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On the safety studies of the antiestrogens toremifene and tamoxifen

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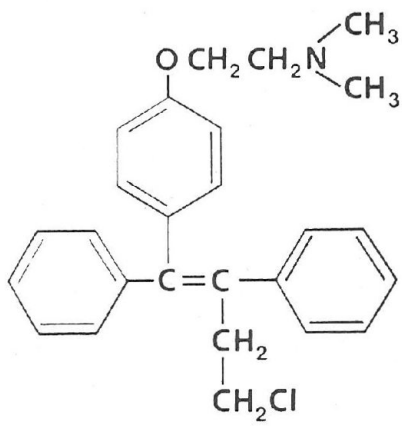
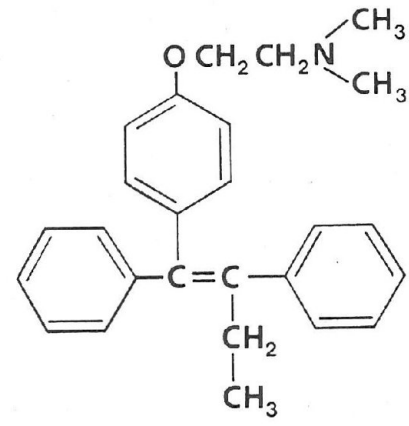
Introduction

Antiestrogens inhibit the binding of estrogens to the estrogen receptor (*Furr & Jordan* 1984, *Kallio et al.* 1986). Tamoxifen and toremifene are non-steroidal triphenylethylene derivatives which have both antiestrogenic and estrogenic properties (*Kangas* 1990, *Kendall & Rose* 1992) depending on species and tissue type studied. The new antiestrogen toremifene differs from tamoxifen only by the substitution of chlorine atom for a hydrogen atom in an ethyl group (Figure 1). Tamoxifen has been used in the therapy of advanced breast cancer for approximately 20 years. It has been considered as a safe drug and even preventive trials have been initiated in which tamoxifen is given to healthy women at an increased risk of developing breast cancer (*Fugh-Berman & Epstein* 1992). However, during recent years stong evidence has been accumulated that tamoxifen is a genotoxic hepatocarcinogen in the rat. Hepatocellular carcinomas have been reported both in female and male rats receiving tamoxifen (*Hirsimäki et al.* 1993, Greaves et al. 1993, *Williams et al.* 1993, *Carthew et al.* 1995a) but not with toremifene (*Hard et al.* 1993, *Hirsimäki et al.* 1993). In humans long-term medication with tamoxifen increases the risk of endometrial carcinoma of the uterus (*Fornander et al.* 1989, *Friedl & Jordan* 1994).

In the present review we will deal with the safety studies of the both antiestrogens with rodents, especially in rats. The risk to humans is also discussed.



TOREMIFENE



TAMOXIFEN

Figure 1. The formulas of tamoxifen and toremifene.

Effects on the liver

Hepatocarcinogenesis:

Tamoxifen has shown to be a strong hepatocarcinogen in rats in independent toxicity studies (*Hard et al.* 1993, *Hirsimäki et al.* 1993, *Williams et al.* 1993, *Greaves et al.* 1993, *Ahotupa et al.* 1994, *Carthew et al.* 1995a, 1995b). Mice treated with the same dosing regime did not develop abnormal liver pathology (*Tucker et al.* 1984).

We did two separate comparative studies with female Sprague-Dawley rats in which tamoxifen induced hyperplastic nodules and hepatocellular carcinomas at the dose level of 45 mg/kg after one years daily dosing by oral gavage. After a three month recovery period almost all normal liver was replaced with tumor masses. The equimolar dose level of toremifene (48 mg/kg) was not hepatoproliferative (*Hirsimäki et al.* 1993, *Ahotupa et al.* 1994).

Williams et al. (1993) exposed female Sprague-Dawley rats to tamoxifen for one year. Tamoxifen was given daily by gavage at the doses of 2.8, 11.3 or 42.5 mg/kg. Tamoxifen induced hepatocellular carcinomas between 3 and 6 months of administration. In the high dose group, hepatocellular adenomas and carcinomas were observed in 71 and 29 % of rats respectively. At 12 months the adenomas were observed in 50 % and carcinomas in 75 % of high dose rats. At the mid dose adenomas were observed in 50 % and carcinomas in 10 % of rats at 12 months. After a 3 month recovery period at this dose level, 45 % of rats exhibited adenomas and 45 % carcinomas.

In another comparative study tamoxifen produced 100 % incidence of hepatocellular carcinoma at the dose level of 22.6 mg/kg at the 12 and 15 month (12 month dosing + 3 month recovery) sacrifice intervals and 67 % and 71 % incidence at the 11.3 mg/kg dose. Toremifene did not produce any hepatoproliferative effects at 12 or 24 mg/kg dose levels, nor in a pilot study at 48 mg/kg (*Hard et al.* 1993).

In a conventional 2-year carcinogenicity bioassay in rats of Zeneca Pharmaceuticals tamoxifen was given to female and male rats by gastric intubation 5, 20 and 35 mg/kg/day. The major finding was a dose-related increase in the incidence of hepatocellular tumors which were first observed after 31 weeks of treatment at the high dose. The majority of these tumors were hepatocellular carcinomas with a well differentiated trabecular pattern. Hepatocellular carcinomas were observed at all dose levels (*Greaves et al.* 1993).

Recently it was reported that tamoxifen (420 ppm in the diet) induced hepatocellular carcinomas in all Wistar and Lewis rats in one year, while in Fischer rats the carcinomas developed later, at 20 months (*Carthew et al.* 1995b). In Wistar rats even 3 months exposure to tamoxifen is sufficient to induce liver carcinomas in high frequency (*Carthew et al.* 1995a).

Mechanism of tamoxifen-induced hepatocarcinogenesis

Promotion, initiation or both:

Early arguments proposed that the hepatocarcinogenic action of tamoxifen would be a result of its estrogen agonist action, i.e. tamoxifen would act as a tumor promoter (*Fentiman & Powles* 1987). This was supported by the observation that tamoxifen was able to act as a tumor promoter in initiation-promotion studies where diethylnitrosamine was used as an initiator (*Yager et al.* 1986, *Dragan et al.* 1991, 1994). It was suggested that tamoxifen lacks of initiating activity when given as a single p.o. dose of 40 mg/kg and that the hepatocarcinogenic effect is due to the promotion of spontaneously initiated hepatocytes (*Dragan et al.* 1991). In the work of *Ghia and Mereto* (1989) tamoxifen (400 ppm in the diet for 6 weeks) was suggested to act as a complete liver carcinogen (induction of foci). In a later study tamoxifen was given as an initiator for a longer period (3 months) before promotion with phenobarbital. The results suggested that tamoxifen can act as an initiator of liver cancer (*Carthew et al.* 1995a). The initiating activity was associated with high-level accumulation of DNA-adducts with very slow adduct elimination rate (*Carthew et al.* 1995a, 1995b). Further, the high incidence of tamoxifen induced cancers, up to 100% at one year, argues against the promotion hypothesis (*Williams* 1995), as does also the fact that toremifene and tamoxifen have equal estrogen agonism in the liver (*Kendall & Rose* 1992), but toremifene is non-carcinogen. However, promotion is also critical for cancer induction: in three rat strains (Fischer, Wistar and Lewis) the level of tamoxifen-induced adducts

was equal at the 6 months time point, however the proliferation index in Fischer rats was depressed when compared to other two strains. This resulted to early carcinomas in Wistar and Lewis rats (6-11 months) and late ones (20 months) in Fischer rats (*Carthew et al.* 1995b). To conclude: tamoxifen is likely to be a complete liver carcinogen.

A genotoxic mechanism seems likely:
During recent few years a vast amount of evidence has been cumulated from several independent laboratories to suggest that tamoxifen exposure of the animals leads to marked genotoxicity. DNA adducts are formed *in vivo* and *in vitro*, also human microsomes are active (see genotoxicity chapter). The high level DNA accumulation of adducts formed, their slow disappearence and correlation with the adduct level and tumor induction rate suggest that the DNA adducts are involved in the cancer initiation process (*White et al.* 1992, *Montadon et al.* 1994, *Carthew et al.* 1995a). The noncarcinogenic toremifene produces no significant amounts of adducts (*White et al.* 1992, *Hard et al.* 1993, *Montadon et al.* 1994). *Sargent et al.* (1994) showed that a single *in vivo* injection of tamoxifen even at the very low dose level (0.3 mg/kg) can produce a variety of clastogenic effects. High frequency of p53 mutations have been found in hepatocarcinomas induced by tamoxifen (*Vancutsem et al.* 1994). These findings support the genotoxic mechanism as the cause of hepatocarcinogenicity of tamoxifen.
Evidence is accumulating that the species responsible for the genotoxic effect are reactive metabolites of tamoxifen, although the ultimate molecule(s) bound to DNA have not yet been verified (*Lim et al.* 1994, *Pathak & Bodell* 1994, *Phillips et al.* 1994, *Randerath et al.* 1994b).

CYP enzyme involvement in tamoxifen activation:
Tamoxifen is activated *in vitro* by rat, mouse and human microsomal cytochrome P450 isoenzymes (CYPs) into reactive metabolites that bind covalently to microsomal protein. This activation is mainly mediated by the CYP3A isoenzyme (*Mani & Kupfer* 1991, *Mani et al.* 1994). A higher CYP3A activity in some human individuals can lead to increased activation (*Mani et al.* 1994). When a panel of 12 human liver microsomal preparations was studied the covalent binding to microsomal protein was mostly correlated with CYP3A4 and CYP2B6 isoenzyme content (*White et al.* 1995). Pretreatment with CYP3A inducers, including tamoxifen, increases the *in vitro* covalent binding to the rat microsomal preparations (*Mani et al.* 1994, *White et al.* 1995). The level of covalent binding into mouse microsomal preparations was markedly higher when compared to human and rat microsomes (*Mani et al.* 1994, *White et al.* 1995). This suggests that the covalent binding to microsomes does not directly predict the DNA-damage level as e.g. higher DNA-adduct levels are achieved in rats than in mice (*White et al.* 1992).
The clastogenicity of tamoxifen correlates with e.g. CYP2E and 3A expression (*White et al.* 1992, Styles et al. 1994). Further, liver microsomes from phenobarbital pretreated rats yield 3-6-fold higher DNA-adduct levels *in vitro* than control microsomes and the DNA-adduct level is decreased by CYP inhibitors (*Pathak & Bodell* 1994). However, in mice pretreatment with phenobarbital or β-naphthoflavone before tamoxifen exposure DNA-adduct formation *in vivo* did not increase (*Randerath et al.* 1994a). On the other hand, there is evidence that phase II metabolic enzymes could be involved in tamoxifen activation as sulphotransferase inhibition with pentachlophenol increases DNA adduct formation in mouse liver (*Randerath et al. 1994a).* Additional experimentation, e.g. *in vitro* works with specific CYP inhibitors, is needed to elucidate the CYP isoenzyme(s) responsible for tamoxifen activation to DNA-binding species.

Extrahepatic tumor induction
Experimental endometrial data:
Endometrial histopathologic data which was collected from several long-term comparative toxicity studies with tamoxifen and toremifene in Sprague-Dawley rats (*Mäntylä et al.* 1996) show that a daily dose of tamoxifen (45 mg/kg p.o.) induced endometrial preneoplastic lesions in 10 % and squamous cell carcinomas in 2 % of the rats. The neoplasias occurred during recovery periods. Equimolar dose levels of toremifene did not produce any such lesions although the hormonal effects of the drugs were equal suggesting

that a nonhormonal mechanism in the tamoxifen-induced endometrial carcinogenesis is possible in the rat. *Greaves et al.* (1993) did not report any endometrial changes in their study. The use of different strain of rats (Wistar derived Alderley Park rats), lower dose levels and omission of recovery groups may explain the discrepancy. Anyhow, further large-scale and mechanistic studies are warranted.

Human endometrial data:
Numerous clinical studies show that long-term tamoxifen therapy increases the incidence of secondary endometrial cancer (*Friedl & Jordan* 1994). In a recent report two Swedish and one Danish trial (5000 patients) were evaluated together showing that tamoxifen-therapy increased the risk for uterine endometrial cancer in average 4-fold (*Fornander et al.* 1989, 1993, *Rutqvist et al.* 1995). This tumorigenicity is generally believed to result from the estrogen agonist action of the drug on the endometrium but a genotoxic component in the mechanism is also possible. Thus far there is no evidence that toremifene would cause endometrial neoplasias after long-term therapy however more follow-up data will be needed.

Gastrointestinal cancers:
The recent clinical cumulative data from three Scandinavian tamoxifen trials show that tamoxifen increases the risk of colorectal and stomach cancers, relative risk 2- and 3-fold, respectively (*Rutqvist et al.* 1995). It is possible that metabolism of tamoxifen in the gut forms some reactive species which might cause local damage. No animal data show any increase in gastrointestinal cancers.

Genotoxicity
Mutagenicity:
Although being a potent hepatocarcinogen tamoxifen has not shown mutagenic activity in the conventional Ames test or in several other *in vitro* tests (*Greaves et al.* 1993). Neither has toremifene not shown any mutagenic activity in bacterial tests.
In formal safety studies tamoxifen has not been shown to induce unscheduled DNA synthesis. However, exposure of rat hepatocytes to tamoxifen *in vitro*, resulted in induction of unscheduled DNA synthesis, when cells from tamoxifen-pretreated rats were used (*White et al.* 1992) but toremifene did not have this effect (*White et al.* 1993).

Clastogenicity:
Toremifene has not shown any clastogenic activity in *in vivo* mouse micronucleus test or *in vitro* in cultured human lymphocytes (Orion-Farmos, unpublished results). Tamoxifen induced a significant increase in micronucleus formation in a dose dependent manner in human lymphoblastoid cell cultures that express several human cytochrome P450 isoenzymes (*White et al.* 1992). In this test system toremifene also gave positive results but was weaker in inducing genotoxicity than tamoxifen (*Styles et al.* 1994). *Sargent et al.* (1994) indicated that a single dose of tamoxifen (0.3-35 mg/kg p.o.) induces aneuploidy, premature condensation, chromosomal breakage and mitotic spindle disruption in female Sprague-Dawley rats.

DNA adduct formation:
Mani & Kupfer (1991) were the first to show that tamoxifen is metabolized *in vitro* to a reactive metabolite which is covalently bound to microsomal protein. *Han & Liehr* (1992) reported that tamoxifen induced DNA-adduct formation *in vivo* in rat and hamster liver and rat kidney. Even a single i.p. dose of 5 mg/kg of tamoxifen citrate was able to induce hepatic adduct formation in hamster liver. As this kind of DNA-reactivity is common to several genotoxic carcinogens (*Williams & Weisburger* 1991), this finding opened a new era in tamoxifen studies the target being human risk assessment: what is the relevance of the DNA-reactivity in human therapy?
In rat liver tamoxifen induced high levels of DNA adducts in a dose-response manner (*White et al.* 1992, *Hard et al.* 1993, *Montadon & Williams* 1994). The adducts are slowly eliminated so that daily dosing of the animals caused adduct accumulation (*White et al.* 1992, *Carthew et al.* 1995a, 1995b). Toremifene did not cause adducts (*Hard et al.* 1993, *Montadon & Williams* 1994). In another study it produced one faint adduct spot and insignificant total amount of adducts (140-fold less adducts than with equimolar

tamoxifen dose) (*White et al.* 1992). Preliminary results suggest that in women on tamoxifen therapy no increase in hepatic adduct level can be observed (*Martin et al.* 1994). However, more studies are needed and also other possible target tissues (especially endometrium and gut) must be studied with respect of adduct accumulation. Interestingly, *in vitro* human liver microsomes are also capable activating tamoxifen to DNA-reactive species (*Pathak & Bodell,* 1994). Studies in mice have indicated that there are at least two different metabolic pathways leading to tamoxifen activation to DNA-reactive species (*Randerath et al.* 1994b).

Conclusions and human risk assessment

Tamoxifen is a potent liver carcinogen in the rat. The evidence suggests that tamoxifen is active in both in initiation and promotion phases of carcinogenesis, i.e. tamoxifen is a complete liver carcinogen. The structurally closely related antiestrogen, toremifene, has not carcinogenic property. The mechanism of cancer initiation seems to be mediated by the DNA-reactivity of the products of tamoxifen metabolism via CYP enzymes. The small difference in the chemical structure makes toremifene safe in this respect. Some evidence suggests that endometrium would be another target tissue of tamoxifen´s carcinogenic action in the rat.

As experimental data suggest tamoxifen to be a genotoxic hepatocarcinogen it is potentially also a human cancer hazard. However, up to now there is no published clinical evidence that tamoxifen would cause liver cancer in man in spite of its global use. Instead, tamoxifen increases the risk for endometrial and possibly also gastrointestinal cancers. More studies in patients on tamoxifen therapy will be needed in order to get an answer to the major questions: Is there an increase in covalent binding or DNA-damage in human liver in certain susceptible individuals (e.g. high hepatic CYP 3A activity)? Is there any DNA damage detectable in the suggested extrahepatic target tissues? If a clear DNA-reactivity is verified in human tissues the risk versus benefit of tamoxifen-therapy should be carefully reevaluated. In preventive trials the use of safer antiestrogens would be justified.

Summary

The safety studies of the two triphenylethylene antiestrogen drugs, tamoxifen and toremifene, are discussed. Tamoxifen has been shown to be a strong hepatocarcinogen in rats in several independent toxicity studies but the new antiestrogen toremifene is not a hepatocarcinogen. A genotoxic mechanism is involved in tamoxifen-induced hepatocarcinogenesis and the species responsible for this genotoxic effect are apparently reactive metabolites of the drug. The activation of tamoxifen to genotoxic metabolite(s) may be mediated by cytochrome P450 isoenzymes (mainly CYP3A). Tamoxifen induces high levels of DNA adducts in experimental animals *in vivo* and *in vitro*. Toremifene produces no significant amount of adducts.

In long-term studies tamoxifen induces also endometrial preneoplastic lesions and some squamous cell carcinomas in the rat. Equimolar dose levels of toremifene do not produce these lesions. In humans tamoxifen increases the risk for endometrial cancers and possibly also the risk for gastrointestinal cancers.

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