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Interactions of Oral Molecular Excipients with Breast Cancer Resistance Protein, BCRP

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ABSTRACT: Mechanistic-understanding-based selection of excipients may improve formulation development strategies for generic drug products and potentially accelerate their approval. Our study aimed at investigating the effects of molecular excipients present in orally administered FDA-approved drug products on the intestinal efflux transporter, BCRP (*ABCG2*), which plays a critical role in drug absorption with potential implications on drug safety and efficacy. We determined the interactions of 136 oral molecular excipients with BCRP in isolated membrane vesicles and identified 26 excipients as BCRP inhibitors with IC_{50} values less than 5 μM using ^{3}H-cholecystokinin octapeptide (^{3}H-CCK8). These BCRP inhibitors belonged to three functional categories of excipients: dyes, surfactants, and flavoring agents. Compared with noninhibitors, BCRP inhibitors had significantly higher molecular weights and SLogP values. The inhibitory effects of excipients identified in membrane vesicles were also evaluated in BCRP-overexpressing HEK293 cells at similar concentrations. Only 1 of the 26 inhibitors of BCRP identified in vesicles inhibited BCRP-mediated ^{3}H-oxypurinol uptake by more than 50%, consistent with the notion that BCRP inhibition depends on transmembrane or intracellular availability of the inhibitors. Collectively, the results of this study provide new information on excipient selection during the development of drug products with active pharmaceutical ingredients that are BCRP substrates.

KEYWORDS: *excipient, BCRP, drug−excipient interaction*
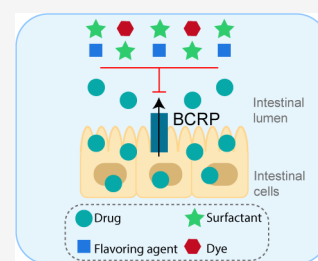
INTRODUCTION

According to the 2018 Generic Drug Access & Savings Report, generic drugs account for 90% of prescriptions dispensed in the United States (https://accessiblemeds.org/resources/blog/2018-generic-drug-access-and-savings-report). However, despite their large and growing use, generic drugs and the generic drug industry face challenges in meeting bioequivalence requirements. Inability to demonstrate bioequivalence between a generic drug product and the reference drug may be due to a range of factors involved in the manufacturing and formulation selection including differences in the quality and quantity of pharmaceutical excipients. Excipients may potentially affect the rate and extent of absorption of the therapeutic drug. This is especially a concern for drugs with narrow therapeutic indices or used to treat life-threatening diseases such as epilepsy, because small variations in the bioavailability of the drug between the brand name product and the generic drug product may have disastrous consequences for patients. To meet this challenge, formulation development strategies for excipient selection for generic drugs must shift from empirical to mechanistic-understanding-based. As highlighted in the FDA's Critical Path Opportunities for Generic Drugs, improving the science underlying quality by design (QbD) is critical for the timely approval of generic drug products while maintaining the same quality as the reference drug from a safety and efficacy perspective.[1]

Our study aims at providing scientific evidence for the rational selection of excipients in the formulation development stage, particularly for drug substances that are substrates of the intestinal efflux transporter, breast cancer resistance protein, BCRP (*ABCG2*). BCRP, which is highly expressed in the human small intestine,[2,3] has been recognized by the International Transporter Consortium (ITC)[4] and regulatory authorities as an important determinant of drug disposition and drug–drug interactions (DDIs)[5] (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/vitro-metabolism-and-transporter-mediated-drug-drug-interaction-studies-guidance-industry). Importantly, pharmaceutical excipients, ubiquitously used in drug products, have been shown in vitro and in vivo to inhibit BCRP-mediated transport of drugs such as mitoxantrone,[6] sulfasalazine,[7] and topotecan.[8] In addition, food additives, including preservatives, colorants, and sweeteners, were reported to inhibit BCRP in vitro.[9]

The goal of the current study was to characterize the interactions of excipients that are present in orally administered FDA-approved drug products (referred to as oral

molecular excipients here) listed on the Inactive Ingredient Database (IID) and on the CERSI Excipient Browser[10] with BCRP. We screened 136 oral molecular excipients using BCRP-overexpressing membrane vesicles and identified 26 BCRP inhibitors among them. Surprisingly, further studies performed in BCRP-overexpressing HEK293 cells showed that some excipients were potent inhibitors of BCRP in vesicles but not in BCRP-overexpressing HEK293 cells. Our study provides important information about excipients that may be incorporated into dosage forms with BCRP substrates, without altering their absorptions. Further, our results suggest the need for additional studies in preclinical animal species, with selected excipients to determine the relevance of our in vitro findings to drug absorptions.

■ MATERIALS AND METHODS

Oral Molecular Excipients and Reagents. Our studies focused on oral molecular (small molecule) excipients listed on the UCSF-Stanford Center of Excellence in Regulatory Science and Innovation (CERSI) excipients database (http://excipients.ucsf.bkslab.org/excipients/molecular/?route=oral). Currently, there are over 292 molecular excipients listed on this database, all of which were considered for screening. This collection was curated by excluding excipients that are no longer used, poorly soluble (defined as less than 1 mM in H_2O, dimethyl sulfoxide (DMSO), or ethanol), commercially unavailable, formulated for delivery by inhalation, or sharing the same anion as the representative excipient (e.g., sodium acetate was tested and calcium/magnesium acetate were not). In total, 136 excipients were screened, and the purchase information is listed in Tables S1 and S2. Ko-143 and bromosulfophthalein (BSP) were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Hampton, NH), respectively. Tritium labeled CCK-8 (^{3}H-CCK8) was purchased from PerkinElmer (Waltham, MA). Tritium labeled oxypurinol was custom-synthesized by IsoSciences (King of Prussia, PA). Human BCRP vesicles were prepared by GenoMembrane (Yokohama, Kanagawa, Japan).

Vesicular Transport Assay. The BCRP vesicular transport assay was performed as reported previously[11] with minor modifications. In brief, ^{3}H-CCK8, a probe BCRP substrate, was mixed with DMSO or BSP, the known inhibitor of BCRP, in vesicle uptake buffer (50 mM MOPS-Tris, pH 7.0 + 70 mM KCl + 7 mM $MgCl_2$) at 37 °C. Into each well, 70 μL of mixture was added into the 96-well flat-bottom uptake plate followed by the addition of 10 μL/well (10 μg/μL) of vesicles expressing human BCRP. Uptake assays were initiated by adding 20 μL of either 25 mM ATP or 25 mM AMP to a final concentration of 5 mM. We performed the experiments at pH 7, as recommended by the commercial supplier of the BCRP vesicles and consistent with other published work.[9,11] The pH value of 7 is within the known pH range of the cytosol (7 to 7.4), in which the ATP binding site of BCRP resides. Plates were incubated at 37 °C for 5 min with orbital shaking (90 rpm). After 5 min, the uptake was quenched by adding 150 μL of ice-cold wash buffer (40 mM MOPS-Tris, pH 7.0 + 70 mM KCl) to each well, and the mixture was transferred to a 96-well filtration plate. Vacuum was applied, and the vesicles were washed three times with wash buffer. Filters were removed from the filter plate, and the radioactivity was determined by liquid scintillation counting.

Excipient Screening and IC$_{50}$ Determination. A total of 136 excipients were screened at 1 mM for all sugars and 200

μM for the rest of the excipients, except the ones with limited solubility, in which case, the studies were carried out at lower concentrations (Table S3). The screening process was performed in triplicate. IC$_{50}$ values for compounds were determined by fitting the uptake results to the Hill equation by nonlinear regression (log (inhibitor) vs normalized response) assuming the Hill slope was −1.0 using GraphPad Prism 7.

Aggregation Test. The test for aggregation was performed according to a previously reported protocol.[12] Dynamic light scattering experiments were carried out in a 384-well plate using a modified DynaPro Plate Reader II system (Wyatt Technology); the width of the 60 mW laser beam (at ~830 nm) was expanded to be appropriate for detecting large colloidal particles. Version 1.7 of the Dynamics software (Wyatt Technology) was used to process the data as well as to automatically adjust the laser power and detector angle (158°). The compounds were each concentrated in DMSO, subsequently diluted with filtered 50 mM potassium phosphate, pH 7.0, leading to a final concentration of 1% DMSO, and then added to the plates for measurement.

Measurement of Fraction Unbound of BCRP-Inhibiting Dyes. Each dye was dissolved in 90 μL of vesicle uptake buffer and 10 μL of TEMP solution (50 mM Tris-HCl (pH 7.0), 50 mM mannitol, 2 mM ethylene glycol−bis(β-aminoethyl ether)−N,N,N',N'-tetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), 8 μg/mL of aprotinin, 10 μg/mL of leupeptin) with or without BCRP membrane vesicles. The final concentration of each dye was aligned with the corresponding screening concentration listed in the Table S3. After 5 min of incubation, the membrane vesicles were centrifuged down at 100 000g for 30 min, and the supernatant (unbound dye) was measured for the UV absorbance. The fraction unbound was calculated by dividing the UV absorbance obtained in the samples that included the membrane vesicles by the UV absorbance in the control samples (without membrane vesicles).

Analysis of Physicochemical Properties and Comparison with Drug Inhibitors of BCRP. Molecular structure files in structure-data format were obtained from the CERSI Excipient Browser at http://excipients.ucsf.bkslab.org/ and PubChem database.[13] MayaChemTools software[14] was used to compute eight one-dimensional and two-dimensional descriptors, such as MW: molecular weight, HA: number of heavy atoms, RB: number of rotatable bonds, MV: molecular volume, HBD: number of hydrogen bond donors, HBA: number of hydrogen bond acceptors, SLogP: Crippen's log of the octanol/water partition coefficient (including implicit hydrogens), and TPSA: total polar surface area.

Boxplots were prepared using the R software package.[32] Boxplots display data distribution using five statistics: minimum, first quartile, median, third quartile, and maximum. The box covers the values from the first quartile to the third quartile. Boxplot whiskers show the locations of the minimum and maximum values. Differences between distributions of molecular descriptors were assessed using the pairwise Student's t-test in R.

FSelector package[15] in R, which functions to select attributes, was used to select two nonredundant and informative physicochemical properties from the eight descriptors, namely, molecular weight and SLogP. Feature selection was performed with the *cfs* function implemented in the FSelector package. More specifically, a subset of features that were independent of each other but correlated with a class
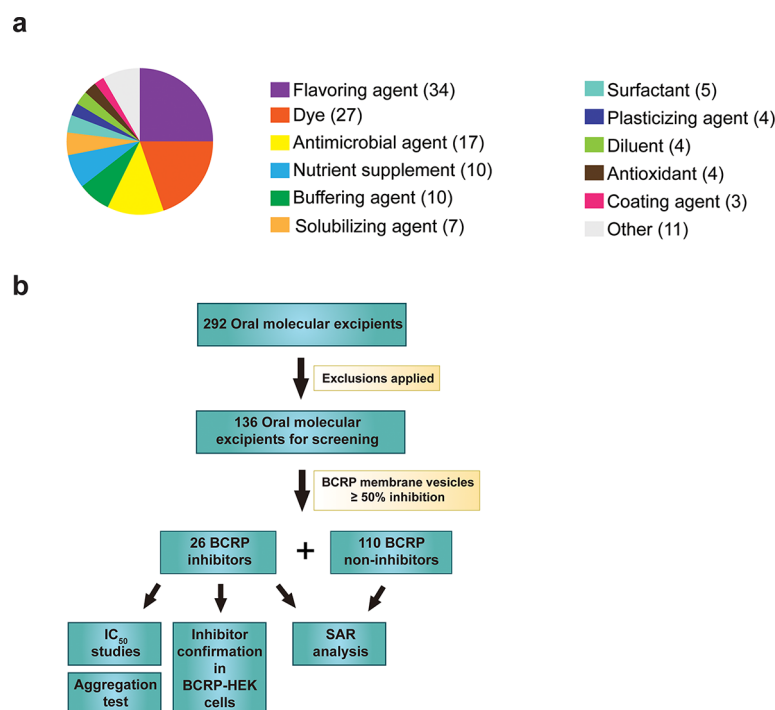
Figure 1. Screening design and establishment. (a) Functional categories of the 136 oral molecular excipients. (b) Overview of the screening procedure in identifying BCRP inhibitors.

label were selected.[16] Multiple regression models using the various physicochemical properties were performed and determined to not be informative (adjusted R^2 value of 0.012 and a p-value of 0.4048 for the F-statistic). Therefore, we decided to use binary models classifying the excipients as inhibitors and noninhibitors.

Cell Lines and Cell Culture. Human embryonic kidney (HEK) 293 cells transfected with empty vector only (pcDNA 3.1) and cells expressing BCRP[17] were maintained in DME-H21 supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM glutamine, and 2 mg/mL of G418 at 37 °C in a humidified incubator with 5% CO_2.

Excipient Screening in BCRP-Overexpressing HEK293 Cells. A total of 26 BCRP-inhibiting excipients identified in BCRP membrane vesicles were tested in BCRP-overexpressing HEK293 cells. Therefore, the ^{3}H-oxypurinol accumulation assay was performed as described previously[17] with minor modifications. In brief, cells transfected with vector only or ABCG2 constructs (1.6×10^5/well) were seeded in 48-well poly-D-lysine-coated plates 24 h prior to experiments. Mixtures containing the following were prepared for cell accumulation studies: Hank's Balanced Salt Solution (HBSS) with a trace amount of ^{3}H-oxypurinol was mixed with BCRP inhibitor, Ko-143 (10 μM), cyclosporin A (50 μM), curcumin (100 μM), and excipients or vehicle ($\leq$1% DMSO). After the cells were washed twice and incubated with HBSS for 15 min, HBSS was removed, and cells were incubated with the mixtures above for 30 min at 37 °C. The accumulation studies were terminated by washing cells twice with ice-cold HBSS, followed by the addition of 800 μL of lysis buffer (0.1% SDS v/v, 0.1 N NaOH). Intracellular radioactivity was determined by scintillation counting and normalized by protein content. The inhibitory effect of tested excipients on BCRP-mediated oxypurinol transport was calculated by the following equation,

in which normalized oxypurinol accumulation refers to the normalization of the uptake in BCRP-expressing cells to uptake in empty-vector-transfected cells

$$\text{normalized oxypurinol accumulation} = \frac{(\text{BCRP} + \text{I})/(\text{BCRP} - \text{I})}{(\text{EV} + \text{I})/(\text{EV} - \text{I})}$$

(1)

"BCRP" represents "BCRP-overexpressing cells". "EV" represents "empty-vector-transfected cells". "I" represents "BCRP-inhibiting excipient identified in membrane vesicles". "BCRP + I" represents "BCRP-overexpressing cells incubated with ^{3}H-oxypurinol plus excipient"."BCRP − I" represents "BCRP-overexpressing cells incubated with ^{3}H-oxypurinol plus DMSO"."EV + I" represents "empty-vector-transfected cells incubated with ^{3}H-oxypurinol plus excipient". "EV − I" represents "empty-vector-transfected cells incubated with ^{3}H-oxypurinol plus DMSO".

RESULTS

Establishment of the Screening Library and Overview of the Screening Procedure. We analyzed a collection of 136 oral molecular excipients identified through the CERSI Excipient Browser (see Materials and Methods).[10] The excipients spanned multiple functional categories including antimicrobial agents, buffering agents, flavoring agents, and dyes (Figure 1a and Tables S1 and S2). Of the included excipients, 65% are also used in food products (Table S2).

The overview of the screening procedure is summarized in Figure 1b. In brief, 136 oral molecular excipients were selected and screened for inhibition of BCRP-mediated ^{3}H-CCK8 uptake in membrane vesicles. IC$_{50}$ studies, aggregation tests, and quantitative structure−activity relationship analysis were conducted on identified inhibitors. The inhibitory effects of BCRP inhibitors identified in membrane vesicles were further evaluated in BCRP-overexpressing HEK293 cells.
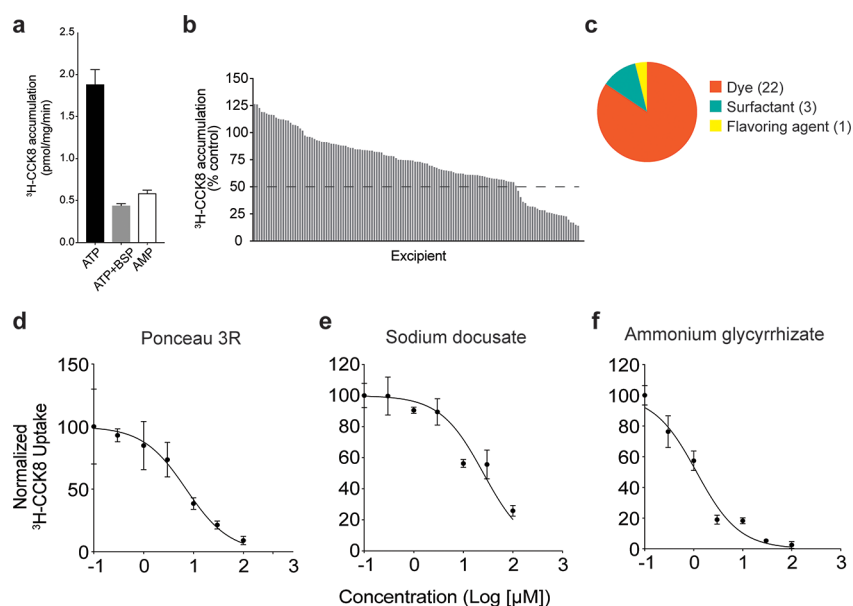
Figure 2. Identification of oral molecular excipients as inhibitors of BCRP. (a) The uptake of ^{3}H-CCK8 by human BCRP. BCRP membrane vesicles (50 μg/well) were incubated with ^{3}H-CCK8 in the presence of 5 mM ATP (black bar), 5 mM ATP and 200 μM BSP (gray bar), or 5 mM AMP (white bar). Plates were incubated at 37 °C for 5 min. Each column represents the mean ± SEM of ^{3}H-CCK8 uptake from three replicates in ≥5 experiments. (b) Screening results for 136 oral molecular excipients. Cells were incubated in HBSS uptake buffer with ^{3}H-CCK8 in the presence of excipients or DMSO. Excipients were screened at 200 μM except for several sugars, surfactants, and those compounds with limited solubility, which were tested at lower concentrations. Inhibitors were defined as those that inhibited BCRP-mediated ^{3}H-CCK8 uptake by more than 50%. There were 26 excipients identified as BCRP inhibitors. (c) Functional categories of excipients identified as BCRP inhibitors, which included 22 dyes, 3 surfactants, and 1 flavoring agent. Representative IC$_{50}$ curves for BCRP-mediated ^{3}H-CCK8 uptake inhibition for the following excipients: (d) ponceau 3R, (e) sodium docusate, (f) ammonium glycyrrhizate. Symbols represent the mean ± SD of N = 3 determinations at each concentration.

## Table 1. Potency of Excipients as Inhibitors of BCRP in Membrane Vesicles

excipient	category	IC$_{50}$ (μM)	95% CI (μM)	IC$_{50, corrected}$ (μM)[a]
FD&C Red No. 3	dye	0.56	0.33–0.93	0.374
Light green CF yellowish	dye	1.00	0.77–1.31	0.943
D&C Red No. 28	dye	1.04	0.59–1.82	0.431
FD&C Blue No. 1	dye	1.97	1.21–3.21	2.02
Ponceau xylidine	dye	2.12	1.12–4.00	1.80
D&C Red No. 27	dye	2.35	1.34–4.06	0.872
D&C Green No. 5	dye	2.47	1.52–4.01	1.71
D&C Red No. 4	dye	2.47	1.79–3.40	2.40
Amaranth	dye	2.72	1.29–5.72	2.58
Guinea green b	dye	2.99	2.16–4.14	1.37
Naphthol blue black	dye	4.34	2.64–7.14	3.01
FD&C Red No. 40	dye	4.42	3.09–6.33	4.31
D&C Orange No. 4	dye	4.77	2.48–9.18	2.78
FD&C Yellow No. 5	dye	5.61	3.39–9.25	5.57
Ponceau 3R	dye	7.12	4.75–10.7	7.05
Rhodamine B	dye	7.78	4.25–14.22	7.02
D&C Brown No. 1	dye	12	6.32–23.0	5.01
D&C Red No. 6	dye	12.3	8.19–18.3	11.6
FD&C Yellow No. 6	dye	14.1	8.58–23.3	13.7
EXT Yellow No. 7	dye	15.2	10.5–22.1	14.9
Indigotindisulfonate sodium	dye	27.8	15.7–49.7	25.8
D&C Red No. 33	dye	34.2	17.0–67.8	32.8
Docusate sodium	surfactant	25.4	17.9–36.2	NA
Sodium lauryl sulfate	surfactant	27.1	12.0–70.7	NA
Sucrose monolaurate	surfactant	57.5	22.2–168	NA
Ammonium glycyrrhizate	flavoring agent	1.15	0.91–1.44	NA

[a]IC$_{50, corrected}$ = IC$_{50}$ * fraction unbound. NA: Not available.
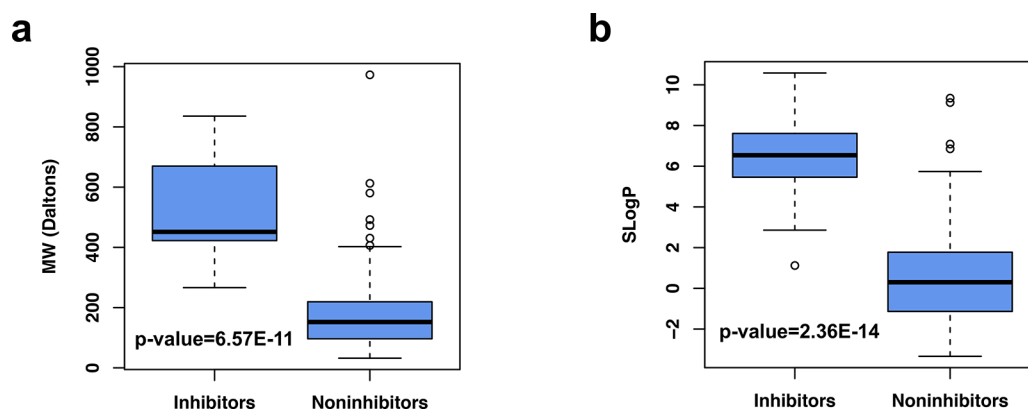
Figure 3. BCRP inhibitors have increased molecular weight and SLogP as compared to noninhibitors. Differences in physicochemical properties between inhibitors (IE: inhibiting excipients) and noninhibitors (NIE: noninhibiting excipients). Boxplots of physicochemical properties are shown: (a) MW: molecular weight, (b) SLogP: Crippen's log of the octanol/water partition coefficient (including implicit hydrogens).
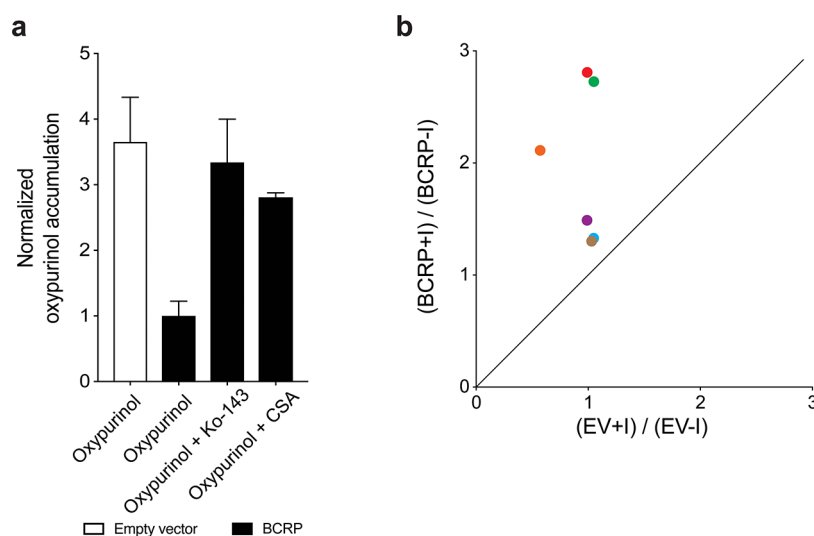
Figure 4. Inhibitory effects of excipients in BCRP-overexpressing HEK293 cells. (a) Empty-vector-transfected cells (white bar) and BCRP-overexpressing cells (black bar) were incubated with ^{3}H-oxypurinol in the presence or absence of 10 μM of Ko-143 or 50 μM of cyclosporin A (CSA) at 37 °C. After 30 min, the accumulation of ^{3}H-oxypurinol was determined by scintillation counting and normalized by protein content. Each column represents the mean ± SD of ^{3}H-oxypurinol accumulation from ≥3 replicates in multiple experiments. (b) The accumulation of ^{3}H-oxypurinol in both empty-vector-transfected cells and BCRP-overexpressing cells. Each dot represents a BCRP inhibitor: Ko-143 (green), CSA (red), curcumine (orange), rhodamine B (purple), docusate sodium (brown), D&C brown No. 1 (blue). The x-axis shows the ratio of the amount of ^{3}H-oxypurinol accumulated in empty-vector-transfected cells incubated with an inhibitor, (EV + I), over the amount of ^{3}H-oxypurinol accumulated in empty-vector-transfected cells incubated with DMSO, (EV − I). The y-axis shows the ratio of the amount of ^{3}H-oxypurinol accumulated in BCRP-overexpressing cells incubated with an inhibitor, (BCRP + I), over the amount of ^{3}H-oxypurinol accumulated in BCRP-overexpressing cells incubated with DMSO, (BCRP − I). The regression line was forced through X = 0 and Y = 0. Each dot represents the mean of normalized ^{3}H-oxypurinol accumulation from three replicates in a single experiment.

Identification of Oral Molecular Excipients as BCRP Inhibitors. ^{3}H-CCK8 was used as the probe substrate of BCRP for screening. ^{3}H-CCK8 uptake in human BCRP membrane vesicles in the presence of ATP (1.88 pmol/mg/min) was 3.2-fold of that in the presence of AMP (0.58 pmol/mg/min) (Figure 2a). Furthermore, ^{3}H-CCK8 uptake was significantly inhibited by bromosulfophthalein (BSP) (0.44 pmol/mg/min), a known BCRP inhibitor (Figure 2a). BCRP-mediated ^{3}H-CCK8 uptake was not affected by DMSO up to 1% (v/v) (Figure S1). Together, these results demonstrate that our assay reliably detects BCRP-mediated ^{3}H-CCK8 uptake in membrane vesicles.

Considering that excipients used as diluents, solubilizing agents, and flavoring agents are commonly used in large quantities in oral drug products, the screening concentration

was 1 mM for all sugars and 200 μM for the rest of the excipients except the ones with limited solubility, in which case the maximum soluble concentration was used (10−50 μM, see Table S3). We selected 50% as the cutoff for inhibition in order to focus on the most potent excipients for follow-up IC$_{50}$ studies. Using this criterion, we detected 26 inhibitors from our primary screen (17.6% of the library; Figure 2b) for follow-up studies. Importantly, based on the classification of screened excipients, the identified inhibitors belonged to three functional classes: 22 dyes, 3 surfactants, and 1 flavoring agent (Figure 2c). Next, IC$_{50}$ studies were conducted for the 26 inhibitors using BCRP-overexpressing membrane vesicles. Representative IC$_{50}$ curves for each functional class of excipients are shown on Figure 2d−f. Notably, 14 excipients were potent inhibitors of BCRP with IC$_{50}$ values lower than 5

μM (Table 1), for example, FD&C Red No. 3 (0.56 μM), light green CF yellowish (1 μM), D&C Red No. 28 (1.04 μM), and ammonium glycyrrhizate (1.15 μM). Finally, aggregation testing on the 26 excipients showed that the IC$_{50}$ values of 11 excipients were more than 10 times less than their aggregation concentration, suggesting specific inhibition on BCRP (Table S4).

Molecular Characteristics of the BCRP Inhibitors. We analyzed the physicochemical properties of excipients that inhibited BCRP-mediated transport and compared them to those of noninhibiting excipients. Eight molecular descriptors were computed, namely, molecular weight, number of heavy atoms, number of rotatable bonds, molecular volume, number of hydrogen bond donors, number of hydrogen bond acceptors, Crippen's log of the octanol/water partition coefficient (including implicit hydrogens), and total polar surface area. Statistically significant, nonredundant, and informative physicochemical properties were molecular weight (Student pairwise t-test p-value $< 6.57 \times 10^{-11}$) and SLogP values (Student pairwise t-test p-value $< 2.36 \times 10^{-14}$) differentiating BCRP-inhibiting from noninhibiting excipients (Figures 3 and S2 and Table S5). Excipients that inhibited BCRP-mediated transport appeared to be more hydrophobic and bulkier than noninhibitors.

Inhibitory Effects of Excipients in BCRP-Overexpressing HEK293 Cells. Since the BCRP-inhibiting excipients were identified in "inside-out" membrane vesicles (in which all excipients had access to the transporter from the internal surface), it is possible that in intact intestine, accessing efflux transporters such as BCRP may be challenging for the excipients studied here. Therefore, the inhibitory effects of the 26 excipients that inhibited BCRP in the vesicle system were evaluated in BCRP-overexpressing HEK293 cells. Accumulation of ^{3}H-oxypurinol in BCRP-overexpressing cells was 3.6-fold less compared to that in empty-vector-transfected cells (Figure 4a). Incubation of 10 μM of Ko-143, a known inhibitor of BCRP, resulted in a 3.4-fold increase in the accumulation of ^{3}H-oxypurinol, which was comparable to that in empty-vector-transfected cells (Figure 4a). Notably, incubation with cyclosporin A (50 μM), a known inhibitor of BCRP[18] that is recommended by the FDA for use in clinical drug−drug interaction studies (https://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm#table5-2), increased the accumulation of ^{3}H-oxypurinol by 2.8-fold compared to that in DMSO-treated BCRP-overexpressing cells (Figure 4a). The accumulation of ^{3}H-oxypurinol in BCRP-overexpressing cells was normalized based on eq 1, considering potential nonspecific effects of excipients on the cell membrane. If the ratio of $(EV + I)/(EV - I)$ equals 1, the nonspecific effect of a given excipient on cell membranes was considered minimal. The accumulation of ^{3}H-oxypurinol in BCRP-overexpressing HEK293 cells treated with Ko-143 was 2.6-fold greater than that in DMSO-treated cells $((BCRP + I)/(BCRP - I))$, while there was a minimal nonspecific effect of Ko-143 on cell membranes $((EV + I)/(EV - I) = 1.05)$ (Figure 4b and Table S6). We also tested the inhibitory effect of another known BCRP inhibitor, curcumin, on the accumulation of oxypurinol in HEK293 cells. In contrast to Ko-143 and cyclosporin A, 100 μM curcumin had a nonspecific effect on cell membranes $((EV + I)/(EV - I) = 0.571)$ even though the normalized oxypurinol accumulation $((BCRP + I)/(BCRP - I))/((EV + I)/(EV - I))$ was 3.7

(Figure 4b and Table S6). Notably, of the 26 tested excipients, only rhodamine B increased the BCRP-mediated ^{3}H-oxypurinol accumulation in BCRP-overexpressing HEK293 cells by more than 50%, followed by D&C Brown No. 1 (26.8%) and docusate sodium (26.4%) (Figure 4b and Table S6). The predicted LogD value for rhodamine B (2.34) and other BCRP-inhibiting excipients identified in membrane vesicles could not explain the discrepancy observed in membrane vesicles compared to HEK293 cells (Table S7).

■ DISCUSSION

In this report, we characterized the interactions of oral molecular excipients with the efflux transporter, BCRP. In vitro and in vivo evidence, including clinical drug−drug interaction studies, support a role for BCRP in intestinal drug absorption.[19] Of the 292 oral molecular excipients that are listed in the CERSI Excipient Browser,[10] we characterized the interaction of 136 excipients with BCRP using BCRP-expressing membrane vesicles. A total of 26 excipients were deemed inhibitors of BCRP, whereas 110 were deemed noninhibitors or weak inhibitors at tested concentrations. Of the 26 inhibitors, three excipients (FD&C Red No. 40, FD&C Yellow No. 5, and FD&C Yellow No. 6) had previously been shown to inhibit BCRP with IC$_{50}$ values similar to our results (within 1−5-fold).[9] Importantly, 13 dyes and 1 flavoring agent were potent inhibitors of BCRP with IC$_{50}$ values less than 5 μM (Table 1). The measurement of fraction unbound of BCRP-inhibiting dyes in incubation with BCRP membrane vesicles (Figure S5) showed that the majority of the dyes (17/22) had a fraction unbound of more than 60%, and their IC$_{50, \text{corrected}}$ (IC$_{50}$ * fraction unbound) values were within the 95% confidence interval of IC$_{50}$ values (Tables S8 and 1). These IC$_{50}$ values are in the range of IC$_{50}$ values for established BCRP inhibitors including prescription drugs, which are considered clinically relevant BCRP inhibitors.[20] For example, the IC$_{50}$ value (0.5 μM) for elacridar against human BCRP[20] is comparable to the values for FD&C Red No. 3 (0.374 μM), light green CF yellowish (0.943 μM), and D&C Red No. 28 (0.431 μM). To determine whether CCK8 shares a common binding site with clinical BCRP substrate drugs, we conducted mechanistic kinetic experiments with rosuvastatin and sulfasalazine. In brief, our results showed different inhibition models for the two drugs (Table S9). Clearly, excipients used in formulations of BCRP substrate drugs should be validated for their inhibitory effects on BCRP-mediated transport of that drug. Studies using in vitro systems, animal species, and humans have suggested that excipients may modulate the absorption of therapeutic agents given orally.[21−24] In the case of inhibiting efflux transporters such as BCRP, an increase in the systemic exposure of drugs that are BCRP substrates has been noted when the drugs have been administered concomitantly with BCRP inhibitors such as curcumin and eltrombopag.[25−27] Modeling and simulation tools[28] have also been used to quantitatively predict the impact of excipients on the systemic exposure of the administered drug substance; this may be important for drugs that are substrates of BCRP, which may include drugs in the Biopharmaceutical Classification System (BCS), Class 2, 3, and 4. For generic drugs in BCS Class 3, the results are particularly important in preparing bioequivalent formulations, since clinical studies may be waived.[29] The concentration of the excipient in the gut contents and the enterocyte will ultimately determine the extent of interaction between the excipient and an efflux

transporter such as BCRP. However, the work presented here may aid in the selection of excipients during formulation development for which the potential for interaction is lower when combined with a drug substance that is a BCRP substrate, therefore alleviating a concern on the safety or efficacy for a drug product.

The 26 excipients identified as BCRP inhibitors in our study were from several functional categories (Figure 2c) but had similarities in their physicochemical properties (Figure 3). In particular, excipients that inhibited BCRP transport had higher molecular weights and were more lipophilic than poor or noninhibitors. These two properties are characteristic of many food colors and may explain their high representation as BCRP inhibitors. In fact, 85% (22/26) of the BCRP inhibitors were dyes, whereas only 20% (27/136) of the excipients screened were dyes. The data suggest that molecular weight and hydrophobicity may be used as an aid in predicting whether excipients are likely to be BCRP inhibitors. In particular, the differences in distributions of these two molecular descriptors between inhibitors and noninhibitors were pronounced (Table S5).

Since food dyes were found to be potent inhibitors of BCRP, knowledge of the estimated daily intake (EDI) of food dyes is important to understand and predict whether intestinal BCRP inhibition may occur in people consuming normal diets. A recent study[30] concluded that exposure to food-color additives in the United States by average and even high-intake consumers is well below the acceptable daily intake (ADI) of each color additive as published by the Joint WHO/FAO Committee on Food Additives (JECFA) and allows wide margins of safety. As shown in Table 2, the $[I]/IC_{50}$ value for

Table 2. Estimated Daily Intake and Intestinal Concentrations of Dyes for the US Population

excipient	EDI $(\mu g/kg/day)^a$	$[I]$ $(\mu M)^b$	$IC_{50\text{-}BCRP}$ (μM)	$[I]/$ $IC_{50\text{-}BCRP}$
FD&C Red 40	11.2	6.97	4.42	1.58
FD&C Yellow 6	4.1	2.83	14.1	0.20
FD&C Yellow 5	5.1	3.07	5.61	0.55
FD&C Blue 1	2.1	0.79	1.97	0.40
FD&C Red 3	1.6	0.54	0.56	0.96

^{a}EDI: Estimated daily intake for the US population (the mean of the maximum use level). $^b[I]$: Estimated intestinal concentration.

each of the dyes is much lower than 10 (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/vitro-metabolism-and-transporter-mediated-drug-drug-interaction-studies-guidance-industry), suggesting minimal potential of inhibition of BCRP based on the average EDI for the US population. However, the EDI for children between 2 and 5 years old is about 3−10 times greater than the EDI for adults, suggesting that children may be at increased risk for intestinal BCRP inhibition by excipients such as dyes, present in foods. Further studies are needed to evaluate the effects of excipients that are present in larger amounts in foods on intestinal absorption of BCRP substrates.

An important question remains as to whether the inhibitors identified in our "inside-out" vesicles assays can actually access the target, BCRP, and inhibit the transporter in vivo. In an attempt to address this question, we used BCRP-overexpressing cells and placed the excipients in the media, mimicking the in vivo situation. Importantly, cyclosporin A

and curcumin, which are recommended by the FDA for use in clinical drug−drug interaction studies, increased oxypurinol accumulation in our BCRP-overexpressing cell system (Figure 4a,b), supporting the suitability of the use of a whole-cell system for BCRP inhibitor screening. Absorption of food dyes is generally low,[9] which may be consistent with poor penetration into enterocytes and, thus, poor ability to access inhibition sites on BCRP. Notably, of the 26 excipients identified as BCRP inhibitors in membrane vesicles, only rhodamine B inhibited BCRP transport activity by more than 50% in BCRP-overexpressing HEK293 cells. This could be due to good passive permeability of rhodamine B into cells,[31] which allows it to achieve intracellular or intramembrane concentrations sufficient to inhibit BCRP transport activity. For the other intestinal excipients that were unable to inhibit BCRP in our cellular assays (Table S6), there is limited information on their membrane permeability. However, though many are highly lipophilic (high SLogP values) (Figure 3 and Table S5), at physiologic pH, most of the excipients would be predominantly in the ionized form, limiting their diffusion rates across the lipid bilayer. On the other hand, it is worth noting that in vivo, bile acids and lecithin that are present in the intestinal lumen can form micelles. Though speculatively, some of the excipients might be trapped within these micelles, allowing them to enter intestinal epithelial cells, resulting in increased excipient concentrations in intestinal epithelial cells in vivo. The discrepancy in the inhibitory effects of excipients between membrane vesicles and whole-cell systems can also be explained by the differences in the substrates used in the two assays. Notably, for technical reasons,[3]H-CCK8 was used as the canonical BCRP substrate in our vesicle assays, whereas oxypurinol was used in our whole-cell assays. Because we did not observe reduced accumulation of ^{3}H-CCK8 in BCRP-overexpressing HEK293 cells, CCK8 is not an appropriate probe for the cellular system. Hence, we used oxypurinol, an alternative substrate for BCRP (see Figure S3). On the other hand, oxypurinol was not an appropriate probe for screening BCRP inhibitors in the vesicle system. That is, we did not see enhanced uptake in the vesicles in the presence of ATP (Figure S4). Thus, we were not able to use the same substrates in the two experimental systems, prohibiting a more direct comparison.

Some discrepancies were observed between our results and results obtained in other laboratories in which inside-out vesicles were used, but the canonical substrates differed. For example, in inside-out membrane vesicles from Sf9 cells, FD&C Blue No. 1 inhibited BCRP-mediated ^{3}H-CCK8 uptake in our study but did not inhibit BCRP-mediated uptake of Lucifer yellow in vesicle assays in a previous study.[9] Conversely, neohesperidin DC did not inhibit BCRP-mediated ^{3}H-CCK8 uptake in our study in vesicles but did inhibit BCRP-mediated Lucifer yellow uptake in the previous study.[9] Collectively, these observations suggest that for comparison of results of BCRP inhibition studies, the experimental system including the canonical substrate should be considered, and caution should be exercised in using the data to predict clinical inhibition of the transporter.

In conclusion, a number of oral molecular excipients were identified as inhibitors of the drug transporter BCRP in inside-out membrane vesicles. Larger molecular weight and increased hydrophobicity were characteristics of the BCRP inhibitors. The discrepancy between our results in the two experimental systems suggest that caution should be exercised in

extrapolating results in inside-out vesicles to results in whole cells and to the in vivo situation. Our data demonstrated that many excipients do not interact with BCRP in cells and, therefore, may be used in formulations that include drugs that are substrates of BCRP. Preclinical in vivo studies are warranted to investigate the interactions of foods that contain higher amounts of excipients with drugs that are ligands of BCRP.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.9b00658.

> Five figures and nine tables including the effects of DMSO on CCK8 uptake by BCRP; physicochemical properties between inhibitors and noninhibitors of BCRP; CCK8 accumulation in HEK293 cells; oxypurinol accumulation in BCRP membrane vesicles; supernatant protein concentration after centrifugation; excipient information; excipient aggregation; oxypurinol accumulation in HEK293 cells; predicted LogD values for BCRP-inhibiting excipients; fraction unbound of BCRP-inhibiting dyes with membrane vesicles; kinetic parameters of BCRP-mediated CCK8 uptake in the presence of rosuvastatin and sulfasalazine (PDF)

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Notes

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