



Thiorhodamines containing amide and thioamide functionality as inhibitors of the ATP-binding cassette drug transporter P-glycoprotein (ABCB1)

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ABSTRACT

Twelve thiorhodamine derivatives have been examined for their ability to stimulate the ATPase activity of purified human P-glycoprotein (P-gp)-His₁₀, to promote uptake of calcein AM and vinblastine into multidrug-resistant, P-gp-overexpressing MDCKII-MDR1 cells, and for their rates of transport in monolayers of multidrug-resistant, P-gp-overexpressing MDCKII-MDR1 cells. The thiorhodamine derivatives have structural diversity from amide and thioamide functionality (*N,N*-diethyl and *N*-piperidyl) at the 5-position of a 2-thienyl substituent on the thiorhodamine core and from diversity at the 3-amino substituent with *N,N*-dimethylamino, fused azadecalin (julolidyl), and fused *N*-methylcyclohexylamine (half-julolidyl) substituents. The julolidyl and half-julolidyl derivatives were more effective inhibitors of P-gp than the dimethylamino analogues. Amide-containing derivatives were transported much more rapidly than thioamide-containing derivatives.

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1. Introduction

Multidrug resistance (MDR) often emerges in the treatment of cancer following exposure of the patient to chemotherapeutic agents. While MDR appears from a variety of mechanisms, efflux proteins that are members of the ATP-binding cassette (ABC) super-family are most commonly associated with the emergence of MDR.^{1–3} P-glycoprotein (P-gp, also known as MDR1, encoded by *ABCB1*)^{1–5} is one member of the ABC superfamily and was the first efflux protein identified and associated with MDR in cancer chemotherapy. Many approaches to the development of inhibitors/modulators of P-gp have been examined. While a large number of compounds, possessing diverse chemical structures and biological activities, are able to reverse MDR, there are currently no approved reversal agents available in the clinic.^{3,6,7}

Rhodamine and rosamine dyes with small structural changes have been used to assay P-gp-mediated transport giving large changes (>100-fold) in rates of transport.^{8–10} Efflux of rhodamine 123 from cells was used to define P-gp transport substrates/antagonists in a cross-correlation of drug resistance patterns in the NCI 60 set of cells with the NCI Drug Screen Database of compounds.^{11,12} Interaction of rhodamine and rosamine dyes with

P-gp can also be assayed for their ability to stimulate P-gp ATPase activity. Our work with rhodamine/rosamine-related structures has demonstrated that small structural changes give >1000-fold range of P-gp affinities (as measured by K_M) with some rhodamine/rosamine molecules being highly stimulating for ATPase activity. Some of the rhodamine/rosamine-related compounds were found to be potent inhibitors of P-gp drug transport.^{13,14}

In more recent work, it was demonstrated that essentially single-atom changes—that is, interchanging amide and thioamide functionality—in a small series of rosamine/rhodamine structures gave either molecules with high affinity for P-gp and high stimulation of ATPase activity or molecules with high affinity but low stimulation of ATPase activity.¹⁵ Specifically, tertiary amide groups on the 9-aryl or heteroaryl substituent gave high P-gp ATPase stimulation while tertiary thioamide groups on the 9-aryl or heteroaryl substituent gave low stimulation of ATPase activity. The single-atom change of amide to thioamide also slows the rate of P-gp-mediated transport of the rhodamine derivatives in both absorptive and secretory directions in the cell. In addition, the substitution of a fused azadecalin for a dimethylamino substituent in the xanthylium core also gave higher affinity for P-gp in both isolated protein and in whole cells.

The chalcogenorhodamines are also photosensitizers for treatment of multidrug-resistant cancer cells in vitro with photodynamic therapy (PDT) via the generation of singlet oxygen from a

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biopolymer-bound chalcogenorhodamine.^{16–18} Understanding the parameters that impact ATPase activity and binding affinity in rhodamine-like modulators of P-gp and the factors that influence the drug availability to the pump in the native membrane environment are critical for the development of more potent rhodamine-like inhibitors of P-gp that may have a dual application as photosensitizers for treatment of multidrug-resistant cells by PDT.

To examine further the roles of the amide/thioamide ‘switch’ and the lipophilicity/hydrophobicity of one amino substituent on P-gp ATPase stimulation, inhibition of P-gp-mediated efflux in whole cells, and transport of the chalcogenorhodamines in whole cells, the series of amide- and thioamide-containing thiorhodamines **1–12** (Chart 1) were prepared. The thiorhodamine core was selected (1) to avoid any ambiguities associated with the chalcogen atom and 2) because a thiorhodamine was the best inhibitor in earlier work.¹⁵ The thiorhodamines were evaluated for their ability to stimulate ATPase activity in isolated, wild-type human P-gp, their ability to be transported in multidrug-resistant, P-gp over-expressing cells, and their ability to inhibit P-gp drug transport in multidrug-resistant cells.

2. Results and discussion

2.1. Synthesis of thiorhodamine analogues 1–12

Thiorhodamines **1–12** were prepared from thioxanthenes **13–15** (Chart 2), which were prepared by reported procedures.^{19,20} Compound **14** replaces a dimethylamino substituent with an azadecalin substituent. The fused aniline equivalent of this substitution is known as julolidine (Chart 2) and compounds derived from **14** are referred to as ‘julolidyl’ rhodamines in the remainder of the manuscript. Thioxanthone **15** incorporates a trimethyltetrahydroquinoline group (Chart 2) and compounds derived from **15** are referred to as ‘half-julolidyl’ rhodamines in the remainder of the manuscript. The thioxanthone **15** has the same number of carbon atoms as **14**, but the *gem*-dimethyl group adds the potential for steric interactions absent in **14**.

Thioxanthone **15** is a previously unknown structure and was prepared as outlined in Scheme 1. *N*-Methylaniline was first

substituted with 1-chloro-3-methyl-2-butene in the presence of K₂CO₃, and the aniline product **16** was obtained in 78–87% isolated yields. Cyclization of **16** with concentrated sulfuric acid gave **17** in isolated yields of 70–80%. Subsequent Vilsmeier–Haack reaction with POCl₃ and dimethylformamide (DMF) converted **17** to aldehyde **18** in yields of >96%. Oxidation of **18** to a carboxylic acid oxidation state was problematic as was observed for the oxidation of 9-formyljulolidine.²⁰ A modified Willgerdt–Kindler²¹ reaction oxidized **18** to the thioamide **19** in 75–83% isolated yields using elemental sulfur and piperidine in refluxing DMF.²² Amide **20** was formed from **19** using trifluoroacetic anhydride (TFAA) in isolated yields of >90%.¹⁹

Directed *ortho*-lithiation of **20** in THF at –78 °C with *sec*-butyllithium and *N,N,N,N*-tetramethylethylenediamine (TMEDA) was followed immediately by the addition of 3-dimethylaminophenyl disulfide (**21**)^{19,23} at –78 °C to minimize the amount of self-condensed side product formation that has been seen in similar reactions. The isolated yield of diaryl sulfide **22** was 35–45%. Subsequent cyclization of **22** with POCl₃ in acetonitrile¹⁹ gave the desired thioxanthone **15** in 90–91% isolated yield, putting the overall yield for the synthesis of the half-julolidyl thioxanthone **15** at 20%.

The reaction is regioselective for *ortho*-lithiation at the desired position. None of the isomeric diaryl sulfide **23** (Chart 3) was observed in the product mixture suggesting either that only *ortho*-lithiated intermediate **24** was formed in the reaction or that *ortho*-lithiated intermediate **25**, if formed, then hindered sterically approach of the electrophile and formation of **23** was prevented. We have been unable to *ortho*-lithiate the tetramethyljulolidyl derivative **26**²⁰ (Chart 3) under a variety of conditions suggesting that kinetic deprotonation next to the *gem*-dimethyl group is extremely slow and that anion **24** is likely the only lithiated species formed.

With thioxanthenes **13–15** readily available, the syntheses of dyes **1–12** followed the pathways outlined in Scheme 2. Willgerdt–Kindler oxidation of thiophene-2-carboxaldehyde with elemental sulfur and diethylamine gave thioamide **27** in 49% isolated yield.²⁴ Deprotonation of **27** with sterically bulky lithium diisopropylamide (LDA) gave the 2-thienyl anion **28**, which was

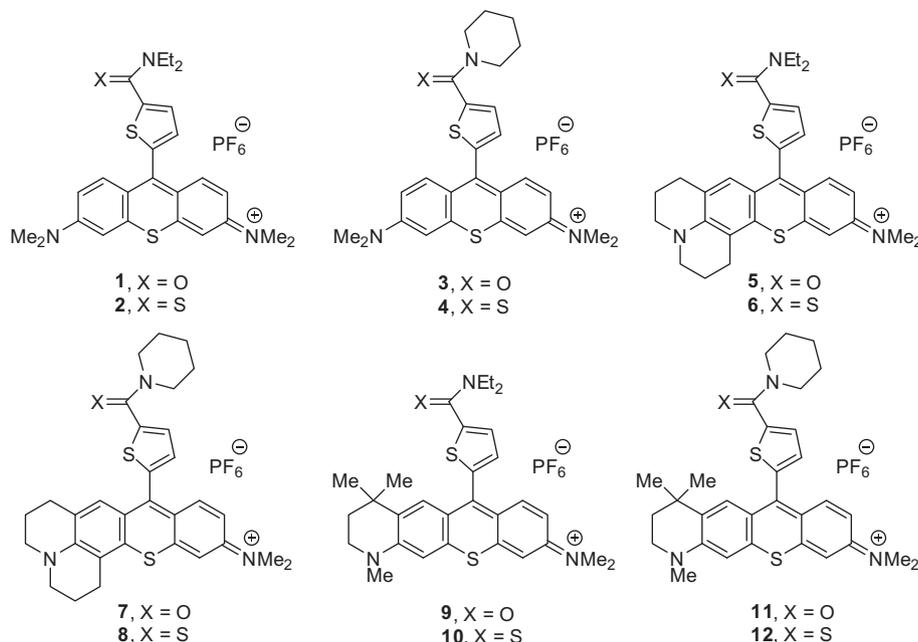


Chart 1. Thiorhodamine analogues **1–12** examined in this study.

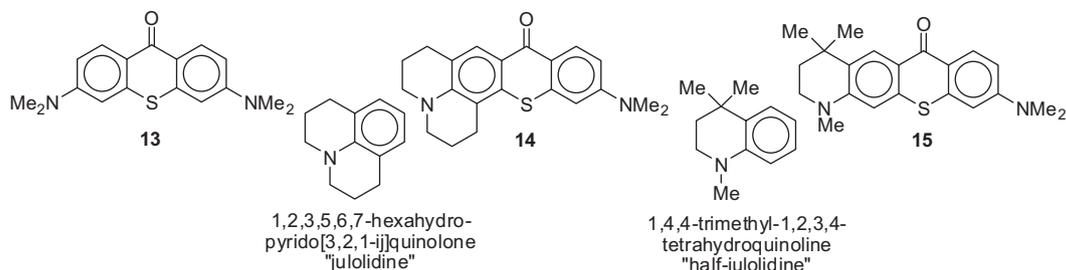
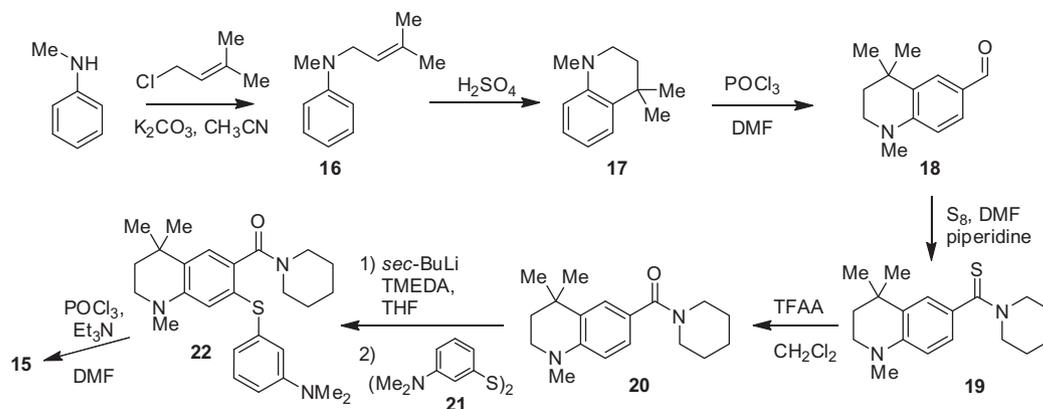


Chart 2. Thioxanthone precursors to thiorhodamines 1–12.



Scheme 1. Synthesis of thioxanthone 15.

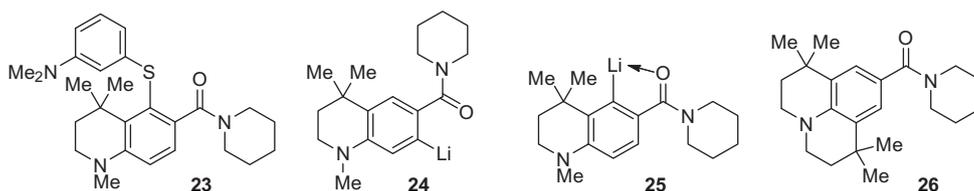
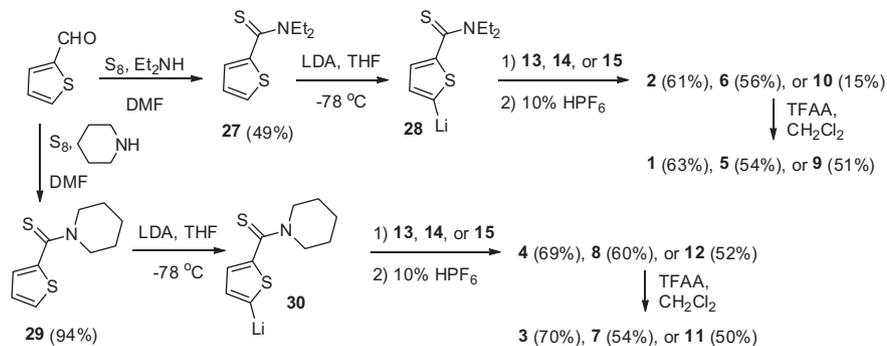


Chart 3. Possible directed lithiation intermediates, products and substrates.



Scheme 2. Syntheses of thiorhodamines 1–12 from thioxanthones 13–15.

then added to solutions of the thioxanthones **13**–**15**. Workup with aqueous HPF₆ gave the diethyl thioamide-containing dyes **2**, **6**, and **10** in 61%, 56%, and 15% isolated yields, respectively.

Unlike the tertiary amide group,²⁵ which is highly directing, the thioamide functionality does not direct lithiation in thiophenes. Only the more acidic α -proton was removed and none of the corresponding 2,3-disubstituted thiophenes were detected in the product mixtures.²⁶

The synthesis of **8** and **12** followed an earlier procedure that was developed for the preparation of thioamide-containing thiorhodamine dye **4**.¹⁵ Willgerodt–Kindler oxidation of thiophene-2-carboxaldehyde with elemental sulfur and piperidine gave thioamide **29** in 94% isolated yield (Scheme 2).²⁷ Deprotonation of **29** with LDA gave anion **30**, which was added to thioxanthone **13** to give thioamide-containing dye **4** in 69% isolated yield following workup with aqueous HPF₆.¹⁵ Similarly, the addition of **30** to

thioxanthenes **14** and **15** followed by workup with aqueous HPF₆ gave piperidyl thioamide-containing dyes **8** and **12** in 60% and 52% isolated yields, respectively.

The amide-containing thiorhodamines **1**, **3**, **5**, **7**, **9** and **11** were prepared from their thioamide counterpart via hydrolysis of the thioamide. Trifluoroacetic anhydride was added to CH₂Cl₂ solutions of thioamides **2**, **4**, **6**, **8**, **10** and **12** to give the corresponding amides in isolated yields of 51–70%.¹⁹

2.2. Measurement of *n*-octanol/water partition coefficients

Experimental values of the *n*-octanol/water partition coefficient ($\log P$) were measured using the 'shake flask' method.²⁸ A saturated *n*-octanol solution of dye **1–12** was shaken with an equal volume of phosphate buffered saline (PBS) at pH 7.4 and the concentrations in the two layers were determined spectrophotometrically. Values of $\log P$ are compiled in Table 1 and covered a range from $\log P = 1.2$ for dyes **1** and **2** to $\log P \sim 2.6$ – 2.7 for dyes **8**, **10** and **12**. Based on these values of $\log P$, thiorhodamine compounds **1–12** would have access to both aqueous and hydrophobic environments in the studies with whole cells described below.

2.3. P-gp ATPase activity with isolated human P-gp-His₁₀

The ATPase activity of thiorhodamine compounds **1–12** (Chart 1) was examined using isolated human P-gp-His₁₀, which was activated with sheep brain phosphatidylethanolamine.^{29–31} The apparent Michaelis–Menten constant (K_M) for ATPase-stimulating compounds was determined as well as the drug-induced stimulation of maximal ATPase activity (V_{max}) using isolated human P-gp-His₁₀ (Table 1). Verapamil (VER) was included as a control compound (generally considered relatively robust and one of the most stimulating drugs known for P-gp).^{32,33} ATPase activity was determined from the release of inorganic phosphate.³³ Representative curves are shown in Figure 1 for compound **1**, which was the most stimulating rhodamine for ATPase activity, and for compound **7**, which had the lowest value of K_M . For compounds **11** and **12**, which were too weakly stimulating for P-gp ATPase activity to permit determination of K_M , the concentration of compound required for 50% inhibition of VER-stimulated (4×10^{-4} M) P-gp ATPase activity (IC_{50}) was determined (Fig. 2 and Table 1).

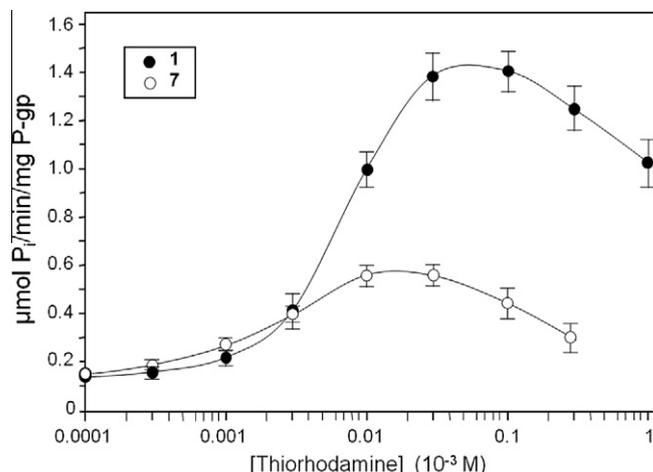


Figure 1. Stimulation of human P-gp ATPase activity by compounds **1** and **7**. Histidine-tagged P-glycoprotein was expressed in BHK cells, isolated by nickel-chelate chromatography and mixed with lipid. P-glycoprotein ATPase activity was then measured in the presence of various concentrations of thiorhodamine derivative **1** or **7**. Error bars represent one standard deviation from the mean.

Several trends emerged from the data shown in Table 1. With respect to V_{max} , values were significantly larger ($p < 0.05$, Student *t*-test) for dimethylamino analogues **1–4** (6.6–12.1-fold stimulation) relative to their corresponding julolidyl analogues **5–8** (3.5–5.6-fold stimulation) and half-julolidyl analogues **9–12** (≤ 4.7 -fold stimulation). The latter two groups did not show significant differences. There were no statistically significant differences in values of V_{max} for any specific amide/thioamide pair. With respect to K_M , values were significantly higher ($p < 0.03$ for amides, $p < 0.01$ for thioamides) for the dimethylamino analogues **1–4** [$(5.5$ – $6.4) \times 10^{-6}$ M] relative to either their corresponding julolidyl analogues **5–8** [$(1.9$ – $3.6) \times 10^{-6}$ M] or the corresponding half-julolidyl analogues **9** and **10** (2.2×10^{-6} M and 3.1×10^{-6} M), respectively). For **11** and **12** where values of K_M could not be determined, values of IC_{50} for 50% inhibition of VER-stimulated (4×10^{-4} M) P-gp ATPase activity were 1.1×10^{-6} M and 1.7×10^{-6} M, respectively. It should be noted that compound **8** showed a lower K_M (8.7×10^{-8} M) in a previous study¹⁵ because those assays were

Table 1

Stimulation of ATPase activity, inhibition of verapamil-induced ATPase activity, IC_{50} 's for calcein AM (CAM) uptake and tritiated vinblastine (VIN) efflux by thiorhodamine amide and thioamide analogues, and *n*-octanol/water partition coefficients ($\log P$) for thiorhodamines **1–12** and VER.

Compd	Isolated V_{max} , ^a fold stimulation	Human K_M , ^b 10^{-6} M	P-gp-His ₁₀ IC_{50} , ^c 10^{-6} M	MDCKII-MDR1 IC_{50} CAM uptake, 10^{-6} M ^d	Cells IC_{50} VIN efflux, 10^{-6} M ^d	$\log P$
VER	17.9 ± 2.0	24 ± 2.6	—	—	—	—
1	12.1 ± 1.6	5.7 ± 1.0	—	>100	—	1.2
2	12.1 ± 1.9	5.8 ± 0.6	—	14 ± 1	—	1.2
3	8.6 ± 2.3	6.4 ± 0.4	—	>100	—	1.5
4	6.6 ± 0.7	5.5 ± 1.3	—	ND ^f (19 ± 2) ^g	—	(1.7) ^g
5	5.6 ± 0.6	3.6 ± 0.4	—	10.0 ± 1.6	—	1.9
6	4.6 ± 1.6	2.3 ± 0.4	—	9.6 ± 1.2	—	1.7
7	4.1 ± 0.6	1.9 ± 0.4	—	56 ± 1	—	1.4
8	3.5 ± 0.9	2.5 ± 0.4	—	12.0 ± 1.1 (2.1 ± 1.2) ^g	—	(2.7) ^g
9	4.7 ± 0.3	2.2 ± 1.0	—	7.1 ± 1.2	8.0 ± 1.2	1.4
10	4.3 ± 0.5	3.1 ± 0.7	—	8.8 ± 1.1	8.7 ± 1.1	2.7
11	<2	ND ^e	1.1 ± 0.2	13.0 ± 1.1	5.6 ± 1.1	1.7
12	<2	ND ^e	1.7 ± 0.3	9.3 ± 1.1	8.9 ± 1.1	2.6

^a V_{max} is the ratio of maximum stimulation in the presence of compound to that without compound (basal activity).

^b K_M is the concentration of compound required to achieve 50% maximal stimulation.

^c IC_{50} is the concentration of compound required to inhibit 50% of verapamil-stimulated ATPase activity.

^d Details for methods are provided in Section 4. Error limits represent ± standard deviation.

^e ND, not determined because stimulation of ATPase activity too low.

^f ND, not determined because of limited solubility in BSA-buffer.

^g Values in parentheses from Ref. 15.

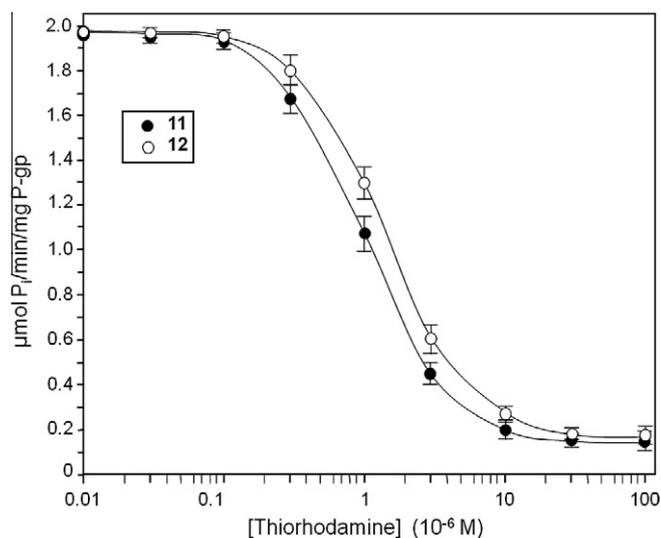


Figure 2. Inhibition of human P-gp VER-stimulated ATPase activity. Histidine-tagged P-glycoprotein was expressed in BHK cells, isolated by nickel-chelate chromatography and mixed with lipid. P-glycoprotein ATPase activity was then measured in the presence of 4×10^{-4} M VER with various concentrations of thiorhodamines **11** and **12**. Data represent the average for triplicate measurements and error bars represent the standard deviation.

performed in the presence of 10-fold lower lipid (0.5 mg/ml) and detergent (2.0×10^{-4} M dodecyl- β -D-maltoside) concentrations.

2.4. Enhancement of CAM uptake into MDCKII-MDR1 transfected cells

Thiorhodamine compounds **1–12** were also evaluated for their ability to facilitate the uptake of calcein AM (CAM) into MDCKII-MDR1 transfected cells, which over express P-gp.³⁴ The CAM uptake in these cells was determined at concentrations of 5×10^{-6} M and 2.5×10^{-5} M in the thiorhodamine and the inhibition of P-gp was compared as a percentage of the inhibition observed with 5×10^{-6} M (R)-4-[(1a,6,10b)-1,1-dichloro-1,1a,6,10b-tetrahydrodibenzo[a,e]-cyclopropa[c]cyclohepten-6-yl]-[(5-quinolinyl-oxy)methyl]-1-piperazineethanol (**31**, LSN 335984, $IC_{50} = 0.4 \times 10^{-6}$ M, Chart 4), which completely inhibits P-gp. LSN 335984 is structurally related to the P-gp-specific inhibitor (R)-4-[(1a,6,10b)-1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cyclohepten-6-yl]-[(5-quinolinyl-oxy)methyl]-1-piperazineethanol (**32**, LSN 335979 or zosuquidar, Chart 4).^{7,35} Values of the percentage inhibition at the two concentrations are shown in Figure 3.

As shown in Figure 3, all of the compounds **1–12** were weak inhibitors at 5×10^{-6} M (0–41%). At 2.5×10^{-5} M in this assay,

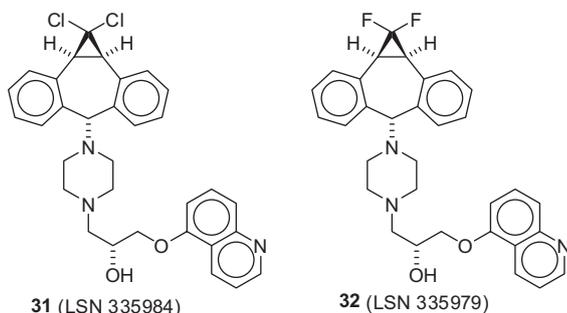


Chart 4. Structures of LSN 335984 (**31**) and LSN 335979 (**32**).

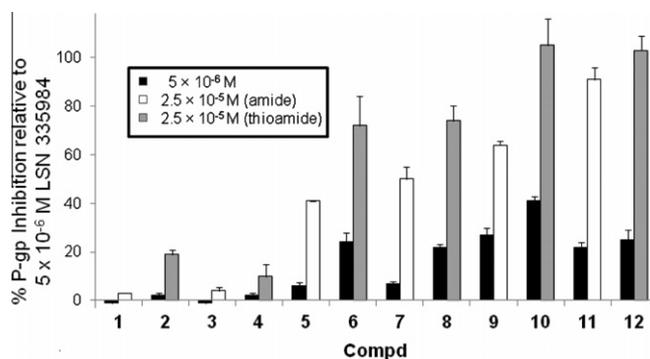


Figure 3. % Inhibition of CAM uptake by 5×10^{-6} and 2.5×10^{-5} M thiorhodamines **1–12** relative to MDCKII-MDR1 cells fully-inhibited by LSN 335984. Values shown are the average of duplicate runs and error bars show the reproducibility of the two runs. White bars show values for amides **1**, **3**, **5**, **7**, **9** and **11** at 2.5×10^{-5} M while gray bars show values for thioamide **2**, **4**, **6**, **8**, **10** and **12** at 2.5×10^{-5} M. The black bars are for the adjacent thiorhodamine at 5×10^{-6} M.

several trends emerged. In pair-wise comparisons, the amides were weaker inhibitors than the corresponding thioamides. With a given amine functionalization (dimethylamino, julolidyl, half julolidyl), the piperidyl thioamides and diethyl thioamides displayed comparable inhibition. Within the different amine functionalization, the dimethylamino analogues **1–4** were weaker inhibitors (3–19% inhibition) than their corresponding julolidyl analogues **5–8** (41–74% inhibition), which in turn were weaker inhibitors than the corresponding half-julolidyl analogues **9–12** (64–105% inhibition).

Values of IC_{50} for the enhancement of CAM uptake into MDCKII-MDR1 transfected cells by thiorhodamines **1–12** (Table 1) were determined by measuring relative fluorescence values obtained after 20-min incubation with CAM at 37 °C. Typical curves are shown in Figure 4 for CAM uptake in the presence of various concentrations of thiorhodamines **1–12**. Among the dimethylamino analogues **1–4**, the amides **1** and **3** were weaker inhibitors (IC_{50} 's for CAM uptake $>1 \times 10^{-4}$ M) than thioamide analogues **2** and **4** (IC_{50} 's of 1.4×10^{-5} M and 1.9×10^{-5} M,¹⁵ respectively). Among the julolidyl analogues **5–8**, diethyl amide **5**, diethyl thioamide **6**, and piperidyl thioamide **8** all had comparable values of IC_{50} for CAM uptake [9.6 – 12.0] $\times 10^{-6}$ M], which were lower than the IC_{50} for piperidyl amide **11** (5.6×10^{-5} M). All of the half-julolidyl amides and thioamides **9–12** had comparable values of IC_{50} for CAM uptake [(7.1–13.0) $\times 10^{-6}$ M], which were comparable to those observed for **5**, **6** and **8**. With the exception of IC_{50} for CAM uptake for half-julolidyl amide **11** relative to julolidyl amide **7** where there was a >4-fold difference in IC_{50} , there were no significant differences in the other three amide/thioamide pairs for the julolidyl and half-julolidyl derivatives **5–12** with respect to IC_{50} .

2.5. Inhibition of vinblastine efflux by thiorhodamines 9–12 in MDCKII-MDR1 cells

Vinblastine (VIN) is a clinical chemotherapeutic drug and inhibition of its transport by the thiorhodamine compounds is indicative of potential translational value. Inhibition of P-gp-mediated VIN efflux by the half-julolidyl series **9–12** was examined in MDCKII-MDR1 transfected cells. [³H]-Vinblastine, in an appropriate dilution series with **9–12** and BSA, was introduced to the basolateral chamber of a monolayer of MDCKII-MDR1 transfected cells. The appearance of [³H]-VIN in the apical chamber was monitored by scintillation counting and gave comparable values of IC_{50} of (5.6–8.9) $\times 10^{-6}$ M for **9–12** (Table 1) for inhibition of VIN efflux.

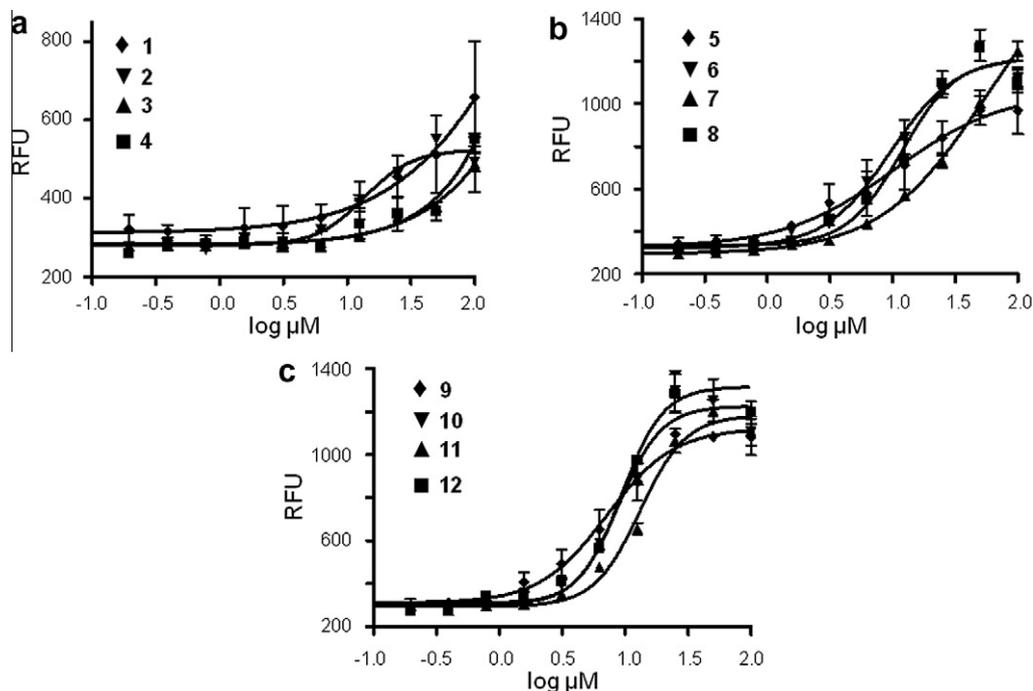


Figure 4. Uptake of CAM into MDCKII-MDR1 cells as a function of concentration of thiorhodamines 1–4 (Panel a), 5–8 (Panel b) and 9–12 (Panel c). Values of IC_{50} (Table 1) were determined by a sigmoidal dose-response (variable slope) analysis. Data represent the average and standard error mean for triplicate measurements.

2.6. Transport across monolayers of MDCKII-MDR1 cells

The transport of thiorhodamine amide and thioamide derivatives 1–12 was measured in monolayers of MDCKII-MDR1 trans-

fect cells (Table 2).³⁴ Bovine serum albumin (BSA) addition to the buffer was required because a marked fraction of mass added to the donor equilibrated with the cell monolayer for some of the compounds and this resulted in gross underestimation of the

Table 2
Transport and cell association studies of thiorhodamine amide and thioamide analogues 1–12 with MDCK-MDR1 cells^a

Compd	P_{AB} , 10^{-9} m s ⁻¹	P_{BA} , 10^{-9} m s ⁻¹	$P_{BA}(-inh)/P_{BA}(+inh)$	$P_{Passive}$, ^b 10^{-9} m s ⁻¹	% Cell associated ^c	Ratio (+/-inh) ^d
1	≤ 1	280 ± 35	22	~ 7	2.1 ± 0.4	5.7
(+inh)	≤ 1	13 ± 2			12 ± 3.5	
2	≤ 1	210 ± 17	41	~ 3	11 ± 3	5.1
(+inh)	≤ 1	5.1 ± 0.1			56 ± 11	
3	≤ 1	290 ± 9	38	~ 4	2.8 ± 1.0	3.9
(+inh)	≤ 1	7.7 ± 0.1			11 ± 3	
4	≤ 1	110 ± 35	73	~ 1	12 ± 1	4.8
(+inh)	≤ 1	1.5 ± 0.1			58 ± 2	
5	≤ 1	210 ± 22	12	~ 9	5.8 ± 0.2	4.8
(+inh)	≤ 1	17.0 ± 0.4			28 ± 3	
6	≤ 1	65.0 ± 1.5	24	~ 2	25.0 ± 0.2	1.8
(+inh)	≤ 1	2.7 ± 0.1			45 ± 6	
7	≤ 1	220 ± 29	27	~ 4	6.9 ± 0.1	6.1
(+inh)	≤ 1	8.1 ± 0.1			42 ± 7	
8	≤ 1	83 ± 3	69	~ 1	31 ± 3	2.1
(+inh)	≤ 1	1.2 ± 0.1			64 ± 9	
9	≤ 1	370 ± 17	14	~ 14	10 ± 2	3.3
(+inh)	≤ 1	27.0 ± 0.3			33 ± 6	
10	≤ 1	69.0 ± 2.5	69	~ 1	52 ± 6	1.2
(+inh)	≤ 1	1.0 ± 0.1			64 ± 7	
11	≤ 1	230 ± 24	31	~ 4	8.6 ± 0.1	5.2
(+inh)	≤ 1	7.5 ± 0.1			45.0 ± 0.1	
12	≤ 1	34 ± 22	170	< 1	34.0 ± 2.7	1.8
(+inh)	≤ 1	0.2 ± 0.1			62.0 ± 0.2	

^a Experiments were run with 5×10^{-6} M dye and 4.3 mg mL^{-1} BSA. Values of transport in the absorptive (P_{AB}) and secretory (P_{BA}) mode in the absence or presence of inhibitor, the ratio $[P_{BA}(\text{no inhibitor})/P_{BA}(\text{with inhibitor})]$, the % cell associated rhodamine analogue in the absence or presence of inhibitor, the ratio of cell associated rhodamine in the presence or absence of inhibitor. Details for methods are provided in Section 4. Error limits represent \pm standard deviation.

^b $P_{Passive}$ represents the mean of P_{AB} and P_{BA} in the fully inhibited system.

^c % Cell associated is the fraction of mass extracted from the cell monolayer by methanol wash after 1-h flux in the AB direction.

^d For % cell associated dye.

permeability coefficient.³⁶ The assay was repeated in the presence of **31** (LSN 335984),³⁵ to measure transport when P-gp was fully inhibited.

MDCKII-MDR1 monolayers display apical and basolateral polarized membranes and are considered to be a near-physiological model for studying P-gp drug transport. In the monolayer, P-gp is solely present at the apical membrane. For thiorhodamines **1–12**, transport was measured in the absorptive (apical to basolateral or AB) and secretory (basolateral to apical or BA) transport direction of the cell monolayer. Values of transport in the absorptive (P_{AB}) and secretory (P_{BA}) mode in the absence of inhibitor, passive transport ($P_{Passive}$) in the presence of inhibitor, and the % cell-associated dye in the AB direction in the absence or presence of inhibitor are compiled in Table 2.

Normally, the role of P-gp in the transport of various molecules is determined by comparison of transport in the absorptive (apical to basolateral or AB) and secretory (basolateral to apical or BA) direction of the cell monolayer, and then an efflux ratio ($P_{BA/AB}$) is calculated.³⁷ In the case of thiorhodamine compounds **1–12**, transport in the absorptive direction was too slow ($P_{AB} \leq 1 \times 10^{-9} \text{ m s}^{-1}$) for measurement and an accurate efflux ratio ($P_{BA/AB}$) could not be calculated. Consequently, a ratio of transport in the secretory direction in the absence and presence of inhibitor [$P_{BA}(-\text{inh})/P_{BA}(+\text{inh})$] is included in Table 2 instead.

Large $P_{BA/AB}$ ratios are assumed to be due to efficient P-gp-mediated efflux of the compound and we assume that the large values of $P_{BA}(-\text{inh})/P_{BA}(+\text{inh})$ observed for **1–12** (14–170, Table 2) are also consistent with these compounds being actively transported by P-gp. In every amide/thioamide pair, P_{BA} for the amide is greater than the thioamide. For the amides, P_{BA} is in the range $(2.1\text{--}3.7) \times 10^{-7} \text{ m s}^{-1}$ and, for the thioamides, P_{BA} is in the range $(0.34\text{--}2.1) \times 10^{-7} \text{ m s}^{-1}$. In the fully inhibited system, $P_{Passive}$ is slow for all of the compounds (from $<1 \times 10^{-9} \text{ m s}^{-1}$ to $1.4 \times 10^{-8} \text{ m s}^{-1}$) and values of $P_{Passive}$ are slower for the thioamide in every amide/thioamide pair.

The % cell-associated dye for thiorhodamines **1–12** was higher in the inhibited system (12–64%) relative to the uninhibited system where the pump was active (2–52%). These differences were twofold or greater for all compounds except for diethyl thioamide **10** for which the inhibited and uninhibited systems were quite similar (64% and 52%, respectively). In every amide/thioamide pair, the % cell-associated dye was higher for the thioamide relative to the amide in both the inhibited and uninhibited system. The ratio for % cell-associated dye between inhibited and uninhibited pump was comparable for both amides and thioamides for the dimethylamino analogues **1–4** (3.9–5.7). For the julolidyl analogues **5–8** and the half-julolidyl analogues **9–12**, the ratio for % cell-associated dye between inhibited and uninhibited pump was much greater for the amide analogues **5, 7, 9** and **11** (3.3–6.1) than the thioamide analogues **6, 8, 10** and **12** (1.2–2.1).

2.7. Discussion of biological results

In earlier work, we saw differences among a group of amide- and thioamide-containing chalcogenorhodamine compounds with respect to rates of transport across monolayers of MDCKII-MDR1 transfected cells and with respect to ATPase stimulation/inhibition in isolated protein.¹⁵ The amide and thioamide functionality were located on aryl or heteroaryl substituents at the 9-position of the chalcogenorhodamine core. The compound with the highest affinity for P-gp in isolated protein and with the lowest values of IC_{50} for CAM uptake was thiorhodamine **8** with the piperidyl thioamide at the 5-position of a 2-thienyl group. Compound **8** was transported extremely slowly in both secretory and absorptive directions in monolayers of MDCKII-MDR1 transfected cells while amide analogues related in structure to **8** gave much faster

transport in the secretory direction (P_{BA}). One can ask whether these observations are general for amide- and thioamide-substituted groups at the 9-position of chalcogenorhodamine analogues or whether the observations are specific for the particular compounds examined.

One might assume that molecules with closely related structures would bind to a common site in P-gp, but this assumption is challenged by several observations. The 'H' and 'R' sites Hoechst 33342 and rhodamine 123, respectively, on P-gp have been described by Shapiro and Ling indicating that there are at least two binding sites on the protein.^{29,38} Furthermore, the binding sites may be fluid. The Clarke laboratory described an 'induced fit' model for binding of small molecules to P-gp in which the shape of the drug-binding site changes upon to accommodate the shape of the small molecule.³⁹ Modeling studies of P-gp have also indicated a variety of different conformations for binding of small molecules to P-gp.^{40–42} It is possible that the structurally related amides and thioamides of this study could have different binding to P-gp. Recent studies have shown that thiourea functionality imparted greater inhibitory activity toward MRP1 in a series of drug derivatives relative to P-gp or BCRP, which is consistent with different types of binding.⁴³

The thiorhodamines **1–12** of this study examined a very small structure-activity space: compounds **1–7** and **9–12** were designed as related structures to **8** to compare amide/thioamide-substituted rhodamines with the tertiary amides/thioamides bearing diethylamino or piperidyl groups and to examine the systematic changes of one amino substituent at the 3-position of the thiorhodamine core. Because the structure-activity space is limited, one might not expect great differences among the compounds, but hopefully trends are identified. In isolated protein, the series **1–12** gave a range of values for V_{max} (maximum stimulation of ATPase activity) from <2-fold stimulation for **11** and **12** to 12.1-fold stimulation for **1** and **2** and for K_M from $1.9 \times 10^{-6} \text{ M}$ for **7** to $6.4 \times 10^{-6} \text{ M}$ for **3**. All twelve compounds entered MDCKII-MDR1 cells as indicated by their active transport by P-gp [$P_{BA}(-\text{inh})/P_{BA}(+\text{inh}) > 12$, Table 2] and by their facilitation of CAM uptake (Table 1). Thus, all of the compounds **1–12** bind to transport sites including the half-julolidyl amide **11** and thioamide **12** that have V_{max} values at or near basal levels.

Compounds **11** and **12**, while showing no apparent ATPase stimulation in isolated P-gp, inhibited VER-induced ATPase activity with IC_{50} 's of $(1.1 \pm 0.2) \times 10^{-6}$ and $(1.7 \pm 0.3) \times 10^{-6} \text{ M}$, respectively. Compounds **11** and **12** act as inhibitors in the isolated protein.

In the series of compounds **1–12**, certain structural features appear critical for high affinity and impact on ATPase activity. Thiorhodamine compounds **5–12** bearing the more hydrophobic julolidyl and half-julolidyl fragments displayed significantly higher affinity for P-gp as indicated by lower values of K_M ($p < 0.03$, Student *t*-test) relative to the corresponding thiorhodamine compounds **1–4** bearing dimethylamino fragments (Table 1). Furthermore, the increased affinity for compounds **5–12** bearing the julolidyl and half-julolidyl fragments gave decreased P-gp ATPase stimulation that was statistically significant ($p < 0.05$, Student *t*-test) relative to the corresponding thiorhodamine compounds **1–4** bearing dimethylamino fragments. The presence of a thioamide or an amide group in thiorhodamines **1–12** gave no predictable trend in either V_{max} or K_M in isolated protein.

In whole cell experiments, values of IC_{50} for CAM uptake were fivefold higher for amides **1, 3**, and **7** relative to their corresponding thioamides **2, 4**, and **8** (Table 1). For the remaining six compounds (**5, 6**, and **9–12**), IC_{50} 's for CAM uptake were similar for both amides and thioamides and were similar to IC_{50} values for **2, 4** and **8**. Values of IC_{50} for VIN efflux were also quite similar for both compounds in the amide/thioamide pairs among compounds **9–12** (Table 1).

One would anticipate that the performance of thiorhodamines **1–12** as inhibitors would be similar in isolated protein or in whole cells. These compounds effectively interact with P-gp in a native membrane environment and facilitate the uptake of **CAM** or inhibit the P-gp-mediated efflux of **VIN**. If one compares the magnitude of K_M and the IC_{50} for inhibition of **VER**-induced ATPase activity in isolated protein with the magnitude of the values of IC_{50} for **CAM** uptake (via inhibition of P-gp efflux of **CAM**) and P-gp-mediated **VIN** efflux in whole cells, values for the whole-cell data are 3- to 30-fold higher (*i.e.*, are less effective in cells than in isolated protein) than values in isolated protein for the julolidyl and half-julolidyl derivatives **5–12**.

There are numerous reasons why effective concentrations in the cell may be different than effective concentrations in isolated protein. Differences between the local environment of P-gp as well as lipid composition and fluidity can impact drug interactions with P-gp.^{44,45} These differences are not a simple function of lipophilicity among the thiorhodamines **1–12** since values of $\log P$, the *n*-octanol/water partition coefficient, fall in the range of 1.2–2.7 (Table 1). All compounds should have access to aqueous and lipophilic environments.

Alternatively, these data suggest that the thiorhodamines may have limited availability to the pump in the native environment especially when one considers that rates of transport in the absorptive direction (P_{AB}) were all $\leq 1 \text{ nm s}^{-1}$ and that values of $P_{Passive}$ were all $\leq 14 \text{ nm s}^{-1}$ (Table 2). This is consistent with both active and passive replenishing of the membrane concentration in the native environment lagging behind active removal mediated by the pump [P_{BA} in the uninhibited system in the range $(0.34\text{--}3.7) \times 10^{-7} \text{ m s}^{-1}$]. If the available membrane concentrations are indeed low, then more drug will be required in the whole cell system to raise membrane concentrations effectively to levels that result in pump inhibition.

The rate of secretory transport (P_{BA}) of thiorhodamines **1–12** across monolayers of MDCKII-MDR1 transfected cells was sensitive to both the amine fragment (dimethylamino, julolidyl, half-julolidyl) and the amide/thioamide functionality. In every example, transport of the thioamide was slower than transport of the amide both in the uninhibited and fully inhibited systems (Table 2). In the fully inhibited system, $P_{BA}(+inh)$ was $\leq 5 \times 10^{-9} \text{ m s}^{-1}$ for all of the thioamide derivatives while $P_{BA}(+inh)$ for the amide derivatives were in the range $(7.5\text{--}27) \times 10^{-9} \text{ m s}^{-1}$. In the uninhibited system, transport of the amide/thioamide pair was most similar for thiorhodamines **1** and **2** with the dimethylamino fragment where the ratio of $P_{BA}(\text{amide})/P_{BA}(\text{thioamide})$ was 1.3. For the remaining amide/thioamide pairs, the ratio of $P_{BA}(\text{amide})/P_{BA}(\text{thioamide})$ was in the range 2.6–6.8 and $P_{BA}(\text{amide})/P_{BA}(\text{thioamide})$ ratios were larger for the half-julolidyl derivatives (5.5 for **9/10** and 6.8 for **11/12**) relative to the julolidyl derivatives (3.2 for **5/6** and 2.7 for **7/8**).

All of the thiorhodamines in the series **1–12** were substrates for P-gp as indicated by the large values of $P_{BA}(-inh)/P_{BA}(+inh)$ (>12 , Table 2). An interesting correlation can be found with the % cell-associated dye in the absence of the added inhibitor **31** (LSN 335984). The % cell-associated dye was higher for the thioamide derivatives relative to the amide derivatives in the uninhibited system (Table 2). An active pump should limit the % cell-associated dye and the results are consistent with increased inhibition with the thioamides. More hydrophobic compounds would be expected to have a higher local concentration in the membrane (% cell-associated dye) and have a greater potential both to inhibit and to invoke recognition by P-gp. This is reflected in the higher values of % cell-associated dye for the more hydrophobic thioamides **6**, **8**, **10**, and **12** relative to **2** and **4**, suggesting more inhibition in the former set of compounds relative to the latter.

An active pump should limit the % cell-associated dye relative to the % cell-associated dye in the presence of inhibitor. The amide systems **5**, **7**, **9** and **11** have ratios for % cell-associated dye between inhibited and uninhibited pump that are between 3.3 and 6.1. Clearly, the active pump [absence of added **31** (LSN 335984)] limits the amount of cell-associated dye. For the thioamide analogues **6**, **8**, **10** and **12**, ratios for % cell-associated dye between inhibited and uninhibited pump were much closer to a value of 1 (1.2–2.1, Table 2), which suggests that the pump is inhibited even in the absence of added **31**.

3. Conclusions

An initial lead comparing amide and thioamide functionality in two thiorhodamine derivatives suggested that thioamides might be better inhibitors of P-gp than amide derivatives.¹⁵ We have examined a small segment of structure–activity space surrounding this observation through a series of thiorhodamine derivatives **1–12** varying in amide versus thioamide functionality on a 2-thienyl substituent at the 9-position of the thiorhodamine core and varying in an amine fragment (dimethylamino, julolidyl, half-julolidyl) at the 3-position of the thiorhodamine core. While overall differences are small within this series, certain trends emerge. The net effect of all the compounds **1–12** is to function as an inhibitor of P-gp in the native environment of whole cells as measured by IC_{50} values for the uptake of **CAM**. Compounds **9–12** also have the net effect of functioning as inhibitors of P-gp as measured for IC_{50} values for inhibition of P-gp-mediated efflux of tritiated **VIN**. Derivatives with julolidyl and half-julolidyl fragments have lower values of IC_{50} for **CAM** uptake in whole cells than derivatives with a dimethylamino fragment suggesting that these derivatives have higher affinity for P-gp. Values of K_M for the julolidyl thiorhodamines **5–8** and half-julolidyl thiorhodamines **9–12** are lower than values of K_M for the dimethylamino-derivatives **1–4**, which is consistent with a higher affinity.

The thioamide derivatives are transported quite slowly in both absorptive and secretory directions in monolayers of MDCKII-MDR1 transfected cells suggesting that the thioamide derivatives have limited availability to the pump in the native membrane environment in this model system. Understanding the factors that determine this availability in the amide/thioamide pairs may help in the development of more potent inhibitors of P-gp that may function also as photosensitizers for PDT of multidrug-resistant cells. The slow transport of the thioamide analogues from P-gp-expressing cells suggests that heavy-atom analogues of **6**, **8**, **10**, and **12** should be efficient photosensitizers for the treatment of multidrug-resistant cells with PDT. The corresponding selenorhodamines should be available via similar synthetic chemistry.

4. Experimental

4.1. General methods

Thiorhodamines **4**¹⁵ and **8**¹⁵ and thioxanthenes **13**¹⁹ and **14**²⁰ were prepared by literature methods. Reactions were run under argon. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl prior to use. Reactions were concentrated in vacuo on a Büchi rotary evaporator. NMR spectra were recorded on an Inova 500 instrument (500 MHz for ¹H, 125 MHz for ¹³C) with residual solvent signal as internal standard. Infrared spectra were recorded on a Perkin–Elmer FTIR instrument. UV–vis near-IR spectra were recorded on a Perkin–Elmer Lambda 12 spectrophotometer or on a Shimadzu UV-3600 spectrophotometer in quartz cuvettes with a 1-cm path length. Melting points were determined with a Büchi capillary melting point apparatus and are uncorrected. All

compounds tested have a purity of at least 95%, which was determined by an analysis on a C18 reverse-phase HPLC column [Protein RP] using 75% CH₃CN/H₂O with a flow rate of 0.4 mL/min and monitoring by a UV-visible detector operating at 254 or 442 nm.

4.1.1. Preparation of 3,6-bis(dimethylamino)-9-(5-(diethylcarbamothioyl)thiophen-2-yl)thioxanthylum hexafluorophosphate (2)

n-Butyllithium (0.890 M in hexanes, 5.86 mL, 5.23 mmol, 3.9 equiv) was added to a solution of *N,N*-diisopropylamine (0.889 mL, 6.30 mmol, 4.7 equiv) in THF (10 mL) at -78°C . The resulting mixture was stirred for 0.5 h before it was added to a solution of *N,N*-diethylthiophene-2-carbothioamide (1.07 g, 5.36 mmol, 4 equiv) in THF (40 mL) at -78°C . The resulting solution was stirred for 2 min before it was transferred via cannula into a solution of 3,6-bis-(dimethylamino)-9-thioxanthen-9-one¹⁹ (**13**, 400 mg, 1.34 mmol) in THF (20 mL) at ambient temperature with stirring. The reaction mixture was heated to 40°C for 0.5 h, and then cooled to ambient temperature. Glacial acetic acid (2 mL) was added and the mixture was poured into a 10% v/v aqueous HPF₆ solution at 0°C . The resulting precipitate was collected via vacuum filtration after 3 h of stirring and washed with water (30 mL) and diethyl ether (50 mL). The product was purified via column chromatography (SiO₂, 1:9 Et₂O/CH₂Cl₂, $R_f = 0.40$), followed by recrystallization from CH₂Cl₂/Et₂O to give 514 mg (61.3%) of thioamide **2** as a purple solid, mp $248\text{--}250^{\circ}\text{C}$. ¹H NMR (500 MHz, CD₂Cl₂) δ 7.78 (d, 2H, $J = 10$ Hz), 7.25 (d, 1H, $J = 3.5$ Hz), 7.11 (d, 1H, $J = 3.5$ Hz), 7.09 (d, 2H, $J = 2.5$ Hz), 7.05 (dd, 2H, $J = 2.5, 10$ Hz), 4.12 (br s, 2H), 3.87 (br s, 2H), 3.30 (s, 12H), 1.41 (t, 6H, $J = 2.0$ Hz); ¹³C NMR (75.5 MHz, CD₂Cl₂) δ 188.5, 153.9, 151.9, 149.6, 144.4, 137.9, 136.4, 130.5, 124.7, 120.1, 116.0, 105.8, 45.2 (br), 44.5 (br), 37.2, 10.6 (br), 7.4 (br); IR (film on NaCl) 2933, 1594, 1498, 1446, 1391, 1364, 1343, 1252 cm⁻¹; λ_{max} in CH₂Cl₂ (log ϵ) 308 (4.76), 339 (sh), 568 (sh), 610 nm (5.02); HRMS (ESI) m/z 480.1613 (calcd for C₂₆H₃₀N₃S₃⁺: 480.1596).

4.1.2. Preparation of 3,6-bis(dimethylamino)-9-(5-(diethylcarbamoyl)thiophen-2-yl)thioxanthylum hexafluorophosphate (1)¹⁵

Trifluoroacetic anhydride (0.449 mL, 3.20 mmol, 10 equiv) was added dropwise to a solution of thioamide **2** (200 mg, 0.320 mmol) in CH₂Cl₂ (30 mL). The mixture was heated at reflux for 2 h, and then cooled to ambient temperature. A solution of 10% Na₂CO₃ (20 mL) was added, and the resulting mixture was extracted with CH₂Cl₂ (3 \times 15 mL). The combined organic extracts were concentrated. The crude product was purified via column chromatography (SiO₂, 1:9 Et₂O/CH₂Cl₂, $R_f = 0.20$), followed by recrystallization from CH₂Cl₂/Et₂O, to give 123 mg (63.0%) of thiorhodamine **1** as a purple solid, mp $264\text{--}266^{\circ}\text{C}$ (lit.¹⁵ mp: $264\text{--}266^{\circ}\text{C}$): ¹H NMR (500 MHz, CD₂Cl₂) δ 7.72 (d, 2H, $J = 10$ Hz), 7.48 (d, 1H, $J = 3.5$ Hz), 7.18 (d, 1H, $J = 3.5$ Hz), 7.10 (d, 2H, $J = 2.5$ Hz), 7.01 (dd, 2H, $J = 2.5, 10$ Hz), 3.61 (br s, 4H), 3.30 (s, 12H), 1.31 (br s, 6H); ¹³C NMR (75.5 MHz, CD₂Cl₂) δ 162.4, 153.8, 151.8, 144.2, 142.7, 137.7, 136.2, 130.9, 128.0, 120.0, 115.9, 105.8, 43.3 (br), 40.9, 13.5 (br); λ_{max} in CH₂Cl₂ (log ϵ) 313 (4.85), 343 (sh), 574 (sh), 615 nm (5.13)

4.1.3. Preparation of 3,6-bis(dimethylamino)-9-(5-(piperidine-1-carbonyl)thiophen-2-yl)thio-xanthylum hexafluorophosphate (3)

Trifluoroacetic anhydride (0.272 mL, 1.96 mmol, 5 equiv) and thiorhodamine **4**¹⁵ (250 mg, 0.392 mmol, 1 equiv) in CH₂Cl₂ (30 mL) were treated as described for the preparation of **1** from **2**. The crude product was purified via column chromatography (SiO₂, 1:9 Et₂O/CH₂Cl₂, $R_f = 0.25$), followed by recrystallization

from CH₂Cl₂/Et₂O, to give 171 mg (70.3%) of thiorhodamine **3** as a purple solid, mp $242\text{--}244^{\circ}\text{C}$. ¹H NMR (500 MHz, CD₂Cl₂) δ 7.72 (d, 2H, $J = 9.5$ Hz), 7.43 (d, 1H, $J = 4.0$ Hz), 7.18 (d, 1H, $J = 4.0$ Hz), 7.10 (d, 2H, $J = 2.5$ Hz), 7.02 (d \times d, 2H, $J = 9.5, 2.5$ Hz), 3.74 (t, 4H, $J = 5.0$ Hz), 3.30 (s, 12H), 1.79–1.72 (m, 2H), 1.71–1.66 (m, 4H); ¹³C NMR (75.5 MHz, CD₂Cl₂) δ 162.0, 153.9, 152.0, 144.4, 141.9, 137.4, 136.3, 130.7, 128.5, 120.1, 116.0, 105.8, 47.1 (br), 41.0, 26.5 (br), 24.9; IR (film on NaCl) 2933, 2850, 1593 cm⁻¹; λ_{max} in CH₂Cl₂ (log ϵ) 308 (4.68), 338 (sh), 565 (sh), 609 nm (4.98); HRMS (ESI) m/z 476.1833 (calcd for C₂₆H₃₀N₃S₃⁺: 476.1825).

4.1.4. Preparation of 12-(dimethylamino)-2,3,6,7-tetrahydro-9-(5-(diethylcarbamothioyl)thiophen-2-yl)-1H,5H-thioxantheno [2,3,4-*ij*]quinolizin-14-ium hexafluorophosphate (6)

n-Butyllithium (0.920 M in hexanes, 4.24 mL, 3.90 mmol), *N,N*-diisopropylamine (0.663 mL, 4.70 mmol) in THF (10 mL) and *N,N*-diethylthiophene-2-carbothioamide (797 mg, 4.00 mmol) in THF (80 mL) at -78°C were treated as described in the preparation of thiorhodamine **2**. A solution of thioxanthone **14**²⁰ (350 mg, 0.999 mmol) in THF (30 mL) was treated with the thienyl anion as described for the preparation of thioamide **2**. The crude product was purified via column chromatography (SiO₂ gel, 1:9 Et₂O/CH₂Cl₂, $R_f = 0.50$), followed by recrystallization from CH₂Cl₂/Et₂O to give 0.381 g (56.2%) of thiorhodamine **6** as a purple solid, mp $153\text{--}155^{\circ}\text{C}$: ¹H NMR (500 MHz, CD₂Cl₂) δ 7.65 (d, 1H, $J = 10$ Hz), 7.39 (s, 1H), 7.24 (d, 1H, $J = 4.0$ Hz), 7.09 (d, 1H, $J = 2.5$ Hz), 7.07 (d, 1H, $J = 4.0$ Hz), 6.99 (dd, 1H, $J = 2.5, 10$ Hz), 4.12 (br s, 2H), 3.89 (br s, 2H), 3.56 (t, 4H, $J = 6.0$ Hz), 3.25 (s, 6H), 2.86 (t, 2H, $J = 6.0$ Hz), 2.78 (t, 2H, $J = 6.0$ Hz), 2.18 (quintet, 2H, $J = 6.0$ Hz), 2.01 (quintet, 2H, $J = 6.0$ Hz), 1.41 (t, 6H, $J = 7.0$ Hz); ¹³C NMR (75.5 MHz, CD₂Cl₂) δ 188.6, 153.1, 149.7, 149.4, 149.1, 142.3, 140.0, 139.0, 135.3, 132.9, 130.3, 126.5, 120.3, 119.1, 115.2, 114.4, 105.9, 51.9, 50.9, 48.9 (br), 48.3 (br), 40.7, 28.2, 24.3, 20.6, 20.1, 14.3 (br), 11.1 (br); IR (film on NaCl) 2932, 1593, 1456, 1387, 1364, 1315, 1180 cm⁻¹; λ_{max} in CH₂Cl₂ (log ϵ) 310 (4.74), 339 (sh), 578 (sh), 621 nm (5.04); HRMS (ESI) m/z 532.1917 (calcd for C₃₀H₃₄N₃S₃⁺: 532.1909).

4.1.5. Preparation of 12-(dimethylamino)-2,3,6,7-tetrahydro-9-(5-(diethylcarbamoyl)thiophen-2-yl)-1H,5H-thioxantheno [2,3,4-*ij*]quinolizin-14-ium hexafluorophosphate (5)

Trifluoroacetic anhydride (0.307 mL, 2.21 mmol, 7.5 equiv) and thiorhodamine **6** (200 mg, 0.295 mmol, 1 equiv) in CH₂Cl₂ (30 mL) were treated as described for the preparation of **1**. The crude product was purified via column chromatography (SiO₂, 1:9 Et₂O/CH₂Cl₂, $R_f = 0.25$), followed by recrystallization from CH₂Cl₂/Et₂O, to give 105 mg (54.0%) of thiorhodamine **5** as a purple solid, mp $159\text{--}161^{\circ}\text{C}$. ¹H NMR (500 MHz, CD₂Cl₂) δ 7.58 (d, 1H, $J = 9.5$ Hz), 7.48 (d, 1H, $J = 3.5$ Hz), 7.32 (s, 1H), 7.14 (d, 1H, $J = 3.5$ Hz), 7.10 (d, 1H, $J = 2.5$ Hz), 6.96 (dd, 1H, $J = 2.5, 9.5$ Hz), 3.62 (br s, 4H), 3.56 (t, 4H, $J = 6.0$ Hz), 3.25 (s, 6H), 2.86 (t, 2H, $J = 6.0$ Hz), 2.76 (t, 2H, $J = 6.0$ Hz), 2.18 (quintet, 2H, $J = 6.0$ Hz), 2.00 (quintet, 2H, $J = 6.0$ Hz), 1.38–1.23 (m, 6H); IR (film on NaCl) 2934, 1593 cm⁻¹; λ_{max} in CH₂Cl₂ (log ϵ) 310 (4.65), 341 (sh), 577 (sh), 621 nm (5.01); HRMS (ESI) m/z 516.2144 (calcd for C₃₀H₃₄N₃OS₂⁺: 516.2138).

4.1.6. Preparation of 12-(dimethylamino)-2,3,6,7-tetrahydro-9-(*N*-piperidyl-2-thienyl-5-carboxamido)-1H,5H-thioxantheno [2,3,4-*ij*]quinolizin-14-ium hexafluorophosphate (7)

Trifluoroacetic anhydride (0.754 mL, 5.43 mmol) and thiorhodamine **8**¹⁵ (250 mg, 0.362 mmol) in CH₂Cl₂ (30 mL) were treated as described for the preparation of **1**. The crude product was purified via column chromatography (SiO₂, 1:9 Et₂O/CH₂Cl₂, $R_f = 0.25$), followed by recrystallization from CH₂Cl₂/Et₂O, to give 132 mg (54.2% yield) of thiorhodamine **7** as a purple solid, mp

183–185 °C. ^1H NMR (500 MHz, CD_2Cl_2) δ 7.59 (d, 1H, $J = 10$ Hz), 7.43 (d, 1H, $J = 3.5$ Hz), 7.34 (s, 1H), 7.13 (d, 1H, $J = 3.5$ Hz), 7.10 (d, 1H, $J = 2.5$ Hz), 6.96 (dd, 1H, $J = 2.5, 10$ Hz), 3.74 (t, 4H, $J = 5.0$ Hz), 3.56 (t, 4H, $J = 6.0$ Hz), 3.24 (s, 6H), 2.86 (t, 2H, $J = 6.0$ Hz), 2.76 (t, 2H, $J = 6.0$ Hz), 2.18 (quintet, 2H, $J = 6.0$ Hz), 2.01 (quintet, 2H, $J = 6.0$ Hz), 1.79–1.72 (m, 2H), 1.72–1.66 (m, 4H); ^{13}C NMR (75.5 MHz, CD_2Cl_2) δ 162.1, 153.0, 149.7, 149.5, 142.2, 141.5, 140.0, 138.3, 135.2, 132.9, 130.4, 128.5, 126.5, 120.4, 119.2, 115.2, 114.4, 105.9, 51.9, 50.9, 47.7 (br), 40.6, 28.1, 26.5 (br), 24.8, 24.3, 20.6, 20.1; IR (film on NaCl) 2937, 2854, 1593 cm^{-1} ; λ_{max} in CH_2Cl_2 ($\log \epsilon$) 310 (4.65), 341 (sh), 578 (sh), 622 nm (5.00); HRMS (ESI) m/z 528.2153 (calcd for $\text{C}_{31}\text{H}_{34}\text{N}_3\text{O}_2^+$: 528.2138).

4.1.7. Preparation of 6-(5-(diethylcarbamothioyl)thiophen-2-yl)-9-(dimethylamino)-1,4,4-trimethyl-2,3,4,6-tetrahydro-1H-thiochromeno[3,2-g]quinolin-6-ylum hexafluorophosphate (10)

n-Butyllithium (0.891 M, 2.79 mL, 2.48 mmol), diisopropylamine (0.370 mL, 2.62 mmol), and piperidin-1-yl(thiophen-2-yl)methanethione (495 mg, 2.48 mmol) in THF (20 mL) were treated as described for the preparation of thioamide **2**. A solution of 9-(dimethylamino)-1,4,4-trimethyl-3,4-dihydro-1H-thiochromeno[3,2-g]quinolin-6(2H)-one (**15**, 250 mg, 0.709 mmol) in THF (10 mL) was treated with the thienyl anion as described for the preparation of **2**. The crude product was purified via column chromatography (SiO_2 , 4% MeOH/ CH_2Cl_2) followed by recrystallization from MeOH/ether to give 28.4 mg (15%) of **10** as dark blue crystals, mp 130–132 °C. ^1H NMR (500 MHz, CD_2Cl_2) δ 7.79 (d, 1H, $J = 9.5$ Hz), 7.59 (s, 1H), 7.26 (d, 1H, $J = 3.5$ Hz), 7.10 (d, 1H, $J = 3.5$ Hz), 7.06 (d, 1H, $J = 2.0$ Hz), 7.02 (s, 1H), 7.01 (d × d, 1H, $J = 3.0$ Hz, 10 Hz), 4.12 (br s, 2H), 3.87 (br s, 2H), 3.65 (t, 2H, $J = 6.0$ Hz), 3.27 (s, 9H), 1.82 (t, 2H, 6 Hz), 1.20 (s, 6H); ^{13}C NMR (75.6 MHz, CD_2Cl_2) δ 188.8, 153.5, 151.2, 150.8, 149.3, 143.7, 143.4, 137.9, 136.0, 135.9, 130.3, 130.2, 124.7, 120.5, 119.7, 115.6, 105.7, 105.0, 48.9, 48.1 (br), 40.8, 40.4, 34.5, 32.3, 28.6, 14.7 (br); IR (film, CCl_4) ν_{max} : 2937, 1594, 1480, 1446, 1389, 1330, 1251 cm^{-1} ; λ_{max} in CH_2Cl_2 ($\log \epsilon$) 312 (4.83), 346 (sh), 576 (sh), 619 nm (5.04). HRMS (ESI) m/z 534.2060 (calcd for $\text{C}_{30}\text{H}_{36}\text{N}_3\text{S}_3^+$: 534.2066).

4.1.8. Preparation of 6-(5-(diethylcarbamoyl)thiophen-2-yl)-9-(dimethylamino)-1,4,4-trimethyl-2,3,4,6-tetrahydro-1H-thiochromeno[3,2-g]quinolin-6-ylum hexafluorophosphate (9)

Trifluoroacetic anhydride (0.082 mL, 0.59 mmol) and thiorhodamine **10** (200.0 mg, 0.294 mmol) in anhydrous CH_2Cl_2 (10 mL) were treated as described for the preparation of **1**. The crude product was purified via column chromatography (SiO_2 , 10% $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to give 101 mg (51%) of **9** as dark blue crystals, mp 155–160 °C. ^1H NMR (500 MHz, CD_2Cl_2) δ 7.69 (d, 1H, $J = 9.5$ Hz), 7.54 (s, 1H), 7.50 (d, 1H, $J = 3.5$ Hz), 7.18 (d, 1H, $J = 4.0$ Hz), 7.07 (d, 1H, $J = 2.0$ Hz), 7.03 (s, 1H), 6.98 (dd, 1H, $J = 9.5$ Hz, 2.0 Hz), 3.65 (t, 2H, $J = 6.5$ Hz), 3.61 (br s, 4H), 3.29 (s, 3H), 3.27 (s, 6H), 1.81 (t, 2H, $J = 6.5$ Hz), 1.32 (t, 6H, $J = 6.5$ Hz), 1.18 (s, 6H); ^{13}C NMR (75.6 MHz, CD_2Cl_2) δ 162.6, 153.5, 151.3, 150.9, 143.7, 143.4, 142.8, 138.1, 136.1, 135.9, 130.7, 130.3, 128.1, 120.5, 119.8, 115.6, 105.7, 105.1, 49.0, 42.8 (br), 40.9, 40.4, 34.5, 32.3, 28.6, 13.9 (br); IR (film, CCl_4) ν_{max} : 2935, 1593, 1477, 1448, 1390, 1330, 1254 cm^{-1} ; λ_{max} in CH_2Cl_2 ($\log \epsilon$) 313 (4.81), 346 (sh), 575 (sh), 618 nm (5.08); HRMS (ESI) m/z 518.2299 (calcd for $\text{C}_{30}\text{H}_{36}\text{O}_1\text{N}_3\text{S}_2^+$: 518.2294).

4.1.9. Preparation of 9-(dimethylamino)-1,4,4-trimethyl-6-(5-(piperidine-1-carbonyl)thiophen-2-yl)-2,3,4,6-tetrahydro-1H-thiochromeno[3,2-g]quinolin-6-ylum hexafluorophosphate (12)

A solution of *n*-butyllithium (0.891 M in hexanes, 5.58 mL, 4.97 mmol) and *N,N*-diisopropylamine (0.740 mL, 5.25 mmol) was

stirred 0.5 h at -78 °C and was then added slowly to a solution of piperidin-1-yl(thiophen-2-yl)methanethione (1.05 g, 4.97 mmol) in THF (20 mL) at -78 °C. The resulting mixture was stirred 5 min before it was transferred via cannula into a solution of thioxanthone **15** (500 mg, 1.42 mmol) in THF (10 mL) at ambient temperature. The resulting mixture was stirred for 0.5 h at 35 °C. After cooling to ambient temperature, glacial acetic acid (2 mL) was added, and the reaction mixture was poured into a 10% v/v solution of stirring, cold aqueous HPF₆. The resulting precipitate was collected via filtration after 1 h of stirring and washed with water (10 mL) and diethyl ether (10 mL). The product was purified via column chromatography (SiO_2 , 4% MeOH/ CH_2Cl_2) followed by recrystallization from MeOH/ether to give 511 mg (52.1%) of **12** as dark blue crystals, mp 168–170 °C. ^1H NMR (500 MHz, CD_3CN) δ 7.72 (d, 1H, $J = 9.5$ Hz), 7.50 (s, 1H), 7.25 (d, 1H, $J = 3.6$ Hz), 7.18 (d, 1H, $J = 4.0$ Hz), 7.13 (d, 2H, $J = 2.8$ Hz), 7.07 (d × d, 1H, $J = 2.8, 9.5$ Hz), 4.31 (br s, 2H), 3.99 (br s, 2H), 3.60 (t, 2H, $J = 6.0$ Hz), 3.22 (s, 9H), 1.78 (br s, 6H), 1.75 (t, 2H, 6.0 Hz), 1.13 (s, 6H); ^{13}C NMR (75.6 MHz, CD_2Cl_2) δ 188.9, 153.5, 151.2, 150.8, 149.1, 143.7, 143.4, 138.1, 136.0, 130.4, 125.2, 120.5, 119.7, 115.6, 105.8, 105.0, 52.4 (br), 49.0, 40.9, 40.4, 34.5, 32.4, 28.6, 27.2 (br), 24.5; IR (film, CCl_4) ν_{max} : 2936, 1594, 1479, 1447, 1389, 1330, 1252 cm^{-1} ; λ_{max} in CH_2Cl_2 ($\log \epsilon$) 312 (4.78), 345 (sh), 579 (sh), 620 nm (4.99); HRMS (ESI) m/z 546.2071 (calcd for $\text{C}_{31}\text{H}_{36}\text{N}_3\text{S}_3^+$: 546.2066).

4.1.10. Preparation of 9-(dimethylamino)-1,4,4-trimethyl-6-(5-(piperidine-1-carbonyl)thiophen-2-yl)-3,4-dihydro-2H-thiochromeno[3,2-g]quinolin-1-ylum hexafluorophosphate (11)

Trifluoroacetic anhydride (0.080 mL, 0.58 mmol) and thiorhodamine **12** (200 mg, 0.289 mmol) in anhydrous CH_2Cl_2 (10 mL) were treated as described for the preparation of amide **1**. The product was purified via column chromatography (SiO_2 , 10% $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to give 98.0 mg (50.1%) of **11** as dark blue crystals, mp 142–147 °C. ^1H NMR (500 MHz, CD_2Cl_2) δ 7.72 (d, 1H, $J = 9.5$ Hz), 7.53 (s, 1H), 7.44 (d, 1H, $J = 3.5$ Hz), 7.17 (d, 1H, $J = 3.5$ Hz), 7.06 (d, 1H, $J = 3.0$ Hz), 7.02 (s, 1H), 6.99 (dd, 1H, $J = 2.5, 9.5$ Hz), 3.74 (t, 4H, $J = 6.0$ Hz), 3.65 (t, 2H, $J = 6.5$ Hz), 3.28 (s, 3H), 3.27 (s, 6H), 1.81 (t, 2H, $J = 6.5$ Hz), 1.74–1.77 (m, 2H), 1.66–1.72 (m 4H), 1.18 (s, 6H); ^{13}C NMR (75.6 MHz, CD_2Cl_2) δ 162.2, 153.5, 151.2, 150.7, 143.7, 143.5, 141.7, 137.9, 136.0, 135.9, 130.6, 130.2, 128.4, 120.5, 119.8, 115.6, 105.8, 105.2, 48.9, 47.1 (br) 40.9, 40.4, 34.5, 32.3, 28.5, 26.5 (br), 24.9; IR (film, CCl_4) ν_{max} : 2936, 1594, 1478, 1448, 1389, 1330, 1253 cm^{-1} ; λ_{max} in CH_2Cl_2 ($\log \epsilon$) 313 (4.81), 344 (sh), 576 (sh), 619 nm (5.08); HRMS (ESI) m/z 530.2288 (calcd for $\text{C}_{31}\text{H}_{36}\text{O}_1\text{N}_3\text{S}_2^+$: 530.2294).

4.1.11. Preparation of *N*-methyl-*N*-(3-methylbut-2-enyl)aniline (**16**)⁴⁶

N-Methylaniline (7.13 g, 66.6 mmol) and potassium carbonate (9.20 g, 66.6 mmol) were stirred for 10 min at ambient temperature in CH_3CN (100 mL). 1-Chloro-3-methyl-2-butene (4.64 g, 88.7 mmol) was added slowly over 10 min. The resulting mixture was heated at 40 °C for 20 h. After cooling to ambient temperature, water (100 mL) was added and products were extracted with CH_2Cl_2 (5 × 50 mL). The organic fractions were dried over anhydrous MgSO_4 and concentrated. The resulting red-brown oil was purified via column chromatography (SiO_2 , 30:70 CH_2Cl_2 /hexanes) to give 10.1 g (86.7%) of **16**. ^1H NMR (500 MHz, CDCl_3) δ 7.25–7.20 (m, 2H), 6.74 (d, 2H, $J = 8.0$ Hz), 6.71 (t, 1H, $J = 7.0$ Hz), 5.22 (t, 1H, $J = 5.0$ Hz), 3.89 (d, 2H, $J = 6.0$ Hz), 2.89 (s, 3H), 1.72 (d, 6H, $J = 1.0$ Hz). HRMS (EI) m/z 175.1359 (calcd for $\text{C}_{12}\text{H}_{17}\text{N}_1^+$: 175.1356).

4.1.12. Preparation of 1,4,4-trimethyl-1,2,3,4-tetrahydroquinoline (**17**)⁴⁷

Concentrated sulfuric acid (1.5 mL) was slowly added to **16** (0.500 g, 2.85 mmol) at 0 °C. After 1 h at 0 °C, ice water (150 mL)

was added. The product was extracted with CH_2Cl_2 (5×50 mL). The combined organic extracts were dried over anhydrous MgSO_4 and concentrated. The resulting brown oil was purified via chromatography (SiO_2 , 1:9 $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to give 380 mg (76.0%) of **17**. ^1H NMR (500 MHz, CDCl_3) δ 7.20 (dd, 1H, $J = 2.0, 7.2$ Hz), 7.09 (td, 1H, $J = 1.5, 8.4$ Hz), 6.67 (td, 1H, $J = 1.5, 7.2$ Hz), 6.60 (dd, 1H, $J = 1.5, 8.0$ Hz), 3.23 (t, 2H, $J = 6.0$ Hz), 2.90 (s, 3H), 1.79 (t, 2H, $J = 6.0$ Hz), 1.28 (s, 6H). HRMS (EI) m/z 175.1357 (calcd for $\text{C}_{12}\text{H}_{17}\text{N}_1^+$: 175.1356).

4.1.13. Preparation of 1,4,4-trimethyl-1,2,3,4-tetrahydroquinoline-6-carbaldehyde (**18**)⁴⁸

Phosphorus oxychloride (481 mg, 3.14 mmol) was added slowly to *N,N*-dimethylformamide (DMF) (2.5 mL) at 0 °C. Compound **17** (500 mg, 2.85 mmol) in dry DMF (2.5 mL) was added and the resulting mixture was heated at reflux for 1 h. After cooling to ambient temperature, cold NaOH (100 mL, 1 M) was added, and the product was extracted diethyl ether (5×50 mL). The combined organic fractions were washed with water (4×50 mL) and brine (5×50 mL), were dried over anhydrous MgSO_4 and concentrated. The product was purified via chromatography (SiO_2 , 1:9 $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to give 560 mg (96.6%) of **18** as a yellow solid, mp 48–50 °C. ^1H NMR (500 MHz, CDCl_3) δ 9.69 (s, 1H), 7.71 (s, 1H), 7.57 (d, 1H, $J = 8.5$ Hz), 6.58 (d, 1H, $J = 8.5$ Hz), 3.42 (t, 2H, $J = 6.0$ Hz), 3.03 (s, 3H), 1.76 (t, 2H, $J = 6.0$ Hz), 1.30 (s, 6H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 190.9, 150.6, 131.9, 131.3, 127.2, 125.5, 110.2, 48.1, 39.6, 36.5, 32.4, 30.3.

4.1.14. Preparation of piperidin-1-yl(1,4,4-trimethyl-1,2,3,4-tetrahydroquinolin-6-yl)methanethione (**19**)

A mixture of **18** (3.19 g, 15.7 mmol), elemental sulfur (1.26 g, 39.2 mmol), piperidine (314 mg, 47.1 mmol) and DMF (5 mL) were heated at reflux for 0.5 h. Cold water (100 mL) was added to the resulting mixture and the orange solid that formed was collected by filtration, dissolved in CH_2Cl_2 , and the resulting solution was dried over anhydrous MgSO_4 and concentrated. The product was purified via column chromatography (SiO_2 , CH_2Cl_2) yielding 3.85 g (81.0%) of **19** as a yellow crystalline solid, mp 150–152 °C. ^1H NMR (500 MHz, CDCl_3) δ 7.18 (m, 1H), 7.16 (d, 1H, $J = 2.0$ Hz), 6.49 (d, 1H, $J = 9.0$ Hz), 4.32 (s, 2H), 3.67 (s, 2H), 3.28 (t, 2H, $J = 6.0$ Hz), 2.93 (s, 3H), 1.81 (s, 2H), 1.76 (t, 4H, $J = 6.0$ Hz), 1.60 (s, 2H), 1.26 (s, 6H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 201.5, 146.1, 130.45, 130.0, 126.6, 124.7, 109.7, 53.6, 51.6, 47.5, 39.1, 36.8, 32.0, 30.6, 27.0, 25.7, 24.4; IR (film, CCl_4) ν_{max} : 2934, 2853, 2363, 1603, 1516, 1473, 1440, 1410, 1330, 1310, 1264, 1238, 1209, 1115, 1017, 786 cm^{-1} ; HRMS (EI) m/z 303.1890 (calcd for $\text{C}_{18}\text{H}_{27}\text{N}_2\text{S}_1^+$: 303.1889).

4.1.15. Preparation of piperidin-1-yl(1,4,4-trimethyl-1,2,3,4-tetrahydroquinolin-6-yl)methanone (**20**)

Trifluoroacetic anhydride (4.06 g, 19.3 mmol) was slowly added to a solution of **19** (5.84 g, 19.3 mmol) in dry CH_2Cl_2 (5 mL). The resulting mixture was stirred 10 min at room temperature. A solution of 10% aqueous Na_2CO_3 (100 mL) was added with stirring. The products were extracted with CH_2Cl_2 (7×50 mL), and the combined organic extracts were washed with brine (5×50 mL), dried over MgSO_4 and concentrated. The product was purified via column chromatography (SiO_2 , 1:9 $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to give 5.00 g (90.4%) of **20** as red-orange crystals, mp 66–68 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.30 (d, 1H, $J = 2.0$ Hz), 7.18 (dd, 1H, $J = 2.0, 2.5$ Hz), 6.52 (d, 1H, $J = 8.5$ Hz), 3.56 (s, 4H), 3.29 (t, 2H, $J = 6.0$ Hz), 2.93 (s, 3H), 1.77 (t, 2H, $J = 6.0$ Hz), 1.68–1.64 (m, 2H), 1.59 (br s, 4H), 1.27 (s, 6H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 172.01, 146.94, 130.97, 127.3, 126.3, 123.3, 110.2, 48.0, 47.0, 39.6, 37.3, 32.5, 31.1, 26.7, 25.3. IR (film, CCl_4) ν_{max} : 2932, 2852, 2360, 2341, 1607, 1518, 1468, 1428, 1406, 1331, 1265, 1210, 1115, 1008,

786, 764 cm^{-1} ; HRMS (EI) m/z 287.2119 (calcd for $\text{C}_{18}\text{H}_{27}\text{O}_1\text{N}_2^+$: 287.2118).

4.1.16. Preparation of (7-(3-(dimethylamino)phenylthio)-1,4,4-trimethyl-1,2,3,4-tetrahydroquinolin-6-yl)(piperidin-1-yl)methanone (**22**)

sec-Butyllithium (1.0 M in cyclohexane, 1.05 mL, 1.05 mmol) was added dropwise to a stirred solution of *N,N,N,N*-tetramethylethylenediamine (150 mg, 1.14 mmol) and **21** (250 mg, 0.873 mmol) in 90 mL of THF at -78 °C. A solution of bis-3-(*N,N*-dimethylamino)phenyldisulfide (1.38 g, 4.54 mmol) in tetrahydrofuran at -78 °C was immediately added, and the resulting mixture was stirred at -78 °C for 1 h and was then stirred at room temperature for 1 h. Aqueous saturated ammonium chloride (15 mL) was added to the resulting mixture and the products were extracted with CH_2Cl_2 (3×50 mL). The organic extracts were dried over anhydrous MgSO_4 and concentrated. The resulting yellow oil was purified via column chromatography (SiO_2 , 1:9 $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to give 55.5 mg (37.4%) of **22** as a yellow-brown oil. ^1H NMR (500 MHz, CDCl_3) δ 7.12 (t, 1H, $J = 8.0$ Hz), 7.02 (s, 1H), 6.80 (t, 1H, $J = 2.0$), 6.71 (d, 1H, $J = 7.6$ Hz), 6.59 (d \times d, 1H, $J = 2.8, 8.4$ Hz), 6.50 (s, 1H), 3.67 (br s, 2H), 3.21 (t, 4H, $J = 6.0$ Hz), 2.91 (s, 6H), 2.73 (s, 3H), 1.72 (t, 2H, $J = 6.0$ Hz), 1.60 (br s, 4H), 1.46 (br s, 2H), 1.24 (s, 6H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 169.4, 151.0, 145.8, 136.3, 130.9, 130.5, 129.4, 126.2, 124.5, 119.6, 115.5, 113.7, 111.2, 48.2, 47.5, 42.9, 40.5, 39.0, 36.8, 31.9, 30.6, 26.0, 24.7; HRMS (EI) m/z 438.2575 (calcd for $\text{C}_{26}\text{H}_{36}\text{O}_1\text{N}_3\text{S}_1^+$: 438.2574).

4.1.17. Preparation of 9-(dimethylamino)-1,4,4-trimethyl-3,4-dihydro-1H-thiochromeno[3,2-g]quinolin-6(2H)-one (**15**)

Phosphorus oxychloride (496 mg, 3.24 mmol) was added dropwise to a mixture of triethylamine (328 mg, 3.24 mmol) and **22** (118 mg, 0.270 mmol) in acetonitrile (25 mL). The resulting red mixture was stirred at ambient temperature for 5 min before stirring for 2 h at 80 °C. After cooling to ambient temperature, the resulting mixture was added to stirring, cold aqueous NaOH (200 mL, 1 M). The products were extracted with dichloromethane (5×50 mL), and the combined organic extracts were dried over MgSO_4 and concentrated. The product was then purified via column chromatography (SiO_2 , 1:9 $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to give 80.0 mg (90.3%) of desired product, mp 204–206 °C. ^1H NMR (500 MHz, CDCl_3) δ 8.44 (d, 1H, $J = 9.0$ Hz), 8.39 (s, 1H), 6.79 (dd, 1H, $J = 2.5, 9.0$ Hz), 6.57 (s, 1H), 6.45 (s, 1H), 3.41 (t, 2H, $J = 6.0$ Hz), 3.07 (s, 6H), 3.02 (s, 3H), 1.78 (t, 2H, $J = 6.5$ Hz), 1.35 (s, 6H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 179.8, 178.5, 165.5, 152.2, 148.3, 139.5, 137.5, 131.4, 126.9, 119.7, 119.1, 111.7, 105.7, 104.3, 48.0, 40.6, 39.6, 36.7, 32.6, 30.4; IR (film, CCl_4) ν_{max} : 2956, 1594, 1514, 1320 cm^{-1} ; HRMS (EI) m/z 353.1687 (calcd for $\text{C}_{21}\text{H}_{25}\text{O}_1\text{N}_2\text{S}_1^+$: 353.1682).

4.1.18. Preparation of *N,N*-diethylthiophene-2-carbothioamide (**27**)

Thiophene-2-carbaldehyde (1.00 g, 8.92 mmol), elemental sulfur (713 mg, 22.3 mmol), and diethylamine (2.76 mL, 26.8 mmol) and DMF (5 mL) were combined in a three-neck flask fitted with reflux condenser. The resulting mixture was allowed to stir 3 h at 110 °C. Ice cold water (100 mL) was added with stirring, and the mixture was extracted with diethyl ether (8×50 mL) and washed with brine (5×50 mL). The organic fractions were combined, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The product was purified via chromatography (silica, 10% ethyl acetate/hexanes) to give 0.87 g (49%) of the desired product as an orange oil. ^1H NMR (500 MHz, CDCl_3) δ 7.35 (dd, 1H, $J = 1.0, 5.5$ Hz), 7.08 (dd, 1H, $J = 1.0, 3.5$ Hz), 6.96 (dd, 1H, $J = 3.5, 5.5$ Hz), 4.10 (br s, 2H), 3.70 (br s, 2H), 1.34 (br s, 6H); ^{13}C

NMR (75.5 MHz, CDCl_3) δ 190.9, 145.0, 127.9, 126.3, 14.9, 48.0 (br), 14.1 (br), 11.1 (br); HRMS: m/z 199.0477 (calcd for $\text{C}_6\text{H}_{13}\text{NS}_2$: 199.0484); IR (film) ν_{max} : 3070, 2969, 2931, 2869, 1520, 1489, 1418, 1380, 1354, 1305, 1276, 1245, 1210, 1175, 1131, 1095, 1074 cm^{-1} .

4.2. Determination of partition coefficients

The octanol/water partition coefficients were all measured at pH 7.4 (PBS) using UV–visible spectrophotometry. The measurements were done using a shake flask direct measurement.²⁸ Mixing for 3–5 min was followed by 1 h of settling time. Equilibration and measurements were made at 23 °C using a Perkin–Elmer Lambda 12 spectrophotometer. High-performance liquid chromatography grade 1-octanol was obtained from Sigma–Aldrich.

4.3. Expression of P-gp, purification and measurement of ATPase activity

To facilitate purification by metal-chelate chromatography,³¹ the cDNA for human P-glycoprotein (P-gp)³⁰ was modified to contain a 10-histidine tag at the COOH-terminal end (P-gp-His₁₀). Histidine-tagged P-gp was then stably expressed in Baby hamster kidney (BHK) cells by co-transfecting with P-gp-His₁₀ and pWL-neo (Stratagene, Cedar Creek, TX). The transfected cells were then treated with the cytotoxic agent G418 as described previously.⁴⁹ Clones over-expressing P-gp were identified by subjecting whole cell extracts of G418-resistant colonies to immunoblot analysis with a rabbit polyclonal antibody to P-gp.⁵⁰ Histidine-tagged P-gp was then isolated by nickel-chelate chromatography as described previously.³¹ A sample of the isolated histidine-tagged P-gp was mixed with an equal volume of 10 mg/ml sheep brain phosphatidylethanolamine (Type II-S, Sigma) and ATPase activity was determined in the absence or presence of various concentrations of thiorhodamine compounds. The samples were incubated for 30 min at 37 °C and the reactions were stopped by addition of SDS. The amount of inorganic phosphate released was then determined.³³

To test for inhibition of P-gp **VER**-stimulated ATPase activity, samples of P-gp-His₁₀ in lipid were pre-incubated with various concentrations of thiorhodamine compounds for 15 min at 20 °C. Verapamil was used as a substrate to test for inhibition because it is one of the most potent activators of P-gp ATPase activity.³² The reactions were started by addition of ATPase reaction mix containing **VER** (final concentration of 4×10^{-4} M) and ATPase activity determined as described above.

4.4. Enhancement of calcein-AM uptake into MDCKII-MDR1 cells by thiorhodamine analogues

MDCK cells transfected with wild-type MDR1 (ABCB1) were obtained at passage number 12 from Dr. Piet Borst at The Netherlands Cancer Institute. Cell growth was maintained in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in 75- cm^2 flasks. Cultures were passaged by trypsinization 1:10 twice a week and used at passage number 16–42. Cells were seeded at 40,000 cells/well in 96-well flat bottom plates (Falcon) using a medium volume of 2×10^{-4} L, which was replaced on day 3 prior to their use on day 4.

Cells were washed once with Dulbecco's phosphate-buffered saline containing 10^{-2} M Hepes buffer at pH 7.4 (DPBSH) (Gibco) and incubated with solutions of the thiorhodamine analogue or control compound in DPBSH containing 4.3 mg mL^{-1} bovine serum albumin (BSA) at 37 °C in room atmosphere. IC_{50} values were calculated from 1:1 serial dilution series. After 30 min, the test

compound was replaced to include 0.5 $\mu\text{g mL}^{-1}$ **CAM** and incubated an additional 20 min. Calcein fluorescence was read on a Cytofluor series 4000 Multi Well Plate Reader (PerSeptive Biosystems) with λ_{EX} and λ_{EM} set at 485 nm and 530 nm, respectively. Negative (0.25% DMSO in DPBSH), and positive [5×10^{-6} M LSN 335984] controls were included in each plate. IC_{50} values were calculated from the serial dilution curves using GraphPad PRISM version 4.03 software. Briefly, compound concentration was plotted as log μM concentration versus relative fluorescence units (rfu) and a sigmoidal dose-response (variable slope) analysis with no weighting or restrictions was applied.

4.5. P-gp-transport studies across MDCKII-MDR1 monolayers

MDCKII-MDR1 cells that were seeded at 50,000 cells cm^{-2} onto 12-well (1.13 cm^2 surface area) Transwell polycarbonate filters (Costar) were fed on days 3 and 5, and used on day 6. The upper and lower chamber volumes were 0.5 mL and 1.0 mL, respectively. Cells were rinsed 10 min in DPBSH at 37 °C with mixing on a nutator (Clay Adams). Cells were pre-incubated with 4.3 mg mL^{-1} BSA in DPBSH alone or containing 5×10^{-6} M LSN 335984. After 30 min, 5 μM test compound (**1–12**) in BSA/DPBSH with or without inhibitor was added to the donor chamber (0.5 mL upper or apical, 1.0 mL lower or basolateral). Initial donor samples were taken at $t = 0$. For apical-to-basolateral (AB) flux, D_0 was taken from the mixing tube before addition to the cell monolayer. For basolateral-to-apical (BA) flux this sample was taken from the 12-well plate 10 min after transfer, but before cell wells were added. Samples were taken from both the donor and receiver chambers following a 1-h incubation at 37 °C with constant mixing by nutation. Cell monolayers were rinsed briefly two times using cold DPBS and extracted with 5×10^{-4} L methanol for 3 min. Samples (5×10^{-5} L) were combined into $n = 3$ cassettes in a 96-deep well assay plate and protein was precipitated by adding 4.5×10^{-4} L acetonitrile, shaken to mix. Plates were centrifuged 5 min at 5000 rpm. Compound concentrations were determined with an LC-MS/MS assay. Chromatography was performed using a Betasil C18 2 \times 20 mm 5 micron Javelin column; (Thermo Scientific, Waltham, Massachusetts) and 1 of 2 mobile phase systems. System 1 consisted of 5×10^{-3} M ammonium bicarbonate in water (Mobile Phase A), and, 5×10^{-3} M ammonium bicarbonate in methanol (Mobile Phase B), with elution accomplished by a methanol gradient at 1.5 mL/min. System 2 consisted of 0.4% trifluoroacetic acid (TFA), 1×10^{-3} M ammonium bicarbonate in water (Mobile Phase A), and, 0.4% TFA/ 1×10^{-3} M ammonium bicarbonate in acetonitrile (Mobile Phase B), with elution accomplished by an acetonitrile gradient at 1.5 mL/min. Mass spectrometric detection was performed with an API4000 mass spectrometer (Applied Biosystems, Foster City, California) equipped with a turbo ion spray source, using selected reaction monitoring in positive ion mode with precursor and product ion transitions specific to each analyte.

4.6. Inhibition of vinblastine efflux in MDCKII-MDR1 cells by thiorhodamine compounds 9–12

MDCKII-MDR1 cells were seeded onto Costar Transwell polycarbonate membranes and maintained as described above. On day 6, cells were rinsed 1×10 min in DPBSH with nutation at 37 °C, and then conditioned in 1×10^{-10} , 1×10^{-8} , 5×10^{-8} , 1×10^{-7} , 5×10^{-7} , 1×10^{-6} or 1×10^{-5} M **9–12** in BSA/DPBSH. After 30 min at 37 °C, 1×10^{-3} L [^3H]-**VIN** (0.25 $\mu\text{Ci mL}^{-1}$ from 0.1 mCi mL^{-1} EtOH stock) in appropriate solution of **9–12** was introduced to the basolateral chamber and 5×10^{-4} M fresh thiorhodamine compound was added to the apical chamber. Initial donor samples were taken from the basolateral chamber at $t = 0$. The apical solution was replaced every 10 min with fresh buffer and

appearance of [^3H]-VIN in the apical chamber was measured by scintillation counting. An IC_{50} was calculated using XLfit software.

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Supplementary data

Supplementary data (^1H and ^{13}C NMR spectra and HPLC data for compounds **2**, **3**, **5–7**, and **9–12** and ^1H and ^{13}C NMR spectra for thioxanthone **15**) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2012.05.075>.

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