Xenobiotica, 2009; 39(10): 711–721

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RESEARCH ARTICLE

Decreased urinary secretion of belotecan in folic acid-induced acute renal failure rats due to down-regulation of Oat1 and Bcrp

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Abstract

1. The effects of folic acid-induced acute renal failure on the renal excretion of belotecan were investigated in rats after intravenous administration.
2. Both glomeruli and renal tubules were seriously damaged by folic acid-induced acute renal failure. The renal excretion clearance, CL_r, of belotecan was significantly decreased by folic acid-induced acute renal failure. Furthermore, glomerular filtration rate and secretion clearance of the drug were dramatically decreased by folic acid-induced acute renal failure.
3. *In vivo* renal uptake of belotecan was inhibited by *p*-aminohippurate, whereas renal excretion was inhibited by GF120918, but not by verapamil and bromosulphalein. This indicates that Oat1/3 and Bcrp are involved in the renal uptake and urinary excretion of belotecan, respectively.
4. Both mRNA and protein levels of Oat1, Oat3 and Bcrp were significantly decreased in folic acid-induced acute renal failure rats. Based on the finding that belotecan is a substrate of OAT1 but not of OAT3, the decrease in CL_r of belotecan in folic acid-induced acute renal failure could, therefore, mainly be attributed to the down-regulation of Oat1 and Bcrp, in addition to the decrease in glomerular filtration rate.

Keywords: *Acute renal failure; folic acid; renal clearance; secretion clearance; Oat1; Bcrp*

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Introduction

The kidney is a major organ for the removal of metabolic waste products and it plays an important role in balancing normal fluid volumes and electrolyte composition (Leon et al. 2005). Therefore, when severe renal impairment, such as acute renal failure (ARF), occurs, the process of renal elimination of xenobiotics is unavoidably affected. To study the nature of this disorder, researchers have used folic acid-induced acute renal failure (FA-ARF) rats (Fiaschi-Taesch et al. 2004; Cheng et al. 2005; Ortega et al. 2005; Szczypka et al. 2005). Characteristically, FA-ARF is associated with the rapid appearance of folic acid crystals within renal tubules and subsequent acute tubular necrosis, followed by epithelial regeneration and renal cortical scarring (Mullin et al. 1976; Bosch et al. 1993). FA-ARF is characterized by tubular injury, including tubular cell apoptosis, as well as tubular cell proliferation, inflammatory cell infiltration, and mild fibrosis in the chronic phase (Ortiz et al. 2000; Dai et al. 2002; Fang et al. 2005; Doi et al. 2006; Ortega et al. 2006). Interestingly, all these features are also found in human ARF, suggesting that FA-ARF is an excellent model that mimics human ARF.

While ARF itself is without doubt a serious disease, ARF in cancer patients is a much more complicated syndrome. Since ARF mostly alters pharmacokinetic elimination profiles of certain drugs due to renal dysfunction,

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(Received 17 March 2009; revised 06 May 2009; accepted 07 May 2009)

ISSN 0049-8254 print/ISSN 1366-5928 online © 2009 Informa UK Ltd
DOI: 10.1080/00498250903026458

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it may prevent cancer patients with ARF from receiving appropriate cancer therapy. Indeed, it has been reported that ARF significantly jeopardizes the chances of cancer patients receiving optimal treatment and, potentially, a cure (Lameire et al. 2005).

It is apparent, then, that the use of an anti-cancer agent such as belotecan should be optimized and carefully administered in cancer patients with ARF. Belotecan, a camptothecin-derivative anti-cancer agent (Figure 1) developed by Chong Kun Dang Pharmaceutical Corporation (Seoul, Korea), is substantially excreted via the urinary route and is known to be minimally metabolized (Crul 2003). Belotecan is currently used for the treatment of small-cell lung cancer and ovarian cancer (Crul 2003), which may induce renal failure due to its abdominal malignancy (Jones & Warren 1996). Therefore, the possibility cannot be ruled out that belotecan may be needed for use in cancer patients with ARF.

Therefore, to circumvent this problem, identifying the pharmacokinetic profiles of belotecan in ARF is mandatory. In our previous studies, it was found that belotecan is a substrate of OAT1, but not of OAT3 (see the Discussion section for more details). Also, it is well known that belotecan is a substrate of P-gp, BCRP and MRP2 (Li et al. 2008). It would therefore be desirable if the effect of ARF on the pharmacokinetics of belotecan could be explained in association with the expressional and/or functional change of these transporters. However, there has not yet been a study done from this perspective.

The purpose of the present study is, therefore, to investigate the pharmacokinetic profiles of belotecan in FA-ARF rat models with a focus on renal excretion, and to uncover the molecular underpinnings involved in belotecan transport.

Materials and methods

Materials

Belotecan was kindly provided by Chong Kun Dang Pharmaceutical Corporation (Seoul, Korea). Folic acid, *p*-aminohippurate (PAH), bromosulphalein (BSP), verapamil, and ofloxacin were purchased from Sigma-Aldrich (St Louis, MO, USA). GF120918 was kindly provided by GlaxoSmithKline (London, UK). A protein-binding kit was purchased from GENESYS (Millipore, Bedford, MA, USA). The primary antibodies for Oat1 (OAT11-A) and Oat3 (OAT31-A) were purchased from Alpha Diagnostic International (San Antonio, TX, USA), and Bcrp (sc-25156), Mrp2 (sc-59611), and P-gp (sc-8313) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from Sigma-Aldrich.

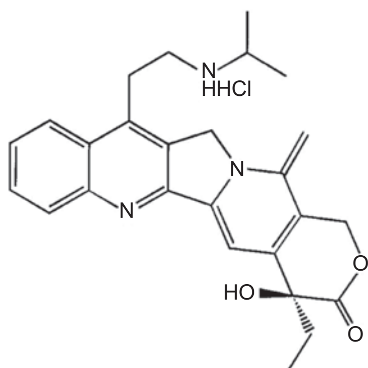

Figure 1. Chemical structure of belotecan.

Animals

Male Sprague–Dawley rats weighing 260–290 g were purchased from Orient Bio, Inc. (Ga-pyeong, Kyoung-gi, Korea). All rats were provided food (Sam Yang Company, Seoul, Korea) and water *ad libitum*, maintained in a light-controlled room (light: 07.00–19.00, dark: 19.00–07.00 hours), and kept at a temperature of 22 ± 2°C and relative humidity of 55% ± 5%. Experimental protocols involving animals were approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University, according to the guidelines of the National Institute of Health (NIH Publication Number 85-23, revised 2002).

Induction of ARF by folic acid

ARF was induced by injecting a solution of folic acid (25%, w/v) in 150 mM sodium bicarbonate intraperitoneally at a folic acid dose of 1 ml kg^{-1} (250 mg kg^{-1}) (Santos et al. 2001). Control rats were injected with 1 ml kg^{-1} of 150 mM sodium bicarbonate. One day after administration, the arterial plasma was collected, and creatinine, blood urea nitrogen (BUN), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) were analysed by an automated analyser (Fuji dri-chem 3500, Tokyo, Japan). Serum testosterone level was assayed by a radioimmunoassay kit (Coat-A-Court1 Testosterone, Diagnostic Products Corporation, Los Angeles, CA, USA). Urine was collected in metabolic cages for 24 h and assayed for *N*-acetyl-β-glucosaminidase (NAG) using a NAG kit (Nittobo, Tokyo, Japan). The rats were euthanized by decapitation and the kidneys excised, rinsed with normal saline, and fixed with 4% neutral buffered formaldehyde solution, and paraplast sections were stained with haematoxylin and eosin. Light microscopy of kidney specimens was then conducted.

Plasma pharmacokinetic study of belotecan

The femoral arteries, veins, and bladders of rats were cannulated with PE-50 polyethylene tubing (Clay

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Adams, Parsippany, NJ, USA) while under anaesthesia with ketamine (50 mg kg^{-1}, intramuscular injection). Body temperature was maintained with a heat lamp. The animals received belotecan at a dose of 5 mg kg^{-1} via the femoral vein. Blood samples (110 μl) were collected from the femoral artery at zero, 1, 10, 30, 60, 120, 240, 360, 480, and 600 min. The blood volume was replaced with an equal volume of saline to compensate for fluid loss. The urine was collected up to 600 min. The plasma samples were separated by centrifugation at 13 000*g* for 3 min at 4°C and stored at −80°C until high-performance liquid chromatography (HPLC) analysis. A total of 50 μl aliquots of plasma and urine samples were spiked with 15 μl of internal standard (ofloxacin solution, 200 μg ml^{-1}, in distilled deionized water [DDW]) and 130 μl of acetonitrile, and mixed. The mixture was then centrifuged (13 000*g* for 3 min at 4°C) and evaporated to dryness. The resulting pellets were dissolved in 130 μl of mobile phase for HPLC analysis. A total of 50 μl of the supernatant were injected for HPLC analysis.

The area under the plasma concentration–time curve from zero to infinity (AUC) was calculated using the trapezoidal-extrapolation method (Chiou 1978). For extrapolation, the area from the last data point to time infinity was estimated by the terminal-phase rate constant. Standard methods (Gibaldi & Perrier 1982) were used to calculate the time-averaged total body clearance (CL_t) and non-renal (CL_{nr}) clearance, and the apparent volume of distribution at steady-state (V_{dss}) by a non-compartmental analysis using WinNonlin® (version 3.1, Pharsight, Mountain View, CA, USA). Renal clearance of belotecan, CL_r, was estimated using equation (1):

$$CL_r = X_{u\ 0-t}/AUC_{0-t} \quad (1)$$

where $X_{u\ 0-t}$ represents the total amount of belotecan excreted into urine for a 0–10 h period; and AUC_{0-t} represents the area under the plasma concentration–time curve for the period, respectively.

Glomerular filtration rate (GFR) was estimated by calculating the creatinine clearance (CL_{cr}), assuming that the kidney function was stable during the experimental period. CL_{cr} was calculated by dividing the total amount of unchanged creatinine excreted in urine over 10 h by the mean plasma concentration of creatinine in each rat. Creatinine was assayed by the Green Cross Reference Laboratory (Seoul, Korea). Secretion clearance (CL_{sec}) was then estimated using equation (2) (Kino et al. 1999):

$$CL_{sec} = CL_r - (f_u \times GFR) \quad (2)$$

where f_u is the unbound fraction of belotecan in plasma.

The plasma protein binding of belotecan in control and FA-ARF rats was determined as follows. The femoral arteries and veins were similarly cannulated and belotecan was administered intravenously at a dose of 5 mg kg^{-1}. Plasma samples (2 ml) were collected twice, at 5 min and 2 h, because those time points are known to represent a disposition phase (5 min) and an elimination phase (2 h), respectively. A total of 500 μl of plasma samples were added into the Ultrafree®-MC ultrafiltration kit (Millipore, Bedford, MA, USA) and centrifuged at 5000 rpm (2300*g*) for 10 min at 37°C. After centrifugation, the concentration of belotecan in the filtrate was determined to be the unbound concentration. The concentration of belotecan in the plasma and filtrate was determined by HPLC analysis. The mean values at 5 min and 2 h served as f_u for control and FA-ARF rats.

Effect of PAH on the renal uptake of belotecan in normal rats

Oat1 is a transporter that mediates the uptake of organic anions from the plasma to the renal tubules of the kidney across the basolateral membrane. Belotecan, in a previous study by the authors, was found to be a substrate of Oat1 *in vitro* (unpublished data). In order to identify the involvement of Oat1 in the renal transport of belotecan *in vivo*, the effect of a representative Oat1 inhibitor, *p*-aminohippurate (PAH), on the uptake of belotecan to the kidney tissue was examined in normal rats. The early-phase tissue *in vivo* uptake clearance (CL_{uptake}) by the kidney was determined *in vivo* within 3 min after the intravenous administration of belotecan. Rats were anaesthetized with ketamine (50 mg kg^{-1}, intramuscular injection), and the femoral artery and vein were cannulated with PE-50 polyethylene tubing (Clay Adams, Parsippany, NJ, USA), filled with heparinized saline (20 IU ml^{-1}). Normal saline or PAH (dissolved in normal saline) was infused via the femoral vein for 4 h. The dose of PAH was 4 mg kg^{-1} h^{-1}. Belotecan in saline was then administered via intravenous at a dose of 0.1 mg kg^{-1}. Blood samples (220 μl) were then collected from the femoral artery at 0, 0.5, 1, 2 and 3 min after the administration of belotecan, and relevant plasma samples were obtained by centrifugation (13 000*g* for 3 min at 4°C). The animals were euthanized at 3 min after the administration of belotecan, and the kidneys were immediately dissected. Tissues were weighed and homogenized with 4 vols of DDW. Aliquots (100 μl) of both the homogenized tissues and the plasma samples were spiked with 30 μl of internal standard (ofloxacin solution, 200 μg ml^{-1}) and 260 μl acetonitrile, and mixed. The mixture was then centrifuged (13 000*g* for 3 min at 4°C) and evaporated to dryness. The resulting pellets were dissolved in 130 μl of mobile phase for HPLC analysis. 50 μl of the supernatant was injected for HPLC analysis of belotecan.

The total volume of blood withdrawn was 1.1 ml, and fluid loss due to the withdrawal was compensated

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for with an injection of saline via the femoral vein. The body temperature of the rats was maintained with a heat lamp. *In vivo* uptake clearance, CL_{uptake}, was estimated using equation (3):

$$CL_{uptake} = X_{3\ min}/AUC_{0-3\,min} \quad (3)$$

where $X_{3\ min}$ represents the amount of belotecan in the tissue at 3 min; and $AUC_{0-3\ min}$ represents the area under the curve up to 3 min.

Effect of GF120918, BSP and verapamil on the renal excretion of belotecan in normal rats

Belotecan was found to be a substrate of P-gp, BCRP and MRP2 (Li et al. 2008). In order to identify their involvement in the renal excretion of belotecan *in vivo*, the effects of these inhibitors on the renal excretion of belotecan were examined. The femoral arteries, veins, and bladders of rats were cannulated with PE-50 polyethylene tubing (Clay Adams) while under anaesthesia with ketamine (50 mg kg^{-1}, intramuscular injection). Body temperature was maintained with a heat lamp. Belotecan was administered via intravenous to rats at a dose of 2 mg kg^{-1} immediately after the intravenous administration of GF120918 (Bcrp and P-gp inhibitor, 3 mg kg^{-1}), BSP (Mrp2 inhibitor, 5 mg kg^{-1}), and verapamil (P-gp inhibitor, 3 mg kg^{-1}). Blood samples (110 µl) were collected from the femoral artery at 0, 1, 10, 30, 60, 120 and 240 min and centrifuged for plasma separation. The total volume of blood withdrawn was 0.77 ml and fluid loss due to the withdrawal was compensated for with an injection of saline via the femoral vein. Urine was collected from the start of the bolus injection up to 4 h. Plasma and urine samples were analysed for belotecan by HPLC. Renal clearance of belotecan, CL_r, was estimated using equation (1), where $X_{u\ 0-t}$ and AUC_{0-t} represent the total amount of belotecan excreted into urine for a 0–4 h period and the area under the plasma concentration–time curve for the period, respectively.

RT-PCR assay

Total RNA from the kidney was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). First-strand cDNA was generated by Superscript III (Takara, Shuzo Co., Kyoto, Japan) following the manufacturer's protocol. The polymerase chain reaction (PCR) amplification protocol was 94°C (15 s), 55°C (30 s), and 72°C (60 s) for 35 cycles, with a final elongation step at 72°C for 10 min with a GeneAmp PCR system 2400 (Applied Biosystems, Foster, CA, USA). Custom-designed oligomers were used for detection of genes of interest. Detailed information is shown in Table 1. PCR products were separated by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide staining. Band intensities were analysed by densitometry using Quantity One (version 4.1, Bio-Rad, Hercules, CA, USA).

Western blot analysis

Western blot analysis of Oat1, Oat3, P-gp, Mrp2 and Bcrp was performed on protein samples prepared from kidney extracts. Kidney samples from control ($n = 4$) and FA-ARF rats ($n = 4$) were homogenized in homogenization buffer comprised of 0.23 M sucrose, 5 mM Tris-HCl (pH 7.5), 2 mM ethylenediamine tetraacetic acid (EDTA), and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), using a Dounce tissue grinder® (Wheaton Science Products, Millville, NJ, USA). The homogenates were centrifuged twice at 3000*g* for 15 min at 4°C, and the supernatants were centrifuged at 100 000*g* for 30 min at 4°C. The pellets (membrane fraction) were collected and resuspended in a small volume of homogenization buffer. Protein contents were measured by bicinchonic acid (BCA) assay with bovine serum albumin as a standard. The crude fractions (50–100 µg) were separated on 5% (Oat1 and Oat3) and 7.5% (P-gp, Mrp2, Bcrp) polyacrylamide gels with 0.1% sodium dodecyl sulfate, then electrotransferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat dry milk in phosphate buffered saline (PBS, pH 7.4) for 1 h, then washed with PBS-Tween buffer. The membrane was probed for 12 h at 4°C with the primary antibodies OAT 11-A (dilution 1:1000), OAT 31-A (dilution 1:1000), H-241 (dilution 1:1000), M2111-5 (dilution 1:300) and ABCG2 (dilution 1:200) to recognize Oat1, Oat3, P-gp, Mrp2 and Bcrp, respectively. The membrane was then washed in PBS-Tween buffer three times,

Table 1. Summary of gene-specific primers used in reverse transcriptase-polymerase chain reaction (RT-PCR).

Gene	Sense strand	Antisense strand	Product size (bp)
Rat Oat1 (Slc22a6)	aga gtc aca gag ccc tgc at	gcc cag gct gta gac ata gc	402
Rat Oat3 (Slc22a8)	tcc tgg tgg gta cca gag tc	ctg cat ttc tga agg cac aa	468
Rat Bcrp (Abcg2)	caa tgg gat cat gaa acc tg	gag gct ggt gaa tgg aga a	536
Rat Mrp2 (Abcc2)	acc ttc cac gta gtg atc ct	gag gct ggt gaa tgg aga a	1084
Rat P-gp (Abcb1)	gga cca tca atg tga ggt at	gta gac aag cgg tga gct at	397
Rat gapdh	aac ttt ggc att gtg gaa gg	ccc tgt tgc tgt agc cgt at	472

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incubated with ImmunoPure® secondary antibody (Pierce Biotechnology, Rockford, IL, USA) for 1 h, and then washed with PBS-Tween buffer. Signal detection was done by enhanced chemiluminescence substrate for detection of horseradish peroxidase (Meridian Rd., Rockford, IL, USA).

HPLC analysis of belotecan

HPLC analysis was performed according to Jin et al.'s (2009) method. Briefly, the concentrations of total belotecan (that is, lactone plus carboxylate forms) in the plasma, urine and kidney homogenates were determined by a reverse-phase HPLC. The HPLC system (Yong Lin Co. Ltd, Kyoung-gi, Korea) consisted of a UV730D absorbance detector, a MIDAS auto sampler and a SP930D pump. A 50 μl aliquot of each sample (plasma, urine and kidney homogenates) was injected into a reverse-phase Microsorb-MV100™ Spherical C_{18} column (4.6 × 250 mm, 5 μm, Varian, Inc., Walnut Creek, CA, USA) at room temperature. The mobile phase was a mixture (25:75, v/v) of acetonitrile and 0.1 M potassium phosphate buffer in Milli-Q-purified water (pH adjusted to 2.4 with 85% phosphoric acid), which contained trifluoroacetic acid (final concentration of 0.2%, v/v, in the mixture). The flow rate was maintained at 1 ml min^{-1}. A Jasco FP-2020 plus fluorescence detector (Tokyo, Japan) was operated at an excitation wavelength of 370 nm and an emission wavelength of 435 nm. Calibration curves for belotecan were linear over the range of 10–2000 ng ml^{-1} and 5–200 μg ml^{-1} for plasma and urine with a correlation coefficient of 0.999. The limit of quantification was 10 ng ml^{-1} and 5 μg ml^{-1} for plasma and urine, respectively.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). A Student's *t*-test was applied for comparison of the two groups.

Results

Successful induction of FA-ARF rats

One day after intraperitoneal injection of folic acid into the rats, the kidneys were examined to determine whether renal failure had occurred. Light microscopy of kidney specimens revealed that both glomeruli and renal tubules were, indeed, extensively damaged (Figure 2). The levels of creatinine and blood urea nitrogen were also investigated, since renal failure is often diagnosed by marked elevation of creatinine and blood urea nitrogen. As expected, serum concentrations of creatinine increased about five-fold when compared with the control group (2.5 ± 1.0 versus 0.6 ± 0.1 mg dl^{-1}; Table 2), and blood urea nitrogen (BUN) levels also increased about nine-fold in FA-ARF rats (123.3 ± 4.7 versus 14.2 ± 4.2 mg dl^{-1}; Table 2). Obviously, the urinary level of *N*-acetyl-β-glucosaminidase, a biomarker for tubular damage, was greatly increased in FA-ARF rats compared with control rats (15.6 ± 4.6 versus

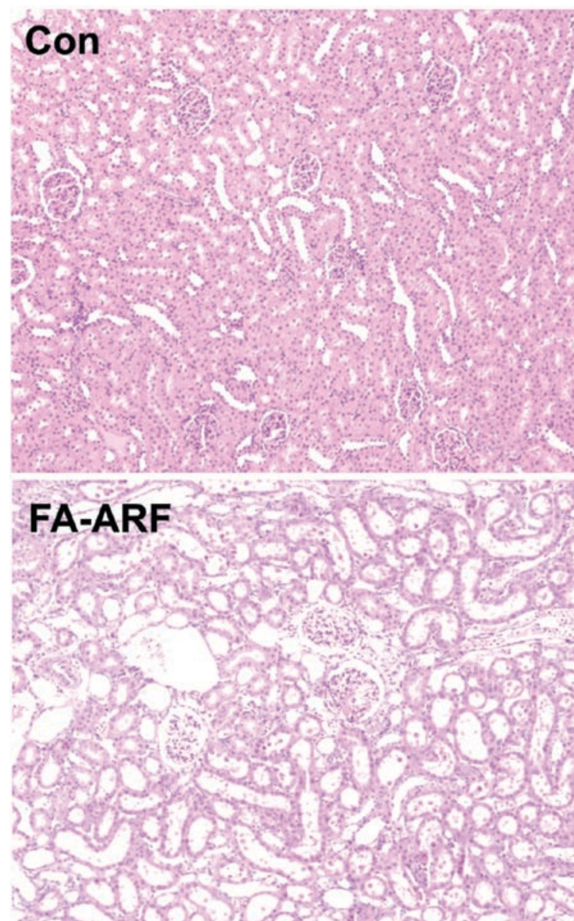

Figure 2. Representative histological images of the kidney (100x) from control (top) and FA-ARF rats (bottom). Necrosis and disruptions are clearly found in the kidneys of FA-ARF rats.

Table 2. Various physiological parameters in control ($n = 4$) and folic acid-induced acute renal failure (FA-ARF) rats ($n = 4$).

	Control	FA-ARF
Serum		
Creatinine (mg dl^{-1})	0.6 ± 0.1	2.5 ± 1.0**
BUN (mg dl^{-1})	14.2 ± 4.2	123.3 ± 4.7***
GOT (U l^{-1})	115.3 ± 34.3	112.5 ± 38.9
GPT (U l^{-1})	33.5 ± 5.8	43.3 ± 9.5
Testosterone (ng ml^{-1})	1.3 ± 0.8	2.5 ± 1.5
Urine		
N-acetyl-β-glucosaminidase (IU g^{-1}·creatinine)	5.7 ± 2.8	15.6 ± 4.6*

Notes: Data are expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.
BUN, blood urea nitrogen; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase.

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5.7 ± 2.8 IU g^{-1}·creatinine; Table 2). The serum levels of testosterone were also checked, since there was a previous report that some specific acute renal failure models are not dependent on the serum testosterone level, inferring that the cause of acute renal failure is different from that of chronic renal failure models (Shim et al. 2008). It was found that serum testosterone levels of the FA-ARF rats were similar to the control groups (2.5 ± 1.5 versus 1.3 ± 0.8 ng ml^{-1}; Table 2), supporting the idea that the aetiology of acute renal failure might indeed be different from that of chronic renal failure.

On the other hand, many studies have also shown that loss of renal function may lead to the decreased hepatic clearance of drugs (Sun et al. 2006). Hence, it was essential to confirm GOT and GPT levels to determine whether liver function was normal in FA-ARF rats. It was found that serum concentrations of GOT (112.5 ± 38.9 versus 115.3 ± 34.3 U l^{-1}; Table 2) and GPT (43.3 ± 9.5 versus 33.5 ± 5.8 U l^{-1}; Table 2) were not significantly different between the groups. It appears, therefore, that folic acid did not cause any significant damage to the liver. Overall, it can be concluded that FA-ARF rats were successfully induced without any noticeable symptoms.

Pharmacokinetic analysis of belotecan in FA-ARF rats

The next step was to check whether the pharmacokinetic profile of belotecan was altered in FA-ARF. After intravenous administration of belotecan into both the FA-ARF and control groups at a dose of 5 mg kg^{-1}, pharmacokinetic profiles and parameters were obtained (Figure 3 and Table 3). It was found that the plasma concentration of belotecan in the FA-ARF group was generally higher than that in control rats. In particular, AUC of belotecan was increased by almost two-fold and the total clearance (CL_t) was reduced by half in FA-ARF rats (Table 3). Because belotecan is known to be substantially excreted via the urinary route (Crul 2003), renal clearance (CL_r) of the two groups was computed for comparison. As expected, CL_r was greatly decreased, by almost six-fold, in FA-ARF rats compared with the control group. The creatinine clearance (CL_{cr}) was also found to be significantly decreased, by seven-fold, in FA-ARF rats. Because the creatinine clearance refers to the approximate GFR, this result indicates that glomerular filtration is significantly damaged in FA-ARF rats. Furthermore, CL_{sec}, which was estimated by equation (2), was seriously decreased by FA-ARF. Taken together, these findings indicate that both renal clearance of belotecan (CL_r) and creatinine clearance (CL_{cr}) were greatly reduced.

On the other hand, it was found that the degree of protein binding did not differ in control and FA-ARF rats, either at 5 min (f_u = 0.64 versus 0.63) or at 2 h (0.65 versus 0.64). The mean f_u value of 0.64 was therefore adopted in the calculation of CL_{sec} (Table 3) according to

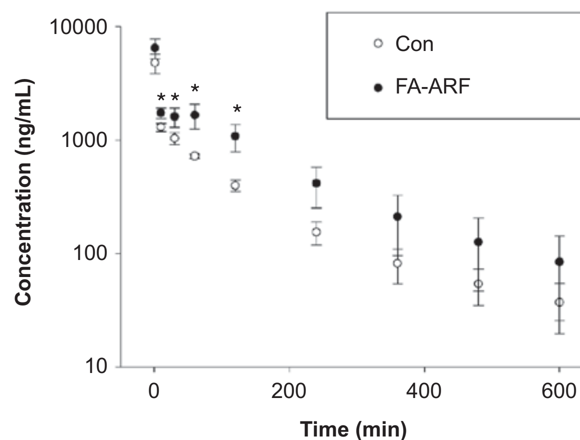

Figure 3. Time-concentration profiles (mean ± SD) of belotecan in control (empty circle, $n = 4$) and FA-ARF rats (filled circle, $n = 4$) following intravenous administration of 5 mg kg^{-1} belotecan. $^*p < 0.05$.

Table 3. Pharmacokinetic parameters after intravenous administration of belotecan in control ($n = 4$) and folic acid-induced acute renal failure (FA-ARF) rats ($n = 4$).

	Control	FA-ARF
AUC (min·μg ml^{-1})[a]	188.8 ± 26.2	386.9 ± 109.7*
CL_t (ml min^{-1} kg^{-1})	26.9 ± 3.8	13.7 ± 3.9**
V_{dss} (l kg^{-1})	4.3 ± 0.9	2.1 ± 0.3*
CL_r (ml min^{-1} kg^{-1})	10.3 ± 2.8	1.7 ± 1.1**
CL_{nr} (ml min^{-1} kg^{-1})	17.9 ± 1.5	10.4 ± 3.0*
CL_{cr} (ml min^{-1} kg^{-1})	4.3 ± 0.9	0.6 ± 0.5***
CL_{sec} (ml min^{-1} kg^{-1})	6.2 ± 2.0	1.3 ± 1.1**

Notes: [a]Calculated using the trapezoidal-extrapolation method (Chiou 1978). The extrapolated values from 600 min to infinity were less than 7%.

Data are expressed as mean ± standard deviation (SD). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

equation (2). These results indicate that protein binding is probably not the reason for the change in belotecan pharmacokinetics in FA-ARF rats.

Involvement of Oat1/3 and Bcrp in the renal transport of belotecan in vivo

It can be easily assumed that various transporters on tubular epithelial cells may contribute to the urinary excretion of belotecan. In fact, although belotecan seems to be a substrate of Oat1 *in vitro* (see the Discussion section for more details), it is not clear whether Oat1 participates in the uptake of belotecan *in vivo* across the basal membrane of renal tubular cells. Likewise, while it has been reported that the transport process of belotecan is associated with Bcrp, Mrp2 and P-gp transporters *in vitro* (Li et al. 2008), it is still unclear if these transporters are involved in the excretion of belotecan *in vivo* across the brush border membrane of renal tubular cells. Therefore, before examining the effect of ARF on belotecan, it is necessary to determine whether belotecan

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transport is mediated by these transporters *in vivo*. To check whether the renal uptake of belotecan is mediated by Oat1 in rats, PAH (Oat1/3 inhibitor) was used for pre-treatment and the uptake clearance of belotecan was observed. The free plasma concentration of PAH at the steady state achieved in the present study was high enough to inhibit the uptake of belotecan via Oat1 (that is, 11.6 ± 0.5 μM, $n = 4$ versus $K_i = 6.0$ μM, human; Jung et al. 2001). Under these PAH conditions, the uptake clearance of belotecan (CL_{uptake}) was greatly decreased (that is, 1.2 ± 0.04 versus 0.9 ± 0.09 ml min^{-1} g^{-1} kidney; Figure 4A), indicating the involvement of Oat1/3 in the renal uptake of belotecan.

Next, GF120918, a dual inhibitor for Bcrp and P-gp (Ose et al. 2008), was used for pre-treatment and the CL_r of belotecan was calculated. As expected, it was found that the CL_r was significantly decreased by the pre-treatment with GF120918 (1.5 ± 0.6 versus 5.7 ± 1.6 ml min^{-1} kg^{-1}; Figure 4B), indicating the involvement of either Bcrp or P-gp in the transport of belotecan. In order to check whether P-gp is involved in the renal transport of belotecan, another inhibition study was performed with verapamil (P-gp inhibitor). The plasma concentration of verapamil (for example, 3.0 ± 0.3 μM at 4 h after the administration) was believed to be sufficient to inhibit P-gp-mediated transport, since the ratio of verapamil concentration over the K_i value (that is, 8 μM; USFDA 2006), even at 4 h after the verapamil administration, was 0.38, which is much greater than 0.1 (a criteria for significant inhibition; USFDA 2006).

It was found that the CL_r was not changed significantly by the verapamil treatment (5.0 ± 1.1 versus 5.7 ± 1.6 ml min^{-1} kg^{-1}; Figure 4C), suggesting that P-gp may be minimally involved in the renal transport of belotecan. In addition, the CL_r of belotecan was not changed by BSP (an Mrp2 inhibitor) (Figure 4D), suggesting that Mrp2 may also be minimally involved in the renal transport of belotecan in rats. Although the influence of verapamil and BSP on the CL_r of belotecan may vary depending on the dose of these inhibitors, it can be concluded that the involvement of Bcrp is significant while that of P-gp and Mrp2 is minimal, if any. These *in vivo* experiments, therefore, demonstrated that the renal transport of belotecan is mainly mediated by Oat1/3 (uptake) and Bcrp (excretion) in rats.

Down-regulation of renal Oat1, Oat3 and Bcrp in FA-ARF rats

As previously mentioned, and as shown in Table 3, it was found that the secretion process — uptake and/or excretion — of belotecan in kidneys is greatly affected in FA-ARF. However, the molecular underpinnings that led to the decreased secretion in FA-ARF are yet unexplained. The results of the *in vivo* inhibition study demonstrated that belotecan in the kidney is taken up by Oat1/3 and excreted into the urine mainly through Bcrp. It thus seemed reasonable to examine the expression levels of Oat1, Oat3, Bcrp, and other transporters in FA-ARF. Therefore, mRNA levels of Oat1, Oat3, Bcrp, Mrp2, and P-gp in kidneys were first investigated using standard reverse transcriptase-polymerase chain reaction (RT-PCR) technique. It was found that mRNA expression of Oat1, Oat3 and Bcrp was significantly reduced in FA-ARF rats ($n = 4$; Figure 5). Levels of P-gp and Mrp2 were similar, however, between control and FA-ARF rats.

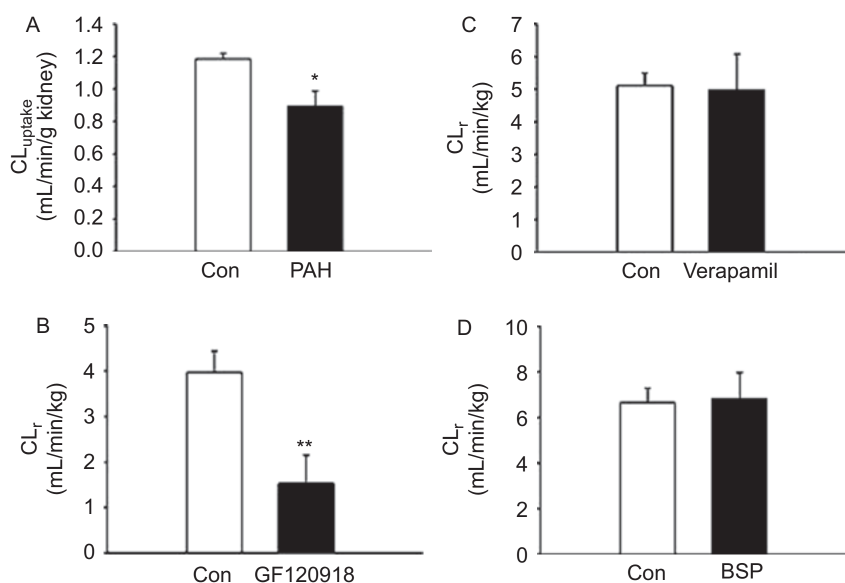

Figure 4. (A) Uptake clearance (CL_{uptake}) of belotecan (0.1 mg/kg) in control (empty bar, $n = 4$) and FA-ARF rats (filled bar, $n = 4$) under the intravenous infusion of PAH at 4 mg kg^{-1} h^{-1}. Renal clearance (CL_r) of belotecan (2 mg kg^{-1}) after pre-treatment with 3 mg kg^{-1} GF120918 (B), 3 mg kg^{-1} h^{-1} verapamil (C), and 5 mg kg^{-1} BSP (D). Data are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$.

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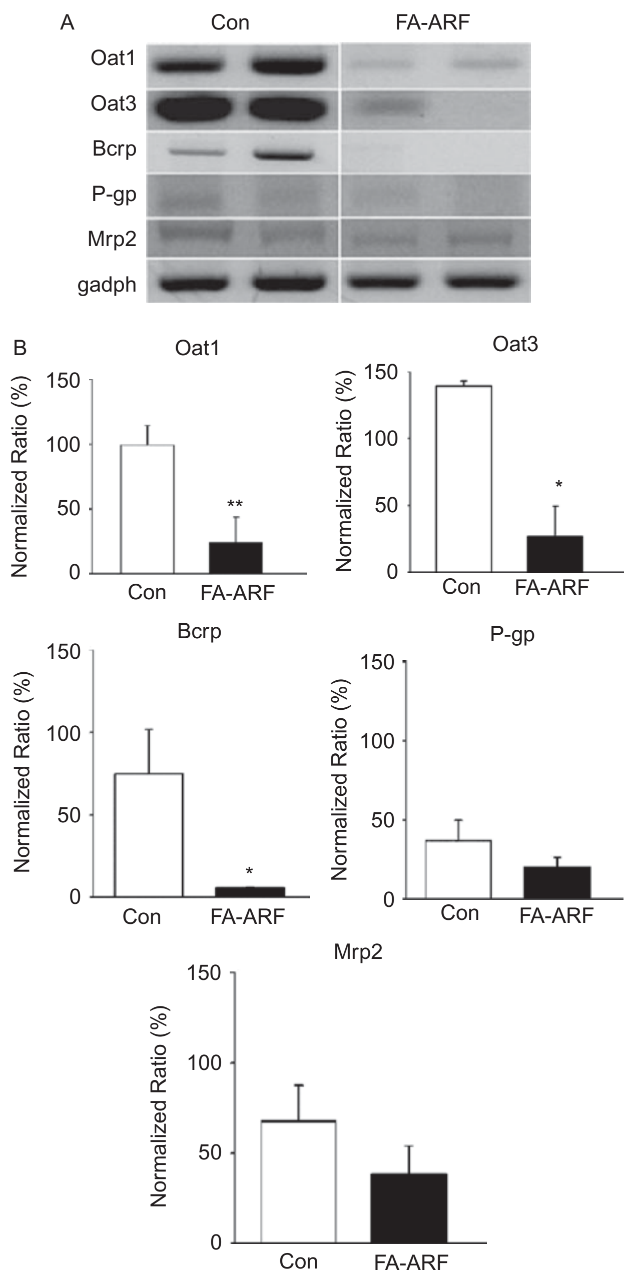

Figure 5. (A) Representative gel electrophoresis images of RT-PCR products in control and FA-ARF rats. (B) Normalized ratios of Oat1, Bcrp, P-gp and Mrp2 for control (empty bar, $n=4$) and FA-ARF rats (filled bar, $n=4$). Band intensities were normalized to the corresponding glyceroaldehyde-3-phosphate dehydrogenase (GADPH) intensity, which were regarded as 100%. Data are expressed as mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$.

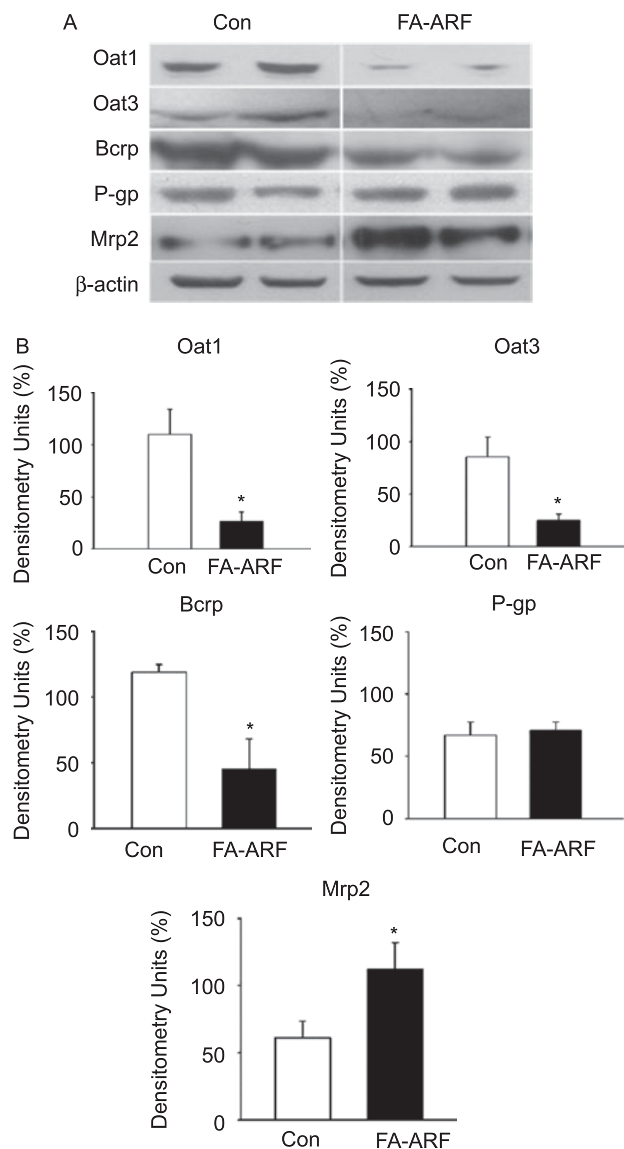

Figure 6. (A) Representative Western blot images from control and FA-ARF rats. (B) Relative densities of renal transporter proteins from control ($n=4$) and FA-ARF rats ($n=4$). Densitometry units were normalized to band densities of β-actin, which were considered as 100%. Data are expressed as mean ± SD. $^{*}p<0.05$.

A further experiment was done subsequently to investigate the levels of corresponding transporters at protein levels. As expected, the expression levels of Oat1, Oat3 and Bcrp proteins in kidneys were greatly decreased in FA-ARF rats when compared with those in control rats ($n=4$; Figure 6). However, it was unexpectedly found that the protein level of Mrp2 was significantly increased in FA-ARF rats ($n=4$; Figure 6), which seemed contradictory to the finding of little change in the Mrp2 mRNA level. On the other hand, in agreement with the mRNA level of P-gp, the protein level of P-gp was comparable between control and FA-ARF rats.

Overall, it can be concluded that the decreased transport of belotecan in ARF is mainly attributable to down-regulation of Oat1/3 and Bcrp transporters.

Discussion

When acute renal failure (ARF) is induced, changes in the elimination of xenobiotics are expected because

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kidneys play an important role in the excretion of xenobiotics. The results presented in this study indicated that folic acid-induced acute renal failure (FA-ARF) affects the pharmacokinetic profile of belotecan (Figure 3). After the intravenous administration of belotecan, as summarized in Table 3, the area under the curve (AUC) was increased two-fold, whereas the time-averaged total body clearance (CL_t) was decreased 50% compared with the control rats. Renal clearance (CL_r) was significantly decreased in FA-ARF rats, indicating that the change in the pharmacokinetics of belotecan caused by FA-ARF is likely to be associated with renal impairment. The non-renal clearance (CL_{nr}) was also decreased in FA-ARF rats, indicating that hepatic clearance of belotecan was probably decreased by FA-ARF, despite unchanged physiological parameters in the livers of FA-ARF rats (Table 2). The mechanism of altered CL_{nr} should be pursued elsewhere.

A previous report mentioned that disrupted brush borders and flattening of epithelia were shown by histological examination in the kidneys of mice receiving FA (Wan et al. 2006). In line with this report, Figure 2 and Table 2 show that glomeruli were seriously damaged in FA-ARF rats. Thus, it may be easily assumed that glomerular filtration is not working properly in FA-ARF rats, shifting loads of the elimination process to tubular cells where the secretion process takes place. Indeed, the secretion clearance (CL_{sec}) of belotecan was dramatically decreased by FA-ARF (Table 3). Since tubular cells have abundant uptake and excretion transporters, it is reasonable to assume that the expression profiles of these transporters are affected by FA-ARF. In the present study, we have demonstrated that Oat1 and Oat3, which are uptake transporters on the basolateral membrane of renal tubular cells, and Bcrp, an excretion transporter on the brush-border membrane of the cell, are down-regulated both at the mRNA and protein levels (Figures 5 and 6). Therefore, it seems evident that the decrease in renal secretion of belotecan in FA-ARF rats is closely associated with the down-regulation of Oat1/3 and Bcrp. Down-regulation might also be associated with the decrease in V_{dss} (Table 3). Since Oat2 is rare in male rat kidneys (Buist et al. 2002), its involvement in the renal uptake of belotecan was not investigated in the present study.

In our separate experiment, uptake assays of *human* OAT1 and OAT3 were carried out by expressing them on *Xenopus* oocytes (unpublished data). As a consequence, it was found that the uptake of belotecan was significantly increased specifically in OAT1-expressed oocytes (1.7-fold, $p < 0.0001$, $n = 10$), while no changes were found in those of OAT3 ($n = 10$).

Generally, orthologous transporters often share common substrates. Indeed, Nakagawa and colleagues revealed that transport characteristics of perfluorooctanoic acid through human and rat OATs/Oats were not much different (Nakagawa et al. 2008). Furthermore, Tahara and colleagues reported that the human OAT1/rat Oat1-mediated transport exhibited a good correlation, indicating that there is a minimal species difference between human OAT1 and rat Oat1 (Tahara et al. 2005). As mentioned above, since belotecan is a substrate of human OAT1 and not of human OAT3, belotecan is more likely to be a substrate of rat Oat1 than of rat Oat3.

With respect to Oat1 down-regulation, several reports have indicated that Oat1 is regulated by protein kinase C (Bringhurst et al. 1993; Uwai et al. 1998). Uwai and colleagues have suggested that Oat1 might be down-regulated by protein kinase C activity (Uwai et al. 1998). In fact, protein kinase C is induced under ischaemic renal injury in rats (Padanilam 2001), suggesting that the level protein kinase C might be elevated in FA-ARF rats, resulting in reduced expression of Oat1. However, further studies are necessary to elucidate the detailed mechanism for the reduction of Oat1 in FA-ARF rats.

Similarly, the present data showed a dramatic decrease of Bcrp in the kidneys of FA-ARF rats. It is now evident, furthermore, that belotecan is transported by human BCRP, both *in vitro* (Li et al. 2008) and *in vivo* (Figure 4B). Moreover, the fact that topotecan (a camptothecin derivative similar to belotecan) transport is inhibited by GF120918 (Jonker et al. 2000), which was also used in the present study to block the Bcrp and/or P-gp-mediated transport, strengthens the finding that belotecan is excreted via Bcrp and/or P-gp. Because it was revealed that P-gp was not much affected in the present study (Figures 4C, 5 and 6), it seems obvious that Bcrp is the major excretion transporter for belotecan in kidneys.

With regard to Mrp2, it was found that the mRNA level was barely changed (Figure 5), while the protein expression was increased in FA-ARF rats (Figure 6). Although the discrepancy did not seem straightforward at first, the increase of Mrp2 protein, rather than mRNA, was also found in other cases in ethynylestradiol-induced cholestasis rats (Trauner et al. 1997), as well as in a cisplatin-induced ARF study (Aleksunes et al. 2008). In the present study, since BSP treatment (Mrp2 inhibition) did not cause any change in belotecan transport in normal rats (Figure 4D), it is likely that Mrp2 is not the major efflux transporter for belotecan in kidneys, especially when compared with Bcrp. Therefore, it seems that the effect of Mrp2 on the renal transport of belotecan would not be significant in FA-ARF rats. Still, the exact mechanism of this post-transcriptional up-regulation of Mrp2 is not known and further studies are required.

Meanwhile, it should be noted that there is a clear species difference between humans and rodents with regard to transporter expression profiles in kidneys. Indeed,

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Huls and colleagues recently reported that the expression level of renal BCRP/Bcrp differs among mice, rats and humans (Huls et al. 2008). Moreover, BCRP is expressed relatively less than MRP2 in human kidneys (Hilgendorf et al. 2007), which is contrary to the case for rat kidneys. Care must therefore be taken when extrapolating these data to human cases. Nevertheless, there is no doubt that belotecan elimination is tightly linked to Bcrp.

The focus of the present study was on the effect of FA-ARF on the renal excretion of belotecan. However, the change in non-renal excretion in FA-ARF, especially hepatic elimination of the drug, is also of interest in association with the involvement of these transporters.

In summary, the present study revealed that the decreased renal secretion of belotecan in FA-ARF rats is mainly due to the down-regulation of Oat1/3 (probably Oat1) and Bcrp in the kidney. Additional concerns, such as reduced dosage and continuous monitoring, may therefore be necessary if belotecan is administered to ARF patients. It is hoped that these results may provide some valuable insights into how to treat cancer patients with ARF.

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Acknowledgements

This study was supported by a grant from the Korean Ministry of Science and Technology through the National Research Laboratory Program (Grant Number ROA-2006-000-10290-0). The authors express their gratitude to Chong Kun Dang Pharmaceutical Corp. (Seoul, Korea) for providing belotecan.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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