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Reduction in Tamoxifen-Induced CYP3A2 Expression and DNA Adducts Using Antisense Technology

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Tamoxifen (TAM) is widely used in the treatment and prevention of breast cancer. There is clear evidence that cytochrome P450 (CYP) 3A enzymes play an important role in TAM metabolism, resulting in metabolites that lead to formation of TAM–DNA adducts. We have investigated the effect of CYP3A2 antisense (AVI-4472) exposure on CYP3A2 transcription, enzyme activity, translation, and TAM–DNA adducts, in livers of rats administered TAM (50 mg/kg body weight [bw]/day) for 7 days. The study design included administration of 0, 0.5, 2.5, or 12.5 mg AVI-4472/kg bw/day for 8 days, beginning 1 day before TAM exposure. The specific activity of CYP3A2 was increased after TAM administration, and decreased significantly (~70%) in the presence of 12.5 mg AVI-4472. CYP3A2 protein levels, determined by immunoblot analysis, showed a similar pattern. Hepatic TAM–DNA adduct levels were measurable in all TAM-exposed groups. However, when rats were co-treated with 2.5 and 12.5 mg AVI-4472/kg bw/day, statistically significant (~50%) reductions in TAM–DNA adduct levels (2.0–2.8 adducts/10^8 nucleotides) were observed compared to rats treated with TAM alone (5.1 adducts/10^8 nucleotides). Rat toxicology U34 arrays (Affymetrix) were used to investigate the modulation of gene expression patterns on co-administration of TAM with AVI-4472. Results indicated that several CYP genes were down regulated although no significant induction of *CYP3A2* was observed in the TAM-exposed rats co-treated with AVI-4472. Overall the data suggest the utility of antisense technology in the redirection of TAM metabolism thereby lowering TAM genotoxicity in rat liver. Published 2005 Wiley-Liss, Inc.†

Key words: DNA adducts; cytochrome P450; CYP3A2; antisense; microarray

INTRODUCTION

Tamoxifen (TAM), a nonsteroidal antiestrogen chemotherapeutic agent, is widely used in the treatment of all stages of estrogen-dependent mammary tumors and for chemoprevention in at-risk women [1]. The genotoxicity of this anticancer agent has been a subject of extensive concern due to its association with increased risk of endometrial cancer [2,3]. The use of TAM as a prophylactic chemotherapeutic agent is of high concern since it is administered to healthy premenopausal women. This indicates that development of better and more efficacious therapies requires strategies that render chemotherapeutic agents safer.

TAM is a potent hepatic carcinogen in rats, it acts as a classic genotoxin through metabolism and DNA adduct formation [4–7]. Cytochrome P450 (CYP) phase I drug metabolism enzymes play a key role in TAM metabolism, resulting in several metabolites including α-hydroxy-tamoxifen, 4-hydroxy-tamoxifen and *N*-desmethyl-TAM [8–10]. α-OH-*N*-desmethyl-TAM is sulfonated by hydroxysteroid sulfotransferases [11] to form (*E*)-α-(deoxyguanosin-N^2-yl)-*N* desmethyltamoxifen [12,13]. It has been reported and recently demonstrated that the CYP3A subfamily of enzymes is largely responsible for TAM α-hydroxylation [14–16], establishing its role in the genotoxicity of this drug. The CYP3A isozymes are expressed in liver mostly; however, they

Abbreviations: TAM, tamoxifen (Nolvadex); TAM–DNA, DNA modified with tamoxifen; CYP, cytochrome P450 enzyme; PMO, phosphorodiamidate morpholino oligomer; PBS, phosphate buffered saline; CIA, chemiluminescence immunoassay; dG-TAM, (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen.

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are also present in kidney, small bowel, and skin. Among them, CYP3A2 is the major isoform in rats, while its homolog CYP3A4 is the major isoform in humans. We hypothesized that inhibition of CYP3A concomitantly with TAM administration results in a reduction of TAM–DNA adduct formation. In this study, we have mainly focused on validating the principle of adduct reduction by abrogation of CYP3A2 protein expression using antisense technology. This approach of ''metabolic redirection'' [17] builds on literature reports implicating the metabolic products of the CYP3A subfamily of enzymes in the genotoxicity of TAM.

The utility of antisense phosphorodiamidate morpholino oligomers (PMO) in inhibiting the major CYP3A isoforms in rats and humans has been demonstrated previously [18–20]. PMO represent a significantly modified DNA molecule in which the deoxyribose moieties are replaced by morpholine moieties, and the charged phosphodiester linkages are replaced by anionic phosphorodiamidate linkages [21]. These oligomers possess several highly desirable pharmacological qualities, including high efficacy for target gene inhibition in multiple organs in vivo, extremely low systemic toxicity, no genotoxicity, long half-life, and resistance to metabolism [22,23]. The lack of inter-nucleoside charge allows the PMO to avoid nonspecific effects such as binding to cellular and extracellular proteins. Furthermore, PMO is highly resistant to various nucleases and proteases [24], as well as extremely efficient inhibitors of translation via a non-RNase H [25], sequence-specific steric-blockade process [26]. The safety and high efficacy of antihuman CYP3A4 has been previously demonstrated [19]. Therefore, PMO represent a clinically viable approach for development as adjuvant therapy to be used with TAM.

The current studies were conducted in rats. The rat model was selected because TAM administration in this species has been previously reported to show good correlations between dose-dependent elevations in hepatic TAM–DNA adducts [27–29] and hepatocellular carcinoma incidence [4,30]. Similarly, women treated with TAM have an increased incidence of uterine endometrial tumors [31,32], though evidence that exposure to TAM results in formation of uterine and endometrial TAM–DNA adducts [33,34] is controversial [2]. The structures of TAM–DNA adducts formed in human and nonhuman primate endometrium have been reported to be identical to those formed in rat liver [14,35]. Thus, the examination of this approach in rats may eventually yield information that would be applicable to human use.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (Simonsen, Gilroy, CA) weighing between 240 and 260 g were housed in plastic cages in the Laboratory Animal Resources Facility at Oregon State University (OSU) in Corvallis, OR. Male rats were used in this study as a previous study with an antisense phosphorothioate oligodeoxynucleotide against CYP3A2 showed effective inhibition of CYP3A2 expression [36]. The animals were maintained in a climate-controlled room with 12 h light/dark cycle and allowed access to a commercial rat chow and tap water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Oregon State University.

Oligomer Synthesis

Oligomers were synthesized at AVI BioPharma (Corvallis, OR) as previously described [21]. Purity was greater than 90% full length as determined by reverse phase HPLC and MALDI TOF mass spectroscopy. The antisense PMO targeted to rat CYP3A2 mRNA (Genbank accession number U09742) translation initiation region and was named AVI-4472. It has the following base sequence: 5′-GAGCTGAAAGCAGGTCCATCCC-3′. The base sequence of AVI-4557 is 5′-CTGGGATGAGAGCCATCACT-3′, which is targeted to the translation initiation region of the human CYP3A4 mRNA (Genbank accession number M14096).

Animal Treatments

Rats were treated with various doses of AVI-4472 (0.5, 2.5, and 12.5 mg/kg/day) or AVI-4557 (12.5 mg/kg/day) starting on study day 0. All PMO injections used PBS as vehicle and were administered intraperitoneally. TAM treatment at 50 mg/kg/day was initiated on study day 1. All TAM administrations used Tricaprylin (Sigma, St. Louis, MO) as vehicle and were administered via bolus gavage. The animals were euthanized and their livers harvested on study day 8. This study examined hepatic TAM–DNA adduct formation following co-administration of TAM with AVI-4472 (5′-GAGCTGAAAGCAGGTCCATCCC-3′), an antisense PMO targeted to the translation initiation region of the rat CYP3A2 mRNA. In addition to vehicle-treated groups, the study also utilized co-administration of control PMO AVI-4557 (5′-CTGGGATGAGAGCCATCACT-3′), antisense to human CYP3A4, to eliminate bias.

The study plan is summarized in Table 1. Six groups of three male Sprague Dawley rats each were administered phosphate buffered saline (PBS) alone or increasing doses of the antisense PMO AVI-4472 (0.5, 2.5, or 12.5 mg/kg/day in PBS intraperitoneally). One control group was administered human PMO AVI-4557 (12.5 mg/kg/day in PBS intraperitoneally) at the highest dose. Dosing with the PMO lasted eight days. TAM (50 mg/kg/day gavage) or vehicle (Tricaprylin) co-administrations lasted 7 days, starting 1 day following the initiation of PMO treatments, to allow for CYP3A2 inhibition prior to

Table 1. Study Design and Data Obtained From Rat Liver Tissue

Group (n)	Tamoxifen [50 mg/kg/day]	CYP3A2 antisense AVI-4472 [mg/kg/day]	Control PMO AVI-4557 [mg/kg/day]	CYP3A2 enzyme activity[a]	CYP2B1 enzyme activity[b]	TAM–DNA adduct[c] (log mean ± SE)
1 (3)	–	–	–	82.6 ± 6.0*	181.7 ± 10.2	–
2 (3)	+	–	–	129.5 ± 9.9	188.3 ± 26.8	5.06 (0.70 ± 0.036)
3 (3)	+	0.5	–	95.0 ± 10.0	160.0 ± 7.6	3.42 (0.53 ± 0.023)
4 (3)	+	2.5	–	102.2 ± 7.3	160.7 ± 7.2	2.08** (0.32 ± 0.073)
5 (3)	+	12.5	–	40.5 ± 5.8**	146.6 ± 6.0	2.81* (0.45 ± 0.079)
6 (3)	+	–	12.5	110.4 ± 9.5	165.0 ± 8.8	5.77 (0.76 ± 0.059)

[a]CYP3A2 enzyme activity was measured by BFC hydroxylation and data is in units of nanomoles of HFC/200 μg S9 protein/h. Data is presented as simple mean ± standard error of the mean.
[b]CYP2B1 was measured as a control enzyme pentoxyresorufin O-dealkylation and data is in units of pmol resorufin/2 mg liver S9 protein/15 min. Data is presented as simple mean ± standard error of the mean.
[c]TAM–DNA adducts are per 10^8 nucleotides. Data is presented as back-transformed mean from analysis of the log transformed data (log mean ± standard error from n = 3 rats after adjusting for multiplicative assay effects). 3 to 4 assays were performed for each group.
*Indicates $P < 0.05$.
**Indicates $P < 0.01$ compared to Group 2, Dunnett adjusted in full model.

initiation of TAM exposure. The in-life portion of the study was concluded 1 day following the last PMO/TAM administration.

CYP3A Enzyme Activity Assay

The enzyme activity was assayed according to manufacturer's instructions with minor modifications (Gentest, Woburn, MA). Briefly, 200 μg aliquot of the S9 fraction protein was diluted in 0.5 M potassium phosphate (Sigma) to a volume of 200 μl in a black polystyrene flat-bottom 96-well plate. This was followed by addition of 20 μl of 2.5 mM substrate 7-benzyloxy-4-[trifluoromethyl]-coumarin (BFC) (Gentest) and β-NADPH regenerating reaction mixture. The plates were incubated for 60 min at 37°C. The reaction was terminated by addition of 50 μl of stop solution (80% acetonitrile, 20% tris buffer pH 7.4). Determination of the fluorescent product, 7-hydroxy-4-[trifluoromethyl]-coumarin (HFC) was performed at the excitation wavelength of 410 nm and emission wavelength of 530 nm. All readings were compared to a standard curve prepared from 5.0 μM stock solution of HFC. The limit of detection (LOD) for this assay is 15 nanomoles of HFC/200 μg S9 protein/h.

CYP2B1 Assay

This assay was performed as per previously published protocol [3] by measuring O-dealkylation of pentoxyresorufin to resorufin. The data is presented as pmol resorufin/2 mg S9 protein/15 min. The LOD for this assay is 35 pmol resorufin/2mg S9 protein/15 min.

Immunoblot Analysis

Levels of CYP3A2, CYP2B1, and β-actin proteins were determined by western immunoblots in liver S-9 fractions. Eighty μg total protein was separated on a 12% sodium dodecylsulfate/acrylamide gel and immunoblotted according to standard techniques (*see user guide P15372H at www.millipore.com*). Polyclonal primary antibodies for CYP3A2 and CYP2B1 were obtained from Gentest and primary monoclonal antibodies for β-actin (clone AC-40) were from Sigma. Densitometry was performed on a Kodak Image Station 440 (NEN, Boston, MA).

TAM–DNA Chemiluminescence Immunoassay (CIA)

DNA from liver tissue of all animals was subjected to the quantitative TAM–DNA CIA for determination of TAM–DNA adducts [29]. Genomic DNA was extracted from rat liver using the Wizard Kit (Promega, Madison, WI) as per the protocol supplied. Rabbit antiserum elicited against DNA modified with (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen (dG-TAM) (2.4% modification) was employed in the TAM–DNA CIA as previously described [37]. Each well of a 96-well microtiter plate was coated with 8.2 pg TAM–DNA (0.073 fmol TAM–DNA/pg DNA) or unmodi-

fied DNA (top and bottom rows) and plates were stored frozen until use. At the time of assay, plates were incubated with 0.33% casein (I-Block, Tropix, Bedford, MA). Microtiter plates contained a TAM–DNA standard curve as well as biological sample DNA (10 μg/well). Each biological sample was assayed three to four times on different days. Competition was achieved by mixing and preincubating equal volumes of anti-TAM–DNA antiserum (1:1 000 000) with either TAM–DNA standard or biological sample DNA prior to adding to each well. Subsequent steps include incubations with biotinylated antirabbit IgG (1:3000; Jackson ImmunoResearch, City, State), streptavidin-alkaline phosphatase both from Bio-Genex (1:3750; San Ramon, CA) and finally CDP-Star containing Emerald II enhancement solution (Tropix) was added and the light emission was measured at 542 nm using a TR717 Microplate Luminometer (PE Applied Biosystems, Foster City, CA). Sample quantitation was achieved by comparison of unknown samples with a TAM–DNA standard curve, in which 50% inhibition was at 0.73 ± 0.22 fmol dG-TAM (mean $\pm$ SE, $n = 6$). Since up to 20 μg of DNA could be analyzed per microtiter well, the lower limit of detection (LOD) was calculated to be ~10 amol of dG-TAM adduct/μg DNA or ~0.3 dG-TAM/10^8 nucleotides.

High Density Oligonucleotide Array Expression Analysis

Following exposure, total RNA was isolated from liver tissue using RNeasy mini kit (Qiagen, Valencia, CA). Further sample preparation for microarray analyses was carried out in accordance with the manufacturer's instructions (Affymetrix, Santa Clara, CA). Briefly, total RNA (12 μg) was used for the preparation of double stranded cDNA using an oligonucleotide $(dT)_{24}$ primer with a T7 RNA polymerase promoter sequence at its 5′ end. Following second strand synthesis, a labeled cRNA transcript was synthesized from the cDNA in an in vitro transcription (IVT) reaction using Enzo BioArray high yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). The labeled antisense cRNA was purified using RNeasy (Qiagen) and each cRNA sample (20 μg) was fragmented [(Mg^{2+}, heat (94°C)]. The fragmented cRNA (15 μg) together with control oligonucleotides were hybridized for 16 h to Affymetrix® rat toxicology U34 arrays. Following hybridization, the arrays were washed, stained and scanned using the Affymetrix GeneArray® 2500 Scanner. All experiments were performed in duplicate.

Data Analysis Using Affymetrix Software

Scanned output images were analyzed with the Affymetrix Microarray Suite (MAS) 5.0 and normalized by global scaling to 1500 and to the average fluorescence intensity for the entire microarray. Absolute analysis was performed for each array prior to comparative analysis. To identify differentially expressed transcripts, pairwise comparison analyses were carried out with MAS 5.0. *P*-values were determined by the Wilcoxon's signed rank test and denoted as increase, decrease, or no change. Analysis using MAS 5.0 provides the signal log ratio (SLR), which estimates the magnitude and direction of change of a transcript when two arrays are compared (experimental versus baseline). All values are expressed as average fold change with standard deviations.

Statistical Analysis

For the CYP3A2 enzyme activity data, 4 of the 18 animals had fewer than three replicate wells. The data were analyzed in two ways that gave very similar results: (1) Using a nested linear mixed model accounting for the unequal number of replicate wells and (2) simple one-way ANOVA of the animal means (over the replicate wells). For simplicity, the latter analysis is presented in the results with pairwise *P*-values adjusted using Dunnett's method for all pairwise comparisons. For TAM–DNA, the adduct data from six total assays were analyzed. Each assay included the animals from a subset of the treatments (incomplete blocks). Adduct data were analyzed on the log transformed scale because (1) overall response varied multiplicatively between assays (multiplicative block effects) and (2) from assay to assay there were consistent within-assay treatment ratios (relative treatment effects). To compare treatments, a linear mixed model with random assays and random animals within groups was used. In order to display the biological (animal-to-animal) variation, Table 1 shows group means and standard errors ($n = 3$ rats per group) after adjusting for the multiplicative assay effects only. CYP3A2 and TAM–DNA adduct data were analyzed with SAS v8.2 (SAS Institute, Cary, NC). The Mixed procedure was used for the simple linear models and linear mixed models with the REML fitting method and the Kenward-Rogers adjustments for unbalanced data [*Littell R, Stroup W, Freund R (2002) SAS for Linear Models, Fourth Edition. SAS Institute, Inc.*]. CYP2B1 data were analyzed by simple one-way ANOVA of the animal means (over the replicate wells) followed by use of Dunnett's method for all pairwise comparisons. These tests were performed using InStat v3.0 (Graphpad Software, San Diego, CA).

RESULTS

In order to examine the liver CYP enzyme activities and the effect of PMO co-treatment with TAM, hepatic tissue was subjected to functional enzyme assays for CYP3A2 and CYP2B1. The functional enzyme assay indicated that TAM exposure resulted in an elevation of CYP3A2 activity from 82.6 ± 6.0 to 129.5 ± 9.9 nanomoles of HFC/200 μg S9 protein/h ($P < 0.05$) (Table 1). Increasing doses of CYP3A2

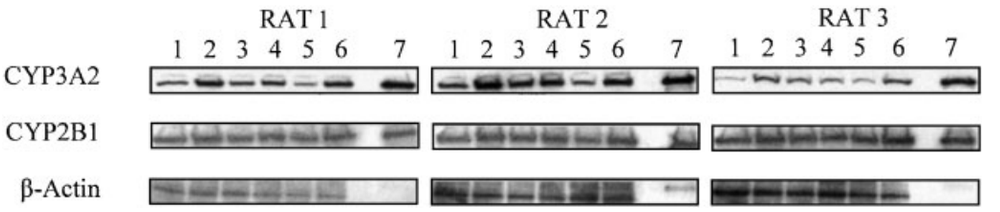

Figure 1. Immunoblot analysis of rat liver samples for CYP enzymes. S9 fractions from rat liver tissue harvested at the time of the study termination were subjected to immunoblot analysis for CYP3A2, CYP2B1, and β-actin. Lane numbers are same as study group number (Table 1). The blots were performed for all three rats from each treatment group and therefore the panels Rat 1, Rat 2, and Rat3 refer to the first, second and third rat of each treatment group. Immunoblot positive controls (lane 7) are liver microsomes from rats induced for CYP3A2 or CYP2B1. β-actin was used as a loading control.

antisense PMO AVI-4472 resulted in lowering of CYP3A2 activity in hepatic S9 fractions but the reduction was statistically significant ($P < 0.001$) only at the highest antisense PMO dose of 12.5 mg/kg/day AVI-4472 in comparison to TAM-only group. There was a reduction in CYP2B1 activity in all treatment groups with varying doses of ABI-4472 (including 12.5 mg/kg/day AVI-4472) but none of them were statically significant in comparison to TAM-only group.

Western analyses were performed to determine the protein expression of the liver CYP enzymes, CYP3A2 and CYP2B1 upon PMO co-treatment with TAM. An increasing dose of CYP3A2 antisense PMO AVI-4472 revealed a decrease in the protein expression in all the three replicate animals (Figure 1). A decrease in protein expression was evident in the 12.5 mg/kg/day AVI-4472 in comparison to TAM-only group. However, no reduction in CYP2B1 expression was observed any of the doses. Also, rats co-treated with 12.5 mg/kg/day of the human CYP3A4 antisense PMO, AVI-4557, showed no changes in CYP3A2 or CYP2B1 protein expression (Figure 1).

The TAM–DNA adduct data demonstrated that rats exposed to TAM alone had high levels of TAM–DNA adducts compared to rats co-treated with TAM plus AVI-4472 (Table 1). Although a dose dependant CYP3A2 antisense-associated reduction in TAM–DNA adduct formation was not evident, a significant decrease for the antisense (AVI-4472) co-treated group with a dose of 2.5 mg/kg ($P < 0.01$) and 12.5 mg/kg ($P < 0.05$) was observed.

Rat toxicology U34 arrays, that were known to interrogate more than 850 mRNA transcripts and estimated sequence tag (EST) clusters, were used to investigate modulation of gene expression patterns in response to TAM treatment, or co-treatment with PMO AVI-4472 (complimentary to CYP3A2 mRNA). TAM treatment alone resulted in induction of several *CYP* genes, specifically, *CYP3A9* (U46118), *CYPB1* (M11251), *CYPB9* (L00320), and *CYPE9* (K00996). However, no significant induction of *CYP3A2* was observed ($P < 0.05$, Table 2). Confirmation of *CYP3A2*, *CYPB9*, and *CYPE9* by real-time PCR indicated very good correlation with the microarray data observed. In the presence of AVI-4472, there was a dose dependent suppression of *CYP3A9* induction and TAM failed to induce *CYPB1*, *CYPB9*, or *CYPE9* at all doses of AVI-4472. While AVI-4557 (complimentary to CYP3A4 mRNA) revealed no significant effect ($P < 0.05$) on inhibition of TAM-induced *CYP3A9*, *CYPB1*, *CYPB9* or *CYPE9* (data not shown).

DISCUSSION

TAM is a major drug used for adjuvant chemotherapy of breast cancer; however, its use has been associated with a small but significant increase in risk of endometrial cancer. In rats, tamoxifen is a hepatocarcinogen, and DNA adducts have been

Table 2. Effect of AVI-4472 Antisense Oligonucleotide on Tamoxifen-Induced CYP Transcripts

Accession number	Gene symbol	Treatment groups (average fold change ± SD)			
		T[a]	T[a] + 0.5 mg A	T[a] + 2.5 mg A	T[a] + 12.5 mg A
M13646	CYP3A2	0.73 ± 0.11	1.10 ± 0.00	0.84 ± 0.04	1.1 ± 0.10
U46118	CYP3A9	2.93 ± 0.43	1.74 ± 0.17	1.04 ± 0.16	0.76 ± 0.10
M11251	CYPB1	2.50 ± 0.07	0.59 ± 0.09	0.57 ± 0.21	0.47 ± 0.10
L00320	CYPB9	3.61 ± 0.54	0.59 ± 0.03	0.58 ± 0.16	0.41 ± 0.10
K00996	CYPE9	2.93 ± 0.16	0.68 ± 0.03	0.71 ± 0.14	0.76 ± 0.20

[a]T, tamoxifen.
A, AVI-4472 antisense oligo nucleotide to *CYP3A2* gene.
The data represents average fold change ± standard deviation, where n = 2 arrays per treatment.

observed in both rat and human tissues. In the present study, we have shown that inhibition of CYP3A2 concomitantly with TAM and PMO administration results in a reduction of TAM–DNA adduct formation (Table 1). The TAM–DNA adduct quantity measured in the groups with the two highest levels of PMO were not significantly different. This could be due to an underestimation of the total TAM–DNA adducts as the *N*-desmethyl adduct is not recognized well (only about 20%) by the antibody used compared to the dG-N2-TAM adduct [38]. Therefore, although we observed a reduction in TAM–DNA adduct level on administration of the PMO, we plan in the future to include an additional but different TAM–DNA adduct measurement to clarify the underestimation of the total DNA adducts that may have resulted. We also propose to explore the phenomenon of adduct reduction on PMO treatment in greater detail, where we plan to include the measurements of 4-OH-TAM, the important metabolite responsible for TAM related antiestrogenic activity.

The results from the functional enzyme assay indicated that TAM exposure resulted in elevated levels of CYP3A2 activity. This change, suggestive of CYP3A2 induction, was also readily observed in our immunoblots. Kasahara et al. [39] have also reported that exposure of rats to tamoxifen increases the expression of hepatic CYP3A2. Examination of CYP2B1 levels, another mixed function oxidase used as control, revealed no significant changes with TAM treatment in either the functional assay or immunoblot. The CYP3A2 induction with TAM-only treatment adds an important layer of value to a previous report linking TAM genotoxicity to metabolism by the CYP3A subfamily [14]. The reported study was carried out in a cell-free in vitro setting where such induction could not have been observed. Our data indicating CYP3A2 induction suggests a greater role for the CYP3A family in TAM metabolism than previously reported, possibly making it a more important player in terms of activation of TAM to its genotoxic metabolites. Despite the lack of a classic dose-response for the doses selected in our study, AVI-4472 co-treatment inhibited hepatic CYP3A2 in a sequence-specific and consistent manner.

Previously, DNA microarray assays have been used to compare transcriptional changes caused by drugs to identify therapeutic mechanisms and potential side effects [40,41]. In this study, we have used DNA microarray assay as a complement to the enzymatic assays, as they may help provide an overview of which *CYP* genes are altered in response to TAM or TAM co-treated with AVI-4472. The microarray data failed to register a significant expression of CYP3A2 on exposure to TAM alone. The sensitivity of the microarray technology allows detection of gene transcripts down to ~1 part in 300 000 [42]. This detection threshold may account for the differences in apparent induction ratios of CYP3A2 by TAM. Another possibility is that in the microarray data, TAM-induced CYP3A2 was not detected may be due to the RNA turnover while in the case of protein, it is cumulative and therefore the protein and enzyme activity was detected using Western blots and enzyme activity assays respectively. Furthermore, it could be due to the fact that male rats may not show the expression of CYP3A2 following a single dose of tamoxifen as reported by Davis et al [43]. Although, the microarray data of TAM-induced CYP3A2 was confirmed using real-time PCR (data not shown) that indicated 58% induction of CYP3A2 compared to the enzyme activity data where a 57% induction was observed, confirming the microarray data observed.

A change in the gene expression of certain *CYP* enzymes on exposure to TAM was reported by Kasahara et al. [39], although the roles of the enzymes in the metabolism of tamoxifen have not yet been clarified. Kawai et al. [44] demonstrated that neonatal treatment of rats with TAM resulted in a long-lasting alteration in the hepatic expression of several *CYP3A* subfamily genes, including *CYP3A9* (exhibits testosterone 6β-hydroxylase activity). They conclude that although relatively little is known about the hormonal regulation of *CYP3A9*, it has been reported that continuous growth hormone infusion to male rats increases hepatic CYP3A9 mRNA level, whereas androgen does not appear to have a suppressive influence in *CYP3A9* expression because castration does not increase *CYP3A9* mRNA level in male rats [45]. *CYP3A9* being one among the *CYP* genes that was induced by TAM in our study, the conclusion arrived at by Robertson et al [45] on the mRNA expression of CYP3A9, might explain the alteration we observed as well. On the other hand, Kupfer et al. [46] reported that exposure of women undergoing TAM therapy to agents that induce *CYPB1* analogues may produce increased levels of 4-hydroxytamoxifen and that it may affect the therapeutic potency of TAM. In our study we have shown that *CYPB1* that was induced on exposure to TAM was suppressed when co-treated with AVI-4472 in a dose dependent manner. The suppression of *CYPB9* and *CYPE9* on co-treatment with AVI-4472 and TAM was confirmed by real-time PCR. Our findings and previous reports indicate that further study on the changes in the expression of endometrial enzymes responsible for TAM metabolism in TAM-administered humans would be important for understanding the correlation between the use of TAM and uterine carcinogenesis.

The CYP3A2 antisense-mediated reductions in TAM–DNA adduct levels (~60%) appeared to be commensurate with the degree of enzyme inhibition (Table 1). However, it is unclear from the data if the remaining TAM–DNA adducts resulted from less than complete inhibition of CYP3A2, or through the activity of other CYPs. In any event, this study

demonstrates a clear reduction in CYP3A2 enzyme activity and protein expression using different doses of AVI-4472 that resulted in suppression of *CYP* genes as well as the inhibition of TAM–DNA adduct formation. The data therefore suggest that a significant reduction in TAM genotoxicity can be achieved through the use of this reagent and still maintain the therapeutic efficiency of TAM.

Although the present study was conducted with the aim of providing evidence to the hypothesis that inhibition of CYP3A2 concomitantly with TAM would decrease TAM–DNA adduct formation using antisense technology, we realize that it could influence/modulate the efficacy of the metabolism of TAM. Hence in future studies, we plan to address the implications of inhibiting CYP3A4 in women receiving tamoxifen therapy. We conclude that there is fundamental value in further pursuit and exploitation of this metabolic redirection strategy by combining TAM with antisense oligomers targeting CYP3A subfamily. This approach takes an immensely useful chemotherapeutic agent, with wide use in chemoprevention, and measurably decreases its genotoxicity.

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REFERENCES

1. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–1388.
2. Poirier MC, Schild LJ. The genotoxicity of tamoxifen: Extent and consequences, Kona, Hawaii, January 23, 2003. Mutagenesis 2003;18:395–399.
3. Curtis RE, Freedman DM, Sherman ME, Fraumeni JF Jr. Risk of malignant mixed mullerian tumors after tamoxifen therapy for breast cancer. J Natl Cancer Inst 2004;96:70–74.
4. Greaves P, Goonetilleke R, Nunn G, Topham J, Orton T. Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 1993;53:3919–3924.
5. Carthew P, Rich KJ, Martin EA, et al. 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 1995;16:1299–1304.
6. Brown K. Breast cancer chemoprevention: Risk-benefit effects of the antioestrogen tamoxifen. Expert Opin Drug Saf 2002;1:253–267.
7. Phillips DH. Understanding the genotoxicity of tamoxifen? Carcinogenesis 2001;22:839–849.
8. Phillips DH, Carmichael PL, Hewer A, Cole KJ, Poon GK. alpha-Hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. Cancer Res 1994;54:5518–5522.
9. Potter GA, McCague R, Jarman M. A mechanistic hypothesis for DNA adduct formation by tamoxifen following hepatic oxidative metabolism. Carcinogenesis 1994;15:439–442.
10. Poon GK, Walter B, Lonning PE, Horton MN, McCague R. Identification of tamoxifen metabolites in human Hep G2 cell line, human liver homogenate, and patients on long-term therapy for breast cancer. Drug Metab Dispos 1995;23:377–382.
11. Shibutani S, Shaw PM, Suzuki N, Dasaradhi L, Duffel MW, Terashima I. Sulfation of alpha-hydroxytamoxifen catalyzed by human hydroxysteroid sulfotransferase results in tamoxifen–DNA adducts. Carcinogenesis 1998;19:2007–2011.
12. Osborne MR, Hewer A, Hardcastle IR, Carmichael PL, Phillips DH. Identification of the major tamoxifen–deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. Cancer Res 1996;56:66–71.
13. Dasaradhi L, Shibutani S. Identification of tamoxifen–DNA adducts formed by alpha-sulfate tamoxifen and alpha-acetoxytamoxifen. Chem Res Toxicol 1997;10:189–196.
14. Kim SY, Suzuki N, Santosh Laxmi YR, Rieger R, Shibutani S. Alpha-hydroxylation of tamoxifen and toremifene by human and rat cytochrome P450 3A subfamily enzymes. Chem Res Toxicol 2003;16:1138–1144.
15. Notley LM, De Wolf CJ, Wunsch RM, Lancaster RG, Gillam EM. Bioactivation of tamoxifen by recombinant human cytochrome p450 enzymes. Chem Res Toxicol 2002;15:614–622.
16. Boocock DJ, Brown K, Gibbs AH, Sanchez E, Turteltaub KW, White IN. Identification of human CYP forms involved in the activation of tamoxifen and irreversible binding to DNA. Carcinogenesis 2002;23:1897–1901.
17. Arora V, Iversen PL. Redirection of drug metabolism using antisense technology. Curr Opin Mol Ther 2001;3:249–257.
18. Arora V, Hannah TL, Iversen PL, Brand RM. Transdermal use of phosphorodiamidate morpholino oligomer AVI-4472 inhibits cytochrome P450 3A2 activity in male rats. Pharm Res 2002;19:1465–1470.
19. Arora V, Cate ML, Ghosh C, Iversen PL. Phosphorodiamidate morpholino antisense oligomers inhibit expression of human cytochrome P450 3A4 and alter selected drug metabolism. Drug Metab Dispos 2002;30:757–762.
20. Arora V, Knapp DC, Reddy MT, Weller DD, Iversen PL. Bioavailability and efficacy of antisense morpholino oligomers targeted to c-*myc* and cytochrome P-450 3A2 following oral administration in rats. J Pharm Sci 2002;91:1009–1018.
21. Summerton J, Weller D. Morpholino antisense oligomers: Design, preparation, and properties. Antisense Nucleic Acid Drug Dev 1997;7:187–195.
22. Iversen PL. Phosphorodiamidate Morpholino oligomers. In: 'Antisense drug technology—principles, strategies and applications'. 2001. pp 375–389.
23. Iversen PL, Arora V, Acker AJ, Mason DH, Devi GR. Efficacy of antisense morpholino oligomer targeted to c-*myc* in prostate cancer xenograft murine model and a Phase I safety study in humans. Clin Cancer Res 2003;9:2510–2519.
24. Hudziak RM, Barofsky E, Barofsky DF, Weller DL, Huang SB, Weller DD. Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev 1996;6:267–272.
25. Summerton J. Morpholino antisense oligomers: The case for an RNase H-independent structural type. Biochim Biophys Acta 1999;1489:141–158.
26. Giles RV, Spiller DG, Clark RE, Tidd DM. Antisense morpholino oligonucleotide analog induces missplicing of C-*myc* mRNA. Antisense Nucleic Acid Drug Dev 1999;9:213–220.

27. Phillips DH, Hewer A, White IN, Farmer PB. Co-chromatography of a tamoxifen epoxide-deoxyguanylic acid adduct with a major DNA adduct formed in the livers of tamoxifen-treated rats. Carcinogenesis 1994;15:793–795.
28. White IN, de Matteis F, Davies A, et al. 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 1992;13:2197–2203.
29. Divi RL, Dragan YP, Pitot HC, Poirier MC. Immunohistochemical localization and semi-quantitation of hepatic tamoxifen–DNA adducts in rats exposed orally to tamoxifen. Carcinogenesis 2001;22:1693–1699.
30. Carthew P, Lee PN, Edwards RE, Heydon RT, Nolan BM, Martin EA. Cumulative exposure to tamoxifen: DNA adducts and liver cancer in the rat. Arch Toxicol 2001;75: 375–380.
31. Kedar RP, Bourne TH, Powles TJ, et al. Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 1994;343: 1318–1321.
32. van Leeuwen FE, Benraadt J, Coebergh JW, et al. Risk of endometrial cancer after tamoxifen treatment of breast cancer. Lancet 1994;343:448–452.
33. Umemoto A, Monden Y, Komaki K, et al. Tamoxifen–DNA adducts formed by alpha-acetoxytamoxifen N-oxide. Chem Res Toxicol 1999;12:1083–1089.
34. Shibutani S, Ravindernath A, Suzuki N, et al. Identification of tamoxifen–DNA adducts in the endometrium of women treated with tamoxifen. Carcinogenesis 2000;21:1461–1467.
35. Shibutani S, Suzuki N, Laxmi YR, et al. Identification of tamoxifen–DNA adducts in monkeys treated with tamoxifen. Cancer Res 2003;63:4402–4406.
36. Desjardins JP, Iversen PL. Inhibition of the rat cytochrome P450 3A2 by an antisense phosphorothioate oligodeoxynucleotide in vivo. J Pharmacol Exp Ther 1995;275:1608–1613.
37. Divi RL, XX MRO, Hewer A, Phillipsand DH, Poirier MC. Tamoxifen–DNA adduct formation in rat liver determined by immunoassay and 33P postlabeling. Cancer Res 1999; 59:4829–4833.
38. Schild LJ, Divi RL, Beland FA, et al. Formation of tamoxifen–DNA adducts in multiple organs of adult female cynomolgus monkeys dosed with tamoxifen for 30 days. Cancer Res 2003;63:5999–6003.
39. Kasahara T, Hashiba M, Harada T, Degawa M. Change in the gene expression of hepatic tamoxifen-metabolizing enzymes during the process of tamoxifen-induced hepatocarcinogenesis in female rats. Carcinogenesis 2002;23: 491–498.
40. Gerhold D, Lu M, Xu J, Austin C, Caskey CT, Rushmore T. Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays. Physiol Genomics 2001;5:161–170.
41. Marton MJ, DeRisi JL, Bennett HA, et al. Drug target validation and identification of secondary drug target effects using DNA microarrays. Nat Med 1998;4:1293–1301.
42. Lockhart DJ, Dong H, Byrne MC, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 1996;14:1675–1680.
43. Davis W, Hewer A, Rajkowski KM, Meinl W, Glatt H, Phillips DH. Sex differences in the activation of tamoxifen to DNA binding species in rat liver in vivo and in rat hepatocytes in vitro: Role of sulfotransferase induction. Cancer Res 2000;60:2887–2891.
44. Kawai M, Bandiera SM, Chang TK, Poulet FM, Vancutsem PM, Bellward GD. Modulation of hepatic CYP2A1, CYP2C11, and CYP3A9 expression in adult rats by neonatal administration of tamoxifen. Drug Metab Dispos 1999; 27:1392–1398.
45. Robertson GR, Farrell GC, Liddle C. Sexually dimorphic expression of rat CYP3A9 and CYP3A18 genes is regulated by growth hormone. Biochem Biophys Res Commun 1998; 242:57–60.
46. Kupfer D, Mani C, Lee CA, Rifkind AB. Induction of tamoxifen-4-hydroxylation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), beta-naphthoflavone (beta NF), and phenobarbital (PB) in avian liver: Identification of P450 TCDDAA as catalyst of 4-hydroxylation induced by TCDD and beta NF. Cancer Res 1994;54:3140–3144.