

Characterization of Intestinal Transport of Vincristine in Rats Applying *in situ* Single Pass Intestinal Perfusion

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Abstract: Objective: The present study was conducted to characterize intestinal transport of the cytostatic drug vincristine in rats, applying *in situ* single pass intestinal perfusion, with a special focus on investigation of the role of P-glycoprotein multidrug transporters in limiting intestinal permeability. Permeability Coefficient and the effect of P-gp modulators on Vincristine transport were studied in anaesthetized rats. **Materials and Methods:** Single Pass Intestinal Perfusion was performed in the jejunum. Vincristine (50 μM) in phosphate buffer saline was perfused at a flow rate of 0.2 mL min^{-1} and co perfused with P-gp inhibitor verapamil (200 μM) and inducer rifampicin pretreated for three weeks (60 mg mg^{-1}) then perfused with vincristine to detect its disposition characteristics. Drug concentrations in perfusate were analyzed by RP HPLC. **Results:** The effective permeability of vincristine in the jejunum found to be $0.692 \pm 0.071 \times 10^{-4}$ cm sec^{-1} due to the efflux mediated by P-gp. When vincristine is coperfused with verapamil its effective permeability is significantly enhanced to $1.575 \pm 0.0240 \times 10^{-4}$ cm sec^{-1} as it is a P-gp inhibitor. Rifampicin pretreated group for three weeks the effective permeability is significantly decreased to $0.344 \pm 0.093 \times 10^{-4}$ cm sec^{-1} is because of P-gp induction. The results of this study reveals that p-gp mediated efflux of vincristine can be reduced by cotreatment with any suitable p-gp inhibitors may reduce P-glycoprotein mediated drug resistance of vincristine and p-gp inducers can increase the efflux so careful monitoring is very important if vincristine is coadministered with any p-gp inhibitors or p-gp inducers.

Key words: Vincristine, verapamil, rifampicin, intestinal permeability, p-glycoprotein, RP-HPLC

INTRODUCTION

Vincristine is the naturally available antitumor alkaloid obtained from the plant *Vinca rosea* and is the important agent finding its applications in Hodgkin's, Nonhodgkins disease and in some of the solid tumors. But the limitation of vincristine is that it suffers from p-gp mediated drug resistance. Multidrug Resistance (MDR) gene encoding the P-glycoprotein found to be one of the major hindrances in successful antitumor therapy (Horio *et al.*, 1988). P-gp is a phosphorylated and glycosylated efflux protein belonging to a family of ATP binding cassette transporter plasma membrane proteins. It functions as a membrane-localized drug transport mechanism that has the ability to actively pump its substrates out of the cell. This could reduce the efficiency of absorption and thus enhance the drug resistance (Endicott and Ling, 1989). One of the usual methods of overcoming P-gp mediated drug efflux is to target it by using monoclonal antibody. It has been demonstrated that the disposition of MRK 16, a monoclonal antibody against P-gp and evidenced the selective accumulation of it in the tumors over expressing this antigen (Mano *et al.*, 1997).

The other method to overcome the MDR is by coadministering a P-gp inhibitor along with the antitumor agent. A number of compounds have been found to possess inhibitor activity both *Invitro* and *Invivo* against P-gp, of which verapamil and cyclosporine are few of them (Slater *et al.*, 1995). Very limited data is available over the concomitant administration of P-gp modulators and antitumor agents which are the substrates of P-gp. The co administration of cyclosporine or verapamil with vincristine or adriamycin has increased the survival time mice indicating the P-gp modulatory activity of them (Song *et al.*, 1999).

It was also evidenced that noncytotoxic dose of verapamil enhanced the cytotoxicity of Vincristine and Vinblastine in leukemia indicating the Multi Drug Resistance reversal (Tsuruo *et al.*, 1981). Based on these evidences, we characterized here the effect of Verapamil and Rifampicin which are inhibitor and inducers of P-gp, respectively on the absorption and disposition of Vincristine in Albino rats. The present study involves the use of well validated rat single pass intestinal perfusion technique (Schanker *et al.*, 1958). It is considered as one of the most reliable technique ensuring the fidelity of absorption and metabolism. This method was also

approved by Food and Drug Administration (FDA) US (Jeong *et al.*, 2004). The possibility of drugs whether they are the substrates for P-gp or not can also be demonstrated (Song *et al.*, 2006). Rat model is very reliable for conducting absorption studies and the influence of P-gp mediated drug efflux mechanisms because of its closer resemblance to human situation with respect to absorption and metabolism (Cao *et al.*, 2006). The *in situ* perfusion experiments offer great advantage over *in vitro* techniques because of intact blood and nerve supply thus simulating the *in vivo* condition.

The aim of the study was to determine the effective permeability of vincristin in jejunal rat segments *in situ* using single pass intestinal perfusion technique.

MATERIALS AND METHODS

Chemicals: Vincristine Sulphate and vinblastine Sulphate were gifted by Cipla Pharmaceuticals (Mumbai, India). Propranolol was gifted by Martix Laboratories Ltd. (Hyderabad, India). Phenol red was purchased from Himedia (Mumbai, India). Solvents used for quantification were of HPLC grade (Merck, India) and all other chemicals and reagents were of analytical grade.

Stability and adsorption test of the samples: Incubation of vincristine and propranolol in the perfusion medium at 37°C for 180 min showed no degradation of the compounds. The stability of vincristine and propranolol in the intestinal perfusate was also tested. Luminal perfusate was incubated for 60 min at 37°C at a pH of 7.4 and no degradation of vincristine and propranolol was detected. No adsorption of vincristine and propranolol to the catheters was found. Vincristine and propranolol was found to be stable in frozen perfusate (-80°C) for at least for six months and the samples were analyzed within 2-3 months after the perfusion experiment.

In-situ permeability studies

Animals: Healthy male Wistar rats (270-350 g, n = 4) used for *in-situ* single-pass perfusion study. Anesthesia, surgical and disposal procedures were justified in detail and were approved by the Institutional Animal Ethics Committee (IAEC, KU, A.P., India).

The surgical procedure and the *in situ* single-pass perfusion experiments were performed according to the methods described previously (Song *et al.*, 2006; Salphati *et al.*, 2001; Varma and Panchagnula, 2005; Jeong *et al.*, 2004; Zakeri-Milani *et al.*, 2007; Balimane *et al.*, 2000). Rats were maintained on a 12 h light-dark cycle and fasted 12-18 h before each experiment. They were anaesthetized by thiopental sodium (50 mg kg⁻¹, i.p.) and placed on a hot pad to maintain normal body temperature. A midline incision was made on the abdomen and a jejunal segment of

approximately 8-12 cm was identified. Semi-circular incisions were made at each end and the lumen was rinsed with saline (37°C). Both ends were cannulated with PE tubing (0.62 inches i.d. and 0.125 inches o.d.) and ligated by using silk suture. Blank perfusion buffer (Phosphate buffer, pH-7.4) was first infused for 5 min at a flow rate of 1 mL min⁻¹ by a syringe pump (NE-1600, New Era Syringe Pumps, Inc. NY, USA), followed by perfusion of phosphate buffer saline (pH-7.4) containing vincristine (50 µM), propranolol (100 µM) and phenol red (50 mg mL⁻¹) (Sutton *et al.*, 2001; Salphati *et al.*, 2001) without and with verapamil (200 µM) at a constant flow rate of 0.2 mL min⁻¹ for 90 min and perfusate collected every 10 min. After cannulation the segment was covered with isotonic saline-wet gauze (37°C). At the end of the perfusion the length of the segment was measured following the last collection. Samples were stored at -80°C until analysis.

P-gp Induction study: Another group of animals were pretreated with rifampicin for about three weeks and followed by perfusion of phosphate buffer saline (pH-7.4) containing vincristine (50 µM), propranolol (100 µM) and phenol red (50 mg mL⁻¹) (Sutton *et al.*, 2001; Salphati *et al.*, 2001) at a constant flow rate of 0.2 mL min⁻¹ for 90 min and perfusate collected every 10 min. After cannulation the segment was covered with isotonic saline-wet gauze (37°C). At the end of the perfusion the length of the segment was measured following the last collection. Samples were stored at -80°C until analysis.

Determination of vincristin and propranolol by HPLC: HPLC was conducted on Shimadzu (Kyoto, Japan). The column used for chromatographic separations was 4.6 mm i.d., 250 mm length and 5 µm particle size C18 (Merck, India). Twenty two microliter of vincristin and propranolol was injected for HPLC analysis. For vincristin the mobile phase consisting of 0.02 M sodium dihydrogen phosphate-methanol (36:64, v/v, pH = 4.7) at a flow rate of 1.0 mL min⁻¹.

The ultraviolet detection wavelength was set at 276 nm (Chen *et al.*, 2011). Vinblastine sulphate was used as internal standard. In case of propranolol mobile phase consisting of 55% methanol and 45% 0.05 mM KH₂PO₄ (pH -6.0) was pumped at a flow rate of 1 mL min⁻¹ and chromatograms were recorded at 227 nm:

$$\frac{-Q \times \ln(C_{out}/C_{in})}{2\pi rL}$$

Determination of phenol red by Spectrophotometry: The phenol red in phosphate buffer pH (7.4) has a characteristic red color which was measured Spectrophotometrically at 560 nm.

Data analysis

Phenol red water flux correction: $C_{out (corr)}$ was calculated from the following equation (Sutton *et al.*, 2001):

$$C_{out (corr)} = C_{out} \times \frac{\text{Concentration of phenolredin (CPR in)}}{\text{Concentration of phenolredin (CPR out)}}$$

Where:

- $C_{out (corr)}$ = Corrected outlet concentration of the drug
- C_{out} = Outlet concentration of the drug
- CPR in = Concentration of phenol red entering the intestinal Segment
- CPR out = Concentration of phenol red exiting the intestinal Segment 2.7.2

Calculation of effective permeability coefficient (P_{eff}).

It is the quantitative estimate of the rate of passage of a solute across a membrane. It is calculated from the steady state concentration of compounds in the collected perfusate which is considered to be attainable when the concentration level of Phenol red is stable. The steady state effective permeability is calculated using the following equation as the buffer solution is perfused from an entrance in one end of the intestinal segment to an exit at the other end of the intestinal segment (Salphati *et al.*, 2001; Griffin and O'Driscoll, 2008).

$$P_{eff} = \frac{-Q \times \ln (c_{out} / c_{in})}{2\pi RL}$$

Where:

- P_{eff} = Effective permeability coefficient
- Q = Perfusion flow rate
- C_{out} = Corrected outlet drug concentration
- C_{in} = Inlet drug concentration
- R = Radius of the rat small intestine
- L = Length of the perfused intestinal segment

Statistical analysis: Difference in permeabilities of vincristine was evaluated by GraphPad prism software version 5.0 at $p < 0.05$ was considered as statistically significant. Difference in permeabilities and difference between the concentration time profiles over the entire range tested were analyzed by two-way ANOVA (Bonferoni post-test).

RESULTS

In situ study

Effect of verapamil and rifampicin on intestinal permeability of vincristin: In the present study (P_{eff}) effective permeability of vincristin was determined in rat jejunum segment using *in situ* single pass perfusion

Table 1: Influence of verapamil (P-gp inhibitor) and rifampicin (P-gp inducer) on P-gp mediated efflux and intestinal permeability of vincristin and propranolol in rat jejunum

Group	$P_{eff} \times 10^{-4}$ (cm sec ⁻¹)
Vincristine	0.692±0.071
Vinc+verapamil	1.575±0.024***
Vinc+rifampicin	0.344±0.093***
Propranolol	0.892±0.072
Propranolol+verapamil	0.921±0.076***
Propranolol+rifampicin	0.592±0.071***

Values are Mean±SD (n = 6), *** $p < 0.001$, in comparison to $P_{eff, control}$ (without P-gp inhibitor and without P-gp inducer)

technique and the samples were analyzed by RP-HPLC. Effective permeability values were calculated from the steady-state concentrations of compounds in the perfusate collected from the outlet. Intestinal permeability coefficient of vincristin in the absence and presence of verapamil was found to be $(0.235 \pm 0.071) \times 10^{-4}$ cm sec⁻¹ and $(0.429 \pm 0.021) \times 10^{-4}$ cm sec⁻¹ ($p < 0.001$), respectively. Verapamil (200 μ M) a P-gp inhibitor co-perfused with vincristin (30 μ M) resulted in significant increase in intestinal permeability by 2.6-fold. Intestinal permeability coefficient of propranolol in the absence and presence of verapamil and rifampicin was found to be $(0.892 \pm 0.072) \times 10^{-4}$ cm sec⁻¹ and $(0.921 \pm 0.076) \times 10^{-4}$ cm sec⁻¹ and $0.592 \pm 0.071 \times 10^{-4}$ cm sec⁻¹, respectively which was found to be statistically insignificant (Table 1). Propranolol is highly permeable marker was shown to have no interaction with P-gp.

DISCUSSION

Permeability studies are routinely performed by using various animal species like rat, rabbit, pig, dog and monkey to study the patterns of drug absorption and influence of various transporters. Among all of these the rat model proved to be a better model and resembles to human situation with respect to paracellular spacer and metabolism and also the expression of *mdr* gene is 99% identical to that expressed in humans (Pang, 2003). Hence, this model is very reliable for conducting absorption studies and the influence of P-glycoprotein mediated drug efflux on absorption and stands as the ideal method for determining the absorption and metabolism of different drug substrates (Jeong *et al.*, 2004). The same was approved by US, FDA. Insitu perfusion experiments offer great advantage over invitro techniques because of intact blood and nerve supply thus simulating the In vivo condition. Phenetoin disposition across the gastrointestinal tract was influenced by P-gp modulators (Neerati *et al.*, 2011) and the functional role of P-gp in diabetes is decreased (Neerati and Gade, 2011) was proved by single pass intestinal perfusion using rat ilium. Jejunal permeability of drugs like propranolol,

naproxen, terbutaline and antipyrine through passive absorption and D-glucose, L-dopa and L-leucine through carrier mediated absorption in rats *in vitro* and then compared to corresponding permeabilities in humans *in vitro* (Lennernas *et al.*, 1997).

The study was motivated by the prevailing information of P-glycoprotein mediated drug efflux in the absorption of vincristine and hence its resistance. Vincristine is an anti cancer drug and is a substrate for P-glycoprotein efflux mechanism which is one of the principal causes of its resistance, hence, requires more doses to get therapeutic benefit as a result of that corresponding adverse reactions may occur. In this study, the interaction of verapamil with vincristine is due to the inhibition of P-gp. The interactions of vincristine and rifampicin is due to induction of P-gp. Vincristine also satisfies all the common structural features required for the substrates of P-gp. In order to verify the role of P-gp in intestinal absorption of vincristine, the experiment was performed in rat jejunum, where the expression of P-gp was high in jejunum (Sababi *et al.*, 2001; Stephens *et al.*, 2001) when compared to the other intestinal segments, using single pass intestinal perfusion technique. If the drug is a P-gp substrate mucosal to serosal transport would be higher. In order to substantiate the above observation the study is focused on intestinal absorption of vincristine following co-perfusion with standard P-gp inhibitor and inducer which are verapamil and rifampicin, respectively.

Expression of P-gp is more significantly found in jejunum (Pang, 2003) and a P-gp substrate is most effectively effluxed in jejunum. This observation was substantiated by the increased absorption of vincristine by verapamil co-perfused group compared with the vincristine alone group. Similarly, absorption was reduced with rifampicin co-perfused group.

In conclusion this is the report of a reduced p-gp mediated efflux of vincristine when concurrently given with p-gp inhibitors may reduce drug resistance of vincristine and increased p-gp mediated efflux may increase drug resistance of vincristine so careful monitoring is very important if vincristine is coadministered with any p-gp inhibitors or p-gp inducers. Hence, co-administration with non therapeutic concentrations of P-gp inhibitors may influence the drug resistance.

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