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Effects of the antiretroviral drug 2'-deoxy-2'-β-fluoro-4'-azidocytidine (FNC) on P-gp, MRP2 and BCRP expressions and functions

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The purpose of the present study was to dig into recent studies designed to characterize the impacts of 2'-deoxy-2'-β-fluoro-4'-azidocytidine (FNC) on P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP). Specifically, the modulation effects of FNC on P-gp, MRP2 and BCRP protein expressions were assessed by western blot methods. 5 (and 6)-Carboxy-2′,7′-dichloroflourescein (CDF) and BODIPY-prazosin were used to provide indications of alterations of MRP2 and BCRP activities. The effects of P-gp, MRP2 and BCRP on FNC were evaluated in the *in situ* single-pass intestinal perfusion model. The results showed that FNC at higher concentrations and with longer incubation times can upregulate the protein expression of P-gp, MRP2 and BCRP in Caco-2 cells. The upregulated proteins were also functionally active, as revealed by a lower degree of CDF and BODIPY-prazosin uptake by the cell monolayers. The intestinal absorptive coefficient (Peff) was observed to significantly increase with the inhibitors of P-gp, MRP2 and BCRP. These results suggested that FNC could modulate the expressions and functions of P-gp, MRP2 and BCRP, while P-gp, MRP2 and BCRP were involved in the efflux transport of FNC. The inductive effects of FNC on P-gp, MRP2 and BCRP suggested the possibility of FNC to contribute to the inter- and intra-individual variability of itself, as well as to alter the absorption of other drugs that may be administered concomitantly.

1. Introduction

Acquired immune syndrome (AIDS), which is caused by human immunodeficiency virus (HIV) infection, is one of the most devastating epidemics in the world. 2'-Deoxy-2'-β-fluoro-4'-azidocytidine (FNC) is a nucleosidic reverse transcriptase inhibitor currently undergoing phase II clinical trials for HIV treatment. FNC is administered orally and has to be absorbed from the small intestine before reaching the bloodstream. Therefore, the intestinal epithelium is the first physiological barrier for FNC absorption, thereby affecting the pharmacological effects of FNC.

Along the intestine, there are many drug transporters expressed both in the apical and basolateral side of intestinal epithelium, which can be classified as influx and efflux transporters according to their functions. The distribution and activity of these transporters can influence the intestinal permeability of many drugs commonly used. The ABC transporter superfamily is one of the largest protein families and widely expressed in the human body (Dean et al. 2001). P-gp, MRPs and BCRP are the best known efflux drug transporters which contribute to the multi-drug resistance (MDR) in cancer cells (Gottesman et al. 1995; Leslie et al. 2005). However, they are also abundantly expressed in normal tissue, such as intestine, liver and kidney (Takano et al. 2006). P-gp is a 170 kDa membrane protein which can modulate membrane permeability of many drugs. The substrates of P-gp include both neutral and positively charged hydrophobic compounds that are structurally and pharmacologically uncorrelated (Takano et al. 2006). MRP2 is a 174 kDa protein which has a relatively hydrophilic substrate profile, including glutathione, glutathione disulfide, and sulfate conjugates (Chan et al. 2004; Suzuki and Sugiyama 2002). BCRP is a 70 kDa transport protein. The substrates of BCRP are similar to P-gp or MRP1 (Doyle and Ross 2003). The activities of P-gp, MRP2 and BCRP can lead to sub-therapeutic plasma concentrations and eventually to failure in treatment as they can use the energy generated by ATP hydrolysis to transport their substrate drugs against the concentration gradient. Drug–drug interactions may also caused by drug transporters, resulting in increased or decreased therapeutic or adverse effects. Moreover, drug can alter the expressions and activities of several transporters, thereby influencing the efficacy and toxicity of the substrate drugs.

Previous evidence showed that FNC is able to upregulate the mRNA expressions of P-gp, MRP2 and BCRP, as well as P-gp activity, which suggested that it can potentially give rise to drug-drug interactions mediated by P-gp, MRP2 and BCRP (Liu et al. 2017). This is of considerable concern because many antiretroviral drugs (ARVs) are the substrates of P-gp, MRP2 and BCRP (Kis et al. 2010). Moreover current treatment of HIV infection requires the inclusion of a combination of three ARVs which have to be from two or more different ARV classes. The combination antiretroviral therapy increases the probability of drug-drug interactions. Given that the upregulation of mRNA may not influence protein expression and may not have functional activities, it is crucial to further assess the effects of FNC on the protein expressions of P-gp, MRP2 and BCRP, as well as the functions of MRP2 and BCRP.

To determine intestinal permeability, several *in silico*, *in vitro* and *in vivo* animal methods have been used. Previous studies on the intestinal absorption mechanisms of FNC using *in vitro* Caco-2 cells method suggested that FNC is well absorbed in the small intestine and is the substrate of P-gp, MRP2 and BCRP (Liu et al. 2017). However, the

Caco-2 cell model has several limitations. When biological relevance and accurate prediction are required instead of high-throughput screening, the *in situ* single-pass intestinal perfusion model is a better approach (Mols et al. 2009). This model is able to maintain the integrity of blood supply and the function of intestinal barrier, thus allowing to study the effects of efflux transporters on intestinal absorption on account of the biological relevant expression of them.

Therefore, the current study aimed to elucidate the effects of FNC on the protein expression levels and functions of P-gp, MRP2 and BCRP in the Caco-2 cell monolayer and to assess the participation of P-gp, MRP2 and BCRP in intestinal absorption of FNC, using the *in situ* single-pass intestinal perfusion model. For this purpose, the differential expression of P-gp, MRP2 and BCRP in Caco-2 cells after exposure to FNC, the uptake of substrate drug in Caco-2 cells after exposure to FNC, and the effects of transporter inhibitors on the intestinal permeability coefficient of FNC were investigated.

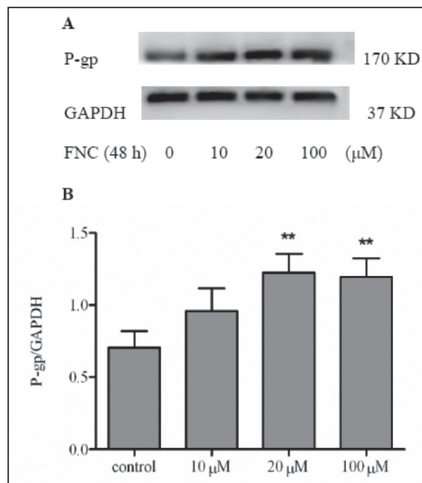

Fig. 1: (A) Effects of different concentration of FNC on the expression of P-gp in Caco-2 cells. (B) Denditometric analysis of immunoblots was performed using Image J and the results were normalized by GAPDH expression. Data are mean±SD of three replicates. $^{**}p < 0.01$ compared with the control group.

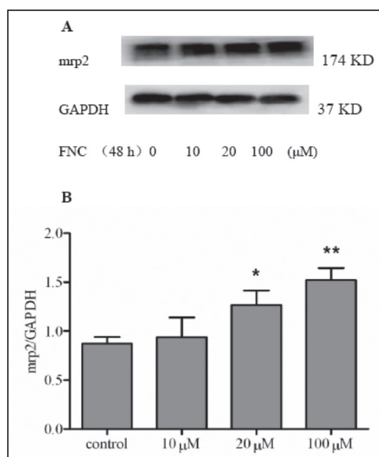

Fig 2: (A) Effects of different concentration of FNC on the expression of mrp2 in Caco-2 cells. (B) Denditometric analysis of immunoblots was performed using Image J and the results were normalized by GAPDHexpression. Data are mean±SD of three replicates. $^{*}p < 0.05$ compared with the control group. $^{**}p < 0.01$ compared with the control group.

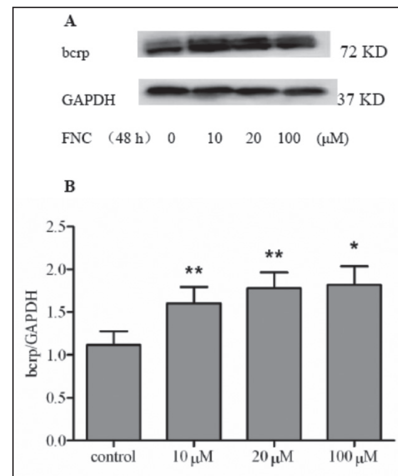

Fig. 3: (A) Effects of different concentration of FNC on the expression of bcrp in Caco-2 cells. (B) Denditometric analysis of immunoblots was performed using Image J and the results were normalized by GAPDH expression. Data are mean±SD of three replicates. $^{*}p < 0.05$ compared with the control group. $^{**}p < 0.01$ compared with the control group.

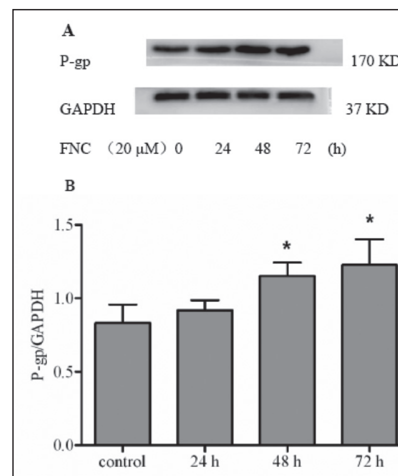

Fig. 4: (A) Effect of FNC (20 μM) on P-gp expression in Caco-2 cells after different incubation time. (B) Denditometric analysis of immunoblots was performed using Image J and the results were normalized by GAPDH expression. Data are mean±SD of three replicates. $^{*}p < 0.05$ compared with the control group.

2. Investigations and results

2.1. *Effects on P-gp, mrp2 and bcrp expressions*

To characterize the expression profile of P-gp, mrp2 and bcrp in the Caco-2 cells, the expression levels of protein were studied by western blot. The expressions of P-gp and mrp2 in control cells and in FNC treated cells are presented in Fig. 1 and Fig. 2, significantly higher expression levels of the P-gp and mrp2 protein were observed after exposure to 20 and 100 μM FNC for 48 h, but at this time point, the expressions of these two protein were not altered by 10 μM FNC. However, bcrp protein levels increased significantly after exposure to all these three concentrations of FNC for 48 h (Fig. 3).

Inductions of the protein expression levels of P-gp, mrp2 and bcrp in Caco-2 cells could also be seen after 72 h incubation with 20 μM FNC, but when reduce the incubation period to 24 h, the inductions were not significant (Figs. 4-6).

2.2. *Effects on MRP2 and BCRP functions*

The effect of FNC on P-gp function was reported in our previous study. The result proved that FNC can alter P-gp function in a concentration-dependent manner. To determine whether the FNC-mediated inductions in mrp2 and bcrp expressions were accompanied by changes in MRP2 and BCRP functions, uptake of CDF and BODIPY-prazosin in the Caco-2 cells which were co-cultured with FNC were examined. To evaluate the effect of FNC on MRP2, CDF uptake in Caco-2 cells was detected. After co-incubation with 20 and 100 μM FNC solutions for 1 h, the uptake of CDF in Caco-2 cells decreased by 21 % and 41 %, respectively, but the uptake did not change

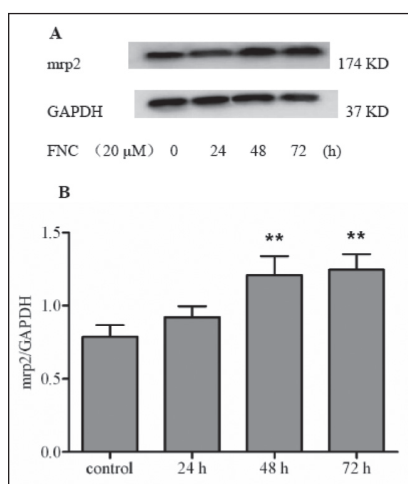

Fig. 5: (A) Effect of FNC (20 μM) on mrp2 expression in Caco-2 cells after different incubation time. (B) Denditometric analysis of immunoblots was performed using Image J and the results were normalized by GAPDH expression. Data are mean±SD of three replicates. $^{**}p < 0.01$ compared with the control group.

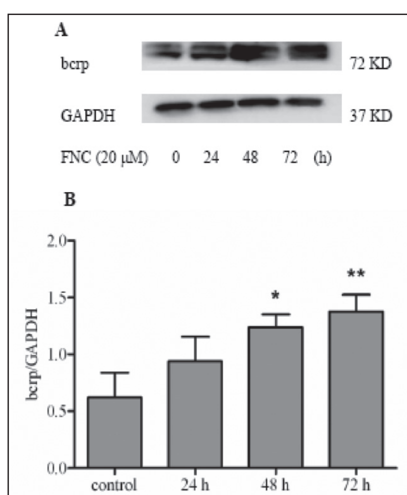

Fig. 6: (A) Effect of FNC (20 μM) on bcrp expression in Caco-2 cells after different incubation time. (B) Denditometric analysis of immunoblots was performed using Image J and the results were normalized by GAPDH expression. Data are mean±SD of three replicates. $^{*}p < 0.05$ compared with the control group. $^{**}p < 0.01$ compared with the control group.

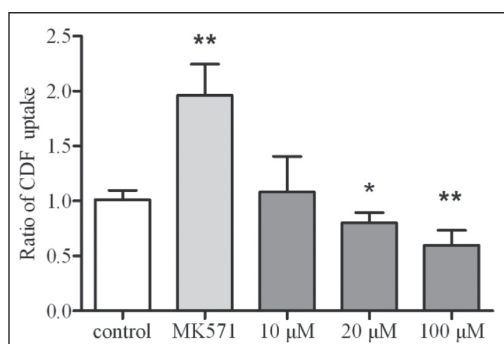

Fig. 7: Effects of different concentrations of FNC and MK571on CDF uptake. Sample fluorescence was standardized by the protein content. The data are the mean±SD of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control group.

significantly when treated with 10 μM FNC. MK-571, which is the inhibitor of MRP2, increased the uptake of CDF by 94%. The results are shown in Fig. 7.

The effect of FNC on BCRP activity was detected by assessing the uptake of BODIPY-prazosin after 1 h incubation with FNC.

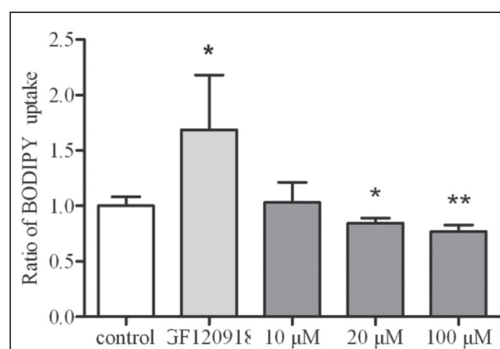

Fig. 8: Effects of different concentrations of FNC and GF120918 on BODIPY FL prazosin uptake. Sample fluorescence was standardized by the protein content. The data are the mean±SD of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control group.

The results are summarized in Fig. 8. FNC reduced the uptake of BODIPY-prazosin in a concentration-dependent manner. 20 and 100 μM FNC decreased the uptake of BODIPY-prazosin by 16 % and 23 %, respectively. FNC (10 μM) did not alter the activity of BCRP significantly. By contrast, GF120918 increased the uptake of BODIPY-prazosin by 69% by inhibiting BCRP.

2.3. The roles of P-gp, MRP2 and BCRP in FNC absorption

Previous *in vitro* studies showed that FNC is a substrate of P-gp, MRP2 and BCRP. However, the expression of the drug transporters in Caco-2 cells is different from that *in vivo*, thus the *in situ* single-pass intestinal perfusion model was selected to further analyze the influences of P-gp, MRP2 and BCRP on FNC intestinal permeability. Three intestinal segments of each rat, duodenum, jejunum and ileum, were used to study the absorption of FNC to test whether the intestinal absorption of drugs was site-dependent. Hence, three intestinal segments were simultaneously perfused to determine the best site for FNC absorption. The results are illustrated in Table 1. There were no obvious differences among duodenum, jejunum, and ileum for FNC absorption ($p > 0.05$). Therefore, the jejunum was chosen as absorptive segment in the following experiments.

Table 1: P_{eff} of FNC (75 μg/ml) in different intestinal segments of rat (n = 3, mean±SD)

Intestinal segment	Peff × 10^{-4} (cm/s)
Duodenum	0.83 ± 0.051
Jejunum	0.84 ± 0.078
Ileum	0.75 ± 0.29

At different starting concentrations (30, 75 and 150 μg/ml), FNC Peff obtained following *in situ* perfusion in the rat jejunum are presented in Table 2. Consistent with *in vitro* studies, FNC exhibited non-linear intestinal absorption. The Peff value first increased and then decreased. At all drug concentrations tested, FNC exhibited high permeability in the rat jejunum.

Table 2: P_{eff} of FNC with different concentrations in rat jejunum (n = 3, mean±SD)

Concentration (μg/ml)	Peff × 10^{-4} (cm/s)
30	0.57 ± 0.12
75	0.84 ± 0.078
150	0.47 ± 0.14

Verapamil, probenecid and GF120918 were studied for their effects on the intestinal absorption characteristics of FNC. The accumu-

Table 3: Effects of the inhibitors of P-gp, MRP2 and BCRP on the absorption of FNC in rat jejunum (n = 3, Mean ± SD). *$p < 0.05$ compared to the control group

Compound	Peff × 10^{-4} (cm/s)
Control	0.84 ± 0.078
Verapamil (100 μg/mL)	1.29 ± 0.087*
Probenecid (100 μg/mL)	2.18 ± 0.047*
GF120918 (5 μg/mL)	1.73 ± 0.15*

lated absorption amount of FNC in the presence or absence of the efflux transporter inhibitors is shown in Table 3. The presence of verapamil, probenecid or GF120918 significantly increased FNC permeability ($p < 0.05$). Hence, it was confirmed that the effects of membrane proteins on FNC intestinal absorption occurs not only *in vitro* in Caco-2 cell model but in the rat as well.

3. Discussion

The purpose of this research is to understand the effects of FNC on P-gp, MRP2 and BCRP and to confirm that FNC is the substrate of P-gp, MRP2 and BCRP, thereby maximizing its antiviral effects and minimizing the toxicity. In this study, experiments have been implemented to make clear the effects of the agent have on ABC drug transporters, and whether it has the potential to alter the intestinal disposition of the substrate drugs. Intestinal ABC drug transporters play significant roles in drug absorption and pre-systemic elimination. Therefore, either from the pharmacological or toxicological perspectives, the study to investigate the factors which can modulate their expressions and functions is critical. Previous studies found that FNC can induce P-gp activity and mRNA expressions of MDR1, MRP2 and BCRP in Caco-2 cells. The objective of present study was to further evaluate the potential of FNC to alter the protein expressions and activities of intestinal transporters.

Upregulation of P-gp, mrp2 and bcrp protein was observed, which suggested a FNC mediated transcriptional modulation. The current study has exhibited that the expression levels of P-gp, mrp2 and bcrp protein in the Caco-2 cells were affected by the FNC concentrations and duration of exposure. 10 μM FNC had no significant effect on the expression of P-gp and mrp2. 20 μM and 100 μM FNC could significantly upregulate the expressions of P-gp and mrp2, while 10 μM, 20 μM and 100 μM FNC could significantly upregulate the expressions of bcrp. The induction of protein expression levels were also observed in Caco-2 cells which were exposed to 20 μM FNC for 48 and 72 h. However, there was no significant alternation in the protein expression levels after 24 h exposure. The results suggest that when the substrates drugs of P-gp, MRP2 or BCRP were used in combination with FNC, FNC can reduce their absorption and increase their clearance.

In addition, CDF and BODIPY-prazosin uptake results suggested that the induction of mrp2 and bcrp protein by FNC is functionally active. Except to be a substrate of MRP2 and MRP3, CDF is also a substrate of OATP2B1. OATP2B1 is an influx transporter which is expressed on the brush border of Caco-2 cell monolayer (Sai et al. 2006). To exclude the possible effect of FNC on OATP2B1, the di-acetate ester prodrug of CDF, CDFDA, was used in the experiment. CDFDA is a non-fluorescent substrate of MRP2 which can be hydrolyzed into CDF by cellular esterases (Zamek-Gliszczynski et al. 2003). In the presence of 20 and 100 μM FNC, the accumulation of CDF in the Caco-2 cells was significantly decreased. In the case of MK571, an inhibitor of MRP2, the CDF retention was significantly increased. BODIPY-prazosin uptake study demonstrated that FNC also decreased the accumulation of this BCRP substrate. In contrast to GF120918, an inhibitor of BCRP, increased the uptake of BODIPY-prazosin. The experimental results show that FNC can regulate the functional activity of P-gp, MRP2 and BCRP, and its effect is concentration-dependent. This study was only a preliminary study to assess the possibility of FNC to increase the potential for drug-drug interactions by affecting the expressions of efflux transporters. Further studies are required to determine if FNC can influence the intestinal absorption and oral bioavailability of substrate drugs of ABC transporters.

Besides investigating the effects of FNC on P-gp, MRP2 and BCRP, studies have also been carried out to examine the effects of P-gp, MRP2 and BCRP on FNC uptake using *in situ* single-pass intestinal perfusion model. Previous study showed that the permeability of FNC, is high in Caco-2 cells. However, the predictive value of this method is not sufficient as the gastrointestinal tract is complex, especially when the interaction of active transport. To combine with *in vivo* permeability assays can help to get a better insight in FNC intestinal permeability and the influence of drug transporters.

In the single-pass intestinal perfusion model, the small intestine absorbs and secrets water while absorbing the drugs. The changes in intestinal water volume will affect the determination of the drug concentrations. Therefore, it is important to evaluate the water absorption and secretion. Commonly used methods include phenol red method, radio-labeled polyethylene glycol method and gravimetric method. Phenol red may interfere with the transport and analysis of some drugs, and phenol red itself can be absorbed. Radio-labeled polyethylene glycol can affect human safety. The gravimetric method was chosen due to its simplicity and precision (Sutton et al. 2001). The flow rate of the perfusion solution also has to be taken into account. High flow rate will damage the intestinal mucosa and lead to increased drug absorption in the intestinal. Moreover, the time of drug stayed in the intestinal will also influence the absorption. In this experiment, the flow rate was only 0.2 ml/min, which resulted in less damage to the intestinal mucosa and good correlation with oral administration (Stewart et al. 1995). The intestinal permeability coefficients were determined in the single-pass intestinal perfusion experiment. FNC was absorbed in rat duodenum, jejunum and ileum, and there was no significant difference in P_{eff} values among different segments. Therefore, the effects of drug concentrations and drug transporters on FNC absorption were investigated in jejunum. The P_{eff} values increased first and then decreased at all three concentrations (30, 75, 150 μg/ml), indicating that FNC was absorbed in a carrier-mediated manner. According to Parvin et al. (2005), P_{eff} values higher than 0.2×10^{-4} cm/s suggest that the absorption of drug is high. P_{eff} values lower than 0.03×10^{-4} cm/s suggest that the absorption of drugs is poor. P_{eff} values between 0.03 and 0.4×10^{-4} cm/s suggest that the drug can be moderately absorbed. The P_{eff} values of FNC in each segments and at all concentrations were above 0.2×10^{-4} cm/s, which indicated that FNC was completely absorbed in the small intestine of rats. When verapamil, probenecid or GF120918 was presented, the P_{eff} values were significantly increased, indicating that FNC was the substrate for P-gp, MRP2 and BCRP, consistent with the results of *in vitro* cell experiments.

In conclusion, the results of this study have shown that FNC has the potential to upregulate P-gp, MRP2 and BCRP protein expressions and enhance MRP2 and BCRP efflux activities in the intestinal epithelium, while the oral absorption of FNC can be affected by P-gp, MRP2 and BCRP. The modifications in intestinal expression and function of P-gp, MRP2 and BCRP not only contribute to the inter- and intra-individual variability in oral absorption of FNC, but also increase the likelihood of drug-drug interactions.

4. Experimental

4.1. Chemicals

FNC (purity 99.77%) was synthesized at Zhengzhou Granlen Pharmaceutical Technology Co., Ltd. (Zhengzhou, China). BODIPY FL prazosin and prestained protein standards were purchased from Thermo Fisher scientific (USA). l-Glutamine and HEPES were purchased from Amresco (USA). MK-571, GF120918, Non-essential amino acids (NEAA), 5(6)-carboxy-2',7'-dichlorofluorescein (CDF) and 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA) were all obtained from Sigma (USA). Probenecid was purchased from Aladdin (Shanghai, China). Rabbit monoclonal anti-P Glycoprotein antibody and mouse monoclonal anti-MRP2 antibody were purchased from Abcam (USA). Mouse monoclonal anti-BCRP antibody was purchased from Santa Cruz Biotechnology (USA). Rabbit polyclonal anti-GAPDH antibody was purchased from Goodhere Biotechnology (Hangzhou, China). Anti-mouse horseradish peroxidase antibody and anti-rabbit horseradish peroxidase antibody were purchased from Dingguo Changsheng Biotechnology (Beijing, China).

4.2. Cell culture

Caco-2 cells used were from passage 30 to 40. The cells were maintained in MEM supplemented with 10% FBS, 1% L-glutamine and 1% NEAA in culture flasks of 25 cm^2. The cells were incubated at 37°C in 5 % CO_2/95% air atmosphere. The medium was replaced on every other day until the cells reached 80–90% confluency.

4.3. Animals

Male Sprague-Dawley rats (200–240 g) were housed in a specific pathogen free (SPF) environment under a 12:12 h light:dark cycle, at 20-26 °C with sterilized food and water *ad libitum*. The animals were treated in accordance to the Guide for the Care and Use of Laboratory Animals (P. R. China) and the experiments were approved by the Animal Ethics Committee of Zhengzhou University.

4.4. Protein preparation

Caco-2 cells were cultured on 6 well culture plates using the MEM medium supplement with FNC (10, 20 and 100 μM) on day 12, 13 or 14. Control cells were cultured with MEM without FNC. On day 15, the culture medium was aspirated and the cells were washed twice with 4 °C PBS. Then 80 μL of Ripa lysis buffer was added into each well and the plates were incubated on ice for 5 min. The cells were moved to Eppendorf tubes by scraping with a cell scraper. After the cells were sufficiently lysed, the lysates were centrifuged at 4 °C for 10 min at 12,000 g with a refrigerated centrifuge (Eppendorf AG, Hamburg, Germany) and the supernatants, containing total proteins, were collected for western blot analysis.

4.5. Western blot analysis

Protein concentrations in the cell lysates were determined using a BCA Protein Assay Kit (Beyotime Biotechnology, China) in line with the manufacturer's instructions. The extracted proteins were solubilized in loading buffer and heated (70 °C, 10 min) to denature. Each protein sample (20 μg) was loaded onto 10 % SDS-polyacrylamide gel to separate. The gels were transferred onto a PVDF membrane and blocked by incubating for 1 h at room temperature with the solution containing Tris buffered saline, 0.1 % Tween 20 and 5 % non-fat dry milk. Then the membranes were incubated over night in the refrigerator with the primary antibody diluted in 5% non-fat dry milk at 1:1000 dilution of rabbit monoclonal antibody to P-gp, or at 1:50 dilution of mouse monoclonal antibody to mrp2 (M2 Ш-6), or at 1:200 dilution of mouse monoclonal antibody to bcrp (B-1), or at 1:1000 dilution of rabbit polyclonal antibody to GAPDH. After washing 10 min for three times in 5% non-fat dry milk, the membranes were incubated with the secondary anti-rabbit or anti-mouse horseradish peroxidase conjugated antibodies (1:2000) for 1 h at room temperature. Then the membranes were washed 10 min for three times again in 5% non-fat dry milk. The intensity of bands was analyzed with the Image J software. The level of P-gp, mrp2 or bcrp expression was normalized to the GAPDH as an internal loading control.

4.6. Effects on MRP2 and BCRP functions

The effects of FNC on the function of MRP2 and BCRP were determined by assessing the uptake of CDF and BODIPY-prazosin in Caco-2 cells by fluorospectroscopy.

Uptake of CDF after exposure to 10 μM CDFDA in Caco-2 cells was used to clarify whether FNC altered MRP2 function. Caco-2 cells were cultured on 24 well culture plates for 14 days. On day 15, the cells were co-cultured with 10, 20 and 100 μM FNC at 37 °C for 1 h. The positive control cells were co-cultured with MK571 and the blank control cells were co-cultured with drug-free HBSS. Then the solutions were removed, 10 μM CDFDA was added to each well and the cells were further incubated for another 1 h. After the incubation, the cells were washed with 4 °C HBSS for three times and then lysed by freeze-thaw cycling. Subsequently, CDF fluorescence was quantified by a microplate fluorescence reader (λ_{ex} = 504 nm, λ_{em} = 520 nm). The protein concentrations were used to standardize the concentration of CDF.

Uptake of BODIPY-prazosin in Caco-2 cells was used to determine if FNC modulated BCRP activity. Caco-2 cells were cultured on 24 well culture plates for 14 days. On day 15, the cells were co-cultured with 10, 20 and 100 μM FNC at 37 °C for 1 h. The positive control cells were co-cultured with 5 μM GF120918 and the blank control cells were co-cultured with drug-free HBSS. Then the solutions were removed, 2.5 μM BODIPY-prazosin was added to each well and the cells were further incubated for another 1 h. After the incubation, the cells were washed with 4 °C HBSS for three times and then lysed by freeze-thaw cycling. BODIPY-prazosin fluorescence was measured as described in the CDF uptake experiment (λ_{ex} = 504 nm, λ_{em} = 512 nm).

4.7. In situ studies on roles of P-gp, MRP2 and BCRP in FNC absorption

The roles of P-gp, MRP2 and BCRP in FNC absorption were investigated by *in situ* single-pass intestinal perfusion model in rats. The samples were analyzed by LC-MS/MS method as previously described (Peng et al. 2014).

Before the experiment, rats were fasted overnight. The experimental procedure was carried out according to Kim et al. (2006). In brief, rats were anesthetized by intraperitoneal injection of urethane (120 mg/100 g) and placed on a surface heated to 37 °C. The abdominal cavity was opened by a midline incision. The duodenum, jejunum and ileum (10 cm) were carefully taken out and both ends of the intestinal segments were cannulated with silicone tubing, avoiding injury to blood vessels. Then the food residual in rat intestine was cleaned out by 37 °C saline. During the experiment, the isolated segments had to be kept moist with saline. The perfusion solution contained FNC (30, 75 or 150 μg/ml), or FNC (75 μg/ml) with verapamil (100 μg/ml), probenecid (100 μg/ml) or GF120918 (5 μg/ml). The flow rate was 0.2 ml/min. At the beginning of the experiment, the perfusion solution was first perfused for 30 min to ensure a steady-state. Then samples were collected in 20 min intervals for 2 h. At the end of the experiment, the length (L) and radius (r) of perfused intestinal segments were accurately measured. The net water flux was determined by gravimetric method. The effective permeability (P_{eff}) was calculated with the following formula:

$P_{eff} = -Q_{in} \times \ln(C_{out}Q_{out}/C_{in}Q_{in})/2\pi rL$

where Q_{in} is the inlet perfusate flux (ml/min), Q_{out} is the outlet perfusate flux (ml/min), C_{in} was the inlet drug concentration, C_{out} was the outlet drug concentration, and $2\pi rL$ was the intestinal area available for absorption.

4.8. Statistical analyses

Data are expressed as the mean±SD. The unpaired two-tailed Student's *t* test and one-way analysis of variance (ANOVA) were used to determine differences for two groups and multiple group comparisons, respectively. $p < 0.05$ was considered as significant. The significance levels were represented as (*) when $p < 0.05$ and (**) when $p < 0.01$.

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