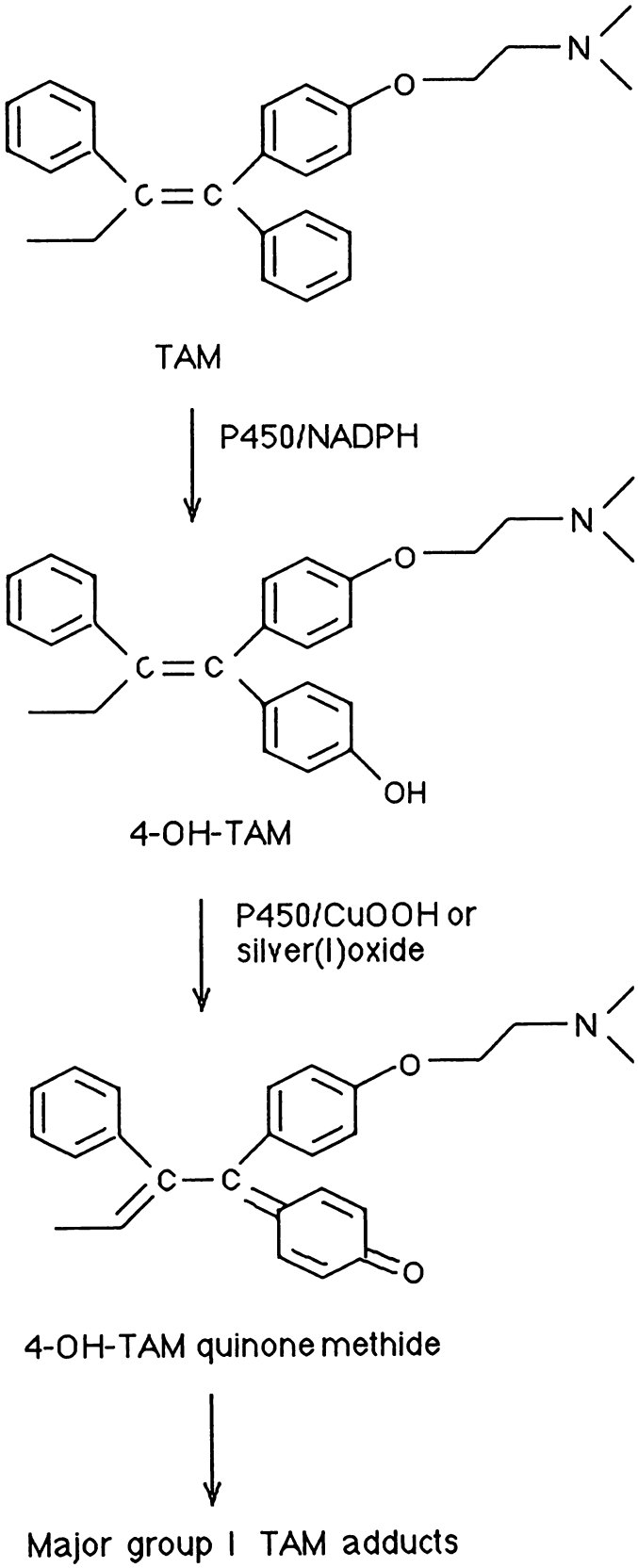

Fig. 4. Proposed mechanism of metabolic activation of TAM leading to group I adducts. 4-OH-TAM, a major TAM *in vivo* metabolite, is known to be formed by P450-catalyzed metabolism in the presence of NADPH (13). Based on our results, 4-OH-TAM is postulated to undergo peroxidative activation to a quinone methide as the ultimate electrophile responsible for group I adduct formation.

is a likely candidate for TAM-mediated genotoxicity, and the current experimental observations support this idea.

Although in confirmation of previous work (9), the intensities of group I adducts in mice were lower than those of group II after i.p. treatment with TAM (Fig. 1*C*), this is not the case when TAM is given p.o. (8), a route of administration that is currently used in women undergoing TAM therapy. In fact, four daily oral doses of TAM to mice lead to the formation predominantly of group I adducts (8), presumably owing to the rapid conversion of TAM to 4-OH-TAM in the gut (9). Since high specific activities of peroxidases are present in human endometrial tissues (25) and 4-OH-TAM accumulates in uteri of rodents exposed to TAM (26, 27), as well as in serum (27) and breast tissues (28) of women undergoing TAM treatment, 4-OH-TAM quinone methide-derived adducts may make a significant contribution to the heightened endometrial cancer risk in these women. Consonant with this hypothesis is the observation from one of our laboratories that 4-OH-TAM can be activated by horse radish peroxidase to form DNA adducts (10). The presence of such adducts in human tissues has not yet been demonstrated, however.

The mechanism of group I TAM adduct intensification in animals co-exposed to PCP is not understood but appears unrelated to inhibition of sulfotransferase activity (9). The recent observation in one of our laboratories[4] that PCP inactivates purified liver glutathione *S*-transferase activities *in vitro* suggests that the PCP-induced intensification of TAM group I adduct formation could occur as a consequence of reduced detoxication of 4-OH-TAM quinone methide to polar nonelectrophilic glutathione conjugates through this mechanism. The fact that allylphenol (*e.g.*, eugenol)-quinone methides have been shown to form glutathione adducts *in vitro* (29) lends support to this hypothesis.

Our finding that microsomal activation of TAM itself with CuOOH gave rise to adduct fraction 5, which cochromatographed with the major *in vivo* group II adduct fraction 5 (Fig. 3, *A* and *B*), demonstrates that microsomal activation led to the formation of this group of adducts also. One of our laboratories reported recently (9) that α-hydroxylation of the ethyl side chain of TAM followed by sulfate conjugation leads to group II adduct formation. According to Phillips *et al.* (15), α-hydroxytamoxifen itself is capable of reacting with DNA. As proposed by Potter *et al.* (24), TAM may undergo a P450-mediated hydride abstraction at the electron-rich α-position adjacent to the olefinic double bond of the molecule to yield an allylic radical carbocation that upon reaction with water gives rise to α-hydroxytamoxifen, the likely precursor of the major group II adduct fraction 5.

In conclusion, the results presented in this study strongly suggest that 4-OH-TAM quinone methide represents an ultimate DNA-binding species responsible for the formation of TAM group I adducts. The relative contribution of these adducts, in comparison with group II adducts, to the development of endometrial cancers and other adverse health effects (3) in women exposed to TAM remains to be explored.

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[4] B. Moorthy and K. Randerath, Pentachlorophenol enhances 9-hydroxybenzo-[a]pyrene-induced hepatic DNA adduct formation *in vivo* and inhibits microsomal epoxide hydrolase and glutathione S-transferase activities, *in vitro*: likely inhibition of epoxide detoxication by pentachlorophenol, manuscript in preparation.

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