The Role of P-glycoprotein in the Bioactivation of Raloxifene

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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 increased significantly ($p < 0.05$) 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 in which raloxifene and naphthalene covalent binding was not altered in the presence of verapamil. In addition, the measure of raloxifene-glutathione 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 decreased significantly ($p < 0.01$). It is interesting that naphthalene covalent binding was not affected. In the presence of the 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.

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 potent than the parent compound or equally potent against a pharmacological target, as with the glucuronidation of morphine to morphine 6-glucuronide against the $\mu$-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 that 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 (P450) 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 P450 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 P450 metabolism in the liver. Pgp is a 170-kDa protein comprising 1280 amino acids and belongs to a superfamily of ATP-binding cassette proteins (Chen et al., 1986). It is expressed in cancerous as well as noncancerous tissues such as the brush-border membrane of the intestine, liver, and kidney and the blood-brain barrier (BBB) (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 noncancerous tissues have shown that the efflux properties of Pgp prevent foreign elements from crossing the Pgp barrier (Matsuo 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 P450 and Pgp activity, including overlap of substrate specificities, common tissue localization, and coinducibility (Cummins et al., 2002). In addition, P450 and Pgp serve a similar purpose, which is detoxification and elimination. Consequently, if Pgp is able to indirectly alter P450 activity, the extent of drug bioactivation may also be modified by drug transporters for compounds that are substrates for both P450 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 the BBB. It has been mentioned that one probable reason that the Pgp substrate loperamide does not elicit neurotoxicity is that it is denied access to the BBB (Kalgutkar and Nguyen, 2004). Second 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 article was to study the contribution of Pgp to the

ABBREVIATIONS: P450, cytochrome P450; Pgp, P-glycoprotein; GSH, glutathione; BBB, blood-brain barrier.
bioactivation of naphthalene and raloxifene in cryopreserved and freshly isolated human hepatocytes. A mechanism of bioactivation for both compounds has been proposed earlier by other laboratories and is illustrated in Fig. 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 a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997). Raloxifene, or Evista, is a selective estrogen receptor modulator, marketed for the treatment of osteoporosis, that can attain peak concentrations of 1 nM in humans (Hardman et al., 1993; Gram, 1997).

Materials and Methods

Materials. Raloxifene, naphthalene, verapamil, ketoconazole, 4-methylpyrazole, reserpine, NADPH, and 20% sodium dodecyl sulfate were purchased from Sigma-Aldrich (St. Louis, MO). [14C]Naphthalene (0.055 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [3H]Raloxifene (21.14 Ci/mmol) was prepared in-house by the Labeled compound Synthesis group at Merck Research Laboratories (Rahway, NJ). Radiopurity of all compounds was >98%. All solvents were of high-performance liquid chromatography grade, purchased from Sigma-Aldrich (St. Louis, MO).

Isolation of Cryopreserved Hepatocytes. Cryopreserved hepatocytes were thawed according to the procedure outlined in the LiverPool product sheet (In Vitro Technologies, Inc.) with minor modifications. In brief, the vials containing the cryopreserved hepatocytes were thawed at 37°C for no longer than 120 s. The contents were transferred to a 50-ml Falcon tube containing 10 ml of prewarmed InVitroGRO HT Medium (In Vitro Technologies, Inc.). 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 min. The supernatant was discarded and resuspended in Williams E buffer containing L-glutamine. The cells were counted, and the resulting viability of the cells was between 78 and 85%. The cells were prepared at 1 x 10⁶/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. (2005) with minor modifications. In brief, 1 mg/ml microsomes were preincubated with 1 mM NADPH in 100 mM potassium phosphate buffer at 37°C for at least 5 min 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 (1–500 nM 4-methylpyrazole). After a 2-h incubation, the reaction was stopped with 400 μl of acetone. The reaction mixture was vortexed carefully, and another 400 μl of acetone was added a second time for a two-step quench process. Precipitated proteins were collected into a 5-ml scintillation vial; 1 ml of 7.5% sodium dodecyl sulfate solution was added and placed into a rotary water bath shaker overnight at 55°C. The following morning, the vials were allowed to cool to room temperature, and 4 ml of Ultima Gold scintillation cocktail (Sigma-Aldrich) was added before counting with 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 triplicate.

Covalent Binding in Hepatocytes. Hepatocytes (250 μl) were aliquoted to a 48-well polystyrene Falcon plate (Fisher Scientific, Pittsburgh, PA) and preincubated for 5 to 10 min. 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 (1–500 μM verapamil), CYP3A (0.05–1 μM ketoconazole), or CYP2E (10–5000 μM 4-methylpyrazole). The reaction was stopped with 400 μl of acetonitrile after 2 h of incubation. The precipitated proteins were recovered using the method outlined above for microsomes. Hepatocyte covalent binding calculations were based on a protein content of 1.7 mg/10^6 cells.

**Intracellular Raloxifene-GSH Levels in Hepatocytes.** Cryopreserved hepatocytes (2 ml) were preincubated for 5 to 10 min. Raloxifene (10 μM) was then incubated for 2 h 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 min and dried down with nitrogen. Acetonitrile (200 μl) containing reserpine as an internal standard was used to reconstitute the reagents mixture. Then, 50 to 100 μl were injected onto a Thermo Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Electron Corporation, Waltham, MA) equipped with Agilent 1100 LC pumps (Agilent Technologies, Palo Alto, CA) and a CTC-PAL autosampler (LEAP Technologies, Carrboro, NC). Chromatographic conditions were adopted from Chen et al. (2002) with modifications. Mobile phases A (0.1% formic acid in 18 MΩ water) and B (0.1% formic acid in acetonitrile) were pumped through a Zorbax Rx-C8 (4.6 mm × 25 cm) column (Agilent Technologies) at a flow rate of 1 ml/min starting with a 7-min isocratic hold at 10% B, followed by an 8-min linear gradient to 20% B. After a 7-min isocratic hold at 20% B, there was a 5-min linear gradient to 30% B. After an isocratic hold for 5 min at 30% B, there was an 8-min linear gradient to 95% B. After an isocratic hold for 2 min, there was a 4-min linear gradient to 10% B. This was followed by a 4-min isocratic hold at 90% B for column equilibration. Raloxifene, the raloxifene-GSH adducts, and reserpine (internal standard) were monitored in the single reaction monitoring mode with transition m/z 474 → 112, m/z 779 → 112, and m/z 609 → 195, respectively. Because 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. Because the highest amount of raloxifene-GSH adduct formed was not greater than 500 pmol of drug equivalent/mg of 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 ± 13 and 1950 ± 144 pmol of drug equivalent/mg of protein for raloxifene and naphthalene, respectively. Covalent binding values for naphthalene are consistent with previously published values by Day et al. (2005). When the reaction was carried out in the absence of NADPH, the extent of covalent binding decreased to 34 ± 5 and 250 ± 6 pmol of drug equivalent/mg of protein for raloxifene and naphthalene, respectively, suggesting that the main route of bioactivation was P450-mediated. These data support previous claims that bioactivation of raloxifene and naphthalene is P450-mediated. In the presence of GSH, the covalent binding attenuated to 92 ± 11 and 35 ± 4 pmol of drug equivalent/mg of protein with raloxifene and naphthalene, respectively.

The effects of P450 and Pgp inhibitors on covalent binding of raloxifene and naphthalene were also examined in microsomes. Ketoconazole is a potent CYP3A inhibitor that can attain peak concentrations of 3.2 μM in humans (Hardman et al., 2001). The extent of covalent binding of raloxifene decreased in a concentration-dependent manner with increasing concentrations of ketoconazole. At 1 μM ketoconazole, the covalent binding was 493 ± 33 pmol of drug equivalent/mg of protein (83% of that of control), suggesting that other P450 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 of drug equivalent/mg of protein.

Verapamil is a potent inhibitor of Pgp that can attain peak concentrations of 600 nM in humans (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 shows that the Pgp inhibitor had no effect on the covalent binding of either compound 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 ± 16 and 210 ± 50 pmol of drug equivalent/mg of 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 five donors were also used 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 article was to examine the role of Pgp in the bioactivation of Pgp substrates. Therefore, to examine whether the
changes in 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 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 P450s. These metabolites may undergo further metabolism by means of conjugation, which helps facilitate elimination through excretion into the urine or the bile. P450s 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 autoimmune 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, where the Pgp creates a gradient favoring excretion into the bile, the extent of metabolism of a Pgp substrate may be lower than that for a non-Pgp substrate. Because 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 used 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 used to block Pgp. In the presence of verapamil, the covalent binding of raloxifene in freshly isolated hepatocytes was significantly higher compared with binding with no inhibitor. On the contrary, 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. To ascertain the role of transporters, covalent binding was examined in microsomes prepared from pooled human livers. The difference between microsomes increases. Verapamil is commonly used to block Pgp. In the presence of verapamil, the covalent binding of raloxifene in freshly isolated hepatocytes was significantly higher compared with binding with no inhibitor. On the contrary, 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. 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 were 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 often used as a potent inhibitor of Pgp, it has also been connected with the inhibition of testosterone 6β-hydroxylase (Yeo and Yeo, 2001), a marker for CYP3A4 activity. However, the mechanism for inhibition was time-dependent, and greater than
of 1-naphthol, which is an intermediate to the formation of the CYP1A2 and CYP3A4 (Wilson et al., 1996). However, the formation CYP2E1, followed by the formation of quinones mediated by oxidation of the 1,2-epoxide mediated by CYP1A2, CYP3A4, and CYP2E1, followed by the formation of quinones mediated by CYP3A4 (Chen et al., 2002). The mechanism proposed for naphthalene bioactivation is the formation of arene oxides or a quinone mediated by CYP3A4 (Chen et al., 2002). 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 (Tables 1 and 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 P450 activities are well intact (Li et al., 1999). Consequently, raloxifene covalent binding significantly decreased in the presence of verapamil. In addition, 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 that in cryopreserved hepatocytes. This difference may be attributed to intracellular glutathione concentrations. Although unknown in humans, the amount of intracellular glutathione in cryopreserved rat hepatocytes is approximately 10% of 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 P450s, Pgp is a defense mechanism. In addition to playing goalkeeper, it may have been selected for in order to help attenuate any adverse reactions that may arise from the generation of unforeseen reactive metabolites. It is interesting to note that although raloxifene and tamoxifen are similar in structure, only tamoxifen is associated with hepatotoxicity. The 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 quinones, is not mediated by P450s but by spontaneous chemical rearrangement (Jerina et al., 1970).

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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 mechanisms of naphthalene bioactivation.

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