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Association of Tamoxifen Biliary Excretion Rate with Prior Tamoxifen Exposure and Increased *mdr1b* Expression

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ABSTRACT. ATPase transporter proteins are commonly found in the hepatocyte canalicular membrane. Some of these, in particular the multidrug resistance (*mdr1b*) gene, have been previously demonstrated to be inducible genes. In this study, we found that tamoxifen induced expression of the *mdr1b* gene in the liver up to 40-fold after 14 days' exposure to tamoxifen in the diet at a concentration of 420 ppm. As tamoxifen and its metabolites are primarily excreted into the bile, we investigated if the increased expression of *mdr1b* in the liver following tamoxifen exposure had any effect on its excretion in rats. We found that the excretion of tamoxifen and its metabolites into bile was increased from 8 ± 1% to 51 ± 18% (mean ± SD) of an administered dose of 180 nmol/kg over a collection period of 3 hr in rats that had received tamoxifen (35 mg/kg) orally for 12 days (plus a 3-day rest) prior to the experiment. These data suggest that prolonged treatment with tamoxifen may result in lower serum and tumour concentrations, due to a self-mediated enhancement of excretion via *mdr1b* gene-encoded P-glycoprotein. This may have implications for other drugs sharing the same route of excretion and co-administered with tamoxifen. BIOCHEM PHARMACOL **60**;2:233–239, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. multidrug resistance; tamoxifen; biliary excretion; gene expression; RT–PCR

Tamoxifen [Z-(1-(4-(2-dimethylaminoethoxy)phenyl)-1,2-diphenyl-1-butene)] (TAM†; for structure, see Fig. 1) is a member of the triphenylethylene class of compounds. It acts as an antagonist of the oestrogen receptor and is effective in the treatment of breast carcinomas. TAM is well tolerated in humans, and there are few short-term side effects [1]. However, an increased risk for the development of endometrial cancer has been associated with TAM administration, one that correlates with the duration of treatment [2, 3]. In rats, TAM forms DNA adducts that lead to hepatocellular carcinomas, although there is no evidence that this occurs in humans [4]. There is some evidence, however, that TAM may form adducts in human endometrium, but this remains controversial [5]. Even if low levels of adducts are formed [6, 7], it does not seem likely that this will be the biochemical basis for the increase in endometrial cancers seen in tamoxifen-treated women [2, 3, 8]. TAM is mainly excreted into the bile in several species, including humans, and undergoes enterohepatic recirculation [9–11]. In untreated rats and mice, approximately 22% of administered TAM (43 μmol/kg i.v.) is excreted into the bile, of which 10% is excreted as α-hydroxytamoxifen glucuronide [12]. Additionally, the tissue distribution of TAM is similar in rats and humans [13].

P-glycoproteins (P-gps) are coded for by the *mdr* genes which, in rats, constitute a family of three, divided into two classes [14]. Class one has two members, *mdr1a* and *mdr1b*, which, when transfected into drug-sensitive cells, confer a multidrug-resistant phenotype [15, 16]. The second class has one member, *mdr2*, which cannot confer a multidrug-resistant phenotype, but is normally expressed in bile canaliculi and may have an important normal physiological role here [17, 18]. Mice made null for this gene show abnormal biliary tree pathology and have a reduced ability to excrete phosphlipids [19]. Mice made null for the *mdr1a* and *mdr1b* genes show a decreased ability to excrete vinblastine and digoxin, resulting in a longer half-life and increased accumulation, particularly in the brain [20, 21]. This leads to a 4-fold reduction in the LD_{50} of vinblastine in null mice [20, 22]. In *mdr1a* null mice, the vinblastine excretory pathway most affected is the faecal route, which correlates with a function for P-gp proteins in biliary excretion [20]. Similar results have been reported for *mdr1b* null mice [21, 23].

Induction of *mdr* gene family (particularly *mdr1b*) expres-

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† *Abbreviations: mdr*, multidrug resistance genes; P-gp, P-glycoprotein; RT–PCR, reverse transcriptase–polymerase chain reaction; and TAM, tamoxifen.

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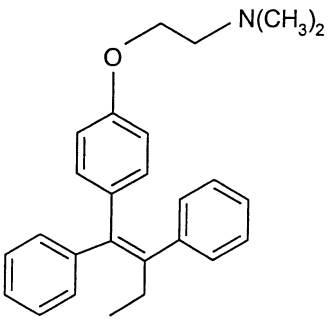

FIG. 1. Structure of tamoxifen.

sion occurs in response to xenobiotics in rat tissues, isolated hepatocytes, and rat liver-derived cell lines. Three characterised inducers are the carcinogens 2-acetylaminofluorene (2-AAF), 3-methylcholanthrene, and aflatoxin B1 [24–27]. In rats, *in vivo*, 2-AAF-induced *mdr* gene expression leads to increased vinblastine excretion into the bile [28]. In monkeys, a link is evident between increased *mdr2* gene expression and administration of drugs that are excreted into the bile such as TAM, erythromycin, and rifampicin [29]. In monkeys and rats, an increased *mdr* gene family expression also results from biliary cholestasis [28]. A relationship has been suggested between the metabolism of xenobiotics by the cytochrome P-4503A family and excretion by P-gp, manifest by their coordinate regulation at the gene level [30]. In response to xenobiotics, there is a coordinated up-regulation in the expression of these two genes in a human adenocarcinoma cell line, suggesting a common mechanism of gene regulation [31]. These data therefore indicate that P-gps in the liver have an important role to play in the excretion of drugs and physiological molecules into the bile, and that there may be a control mechanism by which P-gp levels can be elevated in response to increased need for biliary excretion.

In this paper, we show that TAM can induce its own excretion into the rat bile canaliculus and that this effect is most likely mediated through increased *mdr1b* gene expression.

MATERIALS AND METHODS

Materials

[1-Phenyl-U-^{14}C]tamoxifen (specific activity 2.03 GBq/mmol), of 98% radiochemical purity by HPLC, was obtained from Cambridge Research Biochemicals. [γ-^{33}P]ATP for RT–PCR analysis was from Amersham. Unlabelled TAM was a gift from AstraZeneca Pharmaceuticals PLC. Tricaprylin was obtained from Sigma and ultrapure nucleotides from Pharmacia. All other chemicals were of the highest grade available from local suppliers.

Animals and Dosing

Lewis rats were obtained from Harlan Olac and randomly assigned to cages and maintained on a 12-hr light–dark cycle at 19° with 50% relative humidity in an isolator. The cages were solid polycarbonate and bedded with hardwood chips. After delivery, the rats were kept in isolators for two weeks prior to use. All rats were fed Rodent Maintenance One diet (Special Diet Services, Cheshire, U.K.) containing tamoxifen where appropriate. Distilled water was available *ad lib*.

For the initial induction studies, rats were exposed to 420 ppm TAM in the diet. In the time–course study, exposure to TAM in the diet commenced when the animals were six weeks old. Control rats were given standard diet. The diet was prepared and assayed as previously described [32]. Each experimental group of rats consisted of three individuals.

Analysis of mdr *Gene Expression*

Expression of each *mdr* gene family member was analysed using an RT–PCR-based assay as previously described [33]. For each sample, 100 ng of RNA was subjected to assay in competition with *mdr1a* (0.1 pg), *mdr1b* (0.025 pg), or *mdr2* (3 pg) of internal standard control RNA. Expression was determined by the ratio of the band generated from the internal standard control versus that from the cellular RNA in each case. Band volume was measured using a Molecular Dynamics Phosphorimager. Statistical significance was analysed by the single-factor analysis of variance test, and induced *mdr* gene expression with a P value less than 0.05 or 0.01 is indicated.

Analysis of TAM Excretion into the Bile

Female Lewis rats (150–180 g) were pretreated orally (gavage) with either 1 mL/kg of tricaprylin or TAM (35 mg/kg) in tricaprylin (1 mL/kg) from days 1 to 12. On day 15, the rats were anaesthetised with pentobarbital (60 mg/kg) and the bile duct excised and cannulated. Anaesthesia was maintained with appropriate pentobarbital as required and body temperature maintained using a heat blanket. An initial 20-min bile collection was made before administration of [^{14}C]TAM by intravenous infusion into the tail vein. A total of 180 nmol/kg (0.37 MBq/kg) of [^{14}C]TAM was administered in 709 μL/kg of 33% ethanol in 0.9% saline. Collection of bile was made in aliquots each of 20-min duration for the following 3 hr. The bile flow rate varied from 100 to 150 μL/20 min. From each collection, 20 μL was taken for a gross assessment of radioactivity by scintillation counting and the remainder retained for HPLC analysis of metabolites. After each collection period, the rat was killed and the liver removed and weighed. Six control and six tamoxifen-pretreated rats were cannulated in total. Data from three of each group are presented. Some of those animals not included in the data analysis failed to survive a 3-hr bile collection period and were discounted, while data from the other animals not included were essentially identical to those presented. A piece of liver (approx. 200 mg) was taken for assessment of ^{14}C content by scintillation counting. Excretion of gross ^{14}C radioactiv-

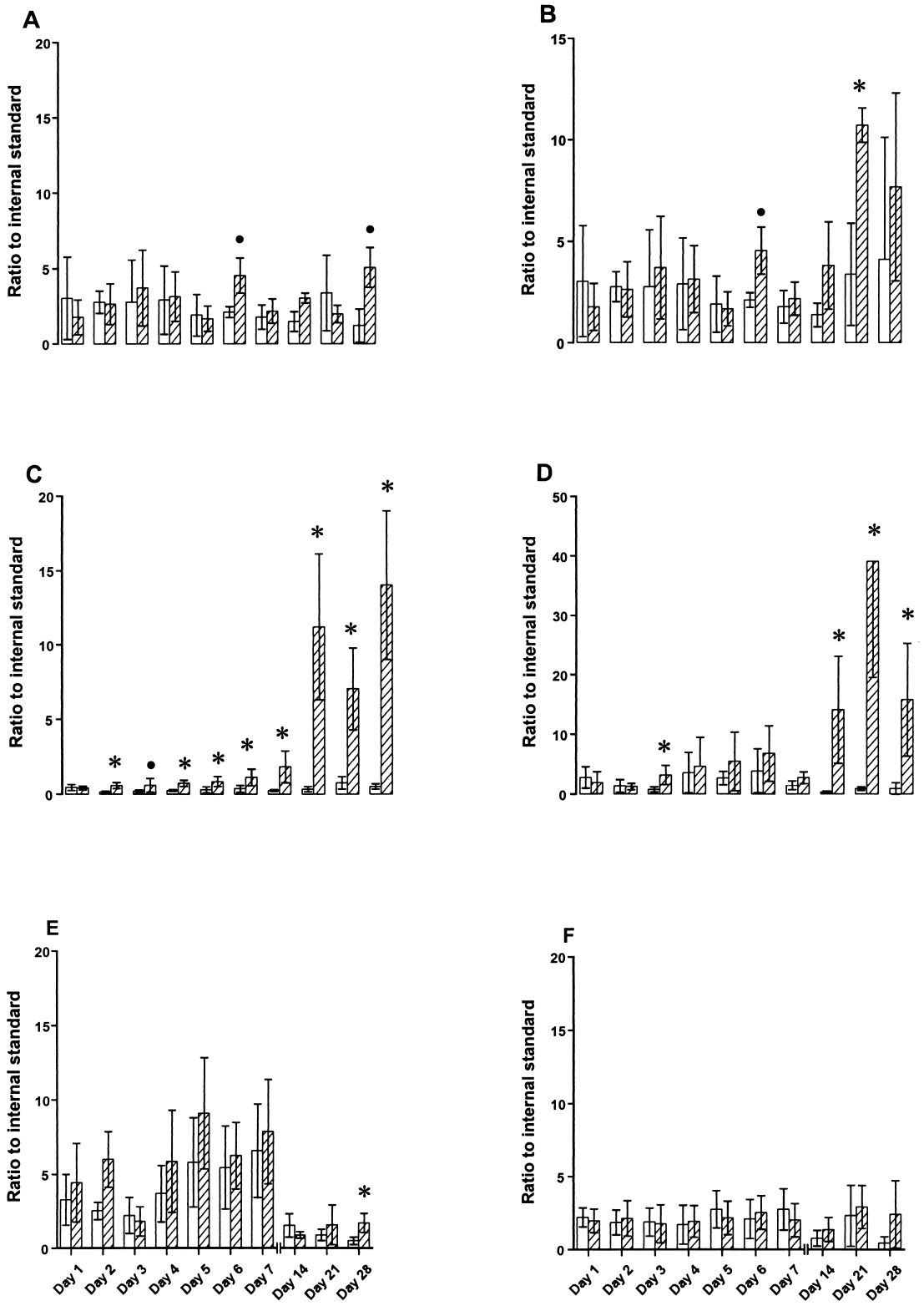

FIG. 2. Induction of *mdr1a, 1b,* and *2* genes over 28 days after commencement of a continuous TAM exposure via the diet. Lewis rats were administered TAM by maintenance on a diet containing 420 ppm TAM. Expression of each *mdr* gene was determined by internally controlled RT–PCR after 1 through 7 days, and 14, 21, and 28 days of TAM exposure in both male and females. A, C, E: *mdr1a, mdr1b,* and *mdr2* mRNA levels in males, respectively; B, D, F: *mdr1a, mdr1b,* and *mdr2* mRNA levels in females, respectively. In each case, the open bars are age-matched controls and the hatched bars rats exposed to TAM. Each bar is the mean ± SD of data from 3 separate rats. Statistical significance was assessed by single-factor analysis of variance and significance is shown by ● = $P < 0.05$ and * = $P < 0.01$.

ity was expressed as the amount contained in each 20-min bile sample period as a percentage of that retained in the liver at termination of the experiment. This method of analysis was employed to avoid compromising the experiment by sampling the liver at the commencement of the experiment and to take account of the minor variations in i.v. injection efficiency.

HPLC Analysis of Biliary Metabolites

For the assessment of TAM metabolites, 20 μL of bile was added to 40 μL of 25% DMSO in methanol. After centrifugation (15,000 × *g*, 5 min, 4°), 45 μL of the supernatant was loaded onto the column. Over the 30-min HPLC run, fractions were collected and ^{14}C content assessed by scintillation counting. HPLC was carried out as previously described [32]. Unlabelled TAM in the bile, and included as a standard, was detected by UV absorption.

RESULTS

Induction of mdr *Gene Expression by TAM in Lewis Rats*

Induction of the *mdr1a*, *1b*, and *2* genes by TAM in Lewis rats was measured at 1 to 7, 14, 21, and 28 days after commencement of dosing on a continuous TAM exposure regime administered in the diet (420 ppm). Male rats showed an induction of *mdr1b* gene expression from day 2 that reached 8-fold over control by day 7 (Fig. 2C). Consistent induction of either *mdr1a* or *mdr2* gene expression was not observed in the livers of the TAM-treated male rats (Fig. 2A and E). Induction of *mdr1b* gene expression was not consistently observed in the female TAM-exposed rats until day 14 (Fig. 2D). By day 28, expression of *mdr1b* was increased 29- and 25-fold over control in male and female rats, respectively (Fig. 2C and D). In female rats, as in males, there was no significant increase in expression of the *mdr2* gene. There were two significant increases in *mdr1a* gene expression at days 6 and 21 in the female rats, but the increases seen here were not maintained in adjacent time points (Fig. 2B and F).

Excretion of TAM into the Bile by P-gp

Rats were pretreated for 12 days with TAM (35 mg/kg) or vehicle (tricaprylin 1 mL/kg). Three days after the last dose, the excretion rate of intravenously administered [^{14}C]TAM in the bile was assessed. The TAM-pretreated rats showed an average increase of 12-fold in *mdr1b* expression, and a concurrent marked increase in biliary [^{14}C]TAM excretion from 8 ± 1% to 51 ± 18% (mean ± SD) of the administered dose of 180 nmol/kg over a collection period of 3 hr (Fig. 3). HPLC analysis of the bile collected before the administration of [^{14}C]TAM showed no trace of unlabelled TAM or metabolites, detected by UV absorption, from the TAM-pretreated rats, confirming that unlabelled TAM administered to induce *mdr* gene family expression had been cleared (data not shown). HPLC analysis of radioactive biliary excretion products showed that the radioactivity was contained in two peaks. The major peak that eluted first was polar and probably contained glucuronidated metabolites, while the second peak was confirmed as unchanged TAM by comparison to a standard (Fig. 4).

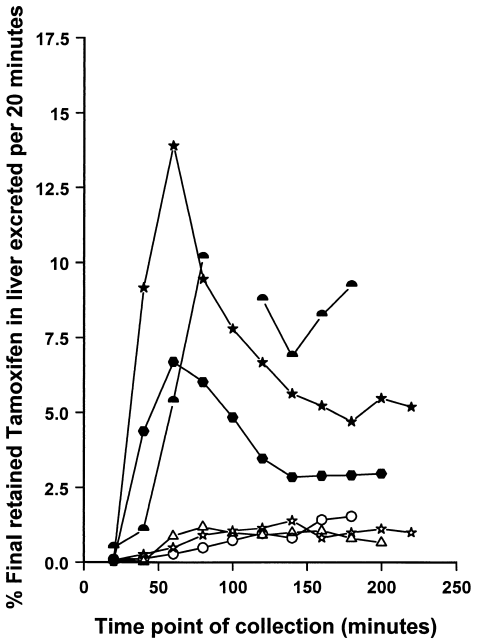

FIG. 3. Biliary excretion of ^{14}C from i.v.-administered [^{14}C]TAM in the bile of control and TAM-pretreated rats. Female Lewis rats (150–180 g) were pretreated with TAM (35 mg/kg) or vehicle control (1 mL/kg) by gavage for 12 days prior to use. Bile duct cannulations were performed on day 14. At time 0, the bile duct was cannulated and collection of bile in 20-min aliquots begun. At 20 min, [^{14}C]TAM (180 nmol/kg − 0.37 kBq/kg) was administered via the tail vein in a volume of 709 μL/kg. Bile was then collected in 20-min aliquots for the next 3 hr. After assessing the volume of each sample, a 20-μL aliquot of the bile was scintillation-counted for total radioactivity. The counts excreted in each 20-min time period were then expressed as a percentage of the total radioactivity in the liver at the termination of the experiment. Open symbols represent control animals and closed symbols TAM-treated animals. One data point is missing due to loss of sample. Each data set shown is from an individual animal.

DISCUSSION

Enhanced Liver Clearance of TAM in Pretreated Rats

We measured the clearance of TAM through the bile in female Lewis rats that had been pretreated with TAM for 12 days to increase P-gp levels. We found an enhanced excretion of both TAM and polar metabolites in the bile from these rats (Figs. 3 and 4). The enhanced excretion correlated with an induction of *mdr1b* gene expression and the *mdr* gene family protein product P-gp (data not shown). Previously, TAM has been shown to be a modulator of P-gp-mediated multidrug resistance *in vivo* and *in vitro*

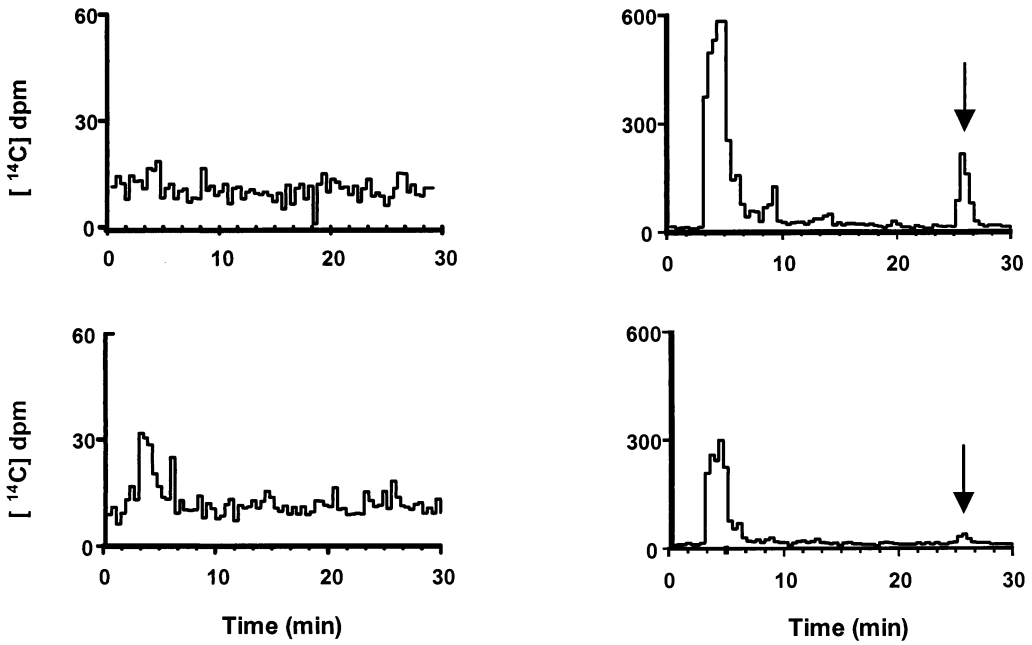

FIG. 4. HPLC analysis of radiolabel excreted in the bile. Bile (20 μL) was added to 40 μL of 25% DMSO in methanol. After centrifugation, 45 μL was loaded onto the column. Over the duration of the 30-min HPLC run period, 0.4-min fractions were collected and scintillation-counted. Results are from one control rat for the 3rd (A) and 7th (C) 20-min bile collections following cannulation. The same time points are shown for one TAM-pretreated rat (B and D, respectively). Unchanged excreted TAM is indicated by the arrow. Note the 10-fold difference in scale for the control and TAM-pretreated plots.

[34–40]. This arises as a result of its ability to interact with P-gp with a binding affinity comparable to that displayed by vinblastine, one of the best P-gp substrates [34, 38]. One study has suggested that TAM is not transported by P-gp and thus acts as a high-affinity but non-transported inhibitor [34]. However, Rao *et al.* [36] have demonstrated that TAM can increase ATPase activity in purified P-gp to the same extent as verapamil, which suggests that it binds both with high affinity to P-gp and is transported. As P-gp is an ATP-utilising protein, the increase in ATPase activity in the presence of TAM [36] suggests that substrate transport is taking place. Verapamil has been shown by direct means to be transported by P-gp, and therefore TAM and verapamil seem to belong to the same class of high affinity, transported P-gp inhibitors [41]. We have attempted to directly demonstrate that P-gp transports TAM using [^{14}C]TAM in cells with a high level of overexpression of *mdr1b*. However, this has proven to be extremely difficult due to the high TAM lypophilicity that causes it to adhere to the plasma membrane.

These data suggest an explanation for the findings of a previous study where, over a six-month period, the amount of TAM retained in the livers of Wistar and Lewis rats, continuously exposed to TAM in the diet, decreased [32]. We tested the livers of these rats for *mdr* gene family expression and found an increase of up to 58-fold in expression, confirming that TAM-induced P-gp was probably responsible for the decrease in TAM retention in the liver (data not shown). In this same study, TAM levels in the livers of Fischer rats similarly exposed were not decreased with time. These livers showed a much lower induction of *mdr* gene family expression compared with that in the Lewis and Wistar rats of the same study.

HPLC analysis of biliary-excreted ^{14}C label showed most of the products to be more polar than the known major phase I metabolites 4-hydroxytamoxifen and *N*-desmethyltamoxifen, and could in part represent products such as the glucuronide of 4-hydroxytamoxifen [9, 12, 42]. However, a significant proportion of the excreted radioactivity was unchanged TAM. TAM is able to induce cytochrome P-450(CYP) 2B and 3A families in rat liver. It is known that CYP3A is responsible for catalysing the *N*-demethylation of TAM, whereas 4-hydroxytamoxifen is metabolised by the non-inducible CYP2C8 [43]. It is therefore possible to hypothesise that the enhanced excretion of TAM metabolites we observed was not due to increased excretion by P-gp, but rather increased TAM metabolism by induced cytochrome P-450s. However, this does not explain the increased excretion rate of unchanged TAM that we observed in the bile of TAM-pretreated rats, which can only be explained by enhanced excretion. P-gp has not been shown to be able to pump polar phase II metabolites. Therefore, while the increased expression of P-gp can account for the increased excretion of unchanged TAM, it is unlikely to be responsible for the substantial excretion of polar metabolites. However, the multidrug resistance-related proteins (MRP) which, like P-gp, are members of the ATPase transporter superfamily of proteins, have a preferential substrate specificity for glutathione, glucuronide, and sulphate conjugates [44, 45]. Additionally, Kauffmann *et al.*

[46] have reported induction of *mrp2* in rhesus monkeys in response to tamoxifen. We therefore checked the expression of the *mrp1* and *2* genes using the B5 and EAG 15 antibodies [47], but found no increased expression in the livers of TAM-treated animals (data not shown). This could indicate either that i) an increase in MRP expression was not necessary to deal with the levels of phase II TAM metabolites being generated or that ii) another unidentified transport protein is responsible for their excretion. Another 3 members of the *mrp* family have been described, suggesting that this could be the case [48].

In humans, TAM is mainly excreted into the bile. The dosing regime used in these rats utilised a dose of TAM that gives a plasma concentration of about 948 ng/mL (2.5 μM) [32]. In high dose chemomodulatory trials, a 4–6-μM serum concentration of TAM is achieved when administered over 13 days [49]. Thus, the exposures used in this study are comparable and suggest that induction of the *MDR1* gene in humans could occur in response to TAM and give rise to an increased TAM clearance, and decreased plasma levels, after short periods of continuous exposure.

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