Evaluation of Biocoat® Intestinal Epithelium Differentiation Environment (3-Day Cultured Caco-2 Cells) as an Absorption Screening Model with Improved Productivity

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INTRODUCTION

Recently, Caco-2 cells have gained enormous popularity as a reliable and high throughput *in vitro* model for evaluation of a large number of drug candidates for their intestinal absorption potential (1,2). The preparation of a fully differentiated confluent Caco-2 cell monolayer, however, generally requires a 3 week cell culture period with 8–9 laborious cell feedings. Recently, Collaborative Biomedical Products (Bedford, MA) reported that Biocoat® Intestinal Epithelium Differentiation Environment (BIEDE) can provide a ready-to-use confluent and fully differentiated Caco-2 cell monolayer with an adequate formation of tight junctions in just 3 days with only one feeding (3). A few differences between the BIEDE and the conventional 3-week systems include 1) type of filter membrane support used to grow monolayer (polyethyleneterephthalate *vs.* polycarbonate, respectively), 2) type of extracellular matrix protein used to facilitate cell adhesion (fibrillar collagen type I *vs.* rat tail collagen type I, respectively), 3) culture medium (butyric acid containing serum-free DMEM *vs.* DMEM supplemented with 10% fetal bovine serum, respectively) and most importantly 4) a number of days in culture require to grow usable monolayers for transport studies (3 days *vs.* 3 weeks, respectively). Therefore, the objectives of present studies were to evaluate the functional performance of the BIEDE and to compare the BIEDE to the conventional 3-week Caco-2 cell monolayer. This would provide data to bridge internal permeability values accumulated in the past five years at Bristol-Myers Squibb PRI (Princeton, NJ). A heterologous series of eighteen passively absorbed drugs (neutral, acidic or basic) with a wide range of absorption in humans was studied as model compounds.

MATERIALS AND METHODS

Materials

[^{14}C]mannitol (specific activity of 55 mCi/mmole) and [^{14}C]methoxyinulin (specific activity of 11.6 mCi/g) were obtained from NEN Research Products (Boston, MA). Acetaminophen, acetbutalol, atenolol, caffeine, desipramine, guanabenz, hydralazine, ibuprofen, ketoconazole, propranolol, salicylic acid, terbutaline, Hank's balanced salt solution and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). BVaraU, nadolol, losartan and pravastatin were obtained from Bristol-Myers Squibb PRI (Princeton, NJ). Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). The BIEDE kit was purchased from Collaborative Biomedical Products (Bedford, MA). The kit consists of pre-coated inserts and cell medium. All solvents were analytical grade.

Cell Culture

Caco-2 cells (cell passage numbers between 35–37) were seeded onto a fibrillar collagen coated insert (6 wells, 4.19 cm^2, 1 μm) at a density of 2.0×10^6 cells/insert. The cells were grown in culture medium consisting of serum free DMEM with MITO+™ serum extender. MITO+™ serum extender is consisted of EGF, transferrin, insulin, ECGS, triiodothyronine, hydrocortisone, progesterone, testosterone, estradiol-17β, selenium and o-phosphorylethanolamine. The culture medium was replaced after 24 hr post-seeding with Entero-STIM™ (butyric acid containing serum free DMEM). The inserts were maintained at 37%C, 90% relative humidity, and 5% CO_2. Permeability studies were conducted with the monolayers two days after medium replacement. The conventional 3-week monolayer was prepared with Transwell™ inserts consist of polycarbonate membrane (4.71 cm^2, 3 μm pore size). The monolayers between 21–25 days in culture with the cell passage numbers between 40 and 50 were used.

Permeability Study

The transport medium was modified Hank's balanced salt solution (MHBS) containing 10 mM HEPES, pH 7.4. Each monolayer was washed twice with MHBS (pH 7.4) and 2.6 mL of MHBS (pH 7.4) was placed on the basolateral side of the monolayer (receiver well). The permeability studies were initiated by adding 1.5 ml of MHBS containing one of the drugs listed in Table I (initial concentration was about 200 μM) and [^{14}C]methoxyinulin (0.2 μCi) to the apical side of the monolayer. [^{14}C]methoxyinulin was co-incubated as a hydrophilic marker to check the integrity of the cell monolayer during the incubation. No monolayer was found to be leaky during permeability experiments. The monolayers were placed on an orbital shaker (50 cycles/min) and incubated up to 4-hour at 37°C. At hourly intervals, samples were taken from each receiver well and the donor compartment was sampled at the end of the 4-hour period.

Apical to basolateral permeability coefficients were calculated according to the following equation: Permeability coefficient (Pc) = $dA/(dt \cdot S \cdot C_o)$, where dA/dt is the flux of drug across the monolayer (nmole/sec), S is the surface area of the cell monolayer (4.19 cm^2), and C_o is the initial concentration (μM) in the apical compartment. The permeability coefficient values are expressed as cm/sec.

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Table I. List of Drugs Evaluated with the BIEDE System

Drugs	BIEDE Pc (mean ± sd, n=3, × 10^{-7} cm/sec)	3-week Pc (mean ± sd, n=3, × 10^{-7} cm/sec)[a]	Oral absorption in humans (%)	Ref.
caffeine	230 ± 9.3	214 ± 5	100	4
ibuprofen	200 ± 25		100	5
salicylic acid	140 ± 6.3		100	6
desipramine	260 ± 5.5		95	7
acetaminophen	220 ± 16		95	8
propranolol	270 ± 1.3	148 ± 10	90	6
hydralazine	160 ± 28		90	9
BVaraU	62 ± 1.9	40 ± 4	82	10
guanabenz	310 ± 6.2	209 ± 11	79	11
ketoconazole	120 ± 18	104 ± 12	76	12
terbutaline	56 ± 21		73	13
atenolol	30 ± 3.9	40 ± 3	50	6
acetbutalol	29 ± 4.3		40	14
nadolol	22 ± 1.1	45 ± 2	35	15
prevastatin	30 ± 3.9	23 ± 2	34	16
losartan	42 ± 5.0	27 ± 4	33	17
mannitol	40 ± 4.0	5 ± 1	16	6
methoxyinulin	12 ± 1.0	10 ± 2	<5[b]	

Note: Both the BIEDE Pc and conventional 3-week Pc values reported above were obtained under the identical experimental conditions.

[a] Data taken from ref. 2 except ketoconazole, losartan and methoxyinulin.

[b] The extent of absorption was estimated to be <5.

Analytical Methods

The concentrations of drugs were analyzed by a HPLC-UV assay. A C_{18} YMC reverse phase column (4.6 mm × 150 mm; YMC, Inc, Wilmington, NC) was used. The mobile phase, consisting of solvent A (water:acetonitrile:trifluoroacetic acid, 95:5:0.1 v/v) and solvent B (water:acetonitrile:trifluoroacetic acid, 20:80:0.1, v/v), was programmed as a linear gradient. The flow rate was 1.0 ml/min and the absorbance was monitored at either 210 or 220 nm. The concentrations of [^{14}C]mannitol and [^{14}C]methoxyinulin were determined by liquid scintillation counting (Model 2500, Packard Instr. Co., Downers Grove, IL).

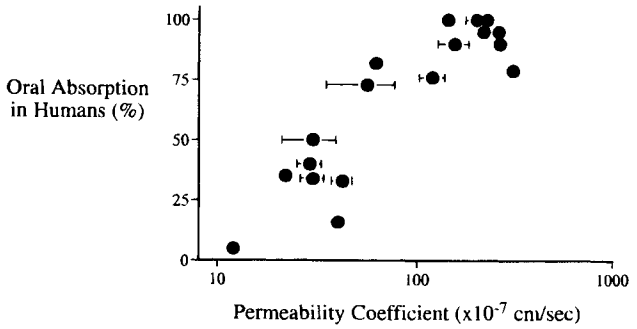

Fig. 1. Correlation between *in vivo* permeability coefficient across the BIEDE Caco-2 cell monolayer. The each data point represents the mean (± sd) value of triplicate experiments.

RESULTS AND DISCUSSION

The permeability coefficients across the BIEDE Caco-2 cell monolayer of a heterologous series of eighteen passively absorbed compounds (Table I) with a wide range of absorption (<5 to 100%) correlated reasonably well with the extent of *in vivo* absorption in humans (Figure 1). The observed variability and the predictive power of the BIEDE model appear comparable to those of the conventional 3-week Caco-2 cell model (2). Figure 2 shows that a fairly good agreement was found when the Pc values of drugs (n=11) determined in the BIEDE monolayer were compared to those in the conventional 3-week monolayer. The slope of the correlation plot was not statistically different from unity suggesting that both systems provided consistent Pc values for the tested model compounds. Previously, the conventional 3-week system was described as a rapid screening tool in support of drug discovery process provided that adequate controls (e.g., compounds with known *in vivo* absorption in humans) are included in each experiment. Similarly, the BIEDE model appears equally acceptable with a much improved productivity (*i.e.*, 3 days with one feeding rather than 3 weeks with multiple feedings).

Table II. Effect of Days in Culture on Pereability (mean ± sd, n=3)

Days in Culture	Propranolol (× 10^{-7} cm/sec)	Losartan (× 10^{-7} cm/sec)	Methoxyinulin (× 10^{-7} cm/sec)
3	260 ± 4.3	36 ± 9.1	16 ± 3.0
4	270 ± 4.2	30 ± 2.8	15 ± 1.6
6	280 ± 3.6	40 ± 2.2	19 ± 0.67
7	280 ± 3.6	47 ± 5.3	14 ± 2.5

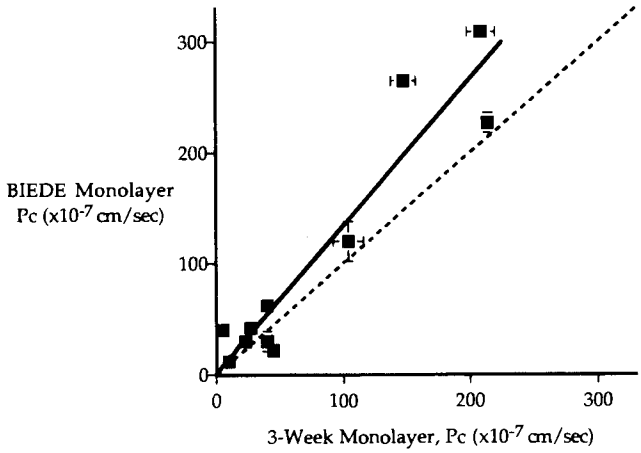

Fig. 2. Correlation between permeability coefficient across the BIEDE Caco-2 cell monolayer and the conventional 3-week Caco-2 cell monolayer. The straight solid line represents the linear regression equation $Y = 1.3\ X + 1.5$ ($r^2 = 0.90$, $p < 0.01$). The 95% confidence interval of the slope ranged from 0.98 to 1.7. The dashed line represents an unity line. The each dara point represents the mean (± sd) value of triplicate experiments.

The permeability of three representative model compounds (i.e., highly-; propranolol, moderately-; losartan, and poorly-permeable; methoxyinulin) was evaluated as a function of number of days in culture. Each compound showed consistent Pc values from 3 to 7 days post-seeding (Table II), suggesting that the functional integrity of the BIEDE monolayer remains reproducible for at least 4 additional days once it becomes usable in 3 days. The observed reproducibility beyond the 3 days post-seeding provides practical flexibility with respect to scheduling experiments.

The Pc value of mannitol which is absorbed exclusively *via* a paracellular route was significantly greater in the BIEDE Caco-2 cell monolayer (40×10^{-7} cm/sec) than the conventional 3-week monolayer (10×10^{-7} cm/sec), suggesting that the BIEDE monolayer has leakier tight junctions than the conventional 3-week system. The conventional 3-week monolayer typically showed considerably higher transepithelial electrical resistance (TEER ~300 Ω cm^2) than human small or large intestine *in vitro* (TEER ~50–100 Ω cm^2) and, therefore, considered to have unphysiologically tight pores (18). A typical TEER value in the BIEDE monolayer ranges from 100 to 150 Ω cm^2 (personal communication with Darwin Asa, Ph.D. at Collaborative Biomedical Products, Bedford, MA). The BIEDE monolayer with leakier tight junctions may provide an advantage over the 3-week system in this regard because it behaves more closely to human intestinal tissues with respect to the TEER value.

In conclusion, a reasonable correlation was demonstrated between the *in vitro* permeability through the BIEDE Caco-2 cell monolayer and the extent of *in vivo* absorption in humans with a heterologous series of eighteen passively absorbed drugs. Both the BIEDE and 3-week systems provided similar Pc values so that the Pc values determined by the BIEDE system can be directly compared with the Pc values accumulated with the conventional 3-week system. Therefore, the BIEDE Caco-2 cell monolayer appears to be a more convenient and productive (i.e., reduced 8 to 9 labor-intensive medium replacement steps down to ONE and was usable in 3 days) absorption screening tool compared to the conventional 3-week system.

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