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Research Article

PACLITAXEL DISPOSITION STUDIES USING P-GP INHIBTOR & INDUCER BY SINGLE PASS INTESTINAL PERFUSION IN RATS

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ABSTRACT

Objective:
The present research work aims to study the intestinal transport of Paclitaxel and to predict its human intestinal permeability and fraction absorbed using SPIP Permeability Coefficient and the effect of P-gp modulators on Paclitaxel were observed in anaesthetized rats.
Methods:
Jejunal segment was used for performing Single Pass Intestinal Perfusion. The rationale for the selection of jejenum is due to the overexpression of P-glycoprotein when compared with other segments. Drug solution (150µg/ml) in phosphate buffer saline was perfused at a flow rate of 0.2ml/min.Besides,P-gp inhibitor verapamil(200 µg/ml) and inducer Rifampicin (60mg/ml) were coperfused with Paclitaxel to detect its disposition characteristics affected by P-gp .Drug concentrations in samples were analyzed using HPLC. Stability studies were conducted to ensure the loss of Paclitaxel due to absorption.
Results:
The effective permeability value of Paclitaxel (150µg/ml) in the jejunal segment was found to be lower due to the efflux mediated by P-gp.When coperfused with verapamil its permeability significantly enhanced as it is a P-gp inhibitor and vice versa with Rifampicin which is a P-gp inducer. Subsequently the human intestinal permeability was estimated considering $P_{eff(human)} = 1.04\ P_{eff(rat)} - 0.0003$.
Conclusion:
P-Glycoprotein mediated drug resistance is one of the serious limitations of Paclitaxel efficacy and jejunal segment is found to have major MDR expression. The Peff value of Paclitaxel was found to be increased upon the Coperfusion with verapamil and similarly reduced with Rifampicin which are inhibitors & inducers respectively indicating Paclitaxel is efficiently transported by P-gp. Hence, Paclitaxel satisfies all the prerequisites to be a P-gp substrate.

Keywords: Paclitaxel, Intestinal permeability, Single pass intestinal perfusion, P-glycoprotein, RP-HPLC.

INTRODUCTION

Paclitaxel is a diterpenoid compound that contains a complex taxane ring as its nucleus.The side chain linked to taxane ring at C-13 is essential for its antitumour activity.Paclitaxel acts by promoting microtubule formation at cold temperatures and in the absence of GTP.It binds specifically to the beta tubulin subunit of microtubules and appears to antagonize the disassembly of this key cytoskeletal protein,with the result that bundles of microtubules and aberrant structures derived from microtubules appear in paclitaxel treated cells.Arrest in mitosis follows.Cell killing is naturally dependent on both drug concentrations and duration of cell exposure. paclitaxel is the important agent finding its applications in Ovarian,Breast,Lung,Head and Neck cancers.eir polymerization required for the formation of mitotic spindle apparatus thereby arresting the cells in the metaphase of the cell cycle. But the serious limitation of Paclitaxel is that it suffers from severe drug resistance.Multidrug resistance (MDR) gene encoding the P-glycoprotein (P-gp) is found to be one of the major hindrances in successful antitumor therapy [3].

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 [1].

P-gp is expressed in both normal cells where its function is to efflux out the bacteria, toxins and drugs. In the tumor cells it is mutated and overexpressed mediating drug resistance. One of the usual methods of overcoming P-gp mediated drug efflux is to target it by using monoclonal antibody. In 1993 Iwahashi *et al* has demonstrated 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 [12].

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 [6].

Very limited data is available over the concomitant administration of P-gp modulators and antitumor agents which are the substrates of P-gp.One of the findings of Watanabe *et al*., 1995 has shown that the co administration of PSC 833, Cyclosporine or Verapamil with Paclitaxel or Adriamycin has increased the survival time of P388/ADR-bearing mice indicating the P-gp modulatory activity of them [9].

It was also evidenced by Takashi Tsuruo et al, that noncytotoxic dose of verapamil, enhanced the cytotoxicity of Paclitaxel and Vinblastine in P388 leukemia indicating the MDR reversal. 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 & disposition of Paclitaxel in Albino rats. The values obtained in these groups are compared with group perfused with Paclitaxel alone.

The main objectives of this study were to

To study the Permeability Coefficient (Peff), absorption rate constant (Ka) and Fraction dose absorbed (Fa) of Paclitaxel in the presence of P-glycoprotein inhibitor, Verapamil & Inducer, Rifampicin.

To predict the Human Peff and Fraction dose absorbed from the rat data obtained.

The present study involves the use of well validated rat single pass intestinal perfusion technique [5]. 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 [2].

One of the significant aspects of this technique is that the data obtained from the rat study Permeability Coefficient(Peff), Fraction dose absorbed(Ka) can be used to predict the human values[8].$P_{eff(human)} = 1.04\ P_{eff(rat)} - 0.0003$ & $Fa_{(human)} = 1 - e^{-38450 \times Peff\ (rat)}$

The possibility of drugs whether they are the substrates for P-gp or not can also be demonstrated by this method [7].

Hence, this method can be fruitful in drug discovery to optimize the balance of properties necessary to convert leads into good medicines.

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 [11].Also the expression of mdr gene is 99% identical to that expressed in humans. The Insitu perfusion experiments offer great advantage over invitro techniques because of intact blood and nerve supply thus simulating the Invivo condition. The goal of this study was to obtain more reliable correlation to predict human intestinal permeability and fraction dose absorbed from the rat intestinal permeability.

MATERIALS & METHODS

Paclitaxel was obtained from (Cipla Pharmaceuticals, Mumbai).Phenol red. A stock of 1mg/ml of Paclitaxel was prepared using distilled water. The final perfusion solution contained 150µg/ml of Paclitaxel, 20 µg/ml of Phenol Red. For perfusion experiments phosphate buffer saline (pH 7.2) was prepared with 10.9g Na_2HPO_4 (anhydrous), 3.2g NaH_2PO_4 (anhydrous), 90g NaCl and 1000ml Distilled water.

All other chemicals were of Analytical grade.

ANIMALS

Male Wistar rats weighing 250 to 300 g; ages 7-9 weeks were obtained from the Central Animal House, Talla Padmavathi College of Pharmacy, Warangal. The animals were placed in polypropylene cages (4 per cage), maintained on 12 hr light; dark cycle and fasted for 20 hr before the experiment with free access to water. The rats were anaesthetized with pentobarbital (60 mg/kg i.p.) 30 min before surgery. The in situ experimental protocol was approved by the Institutional Animal Ethical Committee Kakatiya University, Warangal.

PERFUSION SOLUTION AND COLLECTION OF SAMPLES FROM THE INTESTINE

After overnight fasting, animals were anaesthetized with i.p.Pentobarbital (60 mg/kg).upon verification of the loss of pain reflex, a midline longitudinal abdominal incision was made and a 10-15 cm of Jejenum was identified and cannulated at both ends with plastic tubing. The segment was first rinsed with 37^{o}C saline to clear the segment, the blank phosphate buffer solution was pumped into the segment at a constant flow rate of 0.2ml/min.The blank perfused solution was collected at the outlet and used to prepare Paclitaxel solution.

STABILITY STUDIES

The stability of the compound was tested by its incubation in the perfusion solution and blank perfusate from rat intestine at 37±1^{o}C for 2 hrs.Samples were tested at 0, 1 and 2 h post perfusion by HPLC.The blank perfusate was obtained by passing the blank perfusion buffer through a segment of intestine Insitu at a flow rate of 0.2ml/min.There was no sign of degradation of compound during this period. However Paclitaxel seemed liable, hence, its solution was protected from light.

SINGLE PASS INTESTINAL PERFUSION EXPERIMENT

SPIP studies were performed using previously established methods. Albino rats of either sex weighing 250 to 300 g were fasted overnight for 16 to 22 h with free access to water and anesthetized with an intraperitoneal injection of 60 mg/kg Pentobarbital before surgery. After anesthesia, rats were placed on a heating pad under a surgical lamp to maintain body temperature. A midline longitudinal abdominal incision was made and an inlet Teflon tube (0.42 cm diameter) was inserted into the jejunum. Outlet Teflon tubes were inserted 8 to 10 cm distal to the inlet cannulae.

The perfusion protocol was as follows

- After cannulation, the abdomen was rinsed with isotonic saline, and the entire surgical area was then covered with parafilm to reduce evaporation. Care was taken to maintain inlet perfusion solutions at 37°C and to maintain intact blood supply. Inlet cannulae were connected to syringes that were placed in a perfusion pump
- After allowing 40 min to reach steady-state, drug solution of Paclitaxel was prepared using phosphate buffer saline. The dose was adjusted to be 150µg/ml and perfused through the Jejunal segment at flow rates of 0.2ml/min.
- Outlet perfusate samples were collected every 15 min for 90 min starting from 0 min.The length of the intestinal segment was measured at the end and finally the animal was sacrificed by injecting saturated solution of KCl (10%).
- Samples were stored at -20C until analysis and perfusate concentrations of Paclitaxel was quantified using HPLC.At the end of experiment, the perfused intestinal segment was measured without stretching.

Effect of Verapamil (P-gp inhibitor) and Rifampicin (P-gp inducer) on Intestinal permeability of Paclitaxel

24 rats were divided into 4 groups (n=6) in the present study.SPIP was performed in jejunal segment to test the effect of P-gp influence on the intestinal disposition of Paclitaxel in the presence of P-gp inducer and Inhibitor. The jejunal segment was cannulated for inlet and outlet 25cms away from pylorus. This segment was perfused with solution containing 150µg/ml Paclitaxel and 20 µg/ml phenol red. The experimental setup was performed as described above.

Jejeunal segment was selected as perfused segment, perfusion solution containing 150µg/ml Paclitaxel and 20 µg/ml phenol red were perfused to test its permeability values in the presence of Verapamil (400µg/ml) and Rifampicin (250mg/kg) which are inhibitor and inducer groups respectively.

P-GP Effect on Intestinal Disposition

Paclitaxel suffers with severe drug resistance because of rapid efflux by P-gp and jejunal segment was selected as the P-gp levels are expressed at the maximum levels when compared with rest of the segments [10].

The present study aims to investigate the effect of P-gp inhibitor and inducer on P-gp mediated drug disposition and predicting the same in humans.

Sample Analysis

The absorption and stability of samples were analyzed by HPLC at UV 230 nm.Analytes were separated on a Wakosil II C-18 column (5µm,200mm x 4.6mm I.D).The Mobile Phase constituted Acetonitrile :Water=60:40 (V:V) ,0.2ml Diethylamine, and adjusted to pH 6.5 using O-Phosphoric acid. The flow rate was 1ml/min. Docetaxel (5µg/ml) was used as internal standard. Retention times of Paclitaxel & Docetaxel were 7.1 & 10 respectively.Phenol red was detected at 557nm using UV-Visible Spectrophotometer.

Data Analysis

Calculations were based on outlet perfusate steady state concentrations achieved after approximate 40 min. The steady state intestinal effective intestinal permeability (P_{eff}),cm/sec was calculated according to a parallel tube model[11]. $P_{eff} = Q (1-C''_{out}/C_{in})/2\pi RL$, where Q is perfusion flow rate(0.2ml/min),C_{in} and C''_{out} are inlet and Corrected outlet drug concentrations respectively and the latter was corrected by multiplying inlet concentration with Phenol red[in]/Phenol red[out].The absorption rate constant was calculated from $Ka = (1-C_{out}/C_{in}).Q/V$, and the fraction drug absorbed was determined as

$F^{a}_{b}=1-\frac{C''_{out} X PR_{in}}{C_{in} X PR_{out}}$, where V = volume of the perfused segment

PR_{in}= Inlet phenol red concentration

PR_{out} = outlet phenol red concentration.

The human values were predicted from this study by using following equations

$P_{eff\ (human)} = 1.04\ P_{eff\ (rat)} - 0.0003 \times 10^{-4}$

$Fa_{(human)} = 1-e^{-38450 \times Peff\ (rat)}$

Standard Curve

A stock solution of 1mg/ml of Paclitaxel was prepared and subsequently diluted with phosphate buffer to produce standard dilutions of 500ng/ml,1,10,30,50,100 and 150µg/ml and Phenol red standard solutions of (10,20,40,60,80 & 100µg/ml) were accurately prepared from the stock of 1mg/ml.The solutions were injected for determination of Standard Curve. The regression curve for Paclitaxel and Phenol red were Y=0.0534X+0.6827(r=0.9928) and Y= 0.0059X+0.1678(r=0.9972) respectively indicating good linear relations.

Statistical Analysis

The results reported were expressed as means±SD.The statistical difference between treatment groups were evaluated using one way ANOVA and the identification of significance was carried out with Bonferroni post test.$P < 0.05$ was considered statistically significant.

RESULTS

Intestinal permeability of Paclitaxel was determined in rat jejunal segment using Insitu single pass perfusion and the samples were analyzed by the proposed method. Effective [permeability values were calculated from the steady state concentration of compounds in perfusate collected from the outlet. Steady state was confirmed by the ratio of outlet to inlet concentration [corrected for water transport] Vs time. The corresponding results were shown in Fig [] the effective permeability values for jejunal segment are plotted in Figs.

Effect of P-gp on Intestinal permeation of Paclitaxel

The group treated alone with Paclitaxel was showing P_{eff} : 0.23±0.07, Where as the Peff values for Verapamil & Rifampicin co-perfused groups showed significant increase and decrease measuring about 0.42±0.02 & 0.14±0.09 $x10^{-4}$ cm/sec respectively. This has also a direct impact on absorption rate constant and fraction dose absorbed which are shown in table-1

DISCUSSION & CONCLUSION

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, because of its closer resemblance to human situation with respect to paracellular spacer and metabolism**.** Also the expression of mdr gene is 99% identical to that expressed in humans[10]. Hence, this model is very reliable for conducting absorption studies and the influence of P-glycoprotein mediated drug efflux on absorption.

In Situ Single-Pass Perfused Rat Intestinal Model demonstrated that this model stands as the ideal method for determining the absorption and metabolism of different drug substrates[13]. The same was approved by US, FDA. The other group of researchers evidenced that using the rat data obtained, the human values can be predicted for the permeability coefficients and fraction drug absorbed which gives valuable information for the newer entities discovered.

Insitu perfusion experiments offer great advantage over invitro techniques because of intact blood and nerve supply thus simulating the Invivo condition. The present study deals with the application of single pass perfusion in rat jejunum to assess the intestinal permeability in the humans**.**

Distinct Advantages of this model for assessing intestinal Permeability are:

- The experimental set up is very simple and of low cost.
- The system can be coupled to more sophisticated and sensitive analytical methodologies (eg. LC/MS).
- This is the perfect model for determining absorption of drug substrates and is approved by FDA.
- Absorption data can be extrapolated to humans
- Identification of compound as a Pgp Substrates or not.

The only disadvantage of this model is that it is not suitable for High throughput Screening purposes. This is applicable to the other animal models too.

Present study was motivated by the prevailing information of P-glycoprotein mediated drug efflux in the absorption of Paclitaxel and hence its resistance. Paclitaxel is an anti Cancer drug and is a substrate for P-glycoprotein efflux mechanism which is one of the principal causes of its resistance, requiring more doses and corresponding adverse reactions. In this study, the interaction of Verapamil with Paclitaxel is probably due to the inhibition of Pgp. The interactions of Paclitaxel and Rifampicin is probably due to induction of Pgp. Paclitaxel also satisfies all the common structural features required for the substrates of Pgp.

Intestinal absorption of Paclitaxel in different animal groups was studied using single pass intestinal perfusion technique and the effect of Pgp Inducer and Inhibitor on rat jejunal intestinal permeability of Paclitaxel was investigated.

It was observed that Paclitaxel could permeate through intestines of all the different animal groups investigated. The absorption of Paclitaxel was rapid with the Pgp inhibitor pretreated group probably because of the significant inhibition of efflux mediated by Pgp.Hence substantiated by the observations it would be fruitful to co administer Paclitaxel with a Pgp inhibitor to increase its absorption and lower its resistance. Similarly the absorption of Paclitaxel was less with the Pgp Inducer pretreated group.

In order to verify the role of Pgp in intestinal absorption of Paclitaxel, the experiment was performed in rat jejunum where the expression of Pgp was high (Saitoh and Aungst, 1995; Collett et al., 1999; Li et al., 2002) when compared to the other intestinal segments, using single pass intestinal perfusion technique. If the drug is a Pgp substrate mucosal to serosal transport would be higher. Inorder to substantiate the above observation we have further studied intestinal absorption of Paclitaxel following co-perfusion with standard Pgp inhibitor and inducer which are Verapamil and Rifampicin respectively.

Expression of Pgp is more significantly found in jejunum [10] and so for a Pgp substrate, it is most effectively effluxed in jejunum. This observation was substantiated by the increased absorption of Paclitaxel by the Verapamil coperfused group compared with the Paclitaxel alone group. Similarly, absorption was reduced with Rifampicin coperfused group.

The present study demonstrated that Paclitaxel is a substrate for rat intestinal P-gp because the permeability values for Paclitaxel in the jejunal segment were significantly increased and decreased by Coperfusion with P-gp inhibitor & inducer

(verapamil, Rifampicin respectively) suggesting that Paclitaxel is efficiently transported by P-gp in the gut wall.

The intestinal permeability values estimated from in-situ rat intestinal experiment has been shown to be well co-related to the extent of in-vivo human absorption[11]. Even though the in-situ SPIP protocol is time consuming but it shows a greater correlation for intestinal absorption in humans when compared with other conventional methods like Caco-2 &MDCK cell lines. Thus using this principle the human permeability values of Paclitaxel may be predicted from permeability values obtained from this rat SPIP protocol using the equation $P_{eff(human)} = 1.04\ P_{eff(rat)} - 0.0003 \times 10^{-4}$ Eq-1

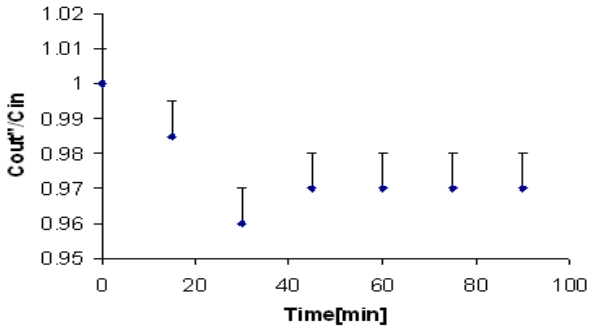
Similarily the fraction absorbed in humans (fa,min) can also be predicted according to $Fa_{(human)} = 1 - e^{-38450 \times Peff\ (rat)}$ Eq-2

Using Eq-1 the permeability value of Paclitaxel alone, in the presence of verapamil and Rifampicin in humans were found to be 2.49±0.7, 15.43±0.21 &1.42±0.1 $X10^{-4}$ cm/sec respectively.Similarily the fraction absorbed for the above mentioned groups were predicted from Eq-2 and found as 59, 87 & 39% respectively.

In conclusion it is demonstrated that Paclitaxel permeation, rate of absorption has been enhanced coadministering with a Pgp inhibitor, Verapamil as it suffers from resistance because of drug efflux. With Rifampicin pretreated group the permeation and rate of absorption were reduced substantiating the increased P-gp expression and the subsequent efflux. These values are even higher compared with Paclitaxel group alone. Hence the same can be predicted in the humans, which further needs evaluation. Paclitaxel a popular anti-cancer agent suffers with the limitation of severe drug resistance mediated by P-gp. Hence co-administration with non therapeutic concentrations of P-gp inhibitors may reduce drug resistance enhancing its potency.

List of Abbreviations: P-gp-P-glycoprotein, SPIP-Single pass intestinal perfusion, RP-HPLC-Reverse phase high performance liquid chromatography, MDR-Multidrug resistance, Peff-Effective permeability, F_{ab}-Fraction dose absorbed.

TABLES & FIGURES



PLOT OF CONCENTRATION RATIO OF THE OUTLET AND INLET TUBING COUT"/CIN VS TIME

Table 1: Showing Experimentally Determined Permeability Values Across The Rat Intestine

group	Rat Peff 10-4cm/sec	Ka min-1	Fab
Paclitaxel	0.23±0.07	0.01	0.93±0.02
Pacli+Verapamil	0.42±0.02	0.44±0.02	1.27±0.08
Pacli+Rifampicin	0.14±0.09	0.3±0.01	0.79±0.06

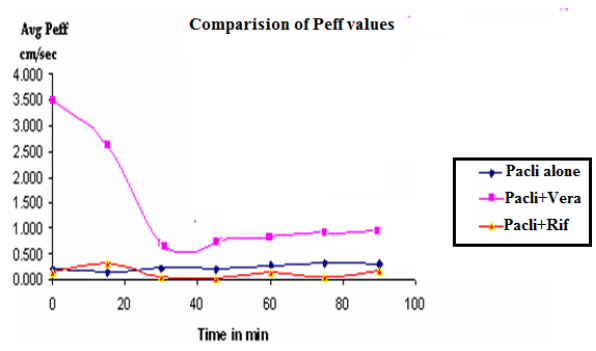
Data are the means ± SD n=6 and are statistically significant

THE PREDICTED HUMAN VALUES WERE GIVEN IN TABLE-2

Table 2: showing predicted Human values from the Rat data.

Group	predicted Peff	predicted Fab
Paclitaxel alone	2.49±0.7	0.59±0.09
Pacli+Verapamil	15.43±2.21	1.95±0.3
Pacli+Rifampicin	1.42±0.1	0.39±0.02

Data are the means ± SD n=6



GRAPH SHOWING THE PEFF VALUES OF PACLITAXEL ALONE AND VERAPAMIL, RIFAMPICIN PRETREATED GROUPS.

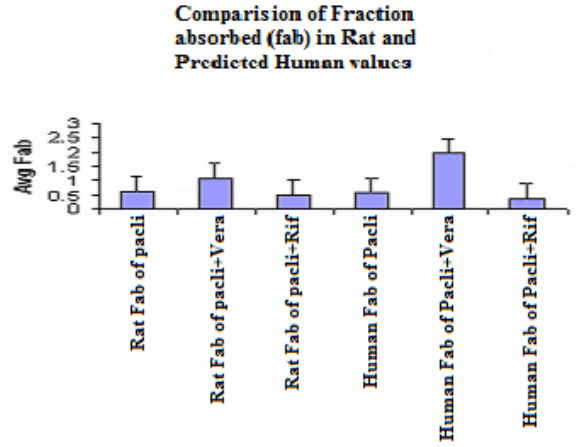


Fig showing the comparison of rat and predicated human Fab values

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