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Report

Intrinsic reactivity of tamoxifen and toremifene metabolites with DNA

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Summary

The antiestrogen tamoxifen is known to cause liver cancer in rats. This may be due to the formation of abundant DNA adducts in rat liver. A likely precursor to some of the tamoxifen adducts in rats is α-hydroxytamoxifen. It is not clear whether the rat data are relevant to human exposure. In the present study, we show that one of the major metabolites in humans reacts with double-stranded DNA *in vitro* in the absence of any metabolizing enzymes or activating chemicals. At least two distinct adduct spots resulting from 4-hydroxy-N-desmethyltamoxifen (metabolite Bx) were detected by ^{32}P postlabeling and thin layer chromatography. The adduct level increases dramatically when metabolite Bx is irradiated with UV light to fuse into a phenanthrene ring system. 4-hydroxy-N-desmethyltoremifene, which differs from Bx by a single chlorine atom,forms fewer DNA adducts without irradiation but similar amounts after irradiation. These results suggest that the chlorine atom may interfere with drug-DNA interactions which facilitate adduct formation.

Introduction

Tamoxifen (TAM) is a triphenylethylene widely used in the treatment of breast cancer. Its use as a chemopreventive agent is somewhat controversial mainly because it is well documented that TAM causes liver cancer in rats. This is presumably due to its ability to form DNA adducts in rat liver after being activated by metabolic enzymes. The genotoxic effects of tamoxifen have been extensively reviewed [1]. Recently, it has been shown that TAM increases the mutation rate in rats [2]. It is not clear to which extent the rat studies apply to humans, because in patients it has been difficult to link occasional incidences of liver cancer to TAM treatment. Instead, an increase in endometrial cancer has been documented and TAM resistant or stimulated breast tumors have been observed in patients and in model systems. Also, intact human hepatocytes, unlike rat hepatocytes, do not form DNA adducts when treated with TAM in culture [3]. Determining the relevance of the rat studies has been difficult because little is known about the pathways leading to TAM adducts in rats. It has been hypothesized that a major mechanism for adduct formation in rats involves α-hydroxylation [4], but, in contrast to rats, α-hydroxytamoxifen levels are often undetectable in human samples. Cultured human hepatocytes synthesize about 50-times less α-hydroxytamoxifen than rat hepatocytes [3]. In mice, two distinct groups of adducts have been identified; one group appears to be derived from α-hydroxytamoxifen or a related metabolite, whereas the other group has been linked to 4-hydroxytamoxifen [5–6].

TAM undergoes liver metabolism to N-desmethyltamoxifen and 4-hydroxytamoxifen. The former

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is the major metabolite in human serum and it is further converted into metabolites Z (didesmethyltamoxifen) and Y (the side chain alcohol). Both N-desmethyltamoxifen and 4-hydroxytamoxifen are precursors to metabolite Bx (4-hydroxy-N-desmethyltamoxifen). Other metabolites identified in humans are metabolite E (without the dimethylaminomethane side chain) and bisphenol. Toremifene (TOR), an alternative antiestrogen which differs from TAM by a single chlorine atom, undergoes a similar metabolic pathway, but additional metabolites have been identified. In contrast to TAM, TOR has not been linked to liver cancer in rats or endometrial cancer in humans. Comparing the reactivity of TAM and TOR metabolites may therefore provide clues regarding the cause for the observed genotoxicity of TAM.

Materials and methods

DNA modification, postlabeling, and thin layer chromatography (TLC)

Salmon testis DNA and human placenta DNA were purchased from Sigma (St. Louis, MO). Spleen DNA from rhesus macaque was purified by standard methods [7]. Different DNA sources were used to rule out the possibility that contaminants oxidize or otherwise activate the drug metabolites. Tamoxifen and toremifene metabolites were obtained from Orion Corp., Finland. 250 or 500 µg DNA were reacted with 100 µg drug in 0.5 ml 20 mM Tris, pH 7.9, 30% ethanol in a rotating incubator at 37 °C. No difference was observed between 4 to 64 hour incubations. After extraction with ether and ethanol precipitation, the DNA pellet was redissolved in water and digested with micrococcal nuclease and spleen phosphodiesterase overnight essentially as described [2]. An aliquot (approximately 10–15 µg DNA) was treated with nuclease P1 and labelled with 95 µCi ^{32}P ATP (Amersham Life Science Inc., Arlington Heights, IL) in a final volume of 20 µl. 2–10 µl were applied to a PEI cellulose sheet (Macherey-Nagel, purchased from Bodman, Aston, PA) and multi-dimensional TLC was performed essentially as described [3]. Adduct spots were detected by exposure of Kodak Biomax film with intensifying screen for 20 hours at – 70 °C. Autoradiographs were superimposed onto the TLC plates to mark the position of a red marker dye from the isotope solution relative to the adduct spots.

UV activation

Photoactivation of all compounds to their phenanthrene derivatives was accomplished by transferring them to an Infrasil quartz cuvette and exposing them to high-intensity ultraviolet light for one minute at a wavelength of 254 nm, as previously reported [8].

Adduct formation in the presence of ethidium bromide

Ethidium bromide (50 µg/ml) was added to the solution containing the DNA and the drug in 30% ethanol. After the incubation, excess ethidium bromide was extracted with butanol.

Results

TAM metabolite Bx reacts with DNA in vitro

We tested the N-desmethyl, the 4-hydroxy, and the 4-hydroxy-N-desmethyl (metabolite Bx) derivatives of TAM and TOR, as well as TAM metabolite Y and the corresponding TOR III. In addition, we examined metabolite E and bisphenol of TAM. The drugs were incubated with salmon testis DNA in Tris buffer without any activating enzymes or chemicals. Only the TAM metabolite Bx consistently gave rise to at least two adduct spots (Figure 1E) which were not seen in the control DNA (Figure 1A). In contrast to endogenous adducts, the spots produced by TAM Bx treatment migrated close to the red marker dye. The appearance of multiple spots suggests multiple reaction products possibly with different bases. Similar results were obtained with human placenta DNA and with spleen DNA

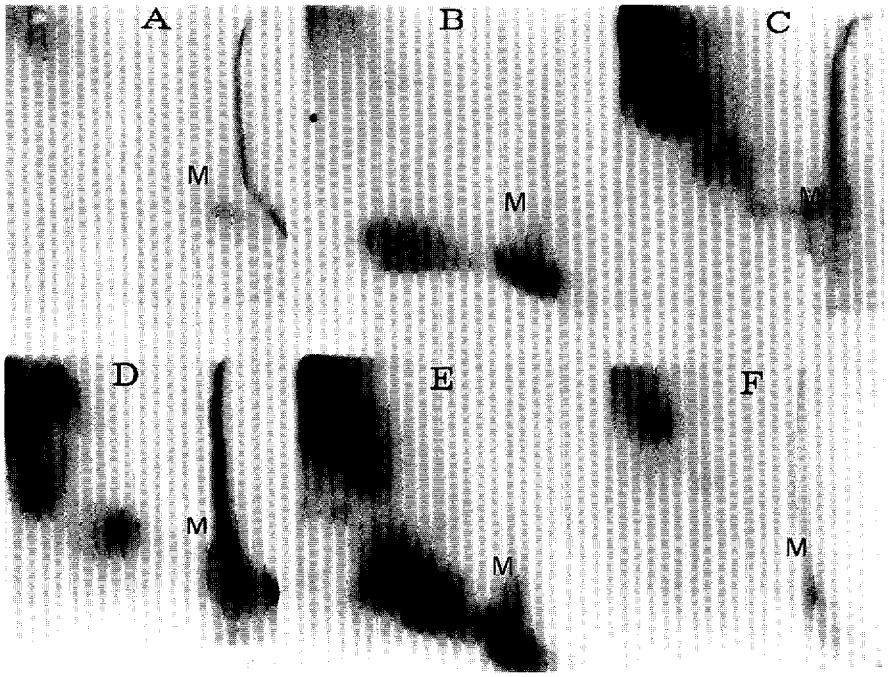


Figure 1. Figure 1 shows chromatograms of postlabeled DNA treated with different tamoxifen metabolites: (A) untreated DNA, (B) 4-hydroxy-tamoxifen, (C) N-desmethyl-tamoxifen, (D) bisphenol, (E) 4-hydroxy-N-desmethyl-tamoxifen (metabolite Bx), (F) metabolite Y. (M) indicates the position of the red dye used as a marker. The origin is in the upper left corner of each chromatogram.

from rhesus macaque. The corresponding TOR metabolite also produced DNA adducts, although the latter were not seen as consistently as the TAM Bx adducts, indicating that TOR Bx (TOR IV) may have a lower intrinsic reactivity than TAM Bx. Occasionally, at least one faint adduct spot was seen with 4-hydroxytamoxifen (Figure 1C). Adducts were not seen with metabolites containing only the N-desmethyl group (Figure 1C), or any other group found downstream in the metabolic pathway. Furthermore, incubation of the metabolite Bx (100 ng) with ^{32}P and polynucleotide kinase did not result in any radioactive spots near the marker (data not shown) ruling out artefacts due to incomplete removal of unreacted drug. The utilization of the red dye from the isotope solution as a marker is a novel feature of the experimental protocol and permits the comparison of spots from different chromatograms.

Adduct levels for the 4-hydroxy-N-desmethyl metabolites of TAM and TOR increase after UV irradiation and in the presence of ethidium

In order to investigate which parts of the molecule might be involved in the reaction with DNA, we irradiated TAM Bx and TOR IV with UV light which causes two of the phenyl groups to fuse into a phenanthrene ring. This reaction has been used for the improved detection of TAM and TOR [8]. The fluorescent TAM derivatives have been shown to possess biological properties similar to the parent drug [9]. The phenanthrene 4-hydroxy-N-desmethyl compounds were reacted with DNA *in vitro* as described above and were found to produce much higher adduct levels than the original metabolites (Figure 2B and 2E). The UV irradiated TOR IV was as reactive as the photoactivated TAM Bx, even though TOR IV adducts had been difficult to detect before photoactivation. In contrast, the parent drugs showed no adduct spots even after they were irradiated (data not shown).

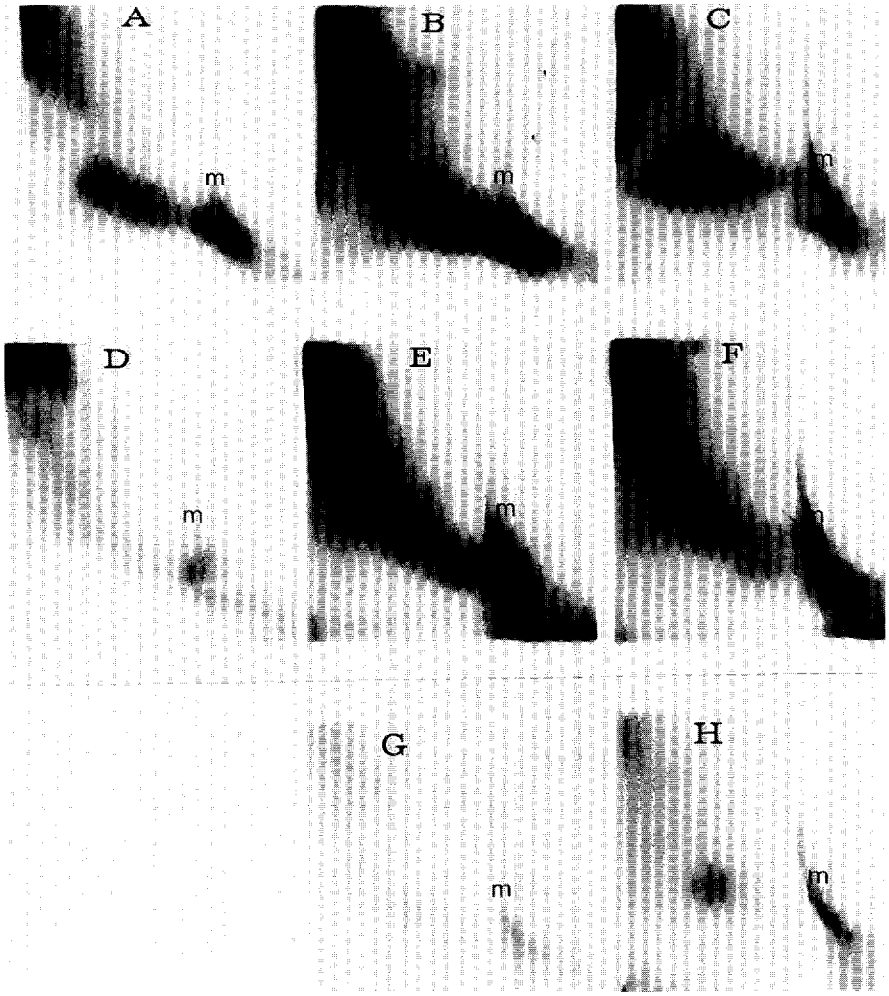
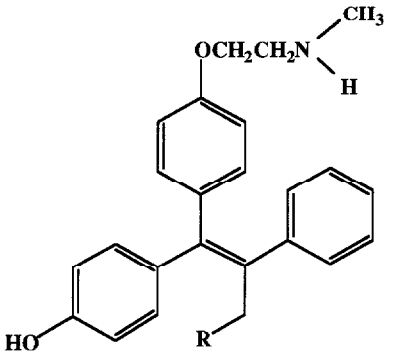


Figure 2. Figure 2 shows chromatograms of tamoxifen metabolite Bx (A–C) and TOR IV (D–F) reacted with DNA (A, D), reacted with DNA after UV irradiation (B, E), and reacted with DNA in the presence of ethidium bromide (C, F). The controls are untreated DNA (G) and DNA treated with ethidium bromide alone (H). The position of the marker dye is marked with (m).

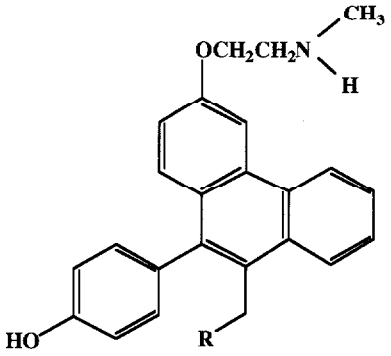
Since the structure of the phenanthrene ring system resulting from irradiation of TAM and TOR metabolites shows some similarities to the intercalator ethidium (Figure 3), we hypothesized that intercalation may play a role in the interaction of TAM Bx and TOR IV with DNA. Intercalation of one drug molecule could alter the structure of DNA to provide a better fit for a second molecule or it could facilitate covalent binding of the same molecule. To distinguish between these possibilities, we reacted TAM Bx and TOR IV in the presence of ethidium bromide and found enhanced adduct formation (Figure 2C and 2E).

Metabolite Bx is present in rodents and humans

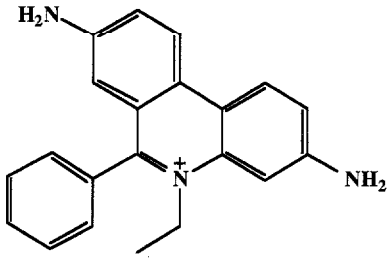
TAM metabolite Bx has been detected in human plasma and is a major metabolite in bile and urine [10]. It is also present in mice [11 and data not shown]. When analyzing previously collected HPLC data of rats treated with TAM or TOR, both TAM Bx and TOR IV were found. These metabolite profiles resulted from long term treatment as reported in [12]; interestingly, the same study analyzed rat liver DNA after short term (7 day) treatment and DNA adducts were detected in TAM treated animals. Metabolite Bx may be responsible for some of these adducts. Other studies confirmed



Metabolite Bx



UV Activated Metabolite Bx



Ethidium

Figure 3. The 4-hydroxy-N-desmethyl metabolite of tamoxifen (R = CH_3) and TOR (R = CH_2Cl), one of the proposed structures of the same metabolite after UV irradiation and, for comparison, the drug ethidium.

that TOR IV is found in humans (data not shown) and rats [13].

Discussion

Our data demonstrate that a human TAM metabolite has carcinogenic potential. 4-hydroxy-N-desmethyltamoxifen (metabolite Bx) reacts with DNA *in vitro* in the absence of any other activating factors. However, adduct levels are fairly low (on the order of 0.5 adducts/10^4 normal nucleotides). During long term treatment with TAM, even relatively rare DNA modifications could accumulate to levels which may affect genome stability. The UV activation data confirm that the 4-hydroxy-N-desmethyl derivative of TOR (TOR IV) potentially forms DNA adducts although the adduct levels are near the detection limit without UV irradiation. The only other TAM metabolite which has been shown to form DNA adducts in normal human cells with a balanced composition of metabolizing and detoxifying enzymes is α-hydroxytamoxifen added in high concentrations [3]. Whether the Bx concentration *in vivo*, and in particular in the nucleus, is sufficient for adduct formation, remains to be shown.

The intrinsic reactivity of TAM metabolite Bx may result from the presence of two potentially reactive groups in appropriate positions to interact with native double stranded DNA. The reaction of Bx with DNA may be catalyzed by structural features of double-stranded DNA which have been implicated in the formation of other adducts [14, 15]. In our model, the lower reactivity of the corresponding TOR metabolite (TOR IV) could be explained by steric or electronic effects of the chlorine atom. Further support for physical interactions between the drug and the DNA preceding and facilitating the formation of a covalent bond comes from the observation that adduct formation is increased by UV irradiation. The resulting phenanthrene ring resembles the structure of ethidium (Figure 3) and may intercalate in a similar fashion, which could alter the rate of reaction of either the intercalated or other nearby drug molecules. Interestingly, ethidium does not seem to compete for the same binding site, because more adducts are obtained in its presence. A similar result with a methylating agent led the investigators to the speculation that the minor groove intercalator ethidium may enhance binding to the major groove of DNA [16].

Since metabolite Bx is a downstream product of 4-hydroxytamoxifen, our data seem consistent with the findings reported by Randerath et al. [5] that 4-hydroxytamoxifen causes group I DNA adducts

in mice. Group I adducts differ in many ways from group II adducts, the predominant adduct species in rat. Interestingly, group I adducts were the main products formed after oral administration of TAM [5]. Both, TAM and 4-hydroxytamoxifen, readily undergo N-demethylation which leads to Bx formation from the latter. In addition, microsomes from treated animals which produced lower levels of group II adducts but normal levels of group I adducts have been shown to efficiently N-demethylate TAM [6], leading the authors to the speculation that 4-hydroxytamoxifen may be resistant to detoxification by N-demethylation. Our data offer an alternative explanation that the ultimate DNA damaging agent is not 4-hydroxytamoxifen but metabolite Bx. The 4-hydroxytamoxifen pathway which includes Bx may be common to humans, mice, and other species, whereas the α-hydroxytamoxifen pathway may be limited to mice and rats. The fact that Bx is stable and readily detectable makes it a good candidate for binding to DNA even if the intrinsic reactivity is relatively low. It has been postulated that the proximate metabolite is a short lived, highly reactive species. However, we suspect that such a compound is more likely to react with nearby proteins after being generated in the cytoplasm [17, 18] than to travel to and enter the nucleus to form adducts with chromosomal DNA. Nuclear Bx concentration may vary with the activity of the estrogen receptor and other individual factors.

Acknowledgements

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