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Advances in Brief

α-Hydroxytamoxifen, a Metabolite of Tamoxifen with Exceptionally High DNA-binding Activity in Rat Hepatocytes[1]

David H. Phillips,[2] Paul L. Carmichael, Alan Hewer, Kathleen J. Cole, and Grace K. Poon

Section of Molecular Carcinogenesis Haddon Laboratories [D. H. P., P. L. C., A. H., K. J. C.] and CRC Centre for Cancer Therapeutics [G. K. P.], Institute of Cancer Research, Cotswold Road, Sutton, Surrey SM2 5NG, England

Abstract

It has been proposed that the antiestrogen tamoxifen induces liver tumors in rats and genotoxic effects *in vitro* through metabolic activation involving, initially, α-hydroxylation of the ethyl group. To test this hypothesis, the extent of DNA adduct formation in primary rat hepatocytes treated with tamoxifen and α-hydroxytamoxifen was investigated. Hepatocytes from female Fischer F-344 rats were treated with 1 or 10 μM concentrations of either α-hydroxytamoxifen or tamoxifen. DNA was isolated and analyzed for the presence of DNA adducts by ^{32}P postlabeling. Chromatography on polyethyleneimine cellulose thin layer chromatography and reverse-phase high performance liquid chromatography revealed that the same pattern of adducts was formed by both compounds. However, the level of adduct formation was 25 and 49 times greater with α-hydroxytamoxifen than with tamoxifen at 1 and 10 μM, respectively. The formation of α-hydroxytamoxifen as a metabolite of tamoxifen was demonstrated by mass spectrometric analysis of the extracted culture medium. α-Hydroxytamoxifen was found to react with DNA in the absence of metabolizing enzymes. These results demonstrate the involvement of α-hydroxylation in the metabolic activation of tamoxifen.

Introduction

Tamoxifen[3] has proved to be effective in the treatment of breast cancer and in the prevention of contralateral breast cancer in women who have already developed a first tumor (1). This has led to the current plans to test the efficacy of tamoxifen in preventing primary breast cancer in healthy women with a family history of the disease (2). However, tamoxifen has been shown to be a potent hepatocarcinogen in rats (3, 4), and there is an increased incidence of endometrial cancer in breast cancer patients who have been treated with tamoxifen (5). These findings have led to differing opinions concerning the wisdom of using tamoxifen prophylactically (2, 6, 7). The controversy emphasizes the need for a thorough investigation of the mechanism(s) through which tamoxifen exerts these deleterious effects.

Evidence is accumulating that tamoxifen possesses genotoxic activity *in vivo* and *in vitro*. Tamoxifen gives rise to DNA adducts in the livers of rats when administered i.p. (8) or by gavage (9), and it induces micronucleus formation in MCL-5 cells (9, 10), a genetically engineered human cell line that expresses five human cytochrome P-450s and epoxide hydrolase (11). It has also been demonstrated that rat and human liver microsomal fractions metabolize tamoxifen to reactive intermediates that bind covalently to protein (12) and DNA (13). Furthermore, a single dose of as little as 0.3 mg/kg of tamoxifen to rats induced a significant frequency of aneuploidy and chromosome exchanges in rat hepatocytes (14).

There have been relatively few studies exploring the pathway(s) of metabolic activation of tamoxifen. However, the hypothesis has been advanced that activation of tamoxifen involves oxidation at the α position of the ethyl group (15). Evidence to support this pathway was recently obtained from studies on the deuterated compound [*ethyl*-D_5]tamoxifen, which showed reduced activity, compared to nondeuterated tamoxifen in two assays for genotoxicity (16). Its DNA binding activity in rat liver *in vivo* was significantly lower; also, it induced significantly fewer micronuclei in MCL-5 cells. These results can be explained in terms of a kinetic isotope effect, whereby metabolism at deuterated positions in a molecule occurs at a slower rate due to the greater bond energy of the C-D bond compared with the equivalent C-H bond (17). The use of [*ethyl*-D_5]tamoxifen did not, in itself, distinguish between the possibility of activation occurring at the α and β positions of the ethyl group. However, mechanistic considerations (15) and the fact that metabolism at the α position, but not the β position, has been demonstrated for both rats (18) and humans (19) strongly implicates the α position of the ethyl group as the critical site of metabolism of tamoxifen through which it is metabolically activated to genotoxic intermediates.

In the current study we demonstrate that α-hydroxytamoxifen is formed as a metabolite of tamoxifen in primary cultures of rat hepatocytes and that this compound gives rise to very high levels of DNA adducts in rat hepatocytes. Because the adducts are chromatographically indistinguishable from those formed in parallel experiments with tamoxifen itself, α-hydroxytamoxifen is deemed to be a major proximate carcinogenic metabolite of tamoxifen.

Materials and Methods

Chemicals. Tamoxifen was purchased from Sigma Chemical Co. (Poole, United Kingdom). α-Hydroxytamoxifen (20) was generously provided by Professor M. Jarman. Reagents and materials for ^{32}P postlabeling were obtained from the suppliers mentioned previously (9).

Isolation and Treatment of Rat Hepatocytes. Female Fischer F-344 rats, ages 8–10 weeks, were used. Livers were perfused *in situ* with solutions of collagenase and hepatocytes isolated according to standard procedures (21). Cell viabilities >80% were achieved, as measured by trypan blue exclusion. Six × 10^6 cells were added to 80-cm^2 tissue culture flasks in 20 ml Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 110 μg/ml sodium pyruvate, 100 μg/ml streptomycin sulfate, and 60 μg/ml benzylpenicillin. After 2–3 h in a humidified incubator at 37°C with a 5% CO_2 atmosphere, unattached cells were removed and the medium was replaced with one lacking fetal calf serum, but otherwise as above. Test compounds were added that were dissolved in DMSO (75 μl) and the cultures were incubated for an additional 18 h, after which the cells were harvested. DNA was isolated by a phenol/chloroform extraction procedure including Pronase and RNase digestions to remove protein and RNA, respectively (22).

Reaction of α-Hydroxytamoxifen with DNA. Salmon sperm DNA (500 μg) dissolved in 10 mM Tris, pH 7.4 (500 μl), was mixed with a solution of

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[1] Supported by the Cancer Research Campaign.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations and trivial names used are: tamoxifen, (*Z*)-1-{4-[2-(dimethylamino)ethoxy]phenyl}-1,2-diphenyl-1-butene; [ethyl-D_5]tamoxifen, [3,3,4,4,4-$^2H_5$]-(*Z*)-1-{4-[2-(dimethylamino)ethoxy]phenyl}-1,2-diphenyl-1-butene; α-hydroxytamoxifen, (*E*)-1-{4-[2-(dimethylamino)ethoxy]phenyl}-1,2-diphenyl-1-buten-3-ol; DMSO, dimethyl sulfoxide; TLC, thin layer chromatography; HPLC, high-pressure liquid chromatography.

α-hydroxytamoxifen (50 μg) in ethanol:DMSO (9:1; 150 μl) and the solution was then incubated at 37°C overnight. It was then extracted with 4 × 1 volume ether, and DNA was precipitated from the aqueous phase by the addition of 0.1 volume 5 M NaCl and 2 volumes ethanol.

^{32}P-Postlabeling Analysis. ^{32}P Postlabeling analysis, using the nuclease P_1 digestion method of sensitivity enhancement, was carried out as described previously (9). Labeled adducts were resolved by polyethyleneimine cellulose TLC (9) and by reverse-phase HPLC (23). For the latter, *cis*-9,10-dihydro-9,10-dihydroxyphenanthrene was used as an internal elution marker.

Mass Spectrometry. Media from hepatocyte incubations (5 ml) were extracted with 2% ethanol in hexane (2 × 5 ml). The organic fractions were combined and concentrated to dryness. The dry sample was reconstituted in acetonitrile (50 μl) and a 10-μl aliquot was used for analysis.

Liquid chromatography-mass spectrometry analysis was performed on a Finnigan MAT TSQ 700 mass spectrometer (San Jose, CA). Samples were introduced via a Waters 600 MS pump interfaced with an atmospheric pressure ionization source operated in electrospray ionization mode. Sample separation was achieved on an LC-18 column (150 x 4.6 mm; Supelco, Ltd., Poole, United Kingdom). The mobile phase consisted of 20 mM ammonium acetate (solvent A) and acetonitrile (solvent B). A linear gradient system was programmed to 20% solvent B for 4 min, 20–40% solvent B for 20 min, and 40–65% solvent B for 16 min, at a flow rate of 0.75 ml/min. The eluent (50%) was directed into the mass spectrometer. The capillary temperature and electrode voltage of the mass spectrometer were set at 220°C and −4.5 kV, respectively, with nitrogen as the drying gas. The full scan mass spectrum was acquired over the mass range of *m/z* 200–600 atomic mass units in the positive ion mode. Tandem mass spectrum for α-hydroxytamoxifen was produced by using argon gas in the collision cell at a pressure of 0.3 mtorr.

Results

When DNA isolated from rat hepatocytes that had been treated with tamoxifen was analyzed by ^{32}P postlabeling, the pattern of 3 major adduct spots on polyethyleneimine cellulose TLC plates shown in Fig. 1*A* was obtained. This pattern is similar to that previously obtained in experiments with DNA from the livers of rats that had been given tamoxifen by gavage (9, 16). A similar pattern of adduct spots was obtained in experiments with α-hydroxytamoxifen, except that adduct formation clearly occurred to a greater extent (Fig. 1*B*). For comparison, the result of analysis of DNA from control hepatocytes, treated with DMSO only, is shown in Fig. 1*C*.

To determine whether α-hydroxytamoxifen possessed any intrinsic reactivity toward DNA in the absence of any metabolizing systems, the compound was incubated with a solution of salmon sperm DNA, which was then analyzed by ^{32}P postlabeling. Thin layer chromatography revealed an adduct pattern, shown in Fig. 1*D*, similar to (but weaker than) that obtained in the experiments with DNA from hepatocytes (Fig. 1, *A* and *B*).

The extent of DNA adduct formation by tamoxifen and α-hydroxytamoxifen in hepatocytes, calculated from the amount of radioactivity detected in the adduct spots resolved on TLC (such as those shown in Fig. 1), is shown in Table 1. At the lower dose of 1 μM, the α-hydroxylated compound gave rise to 25 times the level of adducts formed by tamoxifen; at the higher dose (10 μM), DNA binding by α-hydroxytamoxifen was 49-fold higher than that by tamoxifen.

HPLC analysis of the major tamoxifen-DNA adduct thin layer chromatography spot (Fig. 1*A*, *Spot 2*) revealed that it contained two separable peaks of radioactive material (Fig. 2*A*), similar to previous

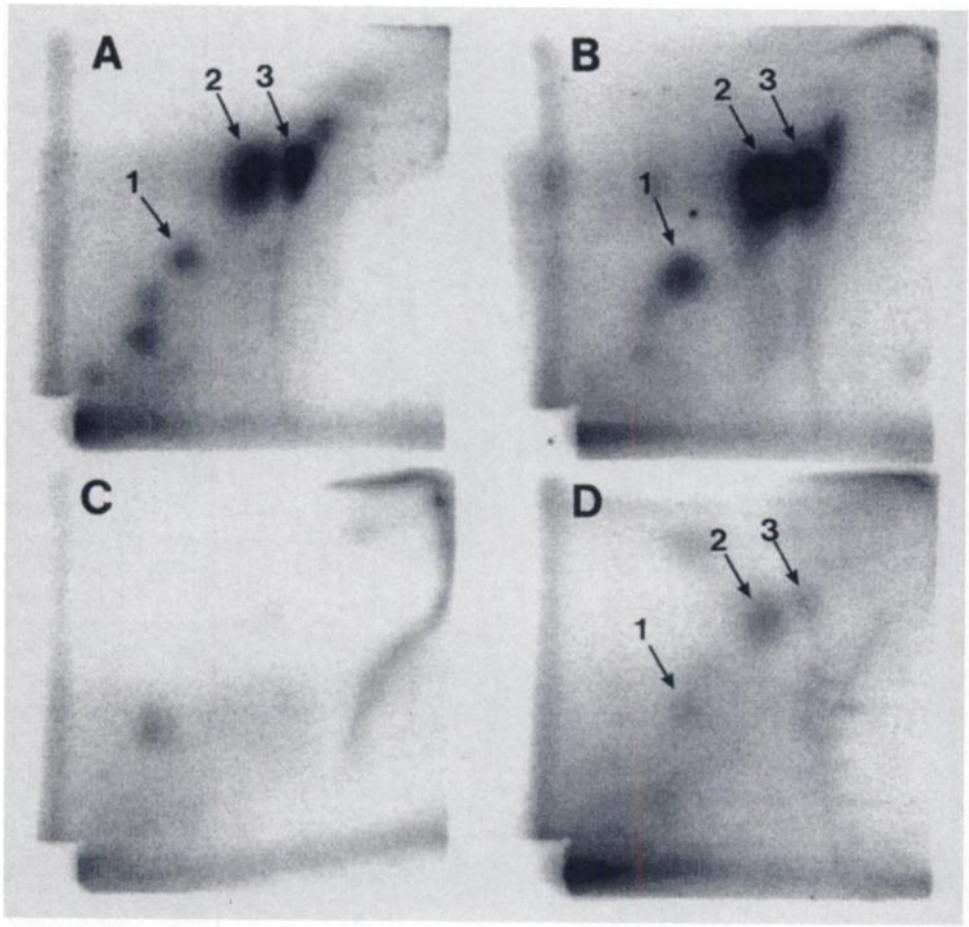

Fig. 1. Autoradiographs of the polyethyleneimine cellulose TLC maps of ^{32}P postlabeled digests of DNA: *A*, from rat hepatocytes treated with 10 μM tamoxifen; *B*, from rat hepatocytes treated with 10 μM α-hydroxytamoxifen; *C*, from control hepatocytes; *D*, from the reaction with α-hydroxytamoxifen *in vitro* in the absence of any metabolizing system. Major adduct spots are numbered.

Table 1 *DNA adduct levels formed in rat hepatocytes and in vitro*

Compound	Experimental system	Concentration (μM)	No. of determinations[a]	Adducts/10^8 nucleotides (mean ± SD)	Ratio tamoxifen: α-hydroxytamoxifen
Tamoxifen	Rat hepatocytes	1	3	18.7±7.5	
Tamoxifen	Rat hepatocytes	10	6	115.1±16.4	
α-Hydroxytamoxifen	Rat hepatocytes	1	2	468.5±15.5	1:25
α-Hydroxytamoxifen	Rat hepatocytes	10	4	5667±1008	1:49
α-Hydroxytamoxifen	Chemical reaction	[b]	3	16.7±1.8	

[a] In the case of experiments with hepatocytes, numbers represent independent experiments on different preparations of cells.
[b] For experimental conditions, see "Materials and Methods."

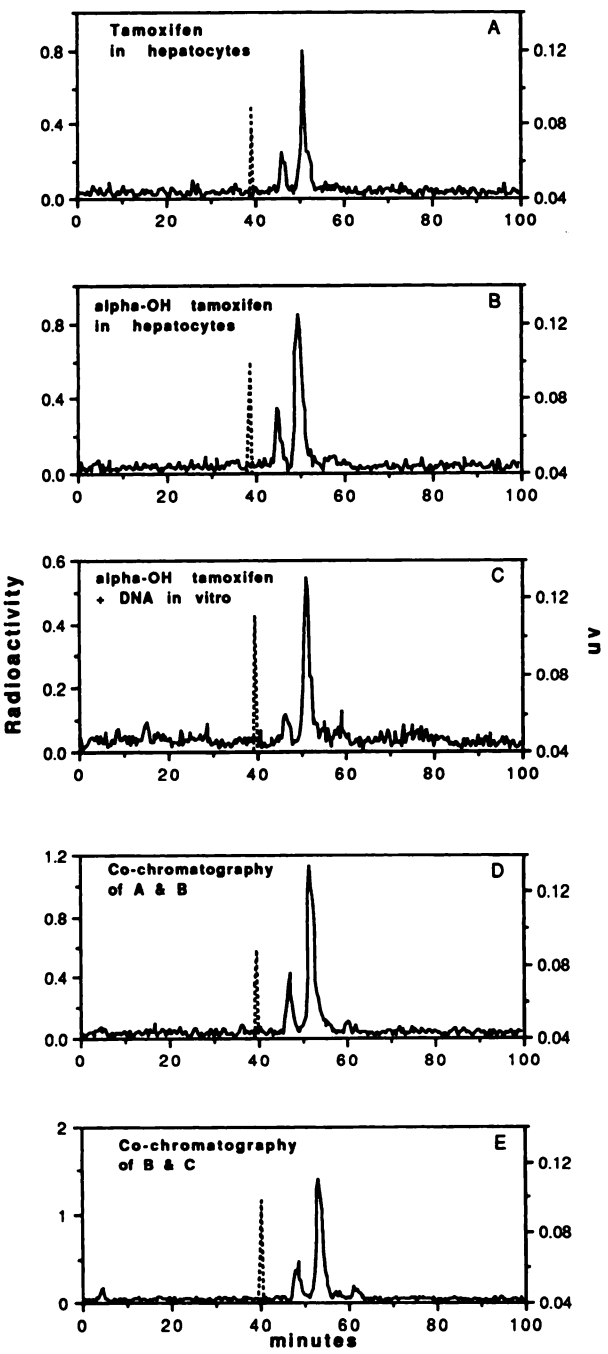

Fig. 2. HPLC analysis of tamoxifen-DNA adducts. The radioactive material present in the major TLC adduct spots shown was eluted with 4 M pyridinium formate and injected onto a phenyl-modified silica column eluted with a high salt, low pH phosphate buffer/methanol gradient. *A*, the major DNA adduct TLC spot from rat hepatocytes treated with tamoxifen; *B*, the major DNA adduct TLC spot from rat hepatocytes treated with α-hydroxytamoxifen; *C*, the major DNA adduct TLC spot from reaction of α-hydroxytamoxifen with DNA *in vitro*; *D*, cochromatography of *A* and *B*; *E*, cochromatography of *B* and *C*. ----, position of elution of the internal standard, *cis*-9,10-dihydro-9,10-dihydroxyphenanthrene, detected by its absorbance at 254 nm.

analyses of rat liver DNA from tamoxifen-treated animals (16, 23), although the elution of the UV marker at 38–40 min indicated that all material was eluting earlier than formerly, owing to changes in HPLC column performance since the earlier studies were conducted. Analysis of the major adduct from hepatocytes treated with α-hydroxytamoxifen (Fig. 1*B*, *Spot 2*) also gave rise to two peaks on HPLC (Fig. 2*B*) which coeluted, in cochromatography experiments, with the adduct material from tamoxifen-treated cells (Fig. 2*D*). Furthermore, the same two peaks were seen with material from the reaction of α-hydroxytamoxifen with DNA *in vitro* (Fig. 2*C*), which also coeluted with the DNA adducts formed by α-hydroxytamoxifen in rat hepatocytes, as shown in Fig. 2*E*.

Fig. 3*a* shows the selected ion chromatograms of extracts of the cell culture media from hepatocytes treated with tamoxifen and analyzed by liquid chromatography mass spectrometry. A major hydroxylated metabolite 16 atomic mass units higher than tamoxifen at *m/z* 388 ($[MH]^+$) was found to have retention time and mass spectrum identical to that of the synthetic standard of α-hydroxytamoxifen. Structural confirmation by tandem mass spectrometry generated the product-ion spectrum of this metabolite and is displayed in Fig. 3*b*. The principal fragment ion at *m/z* 72 represents the alkylamino side chain, which is characteristic for tamoxifen and its analogues (24). These analyses demonstrate that tamoxifen is metabolized to its α-hydroxylated derivative by rat hepatocytes.

Discussion

There is now substantial evidence that tamoxifen is a genotoxic carcinogen in the rat (3, 8, 9, 12, 14, 16). Other rodent species have, in contrast, been studied very little (25). A proper appraisal of the safety of tamoxifen for prophylactic use by humans requires, at the very least, a thorough understanding of the mechanism by which it exerts its genotoxic activity in the susceptible species, the rat. The first stage of this process involves identification of the metabolite(s) that are responsible for the activity. The second stage involves an understanding of the metabolizing enzymes responsible for this biotransformation and whether equivalent processes can or do occur in humans.

The present study contributes information toward the first stage of this process by demonstrating that α-hydroxytamoxifen has a 25–49-fold greater potential to produce DNA adducts in rat hepatocytes than does the parent compound. The identification of the α-hydroxylated derivative as a metabolite of tamoxifen in rat liver cells is also presented. Together, these findings strongly implicate α-hydroxytamoxifen as a proximate carcinogenic metabolite of tamoxifen. Although the compound may well undergo further metabolism at the α position, *e.g.*, to a reactive sulfate or acetic acid ester, the electrophilic activity of α-hydroxytamoxifen, as demonstrated by its covalent modification of DNA in the absence of metabolizing enzymes, shows that it can react with DNA *in vivo* without further metabolic transformation.

The present results further underline the theoretical model for the activation of tamoxifen at the α position of the ethyl group (15), which offers an explanation, *inter alia*, for the lack of carcinogenicity and

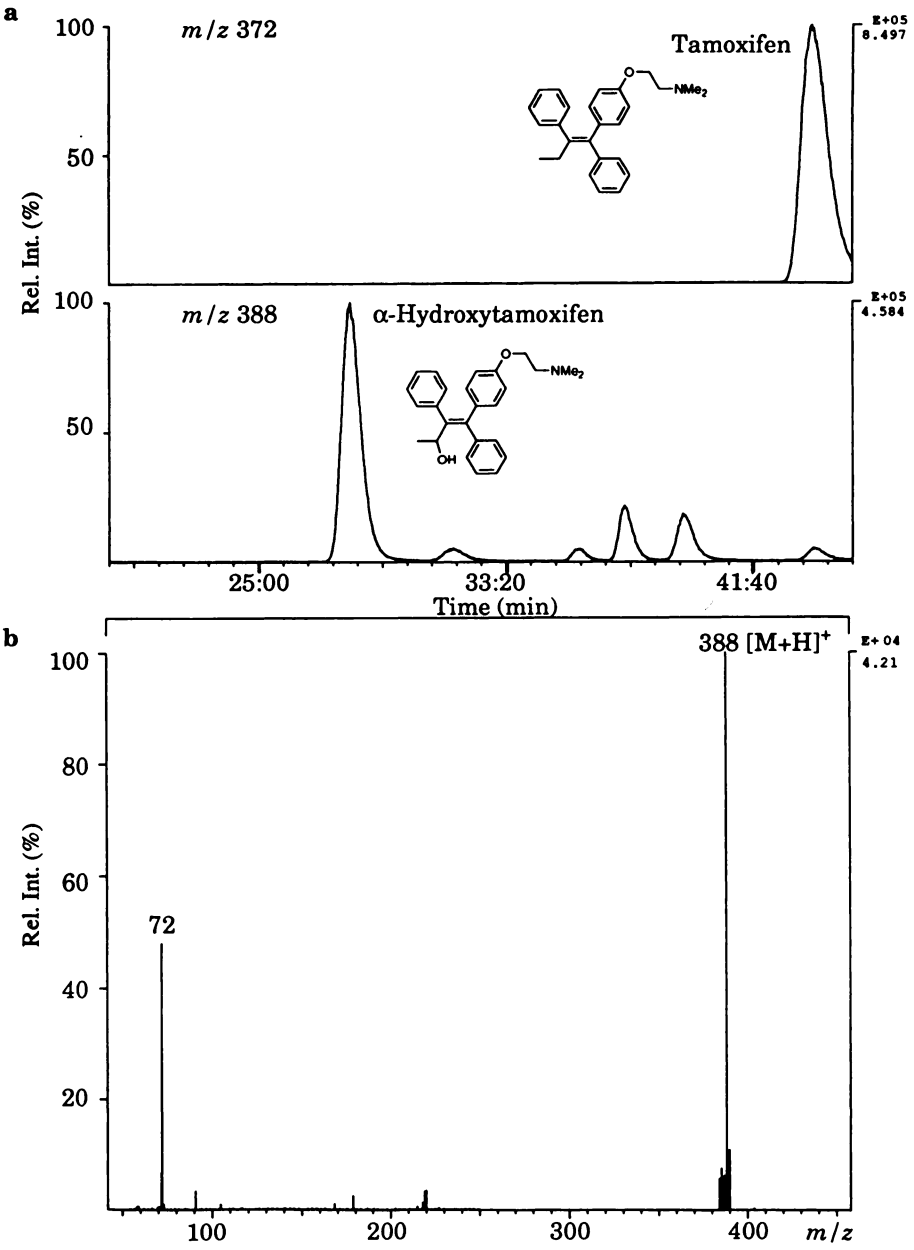

Fig. 3. *a*, liquid chromatography mass spectrometry selected ion monitoring chromatograms of the parent ion (MH^+, *m/z* 372) and α-hydroxytamoxifen (MH^+, *m/z* 388) present in extracts of the cell culture media from hepatocytes treated with tamoxifen (10 μM). *b*, liquid chromatography mass spectrometry product-ion mass spectrum of α-hydroxytamoxifen.

very low level of DNA binding exhibited by the structural analogue toremifene (4, 9). Toremifene differs from tamoxifen in having a chlorine atom at the β position of the ethyl group, which would be expected to interfere with carbocation formation at the α position and thus block the reactivity of the molecule at this position. Furthermore, our recently published study (16), demonstrating that [*ethyl*-D_5]tamoxifen exhibits lower genotoxicity than nondeuterated tamoxifen, also supports the hypothesis that metabolic activation of tamoxifen involves the α position of the ethyl group.

Although the adducts formed from direct reaction of α-hydroxytamoxifen with DNA comigrate on TLC and coelute on HPLC with the adducts formed when rat hepatocytes are treated with tamoxifen or α-hydroxytamoxifen, the possibility that further metabolism at other sites of the molecule cannot be excluded at this stage because the resolving power of our chromatography systems has not been rigorously tested. Thus, it has not been demonstrated that adducts containing additional modifications, *e.g.*, 4-hydroxylation (15), *N*-demethylation (26), or *N*-oxidation (18), would have been resolved on HPLC from those in which only the α position is modified. Experiments are in progress to examine whether metabolites additionally modified at other positions as well as at the α position[4] contribute, in part, to the tamoxifen-DNA adducts formed in rat hepatocytes.

In conclusion, the present study demonstrates that α-hydroxylation is a major pathway of genotoxic activation of tamoxifen in rat liver cells. Other studies have shown α-hydroxytamoxifen to be a metabolite of tamoxifen not only in rat hepatocytes, but also in rat microsomal systems[4] and in breast cancer patients taking tamoxifen.[5] This further underlines the relevance of studies on the genotoxic properties of tamoxifen in rats for assessing the safety of prophylactic tamoxifen in humans.

[4] M. Jarman, G. K. Poon, M. G. Rowlands, R. M. Grimshaw, M. N. Horton, G. A. Potter, and R. McCague. The deuterium isotope effect for the α-hydroxylation of tamoxifen by rat liver microsomes accounts for the reduced genotoxicity of [ethyl-D_5]tamoxifen, submitted for publication.

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