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Induction and Inhibition of Intestinal P-glycoprotein and Effects on Etoposide Absorption

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[OBJECTIVES] P-Glycoprotein actively extrudes a number of anti-cancer drugs, such as etoposide, from cancer cells. Its expression in cancer cells after a period of drug exposure was known as a common mechanism of multi-drug resistance (mdr). At normal physiologic condition, P-glycoprotein also expresses in various organs, such as lung, kidney, liver, adrenal tissues, pancreas, colon, as well as in the brush border membrane of the small intestine.

Etoposide tablet is clinically used as an anti-cancer drug for oral therapy. P-Glycoprotein located at the brush border membrane may pump out orally absorbed etoposide therefore decrease its bioavailability. We hypothesized that induction of intestinal P-glycoprotein might increase the etoposide efflux from blood into the intestinal lumen and, therefore, decrease the etoposide absorption. Similarly, inhibition of intestinal P-glycoprotein might increase the etoposide absorption. The effect of intestinal P-glycoprotein level inflection on the absorption of anti-cancer drugs is of great interest and clinical importance. In this study, we reported that etoposide absorption in the everted gut sacs can be modulated by induction and inhibition of P-glycoprotein in the small intestine. We have also studied the effect of a mdr-reversing agent, quinidine, on the absorption and exsorption of etoposide in the whole animal.

[METHODS] Male Sprague-Dawley rats bred and housed in the animal center of National Cheng Kung University, Medical College, were used. Rats were intraperitoneally injected with 10 µg/kg of sodium arsenite, 0.5 mg/kg of sodium butyrate, or 170 µg/kg of 3-methylcholanthrene (3-MC) daily for 5 days. The inducing effect were monitored by measuring efflux of the rhodamine 123 and confirmed by Western blotting and reverse transcription/polymerase chain reaction (RT/PCR). Etoposide was assayed with an HPLC method modified from a previous report¹. Rhodamine 123 was measured with a fluorescence spectrophotometer (excitation wavelength: 350 nm, emission wavelength 560 nm).

Four segments of the rat intestine of approximately 25 cm were taken and everted sacs were prepared as originally developed by Wilson & Wiseman². The sacs were filled with 3 mL of Tyrode solution and placed in a 50-mL Tyrode solution containing 100 µg/mL of etoposide. Two hundred µL of solution inside the sacs was taken every 10 min. For inhibition studies, 1 mg/mL of quinidine, a P-glycoprotein inhibitor, or 0.1 U/mL of C219 (Centocor Diagnostics, Inc., Malvern, PA, USA), a monoclonal antibody of P-glycoprotein, or 0.2 N of 5'-adenylylimidodiphosphate (AMPPNP), a non-hydrolyzable ATP analog, was pretreated for 30 min before adding etoposide.

Brush-border membrane vesicles (BBMV) were prepared from jejunum and ileum as described in the literature³. The protein content of BBMV was determined by using Bio-Rad protein assay kit. BBMV containing equal amount of protein was loaded and electrophoresed on SDS-PAGE, was then transferred onto nitrocellulose filter paper, and probed with 0.075 µg/mL of C219 monoclonal antibody. Visualization of protein bands was performed with enhanced

chemiluminescence (ECL) detection kit using horseradish peroxidase labelled secondary antibody as suggested by the manufacturer (Amersham International Plc, Buckinghamshire, UK).

RNA of the intestinal tissues was extracted as Chromazynski and Sacchi's method⁴. The sequence of primers for aldolase was GGCAAGGGCATCCTGGCTGCAGA (141-163) and TAACGGGCCAGAACATTGGCATT (583-561). The sequence of primers for *mdr1b* gene was GAGGTGAAGAAGGGCCAGACG (3175-3195) and TTCTGGATGGTGGACAGGCGGTGA (3822-3799)⁵. In RT/PCR reaction, the RNA was preheated at 70 °C for 10 min and chilled in ice. For cDNA synthesis, 200 units of MMLV reverse transcriptase (BRL, Gaithersburg, MD) was added to a 30 µL reaction volume containing RNA, 40 units RNase inhibitor (Promega, Masison, WI), 10 mM dithiothreitol, 333 µM 4 dNTPs, 20 mM TrisHCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 100 µg/mL BSA, and 100 pmol of two forward primers for aldolase and *mdr1b* gene. The reaction mix was incubated at room temperature for 10 min, then at 37 °C for 30 min, and stopped by heating at 95 °C for 5 min. The PCR reaction was performed by adding the RT reaction product to the mixture containing 100 pmol of reverse primers for aldolase and *mdr1b* gene, 6 µL of 5 mM MgCl₂, 2 µL of 5 mM dNTP, 7 µL of 10x Taq buffer (500 mM KCl, 100 mM TrisHCl, pH 9.0, and 15 mM MgCl₂), 55 µL H₂O, and 3.5 units of Taq polymerase. PCR was performed with 40 cycles of 45 sec at 95 °C, 2 min at 55 °C, and 2 min at 72 °C. The reaction product were analyzed on 2% agarose gel with ethidium bromide staining.

An *in situ* single-pass perfusion technique was used for exsorption studies⁶. A similar recirculated perfusion technique was used for absorption studies. The jugular vein was cannulated with silastic tubing and used for drug infusion. Blood samples was collected via the carotid artery cannula. Intestinal perfusate was perfused at approximately 20 mL/hr and collected hourly. The intestinal clearance of etoposide was calculated by dividing the rate of excretion by the mean drug concentration in serum during the sample period.

[RESULTS & DISCUSSION] Etoposide was transported from the mucosal side (bulk solution) to the serosal side (inside sac) after a lag period of approximately 10 min at different segments of the small intestine. When P-glycoprotein inhibitors were added, the etoposide efflux decreased and therefore etoposide concentration in the sacs was increased. The inhibition of etoposide efflux by C219 consisted of a P-glycoprotein-specific and a non-specific components. The negative antibody of C219 revealed non-specific inhibition of etoposide efflux. When the non-hydrolyzable ATP analog, AMPPNP, or quinidine was used, etoposide absorption in both ileum and jejunum was increased. Pretreating rats with sodium arsenite, sodium butyrate, or 3-MC for 5 days, on the other hand, increased the intestinal P-glycoprotein and decreased the rate of etoposide absorption in both jejunum and ileum. In RT/PCR, mRNA of rat *mdr1b* gene was increased in the jejunum, whereas the change was insignificant in the ileum. The inducing agents increased the exsorption of rhodamine 123 in the ileum, but not in the jejunum.

When quinidine was infused simultaneously, the rate of etoposide absorption increased, as reflected in higher etoposide concentration in serum. The increase was dependent on the rate of quinidine absorption. In exsorption studies, the intestinal clearance of etoposide was decreased from 41.7±7.2 mL/hr/kg to 18.4±3.8 mL/hr/kg when quinidine was infused at 1 mg/hr (P<0.05). The total clearance of etoposide was also decreased from 276±28 mL/hr/kg to 170±16 mL/hr/kg by quinidine co-administration (P<0.05).

The *in vitro* data support the hypothesis that etoposide is exsorbed by P-glycoprotein after its absorption. When P-glycoprotein was inhibited by the substrate inhibitor (quinidine), the unhydrolyzable ATP analog (AMPPNP), or the monoclonal antibody of P-glycoprotein (C219), etoposide exsorption was decreased and absorption can be substantially increased. On the other hand, when the intestinal P-glycoprotein was induced, etoposide exsorption increased and absorption decreased. The *in situ* studies further support our hypothesis. Increased etoposide absorption and decreased etoposide exsorption were observed when the P-glycoprotein inhibitor, quinidine, was co-administered.

Sodium arsenite, sodium butyrate, and 3-MC increased *mdr1b* mRNA in the jejunum, but not in the ileum. These inducing agents, on the other hand, increased P-glycoprotein in both the jejunum and ileum. It appeared that the induced P-glycoprotein in the ileum (ileum-type) was not from *mdr1b* gene. Interestingly, rhodamine 123 efflux was also increased in the jejunum, but not in the ileum. The results suggest that rhodamine 123 was transported by the P-glycoprotein encoded by *mdr1b* gene, but not by the ileum-type P-glycoprotein. Because etoposide absorption was decreased in both ileum and jejunum, we might conclude that the ileum-type P-glycoprotein transports etoposide. We, however, do not have data to suggest if there is ileum-type P-glycoprotein present in the jejunum, and whether rat *mdr1b* encoded P-glycoprotein can transport etoposide or not.

An increased serum concentration of etoposide may be due to an increased absorption, or a decreased elimination. Quinidine decreased the total clearance of etoposide. In the exsorption study, quinidine increased the serum concentration of etoposide by 32%. When the same rate of quinidine was infused in the absorption study, the etoposide concentration in serum increased more than 2 folds. It is clear that quinidine not only decreased the rate of etoposide elimination, but also increased the rate of etoposide absorption.

In this study, we have demonstrated that quinidine may increase etoposide absorption as well as inhibit etoposide elimination at a concentration below its therapeutic range. Quinidine may also potentiate etoposide action by inhibiting multidrug resistance of cancer cells. Further study is needed to ensure the clinical benefit of the combination of these two drugs.

[ABSTRACT] P-Glycoprotein (Pgp) actively pumps a number of antineoplastic drugs, such as etoposide, out of cancer cells and causes multidrug resistance. Pgp also expresses at the brush border membrane of the small intestine under the normal physiologic condition. We hypothesized that induction or inhibition of intestinal Pgp might increase or decrease the etoposide efflux from blood into the intestinal lumen and, therefore, decrease or increase the absorption of etoposide, respectively. Etoposide absorption was studied using an everted gut sac preparation of rat jejunum and ileum. In this study, we found that treating rats with sodium arsenite (10 µg/kg), sodium butyrate (0.5 mg/kg), or 3-methylcholanthrene (3-MC, 170 µg/kg) for 5 days decreased the intestinal absorption of etoposide in both jejunum and ileum. Western blotting with C219, a monoclonal antibody of Pgp, confirmed the increase of the intestinal Pgp in both ileum and jejunum. With reversed-transcription/polymerase chain reaction of rat *mdr1b* gene, we found that arsenite and butyrate induction increased *mdr1b* mRNA in the jejunum, whereas the inducing effect of arsenite, butyrate, or 3-MC in the ileum was not significant. Addition of C219 at 0.1 U/ml or 5'-adenylylimidodiphosphate at 0.2 N, a non-hydrolyzable ATP analog, increased the absorption of etoposide. Quinidine, an antiarrhythmics, has been demonstrated to circumvent the multidrug resistance in cell lines, possibly by interfering with Pgp function. Adding quinidine at 1

mg/ml to the everted gut sac preparation also substantially increased the absorption of etoposide. *In situ* absorption of etoposide was also studied by intraluminal perfusion of the drug in anesthetized rats. Intravenous infusion of quinidine at either 1 or 2 mg/hr both increased serum level of etoposide. Such an increase was shown to be dose-dependent. Intravenous infusion of etoposide at 0.2 mg/hr resulted in luminal exsorption of the drug in small intestine. The intestinal clearance rate of etoposide was 41.7 ± 7.2 ml/hr/kg. When etoposide was administered at the same infusion rate with 1 mg/hr of quinidine, the intestinal clearance of etoposide decreased to 18.4 ± 3.9 ml/hr/kg. These *in situ* data confirmed that the intestinal Pgp mediated the efflux of etoposide and the use of Pgp-inhibiting agent such as quinidine may increase the intestinal absorption of etoposide. In addition to etoposide, the efflux of rhodamine 123, which is a common substrate used to measure Pgp function, in the everted gut sacs was also studied. Despite of the Pgp level increase in both ileum and jejunum shown in Western blotting, rhodamine 123 efflux increased only in the jejunum, but not in the ileum. The results suggest that there are multiple members of Pgp family in the rat small intestine. Rhodamine 123 was probably transported by a Pgp encoded by *mdr1b*, and etoposide was transported by another Pgp member.

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