Expression and Regulation of Breast Cancer Resistance Protein and Multidrug Resistance Associated Protein 2 in BALB/c Mice

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Microsomal enzyme inducers are known to influence the expression of many transporter proteins and mRNA. In this study, we examined the effects of microsomal enzyme inducers on the mRNA expression of Breast Cancer Resistance Protein (BCRP) and Multidrug Resistance Associated Protein 2 (MRP2) in BALB/c mice. mRNA expression in liver, duodenum, jejunum and ileum was examined in mice, which were treated with microsomal enzyme inducers–aryl hydrocarbon receptor (AhR) ligands 3-methylcholanthrene (3-MC) and pregnane-x-receptor (PXR) ligand pregenolone-16α-carbonitrile (PCN) and compared with control vehicle. The results suggested that the expression level of bcrp mRNA in the ileum was twice that in the liver, duodenum and jejunum using both semi quantitative PCR and Real-time PCR. Mrp2 mRNA was significantly increased by both PCN and 3-MC treatment. In contrast, bcrp mRNA expression was not significantly affected by these inducers. In summary, this study demonstrated that the expression of mrp2 mRNA is regulated by PCN and 3-MC, however, bcrp mRNA expression was not significantly affected by PCN and 3-MC.

Key words induction; breast cancer resistance protein (BCRP); multidrug resistance protein 2 (MRP2)

Breast cancer resistance protein (BCRP) was first described by Doyle *et al.*,[1] in the human MCF-7 MDR breast cancer cell line that was selected for resistance to doxorubicin. Bcrp is encoded by the ABCG2 gene that was mapped to chromosome 4. The protein consists of 655 amino acids with a molecular weight of 72.6 kDa, it belongs to the ABC transporter family, and is a recently discovered half transporter that probably acts as a homo or heterodimer in transporting cytotoxic agents. BCRP is localized at the canalicular membrane of hepatocytes and the brush border membrane of intestinal epithelial cells,[2,3] like P-glycoprotein (Pgp)[4] and multidrug resistance protein 2 (MRP2).[5] The tissue distribution of human BCRP shows similarities with that of Pgp, suggesting there is an overlap in function. GF120918 is a known potent inhibitor of Pgp, which has no effect on MRP.[6] Studies have shown that GF120918 is also an efficient inhibitor of BCRP, both in human and murine systems.[7—9] Expression of BCRP in capillary endothelial cells suggests that BCRP has a pharmacological and, possibly, toxicological protective role that is comparable with Pgp, and this may be its part physiological functions.

The liver has a number of xenobiotic transporters that are responsible for transport *via* both sinusoidal and canalicular membranes. On the sinusoidal (blood) side of the hepatocyte, the Organic Anion transporting polypeptide (OATP) family of proteins play a primary role in the hepatic uptake of xenobiotics and endogenous organic anions. On the other hand, on the canalicalar side of the hepatocyte, Pgp transports hydrophobic compounds into bile, and the MRP2 is also responsible for the passage of various organic anions into bile. Earlier stage studies have shown that 3-methylcholanthrene (3-MC) mediates mdr1 mRNA and Pgp induction in rat liver epithelial cells[10] while pregenolone-16α-carbonitrile (PCN) has no inductive effect on the expression of mdr1 mRNA and Pgp in rat liver.[11] Previous studies have demonstrated upregulation of Oatp2 mRNA and protein in rat and mouse liver following treatment with Phenobarbital (PB is a constitutive androstane receptor ligand) and PCN. PCN produces a marked elevation of Oatp2 mRNA and protein but, surprisingly, Oatp2 expression is suppressed in response to 3-MC.[12—15] It also has been demonstrated that treatment of human, rat and mouse primary hepatocytes with PB and PCN results in an increased expression of mrp2 mRNA.[16] In addition, the recent studies have been demonstrated that PCN and PB increase mrp2 protein in rat liver in a time-dependent manner, although they have no effect on the expression of mrp2 mRNA.[17,18]

The purpose of present study was to determine whether the mRNA expression of bcrp is under the regulation of microsomal enzyme inducers in BALB/c mice.

MATERIALS AND METHODS

Chemical 3-MC was purchased from Sigma Chemical Co. (St. Louis. MO, U.S.A.). PCN was purchased from Wako Pure Chemical Industries (Osaka Japan). All other chemicals and reagents were of the highest grade and purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals and Surgery BALB/c mice (SLC, Tokyo Japan) were housed at 25 °C with a 12 h light–drark cycle. Mice received water and food ad libitum and were allowed to acclimatize to their environment for 5 d before the study began. BALB/c mice (22—27 g) were given the following chemical treatment: PCN (400 mg/kg), 3-MC (40 mg/kg) or control vehicle (corn oil). PCN and 3-MC were dissolved in corn oil. All chemicals were injected ip in a final volume of 5 ml/kg. Mice were injected i.p. once daily for 4 d. On day 5, the mice were killed under ether anesthesia and the liver and intestine tissues were removed, snap-frozen in liquid nitrogen, and stored at −80 °C (intestinal epithelia were obtained by scraping prior to freezing).

Isolation of Liver and Intestinal RNA Total RNA was extracted from liver and intestinal tissues using Isogen (Nippon Gene, Toyama Japan). Briefly, 50—100 mg of tissue samples was added to 1 ml Isigen and then homogenized for cell lysis. After standing the homogenates at room temperature for 5 min, 0.2 ml chloroform was added and the solutions were mixed thoroughly and centrifuged at 3000 ***g*** for

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15 min. The RNA fraction was recovered from the upper layer and further precipitated with 0.5 ml isopropanol. The RNA thus obtained was washed with 70% ethanol, dried briefly, and dissolved in 0.1 ml of RNase-freed water. The RNA content was determined by spectrophotometry at 260 and 280 nm.

RT-PCR Procedure for the Assay of mRNAs Levels of mRNAs of bcrp, mrp2, Oatp2, CYP3A11 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were measured in the liver, duodenum jejunum and ileum. RNA was determined using an mRNA selective PCR kit (Takara, Tokyo, Japan). Briefly, 1 μg total RNA was reverse transcribed to cDNA and the specific primers for the genes of bcrp (3′ primer 5′-agcagcaaggaaagatccaa-3′; 5′ primer 5′-cccatcacaacgtcatcttg-3′); mrp2 (3′ primer 5′-actggacaagccacaattcc-3′, 5′ primer 5′-ctgcaggagtgctcgtatca-3′), Oatp2 (3′ primer 5′-cctgctgctctaagaggagcaagc-3′; 5′-primer 5′-cctcatcacagcttagttttccgt-3′), cyp3A11 (3′ primer 5′-ttttctgtcttcacaaaccgg-3′; 5′ primer 5′-taacaggcttgcctttctttg-3′) and G3PDH (3′ primer 5′-aacgacccccttcattgac-3′; 5′ primer 5′-tccacgacatactcagcac-3′) were used in PCR reactions. The PCR reaction was performed with AMV optimized Taq DNA polymerase (Takara PCR kit, Tokyo, Japan) under the following conditions for bcrp, Oatp2, CYP3A11, mrp2 and G3PDH (96 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min). PCR amplifications were carried out for bcrp and Oatp2 (24 cycles); mrp2 (22 cycles); CYP3A11 and G3PDH (20 cycles), and the cycles used were in the linear phase for amplifying the respective cDNAs. The resultant products were analyzed by electrophoresis on 8% polyacrylamide gels.

Real Time Quantitative PCR To quantify the expression of the mRNA of bcrp in the liver, duodenum, jejunum and ileum from control and treated mice, real-time quantitative PCR was used. For quantitative PCR, total RNA was isolated from each tissue from control and treated mice using Isogen (Nippon Gene, Toyama Japan). Total RNA was converted to cDNA using a random primer and avian myeloblastosis virus reverse transcriptase (TaKaRa RNA PCR Kit, Takara Shuzo, Tokyo, Japan). The reaction mixture was incubated at 30 °C for 10 min, 42 °C for 30 min, 50 °C for 15 min, and heated to 99 °C for 5 min to denature the reverse transcriptase. Real-time quantitative PCR was performed using a LightCycler™ and the appropriate software (Version 3.53, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for bcrp and G3PDH were used as a housekeeping gene for internal standards.[19] Bcrp primers (3′ primer 5′-aaatggagcacctcaacctg-3′; 5′ primer 5′-cccatcacaacgtcatcttg-3′), and G3PDH primers (3′ primer 5′-aacgacccccttcattgac-3′; 5′ primer 5′-tccacgacatactcagcac-3′) were designed based on the published full sequence of each protein. PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, U.S.A.). An external standard curve was generated by dilutions of the target PCR product, which had been purified and its concentration measured previously. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis and gel electrophoresis. The bcrp gene expression in each reaction was normalized by the expression of G3PDH.

RESULTS

Tissue Distribution of Bcrp mRNA Expression Bcrp mRNA expression levels in liver, duodenum and jejunum were similar. The expression of bcrp in the ileum was about twice as high as that in other tissues. The relative expression levels of bcrp mRNA normalized by G3PDH in the liver, duodenum, jejunum and ileum are shown in Fig. 1.

Effect of Microsomal Enzyme Inducer Treatment on Bcrp mRNA Expression Bcrp mRNA expression after treatment with microsomal enzyme inducers was determined by semi quantitative PCR assay and Real-time quantitative PCR assay (Table 1). No significant difference was observed in the expression of bcrp mRNA between treated mice and control mice.

CYP3A11 mRNA and Oatp2 mRNA Expression Because PCN treatment is known to induce expression of CYP3A11 mRNA and Oatp2 mRNA in mouse liver, we examined the effect of these inducers on the expression of CYP3A11 and Oatp2 mRNA. PCN significantly increased both CYP3A11 mRNA and Oatp2 mRNA expression in liver, however, 3-MC had no effect on the expression of CYP3A11 and Oatp2 mRNA (Figs. 2, 3).

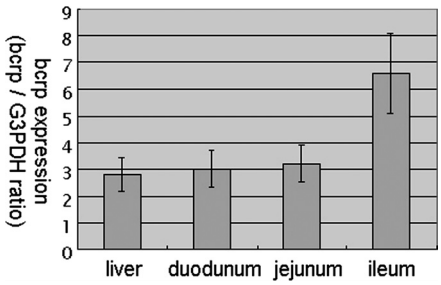

Fig. 1. Tissue Distribution of Bcrp mRNA in BALB/c Mice

Graphical results of semi-quantitative PCR assay are shown. Results are expressed as the ratio of bcrp signal to G3PDH signal. All data are given as mean±S.E. (n=4).

Table 1. Effect of Microsomal Enzyme Inducers 3-MC and PCN on Bcrp mRNA Expression

n=4	Control	3-MC	PCN	p value
Liver	0.28±0.07	0.33±0.12	0.29±0.07	>0.05
Duodenum	0.25±0.14	0.22±0.06	0.24±0.06	>0.05
Jejunum	0.37±0.15	0.36±0.10	0.37±0.03	>0.05
Ileum	0.64±0.33	0.82±0.24	0.63±0.05	>0.05

Statistics: Fisher's LSD.

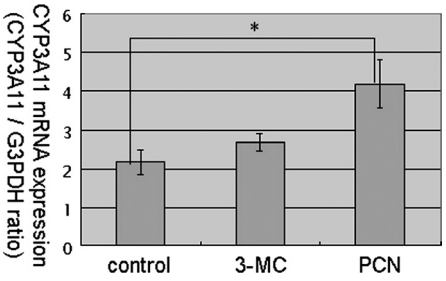

Fig. 2. Graphical Results of CYP3A11 mRNA Expression from Control and Inducer-Treated BALB/c Mice by Semi-Quantitative PCR

Results are expressed as the ratio of CYP3A11 signal to G3PDH signal. All data are given as mean±S.E. (n=5—11). * p<0.05.

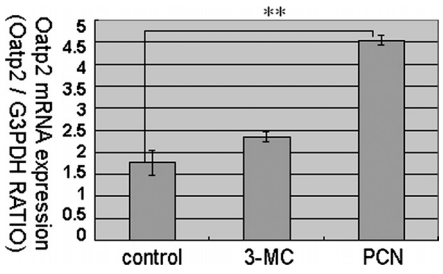

Fig. 3. Graphical Results of Oatp2 mRNA Expression from Control and Inducer-Treated BALB/c Mice by Semi-Quantitative PCR

Results are expressed as the ratio of Oatp2 signal to G3PDH signal. All data are given as mean±S.E. (n=5—11). ** p<0.01.

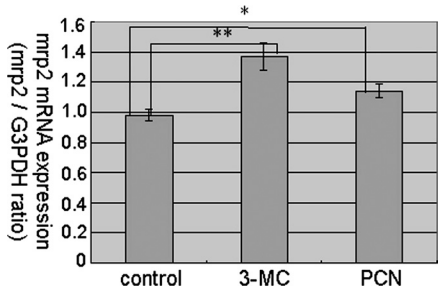

Fig. 4. Graphical Results of Mrp2 mRNA Expression from Control and Inducer-Treated BALB/c Mice by Semi-Quantitative PCR

Results are expressed as the ratio of mrp2 signal to G3PDH signal. All data are given as mean±S.E. (n=5—11). * p<0.05, ** p<0.01.

Mrp2 mRNA Expression Mrp2 mRNA expression from both control and treated BALB/c mice was analyzed by semi quantitative PCR. Mrp2 mRNA was significantly increased by both PCN and 3-MC (Fig. 4).

DISCUSSION

Microsomal enzyme inducers are structurally diverse chemicals that upregulate hepatic enzymes. They are known to upregulate Phase I drug metabolizing enzymes called cytochrome P450s (CYP), which are important for the bioactivation and biotransformation of lipophilic chemicals to more water-soluble compounds. Transporter proteins are also targets of microsomal enzyme inducers. Some studies using human and rodent models have demonstrated that ABC transporter family transporter proteins, such as Pgp, Oatp2, and mrp2, are upregulated by microsomal enzyme inducers.[10—18] In this study, we examined the effects of microsomal enzyme inducers on the mRNA expression of bcrp, which is also responsible for drug resistance and may have a pharmacological role. Bcrp mRNA expression was analyzed by two separate methods, and it was found that neither PCN nor 3-MC affected bcrp mRNA expression in the liver, duodenum, jejunum and ileum in BALB/c mice (Table 1). Recently, Anapolsky *et al.*[20] have reported that PXR plays a role in regulation of BCRP in mice. They used 2-acetylaminofluorene (2-AAF), a kind of hepatocarcinogen, to treat wild-type (PXR(+/+)) and PXR-null (PXR(−/−)) C57BL/6 mice, and evaluated the mRNA alterations of several drug transporters in liver. They found that treatment of PXR(+/+) mice resulted in a dose-dependent 2- to 4-fold induction of MRP2, OATP2, BCRP, CYP3A11 and CYP1A2, but no induction was observed in PXR(−/−) mice. They further demonstrated that 2-AAF does indeed activate PXR. PCN is also a ligand for PXR. In the present study, we found no obvious effects of PCN on bcrp mRNA expression in mouse liver. We speculate that the differences might result from that they used C57BL/6 mice while we used BALB/c mice. However, further studies are needed to clarify this issue.

We also examined the change of Oatp2 mRNA and CYP3A11 mRNA expression by PCN and 3-MC in liver. As expected, Oatp2 mRNA and CYP3A11 mRNA was significantly increased by PCN in liver (Figs. 2, 3). This agrees with the results of Rausch-Derra *et al.* and Staudinger *et al.*[12—14]

In addition, we have demonstrated that mrp2 mRNA expression is induced by PCN and 3-MC in mouse liver (Fig. 4). Mrp2 is a canalicular efflux transporter that pumps endogenous and xenobiotic compounds into bile. This agrees with the results of previous studies which showed that mrp2 expression and protein levels are induced in primary hepatocytes of rat and mouse by PB and PCN.[16] However, some studies have found that mrp2 protein expression is induced following treatment with PB and PCN in rat liver, while mrp2 mRNA expression is not.[17,18] In addition, it was reported 3-MC did not show any effect on the expression of both protein and mRNA of mrp2 in rat.[17,18] There appears to be a discrepancy between the effects of microsomal enzymes on mrp2 mRNA expression. To address this issue, further studies including detection of MRP2 protein are needed. However, we wonder that, at least in part, this may be due to a species difference, or a difference in *in vivo* and *in vitro* experiment conditions since it is known that drug function is very complicated *in vivo*.

In conclusion, we have demonstrated that PCN and 3-MC could upregulate the expression of mrp2 but not bcrp at transcriptional level, suggesting that mrp2 and bcrp have different regulation mechanisms. However, further studies are needed to clarify whether the regulation effects could be achieved at protein level and what are the underlying mechanisms.

REFERENCES

1) Doyle L. A., Yang W., Abruzzo L. V., Krogmann T., Gao Y., Rishi A. K., Ross D. D., *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 15665—15670 (1998).
2) Jonker J. W., Smit J. W., Brinkhuis R. F., Maliepaard M., Beijnen J. H., Schellens J. H., Schinkel A. H., *J. Natl. Cancer Inst.*, **92**, 1651—1656 (2000).
3) Maliepaard M., Scheffer G. L., Faneyte I. F., van Gastelen M. A., Pijnenborg A. C., Schinkel A. H., van De Vijver M. J., Scheper R. J., Schellens J. H., *Cancer Res.*, **61**, 3458—3464 (2001).
4) Juliano R. L., Ling V., *Biochim. Biophys. Acta*, **455**, 152—162 (1976).
5) Cole S. P., Bhardwaj G., Gerlach J. H., Mackie J. E., Grant C. E., Almquist K. C., Stewart A. J., Kurz E. U., Duncan A. M., Deeley R. G., *Science*, **258**, 1650—1654 (1992).
6) den Ouden D., van den Heuvel M., Schoester M., van Rens G., Sonneveld P., *Leukemia*, **10**, 1930—1936 (1996).
7) de Bruin M., Miyake K., Litman T., Robey R., Bates S. E., *Cancer Lett.*, **146**, 117—126 (1999).
8) Allen J. D., Brinkhuis R. F., Wijnholds J., Schinkel A. H., *Cancer Res.*, **59**, 4237—4241 (1999).
9) Maliepaard M., van Gastelen M. A., Tohgo A., Hausheer F. H., van Waardenburg R. C., de Jong L. A., Pluim D., Beijnen J. H., Schellens J. H., *Clin. Cancer Res.*, **7**, 935—941 (2001).
10) Fardel O., Lecureur V., Corlu A., Guillouzo A., *Biochem. Pharmacol.*, **51**, 1427—1436 (1996).

11) Salphati L., Benet L. Z., *Biochem. Pharmacol.*, **55**, 387—395 (1998).
12) Rausch-Derra L. C., Hartley D. P., Meier P. J., Klaassen C. D., *Hepatology*, **33**, 1469—1478 (2001).
13) Staudinger J. L., Goodwin B., Jones S. A., Hawkins-Brown D., MacKenzie K. I., LaTour A., Liu Y., Klaassen C. D., Brown K. K., Reinhard J., Willson T. M., Koller B. H., Kliewer S. A., *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 3369—3374 (2001).
14) Staudinger J., Liu Y., Madan A., Habeebu S., Klaassen C. D., *Drug Metab. Dispos.*, **29**, 1467—1472 (2001).
15) Johnson D. R., Guo G. L., Klaassen C. D., *Toxicology*, **178**, 209—219 (2002).
16) Kast H. R., Goodwin B., Tarr P. T., Jones S. A., Anisfeld A. M., Stoltz C. M., Tontonoz P., Kliewer S., Willson T. M., Edwards P. A., *J. Biol. Chem.*, **277**, 2908—2915 (2002).
17) Johnson D. R., Klaassen C. D., *Toxicol. Sci.*, **67**, 182—189 (2002).
18) Johnson D. R., Habeebu S. S., Klaassen C. D., *Toxicol. Sci.*, **66**, 16—26 (2002).
19) Ball H. J., McParland B., Driussi C., Hunt N. H., *Brain Res. Brain Res. Protoc.*, **9**, 206—213 (2002).
20) Anapolsky A., Teng S., Dixit S. and Piquette-Miller M., *Drug Metab. Dispos.*, **34**, 405—409 (2006).