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A cationic chalcogenoxanthylium photosensitizer effective in vitro in chemosensitive and multidrug-resistant cells

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Abstract—Pentacyclic thio- (**1**) and seleno- (**2**) analogues of tetramethylrosamine (**TMR**) were prepared with a julolidyl fragment replacing the 3-dimethylamino substituent in the xanthylium core. The pentacylic structure increases the lipophilicity of **1** and **2** relative to **TMR-S** and **TMR-Se** and locks the lone-pair of electrons on the julolidyl N atom into conjugation with the xanthylium core. This conformational rigidization leads to longer wavelengths of absorption, but has little impact on other photophysical properties such as quantum yields for fluorescence and singlet-oxygen generation and fluorescence lifetimes in **1** and **2** relative to **TMR-S** and **TMR-Se**. Both **1** and **2** are effective photosensitizers against chemosensitive AUXB1 cells in vitro at 1×10^{-7} M and compound **2** is an effective photosensitizer against multidrug-resistant CR1R12 cells in vitro at 1×10^{-7} M. While the uptake **TMR-S** into CR1R12 cells as measured by fluorescence is significantly lower than uptake into chemosensitive AUXB1 cells, there is no significant difference in the uptake of **1** into either AUXB1 or CR1R12 cells. The addition of 2×10^{-4} M verapamil to the cells prior to treatment with **1** had no significant effect on the uptake of **1** into either AUXB1 or CR1R12 cells. Treating lipid-activated, purified Pgp with **2** and light gave complete inhibition of Pgp ATPase activity.
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1. Introduction

Multidrug resistance (MDR)[1–3] poses a serious threat to human health in a number of areas including cancer chemotherapy, AIDS, bacterial, parasitic, and fungal diseases.[4–7] In cancer, MDR is conferred by multidrug efflux pumps that expel multiple chemically and structurally dissimilar drugs from the cell. The expression of these efflux pumps can be up-regulated by a single exposure to a chemotherapeutic agent. The promiscuity of multidrug-binding pumps is an intriguing problem. Effective clinical intervention will require design of mechanism-based inhibitors of these multidrug-binding proteins. The major proteins responsible for MDR in cancer are: (1) the mammalian plasma membrane protein P-glycoprotein (Pgp, also known as MDR1 or ABCB1), (2) the multidrug resistance protein series (including multidrug resistance protein 1, MRP1 or ABCC1), (3) breast cancer resistance protein (BCRP, MXR, or ABCG2), and (4) lung-resistance related protein (LRP).[8] All four proteins belong to a larger family known as ATP Binding Cassette (ABC) proteins, the majority of which are transporters.[1] In humans, 48 members of this family are divided into seven subclasses based upon degree of homology.[2]

Pgp is the most studied MDR efflux protein. However, the mechanism by which over-expression of Pgp is induced during exposure to chemotherapeutics or chemical agents is not fully understood. Induction may occur at the transcriptional level either due to gene amplification, gene rearrangement, DNA methylation, promoter mutation or chromatin modification.[9] With any one of these factors, transcription is the key for induction of the proteins and in some cases this could be a rapid response to intra/extracellular stimuli.[9a] There is still much to be learned regarding gene regulation of Pgp expression.

Keywords: Tetramethylrosamine; Thiatetramethylrosamine; Selenotetramethylrosamine; Photodynamic therapy; Multidrug resistance.
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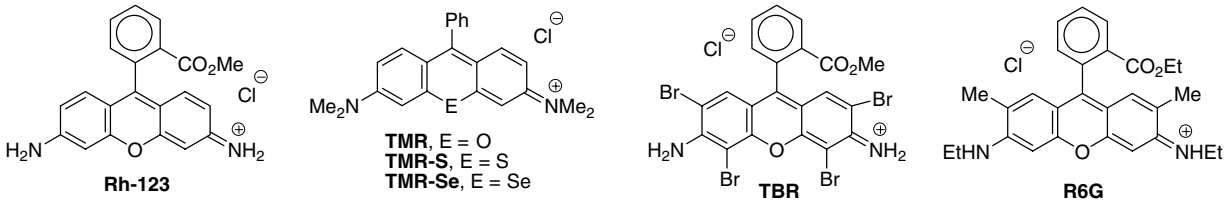


Chart 1.

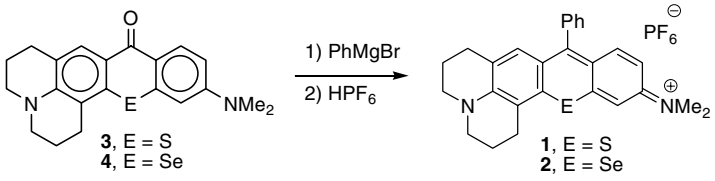
Myriad MDR reversal agents, including verapamil (**VER**), cyclosporin A, and PSC833, have been examined to counteract the mechanisms of drug resistance.[1,3,10] However, these compounds have major drawbacks, such as alterations in cell metabolism and their toxicity toward normal tissues. The therapeutic window for these compounds is also severely restricted because the dose necessary for effective inhibition of Pgp, in many cases, exceeds the minimal toxic concentration in normal tissue.[1,11,12] Ideally, Pgp modulators would be administered in combination with chemotherapeutic agent(s) to increase anti-cancer drug uptake, retention, and effectiveness. Unfortunately, concomitant administration of high doses of modulators and therapeutic doses of anti-cancer agents has resulted in unacceptable toxicity requiring chemotherapeutic dose reduction and ineffective treatment.[12]

Photodynamic therapy (PDT)[13] offers an alternative approach for inhibiting Pgp and other efflux pumps. If the photosensitizer is bound to or transported by the efflux pump in the cancer cells, irradiation may lead to irreversible efflux pump inhibition by photochemical generation of reactive oxygen species such as singlet oxygen, superoxide, or hydroxy radicals. Several photosensitizers have been examined with multidrug-resistant cells. For example, *meta*-tetra(hydroxyphenyl)chlorin (***m*THPC**) shows no difference in uptake and little difference in efficacy in vitro between MCF-7 cells and MCF-7/DXR cells that express Pgp.[14] In vivo, other studies show that there is no significant correlation between tumor ***m*THPC** levels and PDT response, which suggests that PDT effects are most likely associated with vascular damage.[15] Photofrin® also accumulates less in some drug-resistant cells in comparison to chemosensitive parent cell lines.[16] The rhodacyanine dye **Rhodac** is less phototoxic to multidrug-resistant cells in comparison to normal cells.[17] BCRP (ABCG2)-transfected HEK-293 cells are resistant to PDT with pheophorbide a (a BCRP substrate), pheophorbide a methyl ester, chlorin e6, and 5-aminolevulinic acid, but not to ***m*THPC** or *meso*-tetra(3-hydroxyphenyl)porphyrin.[18]

Among the best transport molecules for Pgp are the xanthylium dyes rhodamine 123 (**Rh-123**), rhodamine 6g (**R6G**), and tetramethylrosamine (**TMR**, Chart 1).[19,20] These dyes have been used as fluorescent markers to determine the efficacy of Pgp modulators[20,21] as well as uptake of the dyes in drug-resistant and chemosensitive cells.[16] While none of the rhodamine dyes **Rh-123**, **TMR**, or **R6G** inhibits Pgp function intrinsically either in the dark or with irradiation,[20,22] tetrabromorhodamine 123 (**TBR**, Chart 1) has shown phototoxicity toward Pgp-expressing cell lines.[23] However, the absorption maximum for **TBR** is <550 nm; well removed from the 600 to 900-nm window that is optimal for PDT.[13]

The combination of a Pgp-modulator and photosensitizer has given increased uptake/phototoxicity in drug-resistant cells. For example, in Pgp-expressing cells, the addition of **VER** gives increased uptake of **Rh-123**[16] as well as the heavy-chalcogen photosensitizers **TMR-S** and **TMR-Se**.[24] In contrast, the uptake of Photofrin® is unaffected by the addition of **VER**[16] although a cationic chlorin derivative has shown increased uptake in drug-resistant cells when the transport system is inhibited.[25] Again, co-administration of modulators of MDR efflux pumps and a photosensitizer may give undesirable toxicity.[12] Ideally, a photosensitizer that absorbs light in the 600–900-nm window and that is simultaneously an inhibitor/modulator of Pgp (and/or other efflux pumps) might allow a more effective treatment of multidrug-resistant tumors by PDT.

The synthesis of a library of **TMR** derivatives and their interaction with isolated Pgp have recently been described.[26] Within this library, proper location of hydrogen-bond acceptors gave derivatives that stimulated Pgp ATPase activity while more hydrophobic groups at the 9-position gave little if any stimulation. We report here the synthesis of pentacyclic analogues **1** and **2** (Scheme 1) of **TMR** and the comparison of the uptake of **1** in chemosensitive and drug-resistant cells, as well as the ability of **2** to function as a photoinactivator of Pgp and as a photosensitizer for both chemosensitive and multidrug-resistant cells.



Scheme 1. Synthesis of photosensitizers **1** and **2**.

2. Results

2.1. Synthesis of photosensitizers

Compounds **1** and **2** were prepared by the addition of phenylmagnesium bromide to chalcogenoxanthones **3** and **4**,[27] respectively, followed by the addition of HPF_6 (Scheme 1). Compounds **1** and **2** were isolated in 93% and 94% yields, respectively.

2.2. Physical and photophysical properties of 1 and 2

The N atom of the julolidyl fragment in **1** and **2** is locked into conjugation with the xanthylium core and cannot rotate unlike the dimethylamino substituent in the 6-position, which is free to rotate. Compounds **1** and **2** have absorption maxima, λ_{max}, of 582 and 588 nm in MeOH (Table 1 and Fig. 1), which are 11 and 7 nm longer than λ_{max} for **TMR-S** and **TMR-Se**, respectively.[28] In dichloromethane, values of λ_{max} are 582 and 591 nm and, in water, 581 and 590 nm for **1** and **2**, respectively. While λ_{max} for **2** is still below 600 nm, the molar extinction coefficient, ε, for **2** is 1.26×10^5 M^{-1} cm^{-1} at 588 nm in MeOH and the bandwidth at half-height is 35 nm such that dye **2** absorbs strongly at 600–610 nm ($\varepsilon > 6 \times 10^4$ M^{-1} s^{-1}).

Emission spectra for **1** and **2** are shown in Figure 1 with emission maxima (λ_{EM}) of 600 and 610 nm, respectively, for **1** and **2** in MeOH (Table 1). The conformational locking in **1** and **2** has little impact on values of the quantum yield for fluorescence (ϕ_F) or the quantum yield for the generation of singlet oxygen [$\phi(^1O_2)$] relative to **TMR-S** and **TMR-Se** (Table 1). Values of ϕ_F for **TMR-S** and **1** are 0.44 and 0.36, respectively, and for **TMR-Se** and **2** are 0.009 and 0.19, respectively. Values of $\phi(^1O_2)$ for **TMR-S** and **1** are 0.21 and 0.28, respectively, while the selenoxanthylium analogues **TMR-Se** and **2** have values of $\phi(^1O_2)$ of 0.87 and 0.85, respectively. Excited-state fluorescence lifetime values, τ_F, are nearly identical for **TMR-S** and **1**, and values of τ_F are much shorter for the selenoxanthylium dyes **TMR-Se** and **2**.

The extra four methylenes in the julolidyl fragment of **1** and **2** relative to **TMR-S** and **TMR-Se** also increase the lipophilicity of dyes **1** and **2**. While both **TMR-S** and **TMR-Se** have values of the *n*-octanol/water partition coefficient (log *P*) near 0.0,[26] **1** and **2** have values of log *P* of 2.3 and 2.4, respectively.

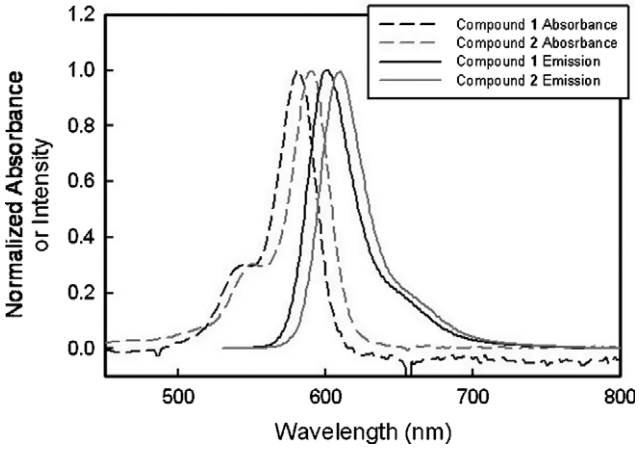


Figure 1. Absorbance and emission of compounds **1** and **2** in MeOH.

2.3. Photoinactivation of Pgp ATPase activity in isolated protein

In a recent study, we examined a small library of chalcogenoxanthylium dyes related in structure to **TMR** for their ability to stimulate Pgp ATPase activity[26] using lipid-activated, mouse MDR3 Cys-less Pgp.[29] From this study, it was clear that many of the **TMR** analogues were strongly protein bound. We hypothesized that irradiation of the dye–Pgp complex where the dye was a heavy chalcogen analogue of **TMR** might give photoinactivation of Pgp.

Lipid-activated, mouse MDR3 Cys-less Pgp was exposed to various concentrations of selenoxanthylium dye **2** in the dark and was then passed through a gel filtration column to remove unbound dye. Light-induced inactivation of Pgp ATPase was performed on the filtered dye–Pgp complex using 3.0 J cm^{-2} of filtered 350–800-nm light delivered at 50 mW (60 s of irradiation). The remaining Pgp ATPase activity was evaluated in the presence of 1.5×10^{-4} M **VER**, which is generally considered to give robust stimulation of Pgp ATPase activity. The combination of **2** and light gave complete inhibition of Pgp ATPase activity as shown in Figure 2a with an EC_{50}, the effective concentration of photosensitizer and 3.0 J cm^{-2} light to give 50% inhibition of 1.5×10^{-4} M **VER**-induced stimulation of ATPase, of $(4.7 \pm 0.6) \times 10^{-7}$ M. The photoinactivation at constant photosensitizer concentration was also linearly dependent on the light dose as shown in Figure 2b. Light alone had no impact on ATPase

Table 1. Absorption maxima (λ_{max}), molar extinction coefficients (ε), emission maxima (λ_{EM}), quantum yields for fluorescence (ϕ_F), excited-state fluorescence lifetimes (τ_F), and quantum yields for singlet oxygen generation [$\phi(^1O_2)$] for **TMR-S**, **TMR-Se**, and dyes **1** and **2**

Compound	λ_{max} (nm)	$\varepsilon_{max} \times 10^{-4}$ (M^{-1} cm^{-1})	λ_{EM} (nm)	ϕ_F[a]	τ_F (ns)	$\phi(^1O_2)$
TMR-S[b]	571	6.3	599	0.44	1.5	0.21
TMR-Se[b]	581	4.4	608	0.009	0.05	0.87
1	582 ± 1	10.5 ± 0.5	600 ± 1	0.36 ± 0.01	2.24 ± 0.02	0.28 ± 0.02
2	588 ± 1	12.6 ± 0.5	610 ± 1	0.019 ± 0.001	<0.1[c]	0.85 ± 0.02

[a] Quantum yields were determined using cresyl violet dissolved in MeOH as the standard; (ϕ_f 0.54).
[b] Data from reference 28.
[c] Too short for measurement.

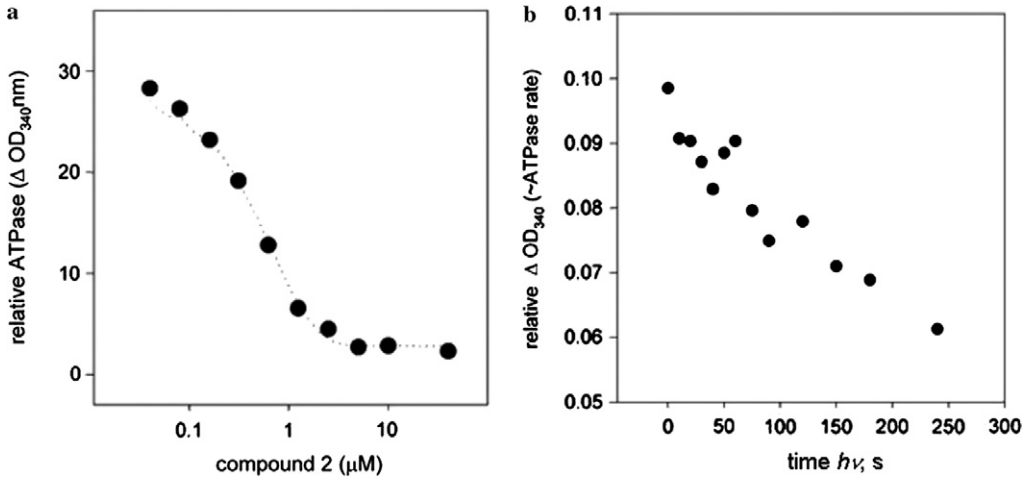


Figure 2. Photoinactivation of Pgp ATPase activity (a) with various concentrations of photosensitizer **2** and 3.0 J cm^{-2} of 350–800-nm filtered light and (b) with 5×10^{-6} M photosensitizer and various quantities of light. Each point represents the mean of at least three experiments performed in duplicate. Error bars have been omitted for clarity, but are all $\leqslant$10%.

activity indicating that the photosensitizer was necessary for photoinactivation. The photosensitizer-induced photoinactivation appeared to be irreversible since residual ATPase activity was unchanged over the ~1-h time course of the ATPase experiments.

2.4. Uptake of TMR-S and 1 into AUXB1 and CR1R12 cells

Ideally, the impact of the pentacyclic structure in **2** on photosensitizer uptake could be compared to the uptake of **TMR-Se**. Unfortunately, ϕ_F for **2** is only 0.019 (Table 1) and ϕ_F for **TMR-Se** is only 0.009,[28] which does not allow easy determination of cellular uptake of either **2** or **TMR-Se** by fluorescence. Fortunately, both **TMR-S** and thioxanthylium dye **1**, which are quite similar to **TMR-Se** and **2**, respectively, in structure, are highly fluorescent, with values of ϕ_F of 0.44 and 0.36, respectively (Table 1), and their presence was easily detected in chemosensitive AUXB1 cells and multidrug-resistant CR1R12 cells.

Drug-sensitive AUXB1 cells or multidrug-resistant CR1R12 cells were exposed to 5×10^{-6} M **TMR-S** or **1** in the dark for 2 h in complete medium. Consistent with previous data, **TMR-S** was taken up by both cell lines. The data displayed in Figure 3a demonstrate that **TMR-S** uptake in multidrug-resistant CR1R12 cells is significantly less in comparison to AUXB1 cells ($P < 0.005$) and is roughly 40% of the uptake of **TMR-S** in the drug-sensitive AUXB1 cells under these conditions. The addition of 2×10^{-4} M **VER** prior to dye exposure did not have a significant effect on the **TMR-S** uptake into the AUXB1 cells but gave a statistically significant increase in uptake ($P < 0.0001$) with a nearly 4-fold increase in **TMR-S** uptake in the CR1R12 cells. In the presence of 2×10^{-4} M **VER**, the uptake of **TMR-S** is comparable in both AUXB1 and CR1R12 cells.

In contrast as shown in Figure 3b, there is no significant difference in the uptake of dye **1** into either CR1R12 cells or AUXB1 cells ($P > 0.05$). The addition of 2×10^{-4} M **VER** prior to dye exposure had no significant impact on the uptake of **1** into either cell line ($P > 0.05$).

2.5. Phototoxicity of chalcogenoxanthylium dye 2 toward chemosensitive and multidrug-resistant cells

In the absence of prior exposure of cells to 5×10^{-5} M **VER**, neither **TMR-S** nor **TMR-Se** showed any significant dark or phototoxicity (>90% cell survival) toward CR1R12 cells at concentrations $\leqslant 5 \times 10^{-6}$ M.[24] In contrast, both **TMR-S** and **TMR-Se** were highly phototoxic

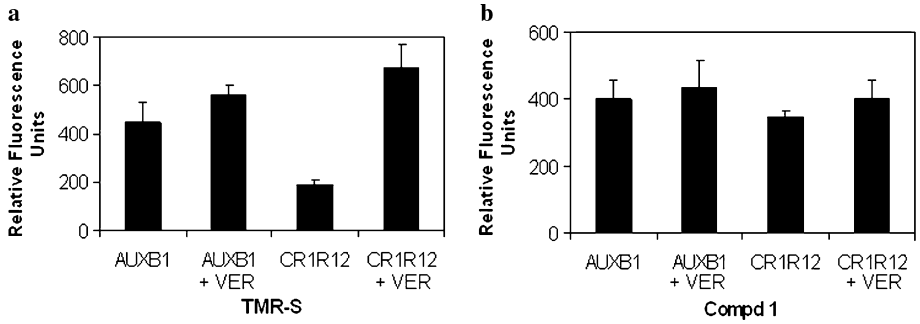


Figure 3. Relative uptake of (a) TMR-S and (b) dye **1** in chemosensitive AUXB1 cells and multidrug-resistant CR1R12 cells in the presence and absence of 2×10^{-4} M **VER**. Each bar represents the mean of at least three experiments performed in triplicate. Error bars indicate one standard deviation.

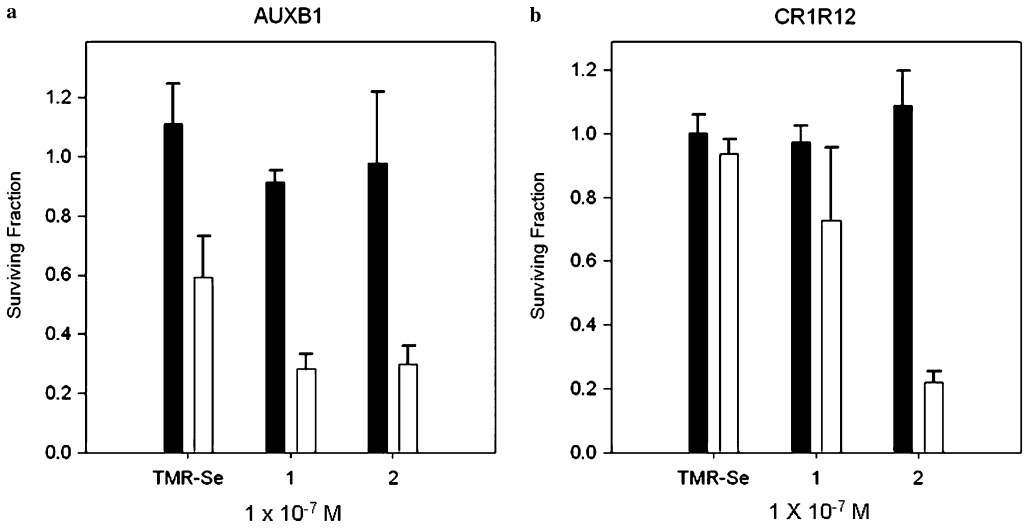


Figure 4. Dark and phototoxicity of selenoxanthylium dye **TMR-Se**, thioxanthylium dye **1**, and selenoxanthylium dye **2** toward (a) AUXB1 cells and (b) CR1R12 cells at 1×10^{-7} M with 5 J cm^{-2} of filtered 350–800-nm light. Data are expressed as the fraction of cell viability compared to control cells, those exposed to neither dye nor light. Each bar represents the mean of at least three experiments performed in triplicate. Error bars indicate one standard deviation.

toward AUXB1 cells with EC_{50}s of 1×10^{-7} and 2×10^{-7} M, respectively.[24]

In the current study, **TMR-Se** was included as a control compound and approximately 50% phototoxicity was observed in AUXB1 cells treated with 1×10^{-7} M **TMR-Se** with 5 J cm^{-2} of 350–800-nm light when compared to dark controls, that is, cells treated with 1×10^{-7} M **TMR-Se** and no light (Fig. 4a). Consistent with earlier results, **TMR-Se** showed no phototoxicity toward CR1R12 cells at 1×10^{-7} M with 5 J cm^{-2} of 350–800-nm light in comparison to dark controls with >90% cell survival (Fig. 4b).[24] Both thioxanthylium dye **1** and selenoxanthylium dye **2** gave 70% phototoxicity toward AUXB1 cells in comparison to dark controls ($P < 0.01$) at 1×10^{-7} M with 5 J cm^{-2} of 350–800-nm light (Fig. 4a). However, only selenoxanthylium dye **2** showed significant phototoxicity (80% phototoxicity) toward CR1R12 cells when compared to dark controls ($P < 0.001$) at 1×10^{-7} M with 5 J cm^{-2} of 350–800-nm light (Fig. 4b).

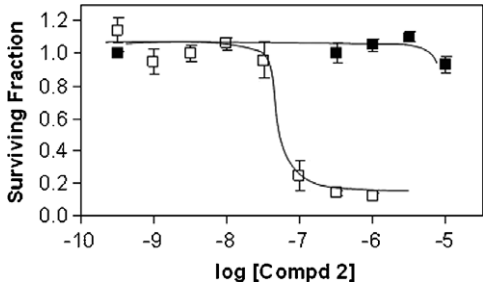


Figure 5. Effect of compound **2** on the viability of CR1R12 cells in culture in the dark (filled squares) or 24 h after exposure to various concentrations of dye and 5.0 J cm^{-2} of filtered 350–800-nm light (open squares). Each point represents three separate experiments performed in duplicate for the viability of cultured CR1R12 cells maintained in the dark or 24 h after exposure of cells to light. Data are expressed as the fraction of control cell viability, exposed to neither dyes nor light, error bars are the SEM.

The dark and phototoxicity for compound **2** at various concentrations toward CR1R12 cells with 5.0 J cm^{-2} of 350–800-nm light is shown in Figure 5. The EC_{50}, the effective concentration of photosensitizer and 5.0 J cm^{-2} of 350–800-nm light to give 50% cell kill, for compound **2** is $\approx 1 \times 10^{-7}$ M toward multidrug-resistant CR1R12 cells and toward chemosensitive AUXB1 cells (data not shown). Compound **2** also displayed minimal dark toxicity (>90% cell survival) at concentrations $\leqslant 1 \times 10^{-5}$ M toward CR1R12 cells (Fig. 5) and $\leqslant 3 \times 10^{-6}$ M toward AUXB1 cells (data not shown).

3. Discussion

The chalcogenoxanthylium dyes **1** and **2** are pentacyclic core ring systems that contain a julolidyl fragment around the 3-amino substituent of the xanthylium core. These dyes are significantly more lipophilic than **TMR-S** and **TMR-Se** due to the additional four methylene carbons in the julolidyl fragment. The lone-pair of electrons on the julolidyl nitrogen is conformationally locked into conjugation with the xanthylium π-framework causing a red shift in λ_{max} for **1** and **2** in comparison to **TMR-S** and **TMR-Se**, respectively. The rigidization of dyes **1** and **2** is also expected to slow the rate of internal conversion, but if this is the case, it has little effect on other photophysical properties such as ϕ_F and $\phi(^1O_2)$ where the thioxanthylium dyes **TMR-S** and **1**, and the selenoxanthylium dyes **TMR-Se** and **2** have similar values of ϕ_F and $\phi(^1O_2)$ (Table 1). **TMR-S** and **1** also have similar excited-state fluorescence lifetimes, τ_F. As photosensitizers, the selenoxanthylium dye **2** absorbs longer wavelengths of light (λ_{max} of 588 vs 582 nm) and has a higher value of $\phi(^1O_2)$ (0.85 vs 0.28) when compared

to **1** and, thus, has better characteristics as a photosensitizer in comparison to **1**.

Ideally, a photosensitizer that is effective against multidrug-resistant cancer cells is either unrecognized by the efflux pump(s) in the cell or acts as an inhibitor of the cellular efflux pump(s). With either condition alone or in combination, the photosensitizer will have a higher intracellular uptake compared to drugs that are recognized and transported by the efflux pumps. The chlorin ***m*THPC** is one example of a photosensitizer that accumulates comparably in multidrug-resistant cells and in chemosensitive cells.[14,18] Although ϕ_F for compound **2** was too small to allow easy determination of cellular uptake, compound **1** is structurally similar with ϕ_F of 0.36 and its presence in AUXB1 and CR1R12 cells is easily determined. While **TMR-S** shows reduced uptake in CR1R12 cells in comparison to AUXB1 cells, compound **1** shows similar uptake in both chemosensitive AUXB1 and multidrug-resistant CR1R12 cells. The addition of 2×10^{-4} M **VER** had no statistically significant effect on the uptake of **1** into either AUXB1 or CR1R12 cells.

As a photosensitizer, compound **2** is effective at a concentration of 1×10^{-7} M with 5 J cm^{-2} of 350–800-nm light both toward chemosensitive AUXB1 cells (70% phototoxicity) and toward multidrug-resistant CR1R12 cells (80% phototoxicity). Dark toxicity (<90% survival) is not observed with **2** at concentrations $\leqslant 3 \times 10^{-6}$ M in AUXB1 cells and $\leqslant 1 \times 10^{-5}$ M in CR1R12 cells. In contrast, while **TMR-Se** and thioxanthylium dye **1** are effective as photosensitizers toward chemosensitive AUXB1 cells at a concentration of 1×10^{-7} M, they show little if any phototoxicity toward CR1R12 cells (>75% cell survival) at concentrations of 1×10^{-7} M with 5 J cm^{-2} of 350–800-nm light.

Compound **2** as a photosensitizer appears to be capable of photoinactivation of lipid-activated, mouse MDR3 Cys-less Pgp.[29] To the best of our knowledge, photoinactivation of the isolated protein has *not* been previously demonstrated. As shown in Figure 1, compound **2** photoinactivates Pgp with an EC_{50} of $(4.7 \pm 0.6) \times 10^{-7}$ M at 3.0 J cm^{-2} of 350–800-nm light and the level of inactivation is proportional to the light dose. The isolated Pgp is unaffected by irradiation in the absence of photosensitizer and the photosensitizer-induced inactivation appears to be irreversible.

In summary, compound **2** represents an exciting lead molecule for a new class of cationic photosensitizers that are effective against multidrug-resistant cells at 1×10^{-7} M concentrations and that can photoinactivate multidrug efflux pumps such as Pgp. Synthetically, we can vary the 9-substituent in these derivatives via the addition of various Grignard and organolithium reagents to the chalcogenoxanthones **3** and **4**. Our challenges are to design derivatives that absorb longer wavelengths of light ($\lambda_{max} > 600$ nm) while maintaining desirable photophysical and biological properties.

4. Experimental

4.1. General methods

Solvents and reagents were used as received from Sigma–Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted. Cell culture media and antibiotics were obtained from Grand Island Biological (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA). Concentration in vacuo was performed on a Büchi rotary evaporator. **TMR-S** and **TMR-Se** were prepared according to Ref. 24a. Chalcogenoxanthones **3** and **4** were prepared according to Ref. 27. NMR spectra were recorded on Varian Gemini 300 or Inova 400 or 500 instruments with residual solvent signal as the internal standard. UV–vis–near–IR spectra were recorded on a Perkin-Elmer Lambda 12 spectrophotometer equipped with a circulating constant-temperature bath for the sample chambers. Elemental analyses were conducted by Atlantic Microlabs, Inc.

4.1.1. Synthesis of thioxanthylium dye 1. Phenylmagnesium bromide (1 M, 3.0 mL, 3.0 mmol) was added to thioxanthone **3** (0.106 g, 0.3 mmol) suspended in dry THF (10 mL). The resulting orange solution was heated at reflux for 1 h. The reaction mixture was cooled to 0 °C. A 12% solution of hexafluorophosphoric acid (5 mL) was slowly added to the reaction mixture producing a deep blue color. Ice water (60 mL) was added producing a dark blue precipitate, which was collected by filtration. The precipitate was washed with cold ether (3×) to give 0.106 g (93%) of **1** as a purple crystalline solid, mp > 300 °C: ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.62 (m, 3H), 7.31 (d, 1H, J = 10.0 Hz), 7.30 (m, 2H), 7.11 (d, 1H, J = 2.5 Hz), 7.06 (s, 1H), 6.87 (dd, 1H, J = 2.5, 10.0 Hz), 3.54 (t, 2H, J = 7.0 Hz), 3.53 (t, 2H, J = 7.0 Hz), 3.23 (s, 6H), 2.88 (t, 2H, J = 6.5 Hz), 2.67 (t, 2H, J = 6.0 Hz), 2.17 (m, 2H), 1.96 (m, 2H); ^{13}C NMR (75.5 MHz, CD_2Cl_2) δ 158.5, 153.0, 149.6, 142.7, 140.4, 136.4, 135.9, 133.6, 129.7, 129.5, 129.0, 125.9, 119.5, 118.8, 114.8, 114.2, 105.9, 51.8, 50.8, 40.6, 28.1, 24.4, 20.6, 20.2; HRMS (ESI) *m*/*z* 411.1890 calcd for $C_{27}H_{27}N_2S^+$: 411.1889. Anal. Calcd for $C_{27}H_{27}F_6N_2PS$: C, 58.27; H, 4.89; N, 5.03. Found: C, 57.94; H, 4.99; N, 4.87.

4.1.2. Synthesis of selenoxanthylium dye 2. Phenylmagnesium bromide (1 M in ether, 3.0 mL, 3.0 mmol) was added to selenoxanthone **4** (0.105 g, 0.26 mmol) suspended in dry THF (10 mL). The resulting orange solution was heated at reflux for 1 h. The reaction mixture was cooled to 0 °C. A 12% solution of hexafluorophosphoric acid (5 mL) was slowly added to the reaction mixture producing a deep blue color. Ice water (60 mL) was added producing a dark blue precipitate, which was collected by filtration. The precipitate was washed with cold ether (3×) to give 0.150 g (94%) of **2** as a purple crystalline solid, mp > 300 °C: ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.60 (d, 1H, J = 2.0 Hz), 7.61–7.58 (m, 2H), 7.32 (d, 1H, J = 10.0 Hz), 7.28–7.26 (m, 3H), 7.08 (s, 1H), 6.78 (dd, 1H, J = 2.5, 10.0 Hz), 3.53 (t, 2H, J = 6.0 Hz), 3.50 (t, 2H, J = 6.0 Hz), 3.21 (s,

6H), 2.78 (t, 2H, $J = 6.5$ Hz), 2.64 (t, 2H, $J = 6.0$ Hz), 2.19 (tt, 2H, $J = 6.0$, 6.5 Hz), 1.96 (tt, 2H, $J = 6.0$, 6.0 Hz); ^{13}C NMR [75.5 MHz, CD_2Cl_2] δ 160.2, 152.6, 149.5, 144.0, 142.9, 137.8(8), 137.8(6), 136.0, 129.6, 129.3, 128.9, 125.3, 120.2, 119.7, 117.0, 114.5, 108.8, 51.9, 51.0, 40.6, 28.0, 26.3, 20.7, 20.4; HRMS (ESI) *m/z* 459.1344 (calcd for $C_{27}H_{27}N_2Se^+$: 459.1334). Anal. Calcd for $C_{27}H_{27}F_6N_2PSe$: C, 53.74; H, 4.51; N, 4.64. Found: C, 54.07; H, 4.51; N, 4.62.

4.2. Determination of quantum yields for the generation of singlet oxygen

The quantum yields for singlet-oxygen generation with chalcogenoxanthylium dyes **1** and **2** were measured by direct methods in MeOH.[24b] A SPEX 270 M spectrometer (Jobin Yvon) equipped with InGaAs photodetector (Electro-Optical Systems Inc., USA) was used for recording singlet-oxygen emission spectra. A diode pumped solid-state laser (Millenia X, Spectra-Physics) at 532 nm was the excitation source. The sample solution in a quartz cuvette was placed directly in front of the spectrometer entrance slit e and the emission signal was collected at 90° relative to the exciting laser beam. An additional longpass filter (850 nm LP) was used to attenuate the excitation laser and the fluorescence from the photosensitizer.

4.3. Determination of fluorescence quantum yields

Fluorescence quantum yields (ϕ_F) and excited-state fluorescence lifetimes (τ_F) were determined using techniques that we have previously described.[30] Briefly, electronic absorbance spectra were recorded by using a HP model 8452A UV–vis spectrophotometer. Steady-state fluorescence spectra were recorded by using an SLM-AMINCO model 8100. A 450 W Xe arc lamp was used as the excitation source. Double and single grating monochromators served as the excitation and emission wavelength selection devices, respectively. The excitation wavelengths were set at 560 and 580 nm for compounds **1** and **2**, respectively. The excitation and emission spectral bandpasses were maintained at 4 nm. All emission spectra were background corrected by using appropriate blanks. Quantum yields were determined by using cresyl violet dissolved in MeOH as the standard; its value is 0.54.[31]

Time-resolved fluorescence experiments were carried out by using an IBH model 5000 W SAFE time-correlated single photon counting fluorescence lifetime instrument. A 595 nm pulsed light emitting diode (LED) (1 MHz) served as the excitation source. Single grating monochromators were used for wavelength selection. The excitation wavelength was adjusted to 595 nm (bandpass of 32 nm) and the emission monochromator was set at 620 nm (bandpass of 16 nm). To avoid pulse pileup, the count rate at each detector was always <2% of the LED repetition rate. The instrument response function and the time-resolved fluorescence intensity decay traces (under magic angle polarization conditions) were simultaneously recorded. All experiments were conducted under ambient conditions (20–22 °C) until there were at least 10^4 counts in the peak multichannel analyzer channel. The time-resolved intensity decay data were analyzed by using Globals WE (Globals Unlimited), a commercially available global analysis software package. All time-resolved intensity decay traces were single exponential over at least four lifetimes.

4.4. Determination of *n*-octanol/water partition coefficients

The octanol/water partition coefficients were all measured at pH 6 (phosphate-buffered) using UV–vis spectrophotometry. The measurements were done using a 'shake flask' direct measurement.[32] 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.

4.5. Cells and culture conditions

Cultured cells used in this study were the Chinese hamster ovary parental cell line AUXB1,[33] a chemosensitive cell line in which Pgp content is very low and the multidrug-resistant cell line CR1R12 which highly constitutively expresses Pgp. Multidrug resistance in CR1R12 cells was established from the CH^RC5 cell line[34] by sequential culturing in increasing concentrations of colchicine with 5×10^{-6} g mL^{-1} being the final concentration used. Cell lines were maintained in passage culture on 60-mm diameter polystyrene dishes in 4.0 mL minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin G, 5.0×10^{-5} g mL^{-1} streptomycin, and 1.0×10^{-6} g mL^{-1} Fungizone® (complete medium). Only cells from passages 1 to 10 were used for experiments. A stock of cells, passages 1–4, were maintained at −86 °C to initiate the experimental cultures. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere (Forma Scientific, Marietta, OH). Passage was accomplished by removing the culture medium, then adding a 1.0 mL solution containing 0.25% trypsin, incubating at 37 °C for 2–5 min to remove cells from the surface followed by seeding new culture dishes with an appropriate number of cells in 4.0 mL of α-MEM. Cell counts were performed using a particle counter (Model ZM, Coulter Electronics, Hialeah, FL).

4.6. Uptake of TMR-S and 1 into AUXB1 and CR1R12 cells

One day prior to uptake experiments, $\sim 1 \times 10^4$ AUXB1 or CR1R12 cells (in 2×10^{-4} L volume) were seeded per well on the inner wells of 96-well sterile tissue culture plates (Corning). (The outer wells were not used to avoid issues associated with evaporation of the aqueous media.) After 24 h and approximately 70% confluency, media were replaced with fresh media containing 5×10^{-6} M of either **TMR-S** or compound **1**. At this point, all manipulations were performed in the dark to avoid phototoxic effects. To avoid dye precipitation, media containing photosensitizers were prepared immediately prior to use by adding concentrated photosensitizer and **VER** (2×10^{-4} M final if present) from DMSO

stocks to 1 mL of media and this was then aliquoted to four 2×10^{-4} mL replicate wells. In all cases DMSO did not exceed 0.2%, and this was not toxic to the cells. After the media/dye mix was added, plates were wrapped in foil and placed at 37 °C with 5% CO_2 for 3 h. Next, plates were removed and wells were rinsed with 2×10^{-4} L sterile 0.9% NaCl (w/v). Finally, 1×10^{-4} L of 50% ethanol (v/v); 1% acetic acid (v/v) was added to each well. The amount of dye present was determined by fluorescence in a microplate reader (SpectraMax Gemini) and compared to a dilution series with excitation appropriate for each dye. For **TMR-S** excitation was at 575 nm and for compound **1** excitation was at 584 nm. In both cases, the emission at 650 nm was recorded.

4.7. Photoinactivation of pure Pgp

Mouse mdr3 CysLess Pgp was purified as described previously.[29] CysLess Pgp displays equivalent activity as wild-type and has already been extensively used to compare the effects of **TMR** analogues in a previous offering.[26] One advantage of using CysLess Pgp is that the need to pre-incubate with a reducing agent such as 1,4-dithiothreitol to prevent inactivating disulfide between P-loop cysteines is not necessary. Moreover, we wanted to avoid inactivation so that photoinactivation by the chalcogenoxanthylium dye is clearly established. CysLess Pgp was activated by incubation with *Escherichia coli* lipids at a 2:1 ratio (w/w) for 20 min at room temperature followed by 30-s sonication in a bath sonicator kept at 4 °C. Next, 1×10^{-5} g of lipid-activated Pgp was incubated in a final volume of 5×10^{-5} L in 4×10^{-2} M Tris–HCl, pH 7.4; 1×10^{-4} M EGTA (TE buffer) with the indicated amount of dye added from a concentrated DMSO stock. The final DMSO concentration did not exceed 2% and this was not inhibitory for Pgp in the absence of dye. All manipulations upon mixture of dye with Pgp were performed in the dark. This mixture was kept at room temperature for 20 min to allow dye binding. Next, samples were placed on ice for a minimum of 5 min and then an additional 5×10^{-2} L of ice-cold TE buffer was added, and the samples were quickly passaged through centrifuge columns at 4 °C (1K rpm spin for 2 min in a clinical centrifuge) as previously described.[26] Columns were home-made and consisted of 1-mL (tuberculin) syringes packed with G-50 Sephadex equilibrated with 5×10^{-2} M Tris–HCl, pH 7.4; 0.001% *n*-dodecyl-β-D-maltopyranoside (DDM; Anatrace). During optimization of this method, we found that addition of 8×10^{-6} M of 1% DDM to the collection tube helped to stabilize Pgp at subsequent steps. Next the entire sample ($\sim 9 \times 10^{-5}$ L) was added to a quartz cuvette (1 cm) and irradiated for 1 min at ambient temperature with 50 mW of 350–800-nm broad band light from a filtered tungsten source. The sample was quickly removed and two 4×10^{-5} L aliquots were placed in a 96-well plate on the inner wells kept on ice in the dark. Upon processing of all samples, 4×10^{-5} L of a 2× concentrated ATPase cocktail containing 2×10^{-2} M NaATP, 2.4×10^{-2} M $MgSO_4$, 3×10^{-3} M phosphoenol pyruvate, 1×10^{-3} M NADH, and 2×10^{-4} g mL^{-1} each of pyruvate kinase and lactate dehydrogenase (Roche), and 1.5×10^{-1} M **VER** was added and ATPase activity was determined by the rate of oxidation of NADH measured at 340 nm at 37 °C in a plate reader as previously described.[26] Relative ATPase rates as a function of dye concentration were plotted and IC_{50} values determined using non-linear regression with Sigmaplot software. Since the centrifuge columns effectively remove unbound photosensitizer, the IC_{50} for photoinactivation obtained by this method is representative of photosensitizer that is tightly bound/directly associated with Pgp, and not the result of indirect damage. Light alone did not cause photoinactivation and the effect of the photosensitizer was irreversible (no reactivation of Pgp was seen after more than 1 h during the kinetic assay). While damage was found to be light dose-dependent, a time of 1 min was chosen for convenience of experimental manipulations.

4.8. Irradiation of cultured cells

After seeding on 12-well plates, AUXB1 or CR1R12 cells were incubated for 24 h to allow cells to attach to the surface. Stock solutions of **2**, prepared at 1×10^{-3} M, were then added directly to the cell culture medium to give a final dye concentration of 3×10^{-9} to 1×10^{-5} M. Cell monolayers were then incubated for 3 h at 37 °C in the dark in a humidified 5% CO_2 atmosphere. The media were then removed and 1.0 mL of α-MEM minus FBS and phenol red (clear medium) was added to each well. One plate, with the lid removed, was then exposed to 350–800 nm light delivered at 1.4 mW cm^{-2} for 1 h (5.0 J cm^{-2}) from a filtered tungsten source for the various dye concentrations. The remaining plate was kept in the dark during the irradiation period. Immediately following irradiation the clear medium was replaced with complete medium and the monolayers were incubated for an additional 24-h period as above. Subsequently, cells were trypsinized and counted to determine cell viability.

4.9. Statistical analysis

Pairwise inter-comparisons among the experimental groups and comparisons with the controls were performed using student's *t*-test. A value of $P < 0.05$ is considered significant.

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References and notes

1. (a) Gottesman, M. M.; Fojo, T.; Bates, S. E. *Nat. Rev./Cancer* **2002**, *2*, 48; (b) Szakacs, G.; Paterson, J. K.;

Ludwig, J. A.; Booth-Genthe, C.; Gottesman, M. M. *Nat. Rev./Drug Discov.* **2006**, *5*, 219.
2. Dean, M.; Rzhetsky, A.; Allikmets, R. *Genome Res.* **2001**, *11*, 1156.
3. (a) Seelig, A.; Gatlik-Landwojtowicz, E. *Mini-Rev. Med. Chem.* **2005**, *5*, 135; (b) Raub, T. J. *Mol. Pharmacol.* **2006**, *3*, 3.
4. Owen, A.; Chandler, B.; Back, D. J. *Fundam. Clin. Pharmacol.* **2005**, *19*, 283.
5. Van Bambeke, F.; Balzi, E.; Tulkens, P. M. *Biochem. Pharmacol.* **2000**, *60*, 457.
6. Borst, P. M.; Ouellette, M. *Annu. Rev. Microbiol.* **1995**, *49*, 427.
7. Wolfger, H.; Mamnun, Y. M.; Kuchler, K. *Res. Microbiol.* **2001**, *152*, 375.
8. Burger, H.; Foekens, J. A.; Look, M. P.; Meijer-van Gelder, M. E.; Klijn, J. G. M.; Wiemer, E. A. C.; Stoter, G.; Nooter, K. *Clin. Cancer Res.* **2003**, *9*, 827.
9. (a) Scotto, K. W. *Oncogene* **2003**, *22*, 7496; (b) Baker, E. K.; El-Osta, A. *Exp. Cell Res.* **2003**, *290*, 177.
10. (a) Tiberghien, F.; Loor, F. *Anti-Cancer Drugs* **1996**, *7*, 568; (b) Hirsch-Ernst, K. I.; Ziemann, C.; Rustenbeck, I.; Kahl, G. F. *Toxicology* **2001**, *167*, 47; (c) Loor, F.; Tiberghien, F.; Wenandy, T.; Didier, A.; Traber, R. *J. Med. Chem.* **2002**, *45*, 4598.
11. Fisher, G. A.; Lum, B. L.; Hausdorff, J.; Sikic, B. I. *Eur. J. Cancer A* **1996**, *32*, 1082.
12. Fojo, T.; Bates, S. *Oncogene* **2003**, *22*, 7512.
13. Detty, M. R.; Gibson, S. L.; Wagner, S. J. *J. Med. Chem.* **2004**, *47*, 3897.
14. Teiten, M. H.; Bezdetnaya, L.; Merlin, J. L.; Bour-Dill, C.; Pauly, M. E.; Dicato, M.; Guillemin, F. *J. Photochem. Photobiol. B* **2001**, *62*, 146.
15. Cramers, P.; Ruevekamp, M.; Oppelaar, H.; Dalesio, O.; Baas, P.; Stewart, F. A. *Br. J. Cancer* **2003**, *88*, 283.
16. Croce, A. C.; Supino, R.; Lanza, K. S.; Locatelli, D.; Baglioni, P.; Bottiroli, G. *Photochem. Photobiol. Sci.* **2002**, *1*, 71.
17. Delaey, E. M.; Vantieghem, A. M.; Derycke, A.; Agostinis, P. M.; de Witte, P. A. *Photochem. Photobiol.* **2001**, *74*, 331.
18. Robey, R. W.; Steadman, K.; Polgar, O.; Bates, S. E. *Cancer Biol. Ther.* **2005**, *4*, 187.
19. (a) Kessel, D. *Cancer Commun.* **1989**, *1*, 145; (b) Eytan, G. D.; Regev, R.; Oren, G.; Hurwitz, C. D.; Assaraf, Y. G. *Eur. J. Biochem.* **1997**, *248*, 104.
20. Loetchutinat, C.; Saengkhae, C.; Marbeuf-Gueye, C.; Garnier-Suillerot, A. *Eur. J. Biochem.* **2003**, *270*, 476.
21. Robey, R.; Bakke, S.; Stein, W.; Meadows, B.; Litman, T.; Patil, S.; Smith, T.; Fojo, T.; Bates, S. *Blood* **1999**, *93*, 306.
22. Shapiro, A. B.; Ling, V. *Eur. J. Biochem.* **1998**, *254*, 189.
23. Kessel, D.; Woodburn, K. *Br. J. Cancer* **1995**, *71*, 306.
24. (a) Gibson, S. L.; Hilf, R.; Donnelly, D. J.; Detty, M. R. *Bioorg. Med. Chem.* **2004**, *12*, 4625; (b) Gibson, S. L.; Holt, J. J.; Ye, M.; Donnelly, D. J.; Ohulchanskyy, T. Y.; You, Y.; Detty, M. R. *Bioorg. Med. Chem.* **2005**, *13*, 6394.
25. Kessel, D.; Woodburn, K.; Skalkos, D. *Photochem. Photobiol.* **1994**, *60*, 61.
26. Tombline, G.; Donnelly, D. J.; Holt, J. J.; You, Y.; Ye, M.; Gannon, M. K.; Nygren, C. L.; Detty, M. R. *Biochemistry* **2006**, *45*, 8034.
27. Holt, J. J.; Detty, M. R. *Synlett* **2006**, submitted for publication.
28. Ohulchanskyy, T. Y.; Donnelly, D. J.; Detty, M. R.; Prasad, P. N. *J. Phys. Chem. B* **2004**, *108*, 8668.
29. Tombline, G. L.; Urbatsch, I. L.; Virk, N.; Muharemagic, A.; White, B.; Senior, A. E. *Arch. Biochem. Biophys.* **2006**, *445*, 124.
30. Baker, G. A.; Bright, F. V.; Detty, M. R.; Pandey, S.; Stilts, C. E.; Yao, H. R. *J. Porphyrins Phthalocyanines* **2000**, *4*, 669.
31. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Plenum Press: York, 1999, Chapter 2.
32. Sangster, J. In *Octanol–Water Partition Coefficients: Fundamentals and Physical Chemistry*; Fogg, P. G. T., Ed.; John Wiley: New York, 1997.
33. Essodaigui, M.; Broxterman, H. J.; Garnier-Suillerot, A. *Biochemistry* **1998**, *37*, 2243.
34. Homolya, L.; Hollo, Z.; Germann, U. A.; Pastan, I.; Gottesman, M. M.; Sarkadi, B. *J. Biol. Chem.* **1993**, *268*, 21493.