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Identification of tamoxifen-DNA adducts formed by 4-hydroxytamoxifen quinone methide

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Tamoxifen is a liver carcinogen in rats and has been shown to increase the risk of endometrial cancer in women. Recent reports of DNA adducts in leucocyte and endometrial samples from women treated with tamoxifen indicate that it may be genotoxic to humans. One of the proposed pathways for the metabolic activation of tamoxifen involves oxidation to 4-hydroxytamoxifen, which may be further oxidized to an electrophilic quinone methide. In the present study we show that 4-hydroxytamoxifen quinone methide reacts with DNA to form covalent adducts. The major products, which result from 1,8-addition of the exocyclic nitrogen of deoxyguanosine to the conjugated system of 4-hydroxytamoxifen quinone methide, are characterized as (*E*)- and (*Z*)-α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen.

Introduction

The nonsteroidal antiestrogen tamoxifen has been used for >15 years as an adjunct chemotherapeutic agent for the treatment of all stages of breast cancer (1,2). While clearly beneficial for the treatment of this disease, tamoxifen has been reported to increase the risk of endometrial cancer (3–9). In addition, it has been shown to be a liver carcinogen in rats (10–12).

Recently tamoxifen has been proposed to be used as a chemoprotective agent in women with elevated risk of developing breast cancer. Toward this end, a large-scale clinical trial has been initiated in which tamoxifen is being administered to inhibit estrogen-stimulated tumorigenesis in women over the age of 35 with the highest risk of developing breast cancer (13). Currently >10 000 women in the US are enrolled and the final goal is 16 000 (14). Similar clinical trials are being conducted in Great Britain (15) and Italy (16). Given the increased incidence of endometrial cancer associated with the use of tamoxifen, plus the observation of its hepatocarcinogenicity in rodents, there has been considerable controversy over the use of tamoxifen as a prophylactic agent in disease-free women (14,17–22).

The mechanisms by which tamoxifen increases the incidence of endometrial cancer are not known. Tamoxifen is known to be both an estrogen antagonist and agonist (23), activities that could be associated with tumor promoting properties. In this regard, tamoxifen, like other estrogens, has been shown to be a hepatic tumor promoter in rats (24,25). Of perhaps greater concern has been the observation that tamoxifen is metabolized to derivatives capable of binding to DNA (11,26–39). Thus, the distinct possibility exists that tamoxifen acts as a complete carcinogen, by initiating tumors through DNA adduct formation and promoting tumors through its estrogenic activity. Should this occur in a suspected target tissue (e.g., endometrium), it would carry a risk profoundly different from that associated with only an estrogen-like tumor promoting mechanism. This concern is reinforced by the recent reports of DNA adducts in endometrial and leucocyte samples from women treated with tamoxifen (40,41), although it must be noted that in another study, DNA adducts were not detected in the endometrium (42).

Three metabolic pathways have been proposed for the activation of tamoxifen to a reactive electrophile. Oxidation of the ethyl side chain can result in the formation of α-hydroxytamoxifen (Figure 1), a metabolite that has been detected in rat hepatocytes (28) and human liver *in vitro* (43), and in the plasma of humans being treated with tamoxifen (43). Subsequent esterification of the hydroxy moiety will create an excellent leaving group and generate a reactive carbocation. Recently, DNA adducts resulting from this pathway (Figure 1) have been characterized (37,44,45). A second pathway involves oxidation of the ethylene linkage to give tamoxifen 1,2-epoxide (Figure 1). While in theory this could give rise to DNA adducts, recent data suggest this is not an important pathway (19). A third pathway involves oxidation of one of the phenyl rings to give 4-hydroxytamoxifen, which may undergo a subsequent oxidation to 4-hydroxytamoxifen quinone methide (Figure 1), an electrophile that can yield DNA adducts through Michael-type addition reactions (30,31,35,36,38). Microsomal, chemical and *in vivo* oxidation of 4-hydroxytamoxifen have been shown to produce DNA adducts, presumably via a quinone methide intermediate, but the structures of these products have not been elucidated (36,38). In this paper, we report the characterization of the major DNA adducts resulting from 4-hydroxytamoxifen quinone methide.

Materials and methods

Instrumentation

Reversed-phase HPLC analyses and separations were conducted using a μBondapak C_{18} column (0.39×30 cm; Waters Associates, Milford, MA) on a Waters Associates system consisting of two Model 510 pumps, a U6K injector, and a Model 660 automated gradient controller. The peaks were monitored at 280 nm with a Hewlett-Packard 1050 diode array spectrophotometric detector.

Mass spectrometry (MS*) was conducted in the fast atom bombardment (FAB) mode on a Finnigan TSQ 700 instrument, using xenon as the FAB gas and a source temperature of 90°C. The samples were dispersed in a thioglycerol matrix. FAB MS/MS analyses were performed using argon at 0.2 mT as the collision gas and a collision energy of 10 V.

^{1}H NMR spectra were recorded on a Bruker AM500 spectrometer operating at 500 MHz. The samples were dissolved in dimethylsulfoxide-d_6 (Me_2SO-d_6) and the proton chemical shifts were referenced to the residual Me_2SO proton resonance at 2.49 ppm.

Chemicals

Salmon testes DNA and bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane (Bis-Tris) were purchased from Sigma Chemical Co. (St Louis, MO). All

***Abbreviations:** Bis-Tris, bis[2-hydroxyethyl]iminotris[hydroxymethyl]-methane; FAB, fast atom bombardment; Me_2SO, dimethylsulfoxide; MS, mass spectrometry.

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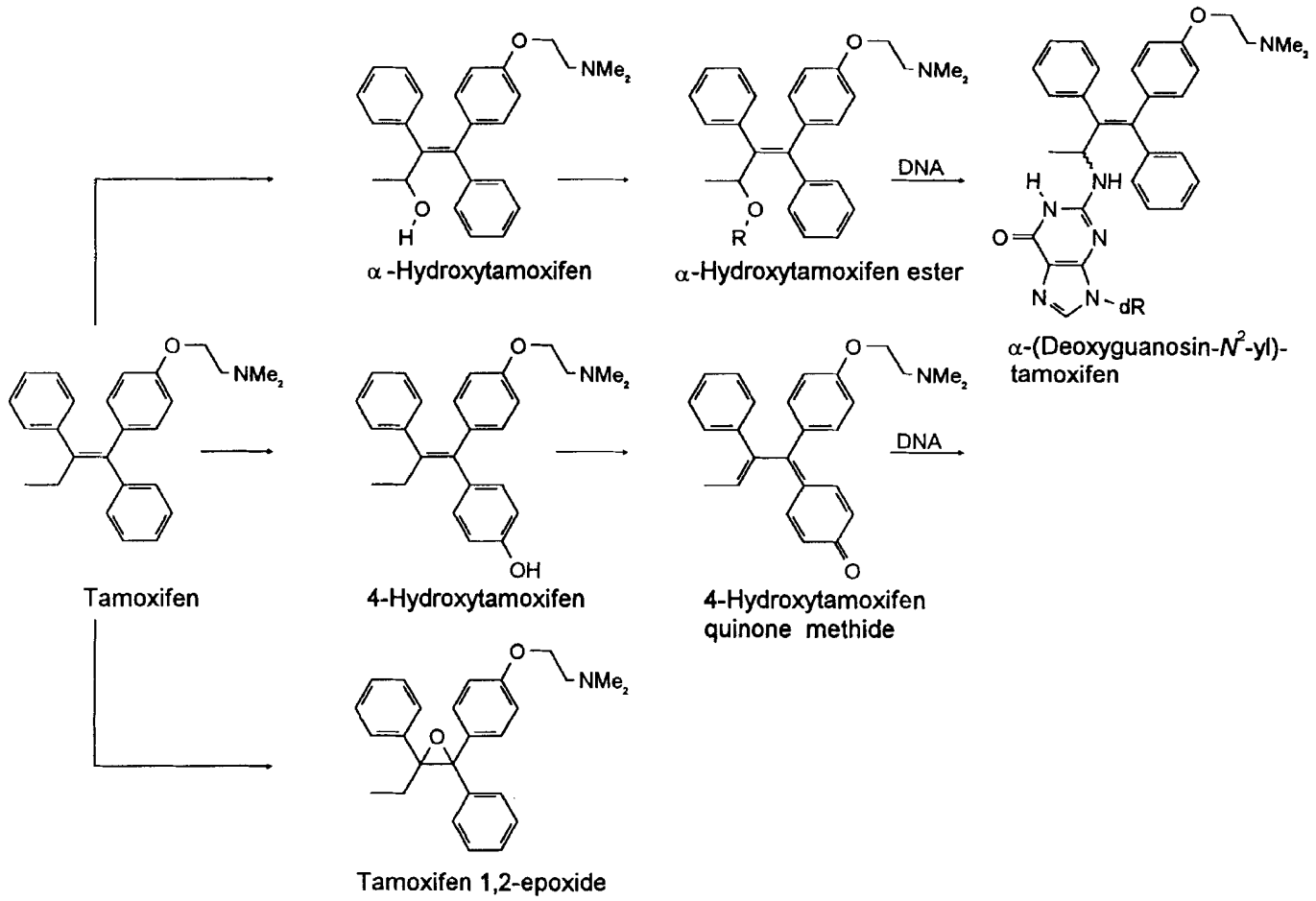

Fig. 1. Proposed metabolic activation pathways for tamoxifen. Only the major DNA adduct resulting from α-hydroxytamoxifen is shown.

other commercially available reagents were obtained from Fluka Chemie AG (Buchs, Switzerland), Aldrich Chemical Co. (Milwaukee, WI), or Sigma-Aldrich Química, S.A. (Madrid, Spain), and were used as received. Whenever necessary, solvents were purified by standard methods (46).

Syntheses

4-Hydroxytamoxifen was obtained as a mixture (*ca*. 1/1) of the *cis* and *trans* isomers by condensation of 4-hydroxy-4′-[2-(dimethylamino)ethoxy]benzophenone with the α-carbon anion from propylbenzene, followed by acid hydrolysis of the resulting diastereomeric carbinols, as described by Olier-Reuchet *et al.* (47).

Active γ-manganese dioxide was prepared by standard precipitation from an aqueous mixture of manganese sulfate and potassium permanganate, as described by Fatiadi (48).

4-Hydroxytamoxifen quinone methide was prepared by oxidation of *cis/trans* 4-hydroxytamoxifen with an excess of freshly prepared active manganese dioxide. Typically, 4-hydroxytamoxifen (20 mg) was dissolved in 1 ml of acetonitrile or acetone, and the solution was protected from light and cooled to 0–5°C. Upon addition of manganese dioxide (100 mg), the mixture was stirred for 20 min in an ice-water bath and centrifuged. The dark brown supernatant containing 4-hydroxytamoxifen quinone methide was removed and used immediately for reaction with DNA.

Treatment of DNA with 4-hydroxytamoxifen quinone methide

To 10 ml of a salmon testes DNA solution (1 mg/ml) in 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1, was added 1 ml of the organic solution containing 4-hydroxytamoxifen quinone methide (*ca.* 26 mmol, *vide supra*), and the mixture was protected from light and incubated overnight at 37°C. Following evaporation of the organic solvent, non-bonded materials were extracted sequentially with diethyl ether (5×1 vol) and *n*-butanol (4×1 vol), which had both been presaturated with 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1. The DNA was precipitated by addition of 5 M NaCl (0.1 vol) and ice-cold absolute ethanol (2 vol), washed twice with 70% ethanol, and redissolved in 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1 at a concentration of *ca*. 1 mg/ml.

Isolation of the adducts

The modified DNA was hydrolyzed to nucleosides by standard treatment with DNase I, followed by alkaline phosphatase and phosphodiesterase (49). The nucleoside adducts were then partitioned into *n*-butanol (3×0.5 vol), the *n*-butanol extracts were combined, back-extracted with *n*-butanol-saturated water (0.5 vol), evaporated to dryness, and redissolved in methanol (200 μl).

The adducts were isolated by HPLC, using a 20-min linear gradient of 20–60% acetonitrile in 100 mM ammonium acetate, pH 5.7, followed by 5 min at 60% acetonitrile, and finally a 10-min linear gradient of 60–100% acetonitrile. The flow rate was 2 ml/min. Upon thorough evaporation to dryness, the materials isolated from 40 mg of DNA were combined, and the adducts were characterized by ^{1}H NMR and mass spectrometry.

Results and discussion

Synthesis and isolation of the adducts

4-Hydroxytamoxifen quinone methide has been proposed as the reactive intermediate in the metabolic activation of 4-hydroxytamoxifen to DNA binding species. Adducts resulting from microsomal, chemical, and *in vivo* oxidation of 4-hydroxytamoxifen, presumably via the quinone methide, have been detected and compared by ^{32}P-postlabeling (36,38) but have not been characterized.

With the aim of elucidating adduct structures resulting from this pathway, we prepared 4-hydroxytamoxifen as a 1/1 mixture of the *cis* and *trans* isomers, following the methodology of Olier-Reuchet *et al.* (47**)**. Since isomerization was expected upon oxidation, due to loss of the double bond character in the ethylenic linkage, the *cis/trans* mixture was used for the chemical synthesis of 4-hydroxytamoxifen quinone methide.

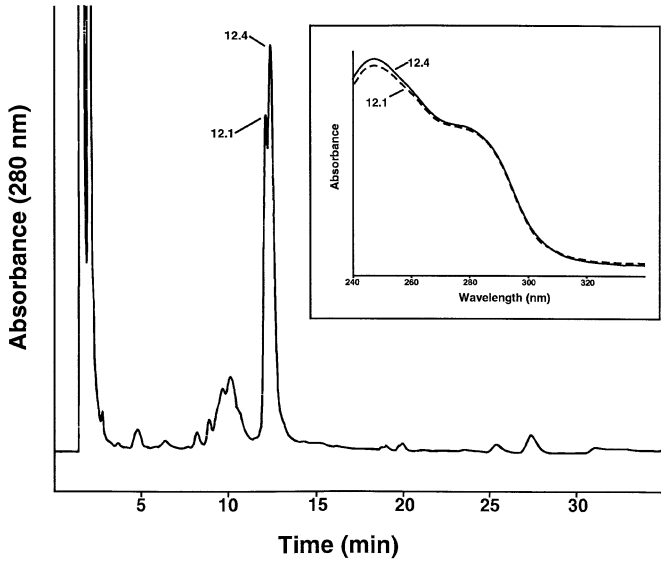

Fig. 2. HPLC of the enzymatic hydrolysate obtained from reacting 4-hydroxytamoxifen quinone methide with DNA. The elution conditions are outlined in Materials and methods. The retention times of the two major adducts are indicated. These adducts were isolated as a single fraction. Inset: UV absorption spectra of the adducts, as detected by HPLC in 100 mM ammonium acetate, pH 5.7/acetonitrile. The broken line corresponds to the 12.1 min peak, and the solid line to the 12.4 min peak.

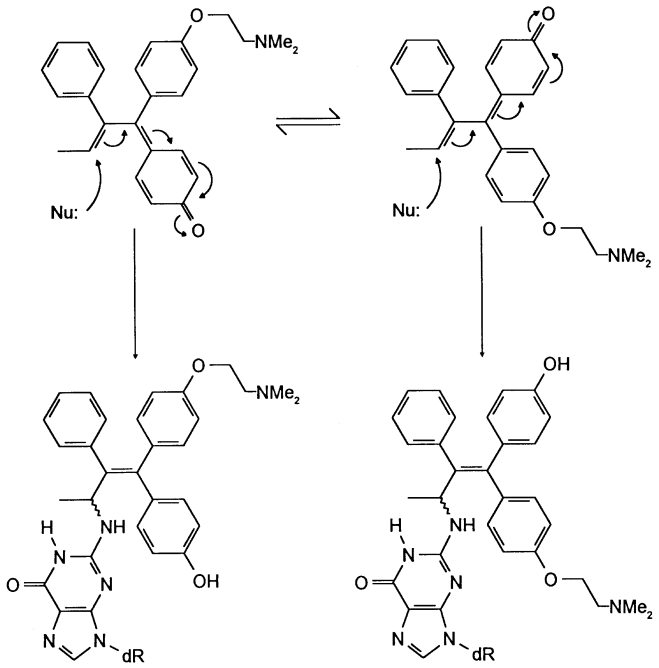

Fig. 3. Proposed mechanism for the formation of (*E*)- and (*Z*)-α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen from 4-hydroxytamoxifen quinone methide. Nu: = dG; dR = 2′-deoxyribosyl.

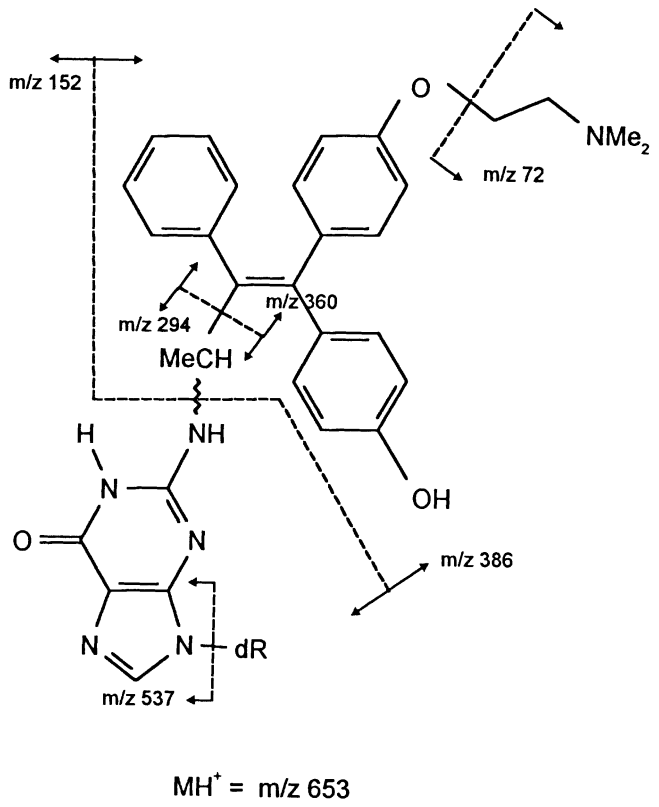

Fig. 4. Proposed MS fragmentation pattern, as illustrated for the *trans* 4-hydroxytamoxifen-deoxyguanosine isomer. The arrows point towards the ions detected from cleavage of each particular bond.

Several mild oxidants, including silver(I) oxide (50), lead dioxide (51), and manganese dioxide (52), have been reported for the synthesis of quinones and quinone methides from a variety of phenolic species. Using an excess of freshly prepared manganese dioxide, we were able to achieve a rapid oxidation of 4-hydroxytamoxifen in a variety of solvents (e.g., chloroform, acetonitrile, and acetone). Although the oxidized species were not characterized, the structures of the adducts obtained upon reaction with DNA (*vide infra*) were consistent with the generation of isomeric quinone methides.

Following enzymatic hydrolysis of the modified DNA to nucleosides, the HPLC profile of the butanol extract (Figure 2) indicated the presence of a series of peaks eluting at 8.2–12.4 min. These were presumably DNA adducts, since they were not detected in control incubations conducted with 4-hydroxytamoxifen quinone methide in the absence of DNA or DNA in the absence of 4-hydroxytamoxifen quinone methide (not shown). We focused on the most intense absorbance, containing two closely eluting peaks at 12.1 min and 12.4 min (Figure 2), which were isolated as a single fraction. The UV spectra of the adducts (Figure 2; inset), showing a maximum at *ca.* 250 nm and a shoulder at 270–280 nm, were almost superimposable and were also similar, although not identical, to those reported for N^2-deoxyguanosyl adducts from α-acetoxy- and α-sulfoxytamoxifen (37,44,45). Based on MS and ^{1}H NMR evidence (*vide infra*), the isolated species were characterized as *cis/trans* isomers resulting from 1,8-addition of the exocyclic nitrogen of deoxyguanosine to the extended conjugated system of 4-hydroxytamoxifen quinone methide, as outlined in Figure 3.

Characterization of the adducts

Mass spectrum. Initial positive ion FAB mass spectrometry of the isolated species yielded product ions at m/z 697 (10%), 675 (30%), and 653 (4%). The ion at m/z 653 ($C_{36}H_{41}N_6O_6$) was consistent with a protonated adduct of 4-hydroxytamoxifen and deoxyguanosine, and the ions at m/z 675 [$(M + Na)^+$] and 697 [$(M + 2Na - H)^+$] were consistent with mono- and di-sodiation of the same adduct.

Product ion analyses were performed in the FAB MS/MS mode for the protonated molecule (m/z 653) and the more

Table I. Proton NMR data for the 4-hydroxytamoxifen-deoxyguanosine adducts

4-Hydroxytamoxifen fragment	δ (ppm)[a]	
	Deoxyribose fragment	Guanine fragment
1.24 (3H, d, J 3.8, C**H**$_3$CH)	2.30 (1H, m, **H**2″)	7.93 (1H, s, **H**8)
1.25 (3H, d, J 3.7, C**H**$_3$CH)	2.45 (1H, m, **H**2″)	7.95 (1H, s, **H**8)
2.12 (3H, s, N(C**H**$_3$)$_2$, *trans*)	*ca.* 2.50[b] (**H**2′)	
2.21 (3H, s, N(C**H**$_3$)$_2$, *cis*)		
ca. 2.50[b] (NC**H**$_2$, *trans*)	*ca.* 3.50[c] (**H**5′, 5″)	
2.62 (2H, t, J 5.8, NC**H**$_2$, *cis*)		
4.04 (2H, t, J 5.8, OC**H**$_2$, *cis*)	3.85 (1H, m, **H**4′)	
4.08 (2H, t, J 6.7, OC**H**$_2$, *trans*)	3.89 (1H, m, **H**4′)	
4.87 (1H, m, CH$_3$C**H**, *trans*)	4.36 (2×1H, m, **H**3′)	
4.92 (1H, m, CH$_3$C**H**, *cis*)		
6.34 (2H, d, J 8.6, HOPh**H**[d], *cis*)	6.21 (2×1H, m, **H**1′)	
6.51 (2H, d, J 8.8, AlkPh**H**[e], *trans*)		
6.61 (2H, d, J 8.6, HOPh**H**, *cis*)		
6.72 (2H, d, J 8.8, AlkPh**H**, *trans*)		
6.85 (2H, d, J 8.5, Ar**H**)		
6.98 (2H, d, J 8.5, Ar**H**)		
7.04 (4H, d, J 8.7, Ar**H**)		
7.10 – 7.25 (8H, m, Ar**H**)		
7.28 (2H, d, J 8.7, Ar**H**)		

[a]Recorded in Me_2SO-d_6. The chemical shifts were referenced to the residual Me_2SO resonance at 2.49 ppm.
[b]Obscured by the solvent resonance.
[c]Obscured by the water resonance.
[d]Protons from the hydroxyphenyl ring.
[e]Protons from the alkoxyphenyl ring.

intense sodiated ion at m/z 675. The product ion spectra of m/z 653 gave prominent fragments at m/z 653 (75%), 537 (44%), and 386 (100%), and additional minor ions at m/z 360, 294, 268, 178, 152, and 72. The product ion spectra of m/z 675 gave prominent fragments at m/z 675 (100%), 559 [(537 + Na), 26%], 290 [(268 + Na), 78%], and 174 [(152 + Na), 37%]. With the exception of fragments m/z 360 and 72, the product ions from MH^+ had corresponding sodiated fragments. The ions at m/z 72 (dimethylaziridinium, $CH_2CH_2N(CH_3)_2^+$) and 386 [(4-hydroxytamoxifen – 1)$^+$] were observed in both spectra. Analysis of the fragmentation pattern, as indicated in Figure 4, strongly suggested that the isolated adducts consisted of a mixture of 4-hydroxytamoxifen-deoxyguanosine diastereomers, with substitution by deoxyguanosine at the allylic carbon of 4-hydroxytamoxifen. Thus, the detection of strong ions at m/z 537 (MH_2^+ – 117) and m/z 559 ($MHNa^+$ – 117) indicated loss of the deoxyribosyl fragment. In addition, the intense fragments at m/z 386 [(4-hydroxytamoxifen – 1)$^+$] and m/z 290 [(deoxyguanosine + Na)$^+$] were consistent with, respectively, the formation of a stable cation and the loss of a stable radical, presumably having extensive conjugation, as would be expected from fragmentation at the allylic carbon. The presence of additional fragments at m/z 152 [(guanine + 1)$^+$] and 174 [(guanine + 1 + Na)$^+$] was also consistent with this interpretation. The detection of an ion at m/z 360 [(4-hydroxytamoxifen – CH_3CH + 1)$^+$] in the product ion spectra of m/z 653, as well as the presence of ions m/z 178 [(guanine + CH_3CH – 1)$^+$], 200 (178 + Na), 294 [(deoxyguanosine + CH_3CH – 1)$^+$], and 316 (294 + Na) further substantiated that attachment of deoxyguanosine to the 4-hydroxytamoxifen moiety must have occurred through the allylic carbon. The same type of allylic fragmentation has been reported previously for the adducts resulting from reaction of the exocyclic amino group of deoxyguanosine at the α carbon of α-acetoxy- and α-sulfoxytamoxifen (37,44).

^{1}H NMR spectrum. The proton chemical shifts obtained for the adduct mixture in Me_2SO-d_6 are listed in Table I. The assignments were based on comparison with proton NMR data for deoxyguanosine and 4-hydroxytamoxifen, combined with homonuclear decoupling experiments. The spectrum indicated ~1:1 mixture of two 4-hydroxytamoxifen-deoxyguanosine *cis*/*trans* isomers. The presence of two isomers was evident in the two H8 signals from the deoxyguanosine moiety (7.93 and 7.95 ppm, respectively), as well as in the doubling of some sugar resonances (e.g., H4′, at 3.85 and 3.89 ppm). Likewise, the resonances for the 4-hydroxytamoxifen segment were fully consistent with the presence of two isomers. Thus, two equally intense *N*-methyl singlets were observed at 2.12 and 2.21 ppm. Two OCH_2 triplets were also observed at 4.04 and 4.08 ppm, and these were coupled to NCH_2 signals at 2.62 and *ca.* 2.50 ppm, respectively. The resonances in the downfield region indicated *trans–cis* isomerization of an otherwise intact aromatic segment. Hence, two sets of upfield mutually coupled doublets (6.34 and 6.61 ppm, and 6.51 and 6.72 ppm) were assigned, respectively, to the AB proton systems of the hydroxyphenyl group in the *cis* isomer and the alkoxyphenyl substituent in the *trans* isomer on the basis of an expected combination of shielding effects in aromatic protons located between two aryl groups (53). The remaining downfield resonances accounted for all the aromatic protons of the two isomers (Table I). Essential information regarding the site of attachment of 4-hydroxytamoxifen to deoxyguanosine was provided by the absence of the typical splitting pattern of the ethyl group of 4-hydroxytamoxifen. The ethyl signals were replaced by two methyl doublets at 1.24 and 1.25 ppm, which were coupled to two methine multiplets at 4.87 and 4.92 ppm. This indicated that both isomers resulted from substitution at the allylic carbon of 4-hydroxytamoxifen. The fact that both methine signals were multiplets suggested the existence of additional coupling of these protons to the N^2 proton of the

deoxyguanosine moiety. Although we were unable to obtain sufficiently intense signals to assign the labile protons unequivocally, the observed splitting pattern of the methine protons was similar to that reported for the *cis* and *trans* N^2-deoxyguanosine adducts from α-acetoxy- and α-sulfoxytamoxifen (37,44,45). It should be noted that there were virtually no differences in the aliphatic region of the NMR spectrum compared to the chemical shift values reported for the adducts derived from α-acetoxy- and α-sulfoxytamoxifen (37,44,45), with the exception of a slight downfield shift (*ca.* 0.2 ppm) of the OCH_2 protons. This provided the basis for the assignment of particular resonances to the *cis* and *trans* isomers (Table I). Conceivably, each of the isomers may correspond to a mixture of epimers, differing in the configuration of the α-carbon; however, such a subtle structural difference would be unlikely to produce a detectable effect in the proton NMR spectrum.

Conclusions

In this paper we show that 4-hydroxytamoxifen quinone methide, the oxidation product of 4-hydroxytamoxifen, reacts with DNA to give two major adducts, (*E*)- and (*Z*)-α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen, at the N^2 position of deoxyguanosine through the allylic carbon of 4-hydroxytamoxifen. The structure of these adducts is nearly identical to that of the major adducts characterized from α-acetoxy- and α-sulfoxytamoxifen (37,44,45), differing only in the presence of a phenolic hydroxyl function. Recently, tamoxifen has been shown to induce mutations in lambda/*lacI* transgenic rats, the majority of which involved G:C base pairs (54). Likewise, the induction of liver tumors in rats (55) and endometrial tumors in women (56) has been associated with mutations in the *p53* tumor suppressor gene. Whether or not the 4-hydroxytamoxifen-deoxyguanosine adduct is involved in the induction of these mutations remains to be established.

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References

1. Cancer Research Campaign Breast Cancer Trials Group (1992) The effect of adjuvant tamoxifen: the latest results from the Cancer Research Campaign Adjuvant Breast Trial. *Eur. J. Cancer*, **28A**, 904–907.
2. Jordan,V.C. (1992) Overview from the international conference on long-term tamoxifen therapy for breast cancer. *J. Natl Cancer Inst*., **84**, 231–234.
3. Magriples,U., Naftolin,F., Schwartz,P.E. and Carcangiu,M.L. (1993) High-grade endometrial carcinoma in tamoxifen-treated breast cancer patients. *J. Clin. Oncol*., **11**, 485–490.
4. Seoud,M.A.-F., Johnson,J. and Weed,J.C.,Jr (1993) Gynecologic tumors in tamoxifen-treated women with breast cancer. *Obstet. Gynecol*., **82**, 165–169.
5. Fisher,B., Costantino,J.P., Redmond,C.K., Fisher,E.R., Wickerham,D.L., Cronin,W.M. and other NSABP contributors (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J. Natl Cancer Inst*., **86**, 527–537.
6. van Leeuwen,F.E., Benraadt,J., Coebergh,J.W.W., Kiemeney,L.A.L.M., Gimbrère,C.H.F., Otter,R., Schouten,L.J., Damhuis,R.A.M., Bontenbal,M., Diepenhorst,F.W., van den Belt-Dusebout,A.W. and van Tinteren,H. (1994) Risk of endometrial cancer after tamoxifen treatment of breast cancer. *Lancet*, **343**, 448–452.
7. Cook,L.S., Weiss,N.S., Schwartz,S.M., White,E., McKnight,B., Moore, D.E. and Daling,J.R. (1995) Population-based study of tamoxifen therapy and subsequent ovarian, endometrial, and breast cancers. *J. Natl Cancer Inst*., **87**, 1359–1364.
8. Cuenca,R.E., Giachino,J., Arrendondo,M.A., Hempling,R. and Edge,S.B. (1996) Endometrial carcinoma associated with breast carcinoma: low incidence with tamoxifen use. *Cancer*, **77**, 2058–2063.
9. Curtis,R.E., Boice,J.D.,Jr, Shriner,D.A., Hankey,B.F. and Fraumeni,J.F.,Jr (1996) Second cancers after adjuvant tamoxifen therapy for breast cancer. *J. Natl Cancer Inst*., **88**, 832–834.
10. Greaves,P., Goonetilleke,R., Nunn,G., Topham,J. and Orton,T. (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res*., **53**, 3919–3924.
11. Hard,G.C., Iatropoulos,M.J., Jordan,K., Radi,L., Kaltenberg,O.P., Imondi, A.R. and Williams,G.M. (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female CrI:CD(BR) rats. *Cancer Res*., **53**, 4534–4541.
12. Williams,G.M., Iatropoulos,M.J., Djordjevic,M.V. and Kaltenberg,O.P. (1993) The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. *Carcinogenesis*, **14**, 315–317.
13. NSABP (1992) National Surgical Adjuvant Breast and Bowel Project (NSABP) NSABP Protocol P1: A Clinical Trial to Determine the Worth of Tamoxifen for Preventing Breast Cancer. University of Pittsburgh, Pittsburgh, PA.
14. Jordan,V.C. (1995) Tamoxifen for breast cancer prevention. *Proc. Soc. Exp. Biol. Med*., **208**, 144–149.
15. Powles,T.J., Jones,A.L., Ashley,S.E., O'Brien,M.E.R., Tidy,V.A., Treleavan,J., Cosgrove,D., Nash,A.G., Sacks,N., Baum,M., McKinna,J.A. and Davey,J.B. (1994) The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. *Breast Cancer Res. Treat*., **31**, 73–82.
16. Vanchieri,C. (1992) Breast cancer prevention study initiated in Italy. *J. Natl Cancer Inst*., **84**, 1555–1556.
17. Love,R.R. (1993) The National Surgical Adjuvant Breast Project (NSABP) Breast Cancer Prevention Trial revisited. *Cancer Epidemiol. Biomarkers & Prev*., **2**, 403–407.
18. Williams,G.M. and Hard,G.C. (1994) Correspondence re: R.R. Love, The National Surgical Adjuvant Breast Project (NSABP) Breast Cancer Prevention Trial revisited. *Cancer Epidemiol. Biomarkers & Prev*., **3**, 185–186.
19. King,C.M. (1995) Tamoxifen and the induction of cancer. *Carcinogenesis*, **16**, 1449–1454.
20. Pitot,H.C. (1995) The tamoxifen controversy—clinical chemopreventive agent and experimental carcinogen. *Proc. Soc. Exp. Biol. Med*., **208**, 139–140.
21. Williams,G.M. (1995) Tamoxifen experimental carcinogenicity studies: implications for human effects. *Proc. Soc. Exp. Biol. Med*., **208**, 141–143.
22. Jordan,V.C. and Assikis,V.J. (1995) Endometrial carcinoma and tamoxifen: clearing up a controversy. *Clin. Cancer Res*., **1**, 467–472.
23. Branham,W.S., Fishman,R., Streck,R.D., Medlock,K.L., DeGeorge,J.J. and Sheehan,D.M. (1996) ICI 182,780 inhibits endogenous estrogen-dependent rat uterine growth and tamoxifen-induced developmental toxicity. *Biol. Repro*., **54**, 160–167.
24. Dragan,Y.P., Fahey,S., Street,K., Vaughan,J., Jordan,V.C. and Pitot,H.C. (1994) Studies of tamoxifen as a promoter of hepatocarcinogenesis in female Fischer F344 rats. *Breast Cancer Res. Treat*., **31**, 11–25.
25. Dragan,Y.P., Vaughan,J., Jordan,V.C. and Pitot,H.C. (1995) Comparison of the effects of tamoxifen and toremifene on liver and kidney tumor promotion in female rats. *Carcinogenesis*, **16**, 2733–2741.
26. Han,X. and Liehr,J.G. (1992) Induction of covalent DNA adducts in rodents by tamoxifen. *Cancer Res*., **52**, 1360–1363.
27. White,I.N.H., de Matteis,F., Davies,A., Smith,L.L., Crofton-Sleigh,C., Venitt,S., Hewer,A. and Phillips,D.H. (1992) Genotoxic potential of tamoxifen and analogues in female Fischer F344/n rats, DBA/2 and C57BL/6 mice and in human MCL-5 cells. *Carcinogenesis*, **13**, 2197–2203.
28. Phillips,D.H., Carmichael,P.L., Hewer,A., Cole,K.J. and Poon,G.K. (1994) α-Hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. *Cancer Res*., **54**, 5518–5522.
29. Phillips,D.H., Potter,G.A., Horton,M.N., Hewer,A., Crofton-Sleigh,C., Jarman,M. and Venitt,S. (1994) Reduced genotoxicity of [D_5-ethy]-tamoxifen implicates α-hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. *Carcinogenesis*, **15**, 1487–1492.
30. Randerath,K., Bi,J., Mabon,N., Sriram,P. and Moorthy,B. (1994) Strong intensification of mouse hepatic tamoxifen DNA adduct formation by pretreatment with the sulfotransferase inhibitor and ubiquitous environmental pollutant pentachlorophenol. *Carcinogenesis*, **15**, 797–800.
31. Randerath,K., Moorthy,B., Mabon,N. and Sriram,P. (1994) Tamoxifen: evidence by ^{32}P-postlabeling and use of metabolic inhibitors for two distinct pathways leading to mouse hepatic DNA adduct formation

and identification of 4-hydroxytamoxifen as a proximate metabolite. *Carcinogenesis*, **15,** 2087–2094.
32. Carthew,P., Rich,K.J., Martin,E.A., de Matteis,F., Lim,C.-K., Manson, M.M., Festing,M.F.W., White,I.N.H. and Smith,L.L. (1995) DNA damage as assessed by ^{32}P-postlabelling in three rat strains exposed to dietary tamoxifen: the relationship between cell proliferation and liver tumour formation. *Carcinogenesis*, **16**, 1299–1304.
33. Davies,A.M., Martin,E.A., Jones,R.M., Lim,C.K., Smith,L.L. and White,I.N.H. (1995) Peroxidase activation of tamoxifen and toremifene resulting in DNA damage and covalently bound protein adducts. *Carcinogenesis*, **16**, 539–545.
34. Pathak,D.N., Pongracz,K. and Bodell,W.J. (1995) Microsomal and peroxidase activation of 4-hydroxytamoxifen to form DNA adducts: comparison with DNA adducts formed in Sprague–Dawley rats treated with tamoxifen. *Carcinogenesis*, **16**, 11–15.
35. Pongracz,K., Pathak,D.N., Nakamura,T., Burlingame,A.L. and Bodell,W.J. (1995) Activation of the tamoxifen derivative metabolite E to form DNA adducts: comparison with the adducts formed by microsomal activation of tamoxifen. *Cancer Res.*, **55**, 3012–3015.
36. Moorthy,B., Sriram,P., Pathak,D.N., Bodell,W.J. and Randerath,K. (1996) Tamoxifen metabolic activation: comparison of DNA adducts formed by microsomal and chemical activation of tamoxifen and 4-hydroxytamoxifen with DNA adducts formed *in vivo*. *Cancer Res.*, **56**, 53–57.
37. Osborne,M.R., Hewer,A., Hardcastle,I.R., Carmichael,P.L. and Phillips, D.H. (1996) Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. *Cancer Res.*, **56**, 66–71.
38. Pathak,D.N., Pongracz,K. and Bodell,W.J. (1996) Activation of 4-hydroxy-tamoxifen and the tamoxifen derivative metabolite E by uterine peroxidase to form DNA adducts: comparison with DNA adducts formed in the uterus of Sprague–Dawley rats treated with tamoxifen. *Carcinogenesis*, **17**, 1785–1790.
39. Li,D., Dragan,Y., Jordan,V.C., Wang,M. and Pitot,H.C. (1997) Effects of chronic administration of tamoxifen and toremifene on DNA adducts in rat liver, kidney, and uterus. *Cancer Res.*, **57**, 1438–1441.
40. Hemminki,K., Rajaniemi,H., Lindahl,B. and Moberger,B. (1996) Tamoxifen-induced DNA adducts in endometrial samples from breast cancer patients. *Cancer Res.*, **56**, 4374–4377.
41. Hemminki,K., Rajaniemi,H., Koskinen,M. and Hansson,J. (1997) Tamoxifen-induced DNA adducts in leucocytes of breast cancer patients. *Carcinogenesis*, **18**, 9–13.
42. Carmichael,P.L., Ugwumadu,A.H.N., Neven,P., Hewer,A.J., Poon,G.K. and Phillips,D.H. (1996) Lack of genotoxicity of tamoxifen in human endometrium. *Cancer Res.*, **56**, 1475–1479.
43. Poon,G.K., Walter,B., Lønning,P.E., Horton,M.N. and McCague,R. (1995) Identification of tamoxifen metabolites in human Hep G2 cell line, human liver homogenate, and patients on long-term therapy for breast cancer. *Drug Metab. Dispos.*, **23**, 377–382.
44. Dasaradhi,L. and Shibutani,S. (1997) Identification of tamoxifen-DNA adducts formed by α-sulfate tamoxifen and α-acetoxytamoxifen. *Chem. Res. Toxicol.*, **10**, 189–196.
45. Osborne, M.R., Hardcastle, I.R., and Phillips, D.H. (1997) Minor products of reaction of DNA with α-acetoxytamoxifen. *Carcinogenesis*, **18**, 539–543.
46. Perrin,D.D. and Armarego,W.L.F. (1988) *Purification of Laboratory Chemicals*, 3rd edn, Pergamon, Oxford, pp. 391.
47. Olier-Reuchet,C., Aitken,D.J., Bucourt,R. and Husson,H.-P. (1995) Synthesis of tamoxifen and 4-hydroxytamoxifen using super-base-metalated propylbenzene. *Tetrahed. Lett.*, **36**, 8221–8224.
48. Fatiadi,A.J. (1976) Active manganese dioxide oxidation in organic chemistry—Part I. *Synthesis*, 65–104.
49. Heflich,R.H., Morris,S.M., Beranek,D.T., McGarrity,L.J., Chen,J.J. and Beland,F.A. (1986) Relationships between the DNA adducts and the mutations and sister-chromatid exchanges produced in Chinese hamster ovary cells by *N*-hydroxy-2-aminofluorene, *N*-hydroxy-*N′*-acetylbenzidine and 1-nitrosopyrene. *Mutagenesis*, **1**, 201–206.
50. Liehr,J.G., DaGue,B.B., Ballatore,A.M. and Henkin,J. (1983) Diethylstilbestrol (DES) quinone: a reactive intermediate in DES metabolism. *Biochem. Pharmacol.*, **32**, 3711–3718.
51. Bolton,J.L., Valerio,L.G.,Jr and Thompson,J.A. (1992) The enzymatic formation and chemical reactivity of quinone methides correlate with alkylphenol-induced toxicity in rat hepatocytes. *Chem. Res. Toxicol.*, **5**, 816–822.
52. Dwivedy,I., Devanesan,P., Cremonesi,P., Rogan,E. and Cavalieri,E. (1992) Synthesis and characterization of estrogen 2,3- and 3,4-quinones. Comparison of DNA adducts formed by the quinones versus horseradish peroxidase-activated catechol estrogens. *Chem. Res. Toxicol.*, **5**, 828–833.
53. Foster,A.B., Jarman,M., Leung,O.-T., McCague,R., Leclercq,G. and Devleeschouwer,N. (1985) Hydroxy derivatives of tamoxifen. *J. Med. Chem.*, **28**, 1491–1497.
54. Davies,R., Oreffo,V.I.C., Martin,E.A., Festing,M.F.W., White,I.N.H., Smith,L.L. and Styles,J.A. (1997) Tamoxifen causes gene mutations in the livers of lambda/*lacI* transgenic rats. *Cancer Res.*, **57**, 1288–1293.
55. Vancutsem,P.M., Lazarus,P. and Williams,G.M. (1994) Frequent and specific mutations of the rat *p53* gene in hepatocarcinomas induced by tamoxifen. *Cancer Res.*, **54**, 3864–3867.
56. Welsh,J.A., Moberger,B., Fornander,T., Malfetano,J.H., Ambros,R.A., Weed,J.C., Deligdisch,L., Cohen,C.J., Silva,E.G., Vincent,F., Jones,R.T. and Bennett,W.P. (1997) Tamoxifen has a characteristic mutation spectrum in human endometrial cancers. *Proc. Amer. Assoc. Cancer Res.*, **38**, 450.

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