Further characterization of the DNA adducts formed in rat liver after the administration of tamoxifen, N-desmethyldtamoxifen or N,N-didesmethyldtamoxifen

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The present study compares the formation of DNA adducts, determined by ³²P-postlabelling, in the livers of rats given tamoxifen and the N-demethylated metabolites N-desmethyltamoxifen and N,N-didesmethyldtamoxifen. Results show that after 4 days treatment (0.11 mmol/kg i.p.), similar levels of DNA damage were seen after treatment with either tamoxifen or N-desmethyldtamoxifen [109 ± 40 (n = 3) and 100 ± 33 (n = 4) adducts/10⁸ nucleotides, respectively], even though the concentration of tamoxifen in the livers of tamoxifen-treated rats was about half that of N-desmethyldtamoxifen in the N-desmethyldtamoxifen-treated animals (51 ± 16 and 100 ± 8 mmol/g, respectively). Administration of N,N-didesmethyldtamoxifen to rats resulted in a 5-fold lower level of damage (19 adducts/10⁸ nucleotides, n = 2). Following ³²P-postlabelling and HPLC, hepatic DNA from rats treated with tamoxifen and its metabolites showed distinctive patterns of adducts. Treatment of rats with N,N-didesmethyldtamoxifen gave a major product that co-eluted with one of the minor adduct peaks seen in the livers of rats given tamoxifen. Following dosing with N-desmethyldtamoxifen, the major product co-eluted with one of the main peaks seen following treatment of rats with tamoxifen. This suggests that tamoxifen can be metabolically converted to N-desmethyldtamoxifen prior to activation. However, analysis of the ³²P-postlabelled products from the reaction between α-acetoxytamoxifen and calf thymus DNA showed two main peaks, the smaller one of which (~15% of the total) also co-eluted with that attributed to N-desmethyldtamoxifen. This indicates that N-desmethyldtamoxifen and N,N-didesmethyldtamoxifen are activated in a similar manner to tamoxifen leading to a complex mixture of adducts. Since an HPLC system does not exist that can fully separate all these ³²P-postlabelled adducts, care has to be taken when interpreting results and determining the relative importance of individual adducts and the metabolites they are derived from in the carcinogenic process.

Introduction

The anti-oestrogen tamoxifen {trans-(Z)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butene} is currently undergoing evaluation as a chemopreventive agent in women at increased risk of developing breast cancer (1). Recent results in healthy, high risk women from the NSABP P1 study demonstrated that this drug resulted in a 49% reduction in breast cancer incidence (2). However, tamoxifen treatment is associated with an increased incidence of endometrial cancer (3,4) and long-term administration to rats results in a dose-dependent increase in hepatic tumours (5,6). Tamoxifen requires metabolic activation to electrophilic species before it can react with DNA, forming adducts detectable using the ³²P-postlabelling assay (7,8). The major phase I metabolites of tamoxifen in both rat and human liver are N-desmethyldtamoxifen and 4-hydroxytamoxifen (Figure 1; 9,10). However, one of the main DNA adducts detected in the livers of tamoxifen-dosed rats is thought to arise as a consequence of α-hydroxylation, followed by hydroxysteroid sulphotransferase-mediated sulphate conjugation (11–13). This adduct, which co-elutes with the major product from the in vitro reaction between α-sulphate tamoxifen or α-acetoxymoxifen and DNA, contains tamoxifen in the trans form covalently linked via the α carbon to the exocyclic amino group of deoxyguanosine (dG-N²-tam) (14,15). This same adduct has recently been identified in DNA extracted from the livers of tamoxifen-treated rats (16). A second major product was N-desmethyldtamoxifen linked covalently to the amino group of deoxyguanosine in a similar manner (16). Previously we have shown that 4-hydroxytamoxifen also forms other minor adducts in rat liver (17).

In this study, we have conducted further detailed investigations into the adducts formed by tamoxifen in rat liver. Using an HPLC system developed previously to separate ³²P-postlabelled tamoxifen adducts (17), we have identified those formed as a result of activation of tamoxifen and its metabolites N-desmethyl and N,N-didesmethyldtamoxifen. This has been achieved using authentic standards of tamoxifen–deoxyguanosine adducts and by dosing rats with the above compounds. Results show that the major peaks contain adducts formed via α-hydroxylation of tamoxifen and N-desmethyldtamoxifen whilst minor peaks contain N-desmethyldtamoxifen and N,N-didesmethyldtamoxifen-derived adducts.

Materials and methods

Chemical methods: general procedures

¹H NMR spectra were determined at 250 MHz using a Bruker 250 ARX spectrometer. Chemical shifts are expressed using the δ system of units relative to the internal standard, Me₂Si. Routine mass spectra of synthesized compounds were obtained with an Autospec Ultima-Q (Micromass, Manchester, UK) spectrometer. Column chromatography was performed on silica gel for flash chromatography (40–63 μm; BDH, Poole, UK). Tamoxifen was a gift from Dr J. Topham (Zeneca plc, Macclesfield, UK) and N,N-didesmethyldtamoxifen was a gift from Dr Ian Hardcastle (Institute of Cancer Research, Sutton, UK). Calf thymus DNA, 2’-deoxyguanosine 3’-monophosphate, tricaprylin, proteinase K, RNase A and RNase T1 were from Sigma Chemical Co. (Poole, UK). Chemical reagents and solvents of analytical grade were purchased from Aldrich Chemical Co. (Poole, UK) or Fisher Scientific Ltd (Loughborough, UK) unless otherwise stated. α-Acetoxytamoxifen was synthesized by the published procedure (14).

Synthesis of N-desmethyldtamoxifen

N-Desmethyldtamoxifen was synthesized from tamoxifen by a novel route using a method adapted from Montzka et al. (18) for the demethylation of tertiary methylamines. To a refluxing solution of tamoxifen (2.30 g, 6.20
mmol) in toluene (100 ml) was added 2,2,2-trichloroethyl chloroformate (2.5 ml, 30 mmol) and potassium carbonate (1.0 g). After 4 h the mixture was cooled to room temperature and extracted with 4 M NaOH (100 ml), 2 M HCl (100 ml), then water (100 ml), dried (potassium carbonate) and concentrated. Crystallization of the residue from heptane afforded the intermediate (3.0 g, 91% yield). Using positive ion electrospray mass spectrometry a protonated molecular ion (M+H)+ was observed with m/z 534 corresponding to the N-desmethylated trichloroethyl carbamate intermediate.

The carbamate intermediate (2.5 g, 4.69 mmol) was dissolved in diglyme (50 ml), then 90% acetic acid (150 ml), and zinc (2.5g, 38.24 mmol) was added and the mixture stirred at room temperature overnight. The reaction mixture was extracted with toluene (3×100 ml), dried (Na2SO4), concentrated and subjected to column chromatography on silica gel (20×20 cm) followed by CH2Cl2/methanol/ammonia (95:5:0.3) to elute any unretracted intermediate, followed by CH3Cl/methanol/ammonia (95:5:0.3) to elute the product. Crystallization from heptane gave white crystals of N-desmethyltamoxifen acetate as the pure trans isomer (1.50 g, 77% yield). 1H-NMR (CDCl3) δ 0.92 (t, 3H, CH3), 1.78 (s, 1H, D2O exchangeable NH), 2.0 (s, 3H, acetate CH3COO), 2.5 (q, 2H, CH2CH3), 2.55 (s, 3H, NCH3), 3.05 (t, 2H, CH2CH2N), 4.0 (t, 2H, CH2CH2N), 6.55 (d, 2H, H-3,5 of C6H4O), 6.8 (d, 2H, H-6,7.5 of C6H4H2O), 7.0–7.5 (m, 10H, ArH). Using positive ion electrospray a protonated molecular ion was observed at m/z 358 representing the molecular weight minus the acetate moiety.

**Reaction of α-acetoxytamoxifen with 2′-deoxyguanosine 3′-monophosphate**

2′-Deoxyguanosine 3′-monophosphate (5 mg) was reacted overnight at 37°C with α-acetoxytamoxifen (10 mg) in 2 ml 100 mM Tris–HCl buffer (pH 8.0). The reaction mixture was centrifuged and the supernatant extracted with 3×1 ml butanol. The pooled extracts were separated by reverse phase HPLC on a Hypersil BDS column (5μ, 250×4.6 mm i.d.) with a 0.05 M ammonium formate (pH 5.4) to methanol gradient (0–80%) in 50 min then to 100% over 10 min at a flow rate of 1 ml/min. Four adduct peaks were collected and subject to analysis by mass spectrometry using a Quattro BIO-Q tandem quadrupole instrument (Micromass, Manchester, UK) fitted with an electrospray source operating in the positive ion mode. Each adduct peak was 32P-postlabelled by treating 10 μl aliquots as 10 μg DNA digest and separated using the HPLC system developed by Martin et al. (17).

**Fig. 1.** Pathways of tamoxifen metabolism and metabolic activation.

32P-postlabelled hepatic DNA can be separated into at least 12 different tamoxifen adduct peaks using the present HPLC system (17). These were numbered in their elution order, with daily by i.p. injection for 4 days. Dosing solutions were made up in tricaprylin (0.11 mmol/ml). The animals were killed 24 h after the last dose. Liver tissue was removed, immediately frozen in liquid nitrogen and stored at –80°C.

**Results**

HPLC separation of 32P-postlabelled adducts formed from in vitro reaction of α-acetoxytamoxifen with calf thymus DNA and 2′-deoxyguanosine 3′-monophosphate

We have previously shown that following long-term administration of tamoxifen in the diet to Wistar rats (35 mg/kg/day), 32P-postlabelled hepatic DNA can be separated into at least 12 different tamoxifen adduct peaks using the present HPLC system (17). These were numbered in their elution order, with
the major adduct peaks 5 and 6 accounting for approximately 45 and 24%, respectively, of the total DNA adducts in the liver after 6 months dosing (17). In the present study, with only 4 days dosing, some of the minor adduct peaks were not detected. Reaction of the model ester trans-α-acetoxytamoxifen with calf thymus DNA in vitro gave three 32P-postlabelled adducts (Figure 2A) which eluted during the isocratic phase and several minor adducts which eluted after 40 min. The major product of this reaction, which accounts for 78% of the total adducts formed, co-elutes with in vivo peak 6 and has previously been identified by Osborne et al. as a trans isomer of dG-N2-tam (14). This group further report that stereoisomers of the cis form of dG-N2-tam and an adenine adduct are also generated in smaller yields (22). The second major peak which co-elutes with peak 5 contributes ~15% of the total adduct level and could contain a diastereoisomer of trans-dG-N2-tam, whilst the minor peak eluting at 23 min which co-elutes with in vivo peak 3 could be an adenine adduct.

To confirm that the products in peaks 5 and 6 contained tamoxifen linked to deoxyguanosine, α-acetoxytamoxifen was reacted with 2′-deoxyguanosine 3′-monophosphate. This incubation yielded four products separable by HPLC, which were collected and analysed by electrospray mass spectrometry. Each peak gave a protonated molecular ion with m/z 717.
or monohydroxylated metabolites were detected after that present in tamoxifen-dosed rats (Table I). No tamoxifen accumulated in the liver giving concentrations approximately twice concentration of than tamoxifen itself. Following dosing with an equimolar N-desmethyltamoxifen detectable in rat liver extracts is higher after 4 days tamoxifen treatment (0.11 mmol/kg) the level of treated rats.

Tamoxifen and N-desmethyltamoxifen metabolites in the livers of treated rats

After 4 days tamoxifen treatment (0.11 mmol/kg) the level of N-desmethyltamoxifen detectable in rat liver extracts is higher than tamoxifen itself. Following dosing with an equimolar concentration of N-desmethyltamoxifen, this compound accumulated in the liver giving concentrations approximately twice that present in tamoxifen-dosed rats (Table I). No tamoxifen or monohydroxylated metabolites were detected after N-desmethyltamoxifen treatment, confirming that any adducts formed must contain an N-demethylated tamoxifen moiety (Figure 4). The concentration of 4-hydroxylated metabolites was similar in both groups of animals. Using LC-ES-SIM-MS analysis, four peaks with [M+H]^+ ions at m/z 374, corresponding to mono-oxygenated derivatives of N-desmethyltamoxifen, were detected in the livers of both tamoxifen- and N-desmethyltamoxifen-dosed animals. If it is assumed that these N-demethylated metabolites elute in the same order as their corresponding tamoxifen metabolites (m/z 388) then A, B, C and D are N-demethylated forms of trans-α-hydroxytamoxifen, cis-α-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen, respectively.

Discussion

The products of the reaction between α-acetoxytamoxifen and the exocyclic amino function of deoxyguanosine in calf thymus DNA in vitro can exist as four stereoisomers (15). In this study the main adduct, a trans form of dG-N^2-tam, accounts for 78% of the total adducts and co-elutes with peak 6 formed in DNA extracted from the livers of tamoxifen-treated rats (Figure 2A and B). The relative importance of each isomer in the carcinogenic mechanism of tamoxifen is currently unknown. As it remains to be confirmed whether the present HPLC system can resolve diastereoisomers of 32P-postlabelled dG-N^2-tam adducts, it is not known whether all four isomers are formed in vivo or whether there is some stereoselectivity in the reaction between the carbocation and double-stranded DNA.

N-Desmethyltamoxifen is a major metabolite of tamoxifen present in liver extracts at a higher concentration than the parent drug (Table I). Relatively higher levels of N-desmethyltamoxifen have also been found both in the liver of rats (23) and in liver and uterine tissues of women (10) following long-term dosing with tamoxifen. Recent results of Rajaniemi et al. have suggested that in rats dosed with tamoxifen, the N-desmethyltamoxifen metabolite undergoes activation to a somewhat greater extent than tamoxifen itself (16). We have found that N-desmethyltamoxifen induces similar levels of adducts as tamoxifen. Dosing with this compound produces three 32P-postlabelled hepatic DNA adducts which co-elute with adduct peaks II, 3 and 5 in tamoxifen-dosed animals. The major
Tamoxifen and tamoxifen metabolite adducts

Fig. 4. Selected ion chromatograms of metabolites in liver extracts from F344 rats treated with tamoxifen and N-desmethyltamoxifen. The metabolites with the [M+H]+ ion at m/z 388 represent trans-α-hydroxytamoxifen (1), cis-α-hydroxytamoxifen (2), 4-hydroxytamoxifen (3) and 4'-hydroxytamoxifen (4). The peaks with [M+H]+ ions at m/z 374 (A, B, C and D) correspond to N-demethylated derivatives of trans-α-hydroxytamoxifen, cis-α-hydroxytamoxifen, 4-hydroxytamoxifen and 4'-hydroxytamoxifen, respectively.

Table I. Hepatic concentrations of tamoxifen and metabolites in F344 rats treated with tamoxifen or N-desmethyltamoxifen (0.11 mmol/kg daily) by i.p. injection for 4 days

<table>
<thead>
<tr>
<th>Compound</th>
<th>Metabolite concentration (nmol/g liver) (means ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Tamoxifen</td>
</tr>
<tr>
<td></td>
<td>N-Desmethyltamoxifen</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxylated metabolites</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>49.9 ± 16.1</td>
</tr>
<tr>
<td>N-Desmethyltamoxifen</td>
<td>0</td>
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<tr>
<td></td>
<td>59.9 ± 20.3</td>
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<td></td>
<td>13.7 ± 1.3</td>
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<td></td>
<td>99.7 ± 8.1</td>
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<td>8.4 ± 0.6</td>
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Values are the mean of three determinations from four rats.

component formed co-elutes with peak 5 and is presumably an N-demethylated derivative of trans-dG-N2-tam (Figure 2C). N,N-Didesmethyltamoxifen is activated to a much lesser extent than tamoxifen or N-desmethyltamoxifen, producing 5-fold lower levels of adducts. An adduct co-eluting with peak 3 is a major product in the livers of N,N-didesmethyltamoxifen-treated rats and a minor product in the livers of N-desmethyltamoxifen-treated rats. Therefore, the major component of this peak is probably an N,N-didemethylated form of trans-dG-N2-tam, arising via α-hydroxylation of N,N-didesmethyltamoxifen. An adduct corresponding to in vivo peak 3 was also produced following reaction of α-acetoxytamoxifen with calf thymus DNA. It is therefore likely that this peak may contain other products such as the adenine adduct reported by Osborne et al. (22). Only low levels of N,N-didesmethyltamoxifen are detected in the livers of rats given tamoxifen but in primates and women this metabolite accumulates in the liver (10,24). N,N-Didesmethyltamoxifen is a potent inhibitor of certain CYP-catalysed reactions and it has been suggested that this metabolite may limit the activation of tamoxifen to the α-hydroxylated reactive metabolite (24).

Past studies using 32P-postlabelling with polyethyleneimine-cellulose TLC separation have failed to detect DNA damage in the livers of women given tamoxifen (20). With the greater resolution and sensitivity of the present analytical system, we have shown the importance of N-demethylated metabolites in tamoxifen–DNA adduct formation. As with tamoxifen, the major adducts are formed as a consequence of α-hydroxylation, but it has previously been shown that activation of 4-hydroxytamoxtifen can also result in DNA adduct formation through oxidation to a quinoine methide (25) or via radical intermediates (26). It is therefore likely that a similar route of activation could also take place with the 4-hydroxy metabolites of N-desmethyltamoxifen and N,N-didesmethyltamoxifen, leading to the potential formation of DNA adducts. We have shown that N-desmethyltamoxifen and N,N-didesmethyltamoxifen are activated in a similar manner to tamoxifen leading to a complex mixture of adducts which at the present time cannot be fully
References


