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USE OF CACO-2 CELLS AS AN IN VITRO INTESTINAL ABSORPTION AND METABOLISM MODEL

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ABSTRACT

The Caco-2 cell line, a human colorectal carcinoma cell line, is an established *in vitro* model for the study of drug transport in the human intestine. We have routinely utilized this *in vitro* model to 1) elucidate intestinal absorption mechanisms of small drug molecules and peptide-like therapeutic agents (e.g. paracellular/transcellular passive diffusion and carrier-mediated active transport), 2) screen and select orally active therapeutic agents, 3) identify optimum luminal pH's for drug absorptions, 4) address dissolution rate-related absorption problems, 5) assess mucosal toxicity of therapeutic agents, and 6) evaluate prodrug approaches for enhanced drug absorptions. We have also utilized this *in vitro* model to assess the metabolic stability of therapeutic agents in the intestinal epithelium. Demonstrated in this report are primarily the techniques for the elucidation of absorption mechanisms. Examples of the characterization of paracellular/ transcellular passive diffusion pathways and carrier-mediated active transport will be given. Application of the Caco-2 model to the process of drug development will also be discussed.

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INTRODUCTION

Enzymatic and cellular barriers in the intestinal tract are the two main obstacles for oral absorption of therapeutic agents. Several *in vitro* and *in vivo* models have been developed to study intestinal absorption and metabolism of drug molecules; these include intestinal loops and everted sacs, vascularly perfused intestines, isolated mucosal cells and brush border membrane vesicles. The Caco-2 cell line is a human colon adenocarcinoma cell line which, at post-confluency, exhibits structural/ morphological as well as biochemical/functional similarities to the small intestinal epithelium (1). Brush border membrane-associated enzymes such as aminopeptidase, alkaline phosphatase, sucrase, dipeptidyl aminopeptidase, and γ-glutamyl transpeptidase, cytosolic phase II enzymes such as glutathione S-transferase, sulfotransferase, and glucuronidase, as well as microsomal cytochrome P450 isozymes are expressed in Caco-2 cells (1-4). Several active transport systems that are located in the small intestinal cells (e.g. sugars, amino acids, dipeptides, bile acids, and cobalamin intrinsic factor) are also expressed in Caco-2 cells (5-8). As such, cultured Caco-2 cells are now being used increasingly as a model for intestinal transport and metabolism studies of therapeutic agents.

The original proposal for the Caco-2 model of intestinal absorption was put forward by Borchardt and workers in 1989 (9). Artusson, Burton, and many others have also demonstrated the utility of the Caco-2 system (10-14). A good correlation between oral drug absorption in humans and apparent drug permeability coefficients in Caco-2 cells has been demonstrated by Artusson and Karlsson (15). There are several advantages to using Caco-2 cell culture model: i) it can serve as a rapid screening tool for the drug absorption studies, ii) it is simpler than the vascularly perfused intestinal model, iii) it provides information on the absorption and transport of drug molecule across intestinal mucosa, an advantage over the intestinal loops and everted sacs which are more suitable for the study of drug uptake into the mucosal cells, iv) it replaces other *in vitro* intestinal absorption models which use animals, v) it provides information on the intestinal absorption and metabolism at cellular level, vi) it provides information on the possible mucosal toxicity caused by therapeutic agents, vii) it needs no interspecies correlation (human origin cells).

We have applied this *in vitro* model to various stages of drug discovery and development processes. We have been routinely using Caco-2 model to elucidate absorption mechanisms of small drug molecules and peptide-like therapeutic agents. We have also used this *in vitro* model to screen and select orally active new chemical entities. We have further used this model to study sites of intestinal absorption (i.e. to address luminal pH and dissolution rate related issues) and to assess the mucosal toxicity of therapeutic agents. In this report, we wish to demonstrate techniques used in the elucidation of absorption mechanisms of small drug molecules and peptide-like therapeutic agents. Example of ondansetron and ranitidine, which are transported across Caco-2 cell monolayers *via* a transcellular and a paracellular passive diffusion, respectively, will be given (16). Metabolism and carrier-mediated active transport of dipeptides L-alanyl-L-alanine (L-Ala-L-Ala) and glycylsarcosine (Gly-Sar) in the Caco-2 system will also be demonstrated (17-19). Characterization of the transport mechanism for the tripeptide-like thyrotropin-releasing hormone (TRH, pGlu-His-Pro-NH_2) being paracellular passive diffusion will be discussed (18,20). Application of the Caco-2 model to the process of drug development will also be discussed.

MATERIALS AND METHODS

Chemicals

[^{14}C]Mannitol (49.3 mCi/mmol) and [^{3}H]Thyrotropin-releasing hormone (83 Ci/mmol) were purchased from New England Nuclear Research Product, Boston, MA. [4-^{14}C]-testosterone (54.5 mCi/mmol), [N-methyl-^{3}H]-ranitidine (6.5 Ci/mmol) and L-alanyl-L-[^{14}C]alanine (1.22 mCi/mmol) were purchased from Amersham, Corp., Arlington Heights, IL. [2(im)-^{14}C]-Ondansetron (53.5 mCi/mmol) was prepared by Glaxo Group Research, Ware, UK. Unlabeled ondansetron and ranitidine were obtained from Glaxo Research Institute, Research Triangle Park, NC. Gly-Sar, glycylglycine (Gly-Gly), L-Ala-L-Ala, glycyl-L-proline, L-phenylalanylglycine, L-carnosine, glycine, L-alanine, L-lysine, L-glutamic acid, mannitol, thyrotropin releasing hormone (TRH), thyrotropin releasing hormone free acid, testosterone, p-Aminobenzoic acid, sodium azide, 2,4-dinitrophenol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP),

ouabain, and amiloride were purchased from Sigma Chemical Co., St. Louis, MO. Naphthalene-2,3-dicarboxyaldehyde (NDA) was purchased from Molecular probes, Inc. Eugenic, OR.

Incubation Media

Eagle's minimum essential medium (mod.) 1X (w/ Earle's salts and L-glutamine) was obtained from Fisher Scientific, Pittsburgh, PA. Fetal bovine serum (FBS), nonessential amino acids (NEAA), Hank's balanced salt solution (HBSS), ethylenediaminetetraacetic acid (EDTA), 0.05% trypsin and 0.02% EDTA in HBSS, glucose, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonic acid (MES), and citric acid were purchased from Sigma Chemical Co., St. Louis, MO.

Caco-2 Cell Culture Model

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured at 37 °C in minimum essential medium, containing 10 % FBS and 1% NEAA, in an atmosphere of 5% CO_2 and 90% relative humidity (9). Cells grown in 75 cm^2 T-flasks (Costar, Cambridge, MA.) were supplied in 25 ml of culture medium. Cells were passaged every 3-4 days at a split ratio of 1 to 5 and confluency was reached within 4-5 days. For transport and cellular uptake studies, cells were seeded on either 4.7 cm^2 or 1.0 cm^2 polycarbonate membranes of Transwells^{TM} (3.0 μm pore size, Costar) at a density of 70,000 cells/cm^2 to late confluency (20-25 days). Media were changed every 2-3 days after seeding and were replaced with transport media (HBSS containing 25 mM glucose and 25 mM HEPES buffer, pH 7.0) an hour before the experiment.

Cellular Transport Studies

Transport experiments were initiated by replacing the AP medium with transport medium (1.5 ml for 4.7 cm^2 Transwells^{TM} and 0.4 ml for 1.0 cm^2 Transwells^{TM}) containing drug to the upper chamber (apical side, AP). The rates of drug transport were monitored by measuring the amount of drug present in the medium

(2.6 ml for 4.7 cm^2 Transwells^{TM} and 1.5 ml for 1.0 cm^2 Transwells^{TM}) of lower chamber (basolateral side, BL) at various time points. For calcium switch assay, calcium free transport medium and the transport medium that contains both EDTA and calcium were used as appropriate (21,22). For substrate specificity study, transport medium containing both drug and putative inhibitor was added to AP side of Transwell^{TM} and the amount of drug transported to the BL medium was measured.

For energy dependence study, cell monolayers were preincubated with transport medium containing active transport inhibitor for 30 min after which the transport of drug was studied. Modified Keiger-Ringer Buffer (61 mM choline chloride, 13 mM choline carbonate, 0.9 mM calcium chloride, 25 mM glucose, and 140 mM mannitol) was used on both AP and BL sides of Transwells^{TM} to investigate the effect of sodium on the transport of drug molecule. Bicarbonate-free HBSS containing either 25 mM citric acid (pH 5.0 and 5.2), 25 mM MES (pH 5.5-6.5), or 25 mM HEPES (pH 6.8 and 7.0) was used to study the effect of pH on the transport of drug (8). Experiments were initiated by adding 0.4 ml of transport medium containing drug molecule at desired pH to the AP side of Transwells^{TM} and the transport rates were monitored by measuring drug present in the BL medium (pH 7.0) at various time points.

All transport experiments were carried out under sink conditions as the concentrations of drug on the BL side remained at least 10-fold lower than those on the AP side of cell monolayers throughout the experiments. At least two measurements were taken for each time point. The integrity of cellular tight junctions was checked by measuring the transepithelial electrical resistance (TEER) of cell monolayers before the experiments. For transport study using unlabeled drug, the integrity of tight junctions during the course of transport was checked by measuring either the TEER value or the amount of the spiked [^{14}C]mannitol (a paracellular leakage marker) transported to the BL side of cell monolayers. The amount of radiolabeled drug transported to the BL side of cell monolayers was quantitated by liquid scintillation counting in a Beckman LS-5801 spectrophotometer and the amount of unlabeled drug transported to the BL side of cell monolayers was quantitated by HPLC assay.

Cellular Uptake Studies

Uptake experiments were initiated by adding radiolabeled drug in transport medium to either the AP side or both the AP and BL sides of Transwells^{TM} (i.e. for compounds such as ranitidine, transport studies were conducted either under sink conditions or at a steady state). Incubation was terminated at various time points and cell monolayers were quickly washed several times with transport medium containing several-fold molar excess of unlabeled drug and incubated with tissue solubilizer at room temperature overnight. Cellular uptake of drugs was then measured by liquid scintillation counting.

Cellular Metabolism Studies

AP and BL samples from the transport studies as well as samples from the incubation of drug molecule with Caco-2 cell homogenate were analyzed by HPLC for metabolite(s) identifications.

Sample Analysis

Ondansetron - An HPLC method consists of a Spherisorb CN column (100 x 4.6 mm) and an isocratic mobile phase of 65% acetonitrile and 35% 25 mM ammonium acetate (pH 4.0) was used with a radiochemical detector and a UV detector at 302 nm. The retention time of ondansetron was 4.1 min.

Ranitidine - An HPLC method consists of a Spherisorb ODS column (250 x 4.6 mm, 5 μm) and an isocratic mobile phase of 35% 50 mM phosphate buffer (pH 6.0), 60% methanol, and 5% tetrahydrofuran was used with a radiochemical detector and a UV detector at 320 nm. The retention time of ranitidine was 6 min.

Dipeptides (Gly-Sar, Gly-Gly, and L-Ala-L-Ala) - A fluorogenic derivatization method was used for the quantitation of dipeptides (23). The optimum pH for the derivatization of these dipeptides and dipeptide internal standards, D-alanyl-D-alanine and B-alanyl-L-alanine, with NDA was investigated and found to be 8.5 (data not shown). For transport studies of Gly-Sar and Gly-Gly, BL samples (1.5

ml) were spiked with 5 μM of standard D-alanyl-D-alanine before filtering through 0.2 μm cellulose acetate centrifuge filters. The filtrates (300 μl) were then incubated with 11.2 μl of 100 mM sodium cyanide, 644 μl of 100 mM borate buffer (pH 8.5) and 45 μl of 6.22 mM NDA at room temperature for one hour. The samples (10 μl) containing derivatized dipeptides were then analyzed by HPLC with a Keystone BDS-Hypersil C8 column (250 x 4.6 mm) and a gradient mobile phase system (70% solvent A to 55% solvent A from 0 to 20 min and 55% solvent A to 30% solvent A from 20 to 25 min where solvent A was 90% 20 mM $AcONH_4$ (pH 4.0) and 10% MeCN; solvent B was 50% 20 mM $AcONH_4$ (pH 4.0) and 50% MeCN). Fluorescence was monitored using an excitation wavelength of 245 nm and an emission filter of 470 nm. For transport study of L-ala-L-ala, β-ala-L-ala was used as internal standard and samples were analyzed on the same stationary phase with a gradient mobile phase system of 50% A from 0 to 9 min and 50% A to 30% A from 9 to 19 min.

TRH - Samples from transport studies and cell homogenate incubations (200 μL) were spiked with 0.36 μM of internal standard p-aminobenzoic acid and were analyzed by a Keystone BDS-Hypersil C18 column (5 μm, 250 x 4.6 mm) with an isocratic mobile phase of 95% solvent A and 5% solvent B where solvent A was 0.05% (w/v) octanesulfonic acid (pH 2.2) containing 4% (v/v) each of MeOH and MeCN and solvent B was 100% MeCN (24). Both radioactivity and uv absorbance at 215 nm were monitored.

Data Analysis

Initial transport rates were calculated by linear regression analysis on the time course plot of amount drug transported. The apparent permeability coefficients (P_{app}, cm/sec) for the passive diffusion process were calculated from the following equation: $P_{app} = (dQ/dt)/C_o/A$ where dQ/dt is the permeability rate (mol/sec), C_o is the initial concentration of drug on the AP side of cell monolayers (mol/ml), and A is the surface area of the porous membrane (cm^2). Permeability rate (dQ/dt) was calculated by plotting the amounts of drug transported to the BL side *vs.* time and determining the slope of the plot. The permeability rates (dQ/dt) were then plotted *vs.* initial concentrations (C_o) to obtain the value of the slope

$(dQ/dt)/C_o$. For cellular transport which involves dual process (i.e. carrier-mediated and passive diffusion), the kinetic parameters V_{max}, K_m, and k_d were calculated from the equation $V_i = \{V_{max} * [S] / (K_m + [S])\} + k_d * [S]$ using the Enzfitter program (Biosoft, Cambridge, U.K.) where [S] is the initial concentration of drug molecule on the AP side of cell monolayers, V_{max} and K_m are the maximum transport rate and the Michaelis constant for the carrier-mediated process, and k_d is the first order rate constant for the passive diffusion process. For a simple carrier-mediated transport process, V_{max} and K_m were calculated from the equation $V_i = V_{max} * [S] / (K_m + [S])$ using the same program.

RESULTS AND DISCUSSION

Mechanism of Drug Transport

I. Passive Diffusion

Ranitidine and Ondansetron: Ranitidine and ondansetron were chosen as model compounds for the demonstration of paracellular/transcellular passive diffusion process (16). A pilot study was performed to investigate the possible metabolism of these two compounds by enzyme(s) present in the Caco-2 cells. Result of HPLC analysis showed no metabolism of these compounds during the course of transport across Caco-2 cells. This enabled us to determine transport rates for [^{14}C]- ondansetron and [^{3}H]-ranitidine using the total radioactivity in the samples. Results of time and concentration dependence studies showed a linear transport for ranitidine and ondansetron over 120 min and 60 min, respectively, at all concentrations studied (i.e. 0.14 μM - 14.3 mM for ranitidine and 3.5 - 111.4 μM for ondansetron). Apparent permeability coefficients (P_{app}) for the transport of ranitidine and ondansetron were unchanged throughout the concentrations studied ($1.03 \pm 0.17 \times 10^{-7}$ cm/sec and $1.83 \pm 0.055 \times 10^{-5}$ cm/sec, respectively), an indication of a simple passive diffusion process for the transport of these compounds across Caco-2 monolayers.

A calcium switch assay was further performed to determine the relative contribution of transcellular and paracellular pathway for the transport of

Table 1

Uptake of 2.5 uM of Ranitidine, Ondansetron, Mannitol, and Testosterone by Caco-2 Cell Monolayers[a]

Compound	Uptake, pmol[b]	Uptake, pmol[c]
Ranitidine	17.8 ± 1.7	3.75 ± 0.24
Ondansetron	99.3 ± 16.8	76.8 ± 7.1
Mannitol	5.0 ± 1.0	0.83 ± 0.16
Testosterone	76.9 ± 8.7	30.3 ± 4.3

[a] 4.7 cm^2 monolayers.

[b] Radiolabeled drugs were added onto both BL and AP sides of monolayers.

[c] Radiolabeled drugs were added onto AP side of monolayers.

ondansetron and ranitidine, respectively. More than an order of magnitude increase in P_{app} was observed for ranitidine when calcium was removed from the transport medium or chelated with the added EDTA. There was no change in P_{app} value for ondansetron under the same conditions. These results suggest that paracellular passive diffusion is the major pathway for the transport of ranitidine across Caco-2 cell monolayers whereas paracellular passive diffusion is not the rate determining step for the transport of ondansetron across Caco-2 cell monolayers.

The more definitive evidence for the transport mechanism of ranitidine and ondansetron was provided by cellular uptake studies. The results in Table 1 showed that ranitidine was taken up into the cells far less efficiently than was ondansetron. When the uptake study was carried out under sink conditions the cellular uptake of ranitidine was 20-fold lower than that of ondansetron. In similar experiments, mannitol, a compound that does not traverse across the Caco-2 monolayers, was taken up 15- to 30-fold less efficiently than the transcellularly transported compound, testosterone. These studies clearly demonstrate the advantage of using the Caco-2 cell culture model to study the mechanism of drug

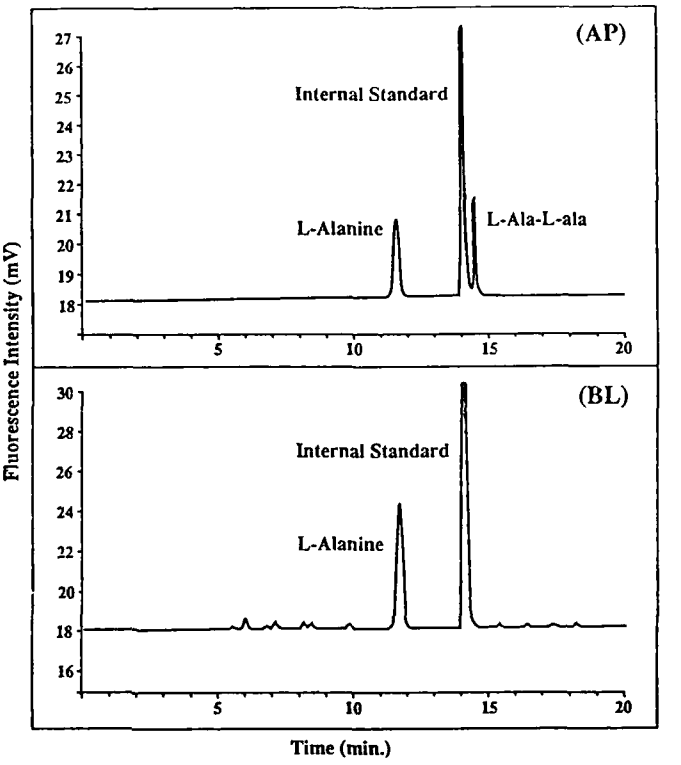

FIGURE 1. HPLC/fluorescence chromatograms of samples from L-Ala-L-Ala (1.56 mM) transport study. (AP), AP sample, (BL) BL sample.

transport, and specifically, to evaluate the contribution of paracellular vs. transcellular pathway to the transport of drug molecules across intestinal mucosa.

II. Carrier-Mediated Active Transport and Passive Diffusion:

L-Ala-L-Ala, Gly-Gly, and Gly-Sar: Three small dipeptides L-Ala-L-Ala, Gly-Gly, and Gly-Sar were chosen as model compounds to demonstrate the metabolic capability of Caco-2 cells as well as the utility of Caco-2 cells in the characterization of the intestinal transport of dipeptide-like therapeutic agents. Results of Caco-2 cell homogenate incubation indicated that Gly-Sar was metabolically stable toward the enzyme(s) present in Caco-2 cells and that L-Ala-L-Ala was hydrolyzed at a rate that was faster than that of Gly-Gly (data not

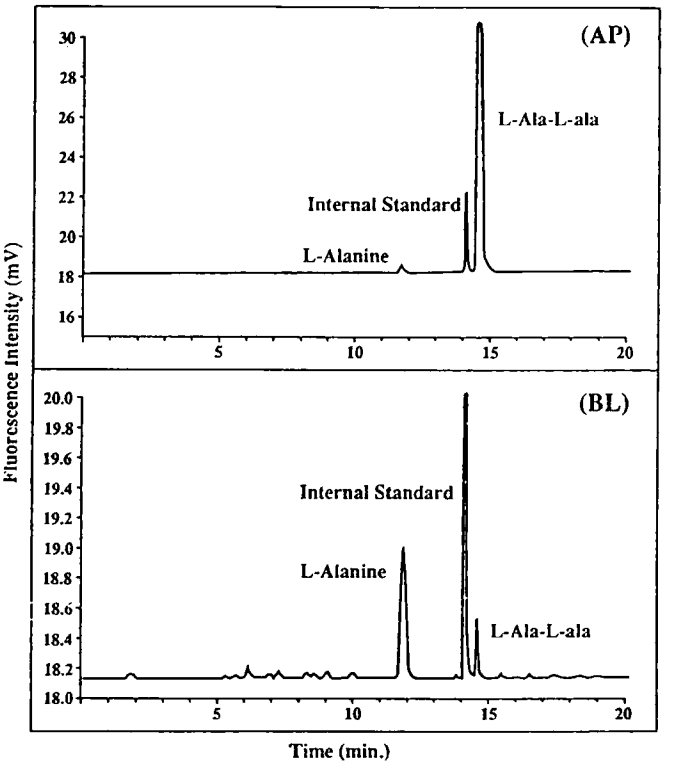

FIGURE 2. HPLC/fluorescence chromatograms of samples from L-Ala-L-Ala (200 mM) transport study. (AP), AP sample, (BL) BL sample.

shown). Results of cellular transport studies of L-Ala-L-Ala (shown in Figure 1) indicated that at relatively low AP concentration, L-Ala-L-Ala was completely hydrolyzed by the enzyme(s) on the brush-border membrane and only its amino acid constituent L-alanine was observed on the BL side of Caco-2 cell monolayers (17). Results also indicated that only at a relatively high AP concentration of L-Ala-L-Ala (e.g. 200 uM), the intact dipeptide could be observed on the BL side of cell monolayers (Figure 2). Similar results were observed for dipeptide Gly-Gly (data not shown). Kinetic analysis on the appearance of both L-alanine and L-Ala-L-Ala in the BL samples indicated a saturable process for the overall transport of this dipeptide (data not shown). In addition, transport of L-Ala-[^{14}C]-L-Ala was found inhibited 20% ~ 50% by dipeptides glycyl-L-leucine and L-Ala-L-Ala, by the Na^+/K^+-ATPase inhibitor ouabain, by the Na^+/ H^+ antiporter inhibitor amiloride,

Table 2

Effect of Dipeptides (10 mM), Amino Acids (10 mM), and Active Transport Inhibitors on the Transport of 1 mM Gly-Sar

Compound	Gly-Sar Transport (% of Control)
Control	100.0 ± 2.3
Glycylglycine	34.8 ± 13.8
Glycyl-L-proline	43.5 ± 3.1
L-Alanyl-L-alanine	15.3 ± 6.4
L-Phenylalanylglycine	19.9 ± 5.9
L-Carnosine	44.8 ± 1.3
Glycine	100.3 ± 16.4
L-Alanine	97.8 ± 13.2
L-Lysine	110.6 ± 11.1
L-Glutamic acid	101.6 ± 4.5
2,4-Dinitrophenol (1 mM)	34.4 ± 7.6
Sodium azide (1 mM)	82.4 ± 3.4
Ouabain (5 mM)	65.6 ± 2.9
Amiloride (1 mM)	53.9 ± 3.4

by the protonophore FCCP, but not by dipeptides β-Alanyl-L-alanine, D-Ala-D-Ala or amino acids indicating a specific dipeptide carrier-mediated active transport of L-Ala-L-Ala across Caco-2 cells.

Detailed kinetic studies are clearly needed to accurately characterize the carrier-mediated L-Ala-L-Ala transport process. For example, a kinetic method which is capable of distinguishing the Ap → BL transported L-alanine from the L-alanine that was generated by the apically absorbed and intracellularly digested L-Ala-L-

Ala in the BL sample. The metabolically stable dipeptide Gly-Sar is, therefore, a better candidate for the characterization of the dipeptide carrier on Caco-2 cells (18,19). Results of HPLC analysis of BL samples from Gly-Sar transport studies indicated a dual mechanism involving carrier-mediated transport and passive diffusion for the transport of Gly-Sar across Caco-2 cell monolayers at pH 7.0 (see ref. 19). Results also indicated that while the dipeptide is transported by both carrier-mediated and passive diffusion mechanisms, the carrier-mediated mechanism clearly contributes predominately to the overall transport of this dipeptide. Biochemical features of this carrier were further characterized and results showed that this carrier was specific for dipeptide as Gly-Sar transport was inhibited by dipeptides but not by amino acids (See Table 2). Transport of Gly-Sar across Caco-2 cells was also found to be an active process as Gly-Sar transport rate was reduced after 30 min preincubation with metabolic inhibitors 2,4-dinitrophenol and sodium azide, with the Na^+/K^+- ATPase inhibitor ouabain, and with the Na^+/H^+ antiporter inhibitor amiloride (see Table 2).

A low transport rate was also observed when transport of 1 mM Gly-Sar was conducted in a sodium free medium indicating sodium dependence on the transport. In addition, Gly-Sar (1 mM) transport rate was reduced by 50% when experiment was conducted at 4 ^oC. The lack of complete inhibition of Gly-Sar transport in the above studies is consistent with the mixed transport mechanism for this dipeptide (i.e. carrier-mediated transport and passive diffusion). This dipeptide carrier-mediated active transport of Gly-Sar was also found to be proton-gradient driven as transport of 1 mM Gly-Sar was reduced 60% after preincubation of cell monolayers with the protonophore FCCP (40 μM) and that transport of Gly-Sar across Caco-2 cells was optimum at pH 5.8 - 6.0 (data not shown). The presence of the dipeptide carrier makes Caco-2 cells a useful *in vitro* model for the intestinal absorption and metabolism studies of small peptide-like therapeutic agents.

III. Passive Diffusion or Carrier-Mediated Active Transport ?

TRH: We have attempted to address the controversial issue on the intestinal absorption mechanism of TRH (18,20). While Yokohama *et al.* demonstrated a saturable process for the absorption of TRH in rats (25,26), our kinetic analysis of

the Caco-2 transport study showed a linear relationship between TRH transport rate and concentration, an indication of a passive diffusion process (P_{app} = 0.95 ± 0.08 x 10^{-6} cm/sec). Using similar approaches in the transport study of the dipeptide Gly-Sar, we have observed that transport of TRH was not an active process as TRH transport was not inhibited by dipeptide Gly-Sar, amino acid glycine, or by active transport inhibitors 2,4-dinitrophenol, sodium azide, ouabain, or amiloride (data not shown).

Results of HPLC analysis of BL samples from the TRH transport studies indicated that TRH was metabolically stable during the course of transport across Caco-2 cells. Incubation of Caco-2 cell homogenate with TRH, however, showed a time-dependent hydrolysis of TRH and the subsequent formation of TRH free acid. These results indicated that either TRH was predominately transported *via* the paracellular route or the metabolites formed during the transcellular transport did not permeate the basolateral membrane. Using the same approach in the calcium switching assay of ranitidine, we have observed a 4-fold increase in the TRH transport with a concomitant increase in mannitol transport when the transport experiment was conducted in the presence of 10 mM EDTA (data not shown). This is indicative of a paracellular passive diffusion as the rate-limiting pathway for the transport of TRH. When the cellular uptake of TRH was examined, a linear relationship between TRH accumulation rate and concentration was also observed. Comparison of relative rates of cellular accumulation *vs.* transport of TRH indicated that the rate of cellular accumulation of TRH was about one third that of TRH cellular transport rate. Together, these results indicated that transport of TRH across Caco-2 cell monolayers was predominately paracellular diffusion with some contribution of transcellular diffusion.

Applications of Caco-2 Cell Culture Model

In addition to elucidate intestinal absorption mechanisms of drug molecules, we have also used Caco-2 model in the following drug development tasks:

1) Screen and select orally active therapeutic agents. We have involved in the process of screening and selecting orally active NCEs from the Research Discovery Program using the Caco-2 system. Result from a computer modeling analysis of more than 70 benzodiazepines indicated that the number of potential H-

bonding and the size of molecule play major role in the intestinal permeability of this series of compounds. The search and design of orally active NCEs was subsequently directed by the good correlation between measured Caco-2 permeabilities and computer calculated permeabilities.

2) Evaluate prodrug approaches for enhanced drug absorptions. We have, in many occasions, compared the permeability of ester prodrugs (e.g. methyl, ethyl, and isopropyl esters of peptide-like therapeutic agents) with that of parent drug. In most cases, ester prodrugs possess ~ two order of magnitude higher permeability. In addition, with the enrichment of esterase(s) in Caco-2 cells, ester prodrugs were found hydrolyzed during the course of transport across Caco-2 cell monolayers.

3) Identify optimum luminal pH's for drug absorptions and address dissolution rate-related absorption problems. We have frequently studied the absorption of charged therapeutic agents at different luminal pH's and at different concentrations in order to address poor bioavailability issues. We have, in many cases, observed relatively high permeabilities for many orally inactive acidic compounds at low pH's (i.e. pH's at upper intestinal tract). Poor bioavailabilities of these compounds were actually owing to their poor aqueous solubilities and poor dissolution rates at low pH's (e.g. precipitated in stomach where pH = 1). We have also observed high permeabilities for many basic compounds at relatively high pH's and yet compounds possess poor bioavailabilities due to their poor aqueous solubilities at high pH's (e.g. precipitated at lower intestinal tract).

4) Assess mucosal toxicity of therapeutic agents. Since we routinely examined the integrity of cell monolayers during the course of transport studies, we have, in many occasions, revealed possible mucosal toxicity effect of tested drugs. In most cases, the mechanism of toxicity was investigated *via* either a mini SAR Caco-2 study or a histopathology study of the tested compound using experimental animals.

5) Assess metabolic stability of therapeutic agents in the intestinal epithelium. The possible intestinal metabolism of tested drugs was often addressed based on the HPLC analysis of samples from transport studies. We have learned, from transport studies of prodrugs, that Caco-2 cells are enriched in esterase(s) and amidase(s). We have also learned, from transport studies of drug molecules with known metabolic pathways, that cytochrome P-450 isozymes and phase II enzymes (3) are present in Caco-2 cells.

CONCLUSIONS

In summary, we have shown that the Caco-2 cell culture system is a good *in vitro* "cell culture" model for the intestinal absorption and metabolism study. It is an excellent tool for distinguishing passive diffusion process from carried-mediated active transport as well as for dissecting paracellular and transcellular transport pathways. As long as the limitations of this model (e.g. cells do not secret mucin, cells possess tighter intercellular junctions, etc.) was acknowledged, intellectual uses of this model to address intestinal absorption and metabolism related problems at various stages of drug discovery and development should prove to be possible.

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