The tamoxifen dilemma

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The anti-oestrogen tamoxifen is widely used for adjuvant therapy in the treatment of women with breast cancer and has a low incidence of serious side-effects. It could also play a role as a breast cancer chemopreventive agent. However, epidemiological studies in both tamoxifen-treated breast cancer patients and in healthy women have shown that treatment results in a small increase in the incidence of endometrial cancers. While the use of tamoxifen in breast cancer patients is clearly justified, the situation for its use as a chemopreventive agent in healthy women is not so clear cut. Reasons for caution come from studies in rats that show that tamoxifen is a genotoxic mutagenic liver carcinogen. Initiation of tumours in the rat is the result of metabolic activation of tamoxifen by CYP enzymes to an electrophile(s) that binds irreversibly to DNA. This is not related to the oestrogen receptor status of the tissue. The extent of DNA damage, detected by 32P-post-labelling is not related to the oestrogen receptor status of the tissue. The anti-oestrogen tamoxifen is widely used for adjuvant therapy in the treatment of women with breast cancer and has a low incidence of serious side-effects. It could also play a role as a breast cancer chemopreventive agent. However, epidemiological studies in both tamoxifen-treated breast cancer patients and in healthy women have shown that treatment results in a small increase in the incidence of endometrial cancers. While the use of tamoxifen in breast cancer patients is clearly justified, the situation for its use as a chemopreventive agent in healthy women is not so clear cut. Reasons for caution come from studies in rats that show that tamoxifen is a genotoxic mutagenic liver carcinogen. Initiation of tumours in the rat is the result of metabolic activation of tamoxifen by CYP enzymes to an electrophile(s) that binds irreversibly to DNA. This is not related to the oestrogen receptor status of the tissue. The extent of DNA damage, detected by 32P-post-labelling is not related to the oestrogen receptor status of the tissue.

Introduction

The anti-oestrogen (Z)-1-[4-(dimethylamino)ethoxy]phenyl]-1,1-diphenyl-1-butene (tamoxifen) (Figure 1) is a drug widely used for adjuvant therapy in the treatment of women with oestrogen receptor (ER)-positive breast tumours and has a low incidence of serious side-effects. Since 1971 it has been used successfully to treat many millions of women (1,2). Treatment results in an increase in disease-free survival (3) and a decrease in recurrence rates of breast cancer (2). Retrospective reviews of adjuvant therapy studies show an ~39% reduction in the incidence of contralateral primary breast carcinoma in tamoxifen-treated women, indicating that this drug could play a role as a breast cancer chemopreventive agent (4,5). A planned 5 year trial involving >13 000 healthy women who were at high risk of developing breast cancer (Breast Cancer Prevention Trial BCPT P1) in the USA was stopped 14 months early when results showed that tamoxifen treatment led to a 49% reduction in breast cancer (6). Two smaller studies carried out in the UK and in Italy failed to show such protection (7,8). Many factors are responsible for the high incidence of breast cancer in the developed world, including the environment and diet. Some familial tendency to develop breast cancer is associated with susceptibility genes, including BRCA1 and BRCA2. Aberrant expression of these genes accounts for only ~6% of the total incidence of breast cancers (9) and is not known to be associated with endometrial cancer.

Carcinogenic risk in women associated with tamoxifen therapy

Epidemiological data indicated that in women taking tamoxifen there was an increased risk of endometrial cancer (10,11). In the studies of Rutqvist et al. (11), involving >4900 Scandinavian breast cancer patients, there was a 4-fold increase in endometrial cancers during a follow-up time of 8–9 years. In the National Surgical Adjuvant Breast and Bowel Program (NSABP B-14) in the USA involving 2823 patients with node-negative, ER-positive breast cancers, tamoxifen treatment over a 5 year follow-up period resulted in a relative risk of 7.5 over the placebo group (3). A case–control study in the USA of the Surveillance, Epidemiology and End Results (SEER) also showed an ~1.6-fold increase in the incidence of uterine tumours in the treated group receiving 20 mg/day tamoxifen (12). In healthy women participating in the Breast Cancer Prevention Trial (BCPT P1) that were treated with tamoxifen, there was a 1.35- to 4.97-fold increased risk of endometrial cancers. In reviewing these data, the IARC concluded that there was sufficient evidence in humans of tamoxifen increasing the risk for endometrial cancer (4). The increased risk occurred predominantly in women aged 50 years or older (6). There is a comparatively rapid onset of these tumours within 2–5 years of the start of treatment, suggesting that the mechanism of action may be via an oestrogen agonist effect rather than as a classical chemical carcinogen. There is some evidence (albeit weak) for an increase in gastrointestinal tract cancers in breast cancer patients (11). This has not been confirmed by other studies (reviewed in ref. 4). It has been pointed out by Jordan (13) that in the order of 30 lives are saved by tamoxifen treatment to each lost through its side-effects. In the case of breast cancer patients, this represents a clear therapeutic advantage.

Carcinogenicity of tamoxifen in rodents

One of the main causes for concern with respect to the chemopreventive use of tamoxifen in healthy women was the...
finding, initially reported by Greaves et al. (14), that lifetime exposure of rats to tamoxifen resulted in an increase in the incidence of hepatocellular tumours. This did not appear to occur via an ER-based mechanism. The incidence of tumours was similar in male and female animals and showed a dose-dependent increase over the range 5–35 mg/kg/day. This is 10- to 100-fold higher than the dose used in humans. A number of studies in many strains of rat has confirmed this original finding (14–17). In contrast, mice were resistant (18,19). It was established that tamoxifen given as a single dose was not able to act as a tumour initiator (20), although it could promote liver cancer initiated by N-nitrosodiethylamine (21). In the rat, lifetime exposure is not necessary for the induction of liver tumours. Following exposure to dietary tamoxifen (equivalent to ~40 mg/kg/day) for only 3 months, after discontinuation of treatment five of 15 rats developed liver tumours after 20 months without any subsequent promotion (20). Promotion of tumour development with phenobarbital after discontinuation of tamoxifen resulted in earlier formation of liver carcinomas and, after 20 months, 12 of 14 animals had liver tumours (20). Of the tamoxifen-induced adenomas and carcinomas examined, 90% showed depleted nuclear ER expression. Prenoplastic foci also showed a progressive higher incidence of ER depletion as they increased in size, indicating that this effect was associated with promotion of foci to tumours (22). It is not presently clear if this down-regulation of the ER is causally related to the promotion process.

A long-term dietary tamoxifen study using three inbred mouse strains has been carried out in an attempt to establish if mice could develop liver tumours. Certain strains (C57Bl/6 and DBA/2) would not tolerate long-term dosing due to the oestrogenic effects of tamoxifen resulting in bone remodelling, leading to kyphosis. However, B6C3F1 mice were more resistant, surviving the full 2 years. These mice did not develop liver tumours even when phenobarbital was used as a promoter (19).

**Response of the mouse or rat uterus after tamoxifen treatment**

No significant increase in uterine tumours was seen following continuous exposure of rats or mice to tamoxifen (18). In rats given dietary tamoxifen for 3 months (420 p.p.m.), uteri were decreased in size, with an absence of glands in the endometrium, reflecting the action of tamoxifen as an oestrogen antagonist. In B6C3F1 mice given tamoxifen for up to 2 years there was hyperplasia of the epithelium of the uterine endometrium for the first 3 months, followed by atrophy of the endometrial component (19). More recently, it has been shown that newborn CD-1 mice were given tamoxifen for the first 5 days after birth, uterine adenocarcinoma were seen in 50% of animals dosed at 10 µg/pup/day tamoxifen at 14–17 months. No similar tumours were observed in corresponding control mice (23). These results raise the exciting possibility that this model may be an appropriate experimental system for studying the mechanisms of tamoxifen-related endometrial tumours in women. It needs to be established if tamoxifen treatment of mice at this early stage results in DNA damage (see below) in the reproductive tract or if the resulting endometrial tumours are the result of alternative mechanisms, such as a loss of imprinting.

**Mechanistic basis for hepatic DNA damage in rats and mice given tamoxifen**

Following dosing of rats with tamoxifen, DNA damage, detected by 32P-post-labelling, was found in their livers (24,25). DNA adducts, separated by TLC or more recently by HPLC, were resolved into at least 12 components (25,26). The extent of DNA damage was related to the time of exposure and to the dose of tamoxifen. In long-term feeding studies of rats given dietary tamoxifen (equivalent to ~40 mg/kg/day), adduct levels increased from ~500 adducts/108 nucleotides at 30 days to almost 3000 adducts/108 nucleotides at 180 days (27). Following long-term dosing of rats, 32P-post-labelled adducts were found in no other organs apart from the kidney (28). The triphenylethylen analogues (Z)-4-chloro-1-[4-[2(N,N-dimethylamino)ethoxy][phenyl]-1-butene (toremifene), droloxifene and idoxifene (Figure 1), when administered to rats at comparative dose levels, caused little or no hepatic DNA damage as determined by 32P-post-labelling (16,25,28–30). Using the more sensitive technique of accelerator mass spectrometry, following a single dose of [14C]tamoxifen, low levels of irreversible binding to DNA from the liver, reproductive and gastrointestinal tracts could be detected (32). In this study, dose-dependent DNA binding in the liver occurred at dose levels equivalent to that used therapeutically in women. When [14C]toremifene was used, irreversible binding to the DNA of liver or other organs was not seen. In the livers of tamoxifen-dosed mice, levels of 32P-post-labelled adducts were about one-third of those detected in the rat, although the pattern of adducts following HPLC or TLC was similar to that seen in rats (16,27,29,33). Following long-term dosing of mice, the level of major DNA adducts did not increase with time of exposure but after the first month decreased, so that by 1 and 2 years it was barely above background. While there are many steps between the initiation of DNA damage and the subsequent development of tumours, the evidence suggests that with tamoxifen there is a causal link between the presence of adducts and the subsequent development of hepatocarcinogenicity.

In rats, loss of 32P-post-labelled adducts from the liver after cessation of dosing with tamoxifen takes many weeks, suggesting either that DNA repair mechanisms became saturated or that removal of tamoxifen adducts is slow. However, when a similar study was carried out using mice, the initial
level of hepatic DNA damage was lower than in the rat. The more rapid hepatic detoxification of tamoxifen in mice, compared with the rat, resulting in a shorter plasma half-life, contributes to the lower level of DNA damage. However, over longer time periods, DNA damage does not accumulate in the liver as seen in rats. It has been suggested that factors such as the induction of multidrug resistance proteins may contribute to this, in addition to the more rapid repair of the damaged DNA in mice (19). These differences almost certainly contribute to the resistance of mice to the hepatocarcinogenic effects of tamoxifen.

If hepatic DNA damage is not repaired, it can be translated into gene mutations. Tamoxifen given to transgenic F344 (Big Blue) rats results in a dose-related increase in the mutation frequency at lacI in the livers following 6 weeks oral dosing (34,35). The spectrum of mutations at lacI showed 60% G:C→T:A transversions compared with 21% G:C→A:T in controls. Toremifene, in contrast, did not induce mutations. No mutations were found at lacI in the DNA extracted from uterine tissues. This pattern of mutations is different from the A→G and C→T transitions found at p53 in liver tumours of rats following tamoxifen treatment (36). Changes in expression of p53 in liver tumours are late events occurring after many months treatment, while mutations at lacI are seen within a few weeks of the start of treatment. This may account for the different pattern of mutations. Although rare in rats, in humans many types of cancer are associated with mutations at p53 (37,38). One preliminary report suggested that mutations at p53 in endometrial tumours associated with tamoxifen therapy in women had a similar spectrum of mutations to those found in the livers of tamoxifen-treated rats (39). This might infer a genotoxic mechanism of tamoxifen associated with tumour formation. However, these data have not been confirmed and a more recent publication showed only a random pattern of p53 mutations in tamoxifen-mediated endometrial tumours in women (40).

**Enzymes involved in the metabolism and activation of tamoxifen**

The pattern of metabolites formed from tamoxifen in the presence of liver microsomal systems in vitro are qualitatively similar in humans, mice and rats (41–43). These detoxification products include the major Phase I detoxification metabolites N-demethyltamoxifen, 4-hydroxytamoxifen and tamoxifen N-oxide (Figure 2). Quantitatively, rates of metabolism by human liver microsomes are about one-third those seen in rats (41,44). The most important enzyme for carrying out the N-demethylation reaction in human liver is CYP3A4 (45,46). The expression of cDNAs for different CYP forms, expressed in Escherichia coli, has confirmed this observation, but additionally has shown that a number of other CYP isoforms have the capability to catalyse this reaction. CYP2D6 was involved in 4-hydroxylation (47), although there is evidence from the use of human liver microsomes that other CYP forms may also participate (48).

In order to be genotoxic, tamoxifen has to undergo metabolic activation by CYP monooxygenases to give a reactive intermediate. In order to establish which human CYP isoforms could carry out such activation reactions, a human lymphoblastoma cell line expressing cDNAs for human P450s has been used in a micronucleus assay. CYP3A4 was the major form catalysing the clastogenic response (49). Toremifene, toremifene and metabolites such as 4-hydroxytamoxifen and 4-hydroxytoremifene all caused the formation of micronuclei (25,49). Droloxifene was negative in this assay.

Irreversible binding of [14C]tamoxifen to microsomal protein or to DNA has been used as a means of comparing the potential of rat or human hepatic systems. The results showed that CYP3A4 catalysed this reaction (50,51). Similar levels of irreversible binding were seen using rat and human liver microsomal mixtures, whereas mice showed ~3-fold higher activities (32). [14C]Toremifene also underwent activation and became covalently bound to DNA and to protein in all three species (51). Microsomal systems are useful for demonstrating the potential for metabolic activation, but do not necessarily accurately reflect the extent to which this will occur in humans.

Many putative reactive metabolites of tamoxifen have been proposed. There is now good evidence that a minor metabolite, α-hydroxytamoxifen, bioactivated as a sulphate ester (52,53), has a role in the formation of the DNA damage in the livers of tamoxifen-treated rats (Figure 3). Originally proposed by Potter et al. (54), α-hydroxytamoxifen has been found in the plasma of women treated with tamoxifen (55) and as the glucuronide in the bile of rats treated with tamoxifen, where it accounts for ~0.1% of the administered dose (45). Evidence for the formation of this intermediate came from the studies of Phillips et al. (56), who showed a reduced genotoxicity of [D5-ethyl]tamoxifen. Subsequently, the same group demonstrated that in isolated hepatocytes DNA damage, determined by 32P-post-labelling, was increased in a concentration-dependent manner when sulphate was added to the culture medium (57). The rates of formation of α-hydroxytamoxifen by human liver microsomal preparations were about half those seen in rats (45). α-Acetoxytamoxifen and α-sulphate tamoxifen prepared synthetically bind to DNA in vitro giving rise to higher levels of adducts than the parent alcohol (58,59). The major product of this reaction has a similar Rf value on TLC and retention time following HPLC to the major adduct.
spot in DNA extracted from the livers of tamoxifen-treated rats. It has been identified as one in which the α position of tamoxifen is covalently linked to the exocyclic amino group of deoxyguanosine to form cis–trans isomers of α-(N²-deoxyguanosinyl)tamoxifen (58,60,61). Reaction with the exocyclic amino function of guanine is unlike that seen with many chemical hepatocarcinogens, such as the nitrogen mustards, nitrosamines or aflatoxins, where ring nitrogens or exocyclic oxygen atoms are the primary targets for attack (62). The possibility of more than one reactive intermediate cannot be excluded. Other reactive species include conversion to 3,4-epoxytamoxifen (42,63), further rearrangement via 4-hydroxytamoxifen to a quinone methide (64), carbon or oxygen centred free radical species derived from 4-hydroxytamoxifen either through the action of CYP or peroxidases (63,65,66) or conversion to 3,4-dihydroxytamoxifen (Figure 3) (67).

DNA damage in tissues of women taking tamoxifen

It is appreciated that there are many steps between the initiation of DNA damage and the subsequent promotion and progression to tumours. However, results using rodent model systems suggested that, following the administration of tamoxifen, DNA damage, determined by 3²P-post-labelling or by accelerator mass spectrometry, was causally related to the subsequent development of tumours. These techniques were therefore used to look at the extent of DNA damage in women given tamoxifen therapeutically. In a study of DNA extracted from the livers of breast cancer patients taking tamoxifen, no tamoxifen-specific DNA damage could be detected by 3²P-post-labelling. Overall levels of DNA damage were not significantly different from those individuals not taking the drug (68). No 3²P-post-labelled adducts could be detected in DNA from human white blood cells obtained from tamoxifen-treated women (69). Human recombinant hydroxysteroid sulphotransferase (SULT2A1) was able to catalyse the formation of tamoxifen–DNA adducts in the presence of α-hydroxytamoxifen, but the activity of this enzyme was 3-fold lower than the equivalent rat enzyme (70). This would offer a degree of protection to humans compared with rats. There is controversial evidence as to the extent that tamoxifen administration to women results in damage to endometrial DNA. The work of Carmichael et al. (71,72) failed to find evidence of genotoxicity in patients treated with either tamoxifen or toremifene, whereas Hemminki et al. (73) detected low levels of ~0.27 adducts/10⁶ nucleotides. This is very much less than the 3000 adducts/10⁶ nucleotides seen in rat livers that develop tumours (27). The long-term biological relevance in humans of such low levels of DNA damage are not known. However, on the basis of the present evidence, the rat liver model of genotoxicity of tamoxifen does not appear as an appropriate one for uterine tumours in women caused by this drug (Table I).

The future: designer selective oestrogen receptor modulators (SERMS)?

Second generation SERMS include 7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]estr-1,3,5(10)-triene-3,17β-diol (ICI 182 780, Faslodex™) and [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl][4-[2-(1-piperidinyl)ethoxy]phenyl]methanone (raloxifene, Evista™) (Figure 4). Wakeling et al. (79,80) designed ICI 182 780 as a 7α-substituted oestradiol analogue with potent pure anti-oestrogen action. It was hoped that this might offer advantages in breast cancer treatment compared with partial agonists like tamoxifen. ICI 182 780 has a high relative affinity for the oestrogen receptor and completely blocked the trophic action of oestradiol on the uterus of ovariectomized adult rats and on bone (81,82). In women, ICI 182 780 did not affect cell proliferation in the uterine or vaginal tissues (83). Short-term treatment of patients with advanced breast cancer resistant to tamoxifen with this drug resulted in significant decreases in tumour proliferation. In oestrogen receptor-positive breast tumours there was also a decrease in the level of receptor protein that could be detected.
The tamoxifen dilemma

Table I. Relevance of rat liver tumour data to endometrial tumours seen in tamoxifen treated women

<table>
<thead>
<tr>
<th>Women</th>
<th>Is this relevant to the effects seen in rat liver?</th>
<th>Rats</th>
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<tbody>
<tr>
<td>Tamoxifen (20 mg/day) results in – 3 fold increase in uterine tumours (16, 11, 74, 6)</td>
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<td>Tamoxifen (5 to 40 mg/kg/day) results in hepatocellular carcinomas (14, 15, 14, 17)</td>
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Human uterus
- Little or no $^{32}$P-postlabelling of DNA (73, 71, 72)
- Little or no irreversible binding of $^{14}$C tamoxifen by AMS (95)
- Little metabolic activation in vitro (75)

Rat liver
- DNA adduct formation: $^{32}$P-postlabelling (25, 16, 29, 26)
- $^{14}$C-irreversible binding by AMS (32)
- UDS in rat liver (dosing in vivo and in vitro) (25)
- Gene mutations at lacI in transgenic ‘Big Blue’ rats (34, 35)
- Chromosomal mutations structural and numerical aberrations (76, 77)
- Metabolic activation by microsomal or hepatocytes in vitro (78, 50, 51, 32, 57)

Genotoxic mechanism does not seem to be relevant

Genotoxic mechanism

Immunocytochemically (84), Fasolodex is usually administered by monthly intramuscular injection rather than orally. Although clinically promising it has so far not gained the widespread acceptance of tamoxifen.

Raloxifene, based on the benzothiaphene structure (Figure 4), is used as an anti-osteoporosis agent for post-menopausal women (85–87). Although it has an oestrogen agonist action on bone, it has little proliferative effects on the endometrium of post-menopausal women (88) and only a weak uterotrophic action in ovariectomised rats (89). Like tamoxifen, raloxifene may be effective as a chemopreventive agent against breast cancer. In early 1999, a comparative Study of Tamoxifen And Raloxifene (STAR) will be carried out to include 22,000 post-menopausal women who are at increased risk of developing breast cancer. Daily doses of tamoxifen (20 mg) and raloxifene (60 mg) or placebo will be given for 5 years.

There is a mechanistic basis for hoping that the endometrial tumours seen following tamoxifen therapy would not occur with raloxifene. Studies by Yang et al. (90) showed that this drug could have different tissue specificities. Using bone-derived MG63 cells they found that the signal transduction mediated via TGF-β3 was not routed through the classical oestrogen response element but rather through adapter proteins and a novel raloxifene response element. X-ray crystallography of the oestrogen receptor has shown that both oestradiol and raloxifene can interact with the ligand-binding domain of the receptor but binding results in different conformational states (91). In oestrogen receptor-negative human breast cancer cells that have been transfected with cDNAs from wild-type or mutant ER, it has been found that mutation of the single Asp351 to Tyr is sufficient to change the actions of raloxifene from an anti-oestrogen to an oestrogen (92). The presence of two ERs, ERα and ERβ, that can either homo or heterodimerize further complicates the potential action of these SERMS. Both ERα and ERβ have a high degree of homology between the amino acid residues in the ligand-binding domain (AF2) and many ligands have a similar affinity for both receptor subtypes (93). However, it has been shown that tamoxifen and raloxifene have oestrogen agonist/antagonist function via ERα but a pure antagonist effect through ERβ (94). The second generation SERMS as well as the triphenylethylene analogues toremifene, droloxifene and idoxifene do not have the clinical track record of tamoxifen. It is presumed, but not proven, that induction of uterine cell proliferation is associated with the subsequent development of endometrial tumours. We will have to await the outcome of long-term clinical trials and of epidemiological studies to demonstrate the long-term safety of these drugs.

Conclusions

The use of tamoxifen as adjuvant therapy in the treatment of breast cancer on balance is beneficial, although there are concerns with regard to the development of endometrial cancer in treated women. There is no epidemiological evidence for an increase in liver tumours in women receiving tamoxifen therapy (4) and on the basis of our mechanistic understanding of the action of this drug in rodents, it seems unlikely that women will be at risk. It is still not clear if the mechanism of formation of uterine tumours is through genetic mechanisms, analogous to those seen in the rat liver, or as a result of the oestrogen agonist actions of tamoxifen. A resolution to this question would be of considerable importance in the ultimate use of tamoxifen or particular analogues of tamoxifen in the clinical setting. There is evidence from the largest clinical trial on the chemopreventive use of tamoxifen in women (6) that this drug provides considerable benefit in reducing the incidence of breast cancer. Tamoxifen is a genotoxic carcinogen in the rat yet in humans it is unlikely that this will be the cause of limitations in the widespread use of this drug. As such it raises interesting issues in the licensing of rodent genocarcinogens for use in a clinical situation. Based on an understanding of its mechanism of toxicity and its proven benefit in the clinic, populations of women at high risk of developing breast cancer may be able to benefit from second or third generation designer oestrogens that provide similar therapeutic benefits to tamoxifen with different toxicological profiles. These may ultimately prove to be of considerable value as chemopreventive drugs.
References


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