行政院國家科學委員會專題研究計劃成果報告

小腸內以 Peptide 做為 P-Glycoprotein 抑制劑之研究

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中文摘要
臨床上癌症病患的化學治療對藥物產生之抗藥性(多重抗藥性)為最主要的問題，而許多研究已證明多重抗藥性與 P 酪蛋白有關。P 酪蛋白為一胞膜運輸體，能將物質由細胞內運送到細胞外，降低其在細胞內的存積，此運輸體表現於癌細胞及許多正常組織中。P 酪蛋白具有廣泛的受質及抑制劑，但抑制劑的毒性在臨床應用上為一大問題，因此，探討其低毒性的抑制劑為目前治療多重抗藥性的一大目標。ALLN 為一個合成的 tripeptide，目前已證明其為 P 酪蛋白的受質及抑制劑，因此選擇 ALLN 為一 model compound 來探討其與 P 酪蛋白受質間的交互作用以進一步評估臨床應用的可行性。以小腸翻腸實驗來探討 peptide 的脂溶性與 P 酪蛋白抑制作用的關係，進一步以老鼠體內實驗來評估藥動學上的變化。同時，以 human mdr1 cRNA 注入 Xenopus oocytes 以增加蛋白質的表現，可進一步了解此 P 酪蛋白抑制劑的特性。由以上結果，可了解 in vitro/in vivo correlation 以預測 P 酪蛋白抑制劑對藥物吸收的影響，這對於治療及預防多重抗藥性具有臨床應用的價值。

關鍵詞：Xenopus oocytes，小腸，P 酪蛋白，ALLN

Abstract
Clinical resistance to chemotherapeutic agents is the major obstacle in the treatment of cancer, which P-glycoprotein (Pgp) plays an important role. P-glycoprotein, a membrane transporter protein, can efflux intracellular compounds outward and thus decrease their intracellular accumulation. This transporter is expressed in tumor cells as well as in a variety of tissues including the small intestine. Pgp exhibits broad substrate specificity with structure-unrelated compounds as modulators. However, the toxicity of modulator may hinder the clinical application. Therefore, investigation into Pgp modulator with low toxicity becomes an important goal to overcome MDR. ALLN, a synthetic linear tripeptide, was shown to be the Pgp substrate and modulator. Thus, ALLN is chosen as a model compound to evaluate the interaction between ALLN and Pgp-mediated drug transport for further assessment of clinical application. The intestinal everted sacs study will be conducted to assess the relationship between the hydrophobicity of linear peptides and their Pgp inhibitory effect. Subsequently, drug interaction in pharmacokinetics will be evaluated using in vivo study in rats. Xenopus oocytes injected with human mdr1 cRNA as an expression system will be applied to understand the characteristic of ALLN as a Pgp modulator. From the study, in vitro/in vivo correlation can be characterized to predict the effect of Pgp modulator on Pgp-mediated drug transport. It is clinically important to choose suitable modulator(s) in the treatment and prevention of drug resistance, which peptide(s) may be a promising candidate.

Keywords：P-glycoprotein, ALLN, Xenopus oocyte, small intestine.
Introduction

P-glycoprotein, the gene product of mdr1 with 170-kDa plasma protein, functions as an energy-dependent drug efflux pump and thus decreases drug accumulation in a variety of systems (Chen et al., 1986; Gros et al., 1986; Endicott and Ling, 1989). Pgp is expressed in a wide range of tissues, including the small intestine and colon in the physiological conditions. This membrane protein has been considered as an absorption barrier for intestinal drug absorption. And MDR-reversing agent can overcome the barrier and increase drug absorption. Several chemicals such as verapamil, cyclosporine A, and PSC 833 have been proved to be potent Pgp inhibitors in vitro; however, the toxicities hindered them for clinical application (Bradshaw and Arceci 1998).

In 1992, a study by Sharma and his colleagues showed ALLN, a semi-synthetic peptide as a Pgp substrate and proposed that the secretion of peptide/protein is related to the physiological function of Pgp (Sharma et al 1992). And a study by Burton et al. also observed an increased transport of AcPhe(NMePhe)NH in the apical to basolateral direction in Caco-2 cells and this transport was also increased in the presence of verapamil (Burton et al 1993). Further studies have shown that hydrophobic peptides and peptide ionophores are substrates/modulators of Pgp (Raymond et al 1992; Eytan et al 1994, 1996; Loe et al 1994; Sarkadi et al 1994; Toppmeyer et al 1994; Sharam et al 1995, 1996, 1998, 1999; Borghia et al 1996; Chen and Pollack 1999). And the interaction mechanism(s) with Pgp remains to be clarified. Most of studies were performed by estimating the ATPase activity or quenching constant (Kd). Therefore, it is interesting to investigate the effect of linear peptide on Pgp-mediated drug transport in vivo and the possibility of linear peptide as Pgp inhibitor for clinical application.

Etoposide, a podophyllotoxin derivative is an antineoplastic agent via inhibition of DNA topoisomerase II activity. It is commonly used in the treatment of neoplastic diseases, such as small-cell lung cancer, and Kaposi’s sarcoma (Clark and Slevin 1987; Belani et al 1994). This anticancer drug exhibits various oral bioavailabilities with a range between 25 to 75%. And considerable intra- and interpatent variation exists (Clark and Slevin 1987). It has been proved that etoposide is a P-glycoprotein (Pgp) substrate (Keller et al 1992; Leu and Huang 1995). Therefore, etoposide is chosen as a model Pgp substrate in the current study.

In this study, in vitro everted sac study was performed to evaluate the effect of ALLN on the transport of etoposide in rat small intestine. And the effect of ALLN on the absorption of etoposide in vivo was further assessed. It is clinically important to understand the interaction between etoposide and linear peptide for the treatment of drug resistance.

Methods

Degradation study in rat BBMV

Intestinal brush-border membrane vesicles from rats were prepared by the Ca precipitation method (Kessler et al. 1978). Total protein concentration of the vesicles was measured by the Bradford method using a Bio-Rad protein assay kit and bovine serum albumin as standard (Bradford 1976). The enrichment of vesicles could be assessed using alkaline-phosphatase as a marker, for which p-nitrophenyl phosphate could be used as a substrate (Walter & Schutt, 1974). The purified BBMV was resuspended in the loading solution (25 mM HEPES/TRIS, pH 6.5, 100 mM mannitol, 100 mM KCl) to make a final concentration of 30 μg protein /10 μl. Ninety μl of 20 μg/ml ALLN in pH 6.5 HEPES/TRIS buffer was incubated with 10 μl of vesicle containing 30 μg of protein at 25°C and the degradative reaction was
stopped by adding 150 µl of ice-cold acetonitrile at different time intervals up to 60 minutes, following vigorous mixing. The mixture was then centrifuged and the supernatant was assayed by a modified HPLC method (Su, unpublished data).

Everted Sacs Study

Male Sprague-Dawley rats were decapitated and either jejunum or ileum with a length of 30 cm was removed from 2–3 cm below the ligament of Treitz or 5 cm above the caecum, respectively. The segment was everted, ligated at both ends, and filled with 3 ml of Tyrode solution. Tyrode solution contained 24 g NaCl, 3 g dextrose, 3 g NaHCO3, 6 ml 10% KCl, 7.8 ml 10% MgSO4·7 H2O, 3.9 ml 5% NaH PO4·2H O, and 5.4 ml 1M CaCl2 in 3 L water (Huang 1990). Subsequently, the sacs were placed in 100 ml of Tyrode solution containing 100 µg/ml of etoposide, which was gassed with air at 37°C. Two hundred microliters of samples were taken every 10 minutes up to 60 minutes. To study the effects of MDR-reversing agents, different concentrations of hydroxyzine and Pgp inhibitors were added outside the sacs with various pre-incubation times. They were 500 µM hydroxyzine for 20 minutes, and 1 mg/ml quinidine for 30 minutes. The following procedure was the same as those above mentioned.

Intestinal Exsorption of Etoposide

The single-pass perfusion study was performed to evaluate the exsorption of etoposide in the intestine. Male Sprague-Dawley rats weighing from 250–300 g were fasted for 16–20 hours prior to the experiment. Water was given at lib. Anesthesia was given by intraperitoneal injection of 50% W/V urethane solution (1.5 g/Kg body weight). The jugular vein cannulated with a heparinized silastic tubing (0.02 inch, ID, and 0.037 inch, OD) (Dow Corning Corp., Midland, MI), was infused with 0.5 mg/ml etoposide and various concentrations of anti-histamines at an infusion rate of 1.0 ml/hr. The blood samples were drawn via carotid artery. Both ends of the intestinal segment were cannulated with Tygon tubings. And the segment was perfused with Tyrode solution at a flow rate of 15-20 ml/hr at 37°C by a syringe pump. The rats' abdomens were covered with paraffin to maintain moisture through the experiment. Both blood and perfusate samples were collected hourly for 8 hours. The total body clearance (CL) and the intestinal exsorption clearance (CLi) were determined.

HPLC Assay

Etoposide was analyzed by a high pressure liquid chromatography (HPLC) system (Leu et al. 1995). A volume of 2 ml chloroform was added to 150 µl sample; vortexed for 1 minute; and centrifuged for 10 minutes. A volume of 1.5 ml of the organic layer was then evaporated under N2 gas; subsequently, was reconstituted by 150 µl of mobile phase for HPLC analysis. The HPLC system consists of a pump (Waters, Model 510, Milford, MA), an automatic injector (Waters, WISP Model 710, Milford, MA), a reverse phase column (µBondapak C18, 300x3.9 mm, Waters), a fluorescence detector (Applied Biosystems Inc., Model 783A, Foster City, CA) with emission and excitation wavelengths at 328 nm and 215 nm, respectively, and an integrator (Hewlett Packard, Model 3395, Avondale, PA). The mobile phase included methanol:water:glacial acetic acid=4:57:0.1. The flow rate was controlled at 1.5 ml/min. The concentration was determined by peak height.

Results and Discussion

When 20 µg/ml ALLN was incubated in rat intestinal BBMV, approximately 93% and 87% of ALLN could be detected after 1 and 2 hours, respectively (Fig. 1). This indicates that enzymatic degradation does not influence the absorption of ALLN in the small intestine.

Figure 2 showed that the addition of
either 1 mg/ml quinidine or 500 μM ALLN could significantly enhance the transport of etoposide from the lumen to the serosal site in both jejunal and ileal sacs. The serosal etoposide concentration increased about two-fold after 90 minutes.

The in vivo study therefore applied to evaluate the effect of ALLN on the absorption of etoposide. Figure 3 showed that the plasma concentration of etoposide increased rapidly at the initial 2 hours, and it reached the steady state with the concentration of 1 μg/ml. No significant difference in plasma or luminal concentration was observed in the presence of ALLN. This suggests that no marked drug interaction between etoposide and ALLN occurs when etoposide is administered intravenously.

References
Figure 1  The enzymatic degradation of ALLN in rat intestinal brush-border membrane vesicles (BBMVs)
Figure 2  Effect of ALLN on the transport of etoposide in rat everted intestinal sacs.
Figure 3  The plasma concentration-time profile of etoposide in the *in vivo* exsorption.

- Control
- ALLN

Concentration (ug/ml)

Time (hr)