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Influence on intestinal mucous permeation of paclitaxel of absorption enhancers and dosage forms based on Electron Spin Resonance Spectroscopy

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The aim of this paper is to investigate the permeation mechanism of the hydrophobic drug, paclitaxel in intestinal membranes of mice in relation to enhancers and preparation factors. The alteration fluidity of lipid and protein in mucous membrane were determined using electron spin resonance (ESR) when the membrane was treated with several enhancers including Pluronic F68, polyethylene glycol (PEG), Brij78 and lecithin. At the same time, the enhanced permeation of paclitaxel across the intestinal intercellular membrane of stratum corneum was studied for three formulations: inclusion complex, microemulsion and injection. The results showed that use of paclitaxel-hydroxypropyl-β-cyclodextrin inclusion complexation and of paclitaxel microemulsion as vehicle and PEG 1500 as enhancer could significantly increase the permeation kinetics of paclitaxel in a fluid diffusion study. The effect on absorption characteristics of enhancing permeation of this hydrophobic drug in the intestinal mucosa was considered in the light of the change in membrane fluid.

1. Introduction

For most oral administration, the intestinal absorption of the drug is important for improving bioavailability and treatment effects. The intercellular bilayer lipid lamellae in the intestinal intercellular membrane constitute the main stratum corneum barrier to the diffusion of a drug. Paclitaxel, thanks to its particular anticancer mechanism (Loprevite et al. 2001; Wilson et al. 1999), has become one of the most effective anticancer drugs against ovarian cancer, breast cancer, head and neck cancer, and non-small lung cancer, as well as melanoma and lymphoma. But because of its strong hydrophobicity, paclitaxel is scarcely absorbed after oral administration. Paclitaxel injection (Taxol®), is currently formulated in a vehicle containing a 50 : 50 (v/v) mixture of ethanol and polyethoxylated caster oil (Cremophor® EL) (Dong et al. 2004). However, the clinical use of paclitaxel injection was initially hampered by hypersensitivity. An urgent need is to investigate the penetration mechanism of hydrophobic drugs and how to increase absorption through biomembranes after oral administration. In modern studies, electron spin resonance (ESR) spectroscopy has been one of the most important techniques to provide information about the dynamic structure of membranes (Szabo et al. 2004). In our work, spin labeled 5- and 16-doxyl stearic acid (5DSA, 16DSA) were used to investigate the effect of hydration on the fluidity of the intestinal mucosa at different depths of the hydrophobic chain and the protein was labeled with 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy (MSL) in order to investigate the influence of enhancing paclitaxel absorption in intestinal mucosa, in addition to determining the permeation coefficients with two alternative dosage forms, paclitaxel-HP-β-CD and paclitaxel microemulsion.

2. Investigations and results

The fluidity of lipids and the conformation of protein in the lipid bilayer are influenced by enhancers. These were evaluated by the parameter of membrane lipid bilayer fluidity S, the C/A value and the variation in membrane protein conformation hw/hs by measuring the ESR signal for each enhancer after membrane labeling (as shown in Fig. 1).

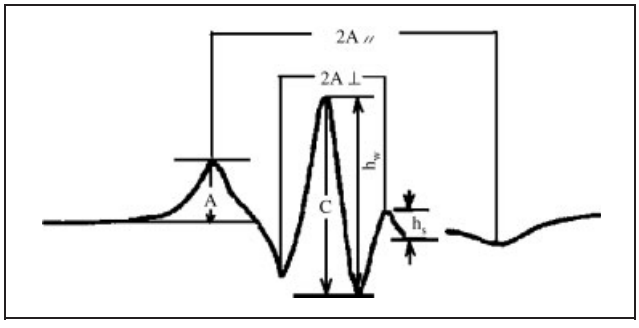

Fig. 1: Measurement on ESR

Table 1: ESR parameters of 5DSA labeled rat intestinal membrane treated with different penetration enhancers ($n = 6$, $\bar{x} \pm s$)

Parameters	Control	SDS	PL	PEG-1500	Brij78	F68	HP-β-CD	Chitosan
$A_{\perp}$	1.73 ± 0.22	1.52 ± 0.20	1.75 ± 0.12	1.65 ± 0.12	1.66 ± 0.11	1.60 ± 0.16	1.58 ± 0.16	1.60 ± 0.11
$A_{//}$	4.4 ± 0.4	4.4 ± 0.3	4.40 ± 0.28	4.0 ± 0.4	4.1 ± 0.3	4.2 ± 0.4	4.23 ± 0.28	4.2 ± 0.3
A'	2.63 ± 0.18	2.48 ± 0.12	2.63 ± 0.18	2.43 ± 0.22	2.46 ± 0.16	2.5 ± 0.3	2.46 ± 0.13	2.45 ± 0.21
S	0.64 ± 0.08	0.65 ± 0.04	$0.62 \pm 0.04^{*}$	0.62 ± 0.05	$0.59 \pm 0.03^{*}$	0.64 ± 0.04	$0.61 \pm 0.02^{*}$	0.64 ± 0.03

Control: phosphate buffer solution (pH = 7.4); * $P < 0.05$ vs control

Table 2: ESR parameters of 16DSA labeled rat intestinal membrane treated with different penetration enhancers ($n = 6$, $\bar{x} \pm s$)

Parameters	Control	SDS	PL	PEG-1500	Brij78	F68	HP-β-CD	Chitosan
C	4.70 ± 0.27	4.2 ± 0.3	5.50 ± 0.10	5.1 ± 0.3	6.4 ± 0.4	5.6 ± 0.4	4.8 ± 0.4	5.8 ± 0.4
A	1.75 ± 0.11	1.25 ± 0.16	2.45 ± 0.12	2.15 ± 0.19	2.90 ± 0.20	2.10 ± 0.16	2.35 ± 0.22	2.25 ± 0.20
C/A	2.69 ± 0.18	$3.36 \pm 0.24^{*}$	$2.14 \pm 0.09^{**}$	$2.37 \pm 0.11^{*}$	$2.22 \pm 0.19^{*}$	2.69 ± 0.21	$2.04 \pm 0.12^{**}$	2.56 ± 0.19

Control: phosphate buffer solution (ph = 7.4); * $P < 0.05$, ** $P < 0.01$ vs control

Table 3: ESR parameters of MLS labeled rat intestinal membrane treated with different penetration enhancers ($n = 6$, $\bar{x} \pm s$)

Parameters	Control	SDS	PL	PEG-1500	Brij78	F68	HP-β-CD	Chitosan
H_w	2.70 ± 0.21	2.50 ± 0.24	2.24 ± 0.12	2.25 ± 0.21	1.80 ± 0.09	2.15 ± 0.22	2.45 ± 0.22	1.50 ± 0.13
H_s	1.40 ± 0.12	1.40 ± 0.09	1.40 ± 0.08	1.60 ± 0.11	1.05 ± 0.11	1.35 ± 0.11	1.75 ± 0.14	1.03 ± 0.08
H_w/h_s	1.93 ± 0.20	$1.79 \pm 0.15^{*}$	$1.40 \pm 0.12^{**}$	$1.41 \pm 0.14^{**}$	$1.71 \pm 0.13^{*}$	$1.59 \pm 0.20^{*}$	$1.40 \pm 0.10^{**}$	$1.48 \pm 0.17^{*}$

Control: phosphate buffer solution (pH = 7.4); * $P < 0.05$, ** $P < 0.01$ vs control

The fluidity rank parameter (S) of 5DSA labeled membrane lipids was calculated by the formula as follows (Bartucci et al. 2003; Wang and Zhang 2004):

$$S = 0.568 \times (A_{//} - A_{\perp})/a \qquad a = (A_{//} + 2A_{\perp})/3 \quad (1)$$

Where $A_{//}$ is A_{max}, half of the outer hyperfine splitting; $A_{\perp}$ is obtained from A_{min}, which is half the inner hyperfine splitting (Griffith and Jost 1976). Values of a were measured at a sufficiently high temperature that constant values were obtained, indicating the absence of slow motional effects (Marsh 1981). The fluidity of the membrane or membrane lipid is reflected by S and an increasing S indicates stronger rigid and weak fluidity of membrane, as shown in Table 1.

The fluidity of 16 DSA labeled membrane lipids was described by the ratio of media field peak (C) and low field peak. The fluidity of membrane protein was represented much better when C/A was smaller, and the results are recorded in Table 2.

There are two different types of sulfhydryl group binding sites on the membrane protein. The bindings of MSL with the deep site and with the surface site are displayed by the strong immobilized spectrum component (hs) and the weak (hw) respectively. Variations in membrane protein conformation are reflected by changes of hw/hs (Table 3).

In addition, the permeation parameter ka was calculated according to data from the *in vitro* permeation diffusion test and the results of influences on paclitaxel permeation are shown in Fig. 2.

Formulation containing PEG, HP-β-CD, PC, Pluronic F68 and chitosan were effective in promoting the permeation of paclitaxel. PEG1500, HP-β-CD and PC showed more significant permeation effects compared with control groups.

The permeation parameters of paclitaxel/HP-β-CD, paclitaxel microemulsion and paclitaxel injection on rat intestinal mucous were determined separately in comparison with paclitaxel (Table 4).

Compared with the control group, the fluidity of membranous protein was enhanced with paclitaxel microemulsion and results for the apparent permeation constants of microemulsion, injection and inclusion complex are shown in Table 5.

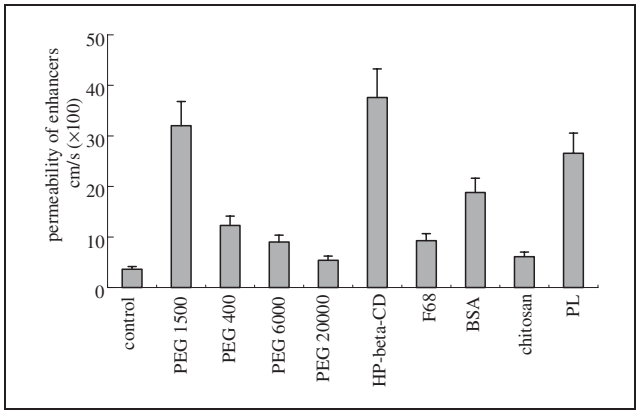

Fig. 2: Effective permeability with enhancers of paclitaxel in intestinal mucous membrane ($n = 6$, $\bar{x} \pm s$)

Table 4: ESR parameters of MLS labeled rat intestinal membrane treated with different penetration enhancers ($n = 6$, $\bar{x} \pm s$)

Parameters	Control	Paclitaxel /HP-β-CD	Paclitaxel microemusion	paclitaxel injection
S	0.61 ± 0.12	0.68 ± 0.08	0.70 ± 0.12	0.70 ± 0.08
C/A	2.61 ± 0.10	$2.25 \pm 0.09^{*}$	$2.19 \pm 0.15^{*}$	$2.22 \pm 0.13^{*}$
H_s/h_w	2.01 ± 0.06	$1.44 \pm 0.07^{*}$	$1.39 \pm 0.09^{*}$	$1.37 \pm 0.11^{*}$

Control: phosphate buffer solution (pH = 7.4); * $P < 0.05$ vs control

Table 5: Relationship of formulation factors and permeation cofficients ($n = 6$, $\bar{x} \pm s$)

Preparations	P_{app} cm/s ($\times 10^5$)
Control	4.70 ± 0.15
Paclitaxel/HP-β-CD	$66.4 \pm 2.8^{***}$
Paclitaxel microemulsion	$73 \pm 5^{***}$
Paclitaxel injection	$69 \pm 4^{***}$

3. Discussion

The spin-labeled ESR results showed that three types of enhancer, PEG1500, HP-β-CD and PC, obviously influence fluidity change of membrane lipids compared with the other enhancers. As regards the thermotropic phase behaviour of PEG and PC, the decrease of $2A_{//}$ suggests more disorder and a looser chain packing density in the hydrocarbon region while the absence of any cooperative chain melting transition is confirmed both by the optical clarity of the samples and by the flat thermal profiles of the absorbance at very low values (Belsito et al. 2000; Montesano et al. 2001). Therefore, it seems very likely that the three enhancers used in carriers of hydrophobic paclitaxel preparations enhance its absorption and oral bioavailability. The results indicated that the permeation rate of paclitaxel across the membrane was remarkably improved in microemulsion, inclusion complex and injection containing Cremophor EL. The solubilization mechanism of paclitaxel was enhanced by the inclusion complex as well as the surface tension of the mucous membrane being reduced by microemulsion, injection containing phosphatidylcholine and Cremophor EL. Further, the fluidity of the membranous lipid and conformation lipoprotein was determined by ESR. The results indicted that for PEG1500,HP-β-CD and PC, their promotion of fluidity and permeability may rely on their mechanism of loosening the structure of the membranous lipid sheet by changing the lipoprotein conformation of the intestinal mucous. In fact they all can increase the solubility of paclitaxel, promote the concentration gradient of the drug between the two sides of the membrane, and enhance the transmembrane diffusion and permeation of paclitaxel. Paclitaxel microemulsion was prepared using tricaproin : tributytin (1 : 1, v/v) as the mixed oil phase. The membranous permeation mechanism of paclitaxel-HP-β-CD showed a relationship with the increase both of solubility and dissolution rate of the hydrophobic drug after being incorporated in the inclusion complex. The results of this experiment provide a basis of theory for further investigations of oral formulations of paclitaxel.

4. Experimental

4.1. Materials

Paclitaxel and paclitaxel injection (Taxol®, containing paclitaxel 30 mg, 5 ml, batch number 20010512) were purchased from Sihuan Pharmaceutical Factory of Beijing Pharmaceutical Factory. Polyethylene glycol (PEG1500), sodium dodecyl sulfate (SDS), Brij78, Pluronic F68 and phosphatidylcholine (PC) were purchased from Sigma Chemical Co. HP-β-CD was obtained from Xi'an Biochemistry Company Limited, China ($M_{av} = 1860$). The fatty acids spin lablelled 5-DSA, 16-DSA and MSL were purchased from Sigma Chemical Co. Paclitaxel microemulsion and paclitaxel-HP-β-CD inclusion complex were prepared separately according to a literature method (Lee et al. 2001; Zhang et al. 2005). SD rats (male, weight $250 \sim 300$g) were provided by the Department of Animals, Beijing University Medicine Health Science Center. All care and handling of animals were performed with the approval of the Institutional Authority for Laboratory Animal Care.

4.2. Influence of fluidity enhancers on intestinal mucous

Hypotonic destruction was used to prepare the mucosal subcellular fractions (Bai and Chang 1995). Rats were euthanized with an overdose intraperitoneal injection of urethane and the small intestinal tract was excised. Mucosal subcellular fractions of the intestine were prepared according to the method of Yan et al. (2004). The intestinal mucosa was scraped off, suspended in phosphate buffer solution (PBS, pH 5.2) and then homogenized by ultrasound for 20 min. The homogenate was centrifuged at $10{,}000 \times g$ for 10 min and the supernatant (15 ml) was collected as a cytosol fraction. The supernatant was divided into 3 parts. Two parts were labeled by adding 100 µl 5-DSA and 100 µl 16-DSA separately, and then diluted with 3 ml saline solution, and cultured in a 37 °C water bath for 30 min. The other part was labeled with 100 µl MSL and kept in water bath at 4 °C overnight. All the samples were centrifuged at $10{,}0000 \times g$ at 4 °C for 30 min and the precipitates were washed three times with PBS (pH 5.2). The same concentration enhancers, 200 µl of 2% chitosan, phosphatidylcholine, PEG 1500, Brij78, Pluronic F68 and hydroxypropyl-β-cyclodextrin and SDS, were added to the tubes and cultured in a 37 °C water bath for 1 h, centrifuged and precipitates collected. Samples were placed in standard 4 mm diameter quartz ESR tubes and ESR spectra were recorded using a microwave power of 10 mW and a magnetic field modulation at a frequency of 100 kHz with a Bruker ESR spectrometer model 200D at room temperature (Nedeianu S, Páli T and Marsh D 2004).

4.3. Penetration of intestinal mucosa

The small intestine tract (5–6 cm) was excised as described in section 4.2. The mucosal subcellular fractions were prepared, spread on a filter paper (0.6 cm diameter, 0.8 µm) and then fixed in the middle of a bi-cell diffuser, sealing with adhesive tape, to form a 6.0 cm effective diffusion area between the donor and acceptor cells (Scherlund et al. 2000). The inside of the intestinal mucous faced the donor cell and the other side the acceptor cell. The diffuser was placed in a 37 ± 1 °C water bath and 5 ml PBS (pH 7.4) was added to both donor cell and accept cell. 200 µl of ethanol solution of paclitaxel ($2\ mg \cdot ml^{-1}$) were added to the donor cell and immediately stirred at $100\ r \cdot min^{-1}$. 0.5 ml samples were withdrawn from the acceptor cell at given times (from 0.25 to 3 h with an interval of 0.25 h) and the same amount of PBS was added to the acceptor cell after each sample collection. After filtering with a 0.45 µm filter 20 µl aliquots were injected into the HPLC column for analysis (Zhang et al. 2006).

4.4. Influence of enhancers on paclitaxel absorption

200 µl of paclitaxel solution ($2\ mg \cdot ml^{-1}$) were placed in the donor cell of a bi-cell diffuser sealed with the intestinal mucosa, and then 200 µl of 10% PEG1500, PEG4000, PEG6000, PEG20000, 2% F68, 50% HP-β-CD, 1% chitosan and 2% phosphatidylcholine respectively were added. 0.5 ml portions of the diffusion solution were withdrawn from the acceptor cell at given intervals. 20 µl aliquots were injected into the HPLC and the paclitaxel concentrations of the samples were determined by a reversed-phase HPLC method.

4.5. Effects of paclitaxel formulation on absorption uptake

Three formulations, paclitaxel microemulsion, paclitaxel-HP-β-CD and paclitaxel injection were diluted with saline solution and adjusted to a paclitaxel content of $0.1\ mg \cdot ml^{-1}$. A free paclitaxel solution (dissolved in anhydrous alcohol) was used as a control group. 0.5 ml aliquots of the solutions were added to the donor cell according to the mucous membrane permeation method, and the paclitaxel content in the samples was determined by HPLC at predetermined times.

4.6. Data analysis

The mechanism of paclitaxel permeation from the donor cell into the acceptor cell across the mucous membrane followed Fick's diffusion law and the rate of permeation was given by the diffusion constant P_{app}:

$$p_{app} = \frac{dc}{dt} \times \frac{V}{AC_0} \qquad (2)$$

Where V is the volume of solution in the acceptor cell, A is the surface area of the membrane, the initial concentration of the drug in the donor cell is Co and dC/dt is the concentration of drugs in the acceptor cell at an unit time.

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