0090-9556/03/3106-773–775$7.00
DRUG METABOLISM AND DISPOSITION
Vol. 31, No. 6
1034/1068329
DMD 31:773–775, 2003
Printed in U.S.A.

INDUCTION OF HEPATIC PHASE II DRUG-METABOLIZING ENZYMES BY 1,7-PHENANTHROLINE IN RATS IS ACCOMPANIED BY INDUCTION OF MRP3

SUI WANG, DYLAN P. HARTLEY, SUZANNE L. CICCOTTO, STELLA H. VINCENT, RONALD B. FRANKLIN, AND MI-SOOK KIM

Department of Drug Metabolism, Merck Research Laboratories, Rahway, New Jersey

(Received December 10, 2002; accepted March 12, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The purpose of the present study was to evaluate the effect of 1,7-phenanthroline (PH), which has been proposed to be a selective phase II enzyme inducer, on the gene expression of xenobiotic transporters, as well as hepatic and renal drug-metabolizing enzymes. After oral administration of PH for 3 days to male Sprague-Dawley rats, mRNA levels in liver (75 and 150 mg/kg doses) and kidney (75 mg/kg dose only) were determined using real-time quantitative polymerase chain reaction. At 150 mg/kg/day, PH treatment resulted in significant increases in hepatic mRNA levels of *Mrp3* (36-fold), *UGT1A6* (20-fold), *UGT2B1* (4-fold), and quinone reductase (*QR*, 5-fold), compared with the vehicle-treated group. Similar increases in *Mrp3* (99-fold), *UGT1A6* (17-fold), *UGT2B1* (3-fold), and *QR* (11-fold) mRNA levels were observed in the liver after PH treatment of rats at 75 mg/kg/day. In contrast, the expression levels of *CYP2C11* and *Oatp2* were decreased by ~80 and 50%, respectively. In addition, PH (75 mg/kg/day) elicited statistically significant changes in renal gene expression of *CYP3A1*, *UGT1A6*, *QR*, and *Mrp3*, but the magnitude of renal *Mrp3* induction was less than 2-fold over control. Although PH is known to modulate hepatic glucuronidation in vivo, these data indicated that PH induced mRNA levels of the efflux transporter, *Mrp3*, which may also affect the disposition of xenobiotics.

Multidrug resistance proteins 1, 2, and 3 (Mrp1,[1] 2, and 3) have gained significance during the last few years because of their function as transporters of organic anions and conjugates, and their involvement in hepatic detoxification and tissue-specific distribution of drugs (Konig et al., 1999; Hirohashi et al., 1999; Borst et al., 2000). Notably, induction of hepatic rat Mrp3 and another transporter, Oatp2, by drugs like phenobarbital was implicated in the altered disposition of acetaminophen and the enhanced uptake of digoxin in rats (Rausch-Derra et al., 2001; Xiong et al., 2002a). Mrp3 induction has been shown also with oltipraz (Cherrington et al., 2002), a dithiolthione phase II enzyme inducer being investigated as a chemoprotectant against aflatoxin carcinogenicity and hepatotoxicity (Kwak et al., 2001a). Oltipraz and other selective phase II inducers, such as 1,7-phenanthroline (PH), offer a new mechanism of protection against carcinogenic and hepatotoxic compounds by inducing the enzymes involved in their metabolism [glutathione *S*-transferase (GST) and UDP-glucuronosyltransferase (UGT)], without increasing cytochrome P450-mediated bioactivation (Franklin and Moody, 1992; Franklin et al., 1993; Buetler et al., 1995; Vargas et al., 1998; Dong et al., 1999; Lamb and Franklin, 2000). Additionally, selective phase II inducers can be used to modulate in vivo exposure to acyl glucuronides. In this regard, a significant increase in the glucuronidation of benoxaprofen, a nonsteroidal anti-inflammatory drug, was observed when rats were treated with PH at 75 mg/kg/day for 3 days, resulting in a 2-fold increase in the biliary excretion of the acyl glucuronide, and an 8-fold increase in the peak plasma concentration (C_{max}) and area under the curve values of the acyl glucuronide (Dong et al., 1999).

In the present study, we report that PH significantly induced not only mRNA levels of hepatic phase II enzymes, but also mRNA levels of *Mrp3*, which is likely to contribute to the altered metabolism and disposition of xenobiotics and their metabolites in PH-treated rats.

[1] Abbreviations used are: Mrp, multidrug resistance protein; Oatp, organic anion transporting polypeptide; PH, 1,7-phenanthroline; GST, glutathione *S*-transferase; UGT, UDP-glucuronosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ct, cycle threshold; QR, quinone reductase; Nrf2, nuclear factor-erythroid 2-related factor.

Address correspondence to: Sui Wang, Department of Drug Metabolism, Merck Research Laboratories, P.O. Box 2000, RY80L-109, Rahway, NJ 07065. E-mail: sui_wang@merck.com

Materials and Methods

Materials. PH was purchased from Sigma-Aldrich (St. Louis, MO). The purity, verified by high-pressure liquid chromatography, was 99.5%. Solvents used for analysis were of analytical or high-pressure liquid chromatography grade (Fisher Scientific, Pittsburgh, PA).

In Vivo Animal Studies. All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Male Sprague-Dawley rats were obtained from Charles River Laboratories Inc. (Wilmington, MA). After an overnight fast, five male Sprague-Dawley rats were dosed orally with PH at 150 mg/kg once daily for 3 days, and four male Sprague-Dawley rats were dosed with vehicle (0.1 M citric acid in 0.5% methylcellulose) in the same manner. Livers were removed from treated and vehicle control rats at 24 h after the last dose and stored at −70°C for quantitation of mRNA. In a separate experiment, three rats were dosed with PH at 75 mg/kg/day for 3 days, and an additional three rats were dosed with vehicle in the same manner. At 24 h after the last dose, the livers and kidneys were removed and stored at −70°C for mRNA quantification.

Development of Specific Primers and Probes for Quantitative Real-Time Polymerase Chain Reaction. Coding sequences for the genes listed in Table 1 were accessed from GenBank. Specific target regions within the coding sequences were determined through nucleotide sequence alignment comparisons of targets within multiple member gene families (e.g., *Oatp2* with *Oatps*, *Mrp2* with *Mrps*, *CYP2C11* with *CYP2C12*, etc.). Primers and probes

TABLE 1

Rat primer-probe sets and gene abbreviations

Gene	GenBank	Forward Primer	Reverse Primer	Probe
CYP3A1	D13912	TGGTCATATTCCATCTTATGCTC	GGCGAAATTCCTCAGGCTCT	TCACCGTGATCCACA
CYP2C11	NM_010184	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA	CATTTGCCCCCTTTTTACGAGGCTTG
GSTA2	NM_01713	CGCTAGACAGTGAAGCACAGTTG	CCGGATGCACTCCATTCTG	CCAGTGCTTCACTACTTCAATGCCCGG
Mrp2	X96393	TGCAGCCTCCATAACCATGAG	GATGCCTGCCATTGGACCTA	AGAGAGAACAGCTTTCGTCGAACACTTAGCC
Mrp3	AF072816	CCAACCCATGAACCCCAA	GCACATCTAGGTCAGCTAGCAGG	CGCTACCAACAGGCTCTGGAGACGTGT
Oatp2	U95011	GTACTCAAGATTTTCCCAGGGAAA	AACATGCTGGTTGAAAATGAAGG	CTTCTATAGTGGCCTTCACCCTTCACCTTAAAGC
QR	NM_017000	CTCGCCTCATGCGTTTTTG	CCCCTAATCTGACCTCGTTCAT	TGCCACGGCTGTGACAACAA
UGT1A1	D38065	CCGTTGTCCTACGTGCCC	TCACCCGCTGCAGGAAGT	AGAGTTTGTCCTCGAACACAGATCGCATG
UGT1A6	NM_057105	CCGCTATCGCTCCTTTGG	CTGTACTCTCTTAGAGGAGCCATCAG	AACAACCACTTTGCTGCTGCCAGTTCCCC
UGT2B1	M13506	GCTGCTTCCAGGAACCTG	TGAGGTCCCAACGCTGTCTT	TGTCATGCCAGTGCCTTCCCTCTAAAA

were designed to the selected target using Applied Biosystems Inc. (Foster City, CA) Primer Express software (v.2.0). All primers and probes were submitted to the National Center for Biotechnological Information for nucleotide comparison using the basic logarithmic alignment search tool (BLASTn) *search for short, nearly exact sequences* to ensure specificity. Primers and probes were synthesized by QIAGEN Operon (Alameda, CA), where primers were 5′- and 3′-labeled with the 6-carboxyfluorescein and 6-carboxytetramethylrhodamine reporter dyes, respectively. The rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer/probe set was purchased from Applied Biosystems Inc. and used per manufacturer's instructions. Each RNA sample was reverse-transcribed before analysis of different gene expression by PCR. Real-time quantitative PCR was performed using an ABI PRISM 7700 Sequence Detector instrument and Sequence Detector v.1.7 software (PerkinElmer Instruments, Skelton, CT).

mRNA Isolation and Quantitative Real-Time Polymerase Chain Reaction. Total RNA from rat tissues was isolated using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. Samples were quantitated by spectrophotometry and diluted to a concentration of 15 ng/μl. Aliquots (500 ng) of RNA were analyzed by agarose/formaldehyde gel electrophoresis to check RNA integrity. Samples were then assayed in triplicate 25-μl reactions using 25 ng of RNA per reaction. Gene-specific primers were used at 7.5 pmol per reaction, and the gene-specific probes were used at 5 pmol per reaction. *GAPDH* was used to normalize gene expression in all samples since it is a highly expressed gene in rat liver and did not change in response to PH treatment [cycle threshold (Ct), control, 21.8 $\pm$ 0.2; PH, 22 $\pm$ 0.3]. Fold induction values were calculated by subtracting the mean difference of gene and *GAPDH* Ct number for each treatment group from the mean difference of gene and *GAPDH* Ct number for the vehicle group and raising this difference to the power of 2.

Statistical analyses were performed using a two-tailed Student's *t* test at α = 0.01 level of significance.

Results and Discussion

As expected from the literature (Vargas et al., 1998; Lamb and Franklin, 2000), significant increases in the amount of mRNA for *UGT1A6* (20-fold) and, to a lesser extent, *UGT2B1* (4-fold) and *QR* (5-fold) were detected in rat liver in response to PH treatment of 150 mg/kg/day (Fig. 1). The mRNA levels of *UGT1A1* were not affected substantially (<2-fold) by this treatment. Similar increases in *UGT1A6* (17-fold), *UGT2B1* (3-fold), *GST* (6-fold), and *QR* (11-fold) mRNA were observed in liver after PH treatment of rats at 75 mg/kg/day for 3 days (Table 2). These results were similar to those reported by Vargas et al. (1998). The observed lack of dose proportionality in the increase of the mRNA of these enzymes between 75 and 150 mg/kg of PH needs further investigation to establish the time-dependence of the induction.

The transcription factor *Nrf2* has been shown to be important for the induction of phase II enzymes. Induction of *QR* and *UGT1A6* by oltipraz was observed in the wild-type, but not *nrf2*-deficient mice, which suggested that *Nrf2* plays a major role in the regulation of these genes (Kwak et al., 2001b). Given that PH causes induction of a

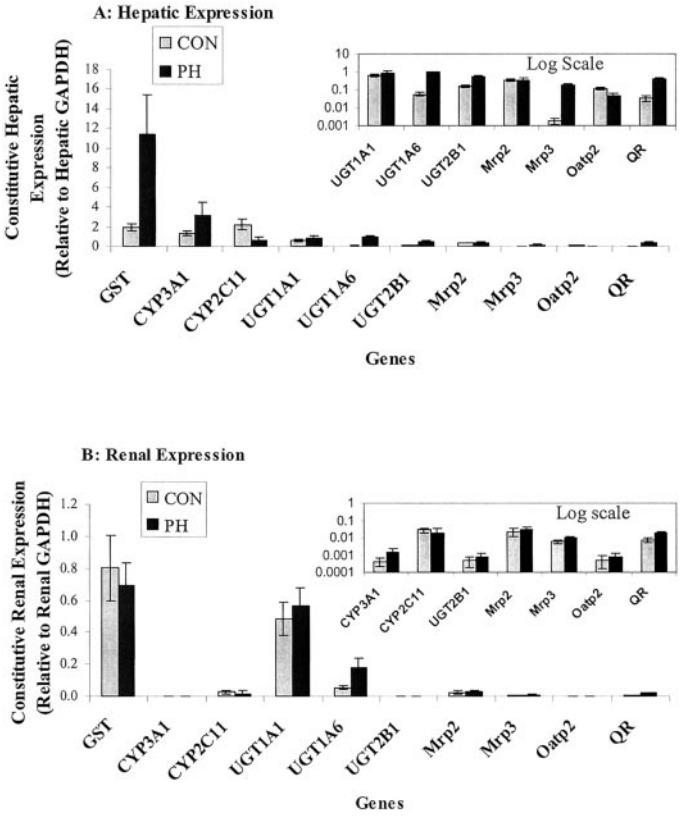

FIG. 1. *Comparison of constitutive (gray) and 1,7-phenanthroline-inducible (75 mg/kg/day for 3 days; black) expression of hepatic (A) and renal (B) drug-metabolizing enzymes and xenobiotic transporters.*

Three animals were in each group; three determinations were performed for each animal. Some of the gene expression levels in log scale are shown in the box on the right in the figure.

battery of genes similar to that of oltipraz, further studies are needed to elucidate the role of *Nrf2*, if any, in the inductive effects of PH.

PH caused a 3- to 4-fold induction of *CYP3A1* gene expression in both liver and kidney; however, this is considered minor in comparison to the induction of *CYP3A1* gene expression by pregnane X receptor agonists (~30-fold) (Hartley and Klaassen, 2000) and may not be associated with increased protein levels (Dong et al., 1999). Significant decreases in the mRNA levels of *CYP2C11* (17% of control) and *Oatp2* (50% of control) were observed by PH treatment at 150 mg/kg/day. Down-regulation of *CYP2C11* and *Oatp2* by some aryl hydrocarbon receptor agonists, such as 3-methylcholanthrene, has been reported previously (Lee and Riddick, 2000; Rausch-Derra et al., 2001; Guo et al., 2002). However, PH does not induce CYP1A2 activity (Dong et al., 1999), suggesting that aryl hydrocarbon receptor

TABLE 2

Effects of 1,7-phenanthroline (75 mg/kg/day and 150 mg/kg/day, 3 days) on hepatic and kidney gene expression in male Sprague-Dawley rats

Data represent mean ± S.D.; $n = 5$ for the 150 mg/kg treatment group and $n = 3$ for the 75 mg/kg group. Three determinations were performed for each animal. Statistically significant differences between treated and control groups were evaluated by the Student's *t* test.

Gene	Liver		Kidney (75 mg/kg treatment)
	150 mg/kg treatment	75 mg/kg treatment	
	fold induction over control		
CYP3A1	3.4 ± 0.5**	2.3 ± 0.7**	3.7 ± 1.5**
CYP2C11	0.2 ± 0.03**	0.3 ± 0.1**	0.7 ± 0.4
UGT1A1	0.7 ± 0.07**	1.4 ± 0.2**	1.2 ± 0.1
UGT1A6	20 ± 1.7**	17 ± 0.8**	3.2 ± 0.6**
UGT2B1	3.7 ± 0.6**	3.3 ± 0.3**	1.6 ± 0.3
Mrp2	1.2 + 0.1	1.0 ± 0.2	1.4 ± 0.1
Mrp3	36 ± 4.9**	99 ± 13**	1.6 ± 0.2**
Oatp2	0.5 ± 0.1**	0.4 ± 0.1**	1.1 ± 0.4
GSTA2	N.D.	5.9 ± 1.3**	0.9 ± 0.1
QR	4.8 ± 1.1**	11 ± 1.9**	2.5 ± 0.3**

N.D., not determined.
** Significantly different ($P < 0.01$) from controls.

may not be involved in the PH down-regulation of *CYP2C11* and *Oatp2*.

In addition to the increases in *QR* and *UGT* mRNA levels, which are consistent with the previous report (Vargas et al., 1998), we also demonstrated that PH markedly increased hepatic *Mrp3* mRNA levels (35-fold at 150 mg/kg and 99-fold at 75 mg/kg), but *Mrp2* levels remained unaffected. Mrp3 is located on the basolateral membrane of polarized cells, and it plays a role in the hepatic elimination by transporting organic anions from liver to blood, which could lead to increased levels of xenobiotics and their metabolites in plasma and possibly an increase in urinary excretion (Konig et al., 1999; Kool et al., 1999). In normal rat liver, constitutive expression of *Mrp3* mRNA is very low; however, the inducible nature of *Mrp3* is well documented (Ogawa et al., 2000; Cherrington et al., 2002). *Mrp3* has been shown to be induced by activators of the constitutive androstane receptor and an antioxidant/electrophile responsive element (Cherrington et al., 2002). However, a recent report by Xiong et al. (2002b) suggests that *Mrp3* regulation occurs independent of constitutive androstane receptor.

In contrast to the robust increase in gene expression levels for *UGT1A6* and *Mrp3* in liver of PH-treated rats, there were much smaller changes in these genes in kidney by PH treatment at 75 mg/kg for 3 days (Table 2). The lack of evidence for a robust renal induction of xenobiotic transporters in rats treated with known hepatic enzyme inducers has been reported previously (Brady et al., 2002; Cherrington et al., 2002). In addition, previous studies have shown minimal inductive effects on drug-metabolizing genes by PH in the small intestine (Vargas et al., 1998).

Thus, PH is a pleiotropic inducer of genes responsible for drug metabolism and transport. These results indicate that in vivo data from PH-induced rats should be interpreted with caution, since up-regulation of *Mrp3* gene expression may result in increased efflux of glucuronides and other xenobiotics from liver into the plasma compartment (Gregus et al., 1990; Xiong et al., 2002a). Further studies are needed to investigate the mechanism by which PH induces *Mrp3* and phase II enzymes, and to determine whether these changes in mRNA levels will correlate with protein levels and/or activity.

References

Borst P, Evers R, Kool M, and Wijnholds J (2000) A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* **92:**1295–1302.

Brady JM, Cherrington NJ, Hartley DP, Buist SC, Li N, and Klaassen CD (2002) Tissue distribution and chemical induction of multiple drug resistance genes in rats. *Drug Metab Dispos* **30:**838–844.

Buetler TM, Gallagher EP, Wang C, Stahl DL, Hayes JD, and Eaton DL (1995) Induction of phase I and II drug-metabolizing enzyme mRNA, protein and activity by BHA, ethoxyquin and oltipraz. *Toxicol Appl Pharmacol* **135:**45–57.

Cherrington NJ, Hartley DP, Li N, Johnson DR, and Klaassen CD (2002) Organ distribution of multidrug resistance protein 1, 2 and 3 (Mrp1, 2 and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats. *J Pharmacol Exp Ther* **300:**97–104.

Dong JQ, Etheridge AS, and Smith PC (1999) Effect of selective phase II enzyme inducers on glucuronidation of benoxaprofen in rats. *Drug Metab Dispos* **27:**1423–1428.

Franklin MR and Moody DE (1992) Concomitant induction of microsomal epoxide hydrolase and UDP-glucuronosyltransferase activities by dipyridine compounds. *Drug Metab Dispos* **20:**26–729.

Franklin MR, Slawson MH, and Moody DE (1993) Selective induction of rat liver phase II enzymes by N-heterocycle analogues of phenanthrene: a response exhibiting high correlation between UDP-glucuronosyltransferase and microsomal epoxide hydrolase activities. *Xenobiotica* **23:**267–277.

Gregus Z, Madhu C, and Klaassen CD (1990) Effect of microsomal enzyme inducers on biliary and urinary excretion of acetaminophen metabolites in rats. *Drug Metab Dispos* **18:**10–19.

Guo GL, Choudhuri S, and Klaassen CD (2002) Induction profile of rat organic anion transporting polypeptide 2 (oatp2) by prototypical drug-metabolizing enzyme inducers that activate gene expression through ligand-activated transcription factor pathways. *J Pharmacol Exp Ther* **300:**206–212.

Hartley DP and Klaassen CD (2000) Detection of chemical-induced differential expression of rat hepatic cytochrome P450 mRNA transcripts using branched DNA signal amplification technology. *Drug Metab Dispos* **28:**608–616.

Hirohashi T, Suzuki H, and Sugiyama Y (1999) Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). *J Biol Chem* **274:**15181–15185.

Konig J, Rost D, Cui Y, and Keppler D (1999) Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology* **29:**1156–1163.

Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, et al. (1999) MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* **96:**6914–6919.

Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K, Yamamoto M, and Kensler TW (2001a) Role of phase 2 enzyme induction in chemoprotection by dithiolthiones. *Mutat Res* **480–481:**305–315.

Kwak MK, Itoh K, Yamamoto M, Sutter TR, and Kensler TW (2001b) Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent 3H-1,2-dimethiole-2-thione. *Mol Med* **7:**135–145.

Lamb JG and Franklin MR (2000) Early events in the induction of rat hepatic UDP-glucuronosyltransferases, glutathione S-transferase, and microsomal epoxide hydrolase by 1,7-phenanthroline: comparison with oltipraz, tert-butyl-4-hydroxyanisole and tert-butylhydroquinone. *Drug Metab Dispos* **28:**1018–1023.

Lee C and Riddick DS (2000) Transcriptional suppression of cytochrome P450 2C11 gene expression by 3-methylcholanthrene. *Biochem Pharmacol* **59:**1417–1423.

Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, Akizawa T, Yoshioka M, and Sugiyama Y (2000) Characterization of inducible nature of MRP3 in rat liver. *Am J Physiol Gastrointest Liver Physiol* **278:**G438–G446.

Rausch-Derra LC, Hartley DP, Meier PJ, and Klaassen CD (2001) Differential effects of microsomal enzyme-inducing chemicals on the hepatic expression of rat organic anion transporters, OATP1 and OATP2. *Hepatology* **33:**1469–1478.

Vargas M, Lamb JG, and Franklin MR (1998) Phase II-selective induction of hepatic drug-metabolizing enzymes by oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione], 1,7-phenanthroline and 2,2′-dipyridyl in rats is not accompanied by induction of intestinal enzymes. *Drug Metab Dispos* **26:**91–97.

Xiong H, Suzuki H, Sugiyama Y, Meier PJ, Pollack GM, and Brouwer KLM (2002a) Mechanisms of impaired biliary excretion of acetaminophen glucuronide after acute phenobarbital treatment of phenobarbital pretreatment. *Drug Metab Dispos* **30:**962–969.

Xiong H, Yoshinari K, Brouwer KLM, and Negishi M (2002b) Role of constitutive androstane receptor in the in vivo induction of Mrp3 and CYP2B1/2 by phenobarbital. *Drug Metab Dispos* **30:**918–923.