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# Site-specific bidirectional efflux of 2,4-dinitrophenyl-*S*-glutathione, a substrate of multidrug resistance-associated proteins, in rat intestine and Caco-2 cells

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## Abstract

The site-specific function of multidrug-resistance-associated proteins (MRPs), especially MRP2 and MRP3, was examined in rat intestine and human colon adenocarcinoma (Caco-2) cells. The MRP function was evaluated pharmacokinetically by measuring the efflux transport of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), an MRP substrate, after application of 1-chloro-2,4-dinitrobenzene (CDNB), a precursor of DNP-SG. The expression of rat and human MRP2 and MRP3 was analysed by Western blotting. The rat jejunum exhibited a higher apical MRP2 and a lower basolateral MRP3 expression than ileum. In accordance with the expression level, DNP-SG efflux to the mucosal surface was significantly greater in jejunum, while serosal efflux was greater in ileum. Site-specific bidirectional efflux of DNP-SG was also observed in in-vivo studies, in which portal and femoral plasma levels and biliary excretion rate of DNP-SG were significantly higher when CDNB was administered to ileum. Caco-2 cells also showed a bidirectional efflux of DNP-SG. Probenecid, an MRP inhibitor, significantly suppressed the mucosal efflux in jejunum and serosal efflux in ileum. In contrast, probenecid significantly suppressed both apical and basolateral efflux of DNP-SG in Caco-2 cells, though the inhibition was of small magnitude. In conclusion, the efflux of DNP-SG from enterocytes mediated by MRPs exhibited a significant regional difference in rat intestine, indicating possible variability in intestinal bioavailabilities of MRP substrates, depending on their absorption sites along the intestine.

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## Introduction

A variety of efflux transporters and metabolic enzymes occur in intestinal epithelial cells, and have important roles in preventing the intracellular accumulation and/or the consequent systemic influx of various toxic xenobiotics. For example, P-glycoprotein, a well-known ATP-dependent efflux transporter, is expressed in the brush-border membrane (BBM) of the small intestine and transports a variety of chemically and pharmacologically unrelated lipophilic compounds (Horio et al 1988; Senior et al 1995). Many clinically important drugs are substrates of P-glycoprotein, including anti-human-immunodeficiency-virus agents, immunosuppressants, antitumour agents, calcium channel blockers, steroids and so on (Hunter et al 1993; Terao et al 1996; Schinkel 1998; Takano et al 2006). Multidrug-resistance-associated proteins (MRPs), another family of ATP-dependent efflux transporters, are also expressed in the intestine, and transport relatively hydrophilic compounds such as the glucuronide, glutathione and sulfate conjugates of endogenous and exogenous compounds, methotrexate, pravastatin, and etoposide (Hunter et al 1993; Terao et al 1996; Hirohashi et al 2000a; Zelcer et al 2001; Suzuki and Sugiyama, 2002; Uhr et al 2002; Chan et al 2004; Takano et al 2006). So far, nine MRP isoforms (MRP1–6 and MRP10–12) have been identified in various human tissues (Kool et al 1997 & 1999; Hopper et al 2001; Tammur et al 2001). MRP1–6 are expressed in the small and large intestine of humans and rodents (Prime-Chapman et al 2004; Zimmermann et al 2005; Johnson et al 2006; Maher et al 2006). In particular, MRP2 and MRP3 have greater roles than other MRPs, because of their higher expression levels (Mottino et al 2000; Rost et al 2002; Zimmermann et al 2005).

MRP2, localised in the BBM, is preferentially expressed at the proximal intestine, and MRP3, localised in the basolateral membrane, is at the distal intestine (Mottino et al 2001; Rost et al 2002; Chan et al 2004). These intestinal MRP2 and MRP3 could act as a host defence mechanism by preventing the influx of substrate compounds from apical and basolateral sides, respectively, and facilitating the efflux of substrate compounds from cells to prevent their intracellular accumulation. It is also reported that intestinal MRP3 could be involved in the enterohepatic circulation, since MRP3 also transports endogenous compounds such as bile salts (Hirohashi et al 2000b).

Because of the site-specific and membrane-specific expression of MRPs (especially MRP2 and MRP3) in the intestine (Gotoh et al 2000; Akita et al 2002; Yokooji et al 2005), they may affect the intestinal bioavailability and/or pharmacokinetics of orally administered substrate drugs, though a detailed study has not yet been reported. In this study we investigated regional differences in the expression of MRP2 and MRP3 between jejunum and ileum in rats by Western blot analysis. Then, the bidirectional (apical vs basolateral, or mucosal vs serosal side) effluxes of 2,4-dinitrophenyl-*S*-glutahione (DNP-SG), an MRP substrate, after application of 1-chloro-2,4-dinitrobenzene (CDNB), a precursor of DNP-SG, was examined in rat intestine in-vitro and in-vivo. The production of DNP-SG from CDNB has been used previously to evaluate MRP function: CDNB is taken up rapidly into enterocytes by passive diffusion, and is metabolised rapidly and completely to DNP-SG, an MRP substrate. Thus, measuring the bidirectional effluxes of DNP-SG allows pharmacokinetic evaluation of the functions of apical MRP2 and/or basolateral MRP3 (Oude Elferink et al 1993; Gotoh et al 2000; Yokooji et al 2005, 2006). However, DNP-SG is transported not only by MRPs (MRP1–3, MRP5 and MRP6), but also by breast cancer resistance protein (BCRP) and organic anion transporting polypeptide (Oatp)1a1, which is a bidirectional transporter expressed in rat liver, kidney and brain (Bergwerk et al 1996; Li et al 1998; Gao et al 1999; Suzuki and Sugiyama 2002; Suzuki et al 2003; Chan et al 2004). Therefore, the contribution of other transporters such as BCRP and Oatp1a1 also should be taken into consideration in transport studies using DNP-SG. The bidirectional efflux of DNP-SG was also evaluated in human colon adenocarcinoma cells (Caco-2) cells, in which the expression levels of MRP1–6 mRNAs correlate well with those in human intestine (Taipalensuu et al 2001).

## Materials and Methods

### Materials

CDNB and reduced glutathione (GSH) were obtained from Wako Pure Chemicals (Osaka, Japan); 1-fluoro-2,4-dinitrobenzene (FDNB) were purchased from Tokyo Kasei (Tokyo, Japan) and Sigma Chemical Co. Ltd. (St Louis, MO, USA), respectively. $M_2$III-6, a monoclonal antibody for MRP2, and D-15, a polyclonal antibody for MRP3, were from Chemicon International, Inc. (Temecula, CA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively. All other chemicals used were of the highest purity available.

### Chemical synthesis of DNP-SG

DNP-SG was synthesised according to the reported method by using FDNB as a lead compound (Hinchman et al 1991; Yokooji et al 2005). The synthesised compound was chromatographically pure.

### Cell culture

Caco-2 cells between passages 65 and 71 were cultured in the same manner as described previously (Takano et al 1998). Briefly, cells were seeded at a density of $10 \times 10^5$ cells on a 100 mm dish for the expression analysis, and at a density of $3.75 \times 10^5$ cells on a polycarbonate filter (4.71 $cm^2$ surface area) in Transwell chambers (Costar, Cambridge, MA, USA) for the transport study. These cells were cultured for 23–24 days after seeding. Transepithelial electric resistance (TEER) of Caco-2-cell monolayers was monitored with a Millicell electrical resistance system (ERS) testing device (Millipore, Bedford, MA, USA). Only monolayers with TEER of 500 $\Omega \cdot cm^2$ of higher were used.

### Animals

Male Sprague–Dawley rats, aged 7–9 weeks, were used. Experiments were performed in accordance with the *Guide for Animal Experimentation* from Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University.

### Expression analysis of MRP2 and MRP3 in rat intestine and Caco-2 cells

The expression of MRP2 and MRP3 in rat intestine and Caco-2 cells was evaluated by Western blot analysis, as described previously, using either BBM or crude membrane (CM) (Yumoto et al 2001; Watanabe et al 2004; Yokooji et al 2006). Briefly, the whole intestine was divided into two parts of the same length and the mucosal surfaces of the upper and lower half were scraped off with a cover glass. The collected intestinal mucosa was homogenised in an appropriate buffer for membrane preparations, and BBM fraction of upper and lower intestinal mucosa was prepared using a magnesium/ethylene glycol bis($\beta$-aminoethylether)-*N, N, N′, N′*-tetra-acetic acid precipitation method, as reported previously (Yokooji et al 2006). The CM fraction of intestinal mucosa was prepared according to the reported method (Yumoto et al 2001) with a small modification (samples were centrifuged at 40 000 *g* for 150 min instead of 24 000 *g* for 30 min). The CM fraction of Caco-2 cells was prepared using the method of Watanabe et al (2004). Protein concentrations in biological samples were determined by the Bradford method (for rat BBM and CM) or Lowry method (for Caco-2 CM) using BSA as the standard (Lowry et al 1951; Bradford et al 1976).

The concentrations of MRP2 and MRP3 proteins in membrane samples were evaluated by Western blot analysis after sodium dodecylsulfate-polyacrylamide gel electrophoresis, as

described previously (Yokooji et al 2006). BBM or CM samples of intestinal mucosa (80 $\mu$g protein) were applied to a gel. For the detection of MRP2 protein, $M_2$III-6 (1:50 dilution) and peroxidase-labelled affinity-purified antibody to mouse IgG (1:1000 dilution) were used as the primary monoclonal and secondary antibodies, respectively. For the detection of MRP3 protein, D-15 (1:50 dilution) and peroxidase-labelled affinity-purified antibody to goat IgG (1:1000 dilution) were used. The optical density of immunoreactive protein was estimated by a computer-aided densitometer with NIH Image (the public-domain program developed at the US National Institutes of Health, Bethesda, MD, USA).

### In-vitro transport studies of DNP-SG in rat everted intestine and Caco-2 cell monolayers

CDNB was dissolved at a concentration of 1 $\mu$M in pH 6.0 isotonic phosphate buffered saline (PBS) containing 4% DMSO. DMSO was used to improve the solubility of CDNB and at this concentration has been shown to have a negligible effect on efflux transporter function and/or membrane integrity in P-gp-mediated efflux transport of rhodamine 123 across rat intestine and Caco-2 cell monolayers (Takano et al 1998; Yumoto et al 1999). The drug solution was kept at 4°C. For transport studies of DNP-SG, 10 cm lengths of everted jejunum and everted ileum were prepared, and both ends of the everted intestines were catheterised with polyethylene tubing to collect the inner serosal (basolateral) fluid of the sac. An aliquot (1mL) of the drug solution was applied to the serosal side and the sac immersed in 20 mL of the same drug solution kept at 4°C for 40 min to preload CDNB. Then, both serosal and mucosal (apical) sides of the everted sac were washed carefully with ice-cold PBS without CDNB. The serosal side of the sac was again filled with 1mL PBS, and then the sac was immersed in 20 mL PBS prewarmed at 37°C and preoxygenated with 5% carbon dioxide/95% oxygen. The incubation medium was bubbled with carbon dioxide/oxygen throughout the transport study. Aliquots of mucosal and serosal media were sampled periodically for 120 min.

In the bidirectional efflux study of DNP-SG in Caco-2 cells, Caco-2 cell monolayers were pre-incubated with CDNB solution (1.6 mL for apical surface, and 2.6 mL for basolateral surface) at 4°C for 40 min to preload CDNB into cells. The cell monolayer and the chamber were carefully washed with ice-cold PBS containing 4% DMSO. Then, the transport medium (PBS containing 4% DMSO) was added to both sides of the cell monolayers, and the Transwell chamber was incubated at 37°C to start the efflux of DNP-SG. The transport medium (100 $\mu$L) in the upper and lower Transwell chambers was sampled periodically, and fresh transport medium was refilled each time.

In the inhibition study, probenecid, a typical MRP inhibitor, was added to both sides of membranes in rat intestine and Caco-2 cells at a concentration of 1 mM in PBS containing 4% DMSO.

### In-vivo study of DNP-SG in rats

Bidirectional DNP-SG efflux to mucosal and serosal sides after application of CDNB was evaluated using the rat intestinal loop method we have described previously (Yokooji et al 2005). Briefly, a 20 cm long loop of jejunum or ileum was made, and CDNB was administered at a dose of 5 $\mu$mol kg$^{-1}$ into the closed intestinal loop. Blood (0.2 mL) and bile samples were then collected periodically. In some experiments, portal-vein blood was collected at consecutive 1 min intervals for 5 min after CDNB administration by using a fishhook-shaped needle. The luminal fluid of the intestinal loop was collected 60 min after administration of CDNB, to measure cumulative mucosal efflux of DNP-SG. Intestinal mucosa was collected by scraping with a cover glass. The liver was also isolated at the same time.

### Analysis

Blood, intestinal perfusate, intestinal mucosa, bile and liver were treated in the same manner as reported previously (Yokooji et al 2006). The transport medium for Caco-2 cells was diluted with an equal volume of 10% perchloric acid. Concentrations of CDNB and DNP-SG in various biological samples were determined by HPLC using a Mightysil RP-18 column (Kanto Kagaku, Tokyo, Japan), as described previously (Yokooji et al 2005). Briefly, mobile phases were a mixture of acetonitrile and 1% acetic acid in a ratio of 15:85 (v/v) for DNP-SG and 35:65 (v/v) for CDNB. Detection was at wavelengths of 365 nm for DNP-SG and 305 nm for CDNB.

### Statistical analysis

Differences between group mean values were assessed by the Kruskal–Wallis test or analysis of variance followed by a post-hoc test (Tukey test) or Student's $t$-test. $P < 0.05$ was considered statistically significant.

## Results

### Western blot analysis for MRP2 and MRP3 expression in rat intestine and Caco-2 cells

Expression of MRP2 and MRP3 proteins in rat intestine and Caco-2 cells was evaluated by Western blot analysis (Figure 1). Bands of approximately 190 kDa, corresponding to the molecular size of MRP2 and MRP3 (Keppler et al 1997; Rost et al 2002), were observed in both jejunum and ileum membranes. The band density of MRP2 in the jejunum was approximately 2.8-fold stronger than that in the ileum, while MRP3 expression was higher in the ileum (approximately 1.6-fold of that in the jejunum). Both MRP2 and MRP3 were detected in Caco-2 cells.

### Bidirectional efflux of DNP-SG in rat everted jejunum and ileum in-vitro

DNP-SG effluxes to mucosal and serosal surfaces after preloading of CDNB were measured in rat everted intestine to evaluate mucosal MRP2 and serosal MRP3 functions simultaneously. When everted sac was incubated in CDNB (1 $\mu$M) solution, approximately 35% of the CDNB dose was taken up by the sac over 40 min at 4°C. The amounts of CDNB taken

up were similar between the jejunum and ileum sacs. Probenecid exerted no significant effect on CDNB preloading. Without probenecid, DNP-SG was effluxed mostly to the mucosal surface of everted jejunum – approximately 70% of the preloaded CDNB effluxed during 120 min' incubation at 37°C, indicating that mucosal MRP2 function is superior to basolateral MRP3 function in jejunum. Probenecid significantly suppressed the mucosal efflux of DNP-SG in jejunum by approximately 30% compared with control (Figure 2A). In everted ileum, approximately 50% of preloaded CDNB was recovered as DNP-SG during 120 min' incubation. Compared with that in the jejunum, mucosal DNP-SG efflux was significantly lower and serosal efflux was higher in ileum (Figure 2B). The serosal efflux in ileum, as well as the mucosal efflux in jejunum, of DNP-SG was significantly suppressed by probenecid. These results suggest that the serosal efflux of DNP-SG in ileum is mediated, at least partly, by MRP3.

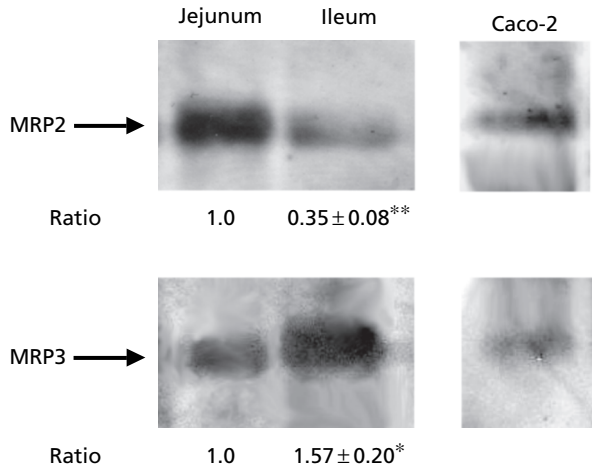


**Figure 1** Western blot analysis of MRP2 and MRP3 proteins in rat intestine and Caco-2 cells. Brush-border membrane and crude membrane were used for the detection of MRP2 and MRP3, respectively, in rat intestine. Crude membrane was used for the detection of MRP2 and MRP3 in Caco-2 cells. Each value of relative staining intensity (Ratio) represents the mean ± s.e. of results from three rats. $*P < 0.05$; $**P < 0.01$ compared with the value for jejunum.

### Pharmacokinetics of DNP-SG after intra-luminal administration of CDNB

To examine the regional difference in MRP function, the pharmacokinetics of DNP-SG after application of CDNB were compared between the jejunum and ileum regions. Some pharmacokinetic parameters of CDNB and DNP-SG uptake are summarised in Table 1. CDNB was almost completely taken up by both the jejunum and ileum regions 60 min after administration.

The in-vivo function of intestinal MRP2 was evaluated by measuring the amount of DNP-SG effluxed into the intestinal lumen (Table 1 and Figure 3). DNP-SG efflux into jejunum lumen was about 2-fold higher than that into ileum lumen (43.7% vs 20.4% of dose). The in-vivo function of intestinal MRP3 was evaluated by measuring the concentrations in portal-vein and femoral-artery plasma, and biliary excretion rates of CDNB and DNP-SG after intraluminal administration of CDNB. CDNB concentrations in portal-vein plasma were low, and no difference in CDNB concentrations was observed between intra-jejunum and intra-ileum administrations. In addition, CDNB was not detected in femoral-artery plasma, bile, intestinal mucosa or the liver. In contrast, DNP-SG concentrations in portal-vein plasma after intra-ileum administration of CDNB were significantly higher than after intra-jejunum administration (Figure 3B). The biliary excretion rate of DNP-SG at the early stages, the peak arterial plasma concentration and the area under the arterial plasma concentration–time curve from 0 to 60 min of DNP-SG were

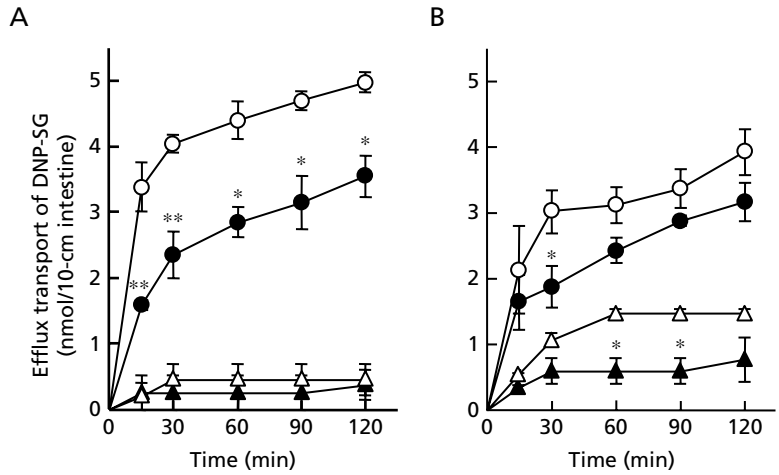


**Figure 2** Bidirectional efflux of DNP-SG after preloading of CDNB in everted rat jejunum (A) and ileum (B) in the absence and presence of probenecid. Everted intestine was incubated in CDNB solution (1 μM) at 4 °C for 40 min to preload CDNB; the efflux transport study of DNP-SG was carried out at 37 °C. Open circles represent the efflux to mucosal side; open triangles represent the efflux to the serosal side in the absence of probenecid. Closed circles and triangles represent the efflux to mucosal and serosal side, respectively, in the presence of probenecid (1 mM). Each value represents the mean ± s.e. of results from three rats. $*P < 0.05$; $**P < 0.01$ compared with control value.

**Table 1** Pharmacokinetic parameters of CDNB and DNP-SG uptake after application of CDNB in rat jejunum and ileum loop

| | Administration route of CDNB | |
|---|---|---|
| | Jejunum | Ileum |
| CDNB | | |
| Amount in intestinal fluid (% of dose) | 1.75 ± 0.80 | 1.67 ± 0.66 |
| Conc. in portal-vein plasma at 4.5 min (μM) | 0.79 ± 0.39 | 0.67 ± 0.02 |
| DNP-SG | | |
| Amount in intestinal fluid (% of dose) | 43.7 ± 6.0 | 20.4 ± 1.6* |
| Concn in intestinal mucosa (nmol (g tissue)$^{-1}$) | 53.7 ± 11.0 | 46.0 ± 9.7 |
| Concn in portal-vein plasma at 4.5 min (μM) | 1.51 ± 0.16 | 10.49 ± 0.99** |
| Cumulative biliary excretion (% of dose) | 11.3 ± 1.6 | 23.1 ± 3.5* |
| Concn in liver (nmol (g tissue)$^{-1}$) | 0.39 ± 0.16 | 0.38 ± 0.06 |
| Concn in femoral-artery plasma | | |
| Peak plasma level (μM) | 1.63 ± 0.19 | 3.21 ± 0.24** |
| $AUC_{0–60}$ (μM·min) | 33.7 ± 3.0 | 50.7 ± 1.2** |

CDNB was administered into a 20 cm long intestinal loop at a dose of 5 μmol kg$^{-1}$. Amounts and concentrations of CDNB and DNP-SG in various biological samples, except in portal-vein plasma, were determined at 60 min after administration of CDNB. Portal-vein blood was collected at 1 min intervals. No CDNB was detected in the intestinal mucosa, bile, liver and femoral-artery plasma samples. Each value represents the mean ± s.e. of results from three rats. *$P < 0.05$; **$P < 0.01$ compared with the value for the jejunum administration. $AUC_{0–60}$, area under the arterial plasma concentration–time curve from 0 to 60 min.

1.5–2-fold higher when CDNB was administered into the ileum region (Figure 4, Table 1).

### Bidirectional efflux of DNP-SG in Caco-2 cells

The efflux transports of DNP-SG after preloading of CDNB were examined in Caco-2 cells (Figure 5). Approximately 40% of the applied dose of CDNB was taken up when Caco-2 cells were incubated with CDNB solution for 40 min at 4°C. Probenecid exerted no significant effect on CDNB preloading. Without probenecid, approximately 40% of preloaded CDNB was effluxed as DNP-SG to the apical side and 50% to the basolateral side after 120 min' incubation. These bidirectional effluxes of DNP-SG were significantly suppressed by probenecid, indicating that both apical and basolateral efflux transports in Caco-2 cells are mediated, at least partly, by MRPs.

## Discussion

MRP-mediated intestinal transport of DNP-SG was studied in-vitro and in-vivo, from the viewpoint of the intestinal absorption of the MRP substrate drugs. We have previously evaluated the mucosal efflux of DNP-SG in rat jejunum and ileum in-vivo and in-vitro, and found a marked regional difference in MRP2 function between jejunum and ileum (Yokooji et al 2005). In the current study, we have studied the contribution of MRP3, expressed on basolateral membrane, together with apical MRP2, in the absorption of MRP substrates in rat intestine and Caco-2 cells.

The localisation and expression of various MRPs in rat intestine have been well studied. Previous reports revealed that MRP2 is localised on the BBM of enterocytes, especially in the proximal intestine, whereas MRP1, MRP3 and MRP6 are localised on the basolateral membrane in rodents (Kool et al 1999; Peng et al 1999; Mottino et al 2001; Rost et al

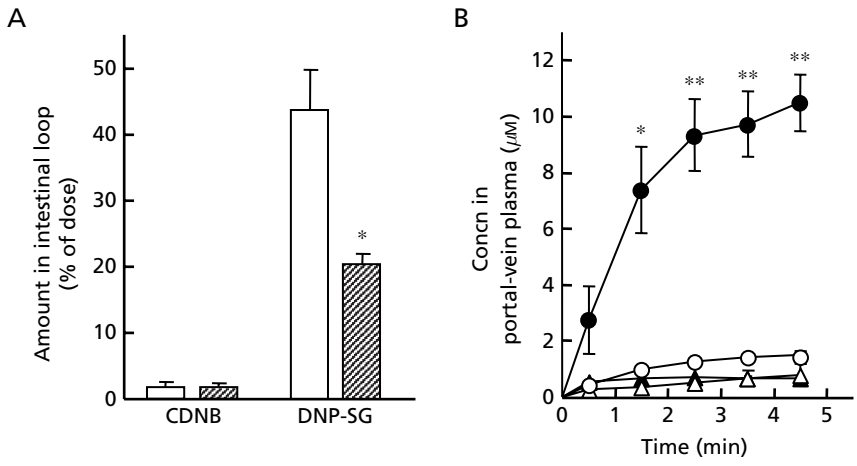


**Figure 3** Recovered amounts of CDNB and DNP-SG in intestinal loop at 60 min (A), and time courses of CDNB and DNP-SG concentrations in portal-vein plasma (B) after intra-jejunum and intra-ileum administrations of CDNB. CDNB was administered into a 20 cm long intestinal loop at a dose of 5 μmol kg$^{-1}$. Portal-vein blood samples were collected at 1 min intervals for 5 min. Concentrations of CDNB and DNP-SG in plasma were plotted at the midpoint of each blood collection. Open columns and symbols represent intra-jejunum administration; closed columns and symbols represent intra-ileum administration. Triangles represent CDNB; circles represent DNP-SG concentrations. Each value represents the mean ± s.e. of results from three rats. *$P$ <0.05; **$P$ <0.01 compared with the value for intra-jejunum administration.

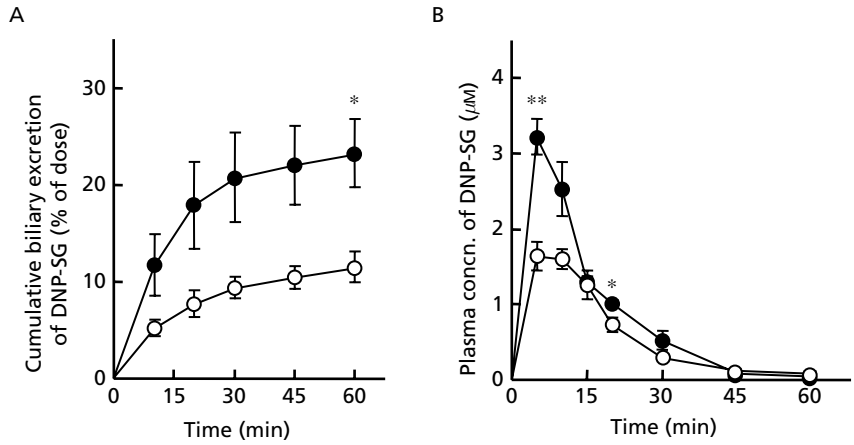


**Figure 4** Time courses of biliary excretion (A) and plasma concentrations in femoral-artery plasma (B) of DNP-SG after intra-jejunum and intra-ileum administrations of CDNB in rats. CDNB was administered into 20 cm long intestinal loops at a dose of 5 $\mu$mol kg$^{-1}$. Open circles represent the intra-jejunum administration; closed circles represent intra-ileum administration. Each value represents the mean ± s.e. of results from three rats. $*P < 0.05$; $**P < 0.01$ compared with the value for intra-jejunum administration.

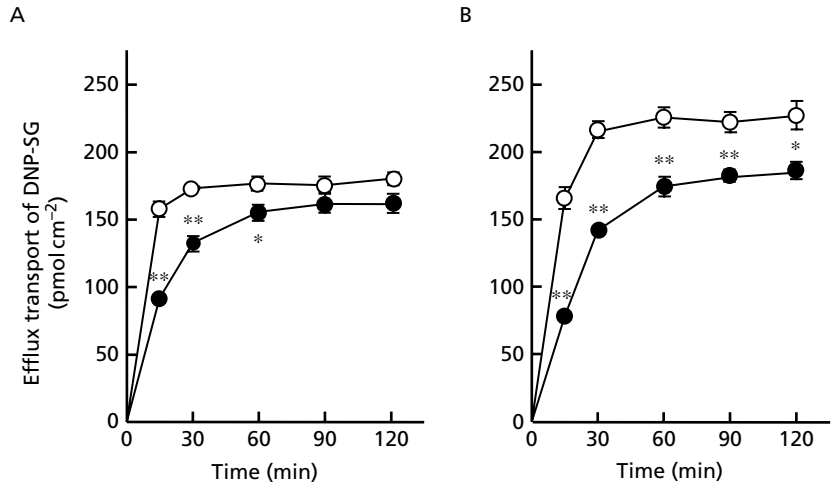


**Figure 5** Apical (A) and basolateral (B) effluxes of DNP-SG after preloading of CDNB in Caco-2 cell monolayers in the absence and presence of probenecid. CDNB (1 $\mu$M) was preloaded into Caco-2 cells at 4°C for 40 min, and efflux transport study of DNP-SG was carried out at 37°C. The concentration of probenecid in the transport medium was 1 mM. Each value represents the mean ± s.e. of three trials. $*P < 0.05$; $**P < 0.01$ compared with control value.

2002). The localisation of MRP4 in enterocytes is less clear. Johnson et al (2006) reported that MRP4 is localised primarily at the basal cytoplasmic region of enterocytes at the villus tips. MRP1 and MRP3 are expressed at a higher level in distal regions, whereas expression of MRP6 is higher at the proximal intestine in rodents. Johnson et al. (2006) reported that the expression of MRP4 in rat small intestine was low. In the current study, the expression of MRP2 and MRP3 was higher at jejunum and ileum, respectively, in good agreement with previous reports (Figure 1). Furthermore, the jejunum showed higher mucosal efflux of DNP-SG than ileum in-vitro and in-vivo, and the efflux was inhibited by probenecid (Figures 2 and 3). DNP-SG is transported by MRP1, MRP3, MRP5 and MRP6 as well as by MRP2 (Suzuki and Sugiyama 2002; Chan et al 2004). However, only MRP2 is expressed on the BBM in the intestine. Thus, the mucosal DNP-SG efflux in jejunum would be preferentially mediated by rat MRP2 (Figure 2A). In contrast, the participation of MRP2-mediated efflux of DNP-SG in rat ileum, where MRP2 expression was minimal, would be low, since probenecid showed no significant inhibitory effect (Figure 2B). Thus, the greater mucosal efflux of DNP-SG in jejunum than ileum could be mostly due to the participation of MRP2-mediated efflux in the jejunum region. DNP-SG is also transported by human BCRP, which is highly expressed in the distal small intestine in rats (Suzuki et al 2003; Tanaka et al 2005). Since a significant portion of DNP-SG efflux remained even in the presence of probenecid (Figure 2A), BCRP may also be involved in the transport. In addition, the regional difference in DNP-SG efflux was not ascribed to the difference in the formation rate of DNP-SG (i.e. glutathione *S*-transferase (GST) activity) because GST activity in rat intestine was quite high and there was no

significant regional difference in GST activity between jejunum and ileum (Yokooji et al 2005).

The function of MRP3 expressed on basolateral membrane was evaluated by measuring the serosal (basolateral) efflux of DNP-SG after preloading with CDNB. The serosal efflux of DNP-SG was higher in ileum, in good agreement with MRP3 expression (Figure 1), and the efflux was significantly suppressed by probenecid (Figure 2). As described above, DNP-SG is transported by MRP1 and MRP6 as well as by MRP2 and MRP3. However, expression of MRP1 in the small intestine is reported to be low and limited to undifferentiated enterocytes at the base of crypts in rodents (Peng et al 1999). MRP6 is highly expressed in rat proximal intestine (Maher et al 2006), although in the current study the serosal efflux of DNP-SG in jejunum was quite low and was not inhibited by probenecid (Figure 2A). Thus, a contribution of MRP1 and MRP6 to DNP-SG efflux transport in rat ileum could be ruled out, and MRP3 would be a predominant transporter for DNP-SG efflux in the ileum. The greater serosal efflux of DNP-SG in ileum than jejunum could be mostly due to the participation of MRP3-mediated efflux in the ileum region.

We have also examined the effect of administration site on the pharmacokinetics of DNP-SG after application of CDNB in rats. As expected, the efflux transport of DNP-SG to the intestinal lumen was greater in jejunum than ileum (Figure 3A). Also, portal-vein and femoral-artery plasma levels and biliary excretion rate of DNP-SG were significantly higher when CDNB was administered to the ileum (Figures 3 and 4). Collectively, these results suggest that the oral bioavailability of MRP substrate drugs could vary depending on the administration site in rat intestine. In this experiment, the portal-vein plasma levels of DNP-SG after intra-ileum administration of CDNB were approximately 7-fold greater than after intra-jejunum administration. In contrast, however, the difference of the peak plasma level of DNP-SG in arterial blood between intra-jejunum and intra-ileum administration was only 2-fold. These results may indicate that the difference in DNP-SG concentrations in portal-vein plasma does not relate directly to the difference in bioavailability. The reason for this is not clear at present. The extents of hepatic first-pass metabolism and/or plasma protein binding of DNP-SG may also be different when portal-vein plasma levels of a drug differ remarkably. Further study is necessary to clarify the mechanism.

Finally, we examined the efflux transport of DNP-SG in Caco-2 cells, a human adenocarcinoma cell line. The mRNAs of MRP1–6 are reportedly expressed in human intestine, and their expression levels in jejunum correlate well with those in Caco-2 cells (Taipalensuu et al 2001; Prime-Chapman et al 2004). However, in the present study, DNP-SG was effluxed to both apical and basolateral sides of Caco-2 cells, and the transport was inhibited by probenecid (Figure 5), in contrast to rat everted jejunum. Thus, there may be some species differences in MRP expression and function in the intestine. Oude Elferink et al (1993) reported that the pretreatment of Caco-2 cells grown on filters with 0.8 mM deoxyglucose led to ATP depletion, and both apical and basolateral DNP-SG secretion was largely inhibited by the ATP depletion (82% inhibition of apical efflux; 69% inhibition of basolateral efflux). Their report may suggest that other transporter(s) such as BCRP should also be taken into consideration, since the efflux transport was not inhibited completely by probenecid in Caco-2 cells (Figure 5), unlike ATP depletion. Further study is necessary to clarify the transport mechanism of DNP-SG in predicting the pharmacokinetics of MRP substrates in human intestine.

## Conclusion

We evaluated regional differences in the intestinal MRP functions pharmacokinetically by using DNP-SG, as well as MRP2 and MRP3 expression, in the rat. In good agreement with regional MRP2 and MRP3 expression levels, a marked site-specific bidirectional efflux pattern of DNP-SG was observed in rat intestine. MRP2-mediated mucosal efflux was greater in jejunum, and MRP3-mediated basolateral efflux was greater in ileum. Bidirectional efflux of DNP-SG was also observed in Caco-2 cells, although the MRP2-mediated and MRP3-mediated efflux transports were less than in rat intestine. As expected, site-specific MRP expression and function affected the pharmacokinetics of DNP-SG in rats. The bioavailability of DNP-SG was significantly higher when CDNP was applied to the ileum region.

There are many clinically important MRP substrates, including methotrexate, pravastatin and etoposide. Our results suggest that the intestinal bioavailability of MRP substrates could vary in humans depending on their absorption site, as demonstrated in rat intestine.

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