行政院國家科學委員會專題研究計畫成果報告

在 Xenopus oocytes 中 Peptide Transporter 特性之研究

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中文摘要

口服給藥是各種給藥方式中最方便的一種，尤其是給長期服藥的病患。雖然 peptide/ protein 具有較高的藥效與較低的毒性，但其口服的生體可用率卻非常低。一般認為 peptide/ protein 是藉由 carrier-mediated transporter 來運送吸收。近年來，兩個 oligopeptide transporters (Pept-1, Pept-2) 已分別自老鼠、兔子、人類的小腸及腎臟組織中分離出來。Pept-1 之 cDNA 由 2921 個 bp 組成，具有 710 個 amino acid，而 Pept-2 cDNA 則有 3938 個 bp，含 729 個 amino acid。Pept-1 與 Pept-2 的作用與 pH 值、溫度、proton-gradient、membrane potential 有關，而與 Na+ 無關。Oligopeptide transporter 的受質包括 di-/tri-peptide 與 β-lactam antibiotics，其抑制劑則有 di-/tri-peptides、cephalosporins、ACE inhibitor，本計劃將 construct 一個 chimera (Pept-1-chl) 以 Gly-Sar 為受質來探討 Xenopus oocyte 中 Pept-1 的作用機轉及結合位置(binding site)，由這些實驗結果可設計藉由 oligopeptide transporter 運送的 peptide/peptidomimetic 藥物，此具有臨床應用的價值。

關鍵詞：Oligopeptide transporter, Xenopus oocyte, β-lactam antibiotics, Gly-Sar

Abstract

Among the routes of administration, oral drug delivery is very convenient, making it the most popular dosing route, especially for long-term treatment. Peptides/proteins usually exhibit high potency and low toxicity. However, the oral bioavailability of peptides/proteins are very low. And it is believed that peptide(s) is transported by a carrier-mediated system. Recently, two different oligopeptide transporters, Pept1 and Pept2 were isolated from intestinal and renal tissues, respectively in rats, rabbits, and humans. The rat Pept1 cDNA consists of 2921 bp with an open reading frame encoding a 710-amino acid protein. And the rat Pept2 has 3938 bp encoding a protein of 729 amino acids. Transport by either Pept1 or Pept2 is pH-dependent, temperature-dependent, proton-gradient dependent, Na+ independent, and electrogenic. The substrates of Pept1 and Pept2 include di-/tri-peptides, β-lactam antibiotics, and aminopenicillins. Transport by either Pept1 or Pept2 can be inhibited by various di-/tri-peptides, cephalosporins, and ACE inhibitors. This study will construct a recombinant chimeric peptide transporter, Pept1-chl to investigate the transport mechanism and functional binding site(s) of Pept1 in Xenopus oocytes using Gly-Sar as a substrate. Therefore, with the information based on the functional studies, a rational design of peptide/peptidomimetic drugs for oligopeptide transporter could be achieved. It is important in clinical use.

Keywords: oligopeptide transporter, Xenopus oocyte, β-lactam antibiotics, Gly-Sar.

Introduction

Clinical resistance to chemotherapeutic
drugs is a major obstacle in the treatment of cancer, which is termed MDR (multidrug resistance). The high level of P-glycoprotein (Pgp) expression was observed in tumors and its expression was higher after treatment than before drug therapy (Goldstein et al. 1989). Therefore, it is considered that Pgp is associated with MDR. Pgp, the product of mdr1 gene with 170-kDa plasma protein, contains 1280 amino acids, and is composed of 2 homologous halves. Each half has 6 transmembrane domains, and one ATP binding site (Pastan et al. 1991). This protein functions as an energy-dependent drug efflux pump and thus decrease drug accumulation in a variety of systems (Chen et al. 1986; Gros et al. 1986). Pgp is expressed in many normal human tissues such as liver, kidney, brain, adrenal tissue, pancreas, colon, and jejunum using immunocytochemistry study (Thiebault et al. 1987; Croop et al. 1989; Weinstein et al. 1990; Pastan et al. 1991). In the small intestine, Pgp is localized in the brush-border membrane but not in the basolateral domain of plasma membrane. Therefore, it is considered to be related to the detoxification of xenobiotics (Fojo et al. 1987; Thiebault et al. 1987).

Etoposide (VP-16) is an important chemotherapeutic agent against a variety of neoplasms, including germ-cell malignant, small-cell lung cancer, leukemia, soft-tissue sarcoma, neuroblastoma, and gastric cancer (Belani et al. 1994). This compound exhibits an erratic oral bioavailability of 50% with the range of 25 to 75%. Clinical studies with the combined use of either dipyridamole or cyclosporin A (CSA) could restore the cytotoxicity of etoposide in cancer patients (Isonishi et al. 1991; Lum et al. 1992; Yahanda et al. 1992). However, the nephrotoxicity of this immuno-suppressive agent may hinder the clinical application of these chemosensitizers. A report by Hait and his colleagues mentioned that a patient treated at Yale Comprehensive Cancer Center developed unexpectedly severe myelosuppression while receiving the chemotherapeutic regimen including doxorubicin and the only other drug this patient received was terfenadine, an antihistamine (Hait et al. 1992). Therefore, they conducted a further study and suggested that terfenadine could enhance cellular accumulation of doxorubicin in MCF-7/ADR cells at a low concentrations of 2–5 µM (Hait et al. 1993). The investigation by Fukuda et al. also demonstrated that another anti-allergic agent, azelastine, could overcome the resistance to vincristine in P388 cells (Fukuda et al. 1993). This provides the promising information which antihistamines result in toxicity to a lesser extent compared to those aforementioned. This implies the feasibility of combined use of etoposide and antihistamines to improve the cytotoxicity of etoposide against tumors with less side effects induced by antihistamines in cancer patients. Therefore, a series of antihistamines with similar chemical structures will initially be screened to determine their abilities to reverse the resistance in the small intestine in vitro. Subsequently, in situ perfusion study in rats will be performed to evaluate the change in pharmacokinetics and thus to understand the interaction between etoposide and antihistamines. It is clinically important in choosing suitable MDR-reversing agents in the treatment and prevention of drug resistance.

Methods

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% NaH2PO4·2H2O, 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 Exorson of Etoposide**

The single-pass perfusion study was performed to evaluate the exorption 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 *ad 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 exorption clearance (CL_in) 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**

The concentration of etoposide inside the sacs increased gradually and it reached 20 μg/ml after 90 minutes (Fig 1). The addition of either 1 mg/ml quinidine or 500 μM hydroxyzine could significantly enhance the transport of etoposide from the lumen to the serosal site in both jejunal and ileal sacs.

To further evaluate the effect of hydroxyzine on the transport of etoposide in the small intestine, the exorption study in the everted sacs was performed. Figure 2 demonstrated that hydroxyzine could markedly inhibit the efflux of etoposide to the lumen. The result from the absorption and exorption study revealed that hydroxyzine could enhance the transport of etoposide in the small intestine and Pgp may play a role.

The *in vivo* study therefore applied to evaluate the effect of hydroxyzine 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. With the co-infusion with either 1 mg/ml quinidine or 500 μM hydroxyzine, the plasma concentration increased approximately two-fold. The results from *in vivo* study also
suggests that hydroxyzine, an antihistamine in the small intestine, could increase the absorption of etoposide in

References


Figure 1  Time course study of etoposide in rat everted intestinal sacs in the presence or absence of Pgp inhibitor.
Figure 2: Exsorption study of etoposide in rat everted intestinal sacs in the presence or absence of Pgp inhibitors.
Figure 3. *In vivo* study of etoposide in rat in the presence or absence of Pgp inhibitors.

**Serum**
- Control N=5
- Quinidine 1mg/ml N=5
- Hydroxyzine 500 µM N=6

**Intestine fluid**
- Control N=5
- Quinidine 1mg/ml N=6
- Hydroxyzine 500 µM N=6