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Chalcogenopyrylium Dyes as Differential Modulators of Organic Anion Transport by Multidrug Resistance Protein 1 (MRP1), MRP2, and MRP4[S]

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ABSTRACT

Multidrug resistance proteins (MRPs) mediate the ATP-dependent efflux of structurally diverse compounds, including anticancer drugs and physiologic organic anions. Five classes of chalcogenopyrylium dyes (CGPs) were examined for their ability to modulate transport of [^{3}H]estradiol glucuronide ($E_2$17βG; a prototypical MRP substrate) into MRP-enriched inside-out membrane vesicles. Additionally, some CGPs were tested in intact transfected cells using a calcein efflux assay. Sixteen of 34 CGPs inhibited MRP1-mediated $E_2$17βG uptake by >50% (IC_{50} values: 0.7–7.6 μM). Of 9 CGPs with IC_{50} values ≤2 μM, two belonged to class I, two to class III, and five to class V. When tested in the intact cells, only 4 of 16 CGPs (at 10 μM) inhibited MRP1-mediated calcein efflux by >50% (III-1, V-3, V-4, V-6), whereas a fifth (I-5) inhibited efflux by just 23%. These five CGPs also inhibited [^{3}H]$E_2$17βG uptake by MRP4. In contrast, their effects on MRP2 varied, with two (V-4, V-6) inhibiting $E_2$17βG transport (IC_{50} values: 2.0 and 9.2 μM) and two (V-3, III-1) stimulating transport (>2-fold), whereas CGP I-5 had no effect. Strikingly, although V-3 and V-4 had opposite effects on MRP2 activity, they are structurally identical except for their chalcogen atom (Se versus Te). This study is the first to identify class V CGPs, with their distinctive methine or trimethine linkage between two disubstituted pyrylium moieties, as a particularly potent class of MRP modulators, and to show that, within this core structure, differences in the electronegativity associated with a chalcogen atom can be the sole determinant of whether a compound will stimulate or inhibit MRP2.

Introduction

ATP-binding cassette (ABC) transporters belong to a superfamily of mostly membrane proteins that mediate the ATP-dependent transmembrane transport of many structurally diverse xenobiotics and endogenous metabolites. Several human ABC transporters have been implicated in the drug resistance that is commonly observed in tumors refractory to chemotherapy. The most frequently implicated in clinical drug resistance are P-glycoprotein, multidrug resistance protein 1 (MRP1), and ABCG2 (Tamaki et al., 2011). Despite their shared function in tumor cell resistance, however, each of these transporters has its own distinct pharmacological and physiologic functions in normal cells (Leslie et al., 2005; Vlaming et al., 2009; Sharom, 2011; Slot et al., 2011). Thus, in addition to conferring drug resistance in tumor cells, the three ABC proteins also have an important influence on the absorption, distribution, and/or elimination of drugs and other xenobiotics from different tissues in the body.

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MRP1 was discovered more than 15 years after P-glycoprotein, and thus it is not surprising that fewer MRP1-specific modulators have been identified (Cole et al., 1992; Chen et al., 2001; Wang et al., 2004; Boumendjel et al., 2005; Norman et al., 2005). Similar to P-glycoprotein, MRP1 can confer resistance to natural product drugs in tumor cells (Cole et al., 1994). It also plays a different but still protective role in normal tissues because it is expressed at the interface of various so-called pharmacological sanctuary sites, including the blood-cerebrospinal fluid barrier (Wijnholds et al., 2000; Kato et al., 2009). Unlike P-glycoprotein, however, MRP1 is an efficient transporter of numerous organic anions, many of which are glutathione or glucuronide conjugates of drug metabolites (Leslie et al., 2005; Slot et al., 2011). Estradiol glucuronide ($E_2$17βG) is a physiologic metabolite effluxed by human MRP1 (Jedlitschky et al., 1996).

MK-571, a cysteinyl leukotriene receptor antagonist, is the most extensively used experimental inhibitor of MRP1 but does not inhibit P-glycoprotein (Gekeler et al., 1995). Several compounds have been identified that modulate both transporters (Narasaki et al., 1997; Kimura et al., 2002; Toppmeyer et al., 2002; Pellicani et al., 2012) despite the fact that MRP1 and P-glycoprotein share <20% sequence identity. Consequently, chemical entities proposed as P-glycoprotein modulators are now often screened for their activity against MRP1 (Wesolowska, 2011; Ebert et al., 2012).

ABBREVIATIONS: ABC, ATP-binding cassette; AU, arbitrary units; CGP, chalcogenopyrylium; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; $E_2$17βG, estradiol glucuronide; FBS, fetal bovine serum; HEK, human embryonic kidney; LTC_4, leukotriene C_4; MAb, monoclonal antibody; MRP, multidrug resistance protein; TSB, Tris-sucrose buffer.

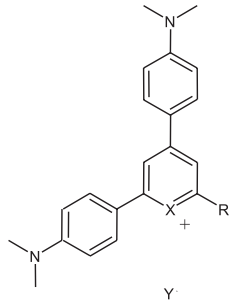
Of eight MRP1 homologs, two (MRP2 and MRP4) are known contributors to xenobiotic disposition and elimination, although neither is thought to play a significant role in resistance in human tumors (Imaoka et al., 2007; Nies and Keppler, 2007; Krishnamurthy et al., 2008; Russel et al., 2008; Slot et al., 2011). Although the substrate specificities of MRP1, MRP2, and MRP4 vary, all three can transport $E_2$17βG, and are inhibited by MK-571. Little is known about the effect of P-glycoprotein or MRP1 modulators on MRP2 and/or MRP4, and further, few (if any) inhibitors specific for the latter transporters have been identified. However, MRP2 and MRP4 transport can be modulated by an array of organic anions, some of which are therapeutically useful agents (Bakos et al., 2000; de Wolf et al., 2007; El-Sheikh et al., 2007). Given the contributions of MRP2 and MRP4 to the pharmacokinetic profiles of numerous therapeutic agents (and hence their efficacy and/or safety) (Russel et al., 2008; Lagas et al., 2009), such information is clinically relevant.

We recently compared the effects of a series of compounds containing different chalcogen atoms (O, S, Se, Te; International Union of Pure and Applied Chemistry group 16) in a pyrylium core with various 2-, 4-, and 6-position substituents on P-glycoprotein and MRP1 function (Ebert et al., 2012). These chalcogenopyrylium (CGP) derivatives are lipophilic cations that were designed based on the ability of related rhodamine-based photosensitizers to modulate P-glycoprotein (Sawada et al., 2008). Although both the rhodamines and CGPs incorporate chalcogen atoms at the 1 position of their trisubstituted pyrylium core, a key structural distinction is the flexibility of the CGP backbone versus the more rigid rhodamines. In our earlier study (Ebert et al., 2012), we examined four classes of CGPs (classes I–IV) and noted similarities and differences in their effects on MRP1 and P-glycoprotein. In the present study, we have identified a fifth structurally distinct class of CGPs characterized by the presence of two symmetrically disubstituted chalcogen-containing pyrylium moieties connected by a methine or trimethine linkage that modulates the vesicular transport activity of MRP1. We subsequently determined the relative potency of a subset of compounds comprising all five classes of CGPs as well as their ability to inhibit MRP1 in a dye efflux assay. Finally, the specificity of the five most potent MRP1 inhibitors was determined by measuring their effects on MRP2 and MRP4 transport.

Materials and Methods

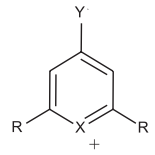
Synthesis and Characterization of Chalcogenopyrylium Dyes. The syntheses of 15 of the 22 CGPs examined in the present study have been described previously, and these compounds have been assigned to one of four structural classes designated CGP I–IV (Fig. 1) (Ebert et al., 2012). Five of 7 compounds (V-1, V-2, V-5, V-6, V-7) comprising a fifth class of structurally distinct CGPs (Fig. 2A) were selected from a library of chalcogenopyrylium methine and trimethine dyes, previously prepared by literature methods for evaluation as photosensitizers (Detty and Murray, 1982; Powers et al., 1989; Detty et al., 1990; Bellnier et al., 1999). Two additional class V CGPs (V-3, V-4) were synthesized as follows [using starting materials as described (Anderson and Stang, 1976; Leonard et al., 1999; Simard et al., 2000)]: 1)

Class I



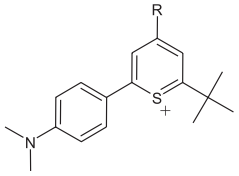
CGP	X	R	% inhibition[a]
I-2	Se	Bu	70
I-3	S	tBu	94
I-4	Se	tBu	96
I-5	Te	tBu	96

Class II



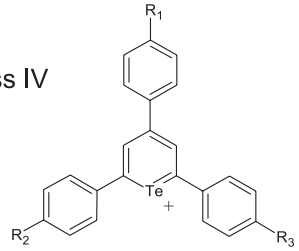
CGP	X	Y	R	% inhibition[a]
II-13	S		tBu	71
II-14	Se		tBu	81

Class III



CGP	R	% inhibition[a]
III-1		93
III-2		96

Class IV

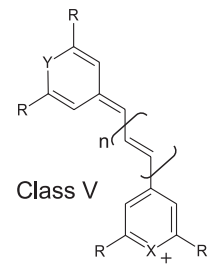


CGP	R1	R2	R3	% inhibition[a]
IV-1		H	NH_2	96
IV-3	H			89

[a]Ebert et al (2012)

Fig. 1. Chemical structures of class I–IV CGPs examined in this study. The structures of 10 CGPs belonging to four distinct chemical classes shown previously to inhibit MRP1 transport activity are illustrated. [a]Percentage inhibition of ATP-dependent [^{3}H]$E_2$17βG uptake by MRP1-enriched inside-out membrane vesicles (tested at a single concentration of 30 μM except for CGP I-2, which was tested at 5 μM; data from Ebert et al., 2012).

A



	X	Y	R	n
V-1	Te	Te	Ph	0
V-2	Se	Se	tBu	0
V-3	Se	O	tBu	0
V-4	Te	O	tBu	0
V-5	Te	S	tBu	0
V-6	Se	Se	tBu	1
V-7	Te	O	tBu	1

B

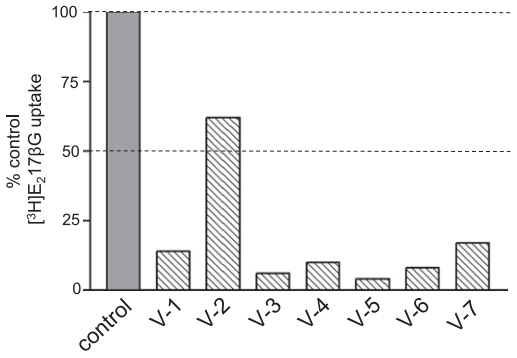


Fig. 2. Chemical structures and effect of class V CGPs on MRP1-mediated $E_2 17\beta G$ uptake into inside-out membrane vesicles. (A) Shown is the backbone chemical structure of the class V CGPs with the functional groups of the seven class V CGPs examined in this study. (B) [^{3}H]$E_2 17\beta G$ uptake was measured in the presence of a single concentration of class V CGP (30 μM except for V-7, which was tested at 5 μM). Bars represent the means of values obtained in two independent experiments. Control: 1% DMSO (vehicle, solid bar).

CGP V-3: (2,6-di-*tert*-butyl-4-((2,6-di-*tert*-butyl-*4H*-pyran-4-ylidene)methyl) selenopyrylium trifluoromethanesulfonate)-2,6-di-*tert*-butyl-4-methylpyrylium trifluoromethanesulfonate (0.077 g, 0.25 mmol) and 2,6-di-*tert*-butyl-*4H*-selenopyran-4-one (0.10 g, 0.25 mmol) in acetic anhydride (2 ml) were heated at 130°C for 2 hours. The reaction mixture was diluted with 2 ml of ether and chilled, precipitating a dark-purple product, which was collected by filtration and washed with ether. The crude product was dissolved in a minimal amount of acetonitrile, and was triturated with diethyl ether, precipitating 0.122 g (80%) of V-3 as a dark-purple solid [m.p. 147–148.5°C; ^{1}H NMR (500 MHz, CD_2Cl_2) δ7.74 (br s, 2 H), 6.98 (br s, 2 H), 6.37 (s, 1 H), 1.535 (s, 18 H), 1.43 (s, 18 H); HRMS (EI) *m/z* 461.2308 (calculated for $C_{27}H_{41}O^{80}Se^+$: 461.2317); λ_{max} (H_2O) 550 nm (ε 9.5 $\times$ 10^4 M^{-1} cm^{-1}); elemental analysis calculated for $C_{27}H_{41}OSe\bullet CF_3SO_3$: C, 55.16; H, 6.78; found: C, 55.13; H, 6.67]. 2) CGP V-4: (2,6-di-*tert*-butyl-4-((2,6-di-*tert*-butyl-*4H*-pyran-4-ylidene)methyl)telluropyrylium hexafluorophosphate)-2,6-di-*tert*-butyl-4-methylpyrylium hexafluorophosphate (0.26 g, 0.75 mmol) and 2,6-di-*tert*-butyl-*4H*-telluropyran-4-one (0.307 g, 0.96 mmol) in acetic anhydride (0.5 ml) were heated at 130°C for 35 minutes. The product was dissolved in a minimal amount of acetonitrile and was triturated with diethyl ether, precipitating 0.42 g (86%) of V-4 as a dark-green solid [m.p. 149–153°C: ^{1}H NMR (400 MHz, CD_2Cl_2) δ7.73 (s, 2 H), 6.96 (s, 2 H), 6.65 (s, 1 H), 1.48 (s, 18 H), 1.39 (s, 18 H); ^{13}C NMR (300 MHz, CD_3CN) δ177.40, 159.37, 156.16, 122.39, 110.98, 43.43, 38.16, 32.42, 28.01; HRMS (EI) *m/z* 511.226 (calculated for $C_{27}H_{41}O^{130}Te^+$: 511.2210); λ_{max} (H_2O) 587 nm (ε 9.2 $\times$ 10^4 M^{-1} cm^{-1}); elemental analysis calculated for $C_{27}H_{41}OTe\bullet PF_6$: C, 49.57; H, 6.32; found: C, 49.63; H, 6.36].

The structures of the CGPs were verified using standard analytical methods (^{1}H NMR, ^{13}C NMR, mass spectrometry, UV-visible near infrared absorption spectroscopy, high-resolution mass spectrometry, and elemental analysis). The full chemical names and quantum fluorescence of the CGPs can be found in Supplemental Table 1. Purity of the CGPs was determined by high-performance liquid chromatography to be ≥94%. *n*-Octanol/water partition coefficients (log P values) were determined experimentally as before (Ebert et al., 2012). Stock solutions of CGPs in dimethylsulfoxide (DMSO) were prepared and kept at −20°C in the dark and diluted as needed.

Cell Culture. The human embryonic kidney (HEK) cell line and the SV40-transformed HEK293T cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine and 7.5% fetal bovine serum (FBS). A stably transfected MRP1 overexpressing the HEK cell line was generated by standard procedures using a pcDNA3.1(−) expression vector containing the entire human MRP1 cDNA transcript and selection in G418 (Ito et al., 2001b; Ebert et al., 2012). The stable cell line was designated HEK-MRP1 and maintained in DMEM/7.5% FBS supplemented with 500 μg ml^{-1} G418. All cultures were grown at 37°C in 5% CO_2/95% air. For membrane vesicle preparations, HEK-MRP1 cells were seeded onto 150-mm plates and collected at confluence.

Cells transiently expressing MRP2 and MRP4 were generated as follows. HEK293T cells were seeded at approximately 18 $\times$ 10^6 cells in 150-mm plates in DMEM/7.5% FBS. Twenty-four hours later, when cells were 90–95% confluent, pcDNA3.1(−) expression vectors containing either human MRP2 or human MRP4 cDNA (20 μg per plate) were transfected into the HEK293T cells using Lipofectamine 2000 (75 μl; Invitrogen, Carlsbad, CA) (Ito et al., 2001a; Hoque et al., 2009). After 6 hours at 37°C, the medium was replaced with fresh medium, and 48 hours later, cells were collected by centrifugation. Cell pellets were overlaid with homogenization buffer consisting of 250 mM sucrose/50 mM Tris pH 7.4/0.25 mM $CaCl_2$ with protease inhibitors (Roche, Mississauga, ON, Canada), snap frozen in liquid nitrogen, and kept at −80°C until needed.

Preparation of Membrane Vesicles. Membrane vesicles were prepared essentially as described by Loe et al. (1996). Cell pellets were thawed on ice, resuspended in homogenization buffer with protease inhibitors, and then disrupted by argon cavitation. After centrifugation at 1400*g*, the supernatant was retained, and the remaining pellet was resuspended in homogenization buffer with 0.5 mM EDTA. Following a second centrifugation at 1400*g*, the supernatants from the first and second centrifugations were combined and overlaid onto a cushion composed of 35% (w/v) sucrose/1 mM EDTA/50 mM Tris, pH 7.4. After centrifugation at 100,000*g*, the interface layer containing membranes was removed and placed in 25 mM sucrose/50 mM Tris, pH 7.4 buffer, and the membranes were collected by centrifugation again at 100,000 $\times$ *g*. The membranes were then washed with Tris-sucrose buffer (TSB; 250 mM sucrose, 50 mM Tris, pH 7.4) and centrifuged at 55,000*g*. The pellet was resuspended in TSB, and vesicles were prepared by passage through a 1-ml syringe with a 27-gauge needle. Vesicles were stored at −80°C until needed, and protein concentrations were determined using the Bradford method.

Immunoblotting for MRP Proteins. The presence of MRP1, MRP2, and MRP4 in the membrane vesicles was confirmed by immunoblot analysis. Proteins were first resolved by electrophoresis on a 7% polyacrylamide gel and then electrotransferred onto polyvinylidene fluoride membranes (Pall Corporation, Ville St. Laurent, QC, Canada). After transfer, the blots were washed in Tris-buffered saline containing 0.1% Tween-20 and blocked in 4% (w/v) skim milk powder in Tris-buffered saline/Tween 20 for 1 hour. Blots were then incubated overnight at 4°C with the human MRP1-specific monoclonal antibody (MAb) QCRL-1 (diluted 1:10,000) (Cole laboratory), MRP2-specific MAb M_2I-4 (diluted 1:5000; Alexis Laboratories, San Diego, CA), or MRP4-specific MAb M_4I-10 (diluted 1:5000; Alexis Laboratories) (Hipfner et al., 1996; Létourneau et al., 2007; Hoque et al., 2009). After washing, MRP1 and MRP2 blots were incubated with horseradish peroxidase–conjugated goat antimouse antibody (Pierce Biotechnology, Rockford, IL), and MRP4 blots were incubated with horseradish peroxidase–conjugated goat antirat antibody (Chemicon) in blocking solution for 1–2 hours and then washed before incubating with chemiluminescence blotting substrate (PerkinElmer, Woodbridge, ON, Canada) and exposing the blot to film.

MRP-Mediated Uptake of ^{3}H-Labeled Organic Anions by Inside-Out Membrane Vesicles. ATP-dependent uptake of [6,7-^{3}H]$E_2 17\beta G$ (45 Ci/$mmol^{-1}$; PerkinElmer) by MRP1-, MRP2-, and MRP4-enriched membrane vesicles was measured using a 96-well rapid filtration method, as previously described (Létourneau et al., 2007; Hoque et al., 2009). Reactions were carried out in duplicate in 96-well round-bottom plates in a final reaction volume of 30 μl in TSB. Stock solutions of CGPs were diluted as needed in TSB and then added to both the reaction mix [containing either AMP or ATP (4 mM), $MgCl_2$

(10 mM), and $E_2 17\beta G$/[^{3}H]$E_2 17\beta G$] and the membrane vesicle preparations. In some experiments, leukotriene C_4 (LTC_4)/[^{3}H]LTC_4 (146 Ci/mmol^{-1}; PerkinElmer) was used instead of $E_2 17\beta G$/[^{3}H]$E_2 17\beta G$. The reaction plate was allowed to acclimatize to 37°C (or 23°C for LTC_4 transport experiments) for 3 minutes prior to initiating the uptake reaction by adding the reaction mix (24 μl) to the membrane vesicles (6 μl). For MRP1-mediated $E_2 17\beta G$ uptake, 2 μg of vesicle protein was incubated with reaction mix containing [^{3}H]$E_2 17\beta G$ (400 nM, 20 nCi) for 3 minutes at 37°C. For MRP1-mediated LTC_4 uptake, 2 μg of vesicle protein was incubated with reaction mix containing [^{3}H]LTC_4 (50 nM, 10 nCi) for 1 minute at 23°C. For MRP2-mediated uptake of $E_2 17\beta G$, 6 μg of membrane vesicle protein was incubated with [^{3}H]$E_2 17\beta G$ (400 nM, 40 nCi) for 4 minutes at 37°C. To measure MRP4-mediated $E_2 17\beta G$ uptake, 5 μg of vesicle protein was incubated with [^{3}H]$E_2 17\beta G$ (1 μM, 60 nCi) for 10 minutes at 37°C.

[^{3}H]$E_2 17\beta G$ (or [^{3}H]LTC_4) uptake was stopped by rapid dilution in ice-cold TSB followed by rapid filtration of the wells' contents onto a Unifilter-96 GF/B filter plate using a 96-well Filtermate Harvester apparatus (Packard BioScience, Meriden, CT). Radioactivity was measured by liquid scintillation counting on a TopCount NXT Microplate Counter (PerkinElmer). To determine ATP-dependent uptake, uptake in the presence of AMP was subtracted from uptake in the presence of ATP and modulator, and was expressed as a percentage of [^{3}H]$E_2 17\beta G$ (or [^{3}H]LTC_4) uptake in the absence of modulator (control). Curve-fitting and IC_{50} values were determined using GraphPad Prism 3.0 software (GraphPad, San Diego, CA). To determine if IC_{50} values were significantly different from one another, one-way analyses of variance with ad-hoc Dunnett's tests or unpaired students *t* tests were performed using GraphPad Prism 3.0; *P* values <0.05 were considered statistically significant.

MRP1-Mediated Calcein Efflux from Intact HEK-MRP1 Cells. The effect of the CGPs on MRP1-mediated calcein efflux from intact HEK-MRP1 cells was measured using the protocol of van Zanden et al. (2005) modified as follows. HEK-MRP1 cells were plated into 96-well clear-bottom, black-sided plates precoated with 0.01% poly-L-lysine (1×10^5 cells per well in 100 μl). After 1.5 hours at 37°C, stock solutions of CGPs in DMSO that had been diluted in OptiMem (GIBCO-Invitrogen, Burlington, ON, Canada) as needed were added to the plates in a volume of 50 μl such that their final concentration was 10 μM. As a vehicle control, 50 μl of TSB was added in place of the CGPs. Reactions were carried out in triplicate. After incubating the plates for 30 minutes at 37°C, 50 μl of calcein-AM (Cedarlane, Burlington, ON, Canada; 24 μM in OptiMem with 0.1% bovine serum albumin) was added to the wells to a final concentration of 6 μM. After 15 minutes at 37°C, the medium was aspirated and replaced with 200 μl of prewarmed OptiMem with 10% FBS. After 10 minutes at 37°C to allow calcein-AM hydrolysis and calcein efflux to occur, the plate was placed on ice and the medium replaced with 200 μl of ice-cold phosphate-buffered saline. Fluorescent calcein remaining in the cells was immediately measured on a Spectramax Gemini XS fluorimeter (Molecular Devices, Sunnyvale, CA; excitation wavelength 495 nm; emission wavelength 515 nm). All of the CGPs tested had little or no fluorescence (Supplemental Table 1), and thus had no impact on the data obtained with this assay. Efflux activity was calculated from the raw fluorescence values as follows. The calcein fluorescence [arbitrary units (AU)] remaining in the vehicle-treated control HEK-MRP1 cells (no CGP) was subtracted from the AU remaining in the HEK-MRP1 cells after incubation with a CGP, and then expressed relative to the maximal value of calcein efflux, which was calculated as the difference between the AU remaining in the HEK cells (no MRP1, no CGP) minus the AU remaining in the vehicle control HEK-MRP1 cells (no CGP). To determine if the effects of the CGPs on MRP1-mediated calcein efflux were different from one another, unpaired Student's *t* tests were performed using GraphPad Prism 3.0. *P* values <0.05 were considered statistically significant.

Results

Class V CGPs Represent a Potent Class of Modulators of MRP1-Mediated [^{3}H]$E_2 17\beta G$ Vesicular Transport. In a previous study, a series of 32 CGPs were tested at a single concentration for their ability to modulate MRP1-mediated [^{3}H]$E_2 17\beta G$ vesicular uptake activity. Ten of these compounds, belonging to four different classes of CGPs, were identified that inhibited MRP1-mediated [^{3}H]$E_2 17\beta G$ uptake by >50% (70–96%) (Ebert et al., 2012) (Fig. 1). In the present study, we tested seven additional CGPs comprising a fifth class of CGPs (Fig. 2A) and found that six of these class V CGPs inhibited [^{3}H]$E_2 17\beta G$ uptake by >80% when tested at a single concentration of 30 μM, or in the case of CGP V-7, 5 μM (Fig. 2B). The only exception was CGP V-2, which inhibited $E_2 17\beta G$ uptake by only 40% at 30 μM.

Concentration-dependent inhibition experiments were then carried out to determine the relative potencies of the 16 CGPs from the five classes of CGPs showing >50% inhibition of MRP1-mediated [^{3}H]$E_2 17\beta G$ uptake. The concentration-response curves obtained exhibited a classic sigmoid shape, and representative graphs for CGPs I-5, III-1, V-3, V-4, and V-6 are presented in Fig. 3 (A–E). The IC_{50} values of the 16 CGPs ranged from 0.7 to 7.6 μM, and are summarized in Table 1. Nine of the 16 CGPs were quite potent, with IC_{50} values <2 μM. These included the two class III CGPs (III-1, III-2) which had comparable IC_{50} values of 1.6 and 2.0 μM, respectively, and two of the four class I CGPs (IC_{50} values of 1.8 μM for CGP I-3 and 0.7 μM for CGP I-4), with CGP I-4 being significantly (2-fold) more potent than CGP I-3 ($P < 0.05$). Five of six class V CGPs had comparable IC_{50} values <2 μM (0.9–1.4 μM), whereas the IC_{50} of CGP V-1 was 4 μM. On the other hand, the IC_{50} values for CGP I-2 and CGP I-5 were comparable at 3.9 and 2.5 μM, respectively ($P > 0.05$), and similarly, the two class II CGPs (CGP II-13, II-14) had IC_{50} values of 7.6 and 4.9 μM, respectively ($P > 0.05$). CGP IV-1 and CGP IV-3 had IC_{50} values of 5.3 and 3.3 μM, with CGP IV-3 being almost 2-fold more potent than CGP IV-1 ($P < 0.05$). Thus, of the nine CGPs that had IC_{50} values ≤ 2 μM, two belonged to class I (CGP I-3, I-4), two belonged to class III (CGP III-1, III-2), and five to class V (CGP V-3, V-4, V-5, V-6, V-7).

Modulation of MRP1-Mediated Calcein Efflux from Intact HEK-MRP1 Cells by CGPs. To further examine the efficacy of the 16 CGPs identified as modulators of MRP1-mediated $E_2 17\beta G$ transport, a calcein efflux assay using intact cells (the HEK-MRP1 cell line and an untransfected HEK cell line as a control) was used. Of the 16 CGPs tested, only five inhibited MRP1-dependent efflux of calcein >25% at 10 μM (Fig. 4). Three of these were from class V (CGP V-3, V-4, V-6) and inhibited calcein efflux by 66% ± 19%, 46% ± 10%, and 59% ± 23%, respectively, whereas CGP III-1 inhibited efflux by 52% ± 9%. None of the four was significantly more effective than another ($P > 0.05$). On the other hand, CGP I-5 was significantly less effective than CGP V-3, V-4, V-6, and III-1 ($P < 0.05$) and inhibited calcein efflux by only 23% ± 4%. The complete structures of these five CGP modulators of MRP1-mediated calcein efflux from intact cells are shown in Supplemental Fig. 1.

Class V CGPs V-3, V-4, and V-6 Are Also Potent Modulators of MRP1-Mediated [^{3}H]LTC_4 Vesicular Transport. Because V-3, V-4, and V-6 were the most active class V CGPs in the calcein efflux assay as well as the [^{3}H]$E_2 17\beta G$ transport assay, it was of interest to determine if these compounds inhibited the transport of LTC_4, an MRP1 substrate with a binding site known to be pharmacologically distinct from the binding site for $E_2 17\beta G$ (Maeno et al., 2009). The IC_{50} values (LTC_4 transport) obtained for V-3, V-4, and V-6 were 3.7 ± 0.6, 7.6 ± 1.5, and 1.3 ± 0.4, respectively.

CGPs I-5, III-1, and V-3, V-4, and V-6 Differentially Modulate MRP2-Mediated Vesicular Transport of [^{3}H]$E_2 17\beta G$. To determine whether the five CGPs that were active in both MRP1 transport assays were specific inhibitors of MRP1, or could modulate the activity of other MRP transporters, membrane vesicles were prepared from transfected HEK293T cells expressing human MRP2 (as confirmed by immunoblotting) (Fig. 5A). The relative potencies of the five CGPs

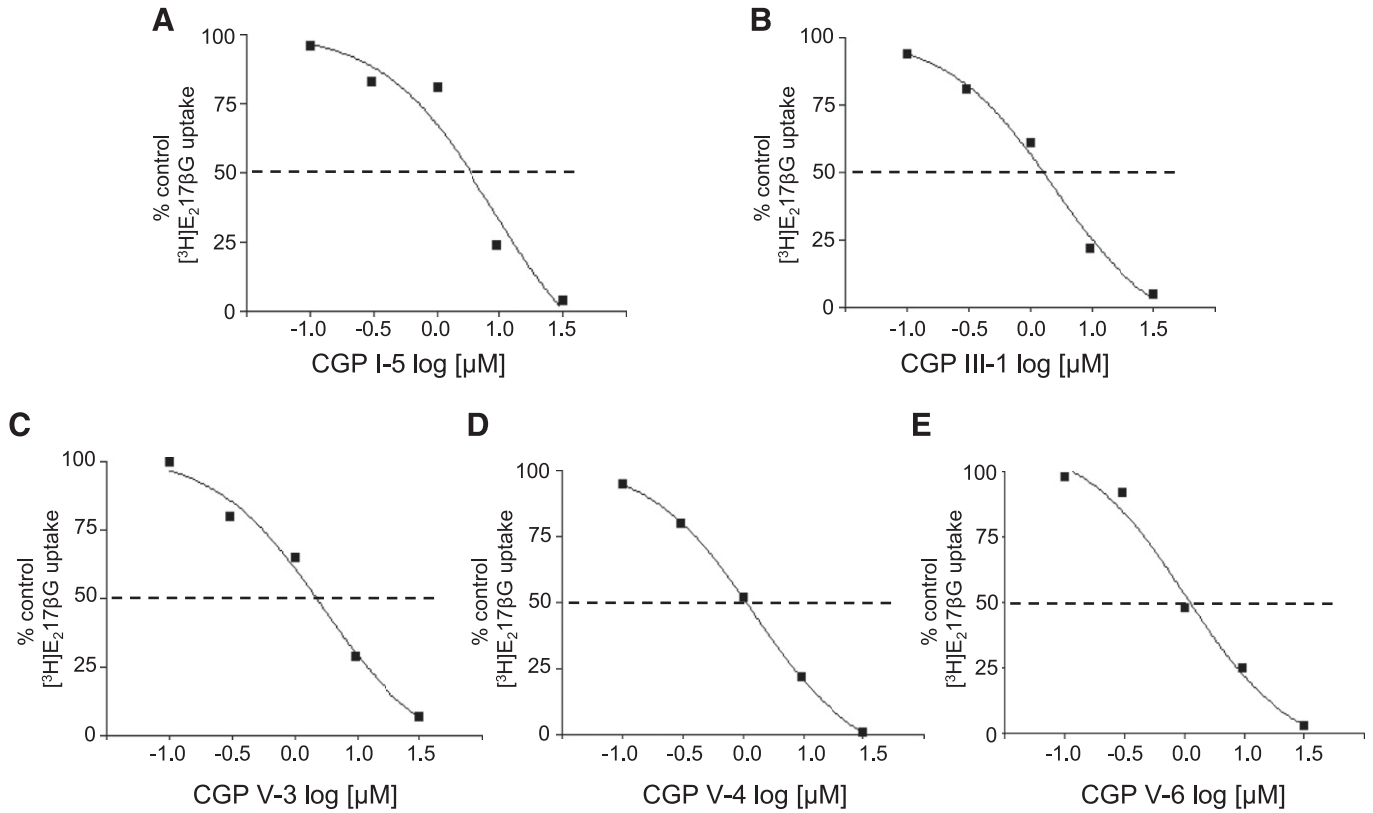


Fig. 3. Concentration dependence of CGP-mediated inhibition of $E_2 17\beta G$ uptake by MRP1. Shown are representative concentration-response curves illustrating the effects of increasing concentrations of selected CGPs on MRP1-mediated uptake of [^{3}H]$E_2 17\beta G$ into inside-out MRP1-enriched membrane vesicles. Each data point represents the mean of duplicate determinations. (A) CGP I-5; (B) CGP III-1; (C) CGP V-3; (D) CGP V-4; and (E) CGP V-6. Similar results were obtained in 2–3 additional independent experiments, and means (± S.D.) can be found in Table 1.

on MRP2-mediated [^{3}H]$E_2 17\beta G$ uptake were then determined by concentration-response experiments in the presence of five different concentrations of CGP (to a maximum of 30 μM). CGP I-5 was ineffective over the entire concentration range tested (0.1–30 μM) (Fig. 5B), whereas the IC_{50} values for CGPs V-4 and V-6 were 2.0 (± 1.3) μM ($n = 3$) and 9.2 (± 2.0) μM ($n = 3$), respectively (Fig. 5, E and F). In contrast, CGPs III-1 and V-3 stimulated [^{3}H]$E_2 17\beta G$ uptake activity to a maximum of 238% (± 31%) ($n = 4$) and 224% (± 44%) ($n = 4$) at approximately 30 μM (Fig. 5, C and D).

TABLE 1

Relative inhibitory potencies of CGPs on MRP1-mediated $E_2 17\beta G$ uptake

CGP	Chalcogen Atom	IC_{50}[a] ($E_2 17\beta G$ Uptake)	Log P[b]
		μM	
Class I			
I-2	S	3.9 ± 1.9 (3)	1.6 ± 0.1
I-3	S	1.8 ± 0.1 (3)	1.5 ± 0.2[c]
I-4	Se	0.7 ± 0.3 (3)	1.6 ± 0.1[c]
I-5	Te	2.5 ± 0.5 (4)	1.6 ± 0.1
Class II			
II-13	S	7.6 ± 3.1 (3)	1.0 ± 0.1
II-14	Se	4.9 ± 3.9 (3)	1.1 ± 0.1
Class III			
III-1	S	1.6 ± 0.4 (3)	2.1 ± 0.1
III-2	S	2.0, 1.8	0.5 ± 0.1
Class IV			
IV-1	Te	5.3 ± 0.7 (3)	1.4 ± 0.1
IV-3	Te	3.3 ± 0.6 (3)	1.1 ± 0.1
Class V			
V-1	Te	4.7, 3.2	2.5 ± 0.2
V-3	Se	1.1 ± 0.4 (4)	1.9 ± 0.1
V-4	Te	1.2 ± 0.2 (3)	1.9 ± 0.1
V-5	Te	1.2, 0.9	1.8 ± 0.2
V-6	Se	0.9 ± 0.1 (3)	1.9 ± 0.1[d]
V-7	Te	1.4, 1.4	2.4 ± 0.1[d]

[a] The values shown represent the means ± S.D. (n) of IC_{50} values obtained in 2–4 independent experiments. When the compound was tested only twice, both values obtained are shown.
[b] The values shown are experimental log P values that were derived as described in *Materials and Methods*.
[c] From Sawada et al. (2008).
[d] From Detty et al. (1990).

CGPs V-3, V-4, V-6, I-5, and III-1 Are Effective Modulators of MRP4-Mediated Vesicular Transport of [^{3}H]$E_2 17\beta G$ at 10 μM but Not 1 μM. The five CGPs that were active in both MRP1 assays (I-5, III-1, V-3, V-4, V-6) were also tested for their ability to modulate [^{3}H]$E_2 17\beta G$ uptake by MRP4. Following transfection of a human MRP4 cDNA expression vector into HEK293T cells, membrane vesicles were prepared and the presence of the transporter confirmed by immunoblotting (Fig. 6A). In contrast to MRP1 and MRP2, two immunoreactive bands were detected with the MRP4-specific MAb M_4I-10. The reason for the two bands is currently unknown, but they have been observed previously and seem likely to be the result of variable MRP4 glycosylation (Hoque et al., 2009).

When tested at 1 μM, none of the five CGPs (I-5, III-1, V-3, V-4, or V-6) inhibited [^{3}H]$E_2 17\beta G$ uptake by MRP4 by >20%. However, when tested at 10 μM, [^{3}H]$E_2 17\beta G$ uptake was inhibited by >75% (77–86%) by all five CGPs (Fig. 6B).

Effect of CGP III-1 Analogs on MRP2- and MRP4-Mediated Vesicular Transport of [^{3}H]$E_2 17\beta G$. In our previous study, five analogs of CGP III-1 (Supplemental Fig. 2) were examined for their ability to modulate MRP1-mediated [^{3}H]$E_2 17\beta G$ uptake activity, but none were shown to be more efficacious than the parent compound (Ebert et al., 2012). In the present study, the five analogs were also tested for their effects on MRP2- and MRP4-mediated [^{3}H]$E_2 17\beta G$

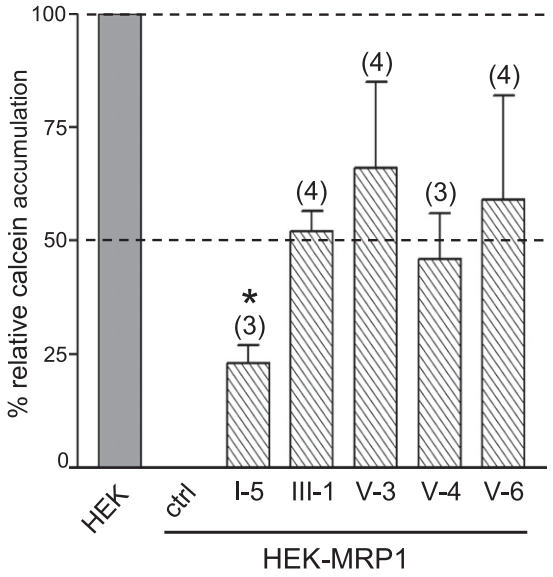


Fig. 4. Effect of CGPs on cellular efflux of calcein from HEK-MRP1 cells. The ability of the indicated CGPs (10 μM) to inhibit calcein efflux from HEK-MRP1 cells was measured (hatched bars) and compared with the relative difference in efflux from control HEK-MRP1 cells (ctrl). The bars represent the means (± S.D.) of 3–4 independent experiments, each performed in triplicate. *II-5 is a significantly less effective inhibitor of calcein efflux than CGP V-3, V-4, V-6, and III-1 ($P < 0.05$); however, there were no significant differences between CGP V-3, V-4, V-6, and III-1 ($P > 0.05$).

uptake (Supplemental Fig. 3). At 1 μM, CGP III-1-1, -2, and -3 had no effect on [^{3}H]$E_2$17βG uptake by MRP2, whereas CGP III-1-4 and -5 stimulated uptake 1.5-fold (Supplemental Fig. 3A). When tested at 10 μM, four of the five CGP III-1 analogs stimulated [^{3}H]$E_2$17βG uptake to varying degrees. The exception was CGP III-1-1, which still had no effect. Stimulation by CGP III-1-2 was weak (1.2-fold at 10 μM), whereas stimulation by CGP III-1-3 and III-1-5 was slightly greater (1.4-fold) at the same concentration. CGP III-1-4 appeared to be the most effective of the five analogs (1.7-fold stimulation) and was comparable to the parent CGP III-1 at 10 μM (1.8-fold stimulation). Taken together, the data indicate that, although some of the CGP III-1 analogs retained the ability to stimulate MRP2-mediated transport, the efficacy of only one of them approached that of parent CGP III-1.

The CGP III-1 analogs were also tested for their effects on MRP4-mediated [^{3}H]$E_2$17βG uptake (Supplemental Fig. 3B). Little or no modulation was seen at 1 μM. However, at 10 μM, three of five CGP III-1 analogs (III-1-1, -3, and -4) inhibited [^{3}H]$E_2$17βG uptake by MRP4 by approximately 60%. The other two (III-1-2 and -5) inhibited uptake by <20% at this concentration. Thus, none of the CGP III-1 analogs were as effective as the parent compound, which inhibited MRP4-mediated [^{3}H]$E_2$17βG uptake by 77% at 10 μM. Overall, the CGP III-1 analogs showed a similar pattern of inhibition of [^{3}H]$E_2$17βG uptake by MRP1 and MRP4, whereas for MRP2, the five analogs either had no effect or stimulated uptake, although to a lesser extent than the parent compound (Supplemental Fig. 3C).

Discussion

Over the past two decades, there has been widespread interest in the identification and development of modulators of MRP1, P-glycoprotein, and other drug transporters because of their influence on cellular accumulation of xenobiotics and hence efficacy and toxicity of antineoplastic and other therapeutic agents. Modulators have typically been identified by either testing drugs already in clinical practice (or derivatives thereof) or screening libraries of natural product or synthetic chemical entities. As rhodamine derivatives originally developed as photosensitizers and subsequently determined to be modulators of P-glycoprotein and/or MRP1, the CGPs are an example of the latter (Tombline et al., 2006; Sawada et al., 2008; Gannon et al., 2009; Ebert et al., 2012). In the present study, six of the seven molecules comprising a new class of CGPs (class V) tested initially at a single concentration inhibited MRP1-mediated $E_2$17βG uptake by >80%, thus identifying class V CGPs with their distinctive methine or trimethine linkage between two disubstituted pyrylium moieties as a particularly effective class of MRP1 modulators (Fig. 2).

When the 16 modulators that were identified in our initial screen and comprised all five classes of CGPs were further investigated, the IC_{50} values obtained for $E_2$17βG transport were all <10 μM (Table 1), demonstrating that, as a whole, this subset of CGPs is a potent group of MRP1 modulators. The majority of the most potent CGPs were the class V compounds described for the first time here. Thus, of nine CGPs with IC_{50} values ($E_2$17βG) <2 μM, five belonged to class V, two to class II, and two to class I.

Distinct from the class I–IV CGPs, class V compounds contain two CGP rings (either the same or different) linked by a methine or trimethine π–framework, and four identical hydrophobic groups at the 2 and 6 position of the two chalcogen-containing rings. The 2,6-tert-butyl substituents appear to impart particular potency on the class V CGPs, since CGP V-1 (which contains 2,6-phenyl substituents) is at least 3-fold less potent than the others. This difference may be related, at least in part, to differences in hydrophobicity, although the experimental log P values indicate that other factors are more important. Indeed, the length of the polymethine bridge connecting the two 2,6-disubstituted pyrylium moieties and the relative chalcogen atom electronegativity are known to impact the charge distribution (Detty et al., 1988; Calitree et al., 2007), and this is likely to influence interactions with MRP1. This may help explain the markedly different activities of the Se-containing CGP V-2 and CGP V-6, which are structurally identical except for their alkenyl bridges (1 carbon versus 3, respectively). On the other hand, the structures of the Te-containing CGP V-4 and CGP V-7 are identical except for the same 2-carbon difference in their alkenyl bridges, and yet their potencies as MRP1 modulators are the same. It may be that, for class V CGPs, charge distribution differences mediated by different chalcogen atoms can supercede charge distribution differences attributed to the linking bridge.

Of the active class I–IV CGPs, it is interesting to note that CGP I-3, I-4, and I-5 differ only in their chalcogen atom (S, Se, and Te, respectively). However, unlike class V CGPs, only one chalcogen atom mediates charge distribution. This suggests that Se confers optimal MRP1 modulating ability on this particular CGP structure, although the difference in potency relative to the S- and Te-containing analogs is modest. Thus, consistent with our previous study (Ebert et al., 2012), the identity of the chalcogen atom in these CGPs does not substantially influence their MRP1 inhibitory potency.

The vesicular transport assay used here is a convenient way to quickly and specifically identify chemical entities that interact with MRP1 (Slot et al., 2011; Ebert et al., 2012). However, because this assay measures substrate uptake into inside-out membrane vesicles, it does not take into account a potential modulator's ability to cross the membrane of an intact cell, which is a prerequisite for activity in an intact organism. Thus, testing the CGPs in an intact cell system was an important next step in their evaluation. However, when the 16 CGPs shown to be inhibitors in the vesicular transport system were investigated for their ability to inhibit calcein efflux from MRP1-expressing cells, most showed little activity. Indeed, only five inhibited calcein efflux by >23%. Four of these (CGP V-3, V-4, V-6,

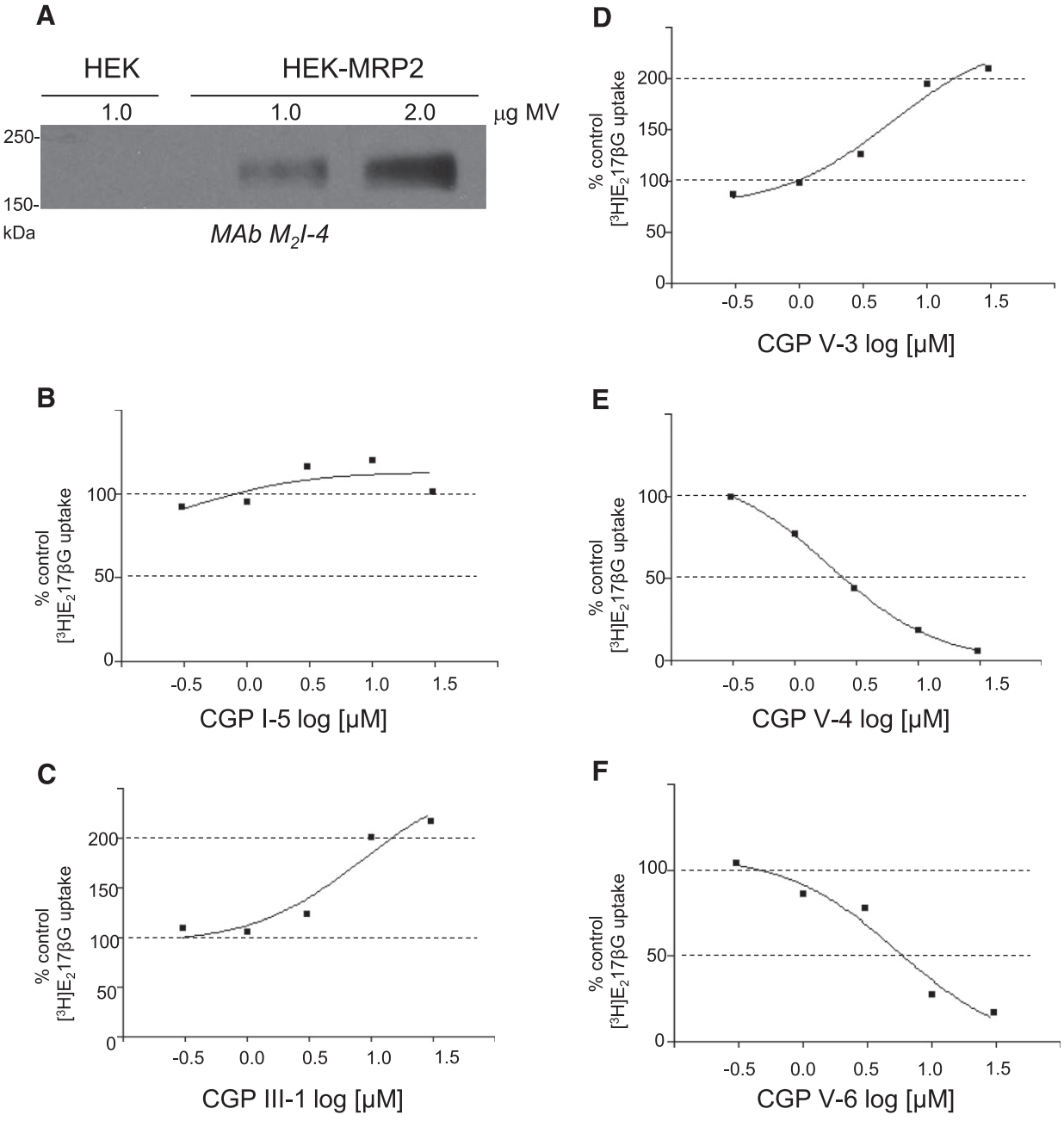


Fig. 5. Concentration-dependent effects of CGPs on MRP2-mediated uptake of [^{3}H]$E_2$17βG into inside-out membrane vesicles. (A) Immunoblot of membrane vesicle proteins prepared from MRP2-transfected and untransfected HEK293T (HEK) cells is shown. MAb M_2I-4 was used to detect MRP2. (B–F) Effect of CGPs on MRP2-mediated [^{3}H]$E_2$17βG uptake activity. Results shown are representative concentration response curves. Data points represent means of duplicate determinations. (B) CGP I-5; (C) CGP III-1; (D) CGP V-3; (E) CGP V-4; and (F) CGP V-6. Similar results were obtained in at least two additional independent experiments. Means (± S.D.) can be found in the text.

and III-1) were significantly more effective than the fifth (CGP I-5) (Fig. 4). Thus, as observed in the vesicular transport assay, the class V CGPs show particular effectiveness against MRP1 in the cellular dye efflux assay. This may be related to the hydrophobicity of these compounds conferred by their four *tert*-butyl substituents, which would be expected to facilitate their uptake across the plasma membrane into the cell. These observations in intact cells support the conclusion that chemical structures composed of two 2,6-di-*tert*-butyl substituted chalcogenopyrylium moieties linked by a methine or trimethine bridge represent a particularly potent group of MRP1 modulators that have a strong potential to be active in vivo.

ABC proteins show little sequence conservation in their membrane, spanning domains which are largely responsible for determining each transporter's distinct substrate profile. Even among the MRPs, the sequence conservation of their membrane spanning domains (MSDs) is rather limited, although they do share the common ability, at least in vitro, to transport various organic anions, a property that distinguishes them from P-glycoprotein, which transports only hydrophobic or neutral compounds. However, each of the MRPs has its own substrate profile, and although there are instances of substrate (and modulator) overlap, this overlap does not necessarily correlate well with sequence similarity among the proteins (Zelcer et al., 2001). For example,

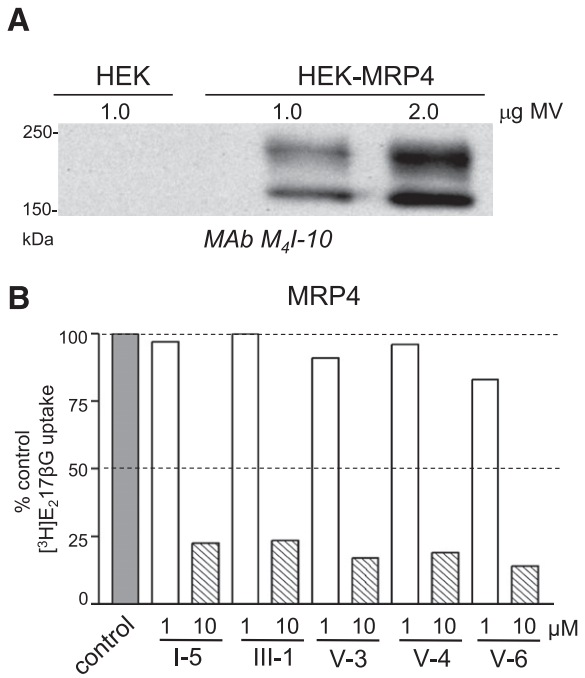


Fig. 6. Effect of CGPs on MRP4-mediated uptake of [^{3}H]$E_2$17βG into inside-out membrane vesicles. (A) Immunoblots of membrane vesicle proteins prepared from MRP4-transfected and untransfected HEK293T (HEK) cells are shown. MAb M_4I-10 was used to detect MRP4. (B) Effect of selected CGPs on MRP4-mediated [^{3}H]$E_2$17βG uptake activity. Bars represent the means of values obtained in two independent experiments. Open bars, 1 μM CGP; hatched bars, 10 μM CGP; solid bar, 1% DMSO (vehicle control). MV, membrane vesicles.

MRP1 and MRP2 are good transporters of the proinflammatory cysteinyl leukotriene C_4, whereas MRP3 and MRP4 are not. Further, the potent glutathione-dependent tricyclic isoxazole inhibitor of MRP1, LY475776, does not interact with MRP2, MRP3, 4 or 5 (Mao et al., 2002; Dantzig et al., 2004; Norman et al., 2005).

In this study, we took advantage of the shared ability of MRP1, MRP2, and MRP4 to transport $E_2$17βG to examine the specificity of the five most potent CGP inhibitors of MRP1. Despite the fact that MRP1 and MRP4 share relatively few common substrates (and relatively less sequence conservation) than MRP1 and MRP2, all five CGPs (I-5, III-1, V-3, V-4, and V-6) were good inhibitors of $E_2$17βG uptake by MRP4, causing >75% inhibition at 10 μM (Fig. 6). In contrast, these five CGPs had some unanticipated effects on $E_2$17βG uptake by MRP2 (Fig. 5) that were quite distinct from their effects on MRP1 and MRP4. First, CGP I-5 had no effect on MRP2-mediated $E_2$17βG uptake even at 30 μM, a concentration that inhibited transport by MRP1 and MRP4 by >80% (Fig. 5B). Thus, since MRP1 and MRP4 are significantly less related to one another than MRP1 and MRP2, CGP I-5 is an example of a modulator whose activity against these three MRPs does not correlate with their relative sequence conservation.

The second distinctive effect of the five CGPs on MRP2 was that, whereas two of them (V-4, V-6) inhibited transport, two others (V-3, III-1) stimulated transport (>2-fold). Previous studies have identified several organic anions that inhibit one MRP transporter while stimulating another, leading to the conclusion that some MRPs contain at least two interacting ligand binding sites (Bakos et al., 2000; Zelcer et al., 2003; Wittgen et al., 2011). For example, both sulfinpyrazone and probenicid inhibit MRP1 and MRP4 but stimulate MRP2 (Bakos et al., 2000; Ito et al., 2001a; Smeets et al., 2004; Huisman et al., 2005). Consequently, the opposite effects observed for CGP V-4 and V-6 on MRP1 (inhibitory) versus MRP2 (stimulatory) are in themselves not surprising. On the other hand, the present observations are quite remarkable when it is noted that CGP V-3 and V-4 have completely opposite effects on MRP2 activity, and yet are structurally identical except for their chalcogen atom (Se versus Te) (Fig. 5, D versus E). This is the first time (to our knowledge) that the ability to stimulate versus inhibit MRP2 transport can be attributed to not only a single atom, but in particular, to a chalcogen atom. The significant difference in Se and Te electronegativity (Pauling scale 2.55 versus 2.10), which changes the inductive character of the carbon-chalcogen bond as well as the distribution of electron density in the π framework linking the two pyrylium moieties, appears to be sufficient, at least in class V compounds, to change dramatically the interactions with MRP2.

In conclusion, we have shown that CGPs are effective modulators not only of MRP1 but also its homologs MRP2 and MRP4. We have further identified class V CGPs with their distinctive linkage between two disubstituted chalcogenopyrylium moieties as a particularly effective class of MRP modulators in both membrane vesicle and intact cell assays, suggesting they are likely to be effective in vivo. Finally, we have shown that within the class V core structure, differences in the electronegativity associated with a chalcogen atom can be the sole determinant of whether a compound will stimulate or inhibit MRP2.

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Authorship Contributions

Participated in research design: Myette, Conseil, Cole.
Conducted experiments: Myette, Conseil, Detty, Ebert, Wetzel.
Contributed new reagents or analytic tools: Conseil, Detty, Ebert, Wetzel.
Performed data analysis: Myette, Conseil, Cole, Detty, Ebert, Wetzel.
Wrote or contributed to the writing of the manuscript: Myette, Conseil, Detty, Cole.

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