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Metabolism of Antiestrogens

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Since the first description of the metabolism of tamoxifen by Fromson and coworkers in 1973 (1,2), there has been an increasing interest in the pharmacology of antiestrogens that has paralleled their expanding clinical applications.

This chapter is a guide to the fundamental advances that have occurred during the past 25 years as a result of increasing knowledge about the metabolism of tamoxifen. Detailed reviews of studies have been published in earlier articles (3–8). Tamoxifen has become the gold standard for the endocrine treatment of all stages of breast cancer (9). The success of tamoxifen, however, has resulted in an intense reexamination of its toxicology because of the proposals to test the drug in the prevention of breast cancer and its current evaluation in clinical trials (10). The finding that tamoxifen produces cancer in the rat liver (11–13), despite there being no evidence of hepatocellular carcinoma after 20 years of clinical use, has caused an urgent reexamination of the metabolism of tamoxifen in animals and humans. Additionally, as a result of the commercial success of tamoxifen, new antiestrogens, based on tamoxifen and its metabolites, are entering clinical trials for a variety of uses from breast cancer treatment to the treatment of osteoporosis (14).

There are, therefore, two major areas of current research interest that are discussed in this review. A close examination of the metabolites of tamoxifen has resulted in (1) the development of new antiestrogens with broad clinical applications and (2) an understanding of rat liver carcinogenesis.

METABOLIC ACTIVATION AND ANTIESTROGEN ACTION

Extensive examination of tamoxifen has identified two principal routes of metabolism: (1) 4-hydroxylation and (2) the progressive degradation of the dimethyaminoethane side chain (Fig. 1).

Tamoxifen is hydroxylated in the 4-position to produce 4-hydroxytamoxifen, a minor metabolite with a high binding affinity for the estrogen receptor (15). The metabolite has been observed as a minor metabolite in rats and humans, but it is a major metabolite in the mouse (16). Metabolic activation seems to be a general principle for most antiestrogens based on triphenylethylene. Antiestrogens that have a methoxy group in an equivalent position, for example, U-23,469 (an analogue of the antiestrogen nafoxidine) (see Chapter 2) (17) or nitromifene (18,19) (Fig. 2), can be demethylated to the hydroxylated metabolite with a high binding affinity for the receptor.

In contrast, the progressive demethylation of the tamoxifen side chain, first to N-desmethyltamoxifen, the principal metabolite in humans (20), and then to didesmethyltamoxifen (21), does not affect the biological actions of the triphenylethylene. However, deamination of didesmethyltamoxifen, first to

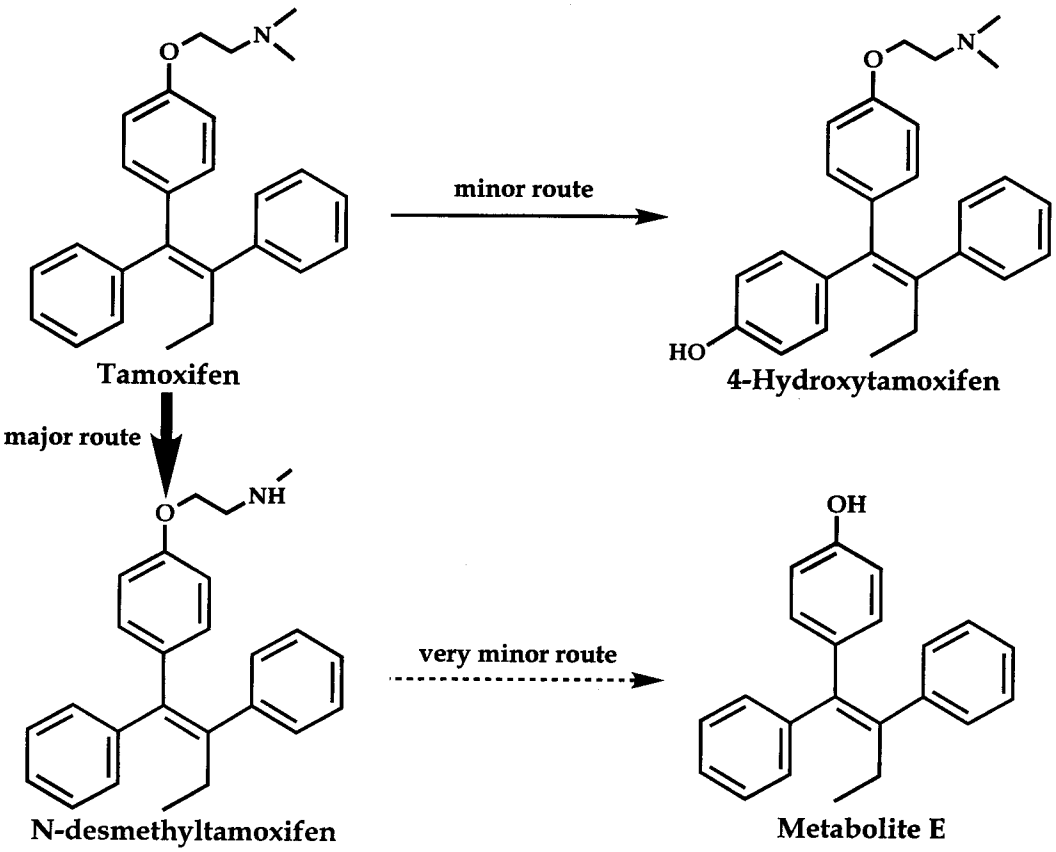


FIG. 1. The principal metabolic routes for tamoxifen in animals and man.

the glycol derivative metabolite Y, and then dealkylation to metabolite E, results in a change in pharmacology from an antiestrogen to an estrogen (22).

ENZYMES INVOLVED IN TAMOXIFEN METABOLISM

There is intense interest in understanding the mechanism of both the metabolic activation of tamoxifen to antitumor agents and the metabolic activation of the drug to a species that will form DNA adducts (see Molecular Mechanism of Carcinogenisis). Numerous research groups (23–30) have identified P_{450}-mediated metabolic routes for tamoxifen in rat and human liver and demonstrated the involvement of flavin-containing mono-oxygenases. It is clear that rat liver enzymes form tamoxifen metabolites at a much higher rate than does human liver enzymes (24,31,32) nevertheless it has been possible to identify the types of P_{450} involved. Tamoxifen N-desmethylation is catalyzed in the rat by CYP1A, CYP2C, and CYP3A enzymes and in the human by CYP3A (23,25). Metabolism to tamoxifen N-oxide, a precursor of N-desmethylation, is mediated by a flavin-containing mono-oxygenase (26), whereas 4-hydroxylation appears to be catalyzed by constitutive P_{450}s. Kupfer's laboratory first identified the covalent binding of tamoxifen to a 52-kilodalton protein (24) and proposed that the CYP3A enzymes activate tamoxifen to the reactive intermediate in rat and human liver microsomes (24).

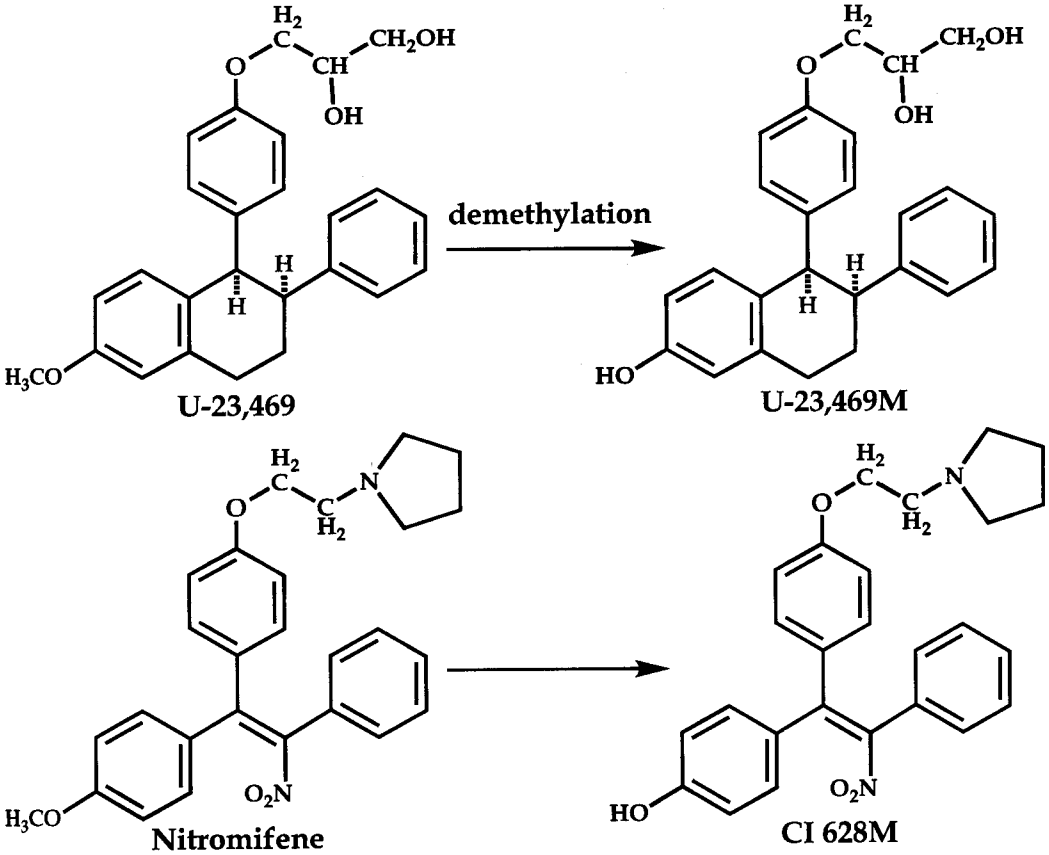


FIG. 2. The demethylation reactions of U-23,469 and nitromifene to form the hydroxylated metabolites with a high binding affinity for the estrogen receptor.

The inducibility of P_{450}s by tamoxifen, toremifene, and droloxifene has been evaluated in the rat and mouse liver. Tamoxifen is a liver carcinogen in the rat but not in the mouse (5), so any differences might help to support a mechanism of carcinogenesis. The relevance of the findings would be confirmed in the rat because toremifene and droloxifene are not potent liver carcinogens (13,33). All the antiestrogens induce CYP2B1 and CYP3A1 in the rat liver, so these may be responsible for the promotion of carcinogenesis rather than initiation. No induction of P_{450}s is observed in mice (27). The studies with tamoxifen in rats have been confirmed (29) and extended with the observation that there is a striking induction of CYP2B2. Additionally, phase II enzyme systems are affected by tamoxifen in the rat liver. Glutathione S-transferase (Ya1 and Ya2) is reduced but other isoforms are unaffected. Tamoxifen also produces a dose-related increase in rat liver UDP-glucuronosyl transferase (34).

PHARMACODYNAMICS

The metabolic activation of tamoxifen is an advantage, but not a requirement, for antiestrogenic activity. Early studies with 4-methyl, chloro, and fluoro tamoxifen demonstrated equivalent antiestrogenic properties in vitro (35) but the compounds were less potent as antiestrogens in vivo (36). The

administration of radiolabeled tamoxifen in immature female rats showed that the 4-hydroxy metabolite is located in the target tissues (37). In general, it is well established that nonsteroidal antiestrogens have a long biological half-life but polar derivatives have a decreased half-life. Nevertheless, studies with radiolabeled 4-hydroxytamoxifen in vivo demonstrate that tissue binding and accumulation is much longer than that of estradiol (38). As discussed in Chapter 2, a number of new antiestrogens are being developed to exploit the knowledge garnered about tamoxifen (14). Droloxifene is a 3-hydroxytamoxifen derivative (39), and TAT-59 is a 4-hydroxytamoxifen derivative that requires dephosphorylation for biological activation (40). In contrast, idoxifene has a 4-iodo group to prevent metabolic activation and a pyrrolidino side chain to prevent degradation (41) (Fig. 3).

One aspect of the pharmacology of tamoxifen is the species differences in estrogenic properties. In short-term tests, tamoxifen can be classified as an estrogen in the mouse, an antiestrogen with partial agonist properties in the rat and human, and a pure antiestrogen in the chick oviduct (4). Although it was natural to suspect that estrogenic properties resulted from differential metabolism to estrogens (i.e., removal of the dimethylaminoethane side chain) in different species, this has not been found to be correct.

A similar line of thinking was proffered to explain the phenomenon of tamoxifen-stimulated breast and endometrial tumor growth as a mechanism of drug resistance. In the laboratory (42,43) and in patients (44,45), tamoxifen will eventually support the growth of estrogen receptor–positive disease. Clones of cells have been selected that perceive tamoxifen as an estrogenic growth stimulus (46).

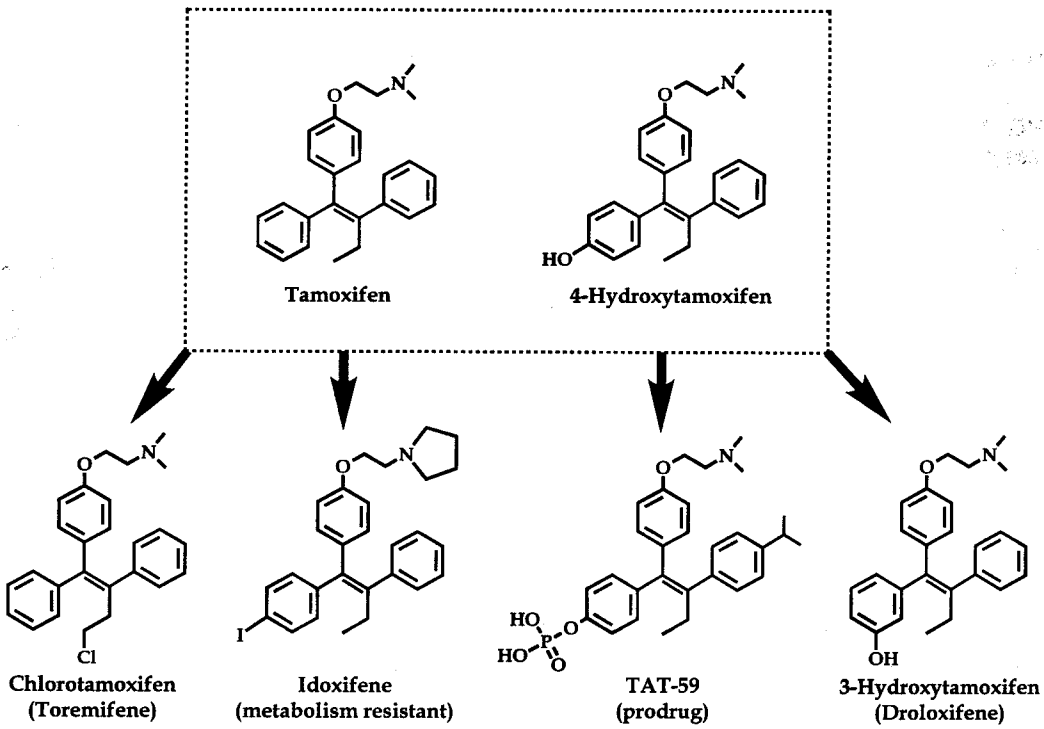


FIG. 3. The new antiestrogens undergoing clinical evaluation based on tamoxifen or its metabolite 4-hydroxytamoxifen.

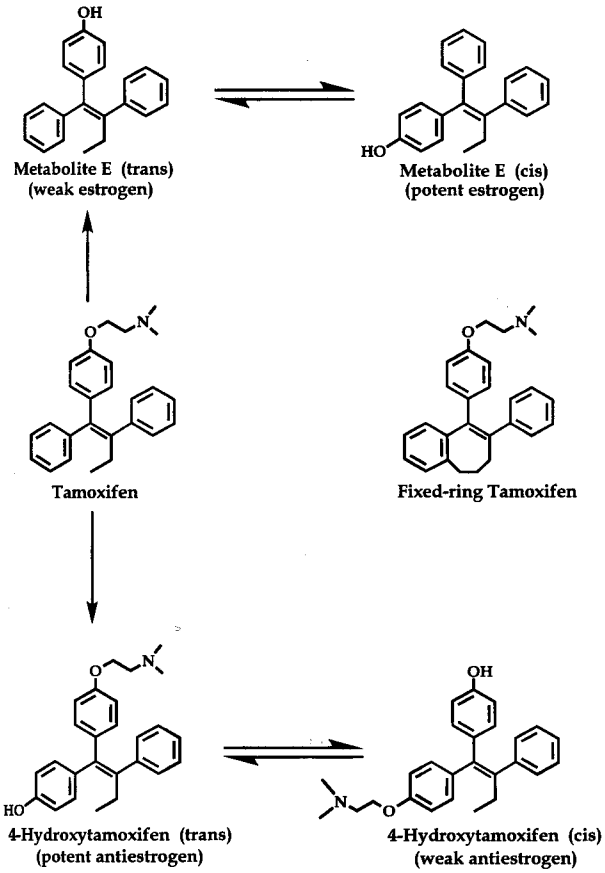


FIG. 4. The proposed local metabolic model to convert tamoxifen from an antiestrogen to an estrogen. (46,47) Tamoxifen is hydroxylated to 4-hydroxytamoxifen, which isomerizes to the weakly antiestrogenic *cis* isomer. Additionally, tamoxifen loses the side chain to form the weak estrogen metabolite E that isomerizes to the potent *cis* isomer. The key is isomerization. However, a derivative, fixed-ring tamoxifen, that cannot isomerize also stimulates breast tumor growth under laboratory conditions. This pharmacologic study makes the proposed hypothesis unlikely (49,50).

One hypothesis (47,48) proposed that tamoxifen was metabolized locally to 4-hydroxytamoxifen, a potent antiestrogen, and metabolite E, a weak estrogen. By a process of isomerization, however, the metabolites would convert to a weak antiestrogen and a potent estrogen (Fig. 4). The key to the metabolic theory of drug resistance was in the isomerization. Without a potent estrogen, the tumor cells could not continue to grow. However, laboratory studies with a tamoxifen analogue that could not isomerize (49) and one that could not be converted to metabolite E (50) demonstrated that tumor growth still occurred in the laboratory model, and other explanations are necessary to describe tamoxifen-stimulated tumor growth.

ANTIESTROGENS AND RAT LIVER HEPATOCARCINOGENESIS

Carcinogenesis is broken down into two phases—initiation and promotion (Fig. 5). Initiation involves DNA damage that cannot be repaired rapidly, and, through a process of promotion, the damaged cells are transformed into a malignancy. Chemicals can be initiators, promoters, or both (complete carcinogens). Tamoxifen has the properties of a complete carcinogen in the rat; however, the role of antiestrogens as promoters has also been pursued to determine risks to humans. Oral contraceptives, which produce a 10-fold increase in the risks of hepatocellular carcinoma in women, are promoters of carcinogenesis in the rat (51).

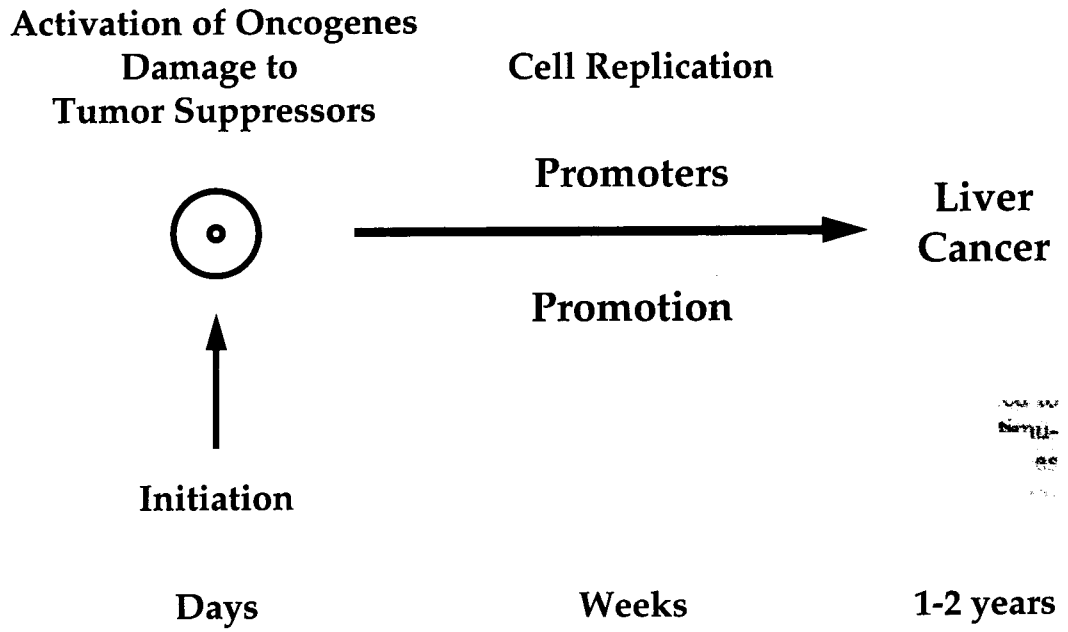


FIG. 5. The proposed pathway for carcinogenesis in the rat liver. An initiating step that damages DNA is followed by promotion to cause liver cancer. Tamoxifen is a complete carcinogen in the rat, whereas toremifene is only a promoter.

The administration of large daily doses of tamoxifen (5–40 mg/kg) in rats from the age of 6 weeks and continuing for the rest of their lives results in hepatocarcinogenesis (52). The process is rat specific and is attenuated by the use of different strains. For example, Fischer 344 rats appear to be more resistant to liver carcinogenesis than Lewis or Wistar rats (53). Tamoxifen produces adducts in rat liver DNA (54), whereas the levels are much lower in the mouse (55), a species that does not form liver tumors in response to tamoxifen administration (5). In contrast, the antiestrogens droloxifene (55) and toremifene (13) do not produce the same high level of DNA adduct as tamoxifen in rat liver and do not appear to be as carcinogenic (33). However, the dose administered is important. For example, toremifene is not as readily absorbed as tamoxifen, and three times the daily dose is required to treat either laboratory (56) or clinical breast cancer (57). Both tamoxifen and toremifene are promoters of rat liver carcinogenesis (58), but three times the dose of toremifene as tamoxifen is required to produce both liver and kidney tumors.

It is known that the *cis* isomer of tamoxifen ICI 47,699 (59) is an estrogen and it was thought possible that isomerization of tamoxifen might be responsible for its ability to promote rat liver carcinogenesis; however, administration of compounds that cannot isomerize still results in tumor promotion (60).

MOLECULAR MECHANISM OF CARCINOGENESIS

During the past 5 years there has been intense interest in discovering the initiating event for tamoxifen-induced rat liver carcinogenesis and determining the relevance for humans. Han and Liehr (54) first noted an accumulation of DNA adducts in the liver of Sprague-Dawley rats on repeated administra-

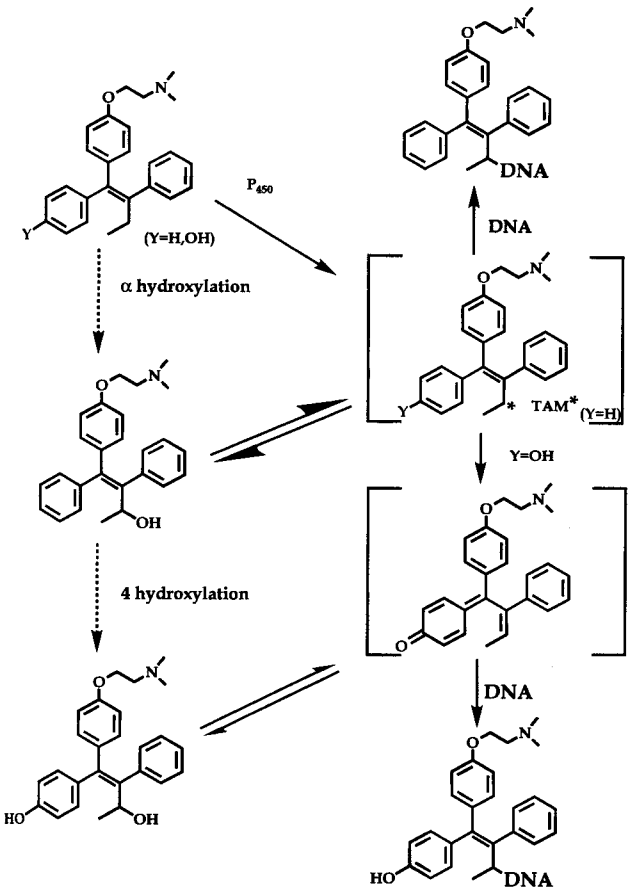


FIG. 6. The proposed intermediates formed from α-hydroxytamoxifen to alkylate DNA (66).

tions of 20 mg/kg (compared with human dose of 0.3 mg/kg). This result has been adequately confirmed by numerous investigators and the focus of investigation has been the identification of the actual DNA adduct. Several candidates have been proposed: an epoxide (31,61,62), 4-hydroxytamoxifen (63,64), metabolite E (65), or α-hydroxytamoxifen (66–68). The main focus has recently been the α-hydroxy metabolite and a potential mechanism has been proposed by Potter and colleagues (66) (Fig. 6). Recently, Osborne and colleagues (69), prepared α-acetoxytamoxifen, which is reacts with DNA to a greater extent (1 in 50 bases) than α-hydroxytamoxifen (1 in 10^5 DNA bases). The products of the reaction were identical to those isolated from DNA of rat hepatocytes or the livers of rats treated with tamoxifen. The adduct of tamoxifen and DNA has been identified at the nucleoside deoxyguanosine in which the α position of tamoxifen is linked covalently to the exocyclic amino of deoxyguanosine (Fig. 7).

RELEVANCE TO HUMAN SYSTEMS

The important observations that have been made provide a framework to study the metabolic activation of tamoxifen in human systems and to identify any DNA adducts in human tissues. The metabolic activation of tamoxifen and its metabolite α-hydroxytamoxifen has been compared using primary cultures of rat, mouse, and human hepatocytes

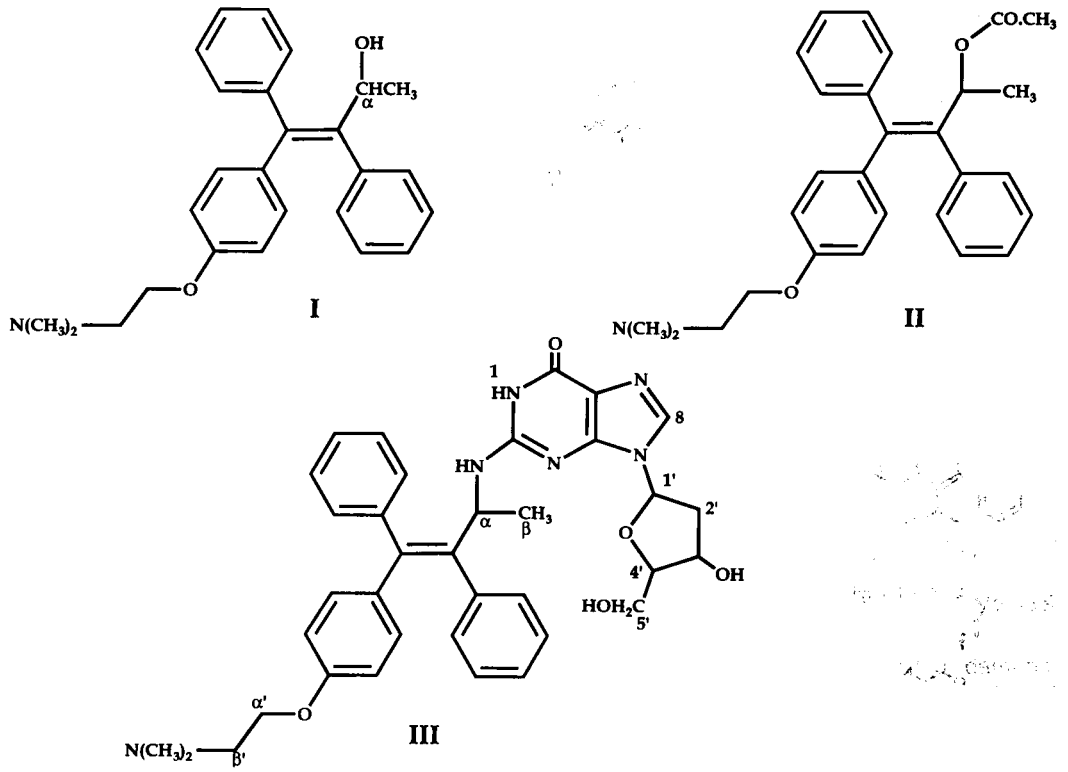


FIG. 7. The formula of α-hydroxytamoxifen (I), α-acetoxytamoxifen (II), and the adduct with deoxyguanosine (III).

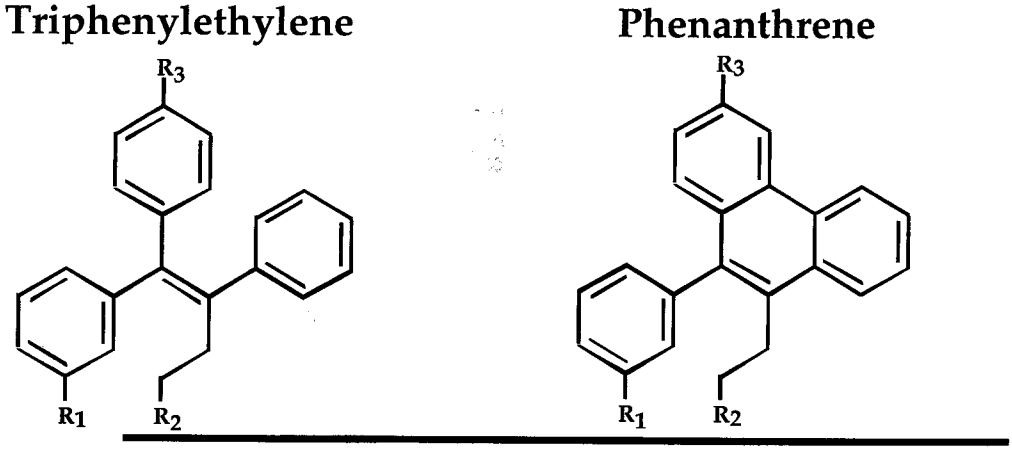
(32). Although DNA adducts are readily identified in rat and mouse hepatocytes (90 and 15 adducts/10^8 nucleotides, respectively), DNA adducts were not detected in tamoxifen-treated human hepatocytes. Additionally, human hepatocytes also appeared to produce 50-fold lower levels of α-hydroxytamoxifen from tamoxifen compared to those produced by rat hepatocytes. Further studies showed that, if cells were treated with α-hydroxytamoxifen, human hepatocytes had 300-fold lower levels of adducts than did rat hepatocytes.

Studies in humans have confirmed that the human is not as susceptible as the rat to DNA adduct formation with tamoxifen. The pattern of DNA adducts found in the rat liver is not found in humans treated with tamoxifen (70), DNA adducts are not found in lymphocytes (71), and there is a lack of genotoxicity of tamoxifen in human endometrium (72). In the latter studies, DNA adducts could be produced in endometrial samples with α-hydroxytamoxifen but not with tamoxifen. The authors proved that tissue was capable of metabolizing tamoxifen to α-hydroxytamoxifen but, apparently, it is incapable of producing adducts. Endometria from patients taking tamoxifen for up to 9 years was analyzed for DNA adducts. No evidence for any DNA adducts induced by tamoxifen was found in any of the patients examined. The authors concluded that the genotoxic events observed with tamoxifen in the rat may not apply to the human endometrium (72). This conclusion supports the previous suggestion that tamoxifen, or indeed any new antiestrogen that has partial agonist actions, will cause the activation and detection of preexisting disease (52).

A COMPARISON OF THE RAT MODEL WITH HUMAN USAGE

Although the value of the rat model to predict the risk of liver carcinogenesis in humans has been described (52,73,74), it is important to summarize the conclusions. In the human, tamoxifen is used at a standard dosage of 20 mg/day (approximately 250 μg/kg for a 70-kg patient) for a duration of at least 5 years (6% of total lifespan). Most patients are in their postmenopausal years. In contrast, rat liver carcinogenesis is started using 6-week-old female rats, with treatment between 5 and 40 mg/kg daily being continued for the rest of the animal's life (2 years). Liver tumors occur during the second year of life and are correlated with DNA adduct formation at the high doses given. Although it is unwise to say there is a threshold dose, there is a report that demonstrates animals to be tumor free using a 3 mg/kg daily dose (11).

It is clear from the aforementioned comparison that rats are receiving an excessive dose and that arguments stating that blood levels in the rat and human are equivalent therefore the rat model is appropriate are misleading (13). It is obvious from laboratory studies that tamoxifen is metabolized rapidly in the rat, which therefore must be given excessively to achieve high blood level (16). Because the therapeutic dosages for breast and mammary cancer are both approximately 0.25 mg/kg daily, the administration of 5



	R_1	R_2	R_3
Tamoxifen	H	H	$OCH_2CH_2N(CH_3)_2$
Toremifene	H	Cl	$OCH_2CH_2N(CH_3)_2$
Droloxifene	OH	H	$OCH_2CH_2N(CH_3)_2$
Idoxifene	I	H	$OCH_2CH_2N(CH_2CH_2)_2$

FIG. 8. The conversion reaction of a triphenylethylene to a fluorescent phenanthrene. Each of the new antiestrogens uses the same methods originally described for tamoxifen (77).

mg/kg daily in rats from age 6 weeks until tumors appear in the second year of life is equivalent to a 14-year-old girl taking 40 tablets (20 times the daily dose) until the age of 40 years. Considering the low levels of α-hydroxylation in the human hepatocyte and the low sensitivity of human cells to DNA adduct formation, it is reasonable to conclude that the therapeutic index for the carcinogenic potential of tamoxifen is hundreds of times safer for the human than the rat.

DETECTION OF TAMOXIFEN AND ITS ANALOGUES

Although there has been a general interest in the metabolism of tamoxifen and metabolites for 2 decades (3,8), the use of tamoxifen in clinical trials as a preventive agent has spurred close scrutiny of phase I and phase II metabolites in breast cancer patients (75,76). The number of metabolites identified in serum is impressive, and these have been compared with metabolites obtained from human HEP G2 cells, human liver homogenates, and urine (76). Serum samples contained N-didesmethyltamoxifen (metabolite I), α-hydroxytamoxifen (metabolite II), 4-hydroxytamoxifen (metabolite III), N-desmethyltamoxifen (metabolite IV), tamoxifen-N-oxide (metabolite V), α-hydroxy-N-desmethyltamoxifen (metabolite VI), and 4-hydroxy-N-desmethyltamoxifen (metabolite VII). Metabolites I through V were noted in human liver homogenates and metabolites II through V in human HEP G2 cells (76). The glucuronides of 4-hydroxytamoxifen, dihydroxytamoxifen, and a monohydroxylated N-desmethyltamoxifen are observed in urine (75). Only very low levels (75 pg/mL) of α-hydroxytamoxifen were observed in serum (76).

The development of routine methods to determine tamoxifen have previously been described (6,8). The routine analysis of tamoxifen and its analogues use automated high-performance liquid chromatography with post-column ultraviolet (UV) activation and fluorescence detection. Tamoxifen assays were first developed using this technology (77), but similar methods are now available for toremifene (78,79) droloxifene (80), and idoxifene (81). The key to detection sensitivity is the conversion of triphenylethylenes to fluorescent phenanthrenes with UV light, which increases detection 1000-fold (Fig. 8).

Serum levels of tamoxifen range between 50 and 250 ng/mL but most studies have a mean serum level of approximately 150 ng/mL (82). The principal metabolite of tamoxifen, N-desmethyltamoxifen is usually observed at 50% to 100% higher levels than tamoxifen. The 4-hydroxylated metabolite 4-hydroxytamoxifen is generally less than 10 ng/mL in serum at steady-state. Tamoxifen accumulates and steady-state levels are generally achieved in patients within 1 month. The half-life of tamoxifen is 7 days and of N-desmethyltamoxifen is 14 days. As a result, approximately 6 weeks is required to clear the drug after treatment is stopped. In contrast, droloxifene, a 3-hydroxytamoxifen, does not accumulate, and conjugated droloxifene metabolites are detected in serum (80,83).

CONCLUSION

The metabolism of tamoxifen in animals and humans has paradoxically provided a remarkable insight into the process of carcinogenesis and antitumor mechanisms. This knowledge is being used to monitor and devise new and novel antiestrogens to treat different diseases of the menopause. Although data are not available on the pure antiestrogen ICI 182,780 and the selective estrogen receptor modulator raloxifene, there is every indication that they are potent antitumor agents but without the complications of complete carcinogenesis in the rat liver.

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