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Structure–Activity Relationships for Triphenylethylene Antiestrogens on Hepatic Phase-I and Phase-II Enzyme Expression

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ABSTRACT. To better understand the mechanism(s) by which tamoxifen induces rat hepatic CYPIIB2 and suppresses GSTA1, structure–activity studies were performed. Compounds employed in these studies included: tamoxifen, fixed-ring tamoxifen, ethylated fixed-ring tamoxifen, pyrrolidino-tamoxifen, 4-iodotamoxifen, idoxifene, and toremifene. With respect to GSTA1 suppression, tamoxifen, fixed-ring tamoxifen, 4-iodotamoxifen, idoxifene, and toremifene were all potent suppressors of GSTA1, while ethylated fixed-ring tamoxifen and pyrrolidino-tamoxifen were completely without activity. The results suggest that the aminoethoxy side chain plays a crucial role in GSTA1 suppression, and that 4-iodination may potentiate this activity. With respect to induction of CYPIIB2, tamoxifen, fixed-ring tamoxifen, and ethylated fixed-ring tamoxifen were inducers of this enzyme, while toremifene and 4-iodotamoxifen were inactive, suggesting that the aminoethoxy side chain is not a structural determinant of CYPIIB2 induction. Because ethylated fixed-ring tamoxifen, toremifene, and 4-iodotamoxifen had differential activities in the two assays, we conclude that CYPIIB2 induction and GSTA1 suppression by triphenylethylenes are the result of two separate and distinct mechanistic pathways. Structure–activity relationships for GSTA1 suppression and CYPIIB2 induction were compared with previously published relationships for triphenylethylene: 1) estrogen receptor relative binding affinity; 2) calmodulin antagonism; 3) antiuterotrophic activity; and 4) antagonism of MCF-7 cell growth. No clear correlation was observed between the effects on CYPIIB2 and these other four activities, suggesting no relationship between the mechanisms responsible for these effects. Similarly, no precise correlation was observed between GSTA1 suppression and these other activities, although rough similarities were observed for relative binding affinity and antiuterotrophic activity. This suggests that the mechanisms responsible for CYPIIB2 induction and GSTA1 suppression are not related to the mechanisms of action for these other documented activities, and may represent different mechanistic pathways. BIOCHEM PHARMACOL **56**;3:321–327, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. tamoxifen; antiestrogen; structure–activity relationship; liver; enzyme induction; gene expression

The antiestrogen TAM†† is the most widely used chemotherapeutic agent for the treatment of ER-positive breast neoplasms [1]. The therapeutic potential of TAM is derived from the ability of the drug to block estrogen-stimulated growth of breast cancer cells via competition with estradiol for binding to the ER [2]. This competition lends TAM a complex pharmacology consisting of both estrogenic and antiestrogenic properties in a variety of tissues [3].

In addition to ER-mediated activities, the drug exhibits a variety of properties that appear to be independent of receptor interaction. Examples of this include regression of ER-negative mammary tumors [4] and breast cancer cell-growth inhibition that cannot be inhibited by an excess of estradiol [5, 6]. Other properties include the ability to inhibit the activity of calmodulin [7–11], protein kinases [7] and cAMP phosphodiesterases [9], and to stimulate the activity of phospholipase D [12], as well as the induction of transforming growth factor-β [13–18] and interaction with antiestrogen-binding sites [19–21]. The effects of TAM on hepatic enzyme expression also appear to be an example of this type of activity [22, 23].

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†† *Abbreviations:* cAMP, cyclic AMP; CYP, cytochrome P450; EFRT, ethylated fixed-ring tamoxifen; ER, estrogen receptor; FRT, fixed-ring tamoxifen; GST, glutathione *S*-transferase; IDOX, idoxifene; ITAM, 4-iodotamoxifen; PTAM, pyrrolidino-tamoxifen; RBA, relative binding affinity; SARs, structure–activity relationships; TAM, tamoxifen; and TOR, toremifene.

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TAM is a potent carcinogen in rat liver [24–27], and a possible human endometrial carcinogen [28–30]. TAM-induced rat liver carcinogenesis is believed to be the result of metabolic activation of the drug to a reactive electrophile [31] that covalently modifies cellular macromolecules, including DNA [32–36]. This metabolic activation is the result of cytochromes P450 [31], sulfotransferases [36, 37], and possibly glucuronosyltransferases [32] and flavin-dependent monooxygenases [31]. Given that TAM can induce its own metabolic activation [38], the effects of the drug on xenobiotic-metabolizing enzyme expression are relevant to its genotoxicity, and possibly its carcinogenicity.

The effects of TAM on hepatic enzyme expression in rat liver can be divided into phenobarbital-like (CYPIIB2 induction) and non-phenobarbital-like (GSTA1 suppression) categories [22, 23]. Based on previously published observations [22, 23], it was reasonable to hypothesize that these effects were the result of two previously uncharacterized mechanisms of action for TAM. Structure–activity studies represent a well documented approach to investigate these phenomena [39]. Numerous structural analogs of TAM have been generated, and these analogs are the basis for the experiments described in this manuscript.

To define these SARs, a series of seven triphenylethylene antiestrogens were administered to F344 rats, and hepatic expression of CYPIIB2 and GSTA1 was examined. The antiestrogens employed in these experiments (see Fig. 1) included: TAM, FRT, EFRT, TOR, ITAM, PTAM, and IDOX. FRT originally was designed to inhibit the isomerization of TAM from the relatively antiestrogenic *trans* conformation to the relatively estrogenic *cis* conformation [40–42]. In EFRT, this modification was combined with replacement of the methyl groups in the aminoethoxy side chain with ethyl groups, in order to specifically block *n*-demethylation of the parent compound [40–42]. ITAM was modified at the 4 position with an iodo group in order to mimic 4-hydroxytamoxifen, the most potent antiestrogenic metabolite of TAM [43, 44]. PTAM was designed with a pyrrolidino group in place of the dimethyl amino, with the intention of reducing metabolism at this site [45]. IDOX was designed with both the 4-OH and pyrrolidino modifications [44] in order to obtain a potent antiestrogen with sustained action. The data from the experiments described in this paper were collected and compared with previously published data for these compounds, which included calmodulin antagonism, antiuterotrophic activity, ER RBA, and antagonism of MCF-7 cell growth.

MATERIALS AND METHODS

Materials

Tricaprylin was obtained from the Sigma Chemical Co. The AIN-76A purified diet was obtained from Harlan Teklad. Radionucleotides (γ- and α-^{32}P) and Hybond N$^+$ nucleic acid transfer membrane were purchased from the Amersham Corp. All other reagents were purchased from the Sigma Chemical Co. and were of molecular biology grade or better when available.

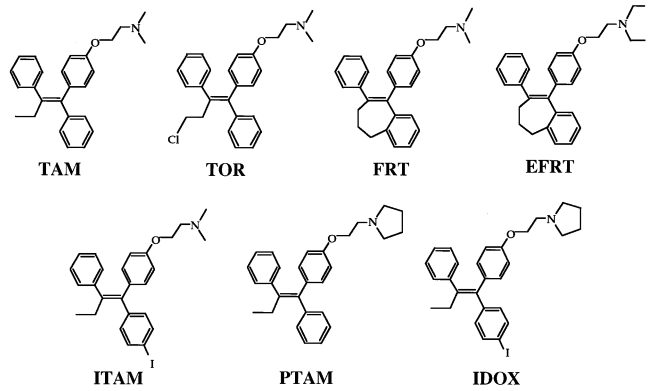

FIG. 1. Structures of seven compounds used to define SARs for the effects of triphenylethylene antiestrogens on hepatic enzyme expression. The Materials and Methods section contains complete details on the sources of all antiestrogens.

Synthesis of TAM Derivatives

TAM (citrate salt and free-base) was purchased from the Sigma Chemical Co. The triphenylethylene antiestrogens FRT and EFRT were synthesized [40–42] and provided by R. McCague (CRC Laboratory, Institute of Cancer Research). TOR was a gift from Dr. Lauri Kangas (Orion Pharmaceuticals). IDOX [44], PTAM [45], and ITAM [43, 44] were synthesized according to published procedures by Drs. Patrice Martin and John Mann of the University of Reading. Figure 1 illustrates the structures of all the triphenylethylene antiestrogens employed in these studies.

Animals and Dosing

For the studies involving comparisons between the triphenylethylene antiestrogens TAM, TOR, ITAM, PTAM, and IDOX, the following protocol was followed. Female F344 rats weighing 130 g were purchased from Harlan Sprague Dawley and were housed three animals per cage on a 12/12 light/dark cycle and fed AIN-76A purified diet and water *ad lib*. The animals were allowed to accommodate to our facility for 1 week prior to the start of the study. Drugs were administered at 9:00 a.m. for 7 days by gavage in 0.7 mL of tricaprylin vehicle. For studies involving comparisons between ITAM, TOR, and TAM-citrate, drugs were administered at 30 mg/kg. These doses corresponded to the following dose, in μmol/kg of body wt/day: TAM-citrate, 53; ITAM, 60; and TOR, 50. For studies involving comparisons between PTAM, IDOX, and TAM-free-base, drugs were administered at 10 mg/kg. This corresponded to the following doses in μmol/kg: TAM-free base, 23; PTAM, 25; and IDOX, 19. This reduced dose was necessary in order to avoid toxicity associated with higher doses of PTAM and IDOX. For the studies involving comparisons between TAM, FRT, and EFRT, the drugs were admixed into the

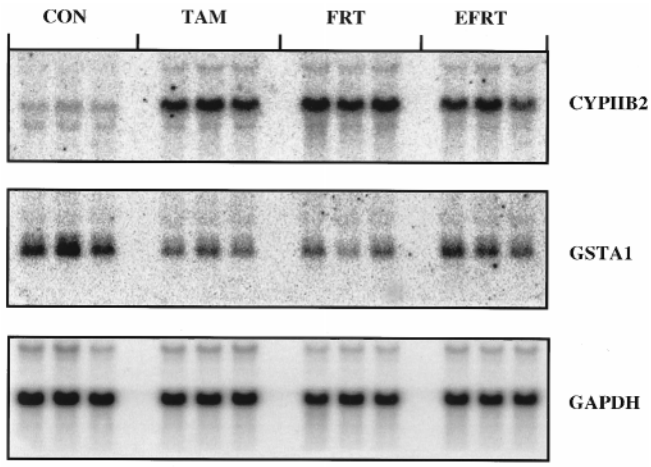

FIG. 2. Northern blot analysis of hepatic GSTA1 and CYPIIB2 after treatment with triphenylethylene antiestrogens. Rats were administered TAM, FRT, or EFRT, and total RNA was prepared and analyzed by Northern blotting as per Materials and Methods. The membrane was serially probed and stripped with DNA probes specific for CYPIIB2, GSTA1, and GAPDH. Each lane represents 15 μg of hepatic total RNA harvested from an individual animal. Images are the result of analysis by a Molecular Dynamics PhosphorImager apparatus.

diet at 250 ppm, and animals were fed this diet *ad lib*. for 6 months. In all cases, animals were decapitated 24 h after the last dose, and their livers were quickly excised and frozen in liquid nitrogen for later RNA analysis.

Preparation of Total RNA and Northern Blot Procedure

Total RNA was prepared [22, 46] and Northern blots were performed [22, 23] as per previously published protocols. Oligonucleotides specific for CYPIIB2 [47] and GSTA1 [48] were employed as probes in these experiments. Specific hybridization was assessed using a Molecular Dynamics PhosphorImager instrument. All blots were first probed with CYPIIB2, stripped and reprobed with GSTA1, and then stripped and reprobed with GAPDH as a loading control. Values are presented as CYPIIB2 or GSTA1 expression divided by the GAPDH signal.

RESULTS

Effect of TAM, FRT, and EFRT on CYPIIB2 and GSTA1

Figure 2 depicts a northern blot analysis for CYPIIB2 (panel A) and GSTA1 (panel B) expression after treatment with TAM, FRT, or EFRT. With respect to CYPIIB2, all three drugs appeared to produced approximately equivalent induction. However, in the case of GSTA1 suppression, TAM and FRT were both potent suppressors of gene expression, whereas EFRT was ineffective or very weakly suppressive. Figure 3 shows the quantitation of Fig. 2 using the PhosphorImager apparatus. All data are the average of three animals per group, normalized to the GAPDH signal. As is visible in the preceding figure, TAM, FRT, and EFRT

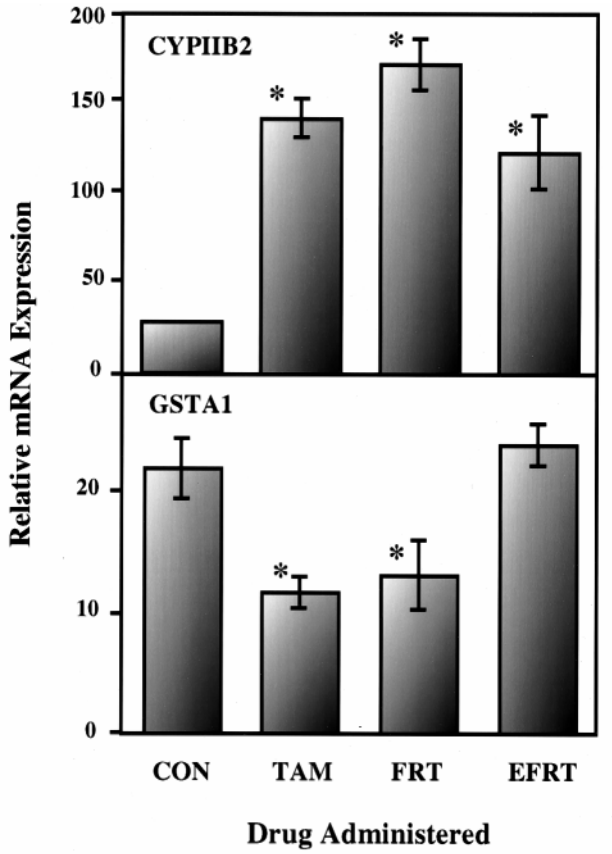

FIG. 3. Comparison of GSTA1 suppression and CYPIIB2 induction for TAM, FRT, and EFRT. The indicated antiestrogens were administered to female F344 rats, and expression of GSTA1 and of CYPIIB2 was analyzed as per Materials and Methods. Each bar (mean ± SD) is the average of three animals, analyzed independently. All data are normalized to GAPDH loading. An asterisk (*) indicates that the value was significantly different ($P < 0.05$) from the respective vehicle-treated control group, as determined by Dunnett's *t*-test.

were inducers of CYPIIB2, all producing approximately 5- to 6-fold increases in mRNA levels for the gene. However, while both TAM and FRT were potent suppressors of GSTA1, reducing mRNA levels by approximately 50%, EFRT was completely ineffective as a suppressor of GSTA1 expression.

Effect of TAM, TOR, and ITAM on CYPIIB2 and GSTA1

The data in Fig. 4 depict the effects of TAM, TOR, and ITAM on GSTA1 and CYPIIB2 expression. Results are the average of three animals per group, normalized to the GAPDH signal. All three triphenylethylene antiestrogens dramatically suppressed GSTA1 mRNA levels. Interestingly, ITAM was a more effective suppressor of GSTA1 than TAM or TOR. ITAM suppressed levels of GSTA1 mRNA to approximately 25% of control, whereas TAM and TOR suppressed expression of the gene to approximately 50% of control. In contrast, only TAM was an effective inducer of CYPIIB2 expression, while both TOR

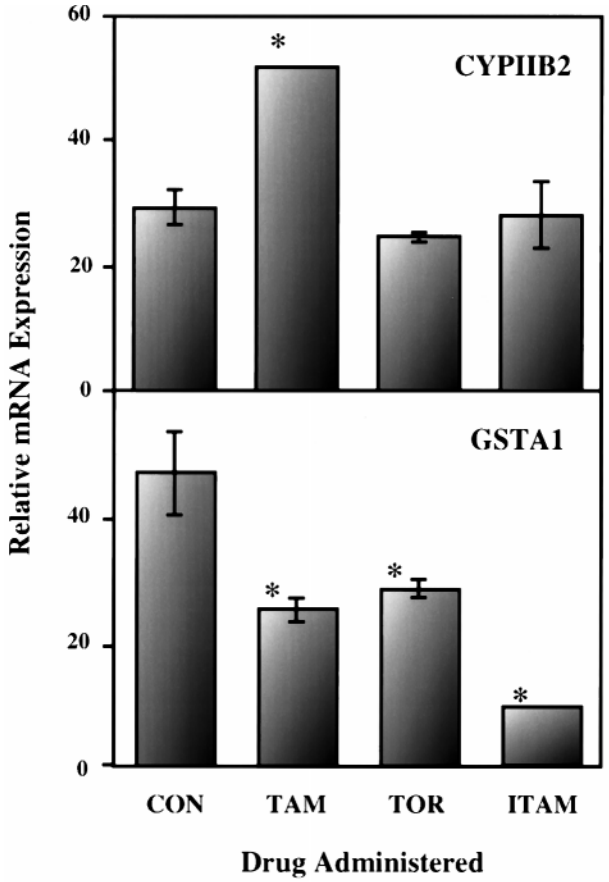

FIG. 4. Comparison of GSTA1 suppression and CYPIIB2 induction for TAM, TOR, and ITAM. Female F344 rats were administered 30 mg/kg of TAM (citrate salt), TOR, or ITAM, and mRNA expression for GSTA1 and CYPIIB2 was analyzed as per Materials and Methods. All data are normalized to GAPDH loading. Each bar (mean ± SD) is the average of three animals, analyzed independently. An asterisk (*) indicates that the value was significantly different ($P < 0.05$) from the respective vehicle-treated control group, as determined by Dunnett's *t*-test.

and ITAM were completely without activity towards this gene.

GSTA1 Suppression by TAM, PTAM, and IDOX

Figures 5 and 6 depict the effects of TAM and PTAM or TAM and IDOX on GSTA1 expression, normalized to the GAPDH control. The drugs in these experiments were administered at a dose of 10 mg/kg, much lower than the dosing regimens used for the other drugs in this series. This was necessary because PTAM and IDOX are acutely toxic, and animals will not tolerate doses higher than 10 mg/kg without exhibiting signs of toxicity. Because of this reduced dose, we were unable to reliably assess the effect of PTAM or IDOX on CYPIIB2 expression. However, because TAM causes greater than 50% suppression of GSTA1 at doses as low as 0.5 mg/kg [23], we were able to examine the effects of PTAM and IDOX on GSTA1 expression at the 10 mg/kg dose. As is evident in Fig. 5, TAM produced a dramatic

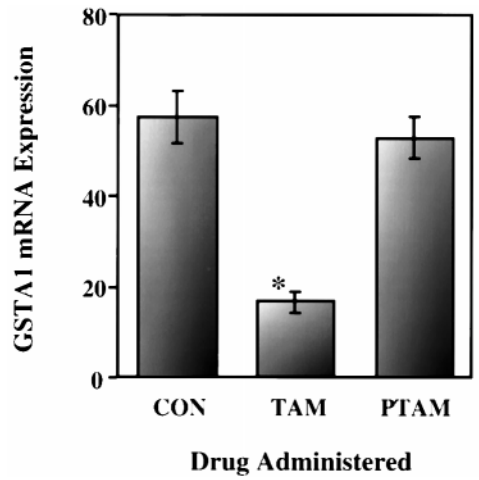

FIG. 5. GSTA1 suppression by TAM and PTAM. Female F344 rats were administered 10 mg/kg of TAM (free-base) or PTAM, and GSTA1 expression was analyzed as per Materials and Methods. Each bar (mean ± SD) is the average of three animals, analyzed independently. All data are normalized to GAPDH loading. An asterisk (*) indicates that the value was significantly different ($P < 0.05$) from the respective vehicle-treated control group, as determined by Dunnett's *t*-test.

suppression of GSTA1, in accordance with results from earlier experiments [23]. However, PTAM had no effect on GSTA1 mRNA levels. Interestingly, IDOX was a more potent suppressor of GSTA1 expression than TAM (see Fig. 6), resulting in approximately 75% suppression of gene expression as compared with 50% by TAM.

Comparison of the SARs for GSTA1 Suppression and CYPIIB2 Induction

A comparison of SARs for effects on GSTA1 and CYPIIB2 expression can be found in Table 1. Treatment with two of

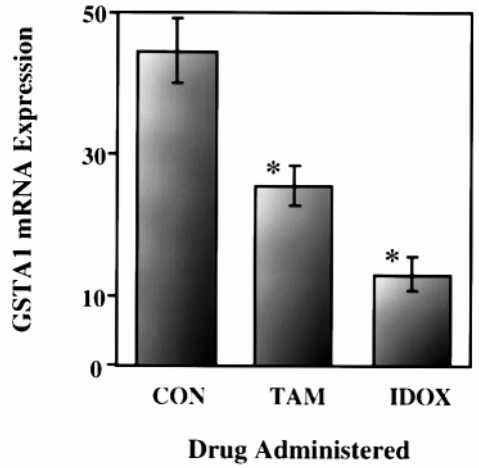

FIG. 6. GSTA1 suppression by TAM and IDOX. Female F344 rats were administered 10 mg/kg of TAM (free-base) or ITAM, and GSTA1 expression was analyzed as per Materials and Methods. Each bar (mean ± SD) is the average of three animals, analyzed independently. All data are normalized to GAPDH loading. An asterisk (*) indicates that the value was significantly different ($P < 0.05$) from the respective vehicle-treated control group, as determined by Dunnett's *t*-test.

TABLE 1. Comparison of SARs for CYPIIB2 induction, GSTA1 suppression, estrogen receptor relative binding affinity (ER RBA), calmodulin antagonism, antiuterotrophic activity, and antagonism of MCF-7 cell growth, for a series of triphenylethylene antiestrogens

	CYPIIB2 induction	GSTA1 suppression	ER RBA	Calmodulin antagonism	Antiuterotrophic activity	Anti-MCF7 growth
TAM	+	+	5	6.8	+	+
FRT	+	+	5	>50	NA	++
EFRT	+	−	NA	NA	NA	NA
ITAM	−	++	12.5	2.3	++	+
PTAM	ND	−	8	NA	+	NA
IDOX	ND	++	12.5	1.5	+++	+++
TOR	−	+	5	NA	+	+

Activities towards CYPIIB2 and GSTA1 were assessed in our laboratory. All the other data in this table were extracted from the specific references cited below. The relative potency of each compound for CYPIIB2 induction or GSTA1 suppression is summarized as: inactive (−), roughly equivalent to the activity of TAM (+), or more active than TAM (++). Calmodulin antagonism is represented as the IC_{50} for inhibition of cAMP phosphodiesterase, an enzyme that is calmodulin dependent and inhibited by certain triphenylethylenes. The antagonistic effect of triphenylethylene on MCF-7 cell growth was assessed using 1 μM of antiestrogens in estrogen replete culture medium. Antiuterotrophic activity is represented as: weakly active (+), moderately active (++), or very active (+++), and is derived from numerical data presented in the references indicated below. Sources for all data are as follows: ER RBA: TAM [44], FRT [41], ITAM [44], PTAM [45], IDOX [44], and TOR [49]; calmodulin antagonism (cAMP phosphodiesterase inhibition): TAM [7], FRT [11], ITAM [7], and IDOX [7]; antiuterotrophic activity: TAM [44], ITAM [44], PTAM [45], IDOX [44], and TOR [50]; antagonism of MCF-7 cell growth: TAM [44], FRT [41], ITAM [44], IDOX [44], and TOR [51]. ND = not determined; NA = not available in current literature.

the seven compounds, PTAM and IDOX, resulted in overt toxicity in the animals at doses above 10 mg/kg. Because CYPIIB2 induction is normally not evident unless doses above 10 mg/kg are administered [22], we were unable to assess the activity of these two compounds for CYPIIB2 induction. In contrast, dramatic suppression of GSTA1 is achieved at doses as low as 0.5 mg/kg [23], thus allowing assessment of this activity for all seven triphenylethylenes. As a result, five compounds were assessed successfully in both assays. Interestingly, three of these five triphenylethylenes, EFRT, ITAM, and TOR, were inactive in one assay but active in the other. The remaining two antiestrogens, TAM and FRT, were consistently active in both assays. Taken together, these results indicate that the structural determinants of these two activities are different, suggesting that the mechanisms that produce these effects are distinct.

DISCUSSION

Examination of the SAR for GSTA1 suppression by triphenylethylene antiestrogens demonstrated that the aminoethoxy side chain is important for this activity. This was demonstrated by the inability of EFRT (diethylated side chain) and PTAM (pyrrolidino side chain) to suppress gene expression as compared with the potent suppression caused by TAM, FRT, TOR, and ITAM. Interestingly, the 4-iodo modification in ITAM and IDOX dramatically increased the ability of these compounds to suppress GSTA1 expression. Because IDOX contains both pyrrolidino and iodo groups, the effect of the iodo derivatization overrides the pyrrolidino modification, resulting in a net potentiation of GSTA1 suppression.

Examination of the SAR for CYPIIB2 induction showed that TAM, FRT, and EFRT were inducers of the gene, whereas TOR and ITAM were without activity. Because of the limited number of compounds available in these studies, no clear correlation was observed between a single structural motif and CYPIIB2 induction. However, these studies suggest that the aminoethoxy side chain is not the primary structural determinant of CYPIIB2 induction because both EFRT and FRT were effective inducers. Further, this activity resides in the central stilbene moiety of triphenylethylene antiestrogens, as demonstrated by the inactivity of TOR and ITAM in this assay. This dependence on the central stilbene moiety is further supported by the ability of *trans*-stilbene oxide to induce CYPIIB2 [52].

A comparison of the SARs for GSTA1 suppression and CYPIIB2 induction, found in Table 1, demonstrates that there is no correlation between these two activities for this series of antiestrogens. Upon close inspection of the data, two distinctions between the two activities are evident: first, the aminoethoxy side chain is not important for CYPIIB2 induction but is important for GSTA1 suppression, and second, 4-iodination potentiates GSTA1 suppression but blocks CYPIIB2 induction. Taken together, these results demonstrate that the mechanisms responsible for these two effects are separate and distinct molecular pathways.

Other researchers have tested triphenylethylenes for a variety of other activities, including: RBA for the ER, calmodulin antagonism, antiuterotrophic activity, and suppression of MCF-7 cell growth. We wished to compare the SARs for these other activities with the SARs for CYPIIB2 induction and GSTA1 suppression, in order to determine if any of these SARs were closely correlated. These comparisons can be found in Table 1. In the case of CYPIIB2, no correlation between enzyme induction and these previously published activities was observed. In every case, at least one compound had a significantly different or opposite effect for any given comparison. For example, TAM and TOR have similar binding affinities, antiuterotrophic activities, and cell-growth antagonism, but TAM was a potent inducer of CYPIIB2 while TOR was inactive in this assay. Similarly, TAM and FRT are both inducers of CYPIIB2, but only

TAM is a calmodulin antagonist. In sum, the data in Table 1 suggest that the mechanism of CYPIIB2 induction is distinct from the mechanisms responsible for ER binding, antagonism of MCF-7 cell growth, calmodulin antagonism, and antiuterotrophic activity for these triphenylethylenes.

Table 1 also compares the structure–activity data for GSTA1 suppression with these other four activities. In all cases, there are compounds that have significantly different activities for a given comparison. For example, TAM and FRT have equivalent activities towards GSTA1, but disparate abilities to act as a calmodulin antagonist or block MCF-7 cell proliferation. However, there is a rough similarity between GSTA1 suppression and ER RBA or antiuterotrophic activity. With the exception of PTAM, most other compounds have roughly consistent activities in these three assays, suggesting that the structural factors of the triphenylethylene molecule that are responsible for GSTA1 suppression, ER RBA, and antiuterotrophic activity may share partial similarity. Overall, the data in Table 1 suggest that the mechanism of action responsible for GSTA1 suppression by triphenylethylene antiestrogens is not identical to the mechanism responsible for any of these other four activities.

In sum, the data from these experiments demonstrate that TAM and other triphenylethylene antiestrogens alter xenobiotic-metabolizing enzyme expression by at least two separate and distinct molecular mechanisms. These mechanisms do not correlate with ER binding affinity, calmodulin antagonism, antiuterotrophic activity, or antagonism of MCF-7 growth. The precise molecular pathways that govern these effects remain to be determined.

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