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METABOLISM OF TAMOXIFEN BY ISOLATED RAT HEPATOCYTES

IDENTIFICATION OF THE GLUCURONIDE OF 4-HYDROXYTAMOXIFEN

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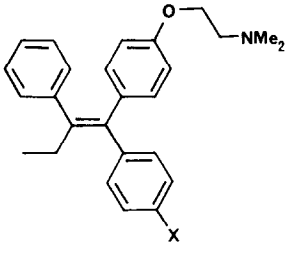
Abstract—Metabolism of 4-hydroxytamoxifen by hepatocytes isolated from rats administered with phenobarbital and examination by TLC of the components not extractable into ethyl acetate revealed 4-hydroxytamoxifen β-glucuronide; its identity was confirmed by comparison of its ^{1}H NMR spectrum with that of synthetic material. This conjugate was also formed on metabolism of tamoxifen. It bound to cytosolic oestrogen receptors with only one thousandth the affinity of 4-hydroxytamoxifen and gave a correspondingly very weak inhibition of growth of the MCF-7 human breast cancer cell line. Therefore, in contrast to reported observations on the 3-glucuronide of oestradiol, the MCF-7 cells were unable to hydrolyse 4-hydroxytamoxifen glucuronide and on this evidence, formation of this metabolite is solely a deactivation pathway.

Tamoxifen (**1**) is an excellent drug for the treatment of advanced breast cancer giving a proportion of patients a period free from disease without significant side-effects [1, 2]. Its principal mode of action is thought to be as an antioestrogen, acting by competitive displacement of the tumour growth promoting hormone oestradiol from its receptor binding site [3]. Metabolism may play an important role in mediating the overall activity of this drug [4]. In particular, the metabolite 4-hydroxytamoxifen (**2**) [5] has an affinity for the oestrogen receptor one hundred times that of tamoxifen and has a correspondingly greater antitumour potency than tamoxifen *in vitro* [6, 7]. However, this high potency is not realised *in vivo* where tamoxifen and 4-hydroxytamoxifen have comparable effectiveness [8] correlating with the low plasma levels that are achieved for the latter (*ca.* 200 ng/mL for tamoxifen in the sera of patients but *ca.* 5 ng/mL for hydroxytamoxifen) [5] and attributable to a rapid clearance mechanism by way of conjugation of the hydroxyl group [9]. By inference from the observation of Fromson *et al.* [10] that 4-hydroxytamoxifen is liberated from faeces of tamoxifen treated patients upon treatment with β-glucuronidase, this conjugate must be a glucuronide.



(**1**) X = H
(**2**) X = OH

We have previously shown that isolated rat hepatocytes will convert the hydroxy group of the aromatase inhibitor 4-hydroxyandrostenedione into the glucuronide [11]. That this glucuronide is also observed as a major urinary metabolite in patients [12] provided evidence that rat hepatocytes provide a faithful model for metabolism *in vivo*. Supporting this conclusion is the finding that rat hepatocytes convert tamoxifen into several of the metabolites identified in the sera of patients [13].

We now report that by examination of the fraction of polar metabolites corresponding to conjugates obtained after incubation of 4-hydroxytamoxifen or tamoxifen with isolated rat hepatocytes, the glucuronide of 4-hydroxytamoxifen can be detected directly.

In addition to the presumed role of 4-hydroxytamoxifen glucuronide in the clearance of tamoxifen it was considered a possibility that the glucuronide might have significant antitumour activity in itself. Aniline mustard [*N*,*N*-di-(2-chloroethyl)aniline], like tamoxifen, undergoes metabolic hydroxylation on the aromatic ring and also a glucuronide is formed [14]. This glucuronide, which is a major serum and bile metabolite of aniline mustard [14], has been shown to be four-fold more cytotoxic than the parent drug [15]. Although we would not anticipate that 4-hydroxytamoxifen glucuronide would have a significant direct action on tumour cells, the finding that oestradiol 3-glucuronide causes a time dependent

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disappearance of oestrogen receptors ("processing") in the human breast cancer cell line MCF-7 in culture, indicated that the cell line possesses glucuronidase activity liberating the potent oestrogen [16]. Moreover, oestradiol 3-glucuronide partially reversed the growth inhibition of MCF-7 cell growth by 4-hydroxytamoxifen despite being unable to reduce [^{3}H]oestradiol uptake in a binding affinity assay, giving further evidence of hydrolysis by the cells. We have consequently examined the glucuronide of 4-hydroxytamoxifen obtained by synthesis for its binding affinity to oestrogen receptors and for its ability to inhibit the growth of the MCF-7 cell line.

MATERIALS AND METHODS

Materials. *Z*-[ring-^{14}C]tamoxifen citrate (sp. act. 21 mCi/mmol and 97% chemically pure) was purchased from Amersham International, (Amersham, U.K.). Unlabelled tamoxifen, 4-hydroxytamoxifen, and methyl tetra-*O*-acetyl β-D-glucuronate were prepared by published methods [17–19].

In vitro *metabolism.* Male adult Wistar rats fed *ad lib.* and given phenobarbital in their drinking water (0.5 g/L) for 10–14 days were used to prepare hepatocytes by a 2-step perfusion method as described previously [11]. The freshly isolated cells (5 mL of 6–8 × 10^6/mL 90% viable) were incubated under an atmosphere of O_2 at 37° with a mixture of [^{14}C]tamoxifen citrate (0.8 × 10^6 DPM) and unlabelled tamoxifen (100 μg/mL) or with unlabelled 4-hydroxytamoxifen (100 μg/mL) in 25-mL conical flasks in a phosphate buffered saline medium at pH 7.5 [20] containing Hepes (20 mmol/L) and glucose (5 mmol/L). After incubation for 60 min the flasks were cooled in ice, the hepatocytes disintegrated by ultrasonication and the protein precipitated with 4 volumes of acetone. The acetone was removed under vacuum and after adjustment of the pH to 9.5, non-utilized substrate and phase I nonpolar metabolites were extracted with ethyl acetate. Conjugates remaining in the aqueous fraction were concentrated on Sep-pak cartridges, eluted with methanol and then subjected to reverse-phase chromatography on Whatman KC 18F plates (20 × 20 cm), eluting with methanol:water:40% tetrabutylammonium hydroxide (80:20:1) and using synthetic 4-hydroxytamoxifen glucuronide as a marker to locate the position of the metabolite glucuronide.

In case of [^{14}C]tamoxifen a Berthold automatic analyser was used to locate the radioactive peaks and to determine the proportion of label in the peak corresponding to 4-hydroxytamoxifen glucuronide.

NMR spectra. Proton NMR spectra (250 MHz; internal tetramethylsilane reference) were recorded on a Bruker AC250 spectrometer. Solutions of metabolites in methanol extracted from the chromatography plates were filtered, concentrated to dryness under reduced pressure, last traces of solvent being removed at 0.01 mm Hg over 1 hr. The resulting residues were redissolved in the appropriate deuterated solvent (0.5 mL) in 5 mm NMR tubes. For metabolites, data was accumulated over 1 hr (1500 scans).

Binding of the glucuronide (**4a**) *to oestrogen receptors* [21]. Calf uterine cytosol was incubated at 18° for 30 min with 5 × 10^{-9} M [^{3}H]oestradiol in the absence and presence of increasing amounts (10^{-9} to 10^{-5} M) of the glucuronide (**4a**) or unlabelled oestradiol (control). Unbound compounds were then removed using dextran-coated charcoal and the amounts of oestrogen receptor bound [^{3}H]oestradiol were measured. The relative concentration of oestradiol and test compound required to achieve 50% inhibition of [^{3}H]oestradiol binding is the relative binding affinity (RBA), i.e. RBA = ([I_{50}]oestradiol/[I_{50}] test compound) × 100.

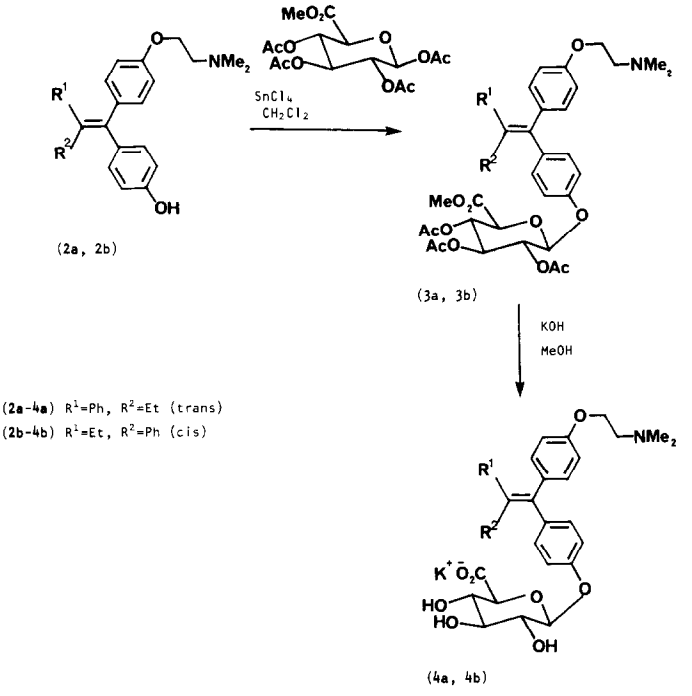
Effect of the glucuronide (**4a**) *on MCF-7 cell growth.* The actions of 4-hydroxytamoxifen glucuronide, 4-hydroxytamoxifen and tamoxifen on MCF-7 cell growth were compared by measuring the amounts of DNA after 5 days of culture according to the protocol described previously [22]. Action of the compounds on ER levels was estimated after 3 days of culture when amounts of receptor were measured in cytosol extracts by multipoint DCC assay using [^{3}H]oestradiol as labelled ligand according to the accepted method [23].

SYNTHESIS

The glucuronide of 4-hydroxytamoxifen was synthesized as shown in Scheme 1; the method being essentially that used by Hadd *et al.* [24]. for the synthesis of the anomeric 17-glucuronides of ethynyloestradiol. Thus, treatment of 4-hydroxytamoxifen as a mixture of *trans* (**2a**) and *cis* (**2b**)-isomers with methyl tetra-O-acetyl-β-D-glucuronate in the presence of excess tin (IV) chloride as Lewis acid gave the glucuronate ethers (**3a, 3b**). There was no advantage in using the pure *trans*-geometric isomer of 4-hydroxytamoxifen since rapid isomerization to a *cis-trans* mixture was catalysed by the tin chloride. The condensation gave high selectivity for the required β-anomer but with extended reaction time there was isomerization to the α-anomer which proved difficult to remove. It was consequently preferable to work up the reaction when it was only about two-thirds complete. The *trans*- and *cis*-β-glucuronates were separated by chromatography on silica and were individually hydrolysed to the potassium salts of the glucuronides (**4a**) and (**4b**). As confirmation of structure, these *trans*- and *cis*-glucuronides were treated with β-glucuronidase (from *Escherichia coli*) in phosphate buffer when there was, in each case, a rapid hydrolysis to give the corresponding isomer of 4-hydroxytamoxifen (identified by reverse phase TLC).

Methyl 1-O-[4-[1-[4-[2-*(dimethylamino)ethoxy*]-*phenyl*]-2-*phenyl*-(Z *and* E)*but*-1-*enyl*]*phenyl*]-2,3,4-*tri*-O-*acetyl*-β-D-*glucuronate.*

To a stirred solution of methyl-1,2,3,4-tetra-*O*-acetyl-β-D-glucuronate (1.72 g, 4.58 mmol) in dichloromethane (10 mL) at 20° was added tin (IV) chloride (1.6 mL, 13.6 mmol). When the solution became mauve (*ca.* 20 min) a suspension of 4-hydroxytamoxifen (1:1 mixture of *Z* and *E* isomers; 1.17 g, 3.03 mmol) in dichloromethane (10 mL) was added. After 24 hr (TLC indicated reaction about



Scheme 1. Synthesis of the glucuronide of 4-hydroxytamoxifen.

two-thirds complete), the mixture was poured into saturated aqueous sodium hydrogen carbonate (125 mL) and the products extracted with ether (2 × 100 mL). The combined extracts were dried with sodium sulphate and concentrated. The residue was applied to a column of silica gel (Merck 15111; 40 g). Elution with dichloromethane-methanol (50:1) gave the methyl triacetyl-β-glucuronate as a 3:4 mixture of *E*-(*trans*) and *Z*-(*cis*) isomers (453 mg, 21%). Separate isomers were obtained by further chromatography on silica but eluting with ether-triethylamine (100:1) giving (i) the *E*-(*trans*)-isomer (**3a**) (44 mg) as a powder, m.p. 120–122° (from light petroleum, b.p. 80–100°), NMR ($CDCl_3$): δ 0.91 (t, *J* 7.4 Hz, 3H, CH_3CH_2), 2.04, 2.05, 2.07 (3 × s, 9H, 3 × $COCH_3$), 2.31 (s, 6H, NMe_2), 2.45 (q, *J* 7.4 Hz, 2H, CH_3CH_2), 2.68 (t, *J* 5.8 Hz, 2H, OCH_2CH_2N), 3.73 (s, 3H, CO_2Me), 3.95 (t, *J* 5.8 Hz, 2H, OCH_2CH_2N), 4.19 (m, 1H, H-5), 5.17 (d, *J* 7.1 Hz, 1H, H-1), 5.25–5.38 (m, 3H, H-2,3,4), 6.54 (d, *J* 8.7 Hz, 2H, Ar*H ortho* to OCH_2CH_2N), 6.74 (d, *J* 8.7 Hz, 2H, Ar*H ortho* to OCH_2CH_2N), 6.96 (d, *J* 8.6 Hz, 2H, Ar*H*), 7.07–7.18 (m, 7H, ArH); Anal. Calc. for $C_{39}H_{45}O_{11}$: C, 66.6; H, 6.4; N, 2.0. Found: C, 66.3; H, 6.5; N, 2.0% and (ii) the *Z*-(*cis*)-isomer (**3b**) (170 mg) as a powder, m.p. 144–146° (from light petroleum, b.p. 80–100°), NMR ($CDCl_3$): δ 0.92 (t, *J* 7.4 Hz, 3H, CH_3CH_2), 2.00, 2.02, 2.02 (9H, 3 × s, 3 × $COCH_3$), 2.39 (s, 6H, NMe_2), 2.46 (q, *J* 7.4 Hz, 2H, CH_3CH_2), 2.79 (t, *J* 5.7 Hz, 2H, OCH_2CH_2N), 3.67 (s, 3H, CO_2Me), 4.07–4.14 (m, 3H, OCH_2CH_2N and H-5) 5.00 (d, *J* 7.0 Hz, 1H, H-1), 5.18–5.31 (m, 3H, H-2,3,4), 6.62 (d, *J* 8.8 Hz, Ar*H*), 6.78 (d, 8.8 Hz, Ar*H*), 6.89 (d, *J* 8.7 Hz, Ar*H ortho* to OCH_2CH_2N), 7.06–7.25 (m, 7H, Ar*H*); Anal. Calc. for $C_{39}H_{45}O_{11}$: C, 66.6; H, 6.4; N, 2.0. Found: C, 66.5; H, 6.5; N, 2.0%.

1-O-[4-[1-[4-[2-*(Dimethylamino)ethoxy]phenyl*]-2-*phenylbut*-1-*enyl*]*phenyl*]-β-D-*glucuronic acid (4-hydroxytamoxifen glucuronide) potassium salt*

Trans-(E)-*isomer* (**4a**). The *trans*-(*E*)-glucuronate (**3a**) (16.5 mg, 0.02 mmol) was added to a solution of 85% potassium hydroxyde (13.3 mg, 0.20 mmol) in methanol (0.25 mL). After 2 hr, ethyl acetate (0.5 mL) was added and the mixture concentrated to dryness. The residue was applied to a column of silica gel (Merck 15111; 1 g). Elution with chloroform–methanol (3:1) gave the hydrated potassium salt of *trans*-4-hydroxytamoxifen glucuronide as a glass (12.7 mg, 87%), NMR (acetic acid-d_4): δ 0.92 (t, *J* 7.4 Hz, 3H, CH_3CH_2), 2.49 (q, *J* 7.4 Hz, 2H, CH_3CH_2), 2.97 (s, 6H, NMe_2), 3.55 (br.t., J 6 Hz, 2H, OCH_2CH_2N), 3.78–3.92 (m, 3H, H-2,3,4), 4.19–4.28 (m, 3H, H-5 and OCH_2CH_2N), 5.18 (d, *J* 7.2 Hz, 1H, H-1), 6.61 (d, *J* 8.7 Hz, 2H, Ar*H ortho* to OCH_2CH_2N), 6.83 (d, *J* 8.7 Hz, 2H, Ar*H meta* to OCH_2CH_2N), 7.06–7.20 (m, 9H, ArH); Anal. Calc. for $C_{32}H_{36}KNO_8.1\frac{1}{2}H_2O$ C, 61.1; H, 6.25; N, 2.2. Found: C, 60.9; H, 6.3; N, 2.3%.

Cis-(Z)-*isomer* (**4b**). Similar treatment of the *cis*-(*Z*)-glucuronate (62.5 mg, 0.089 mmol) with 85% potassium hydroxide (53 mg, 0.80 mmol) in methanol (1 mL) by the method described for the *trans*-isomer gave the potassium salt of *cis*-4-hydroxytamoxifen glucuronide as a glass (48.8 mg, 91%); NMR (acetic acid-d_4): δ 0.93 (t, *J* 7.4 Hz, 3H, CH_3CH_2), 2.49 (q, *J* 7.4 Hz, 2H, CH_3CH_2), 3.04 (s, 6H, NMe), 3.65 (br.t, J 6 Hz, 2H, OCH_2CH_2N), 3.68–3.85 (m, 3H, H-2,3,4), 4.11 (d, 1H, *J* 9.1 Hz, H-5), 4.39 (br.t., *J* 6 Hz, 2H, OCH_2CH_2N), 5.01 (d,

J 7.5 Hz, 1H, H-1), 6.73 (d, *J* 8.8 Hz, 2H, Ar*H*), 6.82 (d, *J* 8.8 Hz, 2H, Ar*H*), 6.96 (d, *J* 8.7 Hz, Ar*H ortho* to OCH_2CH_2N), 7.09–7.22 (m, 7H, Ar*H*); Anal. Calc. for $C_{32}H_{36}KNO_8$: C, 63.9; H, 6.0; N, 2.3%. Found: C, 64.2; H, 6.4; N 2.4%.

Hydrolysis of the β-glucuronides by glucuronidase

To a solution of the glucuronide (**4a**; 1.2 mg) in pH 6.3 phosphate buffer (0.5 mL) at 20° was added a solution of β-glucuronidase (*Escherichia coli* K12; 200 μ/mL; 0.1 mL). Within 30 sec the mixture had become cloudy owing to deposition of 4-hydroxytamoxifen and after 2 min the hydrolysis was complete, and reverse-phase TLC showed only the presence of *trans*-(*Z*)-4-hydroxytamoxifen which could be isolated by ether extraction. Similar treatment of the glucuronide (**4b**) liberated *cis*-(*E*)-4-hydroxytamoxifen. No hydrolysis of (**4a**) or (**4b**) took place in the presence of buffer alone.

RESULTS

Identification of 4-hydroxytamoxifen glucuronide

The synthetic glucuronides could be observed on reverse-phase TLC after elution with a solvent system of 80 : 20 : 1 methanol : water : 40% aqueous tetrabutylammonium hydroxide. The addition of the tetraalkylammonium salt gave rise to improved resolution relative to the more routinely used diethylamine. Indeed, tetrabutylammonium hydrogen sulphate has been used in the reverse-phase TLC analysis of bilirubin glucuronides [25]. There was complete separation of the *trans** and *cis* isomers in this system, the *trans* isomer being the more mobile (*trans* isomer, $R_f = 0.55$; cis isomer $R_f = 0.49$).

Initially hepatocyte metabolism of *trans*-4-hydroxytamoxifen rather than of tamoxifen was studied in order to maximize the production of glucuronide. The fraction of metabolites not extractable into ethyl acetate and corresponding to conjugates was examined on RPTLC revealing by comparison with the synthetic materials, the *trans*-glucuronide as the major metabolite together with a small amount of its *cis*-isomer. The formation of some *cis*-glucuronide is not surprising; it will have formed by isomerization of the 4-hydroxytamoxifen prior to the conjugate formation, a process which is facile owing to conjugation between the hydroxyl group and central olefinic bond and which has been shown to take place under cell culture conditions [26]. The metabolites could be visualized by UV light by virtue of the triarylethylene chromophore or by treatment with a naphthoresorcinol spray followed by heating when a mauve coloration indicated the presence of the glucuronide moiety [27].

In attempting to confirm the identity of the metabolites mass spectroscopy was initially employed. This technique had been used successfully to identify the glucuronide of 4-hydroxyandrostenedione as its permethylated derivative [12]. However, such permethylation of 4-hydroxytamoxifen glucuronide would cause quaternization of the dimethylamino function, and the resultant salt would lack the necessary volatility. A methane chemical ionization (CI) mass spectrum was attainable on the synthetic glucuronide when in the form of the tetrabutylammonium salt. In the CI spectrum, an ion at $m/z = 619$ (0.9% of base peak) corresponds to the butyl ester of the glucuronide (c.f. Ref. 28). Unfortunately, mass spectrometry did not prove sufficiently sensitive in this case since we were not able to obtain a spectrum on the metabolite [29].

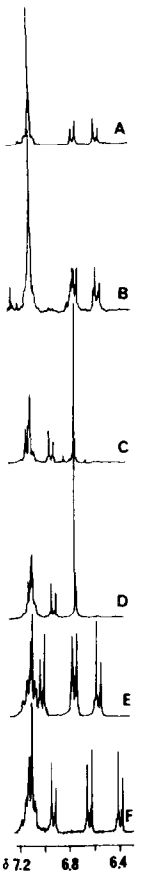


Fig. 1. Part of the proton NMR spectra of *Z* and *E* isomers of 4-hydroxytamoxifen and β-glucuronides recorded at 250 MHz in methanol-d_4. Individual spectra are (A) *trans*-(*E*)-glucuronide-synthetic; (B) *trans*-(*E*)-glucuronide-metabolite; (C) *cis*-(*Z*)-glucuronide-synthetic; (D) *cis*-(*Z*)-glucuronide-metabolite; (E) *trans*-(*Z*)-4-hydroxytamoxifen; (F) *cis*-(*E*)-4-hydroxytamoxifen.

Confirmation of structure was made by proton NMR spectroscopy on both the *trans* and *cis* glucuronide metabolites. Using d_4-methanol as the solvent spectra of the metabolites were obtained and matched the spectra of synthetic material. The glucuronides showed characteristic patterns in the region of the spectra containing the aromatic proton resonances (Fig. 1). The electron withdrawing influence of the glucuronide moiety causes the AB quartet signal seen for the hydroxylated phenyl ring to reduce to a singlet. In the *cis*-glucuronide this singlet is at

* *Trans* and *cis* refer to the relationship of the ethyl group and ring bearing the basic side-chain on the olefinic linkage. For 4-hydroxytamoxifen, the *Z*-isomer is *trans* but for its glucuronide the *Z*-isomer is *cis*.

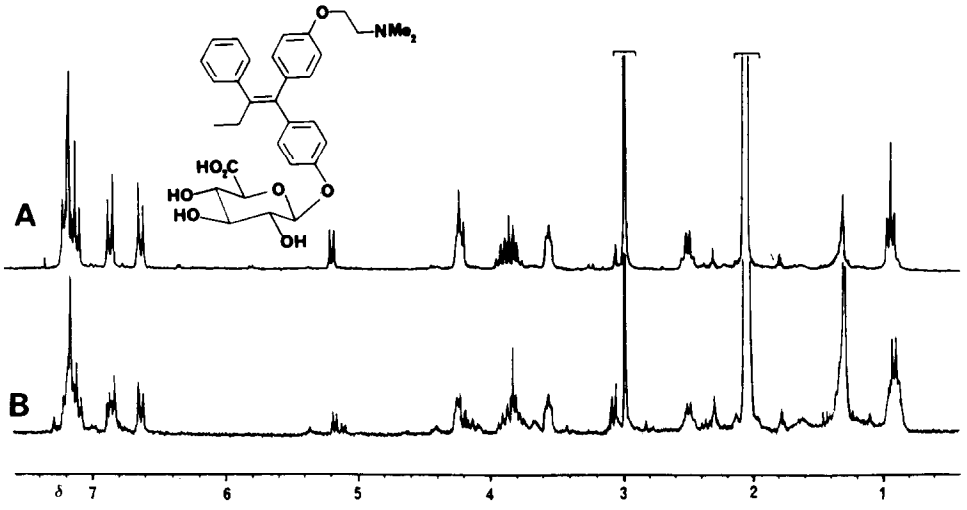


Fig. 2. Full proton NMR spectra of *trans*-4-hydroxytamoxifen glucuronide recorded at 250 MHz in acetic acid-d_4. (A) Synthetic material; (B) metabolite.

lower frequency than the other aromatic signals owing to the through space shielding influence of the adjacent phenyl ring, whereas in the *trans*-glucuronide, the singlet is conversely in a high frequency position. An important feature of the NMR spectra of the glucuronide is the signal for the anomeric proton on the sugar ring for this confirms the presence of glucuronide ether linkage and reveals the stereochemistry. This signal was buried in d_4-methanol under the signal for exchangeable protons. The resonance is however seen when d_4-acetic acid is the solvent (Fig. 2, spectrum A) as a doublet at δ 5.19. The coupling constant of 7.2 Hz is characteristic of a β-glucuronide, an α-glucuronide would give a narrower doublet $J = 3.6$ Hz [24]. The *trans*-glucuronide metabolite (but not the *cis*-isomer) was contaminated by an unknown component that could not be separated by chromatography and was present in each of three occasions that the metabolite was isolated. This contaminant was also a β-glucuronide since it gave a doublet at δ 5.12, $J = 7$ Hz (Fig. 2, spectrum B).

When tamoxifen was the substrate for the hepatocyte metabolism, the glucuronide of 4-hydroxytamoxifen was again observed but only in small quantity. The presence of the radioactive label allowed quantification. The conjugate fraction comprised 16% of the added tamoxifen, and following reverse-phase TLC separation, the 4-hydroxytamoxifen glucuronide, identified by comparison of its chromatographic mobility with a marker of synthetic material, amounted to 23% of the conjugate fraction, a yield of 3.5% from tamoxifen. These figures compare favourably with results from our previous study [13], where the bulk glucuronide fraction was hydrolysed and analysed, and when 4-hydroxytamoxifen was recovered from 20% of the conjugate fraction in a yield of 1.5% from tamoxifen. In addition to 4-hydroxytamoxifen glucuronide, we obtained several bands of lower mobility on RPTLC. By reference to our previous study [13] these will be glucuronides formed from metabolites with the side chain demethylated, N-oxidized, or modified to 1-hydroxyethyl. The identity of the band attributed to 4-hydroxytamoxifen glucuronide was confirmed by β-glucuronidase hydrolysis and repeat RPTLC analysis whereupon the mobility matched that of a 4-hydroxytamoxifen marker.

Endocrinological properties of trans-4-*hydroxytamoxifen β-glucuronide* in vitro

The synthesized glucuronide (**4a**) had an affinity for cytosolic oestrogen receptors (RBA = 0.1, oestradiol reference = 100) that was lower than that of tamoxifen (RBA = 1) and only one thousandth that of 4-hydroxytamoxifen (RBA = 100). Its antitumour potency was determined by measuring its inhibition of the rate of growth of MCF-7 human breast cancer cells in culture. In the absence of oestradiol the glucuronide (**4a**) inhibited growth only at the highest concentration used (10^{-6} M) (Table 1). It is therefore about ten-fold weaker an inhibitor than tamoxifen which gave inhibition at 10^{-7} M. This poor antitumour effect of the glucuronide therefore corresponds to its RBA value. There can be no significant degree of hydrolysis of the glucuronide to 4-hydroxytamoxifen during the 4 days of the assay since 4-hydroxytamoxifen is a potent antioestrogen and even 1% hydrolysis (formation of 10^{-8} M 4-hydroxytamoxifen from 10^{-6} M glucuronide) would have produced a more marked growth inhibition. In the presence of 10^{-8} M oestradiol, the glucuronide was ineffective as an inhibitor of cell growth at 10^{-6} M.

The view that the glucuronide is not hydrolysed is supported by measuring remaining oestrogen receptor levels in cytosol extracts after 3 days of culture in the presence of the compounds. Thus in this measure of "oestrogen receptor processing" [30, 31], 4-hydroxytamoxifen produced a 50% reduction at a concentration between 10^{-10} M and 10^{-9} M, and a total disappearance of receptor at 10^{-8} M. On the contrary, the glucuronide was very much less effective giving a 50% reduction between 10^{-7} M and

Table 1. Effect on growth of the MCF-7 cell line

Compound	Amount of DNA (μg ADN) ± SD*		
	10^{-8}M	10^{-7}M	10^{-6}M
Tamoxifen (**1**)	12.4 ± 0.6 (105)†	7.6 ± 1.3 (64)‖	4.7 ± 1.0 (40)§¶
4-Hydroxytamoxifen (**2**)	4.8 ± 1.4 (41)¶	4.5 ± 0.9 (38)¶	4.9 ± 0.5 (42)¶
Glucuronide (**4a**)‡	12.6 ± 1.5 (107)	12.4 ± 1.6 (105)	7.2 ± 1.2 (61)¶

* Each result is the average of four determinations.

† Percentage of control value, the amount of DNA in control was 11.8 ± 0.7 μg ADN.

‡ In the presence of 10^{-8}M oestradiol, 10^{-6}M glucuronide gave an amount of DNA = 35.2 ± 2.2 μg (196%) whereas tamoxifen gave 26.3 ± 2.8 μg (147%) and oestradiol alone gave 37.7 ± 3.2 μg (210%).

§ One way variance analysis showed that the compounds produce a significant effect on growth; The Newman–Keuls test showed that they were effective at either 10^{-8}, 10^{-7} or 10^{-6}M (‖ $P < 0.05$, ¶ $P < 0.01$).

10^{-6}M which is the same range as for tamoxifen (control oestradiol: 50% between 10^{-11}M and 10^{-10}M). This behaviour of the glucuronide is in contrast to the findings with oestradiol 3-glucuronide [16].

DISCUSSION

We have demonstrated that the β-glucuronide of 4-hydroxytamoxifen is readily observed on reverse-phase TLC after incubation of 4-hydroxytamoxifen or tamoxifen by rat hepatocytes. Further to our lack of success in obtaining a mass spectrum of the metabolite owing to incompatability of the basic side-chain with derivatization procedures, but the ease of obtaining an NMR spectrum, we recommend the greater use of NMR spectroscopy for the identification of glucuronide metabolites in general.

The studies on MCF-7 cell growth inhibition lead to the deduction that MCF-7 cells cannot hydrolyse the β-glucuronide of 4-hydroxytamoxifen. This finding is in marked contrast to an earlier study which provided evidence that the 3-glucuronide of oestradiol was hydrolysed [16]. Yet, the β-glucuronidase derived from *E. coli* was capable of hydrolysing the 4-hydroxytamoxifen glucuronide. It would thus seem that β-glucuronidase in MCF-7 cells has specificity for the glucuronide of the natural hormone and maybe this is also the situation in normal breast tissue, providing a mechanism for control of the concentration of hormone in the breast in addition to the desulphation of circulating oestrogen sulphates [32].

In the absence of any hydrolysis by the tumour cells, if it assumed that MCF-7 cells are representative of breast tumours in general, then the formation of the glucuronide of 4-hydroxytamoxifen by the liver can solely represent a deactivation pathway and fully explains the somewhat poor activity of 4-hydroxytamoxifen *in vivo*. A similar situation affects the aromatase inhibitor 4-hydroxyandrostenedione, and presumably there is a similar problem for other drugs that contain hydroxyl groups where long duration of action is desired, as it is in cancer chemotherapy by continuous endocrine manipulation. Consequently approaches aimed at circumventing the glucuronidation process should be worthwhile in the development of new drugs of increased *in vivo* potency. Three strategies are: (i) addition of a second agent to divert the glucuronidation process, as in the use of sodium valproate to retard conjugation of 4-hydroxyandrostenedione [33]; (ii) structural modifications to impede glucuronidation, possibly by placement of an appropriate function close to the hydroxyl group [29]; and (iii) replacement of the hydroxyl group by a function that lacks the propensity for glucuronidation but possesses the binding capacity of the hydroxyl group or mimics its role in a mechanism based inhibition process. For the purpose of estimating the effectiveness of these strategies, which are presently under continuing investigation, the rat hepatocyte model should prove a valuable tool in determining whether unwanted conjugation is overcome.

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