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A modified fast (4 day) 96-well plate Caco-2 permeability assay

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ABSTRACT

Introduction: The Caco-2 permeability assay is widely used for lead optimization in drug discovery. A 3 to 5-day system using a 24-well plate and a 10 to 21-day system using a 96-well plate have been established. Here, we modified the assay system to provide a ready-to-use Caco-2 cell monolayer using a 96-well plate in just 4 days. **Methods and results:** In our system, collagen-coated inserts and the prolongation of the culture period after seeding leads to greater Caco-2 cell proliferation and sufficient contact-inhibition. The differentiation of Caco-2 cells was enhanced, when the contact-inhibited Caco-2 cells were exposed to the differentiation-inducing agent butyric acid. The permeability to nine well-known compounds showed a statistical correlation between our 4-day system using a 96-well plate and the conventional 21-day system using a 24-well plate. **Discussion:** We conclude that our system is more useful for evaluating many compounds for lead optimization in drug discovery.

1. Introduction

The Caco-2 permeability assay is widely used in drug discovery. A number of reports have demonstrated the possibility of predicting the oral absorption of a drug in humans from its permeability across the Caco-2 monolayer (Bohets et al., 2001; Huebert, Dasgupta, & Chen, 2004; Lennernas, Palm, Fagerholm, & Artursson, 1996). In addition, experiments with Caco-2 cells allow one to explore transport mechanisms with or without carriers (Feng, Pankaj, Ailan, & Patrick, 2002), drug metabolism (Plageman, Pauletti, & Skau, 2002), and the mucosal toxicity of drugs (Shah, Palamakula, & Khan, 2004).

Twenty four well plates have conventionally been used for Caco-2 permeability assays. In the 24-well system, at least 21 days is required for the formation of a well-tight Caco-2 cell monolayer after cell seeding on inserts (Darwin & Paul, 1997). The Caco-2 cell monolayer exhibits features such as the ability to differentiate spontaneously into polarized enterocytes that express high levels of brush border enzymes and form well developed tight-junctional complexes (Moise et al., 1983), as an absorptive intestinal cell. These Caco-2 cells also express a variety of transport proteins including P-glycoproteins (P-gp) (Praveen, Karishma, Anthony, & Saeho, 2004). In the recent decade, BD Biosciences produced a 24-well system in which the cell monolayer is grown for only 3 to 5 days (Darwin, 1998). On the other hand, Millipore supplied another assay system using a 96-well plate, but long-term cell culture (10 to 21 days) is recommended in the 96-well system (Jeanne, Lakshmi, Andrew, & Marcy, 2002).

High-throughput screening has been employed routinely for the past 15 to 20 years as a strategic method to discover compounds that elicit a hit activity in a particular molecular target or cellular screen. However, a tendency towards higher probabilities of failure has been observed. The probability could be attributed to poor pharmacokinetics or toxicity (John, 2001). A shift in strategy for the drug discovery process has occurred. The research for acceptable pharmacokinetics and toxicity is now given equal importance to the goals of potency, efficacy, and selectivity. Hence, in drug discovery, determining the permeability class of compounds is the first screening step. The FDA recommends several intestinal permeability methods for assessing class (Guidance for Industry, 2000). An *in vitro* permeability study using a monolayer of cultured epithelial cells, such as Caco-2 cells, is one of them. However, conventional Caco-2 permeability assays do not have sufficient throughput for the screening of numerous compounds.

In this experiment, we modified the assay system which to provide a ready-to-use Caco-2 cell monolayer using a 96-well plate in 3 to 5 days.

2. Methods

2.1. Materials

Methotrexate, nadolol, ranitidine, atenolol, furosemide, quinidine, carbamazepine, ketoprofen, propranolol, metoprolol, verapamil, caffeine and Lucifer Yellow were purchased from Sigma-Aldrich (St. Louis, MO, USA). Eagle's minimum essential medium (MEM), non-essential amino acid solution, Hank's balanced salt solution (HBSS), Trypsin–EDTA solution were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and phosphate-buffered saline (PBS (−)), pH 7.4, were obtained from Invitrogen (Grand Island, NY, USA). MITO+™ serum extender (containing EGF, human transferring, insulin,

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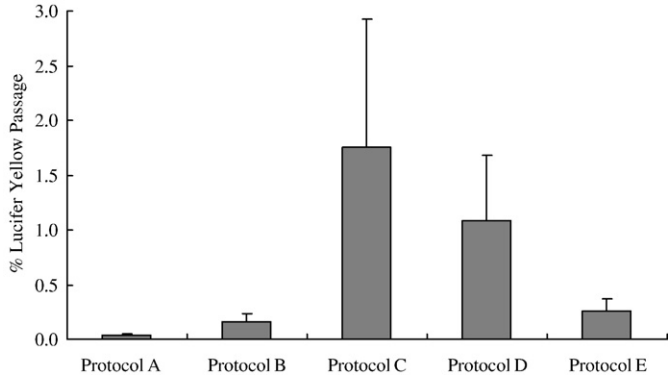

Fig. 1. Passage of Lucifer Yellow Passage through Caco-2 cell monolayers cultured with five different protocols. Lucifer Yellow transport experiments conducted over 1 h at 37 °C with the following five protocols; (Protocol A) the conventional 21-day system using a 24-well plate (n=48); (Protocol B) the 3-day system using a 24-well plate (n=48); (Protocol C) the 3-day system using a 96-well plate without collagen-inserts (n=78); (Protocol D) the 3-day system using a 96-well plate with collagen-inserts (n=75); (Protocol E) the 4-day system using a 96-well plate with collagen-inserts (n=81).

EGGS, Triiodothyronine, hydrocortisone, progesterone, testosterone, 17β-estradiol, selenium and *o*-phosphorylethanolamine), Entero-STEM™ medium (containing butyric acid) and rat tail collagen type I solution (4.7 mg protein/mL) were provided by BD Biosciences (Franklin Lakes, NJ, USA). Caco-2 cells were purchased from ATCC (Rockville, MD, USA). Glucose oxidase, peroxidase, Lys-*p*-nitroaniline, nitroaniline, and *p*-nitrophenylphosphate were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

The Multiscreen® Caco-2 Assay System (96-well plate, 0.11 cm^2/insert) was purchased from Millipore (Billerica, MA, USA). The BIOCOAT® Caco-2 Assay System (24-well plate, 0.31 cm^2/insert) was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

2.2. Preparation of collagen-coated inserts for cell culture

Rat tail collagen type I solution was diluted with a 0.02 mol/L acetic acid aqueous solution, and aliquots of 0.075 mL containing 0.55 μg of protein were spread over inserts of a 96-well plate. The plate was allowed to stand for 1 h at room temperature. The collagen solution was aspirated from the inserts carefully and the inserts were rinsed thrice with PBS(−). The method referred to the technical information from Beckon Dickinson (B.D. website).

2.3. Cell culture

Throughout the experiments, Caco-2 cells were maintained at 37 °C, at 90% relative humidity, and in 5% CO_2. The cells were cultured in MEM containing 10% FBS in 75 cm^2 flasks and grown to high density (>2.5×10^5 cells/cm^2) prior to passage or seeding. The Caco-2 cells were seeded and cultured for the permeability assay as follows:

(1) Conventional 21-day system using a 24-well plate (Protocol A): Caco-2 cells were seeded onto inserts of the 24-well plate at a density of 2.0×10^4 cells/insert and cultured for 21 days in MEM containing 10% FBS (the seeding medium) with media changes of medium twice a week.
(2) Three-day system using a 24-well plate (Protocol B): Caco-2 cells were seeded onto inserts of a 24-well plate at a density of 2.0×10^5 cells/insert and cultured in the seeding medium. At 24 h post-seeding, the seeding medium was replaced with Entero-Stim™ medium containing MITO+™ serum extender (the differentiation-inducing medium) and the cells were incubated for another 48 h.
(3) Three-day system using a 96-well plate (Protocol C): Caco-2 cells were seeded onto inserts of a 96-well plate at a density of 7.2×10^4 cells/insert and cultured in the seeding medium. At 24 h post-seeding, the seeding medium was replaced with the differentiation-inducing medium and the cells were incubated for another 48 h.
(4) Three-day system using a 96-well plate with collagen-coated inserts (Protocol D): Caco-2 cells were seeded onto collagen-coated inserts of a 96-well plate at a density of 7.2×10^4 cells/insert and cultured in the seeding medium. At 24 h post-seeding, the seeding medium was replaced with the differentiation-inducing medium and the cells were incubated for another 48 h.
(5) Four-day system using a 96-well plate with collagen-coated inserts (Protocol E): Caco-2 cells were seeded onto collagen-coated inserts of a 96-well plate at a density of 7.2×10^4 cells/insert and cultured in the seeding medium. At 48 h post-seeding, the culture medium was replaced with the differentiation-inducing medium and the cells were incubated for another 48 h.

2.4. Permeability assay

Each cultured monolayer on the 24-well or the 96-well plate was washed twice with HBSS (pH 7.4). The permeability assay was initiated by the addition of each solution containing Lucifer Yellow into inserts (apical side, A) or receivers (basolateral side, B). Initial concentrations of compounds and Lucifer Yellow were 20 μmol/L and 0.1 mg/mL, respectively. The Caco-2 cell monolayers were incubated for 1 h at 37 °C.

The apical to basal (or basal to apical) apparent permeability coefficients (P_{appa}, cm/s) of each compound were calculated using the equation, $P_{appa}=dQ/dt\times1/AC_0$. The flux of a drug across the monolayer is dQ/dt (μmol/s). The initial drug concentration on the apical side is C_0 (μm). The surface area of the monolayer is A (cm^2).

The apical to basal Lucifer Yellow passage (%) was calculated according to the equation, % Lucifer Yellow passage=$(F_{test}-F_{blank})/(F_0-F_{blank})$. The fluorescence intensity of Lucifer Yellow across the monolayer is F_{test}, the initial fluorescence intensity of Lucifer Yellow on the apical side is F_0, and the fluorescence intensity of the blank sample (HBSS alone) is F_{blank}.

2.5. Analytical methods

The concentration of each compound was measured with a HPLC system (LC-10A, Shimadzu). A reverse phase column (CAPCELLPAK C18 MGII, 2.0 mm×50 mm, 5 μm, Shiseido) was used. The mobile phase, consisting of solvent A (10 mmol/L ammonium acetate) and solvent B (acetonitrile) was programmed as a linear gradient. The flow rate was 0.7 mL/min and the absorbance was monitored at 220 nm.

Table 1
Brush border membrane-associated enzyme activities in the Caco-2 cell monolayers cultured with three different assay systems

Assay system	Activity (U/μg protein)[a]		
	Alkaline phosphatase	Aminopeptidase	Sucrase
Control[b]	1443±57	22±1.3	25±3.2
Conventional 21-day system	9080±444	26±7.4	97±8
24-well (Protocol A)	(6.29)[c] ***	(1.18)[c] **	(3.88)[c] ***
3-day system	8375±831	28±11	92±6.1
24-well (Protocol B)	(5.80)[c] ***	(1.27)[c]	(3.68)[c] ***
4-day system	11479±645	26±0.86	87±3.3
96-well (Protocol E)	(7.95)[c] ***	(1.18)[c]	(3.48)[c] ***

Significance test, t-test was conducted, *$p<0.05$, **$p<0.01$, ***$p<0.001$.
[a] Activity: correspond to the enzyme activities (mean±S.D., n=3).
[b] Control: correspond to the Caco-2 cells before cell seeding.
[c] The up-regulation ratio was calculated from each enzyme activity of each assay system divided with those of control.