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Analogues of tetramethylrosamine as transport molecules for and inhibitors of P-glycoprotein-mediated multidrug resistance

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Abstract—Tetramethylrosamine and its thio- and seleno- analogues (**TMR-O**, **TMR-S**, and **TMR-Se**, respectively) were examined for their ability to be transported by Pgp into chemo-resistant CR1R12 cells. Verapamil (7×10^{-6} M) enhanced the uptake of **TMR-O** and **TMR-S** into CR1R12 cells compared to those cultures not previously exposed to verapamil. The uptake of **TMR-O** and **TMR-S** in CR1R12 cells in the presence of 7×10^{-6} M verapamil was equivalent to its uptake in the chemo-sensitive parent cell line AUXB1 in the absence or presence of verapamil. None of the TMR analogues were effective alone as photosensitizers of CR1R12 cells. However, when either **TMR-S** or **TMR-Se** was added to CR1R12 cells after 7×10^{-6} M verapamil exposure for 2 h, irradiation of cultures with 5.0 J cm^{-2} of 350–750 nm light caused significant phototoxicity. **TMR-O** showed no significant phototoxicity in the presence of verapamil. Chemo-sensitive AUXB1 cells are equally susceptible to phototoxicity using **TMR-Se** with or without previous exposure to verapamil. The Pgp modulators verapamil and CsA increased the uptake of CAM into CR1R12. Exposure of CR1R12 cells to **TMR-S** or **TMR-Se** for 2 h in the dark resulted in no significant change in the intracellular accumulation of CAM. However, 1 h of light exposure after incubation of cells with **TMR-S** or **TMR-Se** resulted in an up to 2-fold increase in CAM uptake.

1. Introduction

Multidrug resistance (MDR), mediated by the plasma membrane protein P-glycoprotein (Pgp), is a major concern for treatment of primary, metastatic, and recurrent cancer.[1–3] Pgp pumps a variety of structurally and mechanistically unrelated chemicals and chemotherapeutic agents from tumor cells, resulting in treatment failures.[1,4–6] Tumor cell resistance to a wide assortment of chemotherapeutic agents can arise from exposure to a single drug making subsequent treatments ineffective.[1]

The mechanism by which Pgp overexpression is induced during exposure to chemotherapeutics or chemical agents is still not fully understood. Induction may occur at the transcriptional level either due to gene amplification, gene rearrangement, DNA methylation, promoter mutation or chromatin modification.[7,8] With any one of these factors, transcription is the key for induction of Pgp and in some cases this could be a rapid response to intra/extracellular stimuli.[7] There is still much to be learned regarding gene regulation of Pgp expression. Development of therapeutic interventions at the transcriptional level could be advantageous. Currently, the most direct approach to inhibiting Pgp function in cancer is at the level of binding and/or the inhibition of ATP hydrolysis that Pgp is dependent upon for drug efflux from cells.

Myriad MDR reversal agents, including verapamil, cyclosporin A, and PSC833, have been examined to counteract the mechanisms of drug resistance.[9–11] 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 severely restricted because the dose necessary for effective inhibition of Pgp, in many cases, exceeds the minimal toxic concentration in normal tissue.[1,12,13] Ideally, Pgp modulators would be administered in combination with chemotherapeutic agent(s) to

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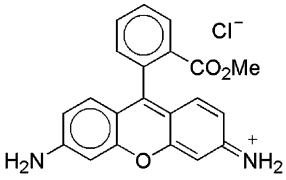
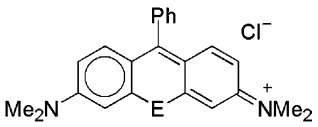
increase anti-cancer drug uptake, retention and effectiveness. However, concomitant administration of high doses of modulators and therapeutic doses of anti-cancer agents have resulted in unacceptable toxicity requiring chemotherapeutic dose reduction and ineffective treatment.[13]

One source of Pgp inhibitors might be derived from the cationic rhodamine dyes, such as rhodamine 123 (**Rh123**) and tetramethylrosamine (**TMR-O**).[14–16] These dyes are transport substrates for Pgp and have been used as fluorescent markers to determine the efficacy of Pgp modulators.[16,17] However, **Rh123** and **TMR-O** do not inhibit Pgp function.[16,18]



Rh123



TMR-O, E = O
TMR-S, E = S
TMR-Se, E = Se

We have recently reported the synthesis and photophysical properties of thio- (**TMR-S**) and seleno-(**TMR-Se**) analogues of **TMR-O** as well as the effectiveness of these materials as photosensitizers toward R3230AC rat mammary adenocarcinoma cells.[19] We initially designed **TMR-S** and **TMR-Se** analogues to be used as photosensitizers of mitochondrial function in cancer cells because their delocalized positive charge should attract them to the strong energy potential and negative internal charge of the mitochondria. Once in the mitochondria, the higher singlet oxygen yields of **TMR-S** and **TMR-Se** relative to either **Rh123** or **TMR-O** upon irradiation make these molecules far more effective as photosensitizers than either **Rh123** or **TMR-O**.[19] Because **TMR-O** is also a transport substrate for Pgp, we surmised that **TMR-S** and **TMR-Se** might have the same properties and in fact might be useful as photosensitizers of Pgp. In this report, we have evaluated the same three compounds for their ability to be transported by Pgp into CR1R12 cells, a highly constitutively Pgp-expressing cell line.[20,21] For comparison, parallel experiments were performed in the chemo-sensitive, parent Chinese hamster ovary cell line, AUXB1.[22] We present data showing that **TMR-S** and **TMR-Se** are, in fact, not only transported by Pgp, but that they can photosensitize the inhibition of Pgp function. These results strongly suggest that these compounds and possibly other rhodamine analogues could be potent photosensitizers for PDT and may simultaneously act as reversal agents of Pgp function.

2. Results

2.1. Time course of uptake of TMR-O into CR1R12 cells

The development of the fluorescence signal from **TMR-O** at 570 nm was followed in cultured CR1R12 cells either exposed to 7×10^{-6} M verapamil 2 h prior to dye addition or in cultures without previous exposure to verapamil. The data in Figure 1 clearly show that verapamil enhances the uptake of **TMR-O** into CR1R12 cells compared to those cultures not previously exposed to verapamil. The increase in intracellular uptake of **TMR-O** in the presence of verapamil becomes statistically significant ($P<0.005$) by 1 h after dye addition to monolayers of cells.

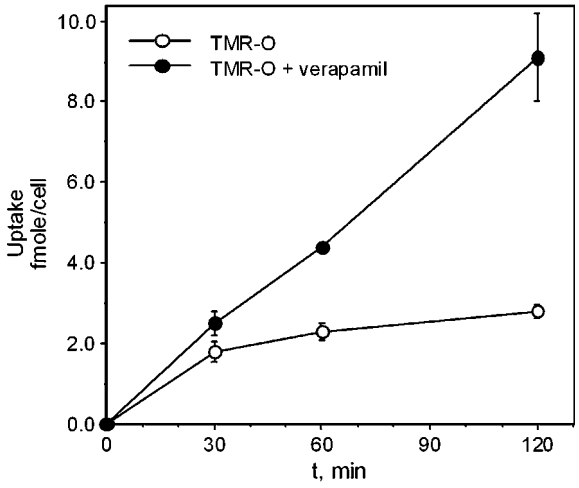


Figure 1. Time course of the uptake of **TMR-O** (1×10^{-5} M) into cultured Pgp-expressing CR1R12 cells in the absence (open circles) or the presence (filled circles) of 7×10^{-6} M verapamil. Experimental conditions are described in detail in the Experimental Section. Each data point, expressed as fmole **TMR-O**/cell, represents the mean of three separate experiments. Data for each experiment was calculated from results obtained from at least four wells of a 96-well culture plate, bars are the SEM.

2.2. Comparison of TMR-O and TMR-S uptake into AUXB-1 or CR1R12 cells

TMR-O or **TMR-S** was added for 1 h at 1×10^{-5} M to cultured AUXB-1 or CR1R12 cells, either alone or after cells had been incubated with 7×10^{-6} M verapamil for 2 h as above. The data in Figure 2 demonstrate that the intracellular accumulation of either **TMR-O** or **TMR-S** was similar in AUXB1 cells in the absence or the presence of verapamil. In CR1R12 cells, the accumulation of both **TMR-O** and **TMR-S** was significantly enhanced in the presence of verapamil compared to cells not previously exposed to verapamil. A more than 3-fold increase in uptake for **TMR-S** was obtained. The uptake of **TMR-S** in the chemo-resistant cell line, CR1R12, in the presence of verapamil was equivalent to its uptake in the chemo-sensitive parent cell line AUXB1 in the absence or presence of verapamil. In AUXB1 cells in the absence or presence of verapamil and in CR1R12 cells in the presence of verapamil, the uptake of **TMR-O** was equivalent to the uptake of **TMR-S**. These data clearly show that verapamil, a strong modulator of Pgp function, significantly effects the uptake of **TMR-O** or **TMR-S** in the chemo-resistant cell line CR1R12 while no effect was seen in the chemo-sensitive AUXB1 cell line. Under the conditions employed, the results strongly suggest that **TMR-S** is a transport substrate for Pgp, similar to **TMR-O**.

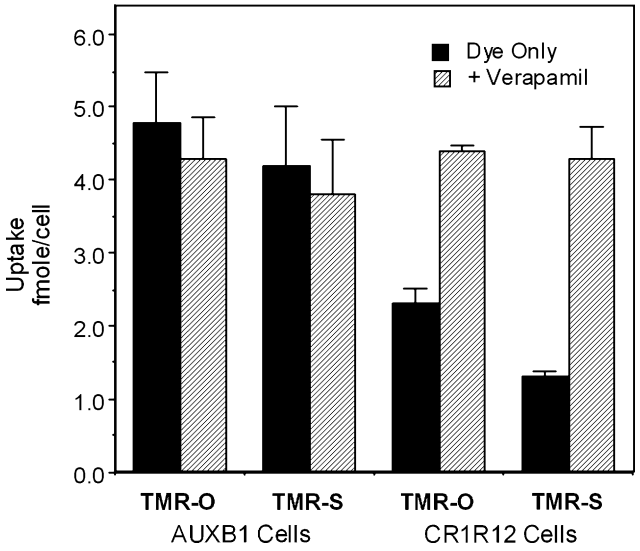


Figure 2. Comparison of the uptake of 1×10^{-5} M **TMR-O** or **TMR-S** after 1 h into cultured, chemo-sensitive AUXB1 and chemo-resistant CR1R12 cells after 1-h incubation in the absence (solid columns) or presence (striped columns) of 7×10^{-6}M verapamil. Experimental conditions are described in detail in the Experimental Section. Each column, expressed as fmole of dye/cell, represents the mean of the data obtained from three separate experiments. Data for each experiment was calculated from results obtained from at least four wells of a 96-well culture plate, bars are the SEM.

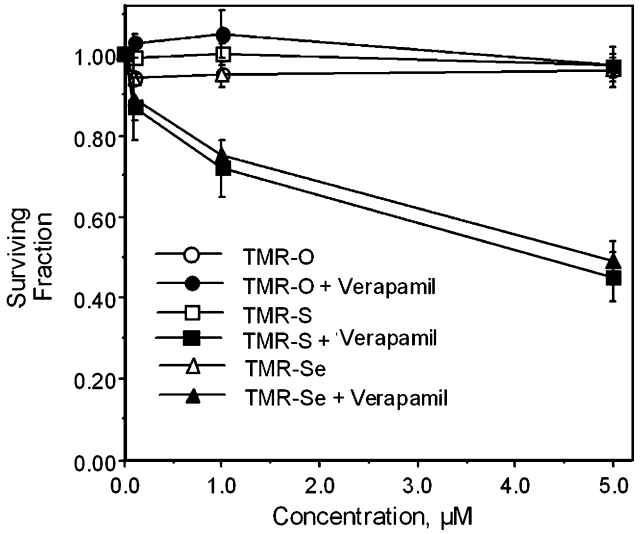


Figure 3. Cell viability of cultured chemo-resistant CR1R12 cells 24 h after a 2-h incubation with (filled symbol) or without (open symbol) 7×10^{-6}M verapamil followed by a 2-h incubation with **TMR-O** (circles), **TMR-S** (squares) or **TMR-Se** (triangles) at indicated concentrations (*x*-axis) and 1-h exposure of the culture plates to light. Experimental and light exposure conditions are described in detail in the Experimental Section. Each data point represents the mean, expressed as percent of control cell viability (cells not exposed to verapamil, dyes or light) of at least three separate experiments performed in triplicate, error bars are the SEM. Cells exposed to light alone or dyes alone in the dark using the same conditions showed no significant loss in cell viability.

2.3. Phototoxicity toward CR1R12 chemo-resistant cells

The data in Figure 3 depict the results obtained when CR1R12 cells are exposed to **TMR** analogues and 5.0 J cm^{-2} light with or without 7×10^{-6} verapamil. The data clearly demonstrate that none of the **TMR** analogues are effective as photosensitizers of CR1R12 cells in the absence of previous exposure to verapamil. However, when **TMR-S** and **TMR-Se** were added to CR1R12 cells after verapamil exposure for 2 h, irradiation of cultures with broad band white light caused significant cytotoxicity. **TMR-O** showed no significant phototoxicity in the absence or presence of verapamil even at the highest concentration, presumably due to its relatively low singlet oxygen production.[19] These data show that **TMR-S** and **TMR-Se** are phototoxic to chemo-resistant CR1R12 cells when a Pgp modulator is present indicating that uptake ensues and that the dyes are able to reach and cause damage to a critical intracellular site during irradiation.

2.4. Comparison of TMR-Se phototoxicity towards AUXB1 or CR1R12 cells

For comparison studies, parallel phototoxicity experiments were performed both with AUXB1 cells, which have low levels of Pgp, and with CR1R12 cells, which constitutively express high levels of Pgp. The data displayed in Figure 4 demonstrate that the chemo-sensitive AUXB1 cells are equally susceptible to phototoxicity using **TMR-Se** with or without previous exposure to 7×10^{-6}M verapamil while significant phototoxicity towards CR1R12 cells only occurs when verapamil is present.

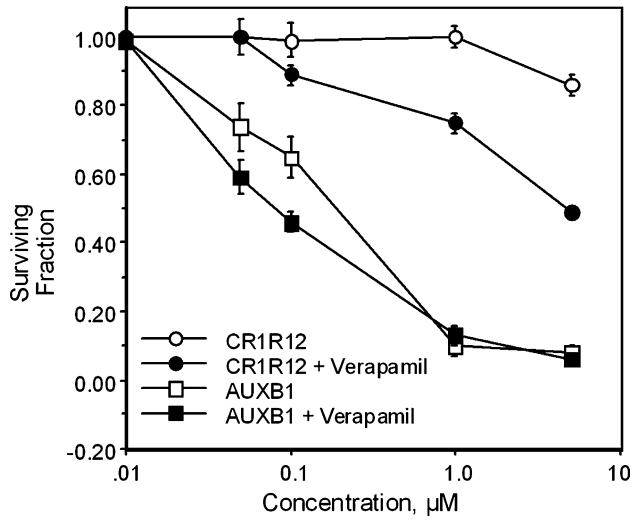


Figure 4. Comparison of the effects of **TMR-Se** and light exposure on the cell viability of cultured chemo-resistant CR1R12 cells (circles) or chemo-sensitive AUXB1 cells (squares) or in the absence (empty symbol) or presence (filled symbol) of 7×10^{-6}M verapamil. Experimental and light exposure conditions are described in detail in the Experimental Section. Each data point represents the mean, expressed as percent of control cell viability (cells not exposed to verapamil, dye or light) of at least three separate experiments performed in triplicate, error bars are the SEM. Cells exposed to light alone or dye alone in the dark using the same conditions showed no significant loss in cell viability.

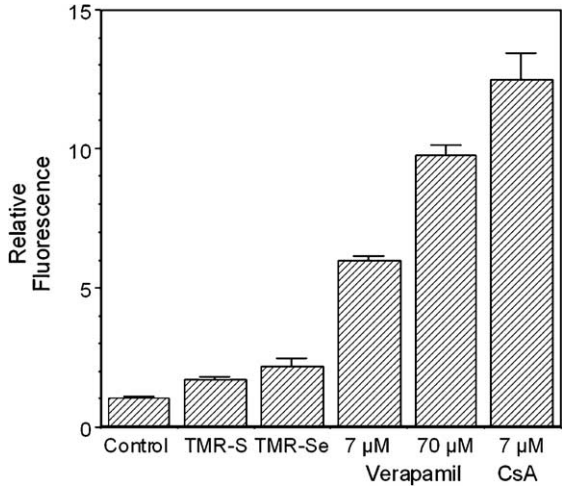


Figure 5. Uptake of Calcein AM (CAM) into cultured chemo-resistant CR1R12 cells after exposure to the Pgp modulators verapamil (7×10^{-6} M or 7×10^{-5} M) or cyclosporin A (7×10^{-6} M) or after cells were exposed to **TMR-S** or **TMR-Se** (1×10^{-5} M) for 2 h in the dark followed by 5.0 J cm^{-2} irradiation. Experimental and light exposure conditions are described in detail in the Experimental Section. Uptake of CAM is measured over a 30-min time period and is expressed as the relative rate of uptake measured in control cells (cells not exposed to verapamil, CsA, dyes or light, relative rate of 1.00 ± 0.03). Each column represents the mean of at least three separate determinations, bars are the SEM.

2.5. Effect of TMR-S or Se photosensitization on the intracellular uptake of Calcein AM

Calcein AM (CAM) is a nonfluorescent, hydrophobic compound that readily crosses the plasma membrane of normal cells. Once inside the cell, the ester bonds of CAM are enzymatically cleaved transforming it into the highly fluorescent, hydrophilic calcein. Appearance of the calcein fluorescence signal following exposure of cells to CAM indicates the intracellular uptake of CAM followed by ester hydrolysis to form calcein and retention of calcein in the cytosol of the cell.

Cells were exposed to **TMR-S** or **TMR-Se** (1×10^{-5} M) with or without light exposure or to the Pgp modulators verapamil or cyclosporin A (CsA) in the dark. Following exposure to the dyes with or without light or to the modulators, CAM was added at 1×10^{-6} M and its intracellular accumulation over 30 min was monitored by measuring the increase in calcein fluorescence at 530 nm with excitation at 485 nm. The data displayed in Figure 5 show that the Pgp modulators verapamil and CsA increased the uptake of CAM into CR1R12 cells from 6 to 13-fold over control CAM uptake in the absence of any modulators. Exposure of CR1R12 cells to **TMR-S** or **TMR-Se** for 2 h in the dark resulted in no significant change in the intracellular accumulation of CAM (data not shown). However, 1 h of light exposure after incubation of cells with **TMR-S** or **TMR-Se** resulted in an up to 2-fold increase in CAM uptake.

Parallel experiments were performed with the parent cell line, AUXB1. In these chemo-sensitive cells, CAM in the presence of Pgp modulators was accumulated intracellularly at a rate equivalent to that obtained for control AUXB1 cells, 1000 ± 30 relative fluorescence units (RFU) of calcein/min/1×10^5 cells. This rate of CAM uptake into AUXB1 cells was 10-fold greater than that measured in control CR1R12 cells at 94 ± 3 RFU/min/1×10^5 cells. Curiously, higher concentrations of **TMR-S** or **TMR-Se** plus light exposure virtually abolished CAM uptake into AUXB1 cells. This result could be due to factors such as damage to the plasma membrane system(s) responsible for intracellular accumulation of CAM or inhibition of intracellular esterases responsible for cleaving the ester bond on CAM that provides a fluorescent compound. These data, taken together, demonstrate that **TMR-S** and **TMR-Se** and light, under the conditions employed, do increase the intracellular accumulation of the Pgp substrate CAM into chemo-resistant CR1R12 cells. The experimental conditions remain to be optimized but these initial experiments demonstrate that highly expressing Pgp cells can be altered by these tetramethylrosamine analogues to take in 2 times more Pgp substrate than cells not exposed to the dyes and light.

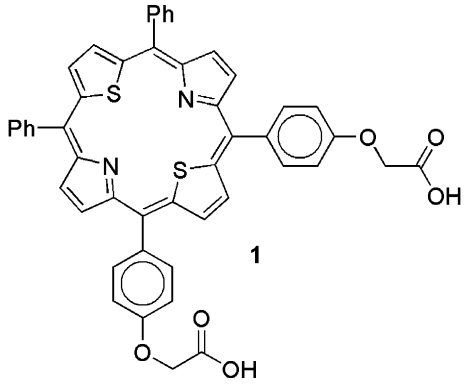
3. Discussion

Multidrug resistance is a phenomenon in cancer therapeutics that continues to elude efforts to reverse its role as a roadblock to successful treatment. The basis for its mechanism of action is the presence of ABC (ATP binding cassette) protein(s) in the plasma membrane that act as efflux pumps for a wide variety of chemically and functionally unrelated molecules.[1,23] The removal of chemotherapeutic drugs by ABC transporters results in the failure of these agents to achieve intracellular levels high enough to induce their expected cytotoxicity. The ABC family includes a number of membrane associated proteins, of which Pgp, a protein encoded by the MDR1 gene, was the first to be identified as a multidrug resistant efflux pump.[24] Of the many transporters that confer the MDR phenotype, Pgp is responsible for eliminating more molecules of different types than all the other MDR proteins combined.[7] Circumventing Pgp action could be of benefit for delivery of cancer chemotherapeutics. Examples of modulators of Pgp include verapamil, phenothiazines, quinine, cyclosporin A, tamoxifen, PSC833 (valspodar) and XR9576 (tariquidar) among many others. A major drawback for these compounds is that their interactions with chemotherapeutics are unacceptable, even though the modulators are supposed to enhance chemotherapeutics' effects. The net result is a reduction in the effectiveness of the therapeutic agent.[25–27] A series of new Pgp modulators that fit the criteria necessary for successful treatment are under investigation.[28–30] These have not yet been studied extensively in the clinic. Results from future investigations into their action will determine their efficacy as a new generation of Pgp modulators.

Others and we have taken a different approach. Photodynamic therapy (PDT) is a cancer treatment regimen that relies on three components for its effectiveness.[31] First, a photosensitizer, such as the porphyrin-derived Photofrin,® has to be in place in the target tissue. Second, light corresponding to the photosensitizer-absorbing wavelength must be delivered to the site at an appropriate fluence rate that will excite the embedded photosensitizer. Third, oxygen must be present in sufficient quantities to allow the photochemical generation of singlet oxygen at cytotoxic levels throughout the light exposure period. This form of therapy has been used successfully for the treatment of a variety of malignancies.[32–34] To enhance the efficacy of PDT, some earlier studies combined PDT with chemotherapeutic intervention. One study of RIF tumors in mice found that PDT using hematoporphyrin derivative (HpD) in combination with cisplatin did not result in increased efficacy beyond that observed with either treatment alone.[35] However, a combination of PDT with adriamycin did result in increased efficacy over single therapy treatment.[35,36]

Those early studies prompted the question as to whether PDT might be successful against cells or tumors expressing the MDR phenotype. It was reported that photosensitization of colorectal carcinoma multicellular spheroids with HpD was potentiated by the addition of the Pgp modulator, verapamil.[37] It was suggested that the increased phototoxicity was due to the binding of verapamil to Pgp thus interfering with HpD binding to Pgp. The net results were reduced efflux of HpD from the cell and increased intracellular concentrations of the photosensitizer. Contrary to these findings, Kessel and Erickson found that the uptake and resultant phototoxicity of mesoporphyrin was no different in drug-sensitive P388 murine leukemia cells than in P388/ADR adriamycin resistant cells—cells, which possess the MDR phenotype.[38] They concluded that cells that express the MDR phenotype are unlikely to be cross-resistant to PDT. In one in vivo study,[39] it was reported that PDT, using aluminum phthalocyanine as a photosensitizer, inhibited tumor growth to a similar degree in both doxorubicin-sensitive and -resistant melanoma, leukemia and lymphoma tumors. This work suggested that PDT might be beneficial for treatment of chemo-resistant malignancies.

In an earlier study, no difference was found in the photosensitizing potency of Photofrin® towards either chemo-sensitive AUXB1 cells or the highly Pgp-expressing CR1R12 cell line.[40] Experiments were also performed to determine whether exposure of CR1R12 cells to Photofrin® and light would effect the ATPase activity of Pgp. The ATPase activity was completely inhibited in CR1R12 cells exposed to $1.0\,\mu g\,mL^{-1}$ Photofrin® for 24 h prior to irradiation with $500\,mJ\,cm^{-2}$ of fluorescent light. In addition, a small but significant increase in adriamycin toxicity was attained in the chemo-resistant CR1R12 cells after their exposure to sub-lethal treatment with Photofrin® and light. These data, taken together, demonstrate that the porphyrins are phototoxic to cells possessing the MDR phenotype and that Pgp function may be impaired, which may allow increased uptake and toxicity of chemotherapeutic agents. As part of this present study, we also found that there was no difference in intracellular accumulation of dicarboxylic acid dithiaporphyrin **1**[41] between CR1R12 cells previously incubated with verapamil and those without verapamil exposure (data not shown).



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These earlier studies demonstrated that photosensitized inhibition of Pgp with porphyrins was effective even though the porphyrins were probably not ligands or transport molecules for Pgp. We synthesized the thio- and seleno-analogues of **TMR-O**, knowing that **Rh123** is a transport substrate for Pgp, and investigated these synthetic rhodamines for their properties as substrate molecules for and photosensitizers of Pgp. Neither **TMR-O** nor **Rh123** generates sufficient quantities of singlet oxygen to be effective photosensitizers with quantum yields for singlet oxygen generation [$\phi(^1O_2)$] of 0.08 and 0.015, respectively, while **TMR-S** and **TMR-Se** have values $\phi(^1O_2)$ of 0.21 and 0.87, respectively.[19] Experiments with the Pgp modulator verapamil in the chemo-resistant CR1R12 cell line demonstrated that **TMR-O**, **TMR-S**, and **TMR-Se** were likely transport substrates for Pgp. Both **TMR-S** and **TMR-Se** were phototoxic to CR1R12 cells only when verapamil was present to block the efflux capabilities of Pgp ($LD_{50} = 4.8\times10^{-6}$ M for **TMR-Se** with $5.0\,J\,cm^{-2}$ of 350–750 nm light), while phototoxicity in the chemo-sensitive AUXB1 cell line was equivalent whether verapamil was present or absent. Using the same experimental conditions 25 times less dye was needed to kill AUXB1 cells ($LD_{50} = 1.9\times10^{-7}$ M for **TMR-Se** with $5.0\,J\,cm^{-2}$ of 350–750 nm light) verses CR1R12 cells. The uptake of CAM, a probe of Pgp transport, was enhanced following exposure of CR1R12 cells to either **TMR-S** or **TMR-Se** and light as determined by calcein fluorescence. These data strongly suggest that this class of rhodamine-related compounds could be potent photosensitizers of Pgp function. Optimization of their chemical, photochemical, and biological properties should lead to agents that, in themselves, are toxic to cells expressing the MDR phenotype. Additionally, analogues should facilitate uptake and therefore toxicity of traditional chemotherapeutics in drug resistant cells and tumors.

4. Experimental section

4.1. General

Chemicals and reagents were purchased 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). Tetramethylrosamine and Calcein AM (calcein acetoxymethyl ester) were purchased from Molecular Probes (Eugene, OR). Both **TMR-S** and **TMR-Se** were prepared as described in Ref. 19. 21, 23-Dithiaporphyrin **1** was prepared as described in Ref. 41.

4.2. Cells and culture conditions

Cultured cells used in this study were the Chinese hamster ovary parental cell line AUXB1,[22] a chemo-sensitive 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 were established from the CH^RC5 cell line[21] by sequential culturing in increasing concentrations of colchicine with $5\,\mu g\,mL^{-1}$ being the final concentration used. Cell lines were maintained in passage culture on 60-mm diameter polystyrene dishes (Corning Costar, Corning, NY) in 4.0 mL Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), $50\,units\,mL^{-1}$ penicillin G, $50\,\mu g\,mL^{-1}$ streptomycin and $1.0\,\mu g\,mL^{-1}$ Fungizone® (complete medium). Only cells from passages 1–10 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, 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).

4.3. Measurement of dye uptake into cell monolayers

Cell lines, AUXB1 or CR1R12, were seeded on 96-well plates in 200 μL/well α-MEM at $1\text{–}4\times10^4$ cells/well. Twenty four hours after seeding, verapamil at 7.0×10^{-6} M was added to selected cells in complete medium and cultures were incubated in the dark at 37 °C for 2 h. Dyes were then added to the cultures at 1×10^{-5} M in complete medium. Cells were incubated in the dark for selected times in the presence of each dye with or without verapamil for the time course study and for 2 h for comparative studies. The medium was then removed and the monolayers washed once with 200 μL 0.9% NaCl and an additional 200 μL 0.9% NaCl was then added. The fluorescence of the intracellular dye was then determined using a multi-well fluorescence plate reader (Gemini, Molecular Devices, Palo Alto, CA). The excitation/emission wavelengths were set at 490/570 for **TMR-O** and 540/600 for **TMR-S**. Because of the weak fluorescence signal emitted by **TMR-Se**, we were unable to obtain uptake data for this analogue. Intracellular dye concentration is expressed as fmole/cell.

4.4. Photoradiation of cell cultures

Cell lines, AUXB1 or CR1R12, were seeded on 96-well plates in 200 μL/well α-MEM at $1\text{-}4\times10^4$ cells/well. Rhodamine analogues were then added directly to the cell culture medium at various concentrations and incubated for 2 h in the dark as above. The medium was then removed and 200 μL α-MEM minus FBS and phenol red (clear medium) were added to each well. One plate, with the lid removed, was then exposed to 350–750 nm light delivered at $1.4\,mW\,cm^{-2}$ for 1 h ($5.0\,J\,cm^{-2}$) from a filtered halogen source while a parallel 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. Subsequently, cells were trypsinized and counted using the Coulter counter to determine cell viability. Determination of cell viability by cell counting is performed according to an earlier method.[40] Briefly, cells that detach from the surface of a culture plate stain with trypan blue, that are nonviable, while all cells that remain attached to the surface exclude trypan blue, 100% viable We were unable to seed new culture plates with cells that detached from the surface after treatment, while cells that remained attached after treatment continued to grow and multiply. Thus, we count only those cells that remain attached to the culture plate surface after treatment and compare those numbers with the cell numbers obtained from control cells, cells not exposed to either dye or light. Data are then expressed as percent cell viability, treated cell counts/control cell counts.

4.5. Calcein AM uptake

CR1R12 or AUXB1 cells were seeded on 96-well plates in 200 μL/well α-MEM at $1\text{–}4\times10^4$ cells/well. Rhodamine analogues at 1.0×10^{-6} M were added to cell cultures on 96-well plates in complete medium for 2 h in the dark at 37 °C. The medium containing the dyes was removed and replaced with clear MEM. A plate, with the lid removed, was then exposed to 350–750 nm light delivered at $1.4\,mW\,cm^{-2}$ for 1 h ($5.0\,J\,cm^{-2}$) from a filtered halogen source. Another plate exposed to the same culture and dye incubation conditions was kept in the dark. Also, in another series of experiments, the Pgp modulators verapamil at 7.0×10^{-6} M or 7.0×10^{-5} M or cyclosporin A at 7.0×10^{-6} M were incubated with the cells in the complete medium for 15 min. Immediately following irradiation or incubation with the modulators, Calcein AM was added to each well at 1.0×10^{-6} M in clear medium. The appearance of a fluorescence signal at 530 nm, excitation 485 nm, was then followed in the plate reader over a 30 min time period. The rate of uptake was calculated from the slope of the line obtained in each experimental protocol and the data are expressed as percent of control Calcein AM uptake, the rate of Calcein AM

taken up into cells that were not exposed to TMR analogues or Pgp modulators.

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