Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 2589–2596
Bioorganic & Medicinal Chemistry

Comparison of the dark and light-induced toxicity of thio and seleno analogues of the thiopyrylium dye AA1

Michael R. Detty,[a,*] Scott L. Gibson[b] and Russell Hilf[b]

[a]*Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY 14260, USA*
[b]*Department of Biochemistry and Biophysics, University of Rochester Medical Center, 601 Elmwood Avenue, PO Box 607, Rochester, NY 14642, USA*

Received 5 February 2004; accepted 13 March 2004

Available online 9 April 2004

Abstract—2,6-Bis(4-anilino)-4-(4-*N*,*N*-dimethylanilino)thiopyrylium chloride (**AA1**) and -selenopyrylium chloride (**AA1-Se**) and 2,6-bis(4-anilino)-4-(4-*N*-morpholinophenyl)thiopyrylium chloride (**1**) and -selenopyrylium chloride (**2**) were prepared via the addition of 4-*N*,*N*-dimethylanilino magnesium bromide and 4-*N*-morpholinophenyl magnesium bromide to chalcogenopyranones **3** followed by treatment with HCl gas then water. Cellular uptake of these dyes varied from 12 ± 3 fmol/cell for **AA1** to 150 ± 40 fmol/cell for **AA1-Se**. upon exposure to 5×10^{-5} M solutions of the dyes for 3 h. Exposure of cell cultures to 1.8 J/cm^2 of 360–750-nm light following incubation with 1×10^{-6} M of either **AA1**, **1**, or **2** for 24 h resulted in no significant additional phototoxicity while **AA1-Se** showed a significant ($P < 0.05$) reduction in cell viability from 81% to 46%. Thiopyrylium dyes **AA1** and **1** showed significant dark toxicity relative to selenopyrylium dyes **AA1-Se** and **2**, respectively. **AA1** was the only one of the four dyes to show inhibition of whole-cell mitochondrial cytochrome *c* oxidase activity in the dark. Irradiation of whole cells or mitochondrial suspensions treated with **AA1**, **AA1-Se**, or **2** gave inhibition of mitochondrial cytochrome *c* oxidase activity. Studies of **JC-1**-efflux indicated that all four cationic dyes accelerated the loss of **JC-1** from the mitochondria, which suggests that all four dyes target the mitochondria.

1. Introduction

Photodynamic therapy (PDT), a relatively recent addition to the armamentarium of cancer treatment regimens, utilizes the combination of a photosensitizer, light, and molecular oxygen to induce cellular toxicity.[1–4] Irradiation of lesions containing the photosensitizer with light that is absorbed by the photosensitizer results in the photochemical generation of singlet oxygen ($^1O_2$), the toxic oxygen species thought to be responsible for the phototoxicity observed for the majority of the photosensitizers utilized in PDT.

Intensive investigation is underway to design a photosensitizer with the most desirable features. Porphyrins and porphyrin-related compounds have attracted the greatest attention because of their apparent affinity for or retention in malignancies, their relatively high quantum yields for the generation of singlet oxygen, and their Q band absorption in the red region of the visible spectrum where light has maximal tissue penetration.[1–4] Other agents have also been studied, although not as intensively as the porphyrins and related compounds. The nonporphyrin photosensitizers possess very different chromophores than the porphyrins but have many of the same desirable features of an ideal photosensitizer including more intense absorption in the red region of the visible spectrum and high quantum yields for the generation of singlet oxygen. These derivatives include Victoria blues,[3] Nile blue dyes,[5–8] methylene-blue-related dyes,[9,10] various cyanine and cyanine-like dyes,[11–14] and a variety of analogues of chalcogenopyrylium trimethine dyes[15–18] (Chart 1). Many of the results of these studies have been encouraging but systematic investigations into these dye classes and associated structure–activity relationships on their photosensitizing properties have not been performed.

We are interested in analogues of triarylchalcogenopyrylium dyes that are phototoxic upon irradiation and offer promise as photosensitizers for PDT.[19–22]

Keywords: Photosensitizers; Photodynamic therapy; Anticancer; Thiopyrylium; Selenopyrylium; Chalcogenopyrylium; Cytochrome *c* oxidase activity; **JC-1** efflux.
* Corresponding author. Tel.: +1-716-645-6800x2200; fax: +1-716-645-6800; e-mail: mdetty@buffalo.edu

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.03.025

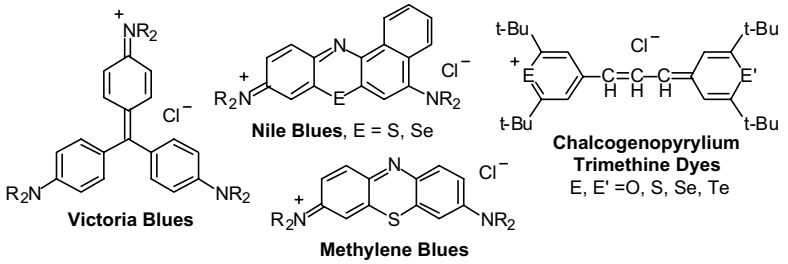


Chart 1. Cationic dyes useful as photosensitizers in photodynamic therapy.

The chemistry of these analogues is based on the thiopyrylium compound **AA1**, whose structure is shown in Chart 2. Compound **AA1** is toxic in the dark to both cultured cancer cells and malignancies in vivo.[23] However, no added phototoxicity was observed with **AA1** in a cultured human colon carcinoma line exposed to **AA1** and light.

The absence of added phototoxicity with **AA1** was intriguing and prompted us to study **AA1** in more depth. Related sulfur-containing chromophores in the Nile blue[5–8] and methylene blue[9,10] series show pronounced phototoxicity. We have recently described increased phototoxicity in S- and Se-containing analogues of tetramethylrosamine (**TMR**, Chart 2).[24] In the chalcogenopyrylium dyes, substitution of Se for S in the chromophore greatly enhances phototoxicity and gives a bathochromic shift in absorption maximum.[15–18] The 4-anilino substituents at the 2- and 6-positions of **AA1** were critical for its activity.[23] We wanted to ascertain whether a Se-containing analogue of **AA1** (**AA1-Se**, Chart 2) would exhibit enhanced phototoxicity and whether substituent changes in the 4-aryl ring (eg., replacing the dimethylamino group of **AA1** or **AA1-Se** with a morpholino group as in compounds **1** and **2**, Chart 2) would greatly impact biological properties.

In an earlier study, we surveyed analogues of **AA1** to determine the effects of a variety of structural modifications on chemical and physical properties such as the quantum yield for the generation of $^1O_2[\phi(^1O_2)]$, the *n*-octanol/water partition coefficient ($\log P$), and the electrochemical reduction potentials, as well as the dark and light-induced toxicity toward cells in culture.[22] The results of these studies demonstrated that a correlation existed between the value of $\log P$ for these dyes and the therapeutic ratio, computed as the ratio of concentrations to give 50% cell kill in the dark and 50% cell kill upon exposure to light. It was suggested that the mitochondria might be an important target of these dyes both in the dark and during irradiation.

In this manuscript, we quantify the impact of changing the heteroatom in **AA1** from S to Se and of changing a single ring substituent from dimethylamino to *N*-morpholino on several biological endpoints. In addition to the standard endpoints of dark- and light-induced toxicity toward cultured cells, we also examined the effects of slight structural variations on cellular uptake and on mitochondrial function both in whole cells, as measured by mitochondrial cytochrome *c* oxidase activity and the retention of the mitochondrial membrane potential probe **JC-1**, and in isolated mitochondrial suspensions as measured by cytochrome *c* oxidase activity.

2. Results and discussion

2.1. Preparation of dyes

Previously, **AA1**, **AA1-Se**, **1**, and **2** were prepared in three steps from chalcogenopyranones **3** via initial addition of the appropriate Grignard reagent, conversion to the hexafluorophosphate salt of the dye, followed by ion exchange to give the appropriate chloride salt in 26%, 38%, 13%, and 14% isolated yields, respectively.[22] We have found that the chloride salts can be prepared directly from the Grignard-addition product in much higher yield as shown in Scheme 1. Following the addition of the Grignard reagent, anhydrous HCl gas was bubbled into the reaction mixture and **AA1**, **AA1-Se**, **1** and **2** were isolated in 75%, 65%, 72%,

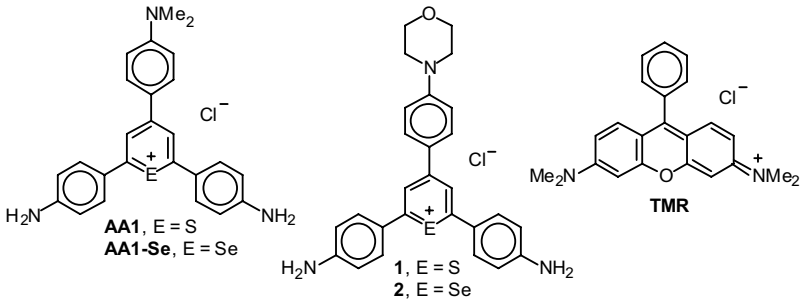
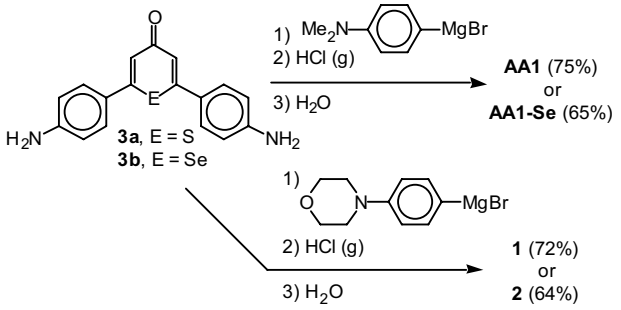


Chart 2. Structures of **AA1**, **AA1-Se**, related morpholino dyes **1** and **2**, and tetramethylrosamine (**TMR**).



Scheme 1. Preparation of **AA1**, **AA1-Se**, **1**, and **2**.

and 64% yields, respectively, following an aqueous workup.

2.2. Intracellular accumulation of AA1, AA1-Se, 1, and 2

The intracellular accumulation of 5×10^{-5} M **AA1**, **AA1-Se**, **1**, and **2** in R3230AC rat mammary adenocarcinoma cells is summarized in Figure 1. For comparison purposes, the uptake of **TMR** was also included. Cells were treated with 5×10^{-5} M dye for 3 h and the intracellular dye uptake was determined from the absorbance of cell lysates. The R3230AC cells contained 12 ± 3 fmol/cell of **AA1**, which was comparable to the 16 ± 3 fmol/cell uptake of **TMR**. In contrast, the uptake of either **AA1-Se** or **1** was roughly a factor of 10 higher at 150 ± 40 fmol/cell for **AA1-Se** and 120 ± 20 fmol/cell for thiopyrylium **1**. The uptake of selenopyrylium analogue **2** was intermediate between the two groupings at 33 ± 6 fmol/cell. These results suggest that while all of the dyes are taken up by R3230AC cells, the level of cellular uptake is not strictly a function of either the chalcogen atom in the ring or the substituent in the 4-position.

2.3. Dark and phototoxicity of AA1, AA1-Se, 1, and 2

As shown in Figure 2, the thiopyrylium dyes **AA1** and **1** were significantly more toxic after 24 h of incubation in

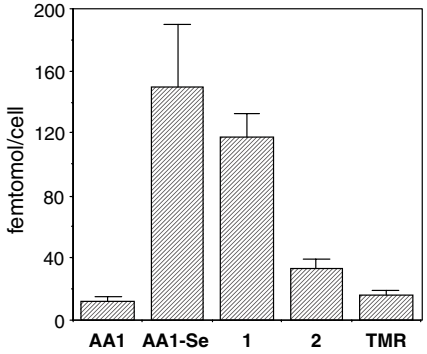


Figure 1. Intracellular accumulation of dyes **AA1**, **AA1-Se**, **1**, **2**, and **TMR** in cultured R3230AC cells. Dyes at 5×10^{-5} M were incubated with cultured R3230AC cells as described in the Experimental Section. Each column represents data accumulated from three separate experiments performed in duplicate. Data are expressed as femtomole dye/cell, error bars are the SEM.

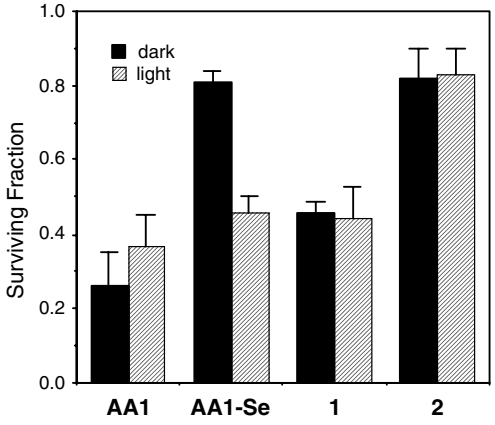


Figure 2. Effect of **AA1**, **AA1-Se**, **1**, and **2** on the viability of R3230AC cells in culture in the dark or 24 h after exposure to 1.8 J/cm^2 of 360–750-nm light. Experimental conditions are described in the Experimental Section. Each bar represents the data obtained from three separate experiments performed in duplicate for the viability of cultured R3230AC cells maintained in the dark (dark bars) or 24 h after exposure of cells to light (striped bars) in the presence of dyes **AA1**, **AA1-Se**, **1**, and **2**. Data are expressed as a fraction of control cell viability, exposed to neither dyes nor light, error bars are the SEM.

the dark toward R3230AC cells in culture than the corresponding selenopyrylium analogues **AA1-Se** and **2**, respectively. Exposure of cell cultures to 1.8 J/cm^2 of 360–750-nm light following incubation with 1×10^{-6} M of either **AA1** or **1** for 24 h resulted in no significant additional phototoxicity, as determined from cell viability, relative to dark controls even though the uptake of **1** was a factor of 10 greater than the uptake of **AA1**. Selenopyrylium dye **2** similarly showed no additional phototoxicity upon irradiation of cell cultures. In contrast, irradiation of R3230AC cells treated with 1×10^{-6} M **AA1-Se** for 24 h followed by 1.8 J/cm^2 of 360–750-nm light reduced cell viability from 81% to 46%, a statistically significant difference of $P<0.05$, when dark control cells were compared with cells whose viability was determined 24 h after light exposure.

2.4. Effects of AA1, AA1-Se, 1, and 2 on whole-cell cytochrome *c* oxidase activity

Cultured R3230AC cells were maintained in the dark or exposed to 1.8 J/cm^2 of 360–750-nm light in the presence of 1×10^{-6} M **AA1**, **AA1-Se**, **1**, or **2** as described above. Following a 24-h incubation period, cells were harvested and mitochondrial cytochrome *c* oxidase activity was measured in sonicated cell suspensions. The results from these experiments, presented in Figure 3, are expressed as the percent of initial, whole-cell, cytochrome *c* oxidase activity. The data clearly show that cytochrome *c* oxidase obtained from cells exposed to light and the dyes **AA1**, **AA1-Se**, and **2** is inhibited to a greater extent than when the enzyme was measured in cultured cells exposed to dye, but maintained in the dark. Cytochrome *c* oxidase activity in whole cells treated with either **1** or **TMR** is unaffected by exposure to light. In pair-wise comparisons, the difference between dark inhibition and photoinhibition of cytochrome *c* oxidase in whole cells

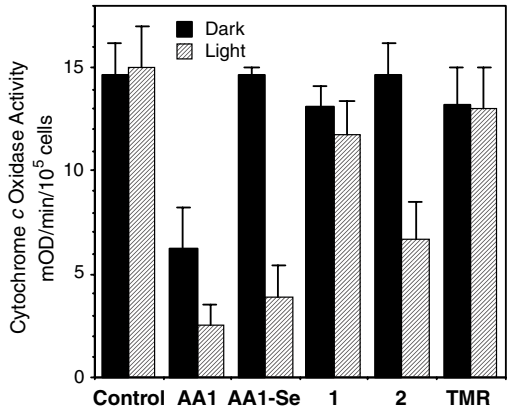


Figure 3. Effect of dyes **AA1**, **AA1-Se**, **1**, and **2** on the whole-cell activity of mitochondrial cytochrome *c* oxidase. Enzyme activity was obtained after cultured R3230AC cells had been exposed to dyes in the dark (dark bars) or 24 h after cells were exposed to 1.8 J/cm^2 of 360–750-nm light (striped bars) as described in detail in the Experimental Section. Data are expressed as percent of control enzyme activity, which was obtained from cells not exposed to either dyes or light. Each bar represents data collected from three separate experiments, error bars are the SEM.

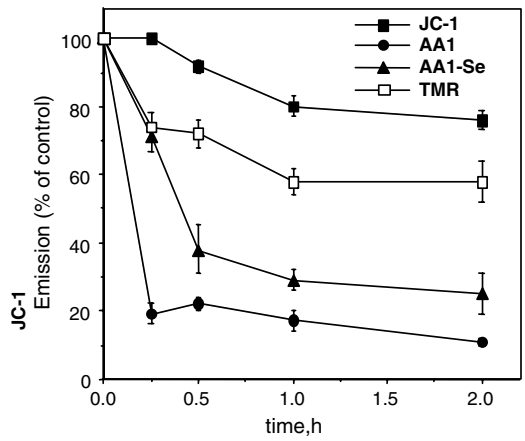


Figure 4. Effect of dyes **AA1**, **AA1-Se**, and **TMR** on the intracellular retention/efflux of the mitochondrial membrane potential probe **JC-1**. Details of experimental conditions appear in the Experimental Section. Each data point represents the mean of three separate experiments performed in duplicate. Data are expressed as percent of initial **JC-1** fluorescence emission, data acquired immediately after a 2 h incubation of cells with 2×10^{-7} M **JC-1** and prior to the addition of cationic dyes. Error bars are the SEM.

treated with selenopyrylium dyes was statistically significant relative to whole cells treated with thiopyrylium dyes: $P < 0.001$ for **AA1** versus **AA1-Se** and $P < 0.02$ for dye **1** versus dye **2**. These data clearly indicate that the substitution of a Se atom for S in these compounds gives increased photoinhibition of cytochrome *c* activity under the conditions employed.

The thiopyrylium dye **AA1** demonstrated >50% inhibition of whole-cell, cytochrome *c* oxidase activity in the dark. Surprisingly, exposure of the **AA1**-treated cells to light gave significantly greater ($P < 0.05$) inhibition of whole-cell, cytochrome *c* oxidase activity.

2.5. Efflux of JC-1 from R3230AC cells

The data displayed in Figure 4 represent the results obtained for the efflux of the mitochondrial membrane potential probe **JC-1** from R3230AC cells in culture in the presence or absence of dyes **AA1**, **AA1-Se**, or **TMR**. The data show that in control samples with dye-free medium, only 25% of the initial intracellular **JC-1** emission disappeared within 2 h with the mitochondrial electrochemical gradient across the inner membrane presumably intact. Cells were incubated with 2×10^{-7} M **JC-1** for 2 h in the dark at 37 °C, the **JC-1**-containing medium was removed, and fresh medium containing 1×10^{-7} M **AA1**, **AA1-Se**, or **TMR** was added. The fluorescence emission of **JC-1** (excitation 485 nm and emission at 600 nm) was monitored as a function of time. Both **AA1** and **AA1-Se** accelerate the loss of emission from **JC-1** with the greatest decrease in **JC-1** fluorescence observed with 1×10^{-7} M **AA1**. The decrease in **JC-1** fluorescence was also significantly greater ($P < 0.05$) for **AA1** and **AA1-Se** relative to 1×10^{-7} M **TMR**. The results for the morpholino analogues **1** and **2** were similar to the results for **AA1** and **AA1-Se**, respectively (data not shown for clarity). These data demonstrate that these cationic compounds interfere with the electrochemical gradient formed across the inner membrane of the mitochondria.

2.6. Cytochrome *c* oxidase activity in irradiated, dye-treated mitochondria

The effects of **AA1**, **AA1-Se**, **1**, and **2** on cytochrome *c* oxidase activity measured in isolated rat liver mitochondria during irradiation as described above are presented in Figure 5. The data are consistent with those obtained for whole-cell enzyme studies in that the selenopyrylium analogues **AA1-Se** and **2** demonstrate

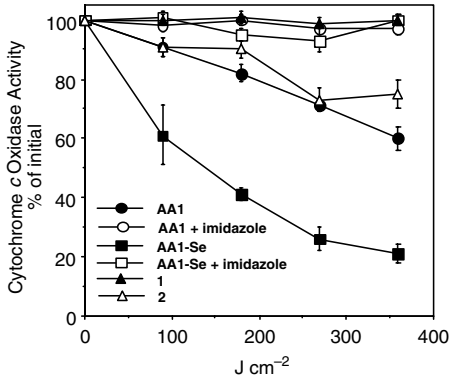


Figure 5. Effect of dyes **AA1**, **AA1-Se**, **1**, and **2** on cytochrome *c* oxidase activity measured in isolated rat liver mitochondria suspensions. Isolated rat liver mitochondria were exposed to 100 mW cm^2 of 500–750-nm light in the presence of 1×10^{-6} M dye as described in the Experimental Section. Each data point represents the mean cytochrome *c* oxidase activity, expressed as percent of initial activity, for dyes **AA1**, **AA1-Se**, **1**, **2**, **AA1** + 0.08 M imidazole, and **AA1-Se** + 0.08 M imidazole obtained from three separate experiments. Error bars are the SEM.

greater photoinhibition of the enzyme than their thiopyrylium counterparts **AA1** and **1**, respectively. These data, in conjunction with the whole-cell mitochondrial cytochrome *c* oxidase inhibition studies, support the hypothesis that introduction of the heavy atom selenium into these cationic compounds increases their photosensitizing potency.

The photodynamic action of **AA1-Se** appears to be a result of singlet oxygen generation. The presence of the $^1O_2$ trap imidazole[25] at 0.08 M in the mitochondrial suspensions, completely eliminated the photoinhibition of cytochrome *c* oxidase activity in mitochondrial suspensions treated with **AA1-Se**.

While irradiation of mitochondrial suspensions treated with thiopyrylium dye **1** displayed no photo-induced inhibition of cytochrome *c* oxidase activity, the effects from irradiation of **AA1**-treated mitochondria were comparable to those from selenopyrylium dye **2**. Even though the total amount of light was more than 100-fold greater than that used in the phototoxicity measurements of Figure 2 and the inhibition of whole-cell cytochrome *c* oxidase in Figure 3, the photodynamic action of **AA1** was unanticipated. Imidazole, when present at 0.08 M in the liver mitochondrial suspension, completely eliminated the photoinhibition of cytochrome *c* oxidase activity in mitochondrial suspensions treated with **AA1**,[25] which suggests that **AA1** can produce singlet oxygen upon irradiation in biological tissues.

3. Summary and conclusions

The direct conversion of the Grignard-addition products from chalcogenopyryanones **3** to the chloride salts of **AA1**, **AA1-Se**, **1**, and **2** gave much higher isolated yields of the dyes than prior procedures that sequentially isolated the hexafluorophosphate salts followed by the chloride salts.[22] The higher synthetic yields make the dyes more accessible for biological studies.

Our findings confirmed the earlier observations[23] that the thiopyrylium analogue **AA1** shows no additional toxicity upon irradiation of **AA1**-treated cells with visible light compared to that observed in **AA1**-treated cells kept in the dark. However, when isolated mitochondria that had been treated with **AA1** were exposed to 360 J cm^{-2} of filtered 500–750-nm light, cytochrome *c* oxidase activity was inhibited by 40% compared to control values. The addition of 0.08 M imidazole, a singlet oxygen trap,[25] to the isolated mitochondria plus **AA1**, reversed the inhibition of cytochrome *c* oxidase activity after exposure of the mitochondrial suspensions to light. In addition, photoinhibition of whole-cell cytochrome *c* oxidase activity was observed in cells treated with **AA1** and light. These data demonstrate that **AA1**, under certain conditions, can be a photosensitizer, which presumably generates singlet oxygen upon irradiation.

At 10^{-6} M, **AA1** was dark toxic to R3230AC cells in vitro with only 25% cell viability (Fig. 2). In contrast, **AA1-Se** showed little dark toxicity at 10^{-6} M even though the cellular uptake of **AA1-Se** was 150 fmol/cell relative to the 12 fmol/cell uptake of **AA1** (Fig. 1). The uptake of **AA1-Se** and dye **1** are comparable (150 fmol/cell and 120 fmol/cell, respectively), but the thiopyrylium dye **1** is more dark toxic than **AA1-Se**. Some structural feature of the thiopyrylium dyes **AA1** and **1** must contribute to their greater dark toxicity toward R3230AC cells relative to the selenopyrylium dyes **AA1-Se** and **2**.

Replacing the S atom of **AA1** with Se as in **AA1-Se** provided a more potent photosensitizer for PDT. **AA1-Se** demonstrated significant phototoxicity toward R3230AC cells in vitro with 1.8 J/cm^2 of 360–750-nm light as shown in Figure 2 in addition to photoinhibition of cytochrome *c* oxidase activity in either whole cells or in mitochondrial suspensions as shown in Figures 3 and 5, respectively. However, one might pose the question of whether the improved performance of **AA1-Se** is due to better photophysical properties relative to **AA1** or due to the 10-fold greater uptake of **AA1-Se** relative to **AA1**. In partial answer to this question, thiopyrylium dye **1** displayed no additional phototoxicity upon irradiation (Fig. 2) even though the uptake of **AA1-Se** and the uptake of **1** were comparable (Fig. 1) and displayed no photoinhibition of mitochondrial cytochrome *c* oxidase in either whole cells (Fig. 3) or mitochondrial suspensions (Fig. 5). Surprisingly, selenopyrylium dye **2** showed no additional phototoxicity upon irradiation of cells treated with **2** even though photoinhibition of mitochondrial cytochrome *c* oxidase was observed both in whole cells (Fig. 3) and in mitochondrial suspensions (Fig. 5). To a first approximation, the improved photophysical properties of the selenopyrylium dyes are most likely responsible for the photodynamic effects observed.

One cannot be unequivocally sure of the subcellular distribution of the various cationic dyes. The inhibition of mitochondrial cytochrome *c* oxidase by **AA1**, **AA1-Se**, and **2** certainly argues for the presence of these dyes in the mitochondria. This argument was further supported by the **JC-1**-efflux studies summarized in Figure 4, which demonstrated that the rate of loss of **JC-1** was accelerated in the presence of all four of the cationic dyes. The increased efflux of **JC-1** suggested that **AA1**, **AA1-Se**, **1**, and **2** all target the mitochondria. The rate of **JC-1**-efflux in the presence of **AA1** was greater than the efflux in the presence of **AA1-Se** even though the cellular uptake of **AA1** was only 8% of the cellular uptake of **AA1-Se**. It is reasonable to ask whether a higher fraction of the intracellular **AA1** is found in the mitochondria than for the other cationic dyes. However, there are no tremendous differences in physical properties among **AA1**, **AA1-Se**, **1**, and **2** that would warrant such a conclusion. Values of log *P* among **AA1**, **AA1-Se**, **1**, and **2** are similar (log *P* of 1.5–1.9) as are values of the electrochemical reduction potential, E° [E° of -0.58 to -0.71 V (vs ferrocene/ferrocinium)].[22] We continue to look for intermolecular interactions between the dyes and biological targets that appear to differentiate the

slight structural differences among these monocyclic chalcogenopyrylium dyes related in structure to **AA1**.

4. Experimental section

4.1. General

All chemicals were purchased from Sigma 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). The mitochondrial membrane potential probe **JC-1** and tetramethylrosamine (**TMR**) were obtained from Molecular Probes Inc. (Eugene, OR). Concentration in vacuo was performed on a Büchi rotary evaporator. Chalcogenopyranones **3** were prepared according to Ref. 22. NMR spectra were recorded on a Varian Inova 500 instrument with residual solvent signal as 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.

4.2a. Preparation of 2,6-bis(4-anilino)-4-(4-*N*,*N*-dimethylanilino)thiopyrylium chloride (AA1)

A solution of Δ-4*H*-2,6-bis(4-anilino)thiopyran-4-one[22] (**3a**, 0.20 g, 0.68 mmol) in THF (10 mL) was added dropwise to the Grignard reagent prepared from *p*-bromo-*N*,*N*-dimethylaniline, (1.02 g, 5.1 mmol) and magnesium turnings, (0.16 g, 6.8 mmol) in THF (5 mL). The resulting mixture was heated at reflux for 0.5 h, allowed to cool to ambient temperature, and anhydrous HCl gas was bubbled into the reaction mixture, which turned blue then gold. The reaction mixture was poured into 60 mL of cold water precipitating a blue solid, which was collected by filtration, washed with water, and dried. The crude product was recrystallized from acetonitrile and a small amount of diethyl ether to give 0.22 g (75%) of **AA1**, whose spectral properties were identical to those of an authentic sample:[22] mp >260 °C; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 8.28 (s, 2H), 7.97 (AA′BB′, 2H, $J = 9.5$ Hz), 7.72 (AA′BB′, 4H, $J = 8.5$ Hz), 6.88 (m, 6H), 4.53 (s, 4H), 3.21 (s, 6H); λ_{max} (CH_2Cl_2) 581 nm, $\varepsilon = 5.55(\pm 0.05) \times 10^4$ M^{-1}/cm.

4.2b. Preparation of 2,6-bis(4-anilino)-4-(4-*N*,*N*-dimethylanilino)selenopyrylium chloride (AA1-Se)

A solution of Δ-4*H*-2,6-bis(4-anilino)-selenopyran-4-one[22] (**3b**, 0.20 g, 0.60 mmol) in THF (10 mL) was added dropwise to the Grignard reagent prepared from *p*-bromo-*N*,*N*-dimethylaniline, (0.87 g, 4.5 mmol) and magnesium turnings, (0.14 g, 5.8 mmol) in THF (5 mL). The resulting mixture was heated at reflux for 0.5 h, and was treated as described for the preparation of **AA1** to give 0.19 g (65%) of **AA1-Se**, whose spectral properties were identical to those of an authentic sample:[22] mp >260 °C; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 8.25 (s, 2H), 7.98 (AA′BB′, 2H, $J = 9.5$ Hz), 7.67 (AA′BB′, 4H, $J = 8.5$ Hz), 6.87 (m, 6H), 4.55 (s, 4H), 3.19 (s, 6H); λ_{max} (CH_2Cl_2) 610 nm, $\varepsilon = 6.90(\pm 0.02) \times 10^4$ M^{-1}/cm.

4.2c. Preparation of 2,6-bis(4-anilino)-4-(4-*N*-morpholinophenyl)thiopyrylium chloride (1)

A solution of **3a**[22] (0.20 g, 0.68 mmol) in THF (10 mL) was added dropwise to the Grignard reagent prepared from *N*-(4-bromophenyl)morpholine (1.23 g, 5.1 mmol) and magnesium turnings, (0.16 g, 6.8 mmol) in THF (5 mL). The resulting mixture was heated at reflux for 0.5 h and was treated as described for **AA1** to give 0.23 g (72%) of **1**, whose spectral properties were identical to those of an authentic sample:[22] mp >260 °C; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 8.29 (s, 2H), 7.95 (AA′BB′, 2H, $J = 9.0$ Hz), 7.75 (AA′BB′, 4H, $J = 8.5$ Hz), 7.07 (AA′BB′, 2H, $J = 9.0$ Hz), 6.87 (AA′BB′, 4H, $J = 8.5$ Hz), 4.60 (s, 4H), 3.86 (t, 4H, $J = 4.8$ Hz), 3.48 (t, 4H, $J = 4.8$ Hz); λ_{max} (CH_2Cl_2) 566 nm, $\varepsilon = 4.58(\pm 0.02) \times 10^4$ M^{-1}/cm.

4.2d. Preparation of 2,6-bis(4-anilino)-4-(4-*N*-morpholinophenyl)selenopyrylium chloride (2)

A solution of **3b**[22] (0.20 g, 0.60 mmol) in THF (10 mL) was added dropwise to the Grignard reagent prepared from *N*-(4-bromophenyl)morpholine (1.02 g, 4.5 mmol) and magnesium turnings, (0.16 g, 6.8 mmol) in THF (5 mL). The resulting mixture was heated at reflux for 0.5 h and was treated as described for **AA1** to give 0.20 g (64%) of **2**, whose spectral properties were identical to those of an authentic sample:[22] mp >260 °C: ^{1}H NMR (500 MHz, CD_2Cl_2): δ 8.25 (s, 2H), 7.95 (AA′BB′, 2H, $J = 9.2$ Hz), 7.70 (AA′BB′, 4H, $J = 8.8$ Hz), 7.06 (AA′BB′, 2H, $J = 9.2$ Hz), 6.86 (AA′BB′, 4H, $J = 8.8$ Hz), 4.63 (s, 4H), 3.87 (t, 4H, $J = 5.1$ Hz), 3.46 (t, 4H, $J = 5.1$ Hz); λ_{max} (CH_2Cl_2) 593 nm, $\varepsilon = 4.48(\pm 0.03) \times 10^4$ M^{-1}/cm.

4.3. Cells and culture conditions

Cells cultured from the rodent mammary adenocarcinoma (R3230AC) were used for all experiments. R3230AC tumors were maintained by transplantation in the axillary region of 100–120 g Fischer female rats, using the sterile trochar technique described earlier.[26] R3230AC cells were cultured from tumor homogenates using the method described earlier.[27] All cell lines were maintained in passage culture on 100-mm diameter polystyrene dishes (Becton Dickinson, Franklin Lakes, NJ) in 10 mL minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin G, 50 μg/mL streptomycin and 1.0 μg/mL Fungizone® (complete medium). Only cells from passages 1 to 14 were used for experiments. A stock of cells, passages 1–4, was 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 mL solution containing 0.25% trypsin, incubating at 37 °C for 2–5 min to remove the cells from the surface and seeding new culture dishes with an appropriate number of cells in 10 mL of MEM. Cell counts were performed using a particle counter (Beckman Coulter, Hialeah, FLA).

Experimental cells were seeded on 12-well culture plates (Corning Costar, Corning, NY) at an appropriate cell density ($1–3\times10^5$ cells/well) for uptake, toxicity and whole-cell cytochrome *c* oxidase studies. The seed density was such that cultures attained 85–95% confluence 48 h after seeding.

4.4. Measurement of dye uptake into cell monolayers

R3230AC cells were cultured on 12-well plates as described above. Twenty-four hours after seeding, **AA1**, **AA1-Se**, **1**, **2**, or **TMR** was added at 5×10^{-5} M in α-MEM plus FBS and phenol red. Cells were incubated for 3 h in the presence of each dye in a humidified atmosphere in the dark as described above. The medium was then removed and the monolayers washed once with 1.0 mL of 0.9% NaCl and 1.0 mL of 50% EtOH/1% glacial acetic acid was added. Cells detached from the surface within 5 min and the dye absorbance in the resulting cell lysates was determined using a diode array UV/visual spectrophotometer (Hewlett Packard, 8452A, Palo Alto, CA). Intracellular dye concentration was calculated by comparing absorbance values obtained from cell lysates with standard curves generated from known concentrations of dyes dissolved in a mixture of 50% EtOH/1% glacial acetic acid. Intracellular dye concentration is expressed in nanomole dye/1×10^5 cells. To determine whether the dyes adhered to the plastic surface of the plates, plates without cells were incubated with each dye in 1.0 mL of complete medium for 3 h and processed using the same conditions for cell lysates. The absorbance values from these cell free experiments were subtracted from the values obtained from the cell lysates to calculate the intracellular dye concentration.

4.5. Irradiation of cell cultures

After seeding on 12-well plates, R3230AC cells were incubated for 24 h to allow cells to attach to the surface. Stock solutions of **AA1**, **AA1-Se**, **1**, **2**, or **TMR**, prepared at 1×10^{-3} M, were then added directly to the cell culture medium to give a final dye concentration of 1×10^{-6} M. Cell monolayers were then incubated for 24 h at 37 °C in the dark in a humidified 5% CO_2 atmosphere. The media was 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 360–750 nm light delivered at 0.5 mW for 1 h (1.8 J/cm^2) from a filtered tungsten source while 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.

4.6. Whole cell cytochrome *c* oxidase measurements

To determine whether dyes photosensitized mitochondrial cytochrome *c* oxidase in cultured cells, R3230AC cells were plated on 12-well culture plates using an initial cell seeding of 1.0×10^5 cells/well in 1.0 mL of complete medium. Cells were incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere as above. Following the 24-h incubation period, dyes were added at 1×10^{-6} M final concentration. Control cells, no added dye, were maintained under the same conditions. Cells were incubated for 24 h in the dark, the medium was removed and replaced with 1.0 mL of clear medium. Monolayers on culture plates with lids removed were then exposed to 360–750-nm light 0.5 mW for 1 h as above (1.8 J/cm^2). Following irradiation of the cultures, the medium was replaced with 1.0 mL of complete medium and irradiated plates and plates that remained in the dark were incubated for 24 h at 37 °C as above. Subsequently 0.1 mL trypsin was added to each well to detach the cells. Cell suspensions from three similarly treated wells were then combined and transferred to 1.0-mL microcentrifuge tubes and centrifuged at 8000*g* for 3 min. The supernatant was aspirated and cell pellets were immediately frozen and stored at −86 °C. Cytochrome *c* oxidase activity was determined on **1** to 1.5×10^6 cells that had been thawed in 0.1 mL 0.9% NaCl and sonicated for 10 s on ice using a Bronson sonicator (Model 185, Brinkmann Ind.) at a setting of 2. Measurement of cytochrome *c* oxidase was performed according to a method described earlier.[28] Data are expressed as percent of control cytochrome *c* oxidase activity, moles of cytochrome *c* oxidized per minute per cell, which was determined from cells not exposed to dye or light. Cytochrome *c* oxidase activity was also determined in cells exposed to dye alone or light alone, drug and light controls, respectively.

4.7. Efflux of JC-1 from cultured R3230AC cells

R3230AC cells were cultured on 96-well plates in complete medium as described above. Twenty-four hours after cells were seeded, the complete medium was removed and the mitochondrial membrane potential probe **JC-1** was added to each well at 2×10^{-6} M in clear medium. The cells then were incubated with **JC-1** for 2 h in the dark at 37 °C as describe above. At the end of the incubation period the medium was removed and fresh clear medium added either with no additions, **JC-1** efflux controls, or with 1×10^{-7} M solutions of **AA1**, **AA1-Se**, **1**, **2**, or **TMR**. The fluorescence emission of **JC-1**, excitation 485 nm and emission at 600 nm, was measured on one strip of wells immediately after the medium change to establish the zero time intracellular concentration of **JC-1**. At various times following the addition of dyes to the wells, the medium was removed, cultures

were washed 1× with 0.9% NaCl and fresh 0.9% NaCl was added. The emission of **JC-1** was determined in these wells at 15, 30, 60, and 120 min after addition of the dyes. The data are expressed as the percent of initial **JC-1** fluorescence emission measured immediately after a 2-h incubation with 2×10^{-7} M **JC-1** and prior to the addition of any other compounds.

4.8. Cytochrome *c* oxidase activity in isolated mitochondrial suspensions

In vitro studies of the effects of photosensitization on mitochondrial function, using cytochrome *c* oxidase as a representative marker, employed mitochondrial suspensions prepared from livers obtained from Fischer female rats according to the method of Gibson and Hilf.[28] Mitochondria were thawed at room temperature and the activity of the cytochrome *c* oxidase adjusted to yield a decrease in 550-nm absorbance of 0.45–0.6 OD units/min when 10 μL of mitochondria suspension were added to the cytochrome *c* reaction mixture. Dye solutions were added at a final concentration of 1×10^{-5} M to 0.5 mL of mitochondrial suspension, vortexed gently and incubated for 5 min at room temperature in the dark. The dye/mitochondria suspension was then centrifuged for 5 min at 8000*g*, the supernatant removed, and the mitochondria were resuspended in 0.5 mL of mitochondrial preparation buffer. The mitochondrial suspension was then transferred to a 3.0-mL quartz cuvette that had been previously positioned in the 1.0-cm diameter beam of a focussed and filtered (500–750 nm) 1000 W tungsten light source. Suspensions stirred magnetically were irradiated at 100 mW/cm^2. At selected times, from 0 to 60 min, 10-μL aliquots were removed for measurement of cytochrome *c* oxidase activity. Separate suspensions of the same mitochondria with or without dye present remained in the dark and samples removed from these served as controls. Three to five separate experiments were performed for each dye and the data are calculated as the percent of initial enzyme activity.

4.9. Statistical analysis

Pair-wise intercomparisons among the experimental groups and comparisons with the controls were performed using the Students' *t* test. A value of $P < 0.05$ is considered significant.

Acknowledgements

This work was done under a research grant to M.R.D. from the NIH (Grant CA 69155).

References and notes

1. Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. *J. Natl. Cancer Inst.* **1998**, *90*, 889.
2. Sharman, W. M.; Allen, C. M.; van Lier, J. E. *Drug Discov. Today* **1999**, *4*, 507.
3. Detty, M. R. *Expert Opin. Ther. Pat.* **2001**, *11*, 1849.
4. Dolmans, D. E. J. G. J.; Fukumura, D.; Jain, R. K. *Nature Rev.* **2003**, *3*, 380.
5. Cincotta, L.; Foley, J. W.; Cincotta, A. H. *Cancer Res.* **1993**, *53*, 2571.
6. Lin, C. W.; Shulok, J. R.; Kirley, S. D.; Bachelder, C. M.; Flotte, T. J.; Sherwood, M. E.; Cincotta, L.; Foley, J. W. *Photochem. Photobiol.* **1993**, *58*, 81.
7. Lin, C. W.; Shulok, J. R.; Kirley, S. D.; Cincotta, L.; Foley, J. W. *Cancer Res.* **1991**, *51*, 2710.
8. Cincotta, L.; Foley, J. W.; MacEachern, T.; Lampros, E.; Cincotta, A. H. *Cancer Res.* **1994**, *54*, 1249.
9. Noodt, B. B.; Rodal, G. H.; Wainwright, M.; Peng, Q.; Horobin, R.; Nesland, J. M.; Berg, K. *Int. J. Cancer* **1998**, *75*, 941.
10. Millish, K. J.; Cox, R. D.; Vernon, D. I.; Griffiths, J.; Brown, S. B. *Photochem. Photobiol.* **2002**, *75*, 392.
11. Modica-Napolitano, J. S.; Brunelli, B. T.; Koya, K.; Chen, L. B. *Cancer Res.* **1998**, *58*, 71.
12. Modica-Nopolitano, J. S.; Koya, K.; Weisberg, E.; Brunelli, B. T.; Li, Y.; Chen, L. B. *Cancer Res.* **1996**, *56*, 544.
13. Ara, G.; Aprille, J. R.; Malis, C. D.; Kane, S. B.; Cincotta, L.; Foley, J.; Bonventre, J. V.; Oseroff, A. R. *Cancer Res.* **1987**, *47*, 6580.
14. Lee, C.; Wu, S. S.; Chen, L. B. *Cancer Res.* **1995**, *55*, 2063.
15. Bellnier, D. A.; Young, D. N.; Detty, M. R.; Camacho, S.; Oseroff, A. R. *Photochem. Photobiol.* **1999**, *70*, 630.
16. Detty, M. R.; Merkel, P. B.; Hilf, R.; Gibson, S. L.; Powers, S. K. *J. Med. Chem.* **1990**, *33*, 1108.
17. Kessel, D. *Photochem. Photobiol.* **1991**, *53*, 73.
18. Powers, S. K.; Walstad, D. L.; Brown, J. T.; Detty, M. R.; Watkins, P. J. *J. Neuro-Oncol.* **1989**, *7*, 179.
19. Leonard, K. A.; Nelen, M. I.; Anderson, L. T.; Gibson, Scott L.; Hilf, R.; Detty, M. R. *J. Med. Chem.* **1999**, *42*, 3942.
20. Leonard, K. A.; Nelen, M. I.; Simard, T. P.; Davies, S. R.; Gollnick, S. O.; Oseroff, A. R.; Gibson, S. L.; Hilf, R.; Chen, L. B.; Detty, M. R. *J. Med. Chem.* **1999**, *42*, 3953.
21. Leonard, K. A.; Hall, J. P.; Nelen, M. I.; Davies, S. R.; Gollnick, S. O.; Camacho, S.; Oseroff, A. R.; Gibson, S. L.; Hilf, R.; Detty, M. R. *J. Med. Chem.* **2000**, *43*, 4488.
22. Brennan, N. K.; Hall, J. P.; Davies, S. R.; Gollnick, S. O.; Oseroff, A. R.; Gibson, S. L.; Hilf, R.; Detty, M. R. *J. Med. Chem.* **2002**, *45*, 5123.
23. Sun, X.; Wong, J. R.; Song, K.; Hu, J.; Garlid, K. D.; Chen, L. B. *Cancer Res.* **1994**, *54*, 1465.
24. Donnelly, D. J.; Gibson, S. L.; Hilf, R.; Detty, M. R. *Biorg. Med. Chem.* **2004**, *12*, in press.
25. Kraljik, I.; El Mohani, F. *Photochem. Photobiol.* **1978**, *28*, 577.
26. Hilf, R.; Michel, I.; Bell, C.; Freeman, J. J.; Borman, A. *Cancer Res.* **1965**, *25*, 286.
27. Hissin, P. J.; Hilf, R. *Cancer Res.* **1978**, *38*, 3646.
28. Gibson, S. L.; Hilf, R. *Cancer Res.* **1983**, *43*, 4191.