Understanding the Genotoxicity of Tamoxifen?

By David H. Phillips, DSc, MRCPath

ABSTRACT

Tamoxifen is an antioestrogenic drug widely used for adjuvant therapy of breast cancer. Its use has caused an increased incidence of endometrial cancer and it is also a potent carcinogen in rat liver. Since the demonstration that tamoxifen forms covalent DNA adducts in rat liver, many investigations of its mechanism of carcinogenic action have focused on the examination of human and animal tissues for the presence of tamoxifen-DNA adducts, the identification of their structures and the determination of the metabolic pathways that lead to their formation. This article reviews the current evidence for genotoxic mechanisms for tamoxifen carcinogenicity, and discusses some inconsistencies in the data.

USE OF TAMOXIFEN

The drug tamoxifen, (Z)-1-{4-[2-(dimethylamino)ethoxy] phenyl}-1,2-diphenyl-1-butene, is a nonsteroidal antioestrogen for the treatment of breast cancer, the most common form of cancer in women world-wide. By virtue of its proven efficacy in extending remission and survival from primary breast cancer and in reducing the incidence of contralateral breast cancer in women who have already had a breast tumour,[1] it is now the world's most widely used cancer chemotherapeutic agent. It has undergone trials in several countries to determine whether its administration to healthy women at high risk of breast cancer can reduce the incidence of malignancy. Results have been mixed, with a US trial showing significant benefits of prophylactic tamoxifen in reducing breast cancer incidence in healthy high-risk women,[2] but with UK[3] and Italian[4] trials showing no such benefit (yet). Other pharmacological properties of tamoxifen, including beneficial effects on bone density and lipid profiles, have led to the development of new selective (o)estrogen receptor modulators (SERMs) that may have the dual benefit of preventing osteoporosis and breast cancer; two such drugs of current interest are the tamoxifen analogue toremifene[5] and the benzothiophene raloxifene.[6]

With the widespread therapeutic and emerging prophylactic use of tamoxifen, there has been much discussion about side-effects of the drug, particularly its carcinogenicity. It is the intention of this article to summarise what is known about the mechanism of tamoxifen carcinogenicity in animals and humans, and to speculate on the extent to which extrapolation from one species to another will be reliable in predicting long-term effects of tamoxifen and other antioestrogens with similar structures and/or properties in humans.

CARCINOGENICITY

Numerous studies have established an increased incidence of endometrial cancer among women taking tamoxifen.[7,8] Recent results confirm not only an increased incidence of endometrial cancer (relative risk up to 7 compared with non-users), but also increased mortality from the disease,[9] implying that the tamoxifen-induced neoplasms are in some way pathologically different from those not associated with use of the drug. Endometrial thickening is evident in a much larger percentage of women.[10] While the benefits of tamoxifen to breast cancer patients far outweigh the risks, findings such as these question the widespread use of tamoxifen by healthy women to prevent breast cancer.

In rats, tamoxifen is a potent hepatocarcinogen in both males and females.[11] Also, when administered to neonatal rats, uterine adenocarcinomas were induced along with a lower frequency of squamous cell carcinomas of the vagina/cervix.[12] In mice, however, liver is not the target tissue for carcinogenesis. Instead, tumours of the testis are induced in males, and of the ovaries in females;[13] tumours also develop in the uterus when tamoxifen is administered neonatally[14] but not when fed in the diet from eight weeks old for 2 years.[15] When administered transplacentally to mice, tamoxifen causes a high incidence of hyperplasia in the reproductive tract, and a lower incidence of tumours, in offspring.[16] Testing of tamoxifen for carcinogenic activity in other species has not been reported.

While it might initially have been thought, or assumed, that the hepatocarcinogenicity of tamoxifen in rats was the consequence of its oestrogenic activity, this view was challenged when it became apparent that the tumours induced were not benign adenomas, but highly malignant carcinomas. Furthermore, tumours were induced in up to 80% of animals of both sexes.[11] Other studies on female rats only have also demonstrated the potency of tamoxifen as a liver carcinogen.[17-19] When it was found that tamoxifen gave rise to DNA adducts in rat liver,[20,21] it was apparent that the compound could undergo metabolic activation to an electrophilic species that binds covalently to cellular macromolecules and that could therefore be carcinogenic by a genotoxic mechanism.[22-24] It should be noted that in all standard short-term tests for carcinogenicity, based on detecting the consequences of DNA damage, tamoxifen gave negative results.[8,13] However, as will be mentioned later, a key metabolite of tamoxifen is mutagenic when suitable conditions for its metabolism are met. A number of other experimental observations are compatible with

Reprinted with permission from Carcinogenesis *2001;22(6):839-849.*

genotoxic activity: tamoxifen induces micronuclei in metabolically competent human cells,[21,25-28] causes aneuploidy[28-30] and chromosomal aberrations[28] in rat liver, and mutations in the lacI reporter gene in the liver of transgenic rats[31]. Furthermore, tumours induced in rat liver by tamoxifen were found to contain mutations in the p53 gene.[32] While no single piece of evidence would be sufficient to designate it a genotoxic carcinogen, taken together with its DNA adduct forming ability these data can be construed as indicating that tamoxifen exhibits the properties of a genotoxin, at least in some circumstances.

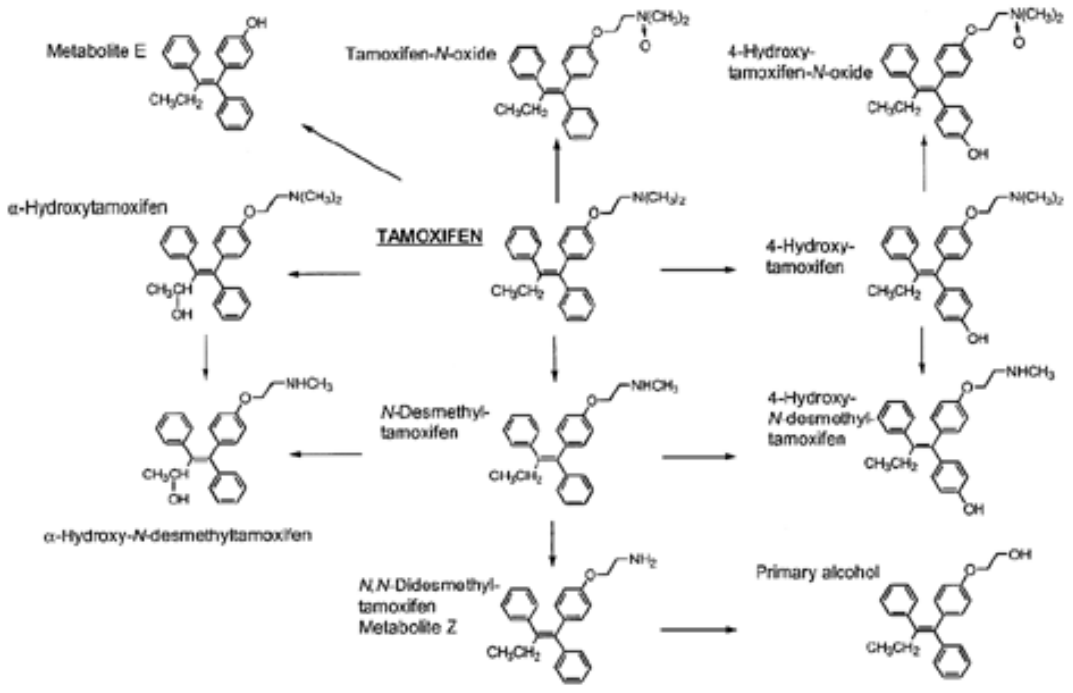
MECHANISM IN RATS

A powerful means of determining the pathways of activation of a carcinogen is to characterise and quantify the DNA adducts it forms, and to determine what factors either enhance or inhibit DNA adduct formation. Much has been learned about the metabolic activation of tamoxifen using this approach, and the method of detection most commonly used has been the highly sensitive 32P-postlabelling method. DNA adduct formation has also been demonstrated by mass spectrometry,[33] accelerator mass spectrometry (AMS)[34] and, using antibodies raised against tamoxifen-adducted DNA, by competitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) and chemiluminescence immunoassay (CIA).[35]

The metabolism of tamoxifen has been widely studied and shows that several positions of the molecule are sites for biotransformation[36,37] (Figure 1). In human metabolism studies, many metabolites are isolated as glucuronyl conjugates.[38] The principal sites of Phase I metabolism are the nitrogen atom of the side chain (*N*-oxidation and demethylation) and the 4-position (hydroxylation). Other positions also subject to metabolism include the α–position of the ethyl side chain (hydroxylation). Although it is a relatively minor site for metabolism, it was proposed on theoretical grounds that the α-position is the primary site of metabolic activation,[39] as oxidative metabolism at this position was predicted to generate a resonance-stabilised carbocation capable of electrophilic attack on nucleophilic centres in DNA, leading to the formation of stable covalent DNA adducts.

Ensuing experimental studies by a number of investigators have, to a large extent, borne out this hypothesis. When substituted with deuterium at the α-position, the DNA adduct forming ability of tamoxifen in rat liver in vivo and in rat hepatocytes in vitro is significantly reduced.[26,40] The extent of the reduction, more than 2-fold, is compatible with the 2-3-fold slower rate of oxidative metabolism at the α-position when it is deuterated, demonstrated in a study using rat liver microsomal fractions.[37] Deuterium substitution at the β-position does not reduce the DNA binding

FIGURE 1. MAIN PHASE I METABOLIC PATHWAYS OF TAMOXIFEN



Adapted from IARC.[8]

activity of tamoxifen.[40] These results indicate the importance of metabolism at the α–position in the metabolic activation of tamoxifen. Indeed, when the DNA binding potential of α–hydroxytamoxifen, the predicted intermediate, was determined in rat hepatocytes[41,42] and, subsequently, in rat liver in vivo,[40,43] it was found to bind to DNA at up to 50 times higher levels than an equal concentration or dose of tamoxifen. As the adduct patterns of the two compounds were indistinguishable, this is further strong evidence that tamoxifen-DNA binding is mediated through α-hydroxylation.

Interestingly, the Potter hypothesis[39] predicted that if α-hydroxylation occurred in combination with 4-hydroxylation, a more stable carbocation would be generated than if α–hydroxylation alone occurred. Indeed, comparison of the reactivity of α–hydroxytamoxifen and α,4-dihydroxytamoxifen shows that the latter reacts more extensively with DNA.[44,45] A number of postulated reactive intermediates derived from 4-hydroxytamoxifen, including the quinone methide,[39] also react readily with DNA to give stable adducts.[45,46] This suggests that 4-hydroxytamoxifen, a major metabolite of tamoxifen, would give rise to DNA adducts in cells. In one study adduct formation by 4-hydroxytamoxifen in rats has been reported,[47] but in subsequent studies adduct formation by this metabolite was not detectable in rat hepatocytes[45] or in rat liver in vivo,[45,48] even though the metabolite can be enzymatically activated to products that bind to DNA[47,49] in cell-free or sub-cellular systems. Furthermore, 4-hydroxytamoxifen activation by the peroxidase/H_2O_2 system in vitro gave a more polar DNA adduct seen only at trace levels in liver DNA from tamoxifen-treated rats.[50] In another study, administration of 4-hydroxytamoxifen to rats for 14 days gave rise to unspecified, but apparently low, levels of DNA adducts in liver that were chromatographically distinct from those formed at much higher levels by tamoxifen itself.[51] Thus tamoxifen activation in rat liver does not proceed via 4-hydroxylation. Presumably an efficient detoxifying mechanism, probably involving Phase II conjugation at the 4-position, is operating in intact cells and it therefore comes as no surprise that 4-hydroxytamoxifen is not carcinogenic when applied topically to rat skin.[52] (The rationale for this seemingly bizarre experiment is that topical application of 4-hydroxytamoxifen to the breast has been proposed as a therapy for breast cancer.)[53] The experience with 4-hydroxytamoxifen should serve as a powerful reminder to all investigators that it is insufficient to demonstrate the DNA binding of a metabolite or putative metabolite in a cell-free or sub-cellular system (where the balance of activation and detoxification pathways may be substantially altered) in order to give credence to a particular theory of metabolic activation. It is necessary also to demonstrate that this binding occurs in whole cells in vitro and/or in mammalian tissues in vivo.

Although α-hydroxytamoxifen exhibits weak DNA binding activity at physiological pH (and increasing reactivity at progressively acidic pH),[54] it is clear from its high DNA binding activity in cells that it undergoes Phase II metabolism to a more reactive intermediate. Tamoxifen bears structural features analogous to those of the naturally occurring carcinogens safrole and estragole, whose pathway of metabolic activation also involves hydroxylation at a carbon atom adjacent to a conjugated allylic position, and which form DNA adducts in which the exocyclic amino group of guanine and adenine are the principal sites of modification in DNA.[55] Using α–acetoxytamoxifen or the sulphate ester of α–hydroxytamoxifen as model electrophiles, the adducts formed by tamoxifen were identified as also consisting of guanine and adenine moieties modified at the exocyclic amino groups.[54,56,57] Rotation of the carbocation about the central bond of the molecule can occur, leading to the formation of both *cis* and *trans* adducts. Once formed, *cis* and *trans* adducts are stable and do not interconvert. Phase II activation of the proximate carcinogen 1'-hydroxysafrole is mediated by sulphotransferase and there is now good evidence that tamoxifen activation also occurs via sulphate ester formation from α-hydroxytamoxifen. When rat hepatocytes were incubated with tamoxifen in sulphate-free media, DNA adduct formation was significantly reduced, but was restored by the addition of sulphate salts.[58] Likewise, co-incubation (in normal media) with dehydroisoandrosterone-3-sulphate (DHEAS), a hydroxysteroid sulphotransferase inhibitor, reduced DNA adduct formation[58]. The fact that tamoxifen and α-hydroxytamoxifen showed entirely similar dependence on sulphate and on sulphotransferase activity suggests that a pathway involving α–hydroxylation dominates over other potential pathways of activation in liver cells.

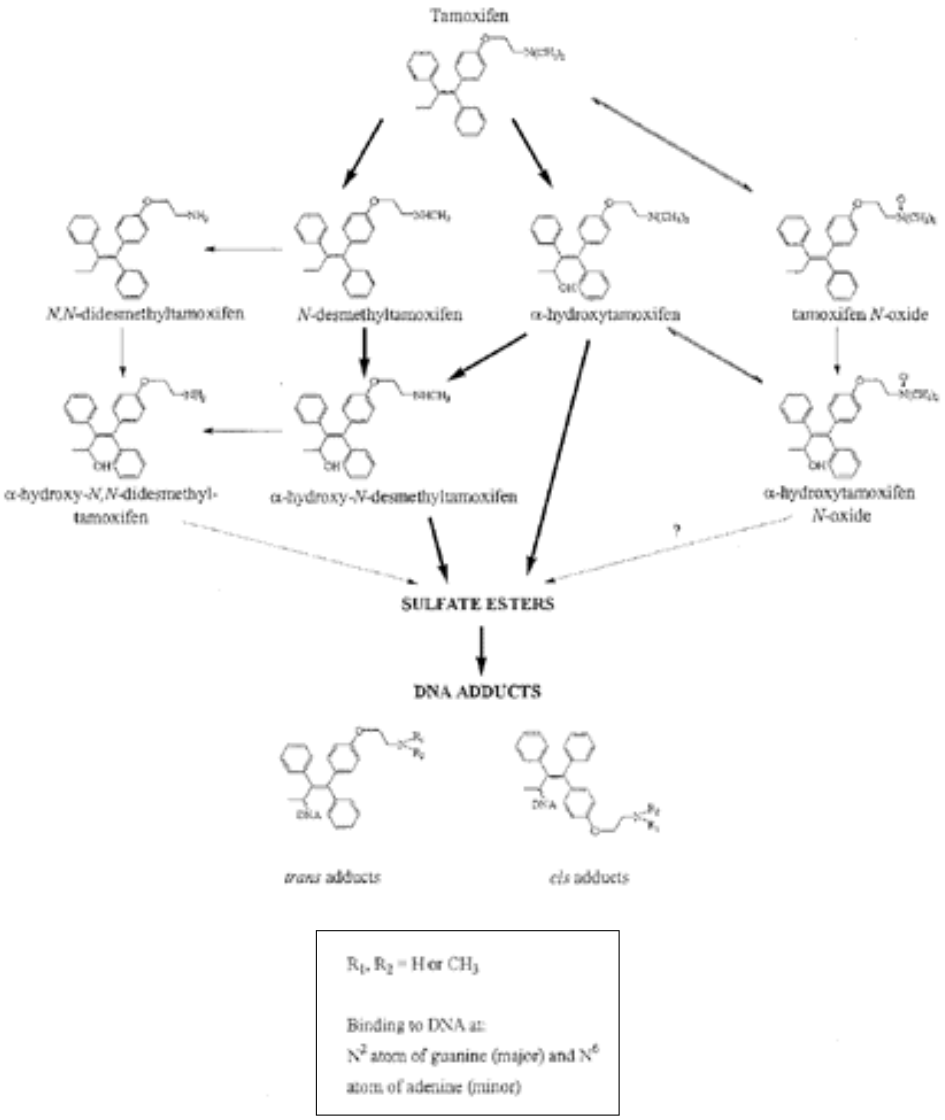
With improvements in the resolution of tamoxifen-DNA adducts by HPLC, it became apparent that the pattern of adducts was more complex than could be accounted for simply by the activation of tamoxifen to a carbocation of tamoxifen itself. It has now been shown that a parallel adduct formation pathway involving *N*-demethylation, in addition to α-hydroxylation, occurs (Figure 2).[33,40,59,60] Examination of the adduct forming potential of a number of metabolites has demonstrated that *N*-demethylation can either precede or follow α–hydroxylation in the activation pathway, with *N*,*N*-didemethylation, again with α-hydroxylation, constituting a minor additional pathway.[40,59]

Another accompanying pathway that has been considered is *N*-oxidation. The available evidence on tamoxifen *N*-oxide or metabolites containing the *N*-oxide function suggests that they are not involved to any great extent in in vivo DNA adduct formation in rats. Tamoxifen *N*-oxide is a major metabolite when tamoxifen is incubated with rat liver microsomes[36] and although tamoxifen-*N*-oxide and α-hydroxytamoxifen-*N*-oxide form adducts in rat liver and in hepatocytes, these are chromatographically indistinguishable from those formed by tamoxifen and α-hydroxytamoxifen, which in turn co-chromatograph with standards not possessing an *N*-oxide function.[40] Thus it would appear that loss of the *N*-oxide occurs prior to further metabolism to activated species, or prior to DNA

binding. The facile reduction of tamoxifen *N*-oxide back to tamoxifen by rat and human liver microsomes suggests that the *N*-oxide may serve as a storage form for tamoxifen in vivo,[61] ie, *N*-oxidation appears to be reversible in the rat. There are several as-yet-unidentified minor tamoxifen-derived DNA adducts detected in rat liver and it is conceivable that some of these are derived from tamoxifen *N*-oxide.

α-Hydroxytamoxifen has a chiral carbon atom, and therefore exists as two enantiomers. These have recently

FIGURE 2. PROPOSED PATHWAYS OF ACTIVATION OF TAMOXIFEN IN RAT LIVER



Adapted from Phillips.[40]

been resolved, their absolute configurations assigned and their potential to form DNA adducts in rat hepatocytes compared: the R-(+)-isomer has a much higher binding activity than the S-(-)-isomer.[62] Both enantiomers will give rise to the same carbocation, from which both epimers of each adduct can arise as chirality at the α-position returns. Thus, for one enantiomer to be more biologically active, it can be assumed that it is a better substrate for activation and/or a poorer substrate for detoxification enzymes than its enantiomeric partner.

Returning to the issue of the Phase II activation step of α-hydroxytamoxifen (with or without accompanying *N*-oxidation and *N*-demethylation), the role of hydroxysteroid sulphotransferase, implicated by the sulphate-dependent and DHEAS-inhibited DNA binding of the metabolite and parent compound in hepatocytes,[58] is reinforced by direct studies with the enzyme. Firstly, recombinant rat liver hydroxysteroid sulphotransferase rHSTa catalyses the binding of α–hydroxytamoxifen to DNA.[63] Also, in *Salmonella typhimurium* TA1538 and Chinese hamster V79 cells genetically engineered to express rHSTa, α-hydroxytamoxifen is mutagenic and forms DNA adducts;[64,65] this is the only example of in vitro mutagenicity of a tamoxifen metabolite.

There are some, including an anonymous reviewer of one of this author's grant applications, who have maintained that the carcinogenicity of tamoxifen in rat liver has nothing to do with DNA adduct formation. Indeed, the proposed pathway of activation by hydroxysteroid sulphotransferase rHSTa creates a paradox: this enzyme, a member of the SULT2A subfamily, is expressed in female rat liver but barely detectable in the male,[66] yet tamoxifen is equipotent in inducing liver tumours in both sexes[11]. Curiously, all the early work on adduct formation had been carried out in only female rats and hepatocytes. When we compared adduct formation in male and female hepatocytes, we found that it was much lower (11-fold) in the male cells.[67] Furthermore, treatment of rats with a single oral dose of tamoxifen resulted in 6-fold lower adduct levels in the liver of males than in females. However, when tamoxifen was administered daily, thereby mimicking the protocol of the animal carcinogenicity experiments[11], adduct levels in males were, by 14 days, similar to the levels in females. The explanation is that in the male rat liver tamoxifen administration induces specifically rHSTa, the sulphotransferase isoform that activates α-hydroxytamoxifen[67] such that tamoxifen-DNA adduct formation is similar in both sexes with prolonged exposure, thereby rendering males equally susceptible to liver tumour induction as females. Thus, the close correlation between DNA adduct formation, sulphotransferase activity and tamoxifen carcinogenicity in the liver is maintained.

With increasing duration of exposure to tamoxifen, there is a change in the relative amounts of the tamoxifen and *N*-desmethyltamoxifen adducts: after 1 day the former outnumber the latter by a factor of 2.3, but after 14 days the latter are predominant by a factor of 1.4[67] and are also the more abundant adducts after 18 months of chronic exposure.[51] This is possibly the result of different rates of DNA repair but a more likely explanation is induction of *N*-demethylation by one or more CYP isozymes.[68,69]

If further proof of the biological significance of tamoxifen-DNA adducts is needed, it is provided by the demonstration that the adducts are miscoding lesions in vitro[70] and are subject to nucleotide excision repair.[71] They induce predominantly GC-TA transversions when modified DNA is replicated in simian kidney (COS-7) cells.[72] This is also the predominant in vivo mutation occurring in the livers of transgenic rats administered tamoxifen.[31,73]

Interestingly, in these experiments with transgenic rats,[31,73] only in the liver were mutations detected. ^{32}P-Postlabelling analysis of DNA from other tissues provides scant evidence for tamoxifen-DNA adduct formation. Most tissues studied appear to be devoid of adducts[21] (A. Hewer and D.H. Phillips, unpublished results). In one study an adduct was reported to be formed in the uterus,[49] but our own attempts to replicate this finding using the identical treatment protocol did not result in the detection of adducts in this tissue (A. Hewer and D.H. Phillips, unpublished results). Several other studies have also found no evidence of adduct formation in rat uterus.[12,43,48,74]

Nevertheless, another technique for adduct determination, accelerator mass spectrometry (AMS) gives somewhat different results on this issue. This method is much the most sensitive method for detecting DNA binding,[75] but does not give structural information on the nature of the binding. Following administration of ^{14}C-tamoxifen to rats, the radioisotope was found to be associated with the DNA isolated from several tissues,[34] at levels that should have been detectable by ^{32}P-postlabelling, assuming the radioisotope is in the form of covalently bound tamoxifen. Therefore, at present there remain uncertainties as to the nature of this observed binding in extrahepatic tissues (a previously unrecognised form of DNA binding by tamoxifen moieties that is not detectable by ^{32}P-postlabelling?), and questions about its biological significance, given the lack of tamoxifen-induced mutations in all tissues except the liver.[31,73,76]

Could tamoxifen and/or its metabolites also be giving rise to DNA damage indirectly, via oxidative and free radical pathways? Both tamoxifen and 4-hydroxytamoxifen induce 8-hydroxyguanine formation in DNA in rat liver microsomal incubations,[77] but the extent to which this may occur in vivo is not known. Conversely, there is extensive documentation of the antioxidant properties of tamoxifen (and 4-hydroxytamoxifen) in a number of experimental systems[5]; these properties may contribute to the chemopreventive action of the drug. It has been shown that peroxidases can activate tamoxifen and 4-hydroxytamoxifen to intermediates that react covalently with DNA *in vitro*,[47,78] but the extent to which this pathway occurs in vivo is unknown, as is the possibility that other (non-tamoxifen) reactive species generated by this pathway could cause DNA damage.[79] It has been suggested[79] that

tamoxifen-induced micronucleus formation in MCL-5 cells[25] and aneuploidy and chromosome exchanges in rat hepatocytes[29] could be the result of oxidative stress rather than 'direct' genotoxicity, and indeed, tamoxifen-DNA adducts were not detected in MCL-5 cells exposed to tamoxifen under conditions that induced micronucleus formation (A. Hewer, F.L. Martin and D.H. Phillips, unpublished results).

MECHANISM IN MICE

Tamoxifen forms DNA adducts in the liver of mice, but at lower levels than in the rat. Short-term treatment of mice with tamoxifen resulted in adducts at a level 30-40% of that induced by comparable treatment of rats.[21] In primary cultures of mouse hepatocytes, adduct formation by tamoxifen was 6 times lower than in rat hepatocytes.[42]

Adducts do not accumulate in mice chronically exposed to tamoxifen.[80] Continuous feeding led to the detection of adducts after 3 months, but not after 2 years of exposure. This suggests the slow induction of a detoxification pathway. This is in contrast to the situation in rat liver where adducts accumulate to a great extent[19]. Earlier studies in which mice were treated with tamoxifen intraperitoneally showed the existence of a major pathway, apparently involving activation via α-hydroxylation, and a minor but inducible pathway proceeding via 4-hydroxytamoxifen.[81-83]

In a recent study of tamoxifen-DNA adducts formed in mouse liver following administration of the compound by gavage, minor adducts (~7% of total) were attributed to binding at the α-position of tamoxifen *N*-oxide[84] and supporting studies on the reactivity and adduct formation by α-acetoxytamoxifen *N*-oxide have been carried out.[85] The major adducts in mouse liver were reported to derive from α-hydroxytamoxifen,[84] and it remains to be determined whether *N*-desmethyltamoxifen is also a precursor of DNA adducts in this tissue.

It has long been a central tenet of chemical carcinogenesis that DNA adduct formation (or DNA damage) is a necessary, but not sufficient, event/stage in the mechanism of action of a genotoxic carcinogen. This is exemplified by the formation of tamoxifen-DNA adducts in mouse liver, which does not give rise to liver tumours. These adducts do not appear to accumulate in mouse liver whereas they do in rat liver, where tumours arise at high frequency. It will be interesting to see whether tamoxifen can induce liver tumours (or any tumours, for that matter) in mice deficient in DNA repair, such as XP knockout mice. However, it is also the case that tamoxifen induces cell proliferation in rat liver providing a tumour promoting influence,[19] but not in mouse liver.[80] While these differences in adduct persistence and cell proliferation go some way to explain why the liver of one species but not the other is susceptible, the reasons for the interspecies differences in response are not clear.

The induction of uterine adenocarcinomas in mice following neonatal treatment,[14] but not following administration to adult animals,[15] suggests a mechanism involving hormonal perturbation of the developing organ. There do not appear to have been any attempts to detect tamoxifen-DNA adducts in the reproductive tract of female mice.

MECHANISM IN HUMANS

Metabolism of tamoxifen in humans appears to be qualitatively similar to metabolism in rodents[38] and it is noteworthy that α-hydroxytamoxifen is a detectable metabolite in the plasma of women on long-term tamoxifen therapy.[86] Also, steady-state serum levels of *N*-desmethyltamoxifen are higher than those of tamoxifen itself in patients on long-term tamoxifen therapy.[87]

Although there have been some case reports of acute liver toxicity of tamoxifen,[88] there have been no findings of increased liver cancer among tamoxifen-treated women,[89,90] albeit with relatively short periods of follow-up given the potentially long latent period for induction of carcinomas in humans. A small ^{32}P-postlabelling study of liver DNA from 7 individuals taking tamoxifen revealed moderate levels of DNA adducts but was not able to distinguish the presence of any resulting from tamoxifen binding, and the total levels were not higher than those found in the liver DNA of 7 control subjects.[91] A single liver sample (post-mortem) from a tamoxifen-treated patient analysed in this author's laboratory did not reveal the presence of any tamoxifen-derived adducts (unpublished result).

In experiments with primary cultures of human hepatocytes, tamoxifen did not form adducts, and DNA binding by α-hydroxytamoxifen was more than 100-fold lower than in female rat hepatocytes;[42] this low level of binding was possibly the result of chemical reaction of the compound without metabolic activation. Concentrations of α-hydroxytamoxifen detected in the media of the human hepatocytes treated with tamoxifen was also significantly lower (~50-fold) than in the media of rat hepatocytes.[42] While these results reveal marked interspecies differences, they do not exclude the possibility that prolonged exposure to tamoxifen may result in induction of a human liver enzyme that activates the compound, analogous to the situation in male rats.[67]

Does the proposed mechanism of activation in rat liver indicate a reason for the lack of adducts (and carcinogenicity) in human liver? There is evidence that α-hydroxytamoxifen is a poorer substrate for sulphotransferase in humans than in rats. Sulphate ester formation (leading to adduct formation) from α-hydroxytamoxifen by the purified recombinant enzymes is at least 3 times more efficient with the rat rHSTa isozyme than with the human isoform,[92] but the difference is considerably greater when the enzymes are expressed in bacterial or mammalian cells: α-Hydroxytamoxifen was mutagenic and formed detectable levels of DNA adducts in the cells expressing the rat rHSTa enzyme, but not in those expressing any of the known human sulphotransferases, indicating at least a 20-fold difference in affinity for the substrate.[65] α-Hydroxytamoxifen can also undergo glucuronidation,[93] which is a likely to be a detoxication (inactivation) pathway. In incubations of α–hydroxytamoxifen with human liver microsomal fractions glucuronidation predominates

over sulphonation, while with rat microsomes sulphate ester formation predominates over glucuronidation.[94] This provides an explanation for the paucity of DNA adduct formation by tamoxifen and α-hydroxytamoxifen in human hepatocytes in vitro[42] and the apparent lack of adduct formation in the liver of women taking tamoxifen[91].

In summary, α-hydroxylation is more prevalent in rodent liver cells than in human cells, as is sulphate ester formation from this metabolite. At the same time, the alternative pathway of glucuronylation, leading to a detoxified conjugate, is more prevalent in human systems than in rodent ones.

The situation regarding tamoxifen-DNA adducts in human endometrium is much less clear. Initially we did not find evidence for DNA adduct formation in endometrial samples from 18 women receiving tamoxifen therapy, with an estimated detection limit of 4 adducts/1010 nucleotides.[95] This lack of adduct formation in vivo was mirrored by the lack of tamoxifen-DNA adduct formation in short-termorgan cultures of endometrial tissue incubated with high concentrations (500 μM) of tamoxifen.[95] α-Hydroxytamoxifen gave rise to low levels of adducts only at concentrations several orders of magnitude higher than would occur *in vivo*, analogous to the situation in primary cultures of human hepatocytes.

These results are in contrast to a subsequent study in which tamoxifen-DNA adducts were reported to be present in the endometrial tissue of 4/6 treated patients at levels of $2.7/10^9$ nucleotides, but were undetectable in 5 controls.[96] The evidence for DNA binding in this study was the appearance of a minor radioactive peak on HPLC seen against a very high background, and the validity of this claim has been questioned.[97] Subsequently, we analysed DNA from a further 34 endometrial samples using the chromatographic conditions of Hemminki and colleagues.[96] Under these conditions we also detected a minor chromatographic peak co-eluting with the major rat liver tamoxifen-DNA adduct in some of the samples, but it was found to be present not only in those from 7/14 tamoxifen-treated women, but also in samples from 3/6 toremifene-treated women and from 5/14 untreated controls.[98] The detection limit for these analyses was estimated at 1 adduct/10^9 nulceotides. We concluded that the peak is either an artefact of the postlabelling procedure or a background (endogenous) adduct that is not derived from tamoxifen. A report of the presence of tamoxifen-DNA adducts in white blood cells of patients, based on similar identification criteria,[99] was similarly not reproduced in our own studies.[100,101]

Using improved ^{32}P-postlabelling and HPLC procedures that result in considerable reductions in background radioactivity, Shibutani *et al.*[102,103] have recently reported the detection of tamoxifen-DNA adducts in endometrial tissue from 8/16 patients, and their absence from all of 15 controls. The adducts were identified by virtue of their co-chromatography with synthetic tamoxifen adducts, and were estimated to be present at levels in the range 0.2-18.0 adducts/10^8 nucleotides. The detection limit was reported to be 2.5 adducts/10^{10} nucleotides.

These adduct levels are clearly much higher than those previously estimated. However, what is highly unusual about these results is that the ratio of *cis* and *trans* tamoxifen-DNA adducts differs widely between individuals, with some samples containing predominantly the *trans* adducts but others containing only the *cis* adducts. This is not what would be expected from the animal experiments, or from the pattern of adducts formed in the chemical reaction of reactive derivatives of α-hydroxytamoxifen with DNA, where the products are predominantly *trans*.[54,56,92] Logically, one would expect to see a spectrum of mainly *trans* adduct peaks, including ones derived from the *N*-desmethyltamoxifen metabolite as well as from tamoxifen itself, but this is not what has been reported.[102,103] The chemistry of the interaction with DNA of reactive intermediates of tamoxifen that give rise to a carbocation at the α-position, and also the profile of adducts formed when tamoxifen is activated in mammalian cells in vitro or in vivo, clearly show that *trans*-tamoxifen adducts predominate over *cis*-tamoxifen adducts. Therefore if tamoxifen-DNA adducts are formed in human tissues it would be expected that they would display the same *cis:trans* ratio as seen under experimental conditions. Thus the apparent detection of variable ratios of *cis:trans* adducts in human endometrium requires an explanation. The suggestion that an as-yet-unidentified polymorphism in a gene encoding a DNA repair enzyme could result in the preferential repair of one type of adduct in some women and the other type in others[102] does not seem plausible if, as has been reported, the adducts are repaired by nucleotide excision repair,[71] a mechanism capable of correcting a very wide spectrum of modifications to the DNA structure and sequence.

Shibutani *et al.*[103] also suggest that our failure to detect tamoxifen-DNA adducts in human endometrium may be due to insufficient sensitivity, but our own published methods have a limit of sensitivity similar to theirs and have not yielded evidence for tamoxifen-DNA adducts in our samples, and even when we replicated their postlabelling procedures[102] adducts were not detected in our samples (A. Hewer and D.H. Phillips, unpublished results). It is entirely conceivable that a low level of adducts could be present in human endometrium, given that α–hydroxytamoxifen has weak intrinsic reactivity towards DNA,[41,54] but this alone would not be likely to result in adduct levels in the endometrium as high as those claimed.[102,103] Using the ultrasensitive method of accelerator mass spectrometry (AMS), a single dose of ^{14}C-tamoxifen to women resulted in as-yet-uncharacterised 'adduct' formation in the endometrium at up to 8 adducts/10^{10} nucleotides (E.A. Martin, personal communication), but this level of binding is close to the limit of detection of the ^{32}P-postlabelling method.

In summary, in vitro studies with human tissues or cells do not show significant DNA adduct formation by tamoxifen in either hepatocytes or endometrium, and binding by α-hydroxytamoxifen occurs only at high

concentration, probably non-enzymically. in vivo, there is no evidence for adduct formation in human liver, but differing results have been obtained with endometrium.

ANALOGUES OF TAMOXIFEN

Toremifene (Figure 3) is not carcinogenic in rat liver[17] and DNA adduct formation by toremifene in rat liver is either undetectable[17] or extremely low.[21,74] When incubated with rat or human microsomal fractions in the presence of DNA, toremifene gave rise to DNA adducts, although at levels lower than those formed by tamoxifen.[104] Also, toremifene induced micronucleus formation in MCL-5 cells[27,28] but it was less active than tamoxifen and it did not show significant clastogenic activity in in vivo experiments.[28] These positive results with toremifene are, perhaps, due to the artificiality of some in vitro test systems and to an imbalance of the activation and detoxification systems operating in vivo or in 'normal' cells.

Insofar as it has been tested (ie, exposure for up to 24 weeks), idoxifene (Figure 3) is not carcinogenic in rat liver.[105] DNA adduct formation by idoxifene in rat liver in vivo is two orders of magnitude lower than with tamoxifen,[105] while in experiments with rat hepatocytes it was found that idoxifene does not form a detectable level of adducts and that the putative metabolite α-hydroxyidoxifene is less active in adduct formation than α-hydroxytamoxifen.[106] Furthermore, α-hydroxyidoxifene was less reactive than α-hydroxytamoxifen towards DNA at pH5[44], and α-acetoxyidoxifene was less reactive than α-acetoxytamoxifen at pH7.[106]

Droloxifene (Figure 3) does not give rise to liver tumours in rats[8] and it did not form detectable levels of DNA adducts in rat liver when investigated by ^{32}P-postlabelling.[21]

Calculations of carbocation stability for a series of triphenylethylenes have shown that the intermediates of toremifene, idoxifene and 4-iodotamoxifen are significantly less stable than that of tamoxifen, suggesting that they are less frequently activated than tamoxifen in this manner.[107]

Thus tamoxifen stands alone in its class. The other therapeutic antioestrogens do not cause tumours in rats, and form few, if any, DNA adducts in vivo.

ILLUMINATION OR CONFUSION?

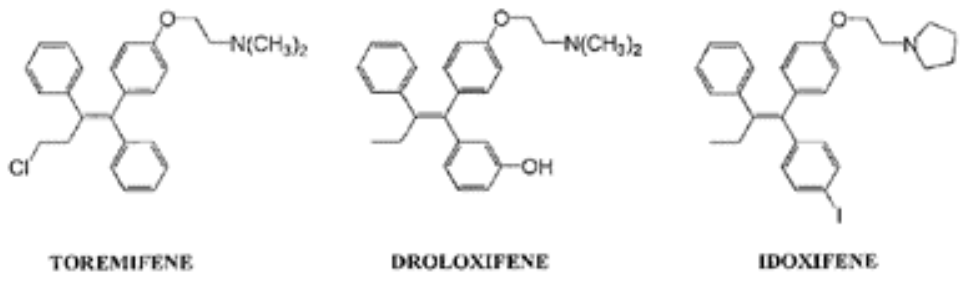
Tamoxifen-DNA adduct formation by the metabolic pathways described herein, followed by cell proliferation, provides a plausible mechanism for tumour formation in rat liver. The mechanism can be defined as a genotoxic one. In mouse liver, adduct formation is less persistent and there appears to be no concomitant stimulation of cell proliferation, so liver tumours do not develop. Tamoxifen-DNA adducts are also formed in the liver of hamsters[20] and rhesus monkeys,[108] in the latter case at levels at least an order of magnitude lower than in rats. However, the drug has not yet been tested for carcinogenicity in hamsters or monkeys. Human liver, in contrast, appears to be better protected against activation of tamoxifen to DNA binding species.

Thus tamoxifen presents something of a problem in the arena of regulatory testing of pharmaceuticals for genetic toxicity: negative in the battery of short-term tests, but demonstrably genotoxic (and carcinogenic) in vivo. The failure of the short-term tests to give positive results for tamoxifen is probably explicable by the low rate of metabolism to α–hydroxytamoxifen and/or the low activity of sulphotransferases in the systems used. Only under special circumstances has a tamoxifen metabolite (α-hydroxytamoxifen) been shown to be mutagenic in vitro.

The nature of apparent DNA binding in extrahepatic rat tissues, detected by AMS but not, it seems, by ^{32}P-postlabelling, clearly requires further investigation and characterisation. The general lack of evidence by ^{32}P-postlabelling analysis for adducts in extrahepatic tissues is compatible with activation of tamoxifen at the α-position by hydroxysteroid sulphotransferase, since this is expressed almost exclusively in the liver. Unfortunately, because AMS measures isotope ratios it requires the use of ^{14}C-labelled drug for detection of DNA binding and thus cannot be used for the routine detection of tamoxifen-DNA adducts in human tissues.

Some studies report formation of tamoxifen-DNA adducts in endometrium, others do not. However, the observed interindividual variation in relative amounts of *cis* and *trans* adducts[102,103] is puzzling. No satisfactory

FIGURE 3. STRUCTURES OF ANALOGUES OF TAMOXIFEN



explanation for this has yet been advanced. We have not found unequivocal evidence for the presence of tamoxifen-DNA adducts in any of 54 samples of endometrial tissue from women taking the drug[95,98] (and unpublished results). Since each human sample is unique, perhaps the question of whether adducts are truly formed in human tissues can only be definitively answered by an inter-laboratory comparison of analyses of coded tissue samples from exposed and control individuals, and when other methods that take a different approach to DNA adduct detection and identification are brought to bear on the problem. Thus, the production of high-affinity antibodies to tamoxifen-DNA adducts[35] may provide a means to shed light on this issue. Ultimately, the question may be not whether or not tamoxifen is capable of DNA adduct formation in humans, but whether the levels of putative adducts are reliably estimated and at a level that would result in observable biological effect. With α-hydroxytamoxifen identified as a human metabolite and possessing the ability to react with DNA without further metabolic activation, the occurrence of tamoxifen-DNA adducts in any human tissue must be considered a possibility. It will be interesting to see whether adducts are detectable in the bladder, since acidic pH catalyses carbocation formation by α-hydroxytamoxifen.[54]

As a general rule, it would seem prudent that, if adducts are thought to be present in human DNA and the method of detection does not provide definitive characterisation but instead relies on co-chromatography with standards (^{32}P-postlabelling is not alone in this regard), the following criteria should be met:

(1) The relative amounts of different adducts should be as expected from the relative amounts formed by reaction of the putative reactive intermediate(s) with DNA (or by reaction of a representative model intermediate); and/or

(2) The relative amounts of different adducts should be as expected from the relative amounts formed by the carcinogen in experimental animals; and/or

(3) The relative amounts of different adducts should not vary dramatically between human individuals.

Where these criteria are testable and not met, then the identification of the adducts, or their origin, should be questioned and a convincing explanation sought.

Can we consider the mechanism of tamoxifen-induced hepatocarcinogenesis in rats relevant to the mechanism of tamoxifen-induced endometrial carcinogenesis in women? If the answer is yes, then analogues of tamoxifen that are not carcinogenic to rat liver, and that do not form DNA adducts therein, would be predicted to be safer alternatives to tamoxifen. If the answer is no, as is suggested by what is *currently* known about the enzymology of tamoxifen activation in rat liver, then there is at present no rational basis on which to make predictions about their long-term safety to human endometrium, or indeed other tissues.

Could tamoxifen therefore be both a genotoxic and a non-genotoxic carcinogen? If it turns out to be the case that tamoxifen is a genotoxic carcinogen in one species (rat) and a non-genotoxic carcinogen in another (human), this would make it a highly unusual, if not unique, carcinogen. Since tumours can be induced in rat uterus following neonatal exposure, but adducts have not been reproducibly detected in this tissue, it may even be the case that tamoxifen is a genotoxic carcinogen in one tissue, and a non-genotoxic carcinogen in another tissue of the same species. In the last decade some very novel and interesting properties of tamoxifen carcinogenicity have been uncovered but the picture remains incomplete. Continued study of this important drug promises further insight into its carcinogenic mechanism(s). Perhaps only then can the title of this article be written as a statement, rather than as a question. **OS**

Acknowledgements: *Studies in the author's laboratory are supported by the Cancer Research Campaign. Mike Jarman and Martin Osborne are thanked for critical reading of the manuscript.*

REFERENCES

1. Early Breast Cancer Collaborative Group (1998) Tamoxifen for early breast cancer: an overview of the randomized trials. *Lancet*, 351, 1451-1467.
2. Fisher, B., Costantino, J.P., Wickerham, D.L., Redmond, C.K., Kavanah, M., Cronin, W.M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L. and Wolmark, N. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl Cancer. Inst.*, 90, 1371-1388.
3. Powles, T., Eeles, R., Ashley, S., Easton, D., Chang, J., Dowsett, M., Tidy, A., Viggers, J. and Davey, J. (1998) Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. *Lancet*, 352, 98-101.
4. Veronesi, U., Maisonneuve, P., Costa, A., Sacchini, V., Maltoni, C., Robertson, C., Rotmensz, N. and Boyle, P. (1998) Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. *Lancet*, 352, 93-97.
5. Wiseman, L.R. and Goa, K.L. (1997) Toremifene. A review of its pharmacological properties and clinical efficacy in the management of advanced breast cancer. *Drugs*, 54, 141-160.
6. Yao, K. and Jordan, V.C. (1998) Questions about tamoxifen and the future use of antiestrogens. *Oncologist*, 3, 104-110.
7. Fisher, B., Costantino, J.P., Redmond, C.K., Fisher, E.R., Wickerham, D.L., Cronin, W.M. and other NSABP contributors (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J. Natl Cancer Inst.*, 86, 527-537.
8. IARC (1996) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 66, Some Pharmaceutical Drugs. *International Agency for Research on Cancer*, Lyon.
9. Bergman, L., Beelen, M.L.R., Gallee, M.P.W., Hollema, H., Benraadt, J., van Leeuwen, F.E. and Comprehensive Cancer Centres' ALERT Group (2000) Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. *Lancet*, 356, 881-887.
10. Kedar, R.P., Bourne, T.H., Powles, T.J., Collins, W.P., Ashley, S.E., Cosgrove, D.O. and Campbell, S. (1994) Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. *Lancet*, 343, 1318-1321.

11. Greaves, P., Goonetilleke, R., Nunn, G., Topham, J. and Orton, T. (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res.*, 53, 3919-3924.
12. Carthew, P., Edwards, R.E., Nolan, B.M., Martin, E.A., Heydon, R.T., White, I.N.H. and Tucker, M.J. (2000) Tamoxifen induces endometrial and vaginal cancer in rats in the absence of endometrial hyperplasia. *Carcinogenesis,* 21, 793-797.
13. Tucker, M.J., Adam, H.K. and Patterson, J.S. (1984) Tamoxifen. In Lawrence, D.R., McClean, A.E.M. and Wetherall, M. (eds), *Safety Testing of New Drugs*. Academic Press, London, pp. 125-161.
14. Newbold, R.R., Jefferson, W.N., Padilla-Burgos, E. and Bullock, B.C. (1997) Uterine carcinoma in mice treated neonatally with tamoxifen. *Carcinogenesis,* 18, 2293-2298.
15. Carthew, P., Edwards, R.E., Nolan, B.M., Martin, E.A. and Smith, L.L. (1996) Tamoxifen associated uterine pathology in rodents: relevance to women. *Carcinogenesis,* 17, 1577-1582.
16. Diwan, B.A., Anderson, L.M. and Ward, J.M. (1997) Proliferative lesions of oviduct and uterus in CD-1 mice exposed prenatally to tamoxifen. *Carcinogenesis,* 18, 2009-2014.
17. Hard, G.C., Iatropoulos, M.J., Jordan, K., Radi, L., Kaltenberg, O.P., Imondi, A.R. and Williams, G.M. (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. *Cancer Res.,* 53, 4534-4541.
18. Hirsimäki, P., Hirsimäki, Y., Nieminen, L. and Payne, B.J. (1993) Tamoxifen induces hepatocellular carcinoma in rat liver: a 1-year study with two antiestrogens. *Arch. Toxicol.,* 67, 49-54.
19. Carthew, P., Rich, K.J., Martin, E.A., De Matteis, F., Lim, C.-K., Manson, M.M., Festing, M.F.W., White, I.N.H. and Smith, L.L. (1995) DNA damage as assessed by 32P-postlabelling in three rat strains exposed to dietary tamoxifen: the relationship between cell proliferation and liver tumour formation. *Carcinogenesis,* 16, 1299-1304.
20. Han, X. and Liehr, J.G. (1992) Induction of covalent DNA adducts in rodents by tamoxifen. *Cancer Res.,* 52, 1360-1363.
21. White, I.N.H., de Matteis, F., Davies, A., Smith, L.L., Crofton-Sleigh, C., Venitt, S., Hewer, A. and Phillips, D.H. (1992) Genotoxic potential of tamoxifen and analogues in female Fischer F344/n rats, DBA/2 and C57Bl/6 mice and in human MCL-5 cells. *Carcinogenesis,* 13, 2197-2203.
22. Osborne, M.R. (1999) Genotoxicity of tamoxifen and other antiestrogens. Recent Res. Devel. *Cancer,* 1, 69-81.
23. White, I.N.H. (1999) The tamoxifen dilemma. *Carcinogenesis,* 20, 1153-1160.
24. Phillips, D.H. (2001) Mechanism of liver carcinogenesis by antoestrogens. In Jordan, V.C. and Furr, B.J.A. (eds), *Endocrine Therapy in Breast and Prostate Cancer.* Humana Press Inc., Totowa, NJ, pp. in press.
25. Crofton-Sleigh, C., Doherty, A., Ellard, S., Parry, E.M. and Venitt, S. (1993) Micronucleus assays using cytochalasin-blocked MCL-5 cells, a proprietary human cell line expressing five human cytochromes P-450 and microsomal epoxide hydrolase. *Mutagenesis,* 8, 363-372.
26. Phillips, D.H., Potter, G.A., Horton, M.N., Hewer, A., Crofton-Sleigh, C., Jarman, M. and Venitt, S. (1994) Reduced genotoxicity of [D5-ethyl]-tamoxifen implicates α–hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. *Carcinogenesis,* 15, 1487-1492.
27. Styles, J.A., Davies, A., Lim, C.K., de Matteis, F., Stanley, L.A., White, I.N.H., Yuan, Z.-X. and Smith, L.L. (1994) Genotoxicity of tamoxifen, tamoxifen epoxide and toremifene in human lymphoblastoid cells containing human cytochrome P450s. *Carcinogenesis,* 15, 5-9.
28. Styles, J.A., Davies, A., Davies, R., White, I.N.H. and Smith, L.L. (1997) Clastogenic and aneugenic effects of tamoxifen and some of its analogues in hepatocytes from dosed rats and in human lymphoblastoid cells transfected with human P450 cDNAs (MCL-5 cells). *Carcinogenesis,* 18, 303-313.
29. Sargent, L.M., Dragan, Y.P., Bahnub, N., Wiley, J.E., Sattler, C.A., Schroeder, P., Sattler, G.L., Jordan, V.C. and Pitot, H.C. (1994) Tamoxifen induces hepatic aneuploidy and mitotic spindle disruption after a single *In vivo* administration to female Sprague-Dawley rats. *Cancer Res.,* 54, 3357-3360.
30. Sargent, L.M., Dragan, Y.P., Sattler, C., Bahnub, N., Sattler, G., Martin, P., Cisneros, A., Mann, J., Thorgeirsson, S., Jordan, V.C. and Pitot, H.C. (1996) Induction of hepatic aneuploidy *In vivo* by tamoxifen, toremifene and idoxifene in female Sprague-Dawley rats. *Carcinogenesis,* 17, 1051-1056.
31. Davies, R., Oreffo, V.I.C., Martin, E.A., Festing, M.F.W., White, I.N.H., Smith, L.L. and Styles, J.A. (1997) Tamoxifen causes gene mutations in the livers of lambda/lacI transgenic rats. *Cancer Res.,* 57, 1288-1293.
32. Vancutsem, P.L., Lazarus, P. and Williams, G.M. (1994) Frequent and specific mutations of the rat p53 gene in hepatocarcinomas induced by tamoxifen. *Cancer Res.,* 54, 3864-3867.
33. Rajaniemi, H., Rasanen, I., Koivisto, P., Peltonen, K. and Hemminki, K. (1999) Identification of the major tamoxifen-DNA adducts in rat liver by mass spectroscopy. *Carcinogenesis,* 20, 305-309.
34. White, I.N.H., Martin, E.A., Mauthe, R.J., Vogel, J.S., Turtletaub, K.W. and Smith, L.L. (1997) Comparisons of the binding of [14C]radiolabelled tamoxifen or toremifene to rat DNA using accelerator mass spectrometry. *Chem.-Biol. Interact.,* 106, 149-160.
35. Divi, R.L., Osborne, M.R., Hewer, A., Phillips, D.H. and Poirier, M.C. (1999) Tamoxifen-DNA adduct formation in rat liver determined by immunoassay and 32P-postlabeling. *Cancer Res.,* 59, 4829-4833.
36. Lim, C.K., Yuan, Z.-X., Lamb, J.H., White, I.N.H., de Matteis, F. and Smith, L.L. (1994) A comparative study of tamoxifen metabolism in female rat, mouse and human liver microsomes. *Carcinogenesis,* 15, 589-593.
37. Jarman, M., Poon, G.K., Rowlands, M.G., Grimshaw, R., Horton, M.N., Potter, G.A. and McCague, R. (1995) The deuterium isotope effect for the α-hydroxylation of tamoxifen by rat liver microsomes accounts for the reduced genotoxicity of [D5-ethyl]tamoxifen. *Carcinogenesis,* 16, 683-688.
38. Poon, G.K., Chui, Y.C., McCague, R., Lønning, P.E., Feng, M.G., Rowlands, M.G. and Jarman, M. (1993) Analysis of Phase I and Phase II metabolites of tamoxifen in breast cancer patients. *Drug Metab. Dispos.,* 21, 1119-1124.
39. Potter, G.A., McCague, R. and Jarman, M. (1994) A mechanistic hypothesis for DNA adduct formation by tamoxifen following hepatic oxidative metabolism. *Carcinogenesis,* 15, 439-442.
40. Phillips, D.H., Hewer, A., Horton, M.N., Cole, K.J., Carmichael, P.L., Davis, W. and Osborne, M.R. (1999) N-Demethylation accompanies α-hydroxylation in the metabolic activation of tamoxifen in rat liver cells. *Carcinogenesis,* 20, 2003-2009.
41. Phillips, D.H., Carmichael, P.L., Hewer, A., Cole, K.J. and Poon, G.K. (1994) α–Hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. *Cancer Res.,* 54, 5518-5522.
42. Phillips, D.H., Carmichael, P.L., Hewer, A., Cole, K.J., Hardcastle, I.R., Poon, G.K., Keogh, A. and Strain, A.J. (1996) Activation of tamoxifen and its metabolite α–hydroxytamoxifen to DNA-binding products: comparisons between human, rat and mouse hepatocytes. *Carcinogenesis,* 17, 88-94.
43. Brown, K., Brown, J.E., Martin, E.A., Smith, L.L. and White, I.N.H. (1998) Determination of DNA damage in F344 rats induced by geometric isomers of tamoxifen and analogues. *Chem. Res. Toxicol.,* 11, 527-534.
44. Hardcastle, I.R., Horton, M.N., Osborne, M.R., Hewer, A., Jarman, M. and Phillips, D.H. (1998) Synthesis and DNA reactivity of putative metabolites of non-steroidal antiestrogens. *Chem. Res. Toxicol.,* 11, 369-374.
45. Osborne, M.R., Davis, W., Hewer, A.J., Hardcastle, I.R. and Phillips, D.H. (1999) 4-Hydroxytamoxifen gives DNA adducts by chemical activation, but not in rat liver cells. *Chem. Res. Toxicol.,* 12, 151-158.
46. Marques, M.M. and Beland, F.A. (1997) Identification of tamoxifen-DNA adducts formed by 4-hydroxytamoxifen quinone methide. *Carcinogenesis,* 18, 1949-1954.
47. Pathak, D.N., Pongracz, K. and Bodell, W.J. (1995) Microsomal and peroxidase activation of 4-hydroxy-tamoxifen to form DNA adducts: comparison with DNA adducts formed in Sprague-Dawley rats treated with tamoxifen. *Carcinogenesis,* 16, 11-15.
48. Beland, F.A., McDaniel, L.P. and Marques, M.M. (1999) Comparison of the DNA adducts formed by tamoxifen and 4-hydroxytamoxifen *in vivo*. *Carcinogenesis,* 20, 471-477.

49. Pathak, D.N., Pongracz, K. and Bodell, W.J. (1996) Activation of 4-hydroxytamoxifen and the tamoxifen derivative metabolite E by uterine peroxidase to form DNA adducts: comparison with DNA adducts formed in the uterus of Sprague-Dawley rats treated with tamoxifen. *Carcinogenesis,* 17, 1785-1790.
50. Martin, E.A., Heydon, R.T., Brown, K., Brown, J.E., Lim, C.K., White, I.N.H. and Smith, L.L. (1998) Evaluation of tamoxifen and α-hydroxytamoxifen ^{32}P-post-labelled DNA adducts by the development of a novel automated on-line solid-phase extraction HPLC method. *Carcinogenesis,* 19, 1061-1069.
51. Firozi, P.F., Vulimiri, S.V., Rajaniemi, H., Hemminki, K., Dragan, Y., Pitot, H.C., DiGiovanni, J., Zhu, Y.H. and Li, D. (2000) Characterization of the major DNA adducts in the liver of rats chronically exposed to tamoxifen for 18 months. *Chem.-Biol. Interact.,* 126, 33-43.
52. Sauvez, F., Drouin, D.S., Attia, M., Bertheux, H. and Forster, R. (1999) Cutaneously applied 4-hydroxytamoxifen is not carcinogenic in female rats. *Carcinogenesis,* 20, 843-850.
53. Mauvais-Javis, P., Baudot, N., Castaigne, D., Banzet, P. and Kuttenn, F. (1986) trans-4-Hydroxytamoxifen concentration and metabolism after local percutaneous administration to human breast. *Cancer Res.,* 46, 1521-1525.
54. Osborne, M.R., Hewer, A., Hardcastle, I.R., Carmichael, P.L. and Phillips, D.H. (1996) Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. *Cancer Res.,* 56, 66-71.
55. Phillips, D.H. (1994) DNA adducts derived from safrole, estragole and related compounds, and from benzene and its metabolites. In Hemminki, K., Dipple, A., Shuker, D.E.G., Kadlubar, F.F., Segerback, D. and Bartsch, H. (eds), *DNA Adducts: Identification and Biological Significance.* IARC Scientific Publications Vol. 125. IARC, Lyon, pp. 131-140.
56. Osborne, M.R., Hardcastle, I.R. and Phillips, D.H. (1997) Minor products of reaction with DNA of α-acetoxytamoxifen. *Carcinogenesis,* 18, 539-543.
57. Dasaradhi, L. and Shibutani, S. (1997) Identification of tamoxifen-DNA adducts formed by α-sulfate tamoxifen and α–acetoxytamoxifen. *Chem. Res. Toxicol.,* 10, 189-196.
58. Davis, W., Venitt, S. and Phillips, D.H. (1998) The metabolic activation of tamoxifen and α–hydroxytamoxifen to DNA binding species in rat hepatocytes proceeds via sulphation. *Carcinogenesis,* 19, 861-866.
59. Brown, K., Heydon, R.T., Jukes, R., White, I.N.H. and Martin, E.A. (1999) Further characterisation of the DNA adducts formed in rat liver after the administration of tamoxifen, *N*-desmethyltamoxifen or *N,N*-didesmethyltamoxifen. *Carcinogenesis,* 20, 2011-2016.
60. Gamboa Da Costa, G., Hamilton, L.P., Beland, F.A. and Marques, M.M. (2000) Characterization of the major DNA adduct formed by alpha-hydroxy-*N*-desmethyltamoxifen *in vitro* and *In vivo*. *Chem. Res. Toxicol.,* 13, 200-207.
61. Mani, C., Hodgson, E. and Kupfer, D. (1993) Metabolism of the antimammary cancer antiestrogenic agent tamoxifen. II. Flavin-containing monooxygenase-mediated *N*-oxidation. *Drug Metab. Dispos.*, 21, 657-661.
62. Osborne, M.R., Hewer, A. and Phillips, D.H. (2000) Resolution of α–hydroxytamoxifen: R-isomer forms more DNA adducts in rat liver cells. *Carcinogenesis,* submitted for publication.
63. Shibutani, S., Dasaradhi, L., Terashima, I., Banoglu, E. and Duffel, M.W. (1998) Alpha-hydroxytamoxifen is a substrate of hydroxysteroid (alcohol) sulfotransferase, resulting in tamoxifen DNA adducts. *Cancer Res.,* 58, 647-653.
64. Glatt, H., Bartsch, I., Christoph, S., Coughtrie, M.W., Falany, C.N., Hagen, M., Landsiedel, R., Pabel, U., Phillips, D.H., Seidel, A. and Yamazoe, Y. (1998) Sulfotransferase-mediated activation of mutagens studied using heterologous expression systems. *Chem.-Biol. Interact.,* 109, 195-219.
65. Glatt, H., Davis, W., Meinl, W., Hermersdörfer, H., Venitt, S. and Phillips, D.H. (1998) Rat, but not human, sulfotransferase activates a tamoxifen metabolite to produce DNA adducts and gene mutations in bacteria and mammalian cells in culture. *Carcinogenesis,* 19, 1709-1713.
66. Rajkowski, K.M., Robel, P. and Baulieu, E.E. (1997) Hydroxysteroid sulfotransferase activity in the rat brain and liver as a function of age and sex. *Steroids,* 62, 427-436.
67. Davis, W., Hewer, A., Rajkowski, K.M., Meinl, W., Glatt, H.R. and Phillips, D.H. (2000) Sex differences in the activation of tamoxifen to DNA binding species in rat liver *In vivo* and in rat hepatocytes *in vitro*: role of sulphotransferase induction. *Cancer Res.,* 60, 2887-2891.
68. Jacolot, F., Simon, I., Dreano, Y., Beaune, P., Riche, C. and Berthou, F. (1991) Identification of the cytochrome P450 IIIA family as the enzymes involved in the N-demethylation of tamoxifen in human liver microsomes. *Biochem. Pharmacol.,* 41, 1911-1919.
69. White, I.N.H., Davies, A., Smith, L.L., Dawson, S. and de Matteis, F. (1993) Induction of CYP2B1 and 3A1, and associated monooxygenase activities by tamoxifen and certain analogues in the livers of female rats and mice. *Biochem. Pharmacol.,* 45, 21-30.
70. Shibutani, S. and Dasaradhi, L. (1997) Miscoding potential of tamoxifen-derived DNA adducts: α-(*N*^{2}-Deoxyguanosinyl)tamoxifen. *Biochemistry,* 36, 13010-13017.
71. Shibutani, S., Reardon, J.T., Suzuki, N. and Sancar, A. (2000) Excision of tamoxifen-DNA adducts by the human nucleotide excision repair system. *Cancer Res.,* 60, 2607-2610.
72. Terashima, I., Suzuki, N. and Shibutani, S. (1999) Mutagenic potential of α-(*N*^{2}-deoxyguanosinyl)tamoxifen lesions, the major DNA adducts detected in endometrial tissues of patients treated with tamoxifen. *Cancer Res.,* 59, 2091-2095.
73. Davies, R., Gant, T.W., Smith, L.L. and Styles, J.A. (1999) Tamoxifen induces G:C—>T:A mutations in the cII gene in the liver of lambda/lacI transgenic rats but not at 5'-CpG-3' dinucleotide sequences as found in the lacI transgene. *Carcinogenesis,* 20, 1351-1356.
74. Komaki, K., Umemoto, A., Suwa, M., Kannno, Y., Suzuki, M., Lin, C.-X., Momen, M.A., Shibutani, S. and Monden, Y. (2000) DNA adducts formed by tamoxifen and toremifene in liver, uterus, vagina and leucocytes of rats. *Proc. Am. Assoc. Cancer Res.,* 41, 96-97.
75. Phillips, D.H., Farmer, P.B., Beland, F.A., Nath, R.G., Poirier, M.C., Reddy, M.V. and Turteltaub, K.W. (2000) Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ. Mol. Mutagen.,* 35, 222-233.
76. Gamboa da Costa, G., Marques, M.M., Hamilton, L.P., Benson, R.W., Smith, B.A., Heflich, R.H. and Beland, F.A. (2000) DNA adduct formation and mutant induction in Sprague-Dawley rats treated with tamoxifen and its derivatives. *Proc. Am. Assoc. Cancer Res.,* 41, 108.
77. Ye, Q. and Bodell, W.J. (1996) Production of 8-hydroxy-2'-deoxguanosine in DNA by microsomal activation of tamoxifen and 4-hydroxytamoxifen. *Carcinogenesis,* 17, 1747-50.
78. Davies, A.M., Martin, E.A., Jones, R.M., Lim, C.K., Smith, L.L. and White, I.N.H. (1995) Peroxidase activation of tamoxifen and toremifene resulting in DNA damage and covalently bound protein adducts. *Carcinogenesis,* 16, 539-545.
79. Day, B.W., Tyurin, V.A., Tyurina, Y.Y., Liu, M., Facey, J.A., Carta, G., Kisin, E.R., Dubey, R.K. and Kagan, V.E. (1999) Peroxidase-catalyzed pro- versus antioxidant effects of 4-hydroxytamoxifen: enzyme specificity and biochemical sequelae. *Chem. Res. Toxicol.,* 12, 28-37.
80. Martin, E.A., Carthew, P., White, I.N.H., Heydon, R.T., Gaskell, M., Mauthe, R.J., Turtletaub, K.W. and Smith, L.L. (1997) Investigation of the formation and accumulation of liver DNA adducts in mice chronically exposed to tamoxifen. *Carcinogenesis,* 18, 2209-2215.
81. Randerath, K., Bi, J., Mabon, N., Sriram, P. and Moorthy, B. (1994) Strong intensification of mouse hepatic tamoxifen DNA adduct formation by pretreatment with the sulfotransferase inhibitor and ubiquitous environmental pollutant pentachlorophenol. *Carcinogenesis,* 15, 797-800.
82. Randerath, K., Moorthy, B., Mabon, N. and Sriram, P. (1994) Tamoxifen: evidence by 32P-postlabeling and use of metabolic inhibitors for two distinct pathways leading to mouse hepatic DNA adduct formation and identification of 4-hydroxytamoxifen as a proximate metabolite. *Carcinogenesis,* 15, 2087-2094.

83. Moorthy, B., Sriram, P., Pathak, D.N., Bodell, W.J. and Randerath, K. (1996) Tamoxifen metabolic activation. Comparison of DNA adducts formed by microsomal and chemical activation of tamoxifen and 4-hydroxytamoxifen with DNA adducts formed *In vivo*. *Cancer Res.,* 56, 53-57.
84. Umemoto, A., Monden, Y., Suwa, M., Kanno, Y., Suzuki, M., Lin, C.X., Ueyama, Y., Momen, M.A., Ravindernath, A., Shibutani, S. and Komaki, K. (2000) Identification of hepatic tamoxifen-DNA adducts in mice: alpha-(N(2)-deoxyguanosinyl)tamoxifen and alpha-(N(2)-deoxyguanosinyl) tamoxifen *N*-oxide. *Carcinogenesis,* 21, 1737-1744.
85. Umemoto, A., Monden, Y., Komaki, K., Suwa, M., Kanno, Y., Suzuki, M., Lin, C.X., Ueyama, Y., Momen, M.A., Ravindernath, A. and Shibutani, S. (1999) Tamoxifen-DNA Adducts Formed by alpha-Acetoxytamoxifen *N*-Oxide. *Chem. Res. Toxicol.,* 12, 1083-1089.
86. Poon, G.K., Walter, B., Lønning, P.E., Horton, M.N. and McCague, R. (1995) Identification of tamoxifen metabolites in human Hep G2 cell line, human liver homogenate, and patients on long-term therapy for breast cancer. *Drug Metab. Disp.,* 23, 377-382.
87. Langan-Fahey, S.M., Tomney, D.C. and Jordan, V.C. (1990) Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. *Eur. J. Cancer,* 26, 883-888.
88. Ching, C.K., Smith, P.G. and Long, R.G. (1992) Tamoxifen-associated hepatocellular damage and agranulocytosis. *Lancet,* 339, 940.
89. Fomander, T., Rutquist, L.E., Cedermark, B., Glas, U., Mattsson, A., Silfversward, C., Skoog, L., Somell, A., Theve, T., Wilking, N., Askergren, J. and Hjalmar, M.-L. (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet,* i, 117-120.
90. Rutqvist, L.E., Johansson, H., Signomklao, T., Johansson, U., Fomander, T. and Wilking, N. (1995) Adjuvant tamoxifen therapy for early stage breast cancer and second primary malignancies. *J. Natl Cancer Inst.,* 87, 645-651.
91. Martin, E.A., Rich, K.J., White, I.N.H., Woods, K.L., Powles, T.J. and Smith, L.L. (1995) 32P-Postlabelled DNA adducts in liver obtained from women treated with tamoxifen. *Carcinogenesis,* 16, 1651-1654.
92. Shibutani, S., Shaw, P.M., Suzuki, N., Dasaradhi, L., Duffel, M.W. and Terashima, I. (1998) Sulfation of alpha-hydroxytamoxifen catalyzed by human hydroxysteroid sulfotransferase results in tamoxifen-DNA adducts. *Carcinogenesis,* 19, 2007-2011.
93. Boocock, D.J., Maggs, J.L., White, I.N. and Park, B.K. (1999) Alpha-hydroxytamoxifen, a genotoxic metabolite of tamoxifen in the rat: identification and quantification *In vivo* and *in vitro*. *Carcinogenesis,* 20, 153-160.
94. Boocock, D.J., Maggs, J.L., Brown, K., White, I.N.H. and Park, B.K. (2000) Major inter-species differences in the rates of *O*-sulphonation and *O*-glucuronylation of α–hydroxytamoxifen *in vitro*: a metabolic disparity protecting human liver from the formation of tamoxifen-DNA adducts. *Carcinogenesis*, 21, 1851-1858.
95. Carmichael, P.L., Ugwumadu, A.H.N., Neven, P., Hewer, A., Poon, G.K. and Phillips, D.H. (1996) Lack of genotoxicity of tamoxifen in human endometrium. *Cancer Res.,* 56, 1475-1479.
96. Hemminki, K., Rajaniemi, H., Lindahl, B. and Moberger, B. (1996) Tamoxifen-induced DNA adducts in endometrial samples from breast cancer patients. Cancer Res., 56, 4374-4377.
97. Orton, T.C. and Topham, J.C. (1997) Correspondence re: K. Hemminki et al., Tamoxifen-induced DNA adducts in endometrial samples from breast cancer patients. Cancer Res., 56: 4374-4377, 1996. *Cancer Res.,* 57, 4148.
98. Carmichael, P.L., Sardar, S., Crooks, N., Neven, P., Van Hoof, I., Ugwumadu, A., Bourne, T., Tomas, E., Hellberg, P., Hewer, A.J. and Phillips, D.H. (1999) Lack of evidence from HPLC 32P-post-labelling for tamoxifen-DNA adducts in the human endometrium. *Carcinogenesis*, 20, 339-342.
99. Hemminki, K., Rajaniemi, H., Koskinen, M. and Hansson, J. (1997) Tamoxifen-induced DNA adducts in leucocytes of breast cancer patients. *Carcinogenesis,* 18, 9-13.
100. Phillips, D.H., Hewer, A., Grover, P.L., Poon, G.K. and Carmichael, P.L. (1996) Tamoxifen does not form detectable DNA adducts in white blood cells of breast cancer patients. *Carcinogenesis,* 17, 1149-1152.
101. Bartsch, H., Phillips, D.H., Nair, J., Hewer, A., Meyberg-Solomeyer, G. and Grischke, E.M. (2000) Lack of evidence for tamoxifen- and toremifene-DNA adducts in lymphocytes of treated patients. *Carcinogenesis,* 21, 845-847.
102. Shibutani, S., Suzuki, N., Terashima, I., Sugarman, S.M., Grollman, A.P. and Pearl, M.L. (1999) Tamoxifen-DNA adducts detected in the endometrium of women treated with tamoxifen. *Chem. Res. Toxicol.,* 12, 646-653.
103. Shibutani, S., Ravindernath, A., Suzuki, N., Terashima, I., Sugarman, S.M., Grollman, A.P. and Pearl, M.L. (2000) Identification of tamoxifen-DNA adducts in the endometrium of women treated with tamoxifen. *Carcinogenesis,* 21, 1461-1467.
104. Hemminki, K., Widlak, P. and Hou, S.-M. (1995) DNA adducts caused by tamoxifen and toremifene in human microsomal system and lymphocytes *in vitro*. *Carcinogenesis,* 16, 1661-1664.
105. Pace, P., Jarman, M., Phillips, D.H., Hewer, A., Bliss, J.M. and Coombes, R.C. (1997) Idoxifene is equipotent to tamoxifen in inhibiting mammary carcinogenesis but forms lower levels of hepatic DNA adducts. *Br. J. Cancer,* 76, 700-704.
106. Osborne, M.R., Hewer, A., Davis, W., Strain, A.J., Keogh, A., Hardcastle, I.R. and Phillips, D.H. (1999) Idoxifene derivatives are less reactive to DNA than tamoxifen derivatives, both chemically and in human and rat liver cells. *Carcinogenesis,* 20, 293-297.
107. Kuramochi, H. (1996) Conformational studies and electronic structures of tamoxifen and toremifene and their allylic carbocations proposed as reactive intermediates leading to DNA adduct formation. *J. Med. Chem.,* 39, 2877-2886.
108. Comoglio, A., Gibbs, A.H., White, I.N.H., Gant, T., Martin, E.A., Smith, L.L., Gamalero, S.R. and DeMatteis, F. (1996) Effect of tamoxifen feeding on metabolic activation of tamoxifen by the liver of the rhesus monkey: does liver accumulation of inhibitory metabolites protect from tamoxifen-dependent genotoxicity and cancer? *Carcinogenesis,* 17, 1687-1693.