A Catenary Model to Study Transport and Conjugation of Baicalein, a Bioactive Flavonoid, in the Caco-2 Cell Monolayer: Demonstration of Substrate Inhibition

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ABSTRACT

The transport and metabolism of baicalein (Ba) was studied in vitro and in Caco-2 cells. Protein binding of Ba with Caco-2 lysate showed that Ba was bound to two classes of sites: a higher affinity, lower capacity site ($K_{\text{a}1} = 27.6 \pm 4.7 \mu M^{-1}$, $n_1 = 10.6 \pm 0.6 \text{ nmol/mg}$) and lower affinity, higher capacity site ($K_{\text{a}2} = 0.015 \pm 0.0013 \mu M^{-1}$, $n_2 = 413 \pm 21 \text{ nmol/mg}$). Incubation studies of Ba with Caco-2 lysate showed substrate inhibition of both glucuronidation and sulfation, with $K_m$ values of 0.14 ± 0.034 and 0.015 ± 0.0053 $\mu M$, and $K_i$ values of 6.75 ± 1.70 and 0.37 ± 0.16 $\mu M$, respectively. In the Caco-2 monolayer, Ba (8–47 $\mu M$) displayed good apparent permeabilities ($P_{\text{app}}$) across the membrane; $P_{\text{app}}$ was found to be increased with elevated loading concentration in both the absorptive and secretory directions. However, the efflux ratio was less than unity, negating the involvement of apical efflux transporters. The concentration ratios of Ba sulfate (BS) and glucuronide (BG) decreased with increased loading of Ba concentration, suggesting that BS and BG are apically excreted via transporters, likely breast cancer resistance protein and multidrug resistance-associated protein 2, respectively. Data fit to the catenary model, composed of basolateral, cellular, and apical compartments, showed a low cellular unbound fraction (0.0019 ± 0.00018), a high passive diffusion clearance (0.012 ± 0.00029 $\text{ml/min/mg}$), and substrate inhibition, with sulfation being more readily saturated and inhibited than glucuronidation, as evidenced by smaller $K_i$ value (0.35 ± 0.078 versus 1.95 ± 0.57 $\mu M$) and $K_i$ value (0.58 ± 0.20 versus 7.90 ± 1.10 $\mu M$); these patterns paralleled those observed in the lysate incubation studies. The results showed that the catenary model aptly predicts substrate inhibition kinetics and offers significant and mechanistic insight into the transport and atypical metabolism of drugs in the Caco-2 monolayer.

Flavonoids are polyphenolic compounds that are widely distributed in both edible plant and derived foods (Yang et al., 2001). Flavonoids possess anticarcinogenic activity and other beneficial effects, including the prevention of oxidation of low-density lipoproteins and development of coronary heart disease. These activities have aroused increasing interest among scientists (Yang et al., 2001). However, several studies have demonstrated that the oral bioavailabilities of flavonoids are low and associated with extensive first-pass metabolism, including glucuronidation, sulfation, and methylation (Kroon et al., 2004; Manach and Donovan, 2004).

ABBREVIATIONS: MRP, multidrug resistance-associated protein; Ba, baicalein; BG, baicalein-7-O-β-glucuronide; UGT, UDP-glucuronosyltransferase; BS, baicalein sulfate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; A, apical; B, basolateral; $P_{\text{app}}$, apparent permeability; EIR, efflux ratio; $n_1$, $n_2$, the number of binding sites in the first and second class of binding sites, respectively; $CL_{\text{int,sec}}$ and $CL_{\text{ad}}$(BG or BS), net efflux clearance of the Ba conjugate (BG or BS) at the apical and basolateral membranes, respectively; $CL_{\text{at1}}$ and $CL_{\text{at2}}$, $CL_{\text{ad1}}$ and $CL_{\text{ad2}}$, influx and efflux, passive diffusion clearance at the basolateral and apical membrane, respectively; $CL_{\text{influx}}$ and $CL_{\text{efflux}}$, transporter-mediated influx and efflux intrinsic clearances at the basolateral membrane; $CL_{\text{abs}}$ and $CL_{\text{int,sec}}$, transporter-mediated intrinsic clearances for absorption and efflux at the apical membrane, respectively; $V_{\text{app}}$, $V_{\text{cap}}$, and $V_{\text{baso}}$, volumes of the apical, cellular, and basolateral compartment, respectively; $f_{\text{app}}$, $f_{\text{cell}}$, and $f_{\text{baso}}$, unbound fractions of drug in apical, cellular, and basolateral compartment, respectively; $V_{\text{Ba--BG}}$ and $V_{\text{Ba--BS}}$, maximum velocity of glucuronidation and sulfation, respectively; $K_m^{\text{Ba--BG}}$ and $K_m^{\text{Ba--BS}}$, Michaelis-Menten constant for glucuronidation and sulfation, respectively; $V_{\text{max}}^{\text{Ba--BG}}$ and $V_{\text{max}}^{\text{Ba--BS}}$, inhibition constant for glucuronidation and sulfation, respectively; ABL, aqueous boundary layer.
Due to its anatomical location, the intestine is the first potential tissue responsible for the first-pass metabolism of flavonoids after oral absorption. Akin to the intestinal tissue, the Caco-2 cell monolayer, an immortalized cell line derived from human colon carcinoma, resembles morphologically the enterocytes of the small intestine, and it exhibits brush-border characteristics at the apical side after confluence. The Caco-2 cell is shown to contain phase I and phase II drug-metabolizing enzymes and efflux transporters such as P-glycoprotein or multidrug resistance protein 1, and members of the multidrug resistance-associated protein (MRP) family (Hunter et al., 1993; Gutmann et al., 1999). Thus, flavonoid metabolism and transport have been investigated quite extensively in Caco-2 cells. Flavonoids such as chrysins, genistein, and epicatechin showed extensive glucuronidation or sulfation during their permeation through the Caco-2 cell monolayer; the formed glucuronide and sulfate metabolites have been implicated as substrates of the MRPs (Galijatovic et al., 2000; Vaidyanathan and Walle, 2001; Liu and Hu, 2002). The metabolic profiles of flavonoids in the Caco-2 cell model and rat intestine perfusion studies indeed showed extensive glucuronidation and sulfation (Liu and Hu, 2002; Zhang et al., 2005). However, there are only a few studies that addressed the kinetics of metabolism and efflux of flavonoids in a quantitative manner.

In the present study, baicalein (Ba) was chosen as a model compound to demonstrate the extensive metabolism of flavonoids. Our previous studies have shown that the low oral bioavailability of Ba could be explained by the extensive intestinal first-pass metabolism to baicalein-7-O-β-glucuronide (BG) (Zhang et al., 2005, 2007b). Glucuronidation of Ba occurs in the rat jejunum (Zhang et al., 2005) and human jejunum and ileum, tissues known to contain a variety of human UDP-glucuronosyltransferases (UGTs) (Zhang et al., 2007a). Moreover, substrate inhibition was observed for UGT1A1 with Ba in vitro, and for sulfation in human and rat liver cytosol (Zhang et al., 2007a). The present study examined the metabolism of Ba and the bidirectional transport of Ba in the Caco-2 cell monolayer model. A catenary model encompassing the apical, cellular, and basolateral compartments was developed to quantitatively appraise the distributional and metabolic behaviors of Ba.

Materials and Methods

Materials

Ba and BG were obtained from Aldrich Chemical Co. (Milwaukee, WI). The solubility of Ba was found to be 70.3 μM in transport buffer and 594 μM in 50 mM Tris buffer, pH 7.4. Thus, the concentrations of Ba used in the transport, protein binding, and metabolism studies were all within the soluble range. Baicalein sulfate (BS) was prepared as described previously (Zhang et al., 2007b). 6-Hydroxyflavanone, the internal standard, was procured from Indofine Chemical Company (Hillsborough, NJ). Uridine 5’-diphosphoglucuronic acid, adenosine 3’-phosphate 5’-phosphosulfate and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methanol (HPLC grade) were obtained from Labscan (Labscan Asia, Bangkok, Thailand).

Cell Culture

Caco-2 cells, purchased from the American Type Culture Collection (Manassas, VA), were cultured as described previously (Zhang et al., 2004). The integrity of the monolayer, monitored by transepithelial electric resistance at 37°C with an epithelial voltohmmeter (WPI, Sarasota, FL), exceeded 600 Ω · cm²² (after subtracting the background value of the Transwell) for each study, and the value remained above 500 Ω · cm²² after the experiment. The permeability of Lucifer yellow, a marker for paracellular transport, was 1.63 ± 0.12 × 10⁻⁷ cm/s. The passage of Caco-2 cells grown in Transwell, was confined to within 32 to 36.

Preparation of Caco-2 Lysate

Caco-2 cells (from eight Transwells) cultured for 21 days were disrupted in 11 ml of 50 mM Tris buffer, pH 7.4, by sonication for 10 min in an ice-cold water bath. The protein content of the lysate was determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Protein Binding of Ba with Caco-2 Lysate

The extent of binding of Ba (5.5–555 μM) to Caco-2 cell lysate was investigated. The solute was incubated with Caco-2 cell lysate (final lysate protein concentration 0.88 mg/ml) at 37°C for 15 min, and the mixture was rapidly filtered through a filter membrane (3000 mol. wt. cut-off; Millipore Corporation, Billerica, MA) by centrifugation at 16,000g for 20 min. The control experiment was carried out with Ba present in 50 mM Tris buffer, pH 7.4. The filtrate was assayed for protein by Bio-Rad protein assay kit to check for leakiness. Protein binding was calculated according to % bound = (1 − Cg/Cu) × 100% (Pendyala and Creaven, 1993), where Cg is the concentration of Ba in the ultrafiltrate, and C is the concentration of Ba obtained from the filtrate in the control experiment. The samples were assayed by HPLC.

Metabolism of Ba Incubated with Caco-2 Lysate

As described previously (Zhang et al., 2007a), Ba (final concentration of 1.11–111 μM) was incubated with Caco-2 cell lysate (final protein concentration 1.34 mg/ml) at 37°C for 10 min. The incubation medium consisted of 50 mM Tris-HCl buffer, pH 7.5, with 8 mM MgCl₂, and 25 μg/ml alamethicin for the study of Ba glucuronidation, and Tris buffer, pH 7.4, with 5 mM MgCl₂ for the study of Ba sulfation. Alamethicin was used to activate the UGTs by causing formation of well defined pores in the endoplasmic reticulum membrane and rendering the UGTs in the endoplasmic reticulum lumen accessible to substrates and cofactors (Fisher et al., 2000). After the addition of 2 mM uridine 5’-diphosphoglucuronic acid or 200 μM adenosine 3’-phosphate 5’-phosphosulfate, the incubation study was carried out for 20 min for glucuronidation and 10 min for sulfation, the predetermined incubation times that showed time linearity in metabolite formation. The reaction was terminated by the addition of 40 μl of ice-cold acetonitrile/acetic acid [9:1 (v/v)] containing the internal standard (6-hydroxyflavanone), followed by the centrifugation at 16,000g for 5 min. The resultant supernatant was directly subjected to HPLC analysis.

Bidirectional Transport Studies in Caco-2 Cell Monolayer

PBS supplemented with Ca²⁺ and Mg²⁺ at pH 6.0 was prepared and used as the transport buffer, as described in our previous study (Zhang et al., 2004). The bidirectional transport study was initiated by loading Ba solutions at various concentrations (Ba, 8–47 μM) into the “donor” side, either the apical (ap or A) (1.5 ml of transport buffer) side or the basolateral (baso or B) compartment (2.6 ml of transport buffer). Aliquots of 0.5-ml samples were taken from the alternate, “receiver” side at different times (30, 60, 90, and 120 min). The removed volume (0.5 ml of blank PBS) was replaced back to the receiver chamber after each sampling. Samples, collected in triplicate, were acidified with 0.1 ml of 20% ascorbic acid solution and 0.1 ml of methanol and stored at −80°C until assay. At the end of the transport study (120 min), amounts of Ba and its metabolites in cells were determined. The monolayer was rinsed with ice-cold saline three times, and 50 μl of the internal standard (6-hydroxyflavanone
at 10 μg/ml was added. The contents were lysed upon the addition of 3 ml of 35% methanol in 25 mM sodium dihydrogen phosphate buffer, pH 2.5, containing 1% ascorbic acid, followed by sonication (30 min). The lysate obtained thereafter was loaded onto an Oasis HLB cartridge, followed by washing with 1 ml of 25 mM sodium dihydrogen phosphate buffer, pH 2.5. Finally, analytes from the cartridge were eluted with 1 ml of methanol. The collected eluent was then evaporated to dryness and reconstituted with 100 μl of 35% methanol in 25 mM sodium dihydrogen phosphate buffer, pH 2.5, containing 1% ascorbic acid. An aliquot of 50 μl was injected into the HPLC for analysis.

**Determination of Ba and Its Metabolites by Liquid Chromatography-Tandem Mass Spectrometry and HPLC**

Samples for protein binding of Ba were assayed by liquid chromatography-tandem mass spectrometry. In brief, the chromatographic separation of Ba and the internal standard was achieved by HPLC using reversed-phase column (BDS, 25 cm × 4.6 mm; Thermo Electron Corporation, Waltham, MA). The column was eluted by mobile phase A (0.05% formic acid in water) and B (acetonitrile) at a flow rate of 1 ml/min, with the gradient as follows: 0 to 3 min, 80% A; 3 to 4 min, 80 to 30% A; 4 to 7 min; 30% A; 7 to 7.5 min, 30 to 80% A; and 7.5 to 10 min, 80% A. Only 20% of the eluent was introduced into mass spectrometer (API Q-Trap; Applied Biosystems, Toronto, ON, Canada). The mass spectrometer was operated in the positive ion mode, with the main working parameters as follows: ionspray voltage, 5500 V; declustering potential, 91 V; entrance potential, 10.5 V; collision cell exit potential 4 V; collision energy, 43 V; curtain gas, 40 psi; nebulizer gases, 70 psi; auxiliary gas, 70 psi; and source temperature, 400°C. For other studies, Ba and the metabolites were analyzed by HPLC/UV detection as described previously (Zheng et al., 2007b). The mobile phase, consisting of a mixture of 20 mM sodium dihydrogen phosphate buffer, pH 4.6 (A), methanol (B), and acetonitrile (C), was run using a linear gradient elution program. The gradient began with 80% A, 5% B, and 15% C, changed linearly to 57% A, 8% B, and 35% C in 10 min; 40% A, 0% B and 60% C in 2 min, and then the condition reverted back to the initial composition in 6 min followed by 7 min thereafter for equilibration.

**Data Analyses**

**Protein binding of Ba to Caco-2 Lysate.** Because the binding proteins in lysate and the molecular weights are unknown, the binding of Ba to Caco-2 lysate of protein concentration [P] was appraised by the Rosenthal plot (Rosenthal, 1967). A two-site binding equation (eq. 1) best fit the data (Rosenthal, 1967),

\[
C_i = C_u + \frac{n_1 [P_i] K_{A1} C_u}{1 + K_{A1} C_u} + \frac{n_2 [P_i] K_{A2} C_u}{1 + K_{A2} C_u}
\]  

(1)

where \(C_i\) and \(C_u\) are the total and unbound drug concentrations, respectively, and the sum of the second and third terms of the right side of the equation denote the bound concentration; \(K_{A1}\) and \(K_{A2}\) are the apparent binding association constants and \(n_1\) and \(n_2\) are the number of binding sites for the first and second classes of noninteracting binding sites, respectively. The unit of \(n_1\) and \(n_2\) is nanomoles per milligram inasmuch as the unknown nature of the binding proteins (Rosenthal, 1967).

**Conjugation of Ba in Caco-2 Lysate.** In view of the suspected substrate inhibition involved in the conjugation kinetics of Ba, the metabolic data generated from the incubation study of Ba with the Caco-2 lysate were fit to eq. 2 (Tracy and Hummel, 2004),

\[
u = \frac{V_{\text{max}}}{K_m + C_u} V_{\text{max}}\left(1 + \frac{C_u}{K_m}\right)^{-1}
\]  

(2)

where \(\nu\) denotes the rate of glucuronidation or sulfation, \(V_{\text{max}}\) is the maximal velocity, \(K_m\) is the Michaelis-Menten constant, and \(K_i\) is the inhibition constant. The unbound concentration of Ba, \(C_u\), is calculated according to eq. 1, where \(C_i\) and \([P]\) are the concentrations of Ba and total lysate protein used and values of \(n_1, n_2, K_{A1}\), and \(K_{A2}\) were taken from the fitting of binding data to eq. 1.

**Transport and Metabolism of Ba in Caco-2 Cell Monolayer.** The apparent permeability (\(P_{\text{app}}\)) is calculated according to eq. 3,

\[
P_{\text{app}} = \frac{dA_p}{dt} \frac{\text{Area} \times C_{D,0}}{P_{\text{app},A-B}}
\]  

(3)

where \(dA_p/dt\) is the rate of drug appearance in the receiver and \(C_{D,0}\) is the initial loading concentration of Ba in the donor side, and Area is the surface area (4.71 cm²) of the filter in the Transwell. The efflux ratio (EFR) is given by the ratio of the apparent permeability from the Ba→A side \((P_{\text{app},B→A})\) to that in A→B \((P_{\text{app},A→B})\) direction.

\[
\text{EFR} = \frac{P_{\text{app},B→A}}{P_{\text{app},A→B}}
\]  

(4)

The total amounts of BG or BS in the donor, cell, and receiver compartments at 2 h were summed. By assigning CL

\[
\text{int}_{\text{BG or BS}}\text{ap}(\text{BG or BS})\text{baso}(\text{BG or BS})\text{baso} / \text{int}
\]  

(5)

and CL

\[
\text{int}_{\text{BG or BS}}\text{baso}(\text{BG or BS})\text{baso} / \text{int}
\]  

(6)

Upon division of eq. 5 by eq. 6 and rearrangement, the concentration ratios of the conjugate (BG or BS) in the apical to basolateral (ap/baso) compartment for each \(C_{D,0}\) was shown in eq. 7. The ratio of the volumes, \(V_{\text{baso}}/V_{\text{ap}}\) is 1.73.

\[
\frac{C_{\text{BG or BS}}(\text{ap})}{C_{\text{BG or BS}}(\text{baso})} = \frac{dC_{\text{BG or BS}}(\text{baso})}{dC_{\text{BG or BS}}(\text{ap})} = \frac{CL_{\text{int}_{\text{BG or BS}}}(\text{BG or BS})\text{baso}(\text{BG or BS})\text{baso}}{CL_{\text{int}_{\text{BG or BS}}}(\text{BG or BS})\text{baso}(\text{BG or BS})\text{baso}} = 1.73
\]  

(7)

Equation 7 offers a simple approach for analyses of the preferential direction of efflux of conjugates that are formed in the cell and normally subjected to efficient secretion into apical and basolateral sides by the different sets of efflux transporters on apical (such as MRP2 and breast cancer resistance protein) and basolateral membrane (such as MRP3 and MRP4), respectively.

**Modeling of Ba Data in the Caco-2 Monolayer.** The transport and metabolic data associated with Ba administration in the Caco-2 monolayer were fit to a catenary model (Fig. 1) that was described previously (Sun and Pang, 2008). The model consists of three compartments: the apical \(V_{\text{ap}}\) of volume 1.5 ml), cellular \(V_{\text{cell}}\), and basolateral \(V_{\text{baso}}\) of volume 2.6 ml compartments (Sun and Pang, 2008), which are interconnected by transfer clearances. The partitioning of drug molecules into plasma membrane, although investigated previously (Raub et al., 1993; Tran et al., 2005), was not incorporated into this model due to the added complexity. The value of \(V_{\text{cell}}\) was estimated to be 3.66 μl/mg protein by geometrical estimation: \(V_{\text{cell}}\) = insert area × cell height that was measured by a pulse height analyzer (Blais et al., 1987). In another approach (Yamaguchi et al., 2000), the estimated \(V_{\text{cell}}\) was 4.05 μl/mg protein by the cellular accumulation study of sulfanilamide, a poorly protein bound compound whose transport was mediated only by passive diffusion. Hence, the average, or 3.86 μl/mg protein, is assigned as \(V_{\text{cell}}\) in our study. Based on 3.86 μl/mg protein and 1.6 mg protein/Transwell, the \(V_{\text{cell}}\) for the Caco-2 monolayer grown on an insert Transwell (surface area 4.71 cm²) is estimated to be 6.2 μl.

The unbound fractions in the apical, cellular, and basolateral compartments are expressed as \(f_{\text{app},A-B}\) and \(f_{\text{int}}\), respectively, for Ba. As shown, Ba is transported into Caco-2 cell with the apical,
Fig. 1. The catenary model for transport and metabolism of Ba to the glucuronide (BG) and sulfate (BS) conjugate in Caco-2 cell monolayer. Apical, cellular, and basolateral compartments are interconnected by transfer clearances. Ba transfer into the cell is associated with the apical, passive diffusive clearance (CL_{ap};), whereas efflux back to apical side is associated with the passive diffusive clearance (CL_{dl};) and secretory intrinsic clearance (CL_{int,sec};) mediated by transporters. Ba transfer between cell and basolateral side is described by influx and efflux intrinsic clearances (CL_{ci},) and CL_{cl},, respectively, the sum of passive and transporter-mediated clearances. The unbound fractions are f_{ap}, f_{cl},, and f_{baso}, for apical, cellular, and basolateral compartment, respectively. Ba is conjugated intracellularly to form BG and BS, with saturable processes denoted by the V_{max} and K_{m} and inhibition constant denoted by K_{i}.

intrinsic passive diffusive clearance (CL_{dl};) and an absorptive clearance mediated by transporters (CL_{cl};). Ba in the cell may be transferred back to the apical side mutually by the passive diffusive clearance (CL_{dl};) and a secretory intrinsic clearance that involves transporters (CL_{int,sec};). Ba transfer between the basolateral and cellular compartments occurs via the influx intrinsic (CL_{ci},) and efflux intrinsic (CL_{cl},) clearances, respectively. Transporter-mediated influx and efflux at the basolateral membrane are assumed to be absent for Ba. Once in the cell, Ba would undergo glucuronidation and sulfation. The total amount of metabolite formed at 2 h was given by the sum of those in the apical, cellular, and basolateral compartments. Because the incubation study of Ba with Caco-2 lysate had demonstrated substrate inhibition kinetics, eq. 2 was used, despite that other modeling schemes, such as absence of inhibition for both or one of the conjugation pathways, were tried and resulted in poor fits (data not shown). The unbound concentration in the cell is given by f_{cell}, C_{cell}, product of the unbound fraction in cell and the cellular concentration of Ba.

Fitting was carried out with the mass balance rate equations shown under Appendix. Several considerations were made to improve the fit. First, drug loss due to sampling could not be neglected. A correction of the volume and mass of Ba due to sampling at 0.5-h intervals (0.5 mL removed at the receiver side) was made (see Appendix). This was described by the pulse function, \( \Sigma_{i=1}^{n} \text{pulserate}(V_{p}/V_{R} \times \Delta_{t}(t_{i},t_{i+1},0)) \), where \( V_{p} \) was the volume of sampling (0.5 mL in this study) at each sampling time point; \( \Delta_{t} \) denoted the amount of drug in receiver side observed at \( t_{i} \) and \( n \) was the number of intervening samplings conducted between time = 0 and at the end of the experiment (see Appendix for the corresponding mass balance equations).

Second, ER was less than, or almost equaled, unity for all loading concentrations of Ba (Fig. 4B; see Results), and it was reasonable to assume that there was a lack of involvement of an apical efflux transporter for Ba, i.e., CL_{int,sec} = 0. This notion was further supported by the pilot fit which showed a low, fitted value for CL_{int,sec} (10^{-15} M/s) (data not shown). Because Ba is a hydrophilic molecule of log P (3.31) that possesses three hydrogen bond donors (<5) and two hydrogen bond acceptors (<10) (Li and Tian, 2004), good passive absorption would be favored. Hence, the assumptions that Ba transport was not predicated on transporters and that identical passive diffusion clearances existed across the membranes (CL_{d1} = CL_{d2} = CL_{ds} = CL_{ds} = CL_{ds} = CL_{ds}) were made. Third, the possibility that trace amounts of soluble protein existed in the apical or basolateral compartment was neglected; accordingly, protein binding in the apical and basolateral compartment was assumed to be negligible and absent, i.e., \( f_{ap} = f_{baso} = 1 \). Cellular binding, however, needed to be considered, since Ba was bound tightly with Caco-2 lysate protein in vitro. Because it is virtually impossible to measure binding of Ba in the intact Caco-2 cell monolayer experimentally, we imported values of \( n_{1}, n_{2}, K_{A1}, \) and \( K_{A2} \) derived from the lysate binding study into the catenary model in pilot fits. However, the results showed nonsensible parameter values that were associated with large sum of squared residuals and unreasonably large covariance (data not shown). This was likely because that the protein concentration in the naïve cytosol differed dramatically from those obtained upon dilution for preparation of Caco-2 lysate (200×, 6.2 μl to 1.4 ml). When the protein concentration changes, values of apparent binding parameters, such as \( K_{A1} \) and \( K_{A2} \), will change accordingly, as shown by Rosenthal et al. (1972). Hence, the binding parameters obtained in the lysate may not reflect that within the intact Caco-2 cell. Thus the unbound fraction of Ba in cell, \( f_{cell} \), was assumed to be a constant within the narrow range of concentration used and was estimated via fitting. Fourth, the distribution of the formed conjugates, BG and BS, was not considered. Instead, the aggregate amounts of BG and BS formed at 2 h in the Caco-2 cell system in both A→B and B→A directions after loading with different C_{D,0} values were used for fitting of the bidirectional data simultaneously.

Fitting. The fitting program, MicroMath Scientist, version. = 2.01 (MicroMath Software, Salt Lake City, UT) was used. Different weighting factors (unity, 1/prediction and 1/prediction^2) were tried. The fitting program, MicroMath Scientist, version. = 2.01 (MicroMath Software, Salt Lake City, UT) was used. Different weighting factors (unity, 1/prediction and 1/prediction^2) were tried. The optimal fit was chosen based on the model selection criteria, the residual plot, standard deviation of the parameter estimates, and the reasonableness of the parameters.

Results

Protein Binding of Ba in Caco-2 Lysate. The binding of Ba to Caco-2 lysate was adequately described by the two-site binding equation (eq. 1), as shown in Rosenthal plot (Fig. 2A). The least square fit revealed two classes of binding sites: a higher affinity (\( K_{A1} = 27.6 \pm 4.7 \mu M^{-1} \)), lower capacity (\( n_{1} = 10.6 \pm 0.6 \text{nmol/mg} \)) and a lower affinity (\( K_{A2} = 0.015 \pm 0.0013 \text{μM}^{-1} \)), higher capacity (\( n_{2} = 413 \pm 21 \text{nmol/mg} \)) site. The unbound fraction in lysate \( f_{cell} \), increased with Ba concentration (Fig. 2B).

Conjugation of Ba in Caco-2 Lysate. As shown in Fig. 3, rates of glucuronidation and sulfation in Caco-2 lysate first increased with Ba concentration, and then they declined upon further increases in concentration, suggesting substrate inhibition for both glucuronidation and sulfation. Upon correction for saturable protein binding, conjugation of Ba was adequately described by eq. 2. The fitted parameters are summarized in Table 1. For both glucuronidation and sulfation, the value of the \( K_{m} \) is less than that for the \( K_{i} \) (0.14 < 6.75 μM and 0.015 < 0.37 μM, respectively). Compared with the glucuronidation, sulfation of Ba was more readily saturated (\( K_{m} = 0.015 \) versus 0.14 μM) and inhibited (\( K_{i} = 0.37 \) versus 6.75 μM).

Transport and Conjugation of Ba in Caco-2 Monolayer. In both the A→B and B→A directions, the \( F_{app} \) for Ba (range 1.0–8.4 × 10^{-6} cm/s) was time-dependent and increased with higher Ba loading concentrations (C_{D,0}) (Fig.
The apparent first-order rate constants for Ba conjugation were consistently less than unity regardless of the value of CD,0 (Fig. 4B), and no succinct pattern was identifiable. The total amounts of BG (Fig. 5A) and BS (Fig. 5B) formed at 2 h increased with dose (CD,0/volume) initially, but these values declined upon further increment of dose, suggesting inhibition kinetics for Ba conjugation. These observations paralleled those found in the incubation study of Ba with Caco-2 cell lysate. The concentration ratio of BG in apical to basolateral side, Cap{BG}/Cbaso{BG}, was initially high (7.4) at lower CD,0 values, but this rapidly declined below 1.73 at higher CD,0 values (Fig. 5C). The Cap{BS}/Cbaso{BS} ratio was high and declined with increasing CD,0 (from 8 to 47 μM), but remained greater than 1.73 (Fig. 5D). The observed trends suggest saturation of transporters for the efflux of BS and BG at the apical membrane, and there was a greater preference for BS to be excreted apically.

Modeling of Ba Transport and Metabolism in the Caco-2 Monolayer. Fitting was performed with different weighting schemes, and a weighting scheme of 1/predicted furnished optimized fits (Table 2). The optimized results (Table 2) showed that the model with substrate inhibition of both conjugation pathways fitted the data adequately (Figs. 6 and 7). The results revealed high cellular binding of Ba (f cell of 0.0019 ± 0.00018) and a rapid passive diffusion clearance (ClD of 0.012 ± 0.00029 ml/min). For both glucuronidation and sulfation, the Km values (1.95 and 0.35 μM, respectively) were less than those for the corresponding Ki values (7.90 and 0.58 μM, respectively). The Km and Ki values for sulfation were smaller than those for glucuronidation, suggesting that sulfation is more prone to saturation and substrate inhibition than glucuronidation in the Caco-2 cell monolayer.
data sets with repetitive sampling of the donor, cell, and receiver compartments at various times. Uptake and/or efflux information was appraised in parallel studies to obtain information on the individual kinetic process (Bourdet et al., 2006). In some cases, data may be obtained from identical Transwells that provide data in the various compartments at each time point (Huang et al., 2006). In other studies, repetitive sampling at various time points in the receiver compartment of the same Transwell with volume replacement was used. In this study, correction of the actual mass removed in the pulse function was found to improve the fit significantly. With proper accounting for the mass and volume removed via sampling, the observations were well predicted by the catenary model (Figs. 6 and 7; Table 2). Values of fitted parameters were quite reasonable, and they were associated with small covariances (<35%).

The present catenary model, however, had not included the aqueous boundary layer (ABL) that reduces $P_{ABL}$ for passive diffusion processes and elevates $K_m$ values for active transport processes (Wilson and Dietschy, 1974). The contribution of the ABL may be unveiled from those of the membrane and paracellular pathways when stirring at different speed was used (Adson et al., 1995). One may turn to the equality that relates the effective ABL-membrane permeability ($P_{ABL,M}$) to the permeability across the ABL ($P_{ABL}$) and cell membrane ($P_M$), described as $1/P_{ABL,M} = 1/P_{ABL} + 1/P_M$ (Ho et al., 2000). In presence of the ABL, the estimated passive diffusion clearance of Ba ($C_{Ld}$) or product of surface area ($S$) and $P_{ABL,M}$ would underestimate the true passive diffusion clearance across the cell membrane. The estimated $C_{Ld}$ equals the true passive diffusion clearance when $1/P_{ABL} = 0$ or $<< 1/P_M$. Although Ba is lipophilic (log $P = 3.31$), the 4-H-benzopyran-4-one group confers considerable acidity ($pK_a = 5.3$) to the three phenolic groups (Yoshizuka et al., 1996), resulting in ~83% ionization of Ba at the pH (6.0) of the transport buffer. The high ionization of Ba will likely reduce the effect of the ABL that tends to retard the transport of nonionized, lipophilic moiety.

The effective $P_{app}$ of Ba was found to be 1.0 to $8.4 \times 10^{-6}$ cm/s (Fig. 4A), a value that is significantly higher than that for mannitol (0.48 $\times 10^{-6}$ cm/s) and smaller than those for other flavonoid aglycones: quercetin, chrysin, kaempferol, isorhamnetin, genistein, daidzein and glycitein, of values ranging from $10^{-6}$ to $50 \times 10^{-6}$ cm/s. The $P_{app}$ pertains only to passive permeability across a single barrier, and it is erroneous when binding and metabolism occur in the system (eq. 3). The high cellular binding ($f_u = 0.0019 \pm 0.00018$) and extensive metabolism of Ba would prevent Ba molecules from reaching the receiver compartment and result in lower, apparent $P_{app}$ estimates. Kalvass and Pollack (2007) had defined the relationship between $P_{app}$ and $C_{Ld}$ of the catenary model ($P_{app} = C_{Ld}/2/Area$) based on absence of cellular binding, metabolism and transporters. According to the fitted $C_{Ld}$, this $P_{app}$ was calculated to be $3.4 \times 10^{-6}$ cm/s, and it is 1 order of magnitude higher than that estimated with eq. 3. From this simple exercise, it can be concluded that the parameter $C_{Ld}$ is superior to $P_{app}$ in evaluating the passive permeability of a solute, since the latter parameter is modified by binding, metabolism and transporters. At higher Ba loading concentrations, higher $P_{app}$ values were obtained in both A→B and B→A directions. Normally, the pattern suggests saturation of metabolism, and/or binding within the

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Time profiles of the apparent permeability of Ba in the A→B ($P_{app,A\rightarrow B}$, open symbols, ---) or B→A ($P_{app,B\rightarrow A}$, solid symbols, ——) direction (A) and EIR, the ratio of apparent permeability ($P_{app,A\rightarrow B}/P_{app,B\rightarrow A}$) (B). In A, data at different loading Ba concentrations ($C_{1,0}$, micromolar) in donor side are denoted by different symbols: □ and ■ for 8.0 μM; □ and ● for 23.7 μM; △ and ▲ for 39.0 μM; and ◻ and ▼ for 47.0 μM. In B, the EIRs at different $C_{1,0}$ are denoted by ■ for 8.0 μM, ● for 23.7 μM, ▲ for 39.0 μM, and ◻ for 47.0 μM, and they are plotted against time. All EIR values were less than unity.

**Discussion**

The Caco-2 cell monolayer is used extensively for the high-throughput screening of drug permeability by treating the system as a single barrier. The resulting constants, $P_{app}$ and EIR, are imprecise since the Caco-2 monolayer contains discrete membranes at opposite sides of the cell, and it is not a single barrier (Sun and Pang, 2008). By contrast, the catenary model encompasses all of the transport and metabolic processes (Fig. 1). The model describes mass transfer across membranes via passive diffusion and transporter mediation, protein binding, and metabolism. Data analyses with the catenary model (Ito et al., 1999; González-Alvarez et al., 2005; Bourdet et al., 2006) and other biophysical models (Ho et al., 2000; Tran et al., 2005) offer more mechanistic insight and accurate depiction of the kinetic process of interest. To demonstrate the robustness of the catenary model, we used the data set on Ba transport and metabolism to reveal substrate inhibition kinetics in metabolism.

The adequate definition of the catenary model requires
monolayer (Sun and Pang, 2008). However, for Ba, this may also be explained by substrate inhibition on metabolism.

The fit confirmed that substrate inhibition kinetics of Ba on BG and BS formation best described the data; absence of substrate inhibition of the pathways presented poor estimates and fits (data not shown). Substrate inhibition was observed both in the lysate incubation (Fig. 3) and transport studies in the intact cell monolayer (Fig. 5). The metabolic data were both poorly predicted when protein binding was nonexistent (unbound fraction in lysate, $f_{u}$ = 1) (data not shown). The fit of the lysate incubation data were improved dramatically upon incorporation of the binding parameters ($n_1$, $K_{A1}$, $n_2$, and $K_{A2}$) derived from the binding study (Fig. 3, fitted line). The same comment holds upon inclusion of the binding constant (unbound fraction in cell, $f_{cell}$) in the intact cell monolayer for fitting of the transport and metabolic data (Figs. 6 and 7; Table 2). The improved fits emphasize the need to consider protein binding, an important variable that modulates the metabolism (Chiba and Pang, 1993).

Substrate inhibition for phase II metabolism is less common than for phase I metabolism. However, more examples are being revealed, including the sulfation of $p$-nitrophenol, 4-hydroxytamoxifen, 2-methoxyestradiol, and genistein by SULT1A1, 4-methylumbelliferone glucuronidation by the UGTs (1A3, 1A8, 1A9, 2B15, and 2B7), and oxazepam glucuronidation by human liver microsomes and UGT2B15 (Court et al., 2002; Uchaipichat et al., 2004; Nagar et al., 2006). The atypical metabolic profiles for Ba glucuronidation in recombinant UGT1A1 and Ba sulfation have been described previously in rat and human liver cytosol (Zhang et al., 2007b) and now in Caco-2 cell lysate (Fig. 3). Results from the optimized fit (Figs. 6 and 7) confirm the view of substrate inhibition of conjugation for Ba. The kinetic parameters for metabolism obtained in the lysate (Fig. 3; Table 1) and trans-

![Fig. 5. Profiles of Ba conjugation in the Caco-2 monolayer. The total amount BG (A) or BS (B) formed at 2 h in Caco-2 monolayer loaded with different amounts of Ba in the apical (□) or basolateral chamber (●) at time 0. Concentration ratios of formed total BG (C) and BS (D) in apical chamber to that in basolateral chamber in Caco-2 monolayer were further plotted against $C_{240}$; the line, 1.73, is the ratio of $V_{baso}/V_{ap}$.

### TABLE 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fitted Parameter ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbound fraction of Ba in Caco-2 cells, $f_{cell}$</td>
<td>0.0019 ± 0.00018</td>
</tr>
<tr>
<td>Passive diffusion clearance for Ba, $CL_{d1}$ = $CL_{d2}$</td>
<td>0.012 ± 0.00029</td>
</tr>
<tr>
<td>Glucuronidation, Ba→BG</td>
<td>0.012 ± 0.00029</td>
</tr>
<tr>
<td>Maximum velocity, $V_{maxBG}$ (nmol/min/mg)</td>
<td>0.079 ± 0.0069</td>
</tr>
<tr>
<td>Michaelis-Menten constant, $K_{mBG}$ (μM)</td>
<td>1.95 ± 0.57</td>
</tr>
<tr>
<td>Inhibition constant, $K_{I_{BG}}$ (μM)</td>
<td>7.90 ± 1.10</td>
</tr>
<tr>
<td>Sulfation, Ba→BS</td>
<td>0.012 ± 0.00029</td>
</tr>
<tr>
<td>Maximum velocity, $V_{maxBS}$ (nmol/min/mg)</td>
<td>0.17 ± 0.048</td>
</tr>
<tr>
<td>Michaelis-Menten constant, $K_{mBS}$ (mM)</td>
<td>0.35 ± 0.078</td>
</tr>
<tr>
<td>Inhibition constant, $K_{I_{BS}}$ (μM)</td>
<td>0.58 ± 0.20</td>
</tr>
</tbody>
</table>

$^a$ Standard deviation of the fitted parameter.
port study in Caco-2 monolayer (Fig. 6 and 7; Table 2) are of the same order of magnitude. The $K_m$ value is less than the $K_I$ value for both BG and BS formation, and it can be inferred that Ba first binds to the higher affinity, catalytic site at low substrate concentration, and then binds to the lower affinity, inhibition site with increasing concentration, resulting in substrate inhibition. The $K_m$ and $K_I$ values for sulfation are much lower than those for glucuronidation, suggesting that glucuronidation is of lower affinity than sulfation and is less prone to substrate inhibition (Tables 1 and 2). However, it was further noted that a higher capacity existed for sulfation than for glucuronidation, suggesting that glucuronidation is of lower affinity than sulfation and is less prone to substrate inhibition (Tables 1 and 2).

The glucuronidated and sulfated metabolites usually exhibit lower $P_{app}$ values ($< 0.15 \times 10^{-6} \text{ cm/s}$) than their parent flavonoids (Walgren et al., 1998; Walle et al., 1999; Chen et al., 2005; Wang et al., 2005), and they are probably substrates of efflux transporters in the Caco-2 cell monolayer (Walle et al., 1999; Galijatovic et al., 2000; Vaidyanathan and Walle, 2001; Liu and Hu, 2002). The same is inferred for BG and BS. BG was preferentially excreted into apical side at lower $C_{D,0}$ values ($C_{ap}(BG)/C_{baso}(BG) \gg 1.73$), although $C_{ap}(BG)/C_{baso}(BG)$ plummeted down below 1.73 upon further increase of $C_{D,0}$, suggesting involvement of apical efflux transporters. This transporter, suggested by Zhang et al. (2007a), was MRP2, whose activities are of higher affinity toward BS and BG compared with those of basolateral efflux transporters (presumably MRP3 and MRP4) that are of lower affinity. In contrast, the BS formed intracellularly was predominantly secreted into the apical side ($C_{ap}(BS)/C_{baso}(BS)$ consistently $>1.73$), indicating a major role of an apical efflux transporter, presumably the breast cancer resistance protein, for secretion of the sulfate conjugate.

As shown in the present example, the catenary Caco-2 model is extremely useful in delineating patterns of atypical metabolism, and it provides mechanistic insight into the roles of binding, transporters, and enzymes on the transport of solutes. Saturation of conjugation and substrate inhibition of BG and BS formation (Fig. 5) will increase the intestinal bioavailability of Ba at higher doses even though a low bioavailability exists at low doses (Zhang et al., 2005, 2007a). The information obtained for Ba may be applicable to other clinically important flavonoids that display similar, extensive phase II metabolism, followed by secretion of the formed metabolites as well as to solutes that exhibit nonlinearity in transport and metabolism (Sun and Pang, 2008).
Appendix

The mass balance rate equations for Ba transport are given below. $A_{ap}$, $A_{cell}$, and $A_{baso}$ denote amounts of Ba in the apical, cellular and basolateral compartments, respectively. Because sampling volume (0.5 ml every 0.5 h from receiver side) was replaced, the removed mass must be considered in modeling by incorporating the “pulse” function into the receiver side, $\sum_{i=1}^{3} \text{pulse}(0.5/V_{ap} \times A_{ap}(t_i), t_i, 0)$ for the $A \rightarrow B$ directions, where $t_i$ is the sampling time with the exclusion of time 0 and the end of experiments, i.e., 0.5, 1.0, and 1.5 h and $A_{ap}(t_i)$ and $A_{baso}(t_i)$ denote the amount of Ba in receiver side observed at $t_i$. The amounts of the conjugate (BG or BS) was summed as $A_{BG}$ and $A_{BS}$, respectively.

Apical Compartment. For the $A \rightarrow B$ direction, where sampling takes place in the basolateral compartment,

$$\frac{dA_{ap}}{dt} = -f_{ap} (CL_{abs} + CL_{d4})(A_{ap}/V_{ap})$$

$$+ f_{cell} (CL_{int,sec} + CL_{d3})(A_{cell}/V_{cell})$$

For the $B \rightarrow A$ direction, where sampling takes place in the apical compartment,

$$\frac{dA_{ap}}{dt} = -f_{ap} (CL_{abs} + CL_{d4})(A_{ap}/V_{ap})$$

$$+ f_{cell} (CL_{int,sec} + CL_{d3})(A_{cell}/V_{cell})$$

$$- \sum_{i=1}^{3} \text{pulse}(0.5/V_{ap} \cdot A_{ap}(t_i), t_i, 0)$$

(9)

Cellular Compartment.

$$\frac{dA_{cell}}{dt} = f_{ap} (CL_{abs} + CL_{d4})(A_{ap}/V_{ap}) + f_{baso} CL_{d3} \frac{(A_{baso}/V_{baso})}{1 + \frac{K_{Ba-BG}}{f_{cell} A_{cell}/V_{cell}}}$$

$$+ f_{cell} (CL_{d3} + CL_{int,sec} + CL_{d2})(A_{cell}/V_{cell})$$

$$- \frac{V_{Ba-BG}}{max}$$

$$1 + \frac{K_{Ba-BG}}{f_{cell} A_{cell}/V_{cell}} + f_{cell} A_{cell}/V_{cell}$$

$$- \frac{V_{Ba-BS}}{max}$$

$$1 + \frac{K_{Ba-BS}}{f_{cell} A_{cell}/V_{cell}} + f_{cell} A_{cell}/V_{cell}$$

(10)
Basolateral Compartment. For the A→B direction, where sampling place takes in the basolateral compartment,
\[
\frac{dA_{\text{baso}}}{dt} = f_{\text{cell}}C_{L42}(A_{\text{cell}}/V_{\text{cell}}) - f_{\text{baso}}C_{L41}(A_{\text{baso}}/V_{\text{baso}}) - \sum_{i=1}^{3} \text{pulses}(0.5V_{\text{baso}}A_{\text{baso}}(t_i, t_i, 0)) \tag{11}
\]
For the B→A direction, where sampling occurs in the apical compartment,
\[
\frac{dA_{\text{ap}}}{dt} = f_{\text{cell}}C_{L42}(A_{\text{cell}}/V_{\text{cell}}) - f_{\text{baso}}C_{L41}(A_{\text{baso}}/V_{\text{baso}}) \tag{12}
\]
References

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