The Role of P-glycoprotein in the Bioactivation of Raloxifene

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Running title:

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Abbreviations:  Pgp, P-glycoprotein; GSH, glutathione; CYP, cytochrome P450; ABC, ATP-binding cassette; BBB, blood-brain barrier; NADPH, dihydronicotinamide adenine dinucleotide phosphate; MRP1, multidrug resistance-associated protein
Abstract

Drug transporters have been shown to alter drug metabolism. Similarly, bioactivation of drugs may also be altered by drug transporters. The aim of this work was to examine the role of P-glycoprotein (Pgp) in the bioactivation of a Pgp substrate, raloxifene, and a non-Pgp substrate, naphthalene. To evaluate the extent of bioactivation, covalent binding was measured. In both freshly isolated and cryopreserved hepatocytes, the extent of raloxifene covalent binding significantly increased (p<0.001) in the presence of verapamil whereas no change was observed with the covalent binding of naphthalene. To ascertain that the change was a Pgp effect, covalent binding was examined in microsomes where raloxifene and naphthalene covalent binding was not altered in the presence of verapamil. In addition, the measure of raloxifene-GSH adducts in the cryopreserved hepatocytes showed that the formation of the adducts increased in the presence of verapamil which supports the idea that blocking Pgp in the liver increases metabolism, and therefore, the bioactivation of raloxifene. Because raloxifene and naphthalene are known to undergo bioactivation mediated by CYP3A4, covalent binding in the presence of ketoconazole was examined. In both hepatocytes and microsomes, raloxifene covalent binding significantly decreased (p<0.001). Interestingly, naphthalene covalent binding was not affected. In the presence of CYP2E inhibitor 4-methylpyrazole, a decrease in naphthalene covalent binding was observed, suggesting that the formation of the 1,2-epoxide may be the main culprit contributing to naphthalene covalent binding. In conclusion, these data suggest that in addition to other “protective” mechanisms, Pgp may attenuate bioactivation of drugs.
Introduction

Drug metabolizing enzymes assist in the deactivation and elimination of xenobiotics from the body. On occasion, the mechanism that is in place to help deactivate foreign elements may produce a metabolite that is more or equally potent than the parent compound against a pharmacological target as with the glucuronidation of morphine to morphine 6-glucuronide against the μ-opioid receptor (Christensen and Jorgensen, 1987; Oguri et al., 1987). In other instances, the metabolic effort to detoxify via elimination may instead produce a reactive species that can bind irreversibly to biological macromolecules to elicit organ toxicity such as with acetaminophen (Jollow et al., 1973; Mitchell et al., 1973; Potter et al., 1973). This process known as bioactivation usually forms a reactive species with low electron density which is capable of forming an adduct with nucleophiles such as proteins (Zhou et al., 2005). Although the progression of covalent binding leading to toxicity is unclear and under investigation, a number of enzyme systems including phase I and phase II pathways have been associated with bioactivation. Among them are cytochrome P450 (CYP) enzymes that are responsible for the metabolism of many structurally diverse compounds and are expressed in many human tissues.

It has been previously demonstrated that CYP metabolism may be coupled to P-glycoprotein (Pgp), a drug transporter (Benet and Cummins, 2001; Cummins et al., 2002; Benet et al., 2003). The hypothesis was that Pgp may decrease the extent of CYP metabolism in the liver. Pgp is a 170 kDa protein comprising of 1280 amino acids and belongs to a superfamily of ATP binding cassette (ABC) proteins (Chen et al., 1986). It
is expressed in cancerous as well as non-cancerous tissues such as the brush border membrane of the intestine, liver and kidney and the blood-brain barrier (Silverman and Schrenk, 1997). Studies conducted in tumor cells have revealed a correlation between Pgp expression and decreased accumulation of cytotoxic compounds (Juliano and Ling, 1976; Goldstein et al., 1989; Choi et al., 1991) whereas studies in non-cancerous tissues have shown that the efflux properties of Pgp prevent foreign elements from crossing the Pgp barrier (Xie et al., 1999; Lee et al., 2000; Mahar Doan et al., 2002; Wang et al., 2004).

There are several arguments that can be made for the relationship that exists between CYP and Pgp activity including overlap of substrate specificities, common tissue localization and coinducibility (Cummins et al., 2002). In addition, CYP and Pgp serve a similar purpose which is detoxification and elimination. Consequently, if Pgp is able to indirectly alter CYP activity, the extent of drug bioactivation may also be modified by drug transporters for compounds that are substrates for both CYP and Pgp. Therefore, by limiting the access of a compound for metabolism through efflux, Pgp may help attenuate adverse reactions caused by reactive intermediates. Pgp may decrease bioactivation by several means. One is that Pgp may prevent access to certain systems such as with the blood brain barrier (BBB). It has been mentioned that one probable reason why the Pgp substrate loperamide does not elicit neurotoxicity is because it is denied access to the BBB (Kalgutkar and Nguyen, 2004). Another possibility is that Pgp may be able to quickly remove the xenobiotic before it has a chance to interact with the metabolizing enzymes in the liver by excreting it into the bile.
The goal of this paper was to study the contribution of Pgp on the bioactivation of naphthalene and raloxifene in cryopreserved and freshly isolated human hepatocytes. Mechanism of bioactivation for both compounds has been proposed earlier by other laboratories and is illustrated in Figure 1. Naphthalene is not a substrate for Pgp and its bioactivation is hypothesized to be the formation of an epoxide and quinone that is mediated by CYP1A, CYP3A and CYP2E1 (Tingle et al., 1993; Gram, 1997). Raloxifene, or Evista®, is selective estrogen receptor modulator marketed for the treatment of osteoporosis which can attain peak plasma concentrations of 1 nM in human (Hardman et al., 2001). It is a substrate for Pgp (Jeong et al., 2004) and its bioactivation is hypothesized to be mediated mainly by CYP3A4 (Chen et al., 2002). To measure the extent of bioactivation, irreversible binding of the reactive intermediate metabolites to proteins and the formation of raloxifene-GSH adducts were determined. By using specific inhibitors for Pgp (verapamil) and CYPs (ketoconazole for CYP3A and 4-methylprazolo for CYP2E), changes in the covalent binding and GSH adducts were monitored to investigate into the roles of Pgp and CYP enzymes in drug bioactivation.
Materials and Methods

Materials. Raloxifene, naphthalene, verapamil, ketoconazole, 4-methylpyrazole, reserpine, NADPH and 20% sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Co. (St. Louis, MO). \[^{14}\text{C}\] Naphthalene (0.055 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). \[^{3}\text{H}\] Raloxifene (21.14 Ci/mmol) was prepared in-house by Labeled Compound Synthesis group at Merck Research Laboratories. Radiopurity of all compounds were >98%. All solvents were of HPLC grade purchased from Sigma-Aldrich Co. Male liver microsomes pooled from 15 donors (Lot QNS) and cryopreserved human hepatocytes pooled from 5 donors (LiverPool\textsuperscript{TM}; Lot MOO) were purchased from In Vitro Technologies, Inc (Baltimore, MD). Freshly isolated human hepatocytes were purchased from In Vitro Technologies, Inc or CellzDirect (Pittsboro, NC).

Isolation of Cryopreserved Hepatocytes. Cryopreserved hepatocytes were thawed according to the procedure outline in the LiverPool\textsuperscript{TM} product sheet with minor modifications. Briefly, the vials containing the cryopreserved hepatocytes were thawed at 37°C for no longer than 120 seconds. The contents were transferred to a 50 mL Falcon tube containing 10 mL pre-warmed InVitroGRO HT Medium (In Vitro Technologies, Baltimore, MD). Another 1 mL of the growth medium was added to the vials to resuspend any remaining cells and the Falcon tube was filled to 50 mL. The hepatocytes were resuspended by gently inverting the tube several times. The cell suspension was then centrifuged at 50g at room temperature for 5 minutes. The supernatant was
discarded and resuspended in Williams E buffer containing L-glutamate. The cells were counted and the resulting viability of the cells was between 78-85%. The cells were prepared at 1 million/mL concentration. All incubations were done at 37°C under 95%/5% Oxygen/Carbon Dioxide atmosphere.

*Covalent Binding in Human Liver Microsomes.* Covalent binding was determined following the “filtrate” protocol by Day et al. with minor modifications (Day et al., 2005). Briefly, 1 mg/mL microsomes were preincubated with 1 mM NADPH in 100 mM potassium phosphate buffer at 37°C for at least 5 minutes on a dry-bath incubator. To start the reaction, [14C]naphthalene or [3H]raloxifene was added to the incubation to give a final concentration of 10 µM in the absence or presence of various inhibitors for Pgp (1 to 500 µM verapamil), CYP3A (0.05 to 1 µM ketoconazole) or CYP2E (10 to 5000 µM 4-methylpyrazole). After 2 hour incubation, the reaction was stopped with 400 µL of acetone. The reaction was vortexed carefully and another 400 µL of acetone was added a second time for a two-step quench process. Precipitated proteins were collected onto a Whatman GF/B brand glass filter mats (Brandel, Gaithersburg, MD) using a Brandel cell/membrane harvester (Brandel, Gaithersburg, MD). The filter and the protein were washed with 80% methanol for at least 5 times. The individual filters were collected into a 5 mL scintillation vial. 1 mL of 7.5% SDS solution was added and placed into a rotary water bath shaker overnight at 55°C. On the following morning, the vials were allowed to cool to room temperature. 4 mL of Ultima Gold scintillation cocktail (Sigma-Aldrich Co., St. Louis, MO) was added before counting on the liquid scintillation counter.
Control reactions were run in the absence of NADPH or in the presence of 10 mM GSH. All reactions were performed in triplicates.

**Covalent Binding in Hepatocytes.** 250 µL of hepatocytes were aliquoted to a 48-well polystyrene Falcon plate (Fisher Scientific, Pittsburg, PA) and preincubated for 5-10 minutes. To start the reaction, [14C]naphthalene or [3H]raloxifene was added to give a final incubation concentration of 10 µM with or without various inhibitors for Pgp (1 to 500 µM verapamil), CYP3A (0.05 to 1 µM ketoconazole) or CYP2E (10 to 5000 µM 4-methylpyrazole). The reaction was stopped with 400 µL acetone after 2 hour incubation. The precipitated proteins were recovered using the above method outlined for microsomes. Hepatocyte covalent binding calculations were based on a protein content of 1.7 mg/million cells.

**Intracellular Raloxifene-GSH Levels in Hepatocytes.** 2 mL of cryopreserved hepatocytes were preincubated for 5-10 minutes. Raloxifene (10 µM) was then incubated for 2 hr in the hepatocytes with either verapamil (100 µM), ketoconazole (1 µM) or methanol (control). The reaction was stopped with 2 mL of methanol. The reaction mixture was centrifuged at 3000g for 10 minutes and dried down with nitrogen. 200 µL acetonitrile containing reserpine, as an internal standard, was used to reconstitute the reaction mixture. 50-100 µL were injected onto a Thermo-Finnigan TSQ Quantum Ultra mass spectrometer equipped with Agilent 1100 LC-pumps and a CTC-PAL autosampler. Chromatographic conditions were adopted from Chen et al. with modifications (Chen et
Mobile phase A (0.1% formic acid in 18 MΩ water) and B (0.1% formic acid in acetonitrile) were pumped through a Zorbax Rx-C8 (4.6mm x 25cm) column (Agilent Technologies, Palo Alto, CA) at a flow rate of 1 mL/min starting with a 7 minute isocratic hold at 10% B followed by a 8 minute linear gradient to 20% B. After a 7 minute isocratic hold at 20% B, there was a 5 minute linear gradient to 30% B. Following an isocratic hold for 5 minutes at 30% B, there was a 8 minute linear gradient to 95% B. Followed an isocratic hold for 2 minutes, there was a 4 minute linear gradient to 10% B. This was followed by a 4 minute isocratic hold at 90% B for column equilibration. Raloxifene, the raloxifene-GSH adducts, and reserpine (internal standard) were monitored in the single reaction monitoring (SRM) mode with transitions of $m/z$ 474 → 112, $m/z$ 779 → 112, and $m/z$ 609 → 195, respectively. Since synthetic standards of raloxifene-GSH adducts were not available, the peak areas were integrated and the ratios of GSH-adduct peak area to internal standard peak area were calculated and compared between each sample. Since the highest amount of raloxifene-GSH adduct formed was not greater than 500 pmol drug equivalent/mg protein, it was assumed that the detector response was linear across the concentration range of GSH-adducts formed.
Results

Covalent Binding in Microsomes. Covalent binding was examined in microsomes prepared from pooled human livers and are summarized in Table 1. The extent of covalent binding was $589 \pm 13$ and $1950 \pm 140$ pmol drug equivalent/mg protein for raloxifene and naphthalene, respectively. Covalent binding values for naphthalene are consistent with previously published value by Day et al. (Day et al., 2005) When the reaction was carried out in the absence of NADPH, the extent of covalent binding decreased to $34.1 \pm 5.2$ and $250 \pm 6$ pmol drug equivalent/mg protein for raloxifene and naphthalene, respectively, suggesting that the main route of bioactivation was P450 mediated. These data support previous claims that bioactivation for raloxifene and naphthalene is P450 mediated. In the presence of GSH, the covalent binding attenuated to $92 \pm 11$ and $35 \pm 4$ pmol drug equivalent/mg protein with raloxifene and naphthalene, respectively.

The effect of CYP and Pgp inhibitors on covalent binding of raloxifene and naphthalene were also examined in microsomes. Ketoconazole is a potent CYP3A inhibitor which can attain peak plasma concentrations of 3.2 µM in human (Hardman et al., 2001). The extent of covalent binding of raloxifene decreased in a concentration dependent manner with increasing concentrations of ketoconazole. At 1µM ketoconazole, the covalent binding was $493 \pm 33$ pmol drug equivalent/mg protein (83% that of control) suggesting that other CYP enzymes in addition to CYP3A may be involved in the bioactivation of raloxifene. Interestingly, ketoconazole had no effect on the covalent binding of naphthalene. Instead, 4-methylpyrazole, which is a potent inhibitor of CYP2E, decreased
the covalent binding of naphthalene in a concentration dependent manner. At a final concentration of 500 µM 4-methylpyrazole, the covalent binding was 190 ± 4 pmol drug equivalent/mg protein.

Verapamil is a potent inhibitor of Pgp which can attain peak plasma concentrations of 600 nM in human (Hardman et al., 2001). Various concentrations of verapamil were coincubated with either raloxifene or naphthalene to determine the effect of verapamil on covalent binding. Table 1 show that the Pgp inhibitor had no effect on the covalent binding of either compounds examined.

Covalent Binding in Hepatocytes. Covalent binding was examined in three separate lots of freshly isolated human hepatocytes. Both raloxifene and naphthalene exhibited measurable covalent binding at 82.3 ± 22.9 and 210 ± 43 pmol drug equivalent/mg protein, respectively. As summarized in Table 2, data show that ketoconazole significantly decreased (p<0.005) the covalent binding of raloxifene. However, consistent with the microsome data, only 4-methylpyrazole, and not ketoconazole, significantly decreased (p<0.005) the covalent binding of naphthalene. In the presence of verapamil, the covalent binding of raloxifene significantly increased (p<0.005). However, verapamil had no effect on the non-Pgp substrate naphthalene.

Cryopreserved human hepatocytes pooled from 5 donors were also employed to examine the covalent binding of raloxifene. As listed in Table 3, the extent of covalent binding in the cryopreserved hepatocytes was similar to the values observed in fresh hepatocytes. In
addition, as observed in fresh hepatocytes, the extent of covalent binding significantly decreased (p<0.005) in the presence of ketoconazole and significantly increased (p<0.005) in the presence of verapamil.

Intracellular Raloxifene-GSH Adducts in Cryopreserved Hepatocytes. The goal of this paper was to examine the role of Pgp in the bioactivation of Pgp substrates. Therefore, in order to examine if the changes of covalent binding in the hepatocytes were a direct result of changes in the bioactivation of raloxifene, intracellular levels of raloxifene-GSH adduct were determined. Three raloxifene-GSH adducts have been previously proposed and have been elucidated with the LC/MS/MS (Chen et al., 2002). Figure 2 shows the total amount of raloxifene-GSH adducts that were produced in the pooled cryopreserved hepatocytes in the absence or presence of various inhibitors. In the presence of ketoconazole, the amount of raloxifene-GSH adduct formed significantly decreased (p<0.005) when compared to the methanol control which is consistent with the observed decrease of covalent binding. In addition, the raloxifene-GSH adduct significantly increased (p<0.005) in the presence of verapamil, which is consistent with the observed increase of covalent binding.
Discussion

There are several defense mechanisms that the body has created to prevent foreign molecules from reaching their intended target. One mechanism is the enzymatic conversion of the intruding molecules to more hydrophilic moieties mediated by drug metabolizing enzymes such as CYPs. These metabolites may undergo further metabolism by means of conjugation which helps facilitate elimination through excretion into the urine or the bile. CYPs have broad substrate specificities and are almost ubiquitously distributed with high expression in the liver and the intestine ensuring that the drug is quickly and efficiently eliminated. However, despite the efforts for detoxification, some compounds may become bioactivated to produce chemically reactive intermediates that can irreversibly bind to DNA or proteins. Once these macromolecules become altered, toxicity may ensue from loss of activity or trigger an auto-immune response.

Another defense mechanism is the plasma membrane that surrounds the cells. In addition to providing a physical barrier, it may express efflux drug transporters such as Pgp to actively prevent access of drugs. When neighboring cells form tight junctions, a protective partition is formed to thwart unwanted intrusions as observed with the BBB and the intestine. Previously, it has been put forward that drug transporters may modulate metabolism (Benet and Cummins, 2001; Cummins et al., 2002; Johnson et al., 2003). It has been hypothesized that if a compound is a substrate for both drug transporter and drug metabolizing enzyme, the access of the particular compound to the enzyme may be determined by the transporter. Therefore, in the liver...
creates a gradient favoring excretion into the bile, the extent of metabolism of a Pgp substrate may be lower than for a non-Pgp substrate. Since the liver is one of the primary sites for metabolism and bioactivation, this work aims to examine the role of Pgp in the bioactivation of a Pgp substrate and a non-Pgp substrate using human hepatocytes. Covalent binding was employed as a tool in an attempt to measure the extent of bioactivation.

It has been previously shown that raloxifene is a substrate for drug transporters including Pgp and MRP (Jeong et al., 2004), whereas naphthalene is not a substrate for active efflux transporters. If the process of bioactivation is coupled with Pgp, the extent of covalent binding is expected to increase for Pgp substrates in the presence of verapamil because as Pgp becomes blocked, the opportunity for the Pgp substrate to interact with the enzyme to form reactive intermediates increases. Verapamil is commonly utilized to block Pgp. In the presence of verapamil, the covalent binding of raloxifene in freshly isolated hepatocytes was significantly higher than when compared with no inhibitor. Contrarily, verapamil had no effect on the covalent binding of the non-Pgp substrate naphthalene. These data summarized in Table 2 suggest that Pgp was capable of modulating the extent of covalent binding and therefore, bioactivation. In order to ascertain the role of transporters, covalent binding was examined in microsomes prepared from pooled human livers. The difference between microsomes and hepatocytes is that hepatocytes are self-sustaining living cells expressing cellular components that microsomes lack, including the plasma membrane and transporters. If verapamil was indeed responsible for the modulation of covalent binding of raloxifene, blocking Pgp
should have no effect in the microsomes. As it turns out, the extent of covalent binding was not altered in microsomes for both raloxifene and naphthalene as shown in Table 1.

Although verapamil is oftentimes used as a potent inhibitor of Pgp, it has also been connected with the inhibition of testosterone 6β-hydroxylation (Yeo and Yeo, 2001), a marker for CYP3A4 activity. However, the mechanism for inhibition was time-dependent and greater than 50% inhibition was only observed when it was preincubated for at least 25 minutes. Furthermore, the IC₅₀ values of testosterone 6β-hydroxylation were 9-fold lower when preincubated compared to coincubation conditions. As outlined in the Materials and Methods section, the inhibitors in these experiments were not preincubated and consequently, the covalent binding did not change in the microsomes, suggesting that the metabolism component was not altered and that changes observed in hepatocytes were most likely due to the blocking of the Pgp component. Yet, even if verapamil had blocked metabolism in these experiments, the increase of the covalent binding values in the hepatocytes would not have been explainable by inhibition of the metabolism component.

One alternate explanation as to how verapamil may be increasing the covalent binding of raloxifene may be that it has been linked to GSH depletion. It has been previously demonstrated that 100 µM verapamil may be able to stimulate MRP1 mediated efflux of GSH (Loe et al., 2000). Therefore, if GSH is completely depleted, the reactive intermediates would be more available to bind irreversibly to macromolecules. There are several reasons why GSH depletion cannot be the reason for increased covalent binding
of raloxifene. First, unlike the T14 (HeLa) cells that were used in the former experiments, hepatocytes are capable of generating glutathione. After 2 hour incubation, it is unlikely that GSH would be completely depleted in the hepatocytes. Second, if GSH depletion resulted in the increase covalent binding of raloxifene, the covalent binding for naphthalene should have increased as well. However, Table 2 shows that the covalent binding of naphthalene was not affected in the presence of verapamil. Finally, Figure 2 shows that in the presence of verapamil, the amount of raloxifene-GSH adduct increased when compared to the control lacking verapamil. The increase with verapamil is consistent with this paper’s hypothesis that Pgp may modulate bioactivation. By blocking Pgp, the metabolism, and therefore bioactivation, is increased as more raloxifene is able to interact with the enzyme.

Blocking metabolism decreases bioactivation and thus, the extent of covalent binding is expected to decrease. The mechanism of bioactivation for raloxifene and naphthalene is illustrated in Figure 1. The mechanism proposed for raloxifene bioactivation is the formation of arene oxides or a quinone mediated by CYP3A4 (Chen et al., 2002). The mechanism proposed for naphthalene bioactivation is the formation of the 1,2-epoxide mediated by CYP1A2, CYP3A4 and CYP2E1, followed by the formation of quinones mediated by CYP1A2 and CYP3A4 (Wilson et al., 1996). However, the formation of 1-naphthol, which is an intermediate to the formation of the quinones, is not mediated by P450s but mediated by spontaneous chemical rearrangement (Jerina et al., 1970).
The extent of covalent binding was examined in the presence of 1 µM ketoconazole. In incubations with microsomes (Table 1) and hepatocytes (Table 2), raloxifene covalent binding decreased as expected. Interestingly however, ketoconazole was not able to change naphthalene covalent binding (Table 1 and Table 3). Instead, 500 µM 4-methylpyrazole, an inhibitor for CYP2E1, significantly decreased the covalent binding of naphthalene. Although more follow-up studies are needed, these data with naphthalene suggest that CYP2E1 may play a major role in naphthalene bioactivation and that 1,2-epoxide may be the critical intermediate in eliciting irreversible binding.

Covalent binding was examined in pooled cryopreserved human hepatocytes (Table 3). Although there may be damage associated with the freeze-thaw cycles of cryopreserved cells, studies have shown that CYP activities are well intact (Li et al., 1999). Consequently, raloxifene covalent binding significantly decreased in the presence of ketoconazole. Additionally, it has been shown that uptake transporters involved in the movement of taurocholate and estradiol 17β-glucuronide were functioning in cryopreserved hepatocytes (Shitara et al., 2003). Although the fate of Pgp in cryopreserved hepatocytes is unknown, increases of covalent binding in the presence of verapamil suggest that the Pgp function is intact as well. Overall, the covalent binding in the fresh hepatocytes seemed to be a bit lower than in cryopreserved hepatocytes. This difference may be attributed to intracellular glutathione concentrations. Though unknown in human, the amount of intracellular glutathione in cryopreserved rat hepatocytes is about 10% that of freshly isolated hepatocytes (Sohlenius-Sternbeck and Schmidt, 2005).
The work presented here examines the role of Pgp in the bioactivation of raloxifene. By measuring covalent binding, the role of Pgp in bioactivation was investigated. As with CYPs, Pgp is a defense mechanism. In addition to playing goalkeeper, it may have been selected for to help attenuate any adverse reactions that may arise from the generation of unforeseen reactive metabolites. It is interesting to note that while raloxifene and tamoxifen are similar in structure, only tamoxifen is associated with heptatotoxicity. Same observation can be made with haloperidol and loperamide, where only haloperidol is linked with neurotoxicity. The difference between the two compounds is that compounds that are Pgp substrates are not associated with any known toxicity. More studies are needed to understand the relationship between Pgp and bioactivation and how this may be relevant in vivo. In summary, blocking Pgp in the hepatocytes increased raloxifene covalent binding and the formation of raloxifene-GSH adducts. The utility of cryopreserved hepatocytes in examining Pgp activity and bioactivation was demonstrated. Finally, the formation of the 1,2-epoxide metabolite via CYP2E1 is suggested to be one of the major mechanism of naphthalene bioactivation.
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References


Christensen CB and Jorgensen LN (1987) Morphine-6-glucuronide has high affinity for the opioid receptor. Pharmacol Toxicol 60:75-76.


Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR and Brodie BB (1973)
Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J

binding of morphine and nalorphine to opioid delta receptor by glucuronate and
sulfate conjugations at the 6-position. Life Sci 41:1457-1464.

Potter WZ, Davis DC, Mitchell JR, Jollow DJ, Gillette JR and Brodie BB (1973)
Acetaminophen-induced hepatic necrosis. 3. Cytochrome P-450-mediated

transporters for taurocholate and estradiol 17beta-D-glucuronide in cryopreserved

Silverman JA and Schrenk D (1997) Hepatic canalicular membrane 4: expression of the
multidrug resistance genes in the liver. Faseb J 11:308-313.

Sohlenius-Sternbeck AK and Schmidt S (2005) Impaired glutathione-conjugating

Tingle MD, Pirmohamed M, Templeton E, Wilson AS, Madden S, Kitteringham NR and
Park BK (1993) An investigation of the formation of cytotoxic, genotoxic,
protein-reactive and stable metabolites from naphthalene by human liver

penetration of methadone (R)- and (S)-enantiomers is greatly increased by P-
glycoprotein deficiency in the blood-brain barrier of Abcb1a gene knockout mice.

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Legend

Figure 1. Proposed mechanism of bioactivation for raloxifene (Chen et al., 2002) and naphthalene (Wilson et al., 1996). Bioactivation of raloxifene has been hypothesized to be mediated by the formation of arene oxide or quinone intermediate via CYP3A4. Bioactivation of naphthalene has been hypothesized to be mediated by the formation of 1,2-epoxide and/or quinones. Data examined in this paper suggest that the formation of 1,2-epoxide via CYP2E1 may be the major cause of irreversible binding of naphthalene.

Figure 2. Measure of raloxifene-GSH adducts found in the cryopreserved hepatocytes. Adducts with m/z 779 were monitored using LC/MS following incubation in the absence (control) or in the presence of verapamil or ketoconazole. The values were normalized to those that lack inhibitors and reported as % Control. All experiments were done in triplicate and the statistics were done using the T-test function in Excel. Star (*) represents p<0.005.
Table 1. Mean covalent binding of $[^{14}C]$ naphthalene and $[^{3}H]$ raloxifene in human liver microsomes. Incubation conditions outlined in Materials and Methods were followed except where specified. All experiments were done in triplicates and the statistics were done using the T-test function in Excel.

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<td>pmol/mg protein</td>
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<td>Control T=120</td>
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<td>2210 ± 38</td>
<td>619 ± 52</td>
</tr>
<tr>
<td>4-Methylpyrazole (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>5000</td>
<td>27 ± 1*</td>
<td>NA</td>
</tr>
<tr>
<td>1000</td>
<td>104 ± 12*</td>
<td>NA</td>
</tr>
<tr>
<td>500</td>
<td>190 ± 4*</td>
<td>NA</td>
</tr>
<tr>
<td>250</td>
<td>311 ± 26*</td>
<td>NA</td>
</tr>
<tr>
<td>100</td>
<td>447 ± 16*</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>622 ± 22*</td>
<td>NA</td>
</tr>
</tbody>
</table>

* p<0.01
NA = Data not available
Table 2. Mean covalent binding of $[^3]$H raloxifene and $[^{14}]$C naphthalene in freshly isolated hepatocytes. All experiments were done in triplicates and the statistics were done using the T-test function in Excel.

<table>
<thead>
<tr>
<th></th>
<th>Fresh Lot 1</th>
<th>Fresh Lot 2</th>
<th>Fresh Lot 3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3]$H Raloxifene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (T=120 min)</td>
<td>74.3 ± 7.3</td>
<td>109 ± 17</td>
<td>63.1 ± 3.7</td>
<td>82.3 ± 16</td>
</tr>
<tr>
<td>100 µM Verapamil</td>
<td>106 ± 8*</td>
<td>165 ± 11*</td>
<td>111 ± 15*</td>
<td>127 ± 22**</td>
</tr>
<tr>
<td>1 µM Ketoconazole</td>
<td>29.2 ± 3.3**</td>
<td>39.3 ± 4.9**</td>
<td>24.4 ± 1.6**</td>
<td>31.0 ± 6.5**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Fresh Lot 4</th>
<th>Fresh Lot 5</th>
<th>Fresh Lot 6</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{14}]$C Naphthalene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (T=120 min)</td>
<td>224 ± 28</td>
<td>165 ± 21</td>
<td>240 ± 38</td>
<td>210 ± 50</td>
</tr>
<tr>
<td>100 µM Verapamil</td>
<td>261 ± 6</td>
<td>223 ± 20</td>
<td>176 ± 18</td>
<td>220 ± 30</td>
</tr>
<tr>
<td>1 µM Ketoconazole</td>
<td>234 ± 2</td>
<td>188 ± 15</td>
<td>163 ± 10</td>
<td>195 ± 20</td>
</tr>
<tr>
<td>500 µM 4-Methylpyrazine</td>
<td>149 ± 4*</td>
<td>96.6 ± 23.1*</td>
<td>109 ± 7**</td>
<td>118 ± 30**</td>
</tr>
</tbody>
</table>

* p < 0.01  
** p < 0.005
Table 3. Mean covalent binding of [$^3$H] raloxifene in pooled cryopreserved human hepatocytes. All experiments were done in triplicates and the statistics were done using the T-test function in Excel.

<table>
<thead>
<tr>
<th>$[^3]$H Raloxifene</th>
<th>Covalent Binding (pmol drug equivalent/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (T = 120 min)</td>
<td>131 ± 5.8</td>
</tr>
<tr>
<td>100 µM Verapamil</td>
<td>181 ± 8.0**</td>
</tr>
<tr>
<td>10 µM Verapamil</td>
<td>152 ± 9.5*</td>
</tr>
<tr>
<td>1 µM Ketoconazole</td>
<td>67 ± 7.6**</td>
</tr>
<tr>
<td>0.1 µM Ketoconazole</td>
<td>109 ± 6.4*</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.001
**Figure 1**

Proposed position of the glutathionyl moiety for each of the three adducts.

Adduct 1: \( \text{R1 OH} \)  
Adduct 2: \( \text{R1 SG, R2 OH} \)

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Figure 2

GSH Formation (% Control)

10 µM Raloxifene + Methanol

10 µM Raloxifene + 100 µM Verapamil

10 µM Raloxifene + 1 µM Ketoconazole

*