ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1335–1342

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Identification and mechanism of formation of potentially genotoxic metabolites of tamoxifen: study by LC-MS/MS[1]

C.K. Lim *, Zhi-Xin Yuan, R.M. Jones, I.N.H. White, L.L. Smith

MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, UK

Received 21 August 1996; accepted 11 November 1996

Abstract

On-line high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI MS) and tandem mass spectrometry (MS/MS) have been applied to the study of tamoxifen metabolism in liver microsomes and to the identification of potentially genotoxic metabolites. The results showed that the hydroxylated derivatives, including 4-hydroxytamoxifen and α-hydroxytamoxifen are detoxication metabolites, while arene oxides, their free radical precursors or metabolic intermediates, are the most probable species involved in DNA-adduct formation.

Keywords: HPLC; Electrospray ionization mass spectrometry; Tamoxifen metabolism in liver microsomes; Reactive and genotoxic metabolites of tamoxifen

1. Introduction

Tamoxifen is an antioestrogenic drug widely used in the treatment of breast cancer. It prevents or delays the recurrence of oestrogen receptor positive breast cancers and increases the survival time of treated patients [1]. Tamoxifen has also been used as a chemopreventative agent for disease-free women who are at high risk from breast cancer [2,3]. It is known that rats given tamoxifen developed liver cancers [4–7] and epidemiological data suggested that breast cancer patients on long-term therapy suffer from an increased risk of endometrial and possibly GI-tract cancers.

It has been shown [8] that tamoxifen is activated by the liver to give DNA-damaging reactive metabolites and DNA-adducts have been detected by ^{32}P-postlabelling [9,10] and accelerator mass spectrometry [11,12]. The nature of the reactive metabolites which cause DNA damage is still a subject of debate and 1,2-epoxytamoxifen [13], α-hydroxytamoxifen [14,15], 4-hydroxytamoxifen [16], 3,4-epoxytamoxifen [17] and 3′4′-epoxytamoxifen [17] (Fig. 1) have all been proposed as possible candidates. This paper reviews evidence for and against each of the above structures using recent data obtained by on-line HPLC-electrospray ionization mass spectrometry (ESI MS) and tandem ESI MS or MS/MS.

* Corresponding author. Tel: +44 116 2525561; fax: +44 116 2525616.

[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August 1996, Osaka, Japan.

0731-7085/97/$17.00
PII S0731-7085(96)02007-9

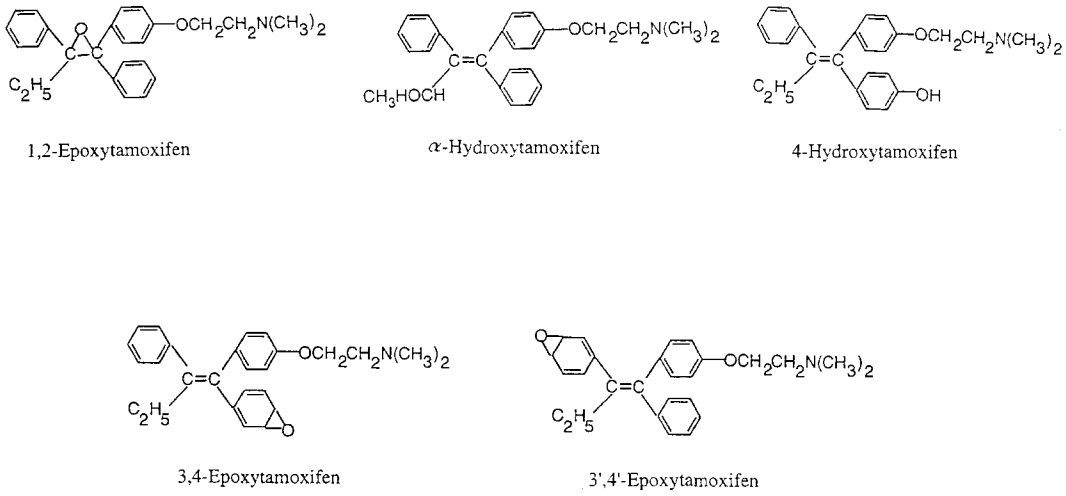

Fig. 1. Structures of tamoxifen metabolites with potential to cause DNA damage.

2. Experimental

2.1. Materials and reagents

Tamoxifen and 4-hydroxytamoxifen were gifts from Dr. J. Topham, Zeneca, Macclesfield, UK. Ammonium acetate, $MgCl_2$, NaOH, Hepes, methanol and DMSO were AnalaR grade from Merck (Poole, Dorset, UK). NADPH was analytical grade from Sigma (Poole, Dorset, UK).

2.2. Liver microsomal metabolism

The method was as previously described [17] as follows. Liver microsomal preparations (0.6 mg protein) were preincubated with tamoxifen or 4-hydroxytamoxifen (50 μM) in 50 mM Hepes-NaOH buffer (pH 7.4) in the presence of $MgCl_2$ (5 mM) at 37°C for 5 min in the dark. The reaction was started by adding NADPH (0.5 mM final concentration) to the mixture and reaction was allowed to continue for 30 min. The reaction was stopped by vortex—mixed with two volumes of methanol/DMSO (4:1 v/v). The supernatant after centrifugation was analysed by on-line HPLC-ESI MS.

2.3. On-line HPLC-ESI MS/MS conditions

A Varian 9012 solvent delivery system (Varian, Walton-on-Thames, UK) coupled to a VG Quattro BQ tandem quadrupole mass spectrometer (VG Organic, Manchester, UK) fitted with an atmospheric pressure ionization electrospray source was used. Sample injection was via a Rheodyne 7125 injector (Cotati, CA, USA) fitted with a 200 μl loop. The separation of the monoxogenated metabolites and the dimers of 4-hydroxytamoxifen were carried out on a 5 μm particle size Hypersil BDS C_{18} reversed-phase column (250 mm × 4.6 mm I.D.) from Hypersil, Runcorn, Cheshire, UK with 70 or 80% (v/v) methanol in 0.5 M ammonium acetate as mobile phases at 1 ml min^{-1}, respectively. The separation of tamoxifen metabolites in human liver microsomes was performed on a 250 × 4.6 mm Res Elut-BD column (Varian) with 70% (v/v) methanol in 0.5 M ammonium acetate as eluent. The HPLC effluent was split in the ratio of 1:6 before entering the mass spectrometer. The ESI capillary and HV electrode potentials were 0.38 and 3.78 kV, respectively. The source temperature was 150°C and the cone and lens two voltages were set at

80–85 V. All spectra were acquired in the positive ion mode, over a mass range of m/z 350–900, at a rate of one scan every 2 s.

The collision gas was argon. The collision cell pressure and collision energy were 4.3×10^{-4} mbar and 100 eV, respectively. Product ions were scanned between the masses 100–800, and the spectra were collected in the form of continuum data.

3. Results and discussion

Studies on the liver microsomal metabolism of tamoxifen suggested that activation was required in order for genotoxicity to be expressed [8]. It was established that tamoxifen could cause DNA damage to rat, even after a single dose, and this damage was selective for the liver target organ [10]. DNA-adduct formation was much lower in the liver of mouse treated with tamoxifen compared to rat, and in humans the level was barely above background [18]. Various structures have been proposed for the identity of the reactive metabolite or metabolites of tamoxifen that formed DNA adducts in the rat liver. Although the structures proposed by different groups differ, all have agreed that it is most likely to involve a monooxygenated derivative of tamoxifen and efforts have been devoted to the identification of such a species.

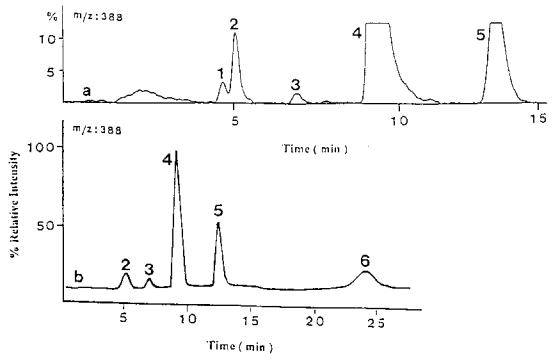

Fig. 2. On-line HPLC-ESI MS analysis of the monooxygenated tamoxifen metabolites formed by (a) mouse and (b) rat liver microsomes. Peaks: 1 = α-hydroxytamoxifen; 2 = 3,4-epoxytamoxifen; 3 = 3′4′-epoxytamoxifen; 4 = 4-hydroxytamoxifen; 5 = 4′-hydroxytamoxifen; 6 = tamoxifen *N*-oxide (not shown for mouse, as the peak was too large). The sensitivity of detection in (a) was 20 × that of (b).

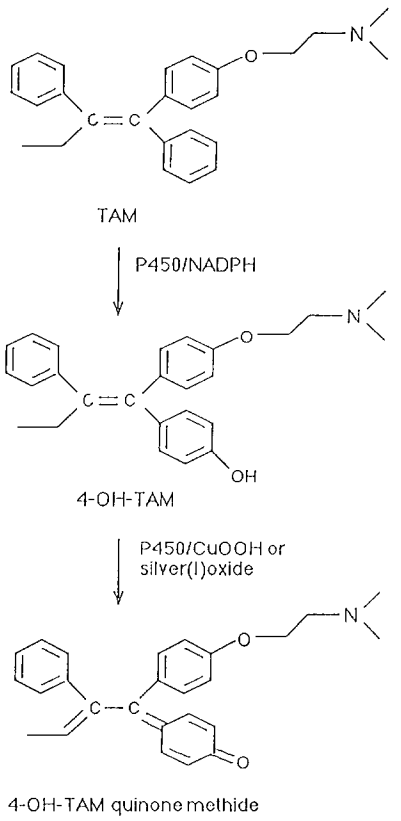

Fig. 3. Proposed mechanism of metabolic activation of tamoxifen and 4-hydroxytamoxifen [16].

Phillips et al. [13] first proposed tamoxifen 1,2-epoxide (Fig. 1) as a reactive metabolite. They have shown that the compound reacted with

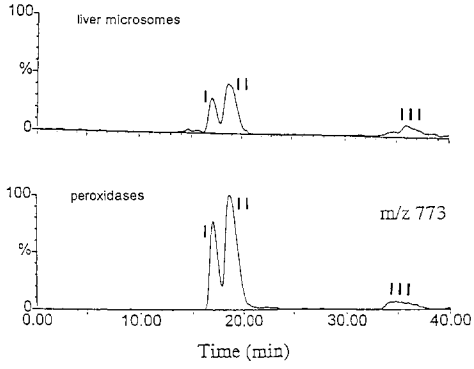

Fig. 4. On-line HPLC-ESI MS analysis of 4-hydroxytamoxifen metabolites with $[M + H]^+$ at m/z 773. See Fig. 5 for peak identification.

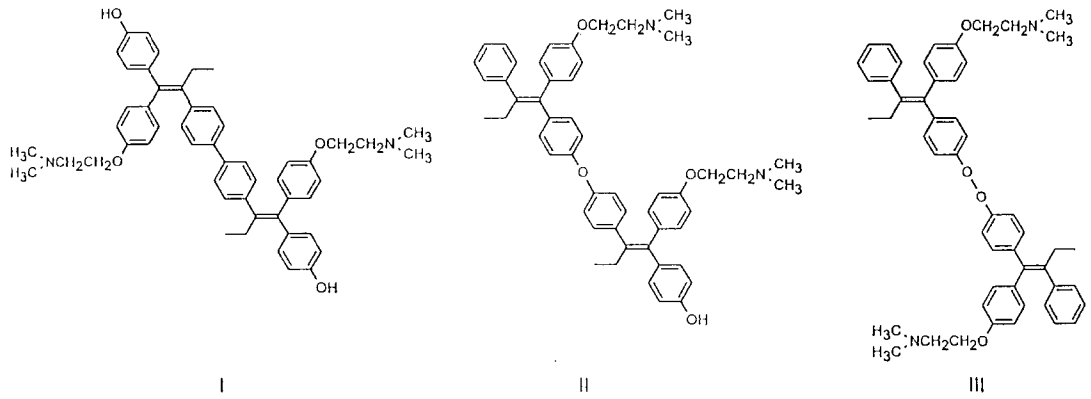

Fig. 5. Possible structures of 4-hydroxytamoxifen dimers derived from free radical intermediates.

DNA and polydeoxyribonucleotides to give adducts which were indistinguishable from those formed in vivo in the liver of tamoxifen-treated rats when analysed by HPLC and two-dimension TLC following ^{32}P-postlabelling. We could not reproduce the results of Phillips et al. [13] and have found tamoxifen 1,2-epoxide to be chemically stable and unreactive. The epoxide was so stable that it could not be hydrolysed even under acid conditions. Using on-line HPLC-ESI MS, we have analysed in detail the monooxygenated metabolites of tamoxifen formed by rat liver microsomes [17]. We were unable to detect tamoxifen 1,2-epoxide as a metabolite. We were also unable to detect the corresponding 1,2-dihydrodihydroxytamoxifen derivative which is expected to be formed by the hydrolysis of the epoxide by epoxide hydrolase in the in vitro microsomal experiments. It is therefore highly unlikely that tamoxifen 1,2-epoxide is a reactive metabolite of tamoxifen.

Phillips et al. [14] then proposed α-hydroxytamoxifen (Fig. 1) as a reactive metabolite. It was reported to be a major metabolite produced by hepatocytes and was detected in the plasma of breast cancer patients treated with the drug [19].

α-Hydroxytamoxifen *N*-oxide and α-hydroxy-*N*-desmethyltamoxifen have also been identified in the urine of patients on tamoxifen therapy [20]. The above results would suggest that α-hydroxytamoxifen is relatively stable and unreactive, particularly with proteins, and is therefore more likely to be a detoxication metabolite rather than a reactive one. Indeed, we have found a much higher level of α-hydroxytamoxifen, although still a minor metabolite, being produced by mouse liver microsomal metabolism compared to rat (Fig. 2). This is consistent with α-hydroxytamoxifen being a detoxication metabolite, since mice are known to be more able to convert tamoxifen into hydroxylated detoxication metabolites [17] and are resistant to liver tumour formation.

It has been shown, however, that α-hydroxytamoxifen could be activated in vitro to reactive species with high DNA-binding capacity [15]. The DNA-adduct was again found to have identical chromatographic behaviours to that formed in vivo in the liver of tamoxifen-treated rats [15]. The in vitro results could be interpreted as demonstrating the potential of α-hydroxytamoxifen or its derivative to be metabolically activated, but do not necessarily reflect the in vivo situation, since Phase II detoxication enzymes are largely excluded.

More recently, Moorthly et al. [16] postulated that the reactive metabolite was a quinone methide formed from tamoxifen via 4-hydroxytamoxifen (Fig. 3). We have also shown that tamoxifen could be activated by a horseradish-H_2O_2 system to reactive metabolites via arene oxide and 4-hydroxytamoxifen [21,22]. Our results showed that free radical intermediates are involved, which could be detected by electron proton resonance spectroscopy in the presence of

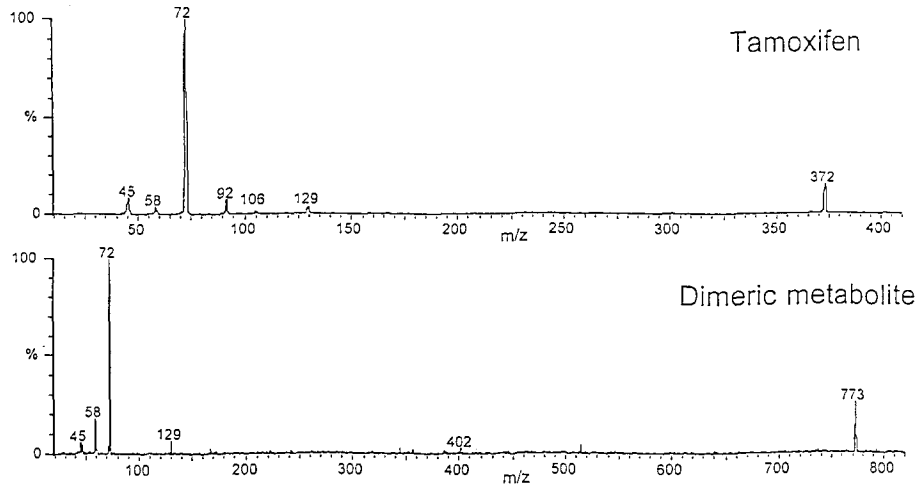

Fig. 6. Tandem ESI MS product ion spectra of (a), tamoxifen and (b), 4-hydroxytamoxifen dimer (I).

reduced glutathione and the free radical trap 5,5-dimethyl-1-pyrroline *N*-oxide. The involvement of free radicals was confirmed by the identification of 4-hydroxytamoxifen dimers (m/z 773) derived from free radical intermediates by HPLC-ESI MS (Fig. 4). The possible structures of the dimers is shown in Fig. 5. That these are dimers derived from 4-hydroxytamoxifen has been confirmed by tandem ESI MS studies. The compounds gave the characteristic side chain fragmentation pattern of tamoxifen derivatives with product ions at m/z 72, 58 and 45 (Fig. 6). The structures were tentatively assigned based on their HPLC elution order. Structure I, with two-OH groups, is the most polar (least hydrophobic) and is thus expected to be the first to elute; structure II, with one-OH group, is of intermediate polarity and was therefore eluted between structure I and the least polar (most hydrophobic) structure III with no-OH group. The fact that structure III was easily dissociated into two equal fragments under the on-line HPLC-MS conditions used also indicated that this compound possessed a weak –O-O-linkage. Structures I and II were stable under similar conditions. Other dimeric structures are obviously possible. However, further characterization was difficult due to the small amounts of metabolites available. Dimers of 4-hydroxytamoxifen have also been identified recently in rat liver microsomal metabolism in vitro of tamoxifen and 4-hydroxytamoxifen [23]. This shows that tamoxifen can also be activated via 4-hydroxytamoxifen to produce free radical intermediates. It must be emphasised again that these in vitro results may not truly reflect what is actually occurring in vivo. There is at present no evidence for the activation of 4-hydroxytamoxifen by endogenous peroxidases in experimental animals or humans and rats and mice dosed with 4-hydroxytamoxifen have shown little in vivo DNA damage [22]. 4-Hydroxytamoxifen is thus also a detoxication metabolite under normal in vivo circumstances and is excreted as the glucoronide conjugate [24].

We have analysed the tamoxifen metabolites corresponding formally to the addition of an oxygen atom to tamoxifen (m/z 388) formed by female rat, mouse and human liver microsomes by an optimised HPLC-ESI MS method [25]. This revealed the formation of arene oxides, 3,4-epoxytamoxifen and 3′4′-epoxytamoxifen, as potential reactive metabolites in addition to the detoxication hydroxy derivatives (Fig. 2). The corresponding hydrolysis products, 3,4-dihydrodihydroxytamoxifen and 3′,4′-dihydrodihydroxytamoxifen, respectively, were also detected in the incubates by HPLC-ESI MS [17]. It has been suggested [20] that the dihydrodihydroxytamoxifen derivative (m/z 406) detected by

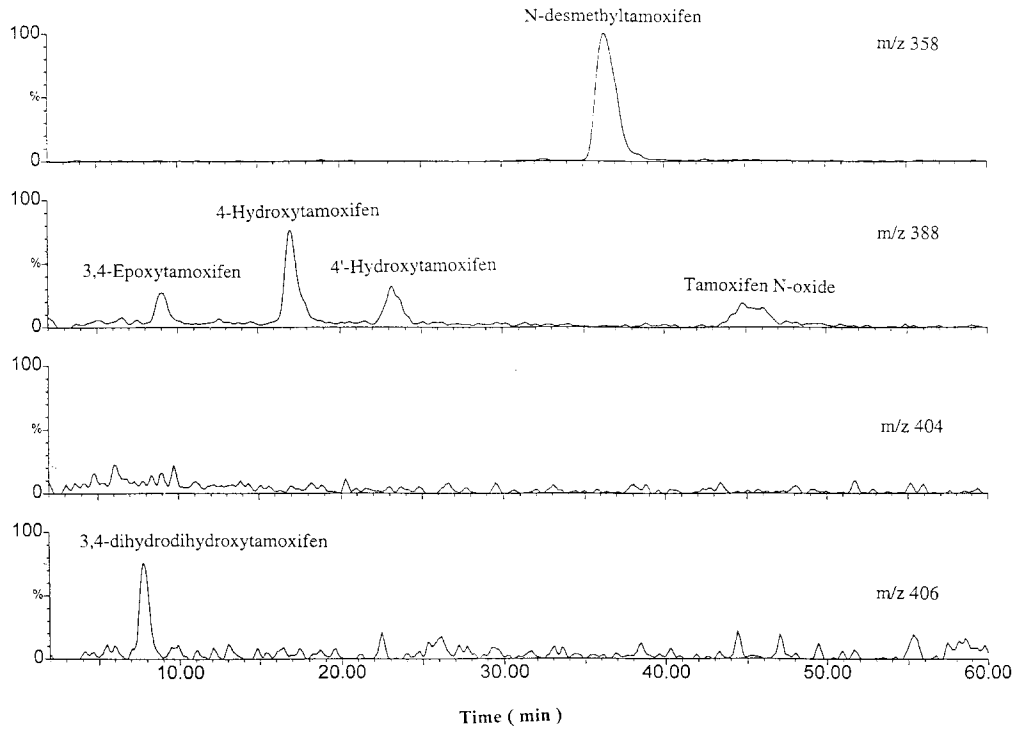

Fig. 7. On-line HPLC-ESI MS analysis of tamoxifen metabolites formed by human liver microsomes.

HPLC-ESI MS could be the isotope peak of a dihydroxy compound, e.g. 3,4-dihydroxytamoxifen (m/z 404). This is only possible if there is inadequate separation between the two compounds. Using the optimised HPLC-ESI MS system [25] we were able to completely resolve 3,4-dihydroxytamoxifen and 4-hydroxytamoxifen *N*-oxide from the dihydrodihydroxytamoxifen derivatives. Furthermore, analysis of the metabolites formed by human liver microsomes where no dihydroxytamoxifen or monohydroxytamoxifen *N*-oxide could be detected, 3,4-epoxytamoxifen and 3,4-dihydrodihydroxytamoxifen were clearly separated and detected (Fig. 7), thus eliminating the possibility of isotopic contamination.

The concentration of epoxide formed by liver microsomes was higher in rats than in mice, with the lowest level being produced by humans. These results are consistent with the fact that DNA-adduct level was the highest in rats, followed by mice and humans.

From the results obtained so far, we believe that arene oxides are the most likely compounds for causing DNA damage. However, epoxides may be derived from free radical intermediates and may also be metabolised to free radical species (Fig. 8). The possibility that an epoxide precursor and/or metabolic free radical intermediates are involved in DNA adduct formation cannot be ruled out.

4. Conclusions

In vitro experiments using liver microsomal systems have all shown that tamoxifen and its hydroxylated metabolites, in particular 4-hydroxytamoxifen and α-hydroxytamoxifen, could be activated to reactive intermediates which react with DNA to form adducts. The results of the in vitro activation of hydroxytamoxifens should not be unquestionably extrapolated to explain in vivo data, since without phase II conjuga-

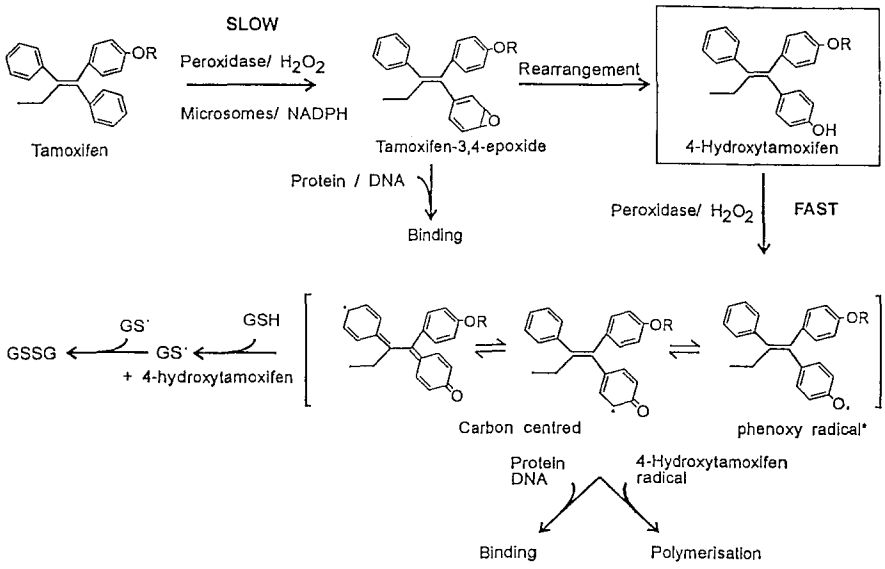

Fig. 8. Proposed metabolic pathway for the formation of tamoxifen arene oxides, free radical intermediates and dimeric metabolites.

tion and excretion, almost all tamoxifen derivatives could be activated in vitro at different degrees.

The identification of reactive metabolites using ^{32}P-postlabelling followed by two-dimension thin-layer chromatography should be treated with caution. The technique, although very sensitive, has insufficient resolving power to allow discrimination between DNA adducts formed by various reactive metabolites. HPLC can be used to improve the resolution, but positive characterisation requires more specific analytical techniques such as HPLC-MS/MS or HPLC-NMR if sufficient quantities of the adducts could be generated.

The nature of the reactive metabolites of tamoxifen in vivo has yet to be conclusively elucidated. Evidence from our laboratories points to the involvement of arene oxides or their free radical precursors or metabolites in causing DNA damage, although further confirmation is required.

References

[1] Anonymous, Lancet, 339 (1992) 1–15.
[2] T.J. Powles, Lancet, 340 (1992) 1145–1147.
[3] R. Stone, Science, 258 (1992) 734.
[4] P. Hirsimaki, Y. Hirsimaki, L. Nieminen and B.J. Pane, Arch. Toxicol., 67 (1993) 49–54.
[5] P. Greaves, R. Goonetilleke, G. Nunn, J. Topham and T. Orton, Cancer Res., 53 (1993) 3919–3924.
[6] G.M. Williams, M.J. Iatropoulos, M.V. Djordjevic and O.P. Kaltenberg, Carcinogenesis, 14 (1993) 315–317.
[7] G.C. Hard, M.J. Iatropoulos, K. Jordan, L. Radi, O.P. Kalterberg, A.R. Imondi and G.M. Williams, Cancer Res., 53 (1993) 4534–4541.
[8] I.N.H. White, F. De Matteis, A. Davies, L.L. Smith, C. Crofton-Sleigh, S. Venitt, A. Hewer and D.H. Phillips, Carcinogenesis, 13 (1992) 2197–2203.
[9] Y. Han and J.G. Liehr, Cancer Res., 52, 1360–1363.
[10] P. Carthew, K. Rich, E.A. Martin, F. De Matteis, C.K. Lim, M.M. Manson, M.F.W. Festing, I.N.H. White and L.L. Smith, Carcinogenesis, 16 (1995) 1299–1304.
[11] E.A. Martin, K.W. Turteltaub, R. Heydon, A. Davies, I.N.H. White and L.L. Smith, The Toxicologist, 15 (1995) 152.
[12] E.A. Martin, K.W. Turteltaub, R. Mauthe, I.N.H. White, J.A. Styles and L.L. Smith, The Toxicologist, 30 (1996) 233.
[13] D.H. Phillips, A. Hewer, I.N.H. White and P.B. Farmer, Carcinogenesis, 15 (1994) 793–795.
[14] D.H. Phillips, P.L. Carmichael, A. Hewer, K.J. Cole and G.K. Poon, Cancer Res., 54 (1994) 5518–5522.
[15] M.R. Osborne, A. Hewer, I.R. Hardcastle, P.L. Carmichael and D.H. Phillips, Cancer Res. 56, (1996) 66–71.
[16] B. Moorthy, P. Sriram, D.N. Pathak, W.J. Bodell and K. Randerath, Cancer Res., 56 (1996) 53–57.

[17] C.K. Lim, Z.-X. Yuan, J.H. Lamb, I.N.H. White, F. De Matteis and L.L. Smith, Carcinogenesis, 15 (1994) 589–593.
[18] E.A. Martin, K. Rich, I.N.H. White, K.L. Woods, T.J. Powles and L.L. Smith, Carcinogenesis, 16 (1995) 1651–1654.
[19] G.K. Poon, B. Walker, P.E. Lonning, M.N. Horton and R. McCague, Drug Metab. Dispos., 23 (1995) 377–382.
[20] G.K. Poon, Y.C. Chui, R. McCague, P.E. Lonning, R. Feng, M.G. Rowlands and M. Jarman, Drug Metab. Dispos., 21 (1995) 1119–1124.
[21] A.M. Davies, E.A. Martin, R.M. Jones, C.K. Lim, L.L. Smith and I.N.H. White, Carcinogenesis, 16 (1995) 539–545.
[22] A.M. Davies, M.E. Malone, E.A. Martin, R.M. Jones, R. Jukes, C.K. Lim, L.L. Smith and I.N.H. White, Free Radical Biol. Med., In press.
[23] R.M. Jones, J.H. Lamb, A. Davies, I.N.H. White, C.K. Lim and L.L. Smith, Proceedings of the 44th ASMS Conference, Portland, Oregan, USA, May 12–16, 1996, p 964.
[24] F. Berthou, Y. Dreano, C. Belloc, L. Kangas, J.C. Gautier and P. Beaune, Biochem. Pharmacol., 47 (1994) 1883–1895.
[25] R.M. Jones, Z.-X. Yuan, J.H. Lamb and C.K. Lim, J. Chromatogr., 722 (1996) 249–255.