Glyceollin Transport, Metabolism, and Effects on P-Glycoprotein Function in Caco-2 Cells

Chukwuemezie Chimezie,¹ Adina C. Ewing,¹ Syeda S. Quadri,² Richard B. Cole,^{2,3} Stephen M. Boué,⁴ Christopher F. Omari,¹ Melyssa Bratton,¹ Elena Glotser,¹ Elena Skripnikova,¹ Ian Townley,¹ and Robert E. Stratford, Jr.¹

¹Department of Basic Pharmaceutical Sciences, College of Pharmacy, Xavier University of Louisiana, New Orleans, Louisiana, USA.

²Department of Chemistry, University of New Orleans, New Orleans, Louisiana, USA.

³Laboratory of Organic and Biological Structural Chemistry, Université Pierre et Marie Curie (Paris 6), Paris, France.

⁴Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, Louisiana, USA.

ABSTRACT Glyceollins are phytoalexins produced in soybeans from their isoflavone precursor daidzein. Their impressive anticancer and glucose normalization effects in rodents have generated interest in their therapeutic potential. The aim of the present studies was to begin to understand glyceollin intestinal transport and metabolism, and their potential effects on P-glycoprotein (Pgp) in Caco-2 cells. At 10 and 25 μ M, glyceollin permeability was $2.4 \pm 0.16 \times 10^{-4}$ cm/sec and $2.1 \pm 0.15 \times 10^{-4}$ cm/sec, respectively, in the absorptive direction. Basolateral to apical permeability at 25 μ M was $1.6 \pm 0.10 \times 10^{-4}$ cm/sec. Results suggest high absorption potential of glyceollin by a passive-diffusion-dominated mechanism. A sulfate conjugate at the phenolic hydroxyl position was observed following exposure to Caco-2 cells. In contrast to verapamil inhibition of the net secretory permeability of rhodamine 123 (R123) and its enhancement of calcein AM uptake into Caco-2 cells, neither glyceollin nor genistein inhibited Pgp (MDR1; *ABCB1*) up to 300 μ M. There was no significant change in MDR1 mRNA expression, Pgp protein expression, or R123 transport in cells exposed to glyceollin or genistein for 24 h up to 100 μ M. Collectively, these results suggest that glyceollin has the potential to be well absorbed, but that, similar to the isoflavone genistein, its absorption may be reduced substantially by intestinal metabolism; further, they indicate that glyceollin does not appear to alter Pgp function in Caco-2 cells.

KEY WORDS: • diet • flavonoids • genistein • glyceollin • induction of Pgp • inhibition of Pgp • isoflavone • phytoestrogen

INTRODUCTION

FLAVONES AND ISOFLAVONES are polyphenolic compounds that occur naturally in many plants; they are produced in abundance in soybeans. Recognition that their antioxidant properties may have beneficial effects in prevention of diseases associated with oxidative stress, such as cancer, diabetes, and cardiovascular, neurodegenerative, and inflammatory diseases, has created widespread interest in their intentional use to promote human health.¹ Awareness of their poor systemic bioavailability has led to investigations of their mechanisms of intestinal transport and metabolic fate.^{2,3} Genistein, an isoflavone found in soybeans, is a noteworthy example. Detailed investigations of its intestinal disposition have revealed an integration of pre- and post-

absorptive metabolism and carrier-mediated transport of metabolites that severely limit its systemic bioavailability following oral ingestion.⁴

Soybeans exposed to stressful conditions, such as fungal or bacterial pathogens, or UV light, produce compounds known as phytoalexins from their flavone and isoflavone precursors. Specifically, three glyceollin isomers (Fig. 1), which are derived from the isoflavone daidzein, have been identified in soybeans exposed to *Aspergillus sojae*.⁵ Initial studies of their biological effects indicated that glyceollins were estrogen receptor antagonists,⁶ with isomer I being the most potent⁷; glyceollins exhibit antiproliferative effects in estrogen-dependent breast cancer cells⁶ and they inhibit cell proliferation in mouse xenograft breast cancer models.⁸ More recently, glyceollins have been shown to improve glucose homeostasis in response to a glucose challenge in a diabetic mouse model⁹ and in a prediabetic rat model of type 2 diabetes.¹⁰ Overall, these pharmacologic effects have generated interest in the use of glyceollin-enriched soy as a nutri-pharmaceutical. The Boué *et al.* report¹⁰ in the

Manuscript received 9 May 2013. Revision accepted 24 October 2013.

Address correspondence to: Robert E. Stratford, Jr., PhD, Department of Basic Pharmaceutical Sciences, College of Pharmacy, Xavier University of Louisiana, 1 Drexel Drive, New Orleans, LA 70125, USA, E-mail: rstratfo@xula.edu

FIG. 1. Structures of glyceollin isomers isolated from soybeans exposed to *Aspergillus sojae* and used in the present studies.

prediabetic rat model also included the first reported measure of glyceollin systemic exposure following oral administration; however, since intravenous administration was not included, it was not possible to measure bioavailability. In a subsequent study, metabolism profiling of the plasma samples collected in the Boué *et al.* study revealed production of several metabolites, including oxidative and direct sulfate conjugation of the phenolic hydroxyl group, suggesting that first-pass intestinal and hepatic metabolism may present a significant barrier to systemic absorption of glyceollin.¹¹ Much like the work done to characterize the causes of the low oral bioavailability of genistein,^{2,3,12} the aim of the present investigation was to begin to understand the intestinal transport and metabolism of glyceollin. For these initial investigations, we used Caco-2 cells. In addition, we sought to determine the potential for glyceollin to inhibit and/or induce P-glycoprotein (Pgp) function in Caco-2 cells. Given its important protective role in limiting the absorption of xenobiotics, we wanted to determine the potential for glyceollin to alter Pgp function.

MATERIALS AND METHODS

Materials

A mixture of glyceollins I, II, and III (Fig. 1) was isolated using a procedure developed at the Southern Regional Research Center (ARS, USDA, New Orleans, LA, USA)^{6,8} and used in recent studies to evaluate glyceollin effects on glucose disposition in fat cells¹⁰ and metabolism in rat plasma.¹¹ UV-Vis spectrophotometry at 285 nm allowed an estimation of the proportions of the three isomers used in all experiments: glyceollin I (68%), glyceollin II (21%), and glyceollin III (11%). Genistein was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Indomethacin was purchased from MP Biomedicals, LLC (Solon, OH, USA). Calcein AM (CAM) was purchased from

AnaSpec, Inc. (Fremont, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Caco-2 cells were obtained from ATCC (Manassas, VA, USA) at passage 27. Cells were maintained in stock cultures in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (HyClone; Thermo Scientific, Waltham, MA, USA), 1% MEM nonessential amino acids (100×; Mediatech, Inc., Manassas, VA, USA), 1 mM sodium pyruvate (Mediatech), 2 mM L-glutamine (Mediatech), and 1% antibiotic/antimycotic solution (Mediatech) at 37°C in a humidified incubator with 5% CO₂. For 96-well plate (cell viability) and 24-well plate (CAM Pgp inhibition) assays, cells were seeded at 1.3 and 1.9 × 10⁵ cells/mL, respectively. For RT-qPCR and Western blot analyses, cells were seeded onto 10-cm² plates at a density of 2 × 10⁵ cells/mL. Cells were seeded onto collagen-coated Transwell-COL[®] permeable supports (12 mm, 0.4-μm PTFE) at a seeding density of 1.2 × 10⁵ cells/mL and cultured for 2.5–3.5 weeks before using in an assay [rhodamine 123 (R123) or glyceollin transport and metabolism]. For all culture formats, cells were fed three times per week and used between passages 30 and 55.

Glyceollin transport and metabolism

A 50 mM stock solution of the isomeric glyceollin mixture was prepared in equal volumes of DMSO and ethanol, and subsequently diluted to either 10 μM or 25 μM in pH 7.4 transport buffer (25 mM HEPES, 5 mM glucose, 145 mM NaCl, 3 mM CaCl₂, and 0.5 mM MgCl₂). Prior to glyceollin exposure, cells were washed twice with transport buffer and incubated for 30 min at 37°C, and their transepithelial electrical resistance (TEER) was measured (EVOM² meter; World Precision Instruments, Sarasota, FL, USA). Filters with TEER values < 200 ohms · cm² were not included in the analysis of glyceollin permeability. Apical side volume was 0.5 mL and basolateral side volume was 1.5 mL. Following addition of glyceollin to the donor compartment, filters were placed on an orbital shaker (150 rpm) set at 37°C. Receiver compartment samples (0.4 mL) were taken every 30 min with volume replacement for 2 h for analysis of cumulative glyceollin mass transport in the apical to basolateral (AB) and basolateral to apical (BA) directions. At the conclusion of the incubation, donor samples (50 μL) were taken to support determination of mass balance. All samples were stored at –70°C until time of analysis. At the end of the 2-h incubation, lucifer yellow (10 μM donor concentration, AB direction) permeability was determined from a 60-min incubation to evaluate monolayer integrity. Filters with lucifer yellow permeability > 3 × 10^{–6} cm/sec were not included in the analysis of glyceollin permeability.

Measurement of glyceollin concentration in donor and receiver compartments was achieved using HPLC–tandem mass spectrometry (LC-MS/MS). An Agilent 1200 series LC system (Agilent, Santa Clara, CA, USA) was coupled with a 3200 QTrap triple quadrupole mass spectrometer

(Applied Biosystems/MDS SCIEX, Foster City, CA, USA). Separation was performed on an Agilent Eclipse-XDB C18 column (4.6×150 mm ID, 5 μ m) at 25°C at 0.5 mL/min. Mobile phase A was water with 0.1% formic acid; mobile phase B was acetonitrile with 0.1% formic acid. The gradient for quantification was 0–5 min 40% B to 100% B. Flow rate was 0.5 mL/min. Mass spectrometry employed electrospray ionization in the negative mode; quantification involved monitoring of the m/z 337→ m/z 148 transition in selected reaction monitoring mode, as described previously.¹¹ Standards (500–20,000 nM) were prepared fresh for each run. Daidzein (1000 nM) was used as an internal standard.

Identification of glyceollin-derived metabolites followed a 3-h incubation of a 25 μ M glyceollin solution in transport buffer (pH 7.4) to both sides of cells grown on Transwell® inserts for 16–23 days. Cells were subsequently exposed to acetonitrile for 5 min and centrifuged, and the supernatant was saved for analysis. Buffer was collected from both sides, pooled, and treated with a 1:2 ratio of buffer:acetonitrile. The acetonitrile phase was separated from the aqueous phase using ammonium acetate followed by centrifugation. Samples were stored at –70°C until time of analysis. Metabolite identification was obtained from direct infusion MS/MS negative electrospray with precursor ion scans of m/z 148 for screening in a first step followed by a second step of product ion scans of each of the precursors detected in the first step. This approach was used previously for the identification of metabolites in rat plasma following a 90 mg/kg oral dose of the glyceollin isomeric mixture.¹¹

Pgp inhibition

R123 is a fluorescent dye that is a Pgp substrate.¹³ The AB and BA directional transport of 5 μ M R123 in the absence versus presence of 100 μ M glyceollin, genistein, or verapamil was evaluated. At this concentration, verapamil was shown to inhibit the transport of digoxin, a prototypical Pgp substrate.^{14,15} Following two wash steps with 37°C transport buffer and preincubation with 100 μ M compound on both sides of the cell barrier for 30 min at 37°C, R123 was added to the donor compartment and the 12-well plate was incubated for 2 h in an orbital shaker set at 150 rpm and 37°C. As with glyceollin transport, receiver compartment samples were taken every 30 min. Initial and final donor samples were taken for determination of mass balance (which averaged 100±4.3%) and calculation of R123 permeability coefficient based on the following equation, where P is permeability in cm/sec, dm/dt is flux (the slope of the amount transported vs. time), A is the Transwell support surface area (1.12 cm²), and C is the average of the initial and final donor concentrations (nmoles/mL).

$$P = \frac{dm/dt}{(A \cdot C)}$$

R123 concentrations were obtained by reference to the fluorescence of freshly prepared standards over the con-

centration range 0.05–2 nmoles/mL and fluorescence was measured in a 96-well plate reader (Biotek Synergy 4, Winooski, VT, USA) with excitation wavelength of 495 nm and emission wavelength of 520 nm. Correlation coefficients (r^2) for the standard curves were >0.99.

Uptake of CAM, another fluorescence-based probe Pgp substrate,¹⁶ into cells grown on 24-well plates was also used to determine whether glyceollin inhibited Pgp function. This assay has higher throughput compared to the R123 transport assay (3-day culture vs. 3-week culture); thus, it more readily permits analysis of concentration dependency of potential Pgp inhibition. Following a 30-min preincubation in the presence of a test compound, Caco-2 cells between 75–95% confluency were exposed to 0.5 μ M CAM for 20 min at 37°C. The concentration range of the three compounds evaluated—glyceollin, genistein, and verapamil—was 1–300 μ M. Indomethacin, a multidrug resistance protein 2 inhibitor (MRP2), at a final concentration of 500 μ M was included in the incubation to promote retention of fluorescent calcein, which has been reported to be an MRP2 substrate¹⁷ (data not shown). At the conclusion of the incubation, cells were washed three times in ice-cold transport buffer and then lysed in 0.1% triton X-100 (15 min at 37°C). Subsequently, calcein fluorescence was measured in a 96-well plate reader at 485 nm/530 nm (excitation/emission wavelengths). Measured fluorescence was normalized to protein content based on the BCA protein assay (Thermo Scientific). Bovine serum albumin was used as a reference protein and standards were prepared over the concentration range 50–1600 μ g/mL (r^2 >0.99 for the absorbance vs. albumin standard concentration relationship).

Cell viability

The effect of exposure time and concentration of glyceollin and genistein on Caco-2 cell viability was measured using the Alamar Blue assay (Thermo Scientific). Upon reaching 80–90% confluency, cells grown in 96-well plates were exposed to Alamar Blue reagent (2 h at 37°C) and absorbance was measured at 560 nm in a 96-well plate reader. Subsequently, cells were exposed for either 24 or 72 h to 1, 10, or 100 μ M test compound. Various controls were also evaluated. These were 1% sodium dodecylsulfate as a positive control for cell death, solvent control (0.1% DMSO/0.1% ethanol), and a solvent blank (growth medium only). Following exposure for a specified time (24 or 72 h), the 2 h of Alamar Blue incubation was repeated. Results were expressed as the ratio of postexposure/pre-exposure absorbance and normalized to the solvent blank.

Alteration of MDRI mRNA expression

Caco-2 cells were exposed to solvent (negative control, 0.1% DMSO/0.1% ethanol), 1, 10, or 100 μ M glyceollin or genistein for 24 h at 80–90% confluency. There were three wells for each treatment condition and each condition was repeated a total of three times with cells from consecutive passage numbers 33–35. Total RNA samples were collected using RNeasy Plus kits (Qiagen, Valencia, CA, USA).

Samples were stored at -70°C until time of analysis. The concentration of total RNA in the samples was measured by an ND-1000 NanoDrop[®] spectrophotometer (Thermo Scientific). The A260/A280 purity of all samples was in the range of 2.0–2.1, and the A260/A230 ratio was 1.8–2.3. The integrity of all samples was confirmed by the Experion Automated Electrophoresis System (BioRad, Inc., Hercules, CA, USA), with the 18S/28S ratio being higher than 1.6. Five micrograms of each RNA sample was reverse transcribed in a 20 μL reaction volume by a commercial first-strand cDNA synthesis kit (SABiosciences-Qiagen). MDR1 primer and those of three housekeeping genes—beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase (HPRT1), and ribosomal protein L13A (RPL13A)—were purchased from SABiosciences-Qiagen, as well as RT SYBR Green Fluor qPCR Mastermix. Two microliters of cDNA was used in 25 μL of qPCR mix using a standard two-step temperature protocol and a Bio-Rad iQ5 Thermocycler. Each 96-well plate contained nontemplate control, calibrator sample for each gene, and all experimental samples, which were each run in duplicate. Melting curve analysis was performed for each PCR plate. Delta delta C_t method was used to calculate the fold change in MDR1 gene expression.¹⁸

Alteration of Pgp expression by Western blot analysis

Caco-2 cells were seeded in 10-cm² plates at a density of 60–70% confluence. The cells were treated with the indicated test compounds/concentrations for 24 h. The media were then removed and the cells were scraped into 1 mL of phosphate-buffered saline (PBS) plus 3 mM EDTA. Cell suspensions were spun for 5 min at 2000 g and the supernatant was aspirated. Pellets were lysed by vortexing in 200 μL of M-PER mammalian protein extraction buffer (Pierce; cat. No. 78501) containing protease and phosphatase inhibitors (Sigma; cat. Nos. P1860-1ML, P0044, and P5726). The samples were then spun in a microcentrifuge for 5 min at 12,000 g and the supernatants were collected. Protein concentrations were determined using a nanodrop spectrophotometer (Thermo Life Sciences) and 100 μg of total protein was loaded into each well and run on a 4–12% polyacrylamide gel (Life Technologies). Subsequently, gels were blotted onto nitrocellulose using the iblot transfer system (Life Technologies). Blots were blocked for 1 h at room temperature in 1 $\times$ PBST (PBS with tween 20; Cell Signaling, Danvers, MA, USA) containing 5% nonfat milk, washed in 1 $\times$ PBST, and incubated overnight at 4 $^{\circ}\text{C}$ in 5 mL of primary antibody [MDR1 (G1); Santa Cruz Biotechnology, Dallas, TX, USA] at a 1:500 dilution in 5% BSA/PBST (Sigma; cat. No. A7906-1KG). Blots were then washed in 1 $\times$ PBST and incubated with fluorescently labeled secondary antibodies (Alexa-Fluor 488; Life Technologies) for 60 min at room temperature. The blots were then washed in 1 $\times$ PBST and scanned using a Versadoc 4000 scanner (BioRad). Bands were quantified using Quantity One software (BioRad) and normalized to bands corresponding to the housekeeping Rho-GDI protein. Three independent samples were prepared for each treatment condition.

Alteration of Pgp function

Caco-2 cells grown on Transwell[®] permeable supports for 24 days were exposed to 1, 10, or 100 μM glyceollin or genistein for 24 h to determine whether such exposure altered Pgp activity. Potential effects on Pgp activity were measured using the R123 assay described earlier with comparison to R123 permeability in control incubations (DMSO/ethanol co-solvent only). Notably, R123 permeability was measured in the BA direction only on the basis of the greater sensitivity of verapamil-mediated alteration of R123 permeability in this direction observed in the Pgp inhibition assay (Fig. 3). This approach has been used by others to measure alteration of Pgp activity in Caco-2 cells.¹⁹

Data analysis

Values are presented as mean $\pm$ standard error of the mean. The EC_{50} for the verapamil-mediated increase in CAM uptake was calculated using Phoenix WinNonlin 6.1 (Pharsight Corporation, Mountain View, CA, USA). Statistical analyses were conducted using IBM SPSS Statistics 19 (IBM Corporation, Armonk, NY, USA).

RESULTS

Glyceollin transport was evaluated in both the absorptive (AB) and secretory (BA) directions across Caco-2 cells cultured for 18 days on Transwell[®] filters at an initial donor concentration of 25 μM . Figure 2 demonstrates that glyceollin transport in both directions was rapid, approaching equilibrium well within the course of the 120-min incubation. Permeability coefficients, estimated using a first-order kinetic process, were $2.1 \pm 0.06 \times 10^{-4}$ cm/sec (six filters) and $1.6 \pm 0.06 \times 10^{-4}$ cm/sec (three filters) in the AB and BA directions, respectively. The calculated mass balance averaged $129 \pm 1.9\%$ (AB direction) and $143 \pm 1.9\%$ (BA

FIG. 2. Transport of glyceollin across Caco-2 cells. Data are presented as the average (mean $\pm$ SEM) of cumulative nmoles of glyceollin transported across monolayers following exposure to 25 μM glyceollin on the apical side (open squares, six filters) or basal side (closed triangles, three filters). SEM, standard error of the mean.

FIG. 3. Effect of verapamil, glyceollin, and genistein on R123 permeability in Caco-2 cells. Results are presented as the mean $\pm$ SEM of R123 permeability coefficients measured from a 5 μ M R123 donor concentration in the apical to basolateral (open bars) or basolateral to apical (BA, closed bars) direction in the absence versus presence of 100 μ M of the indicated compound. Both sides of 17–22-day postseeding Caco-2 cells grown on Transwell inserts were exposed to solvent control or compound during a 120-min incubation. Results are from three to four separate experiments, with $n=3$ filters/experiment for each transport direction. *Statistically different from the no-inhibitor control by a one-sided Student's t -test, $P<.01$. R123, rhodamine 123.

direction). At the conclusion of the 2-h incubation, the AB permeability of lucifer yellow at a 10 μ M donor concentration was determined from a 60-min incubation. Permeability coefficients averaged $2.7 \pm 0.02 \times 10^{-6}$ cm/sec and $2.3 \pm 0.14 \times 10^{-6}$ cm/sec, respectively, for filters subjected to glyceollin transport in the AB and BA transport directions, or roughly 1% of the glyceollin permeability coefficients. Glyceollin permeability was also evaluated in the AB direction at a 10 μ M donor concentration. Permeability at this concentration was $2.4 \pm 0.16 \times 10^{-4}$ cm/sec (three filters).

Metabolism profiling of acetonitrile extracts of transport buffer and cells obtained following a 3-h incubation of 25 μ M glyceollin with Caco-2 cells grown on Transwell® filters identified two metabolites: one with 16 mass units greater than glyceollin, presumably a hydroxylated metabolite, and the second of 80 mass units greater than glyceollin. Both of these metabolites were observed in rat plasma following a 90 mg/kg oral dose of glyceollin, with the second metabolite identified as the sulfate conjugate at the phenolic hydroxyl position.¹¹ These two metabolites, with potential hydroxylation sites of the +16 metabolite, are shown in Figure 4.

In addition to evaluation of the transport and metabolism of glyceollin by Caco-2 cells, we examined whether this phytoalexin was an inhibitor of Pgp function in these cells. As

shown in Figure 4, exposure to 100 μ M verapamil, an inhibitor of Pgp,²⁰ abolished R123 efflux asymmetry through a reduction in BA permeability from $8.9 \pm 0.68 \times 10^{-6}$ cm/sec to $3.5 \pm 0.31 \times 10^{-6}$ cm/sec. This decrease resulted in a reduction in R123 BA/AB permeability in the presence of verapamil (Table 1). In contrast, exposure to 100 μ M glyceollin did not alter R123 directional permeability. Exposure to 100 μ M genistein slightly increased R123 permeability in the BA direction from $7.2 \pm 0.29 \times 10^{-6}$ cm/sec to $9.1 \pm 0.27 \times 10^{-6}$ cm/sec. This increase translated to an increase in the R123 BA/AB permeability ratio (Table 1). To evaluate concentration effects on Pgp function in Caco-2 cells without having to culture cells for 3 weeks, an assay that measured inhibition of CAM efflux by Pgp in cells grown on plastic to 90% confluency in 3 days was developed. Results from this assay are summarized in Figure 5 for glyceollin, genistein, and the positive control verapamil. There was no evidence of Pgp inhibition by glyceollin or genistein over the concentration range 1–300 μ M. In contrast, the EC_{50} estimated for inhibition of CAM efflux by verapamil was 16.9 μ M (coefficient of variation = 23%), which is similar to 14 μ M reported previously for inhibition of CAM efflux in MDR1-transfected MDCK cells.²⁰

The standard set of experiments, that is, MDR1-derived mRNA expression via RT-qPCR, measurement of Pgp

TABLE 1. RHODAMINE 123 BASOLATERAL TO APICAL/APICAL TO BASOLATERAL PERMEABILITY RATIOS IN THE ABSENCE (CONTROL) VERSUS PRESENCE OF 100 μ M OF A GIVEN COMPOUND

Compound	R123 BA/AB permeability ratio	
	Absent	Present
Glyceollin	3.1 $\pm$ 1.27 (3)	2.7 $\pm$ 0.96 (3)
Genistein	2.5 $\pm$ 0.26 (3)	3.2 $\pm$ 0.21 (3)*
Verapamil	3.1 $\pm$ 0.65 (4)	1.2 $\pm$ 0.21 (4)*

Values represent the mean $\pm$ standard error of the mean. Number in parenthesis is the number of experiments, with three filters per experiment in each transport direction.

*Significantly different from the solvent control: $P < .01$, according to a one-sided Student's *t*-test.

AB, apical to basolateral; BA, basolateral to apical; R123, rhodamine 123.

expression via immunoblotting, and finally determination of functional effects on transport, were designed to evaluate whether glyceollin exposure altered Pgp expression and activity in Caco-2 cells. This *in vitro* model of the small intestine has been used to evaluate the regulation of Pgp via nuclear receptor activation.²¹ To support planning and interpretation of these studies, the effects of exposure time and concentration on cell viability were first evaluated. As shown in Figure 6, exposure to 100 μ M glyceollin for 72 h resulted in a significant decline in cell viability. Exposure to genistein resulted in both time- and concentration-dependent loss of Caco-2 cell viability. Based on these viability results, the effect of glyceollin and genistein on Pgp expression was evaluated following 24 h of exposure at three concentrations: 1, 10, and 100 μ M. As shown in Figure 7, MDR1-derived mRNA expression and Pgp protein expression were unaltered following 24 h of exposure to

FIG. 5. Effect of verapamil, glyceollin, and genistein on calcein AM (CAM) uptake into Caco-2 cells. Data are presented as mean $\pm$ SEM of the percentage change (relative to 1 μ M of a given compound) in 0.5 μ M CAM uptake into Caco-2 cells following a 20-min incubation with the indicated concentrations of the various compounds. Cells were seeded into 24-well culture plates at 1.9×10^5 cells/mL and used at 75–95% confluency and 24 h after a media change. Results are from three separate experiments with $n = 4$ wells/concentration in each experiment.

FIG. 6. Effect of glyceollin (Gly) and genistein (Gen) on Caco-2 cell viability. Data are presented as mean $\pm$ SEM ($n = 3$ –4 experiments with four wells/condition/experiment) of cell viability relative to no compound/no solvent (DMSO Blank) control. Open bars represent 24 h of exposure and closed bars represent 72 h of exposure of Caco-2 cells to the indicated condition/concentration. Cells were seeded into 96-well culture plates at 1.3×10^5 cells/mL and exposed when they had reached 80–90% confluency. SDS, sodium dodecylsulfate. *Significantly different from compound blank ($P < .01$) by Tukey's test for multiple comparisons.

glyceollin or genistein up to 100 μ M. Figure 8 demonstrates that 24 h of glyceollin and genistein exposure up to 100 μ M had no functional effect on Pgp activity measured by evaluation of R123 BA permeability.

DISCUSSION

Given their commonplace occurrence in the diet and potential health benefits, several studies have characterized the intestinal disposition of polyphenolic flavonoids,^{22–24} including soy-derived flavones and isoflavones.^{12,25} The results presented herein are the first to report on the intestinal transport and metabolism of glyceollin, a phytoalexin produced in soy from its isoflavone precursor daidzein. These initial studies were conducted in Caco-2 cells, a well-established model of the human small intestine. Permeability of glyceollin was very high, being comparable to other compounds with 100% absorption.²⁶ There was no evidence of asymmetric transport and permeability was constant over the concentration range 10–25 μ M. Given these observations, we conclude that passive diffusion is the dominant mechanism relevant to the intestinal transport of glyceollin.

Based on reported plasma concentrations of glyceollin in rats following oral administration of a 30 or 90 mg/kg

FIG. 7. Effect of glyceollin and genistein on MDR1 mRNA expression (*top*) or Pgp protein expression (*bottom*) relative to solvent control. For each experiment, cells were seeded into 10-cm² culture plates at 2×10^5 cells/well in triplicate and exposed to compound after they had reached 80–90% confluency. Data are presented as mean $\pm$ SEM ($n=3$ experiments) of fold-change following 24-h exposure to glyceollin (open bars) or genistein (closed bars) at the indicated concentrations. Pgp, P-glycoprotein.

dose,¹⁰ estimated exposure (expressed as area under the curve [AUC] from 0 to 240 min) of glyceollin was 20,340 ng·min/mL and 96,570 ng·min/mL, respectively. These exposures translate to apparent oral clearance values of 1475 and 932 mL/(min·kg⁻¹), both of which are well in excess of portal vein plasma flow [23 mL/(min·kg⁻¹)] in a rat²⁷ and suggest that glyceollin bioavailability is much less than unity. In connection with the observed high permeability of glyceollin, this analysis suggests that intestinal and hepatic metabolism may contribute to its low oral bioavailability. These assertions of substantive intestinal and hepatic first-pass metabolism of glyceollin resulting in its low oral bioavailability are in agreement with observations of other dietary flavonoids.^{2,3,28} In a separate study¹¹ we reported that the sulfate conjugate of glyceollin was a major metabolite in the same plasma samples used for quantitative analysis to measure glyceollin exposure,¹⁰ and that, while several samples consistently possessed the sulfate conjugate, not all of them contained intact glyceollin. In the

FIG. 8. Influence of prior exposure to glyceollin and genistein on 5 μ M R123 permeability in the BA transport direction in Caco-2 cells. Both sides of 23-day postseeding Caco-2 cells grown on Transwell inserts were exposed to solvent control or compound for 24 h at the indicated concentrations (glyceollin, open bars; genistein, closed bars). Data are presented as mean $\pm$ SEM ($N=6$ filters).

present study, incubation of 25 μ M glyceollin for 3 h with Caco-2 cells grown on Transwell® filters for 3 weeks also resulted in production of the sulfate conjugate. Altogether, it would appear that glyceollin metabolism in the intestine proper is a major barrier to its systemic absorption. Certainly, future studies will be needed to support these claims and, if they are borne out, to understand the role of efflux transporters in the intestine relative to the disposition of intestinally derived metabolites, as has been done with genistein.^{29,30}

In addition to evaluation of the intestinal absorption of glyceollin, we wanted to begin characterization of its possible effects on intestinal efflux transporters, including the potential for inhibition as well as alteration of expression via influence on transcriptional or post-transcriptional processes. Pgp inhibition is a well-known cause of drug interactions leading to enhanced exposure of substrates co-administered with an inhibitor.^{31,32} In two separate assay platforms (21-day culture Transwell® inserts with measurement of R123 vectorial transport and 3-day culture on plastic to measure calcein-AM uptake), we observed that, in contrast to the positive control verapamil, glyceollin did not inhibit Pgp function up to 300 μ M. With respect to genistein, there was a modest increase in R123 permeability in the BA direction that translated to a statistically significant increase in R123 BA/AB permeability ratio, suggesting that genistein may have enhanced Pgp activity. However, in the CAM uptake assay, there was no change in calcein uptake up to 300 μ M. The literature is mixed regarding genistein's effects on Pgp function. Versantvoort *et al.* concluded that 200 μ M genistein did not alter daunorubicin accumulation in Pgp competent human small cell lung cancer cells.³³ In contrast, R123 accumulation in MDR1-transfected human breast cancer cells was enhanced in the presence of 200 μ M genistein, suggesting that genistein inhibited Pgp at this concentration.³⁴ Given the well-recognized influence of the

cell membrane environment on Pgp activity³⁵ and the use of different cells in these studies, such differences are not unexpected. Similar to our R123 findings, a recent study by Okura *et al.*³⁶ demonstrated that 100 μ M genistein increased R123 efflux in vinblastine-resistant LS180 colon carcinoma cells. However, in this same study, 100 μ M genistein did not alter R123 uptake, a result that is similar to our results with CAM, which are also based on an uptake endpoint. Given the mixed results observed with genistein in these two studies using intestinal cell lines, it is not possible to conclude its effects on intestinal Pgp based on *in vitro* analyses, let alone genistein's ability to influence the intestinal absorption of Pgp substrates *in vivo*. Importantly, genistein interacts with another member of the ABC transporter family, Cystic Fibrosis Transmembrane Regulator (CFTR; *ABCC7*), to potentiate function at low concentrations and inhibit CFTR at higher concentrations.³⁷ The possibility exists, therefore, that genistein interacts with Pgp in a similar concentration-dependent manner and this is the cause of the mixed results observed. Finally, with respect to our findings at 100 μ M genistein in the Caco-2 vectorial transport model, this same concentration has been shown to reduce cimetidine B to A transport in Caco-2 cells.³⁸ The authors suggested that genistein inhibited Pgp-mediated efflux of cimetidine. However, since cimetidine is a substrate for multiple transporters, and the effect of the known Pgp inhibitor verapamil was modest in this study and above a recently reported efflux ratio threshold of ≤ 2 to conclude whether a compound is a Pgp substrate,³⁹ it is quite possible that genistein was inhibiting another transporter(s). Notably, our results with verapamil show that it reduced the R123 BA/AB ratio below 2. Thus, we do not see our results with R123 being at odds with the cimetidine/genistein work. Our results with glyceollin, which are the first to be reported for this phytochemical, suggest that it would not be expected to inhibit Pgp activity in the small intestine; however, given the complexity of drug transport analysis as discussed earlier with genistein, additional experiments are needed to confirm these initial findings.

Pgp serves a protective role in the small intestine by limiting the uptake of xenobiotics that are consumed or that can be formed by resident gut microorganisms.⁴⁰ Increasing evidence links reduced expression and function of Pgp with the pathogenesis of irritable bowel disease,^{41,42} Crohn's disease,⁴³ and ulcerative colitis.⁴⁴ A recent report demonstrated that *Lactobacillus rhamnosis* and *Lactobacillus acidophilus*, two commensal bacterial species commonly found in probiotic foods, increased Pgp expression and function in Caco-2 cells.⁴⁵ In a similar vein, we were interested in determining whether glyceollin, which conceivably could be consumed as a glyceollin-enriched soy product, would have a similar effect on Pgp expression and function in Caco-2 cells. To evaluate this potential effect, we measured MDR1-derived mRNA using standard RT-qPCR and Pgp protein using Western blotting to monitor for possible changes in Pgp expression in Caco-2 cells following 24 h of exposure to 1, 10, and 100 μ M glyceollin. There were no changes in either MDR1-derived mRNA or Pgp at

these concentrations (Fig. 7). We verified absence of an effect of glyceollin on Pgp function by evaluating the BA transport of R123 in 21-day Transwell®-cultured cells. There was no statistically significant difference in R123 permeability among the three glyceollin concentrations and relative to the control (Fig. 8); thus, we conclude that exposure to glyceollin up to 100 μ M for 24 h does not alter Pgp expression and function in Caco-2 cells. To date, only one other study has evaluated glyceollin's ability to influence transporter function through alteration of gene expression.¹⁰ In that study, 3-h exposure of 3T3-L1 adipocytes to 5 μ M glyceollin increased GLUT1 and GLUT4 (*SLC2A1/2A4*, respectively) mRNA expression and GLUT4 protein levels. These alterations correlated with increased adipocyte accumulation of glucose, suggesting a possible cause-effect relationship. Apart from the different exposure times and cell lines used in that study and ours, known mechanisms that regulate GLUT4 and MDR1 gene expression are quite different.^{46,47} The potential for glyceollin to alter transporter function through gene regulation in cancer and noncancer cells in connection with its anticancer and antidiabetic properties, and its potential to influence expression of xenobiotic metabolism and transport proteins in liver and intestine, will likely be fruitful areas of future investigation. Evaluation of genistein under identical conditions also did not alter MDR1 mRNA or Pgp protein levels in Caco-2 cells. Our results with genistein are similar to those reported by Okura *et al.*,³⁶ who did not see an increase in MDR1 mRNA or Pgp protein in vinblastine-resistant LS180 cells following 48 h exposure to 100 μ M genistein. The lack of effects on mRNA and protein translated to no-observable effect of 24-h exposure to 1, 10, and 100 μ M genistein on R123 BA permeability (Fig. 8).

In conclusion, we have determined that glyceollin transport across Caco-2 cells is rapid and appears to be dominated by a passive diffusion mechanism. Sulfation of the phenolic hydroxyl group of glyceollin was observed in Caco-2 cells. This conjugate was a principal metabolite in rat plasma as well.¹¹ High permeability of glyceollin placed in the context of its estimated high oral clearance in rats suggests that first-pass intestinal metabolism is a substantial barrier, perhaps the principal barrier, to glyceollin systemic absorption following oral administration. In this regard, glyceollin is similar to other dietary polyphenols, including the soy isoflavone genistein.²⁵ Glyceollin does not appear to inhibit Pgp function, nor alter the function of this efflux transporter in Caco-2 cells as a consequence of prior exposure; thus, intestinal exposure to glyceollin would not be expected to alter the protective function of Pgp in the intestine, nor interfere with the absorption of drugs that are Pgp substrates.

ACKNOWLEDGMENTS

This work was funded by a Louisiana Cancer Research Consortium Seed Grant (OSP-7011-003) and in part by Grant Number 8G12MD007595-04 from the National Institutes on Minority Health and Health Disparities (NIMHD), National

Institutes of Health (NIH), and Department of Health and Human Services (DHHS). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIMHD or NIH.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

- Martel F, Monteiro R, Calhau C: Effect of polyphenols on the intestinal and placental transport of some bioactive compounds. *Nutr Res Rev* 2010;23:47–64.
- Liu Y, Hu M: Absorption and metabolism of flavonoids in the caco-2 cell culture model and in a perfused rat intestinal model. *Drug Metab Dispos* 2002;30:370–377.
- Chen J, Lin H, Hu M: Metabolism of flavonoids via enteric recycling: Role of intestinal disposition. *J Pharmacol Exp Therap* 2003;304:1228–1235.
- Zhu W, Xu H, Wang SWJ, Hu M: Breast cancer resistance protein (BCRP) and sulfotransferase contribute significantly to the disposition of genistein in the mouse intestine. *AAPS J* 2010;12:525–536.
- Ayers AR, Ebel J, Finelli F, Berger N, Albersheim P: Host-pathogen interactions. IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. *Plant Physiol* 1976;57:751–759.
- Burrow ME, Boue SM, Collins-Burrow BM, Melnik LI, Duong BN, Carter-Wientjes CH, Li S, Wiese TE, Cleveland TE, McLachlan JA: Phytochemical glyceollins, isolated from soy, mediate antihormonal effects through estrogen receptor alpha and beta. *J Clin Endocrinol Metab* 2001;86:1750–1758.
- Zimmermann MC, Tilghman SL, Boué SM, Salvo VA, Elliott S, Williams KY, Skripnikova EV, Ashe H, Payton-Stewart F, Vanhoy-Rhodes L, Fonseca JP, Corbitt C, Collins-Burrow BM, Howell MH, Lacey M, Shih BY, Carter-Wientjes C, Cleveland TE, McLachlan JA, Wiese TE, Beckman BS, Burrow ME: Glyceollin I, a novel antiestrogenic phytoalexin isolated from activated soy. *J Pharmacol Exp Therap* 2010;332:35–45.
- Salvo VA, Boué SM, Fonseca JP, Elliott S, Corbitt C, Collins-Burrow BM, Curiel TJ, Srivastav SK, Shih BY, Carter-Wientjes C, et al.: Antiestrogenic glyceollins suppress human breast and ovarian carcinoma tumorigenesis. *Clin Cancer Res* 2006;12:7159–7164.
- Park S, Kim DS, Kim JH, Kim JS, Kim HJ: Glyceollin-containing fermented soybeans improve glucose homeostasis in diabetic mice. *Nutrition* 2012;28:204–211.
- Boué SM, Isakova IA, Burrow ME, Cao H, Bhatnagar D, Sarver JG, Shinde KV, Erhardt PW, Heiman ML: Glyceollins, soy isoflavone phytoalexins, improve oral glucose disposal by stimulating basal and insulin-mediated uptake mediated by glucose transporters in adipocytes. *J Agric Food Chem* 2012;60:6376–6382.
- Quadri SS, Stratford RE, Boué SM, Cole RB: Screening and identification of glyceollins and their metabolites by electrospray ionization tandem mass spectrometry with precursor ion scanning. *Anal Chem* 2013;85:1727–1733.
- Chen J, Lin H, Hu M: Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model. *Cancer Chemother Pharmacol* 2005;55:159–169.
- Pétriz J, García-López J: Flow cytometric analysis of P-glycoprotein function using rhodamine 123. *Leukemia* 1997;11:1124–1130.
- Shapiro AB, Ling V: Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *Eur J Biochem* 1997;250:130–137.
- van der Sandt IC, Blom-Roosemalen MC, de Boer AG, Breimer DD: Specificity of doxorubicin versus rhodamine-123 in assessing P-glycoprotein functionality in the LLC-PK1, LLC-PK1:MDR1 and Caco-2 cell lines. *Eur J Pharm Sci* 2000;11:207–214.
- Tiberghien F, Loo F: Ranking of P-glycoprotein substrates and inhibitors by a calcein-AM fluorometry screening assay. *Anti-cancer Drugs* 1996;7:568–578.
- Lautier D, Canitrot Y, Deeley RG, Cole SP: Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. *Biochem Pharmacol* 1996;52:967–977.
- Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods* 2001;25:402–408.
- Yue GGL, Cheng SW, Yu H, Xu ZS, Lee JKM, Hon PM, Lee MYH, Kennelly EJ, Deng G, Yeung SK, Cassileth BR, Fung KP, Leung PC, Lau CBS: The role of turmerones on curcumin transportation and P-glycoprotein activities in intestinal caco-2 cells. *J Med Food* 2012;15:242–252.
- Gannon MK, Holt JJ, Bennett SM, Wetzel BR, Loo TW, Bartlett MC, Clarke DM, Sawada GA, Higgins JW, Tomblin G, Raub TJ, Detty MR: Rhodamine inhibitors of P-glycoprotein: an amide/thioamide “switch” for ATPase activity. *J Med Chem* 2009;52:3328–3341.
- Takara K, Tsujimoto M, Ohnishi N, Yokoyama T: Digoxin up-regulates *MDR1* in human colon carcinoma Caco-2 cells. *Biochem Biophys Res Commun* 2002;292:190–194.
- Hollman PCH, Katan MB: Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed Pharmacother* 1997;51:305–310.
- Tian XJ, Yang XW, Yang XD, Wang K: Studies of intestinal permeability of 36 flavonoids using Caco-2 cell monolayer model. *Int J Pharm* 2009;367:58–64.
- Walle T: Absorption and metabolism of flavonoids. *Free Radic Biol Med* 2004;36:829–837.
- Wang SWJ, Chen J, Jia X, Tam VH, Hu M: Disposition of flavonoids via enteric recycling: structural effects and lack of correlations between *in vitro* and *in situ* metabolic properties. *Drug Metab Dispos* 2006;34:1837–1848.
- Artursson P, Karlsson J: Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem Biophys Res Commun* 1991;175:880–885.
- Davies B, Morris T: Physiological parameters in laboratory animals and humans. *Pharm Res* 1993;10:1093–1095.
- Chen J, Wang S, Jia X, Bajimaya S, Tam V, Hu M: Disposition of flavonoids via recycling: comparison of intestinal versus hepatic disposition. *Drug Metab Dispos* 2005;33:1777–1784.
- Álvarez AI, Vallejo F, Barrera B, Merino G, Prieto JG, Tomás-Barberán F, Espín JC: Bioavailability of the glucuronide and sulfate conjugates of genistein and daidzein in breast cancer resistance protein 1 knockout mice. *Drug Metab Dispos* 2011;39:2008–2012.
- Yang Z, Zhu W, Gao S, Yin T, Jiang W, Hu M: Breast cancer resistance protein (ABCG2) determines distribution of genistein

- phase II metabolites: reevaluation of the roles of ABCG2 in the disposition of genistein. *Drug Metab Dispos* 2012;40:1883–1893.
31. Benet LZ, Cummins CL, Wu CY: Transporter-enzyme interactions: implications for predicting drug-drug interactions from *in vitro* data. *Curr Drug Metab* 2003;4:393–398.
32. Lin JH: Drug-drug interaction mediated by inhibition and induction of P-glycoprotein. *Adv Drug Deliv Rev* 2003;55:53.
33. Versantvoort CHM, Schuurhuis GJ, Pinedo HM, Eekman CA, Kuiper CM, Lankelma J, Broxterman HJ: Genistein modulates the decreased drug accumulation in non-P-glycoprotein mediated multidrug resistant tumour cells. *Br J Cancer* 1993;68:939–946.
34. Castro AF, Altenberg GA: Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. *Biochem Pharmacol* 1997;53:89–93.
35. Romsicki Y, Sharom FJ: The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter. *Biochemistry* 1999;38:6887–6896.
36. Okura T, Ibe M, Umegaki K, Shinozuka K, Yamada S: Effects of dietary ingredients on function and expression of P-glycoprotein in human intestinal epithelial cells. *Biol Pharm Bull* 2010;33:255–259.
37. Melani R, Tomati V, Galletta LJV, Zegarra-Moran O: Modulation of cystic fibrosis transmembrane conductance regulator (CFTR) activity and genistein binding by cytosolic pH. *J Biol Chem* 2010;285:41591–41596.
38. Taur JS, Rodriguez-Proteau R: Effects of dietary flavonoids on the transport of cimetidine via P-glycoprotein and cationic transporters in Caco-2 and LLC-PK1 cell models. *Xenobiotica* 2008;38:1536–1550.
39. Mease K, Sane R, Podila L, Taub ME: Differential selectivity of efflux transporter inhibitors in Caco-2 and MDCK-MDR1 monolayers: a strategy to assess the interaction of a new chemical entity with P-gp, BCRP, and MRP2. *J Pharm Sci* 2012;101:1888–1897.
40. Ho GT, Moodie FM, Satsangi J: Multidrug resistance 1 gene (P-glycoprotein 170): an important determinant in gastrointestinal disease? *Gut* 2003;52:759–766.
41. Langmann T, Moehle C, Mauere R, Scharl M, Liebisch G, Zahn A, Stremmel W, Schmitz G: Loss of detoxification in inflammatory bowel disease: dysregulation of pregnane X receptor target genes. *Gastroenterology* 2004;127:26–40.
42. Blokzijl H, Vander Borcht S, Bok LI, Libbrecht L, Geuken M, van den Heuvel FA, Dijkstra G, Roskams TA, Moshage H, Jansen PL, Faber KN: Decreased P-glycoprotein (Pgp/MDR1) expression in inflamed human intestinal epithelium is independent of PXR protein levels. *Inflamm Bowel Dis* 2007;13:710–720.
43. Marzolini C, Paus E, Buclin T, Kim RB: Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance. *Clin Pharmacol Ther* 2004;75:13–33.
44. Annese V, Valvano MR, Palmieri O, Latiano A, Bossa F, Andriulli A: Multidrug resistance 1 gene in inflammatory bowel disease: a metaanalysis. *World J Gastroenterol* 2006;12:3636–3644.
45. Saksena S, Goyal S, Raheja G, Singh V, Akhtar M, Nazir TM, Alrefai WA, Gill RK, Dudeja PK: Upregulation of P-glycoprotein by probiotics in intestinal epithelial cells and in the dextran sulfate sodium model of colitis in mice. *Am J Physiol Gastrointest Liver Physiol* 2011;300:G1115–G1123.
46. Im SS, Kwon SK, Kim TH, Kim HI, Ahn YH: Regulation of glucose transporter type 4 isoform gene expression in muscle and adipocytes. *IUBMB Life* 2007;59:134–135.
47. Chen KG, Kikic BI: Molecular pathways: Regulation and therapeutic implications of multidrug resistance. *Clin Cancer Res* 2012;18:1863–1869.