Published in final edited form as:

Bioconj Chem. 2016 October 19; 27(10): 2424–2430. doi:10.1021/acs.bioconjchem.6b00394.

Synthesis and Evaluation of Tetraarylethylene-based Mono-, Bis-, and Tris(pyridinium) Derivatives for Image-Guided Mitochondria-Specific Targeting and Cytotoxicity of Metastatic Melanoma Cells

Jessica L. Reedy[†], Devin K. Hedlund[†], Moustafa T. Gabr[‡], Grant M. Henning[†], F. Christopher Pigge[‡], and Michael K. Schultz^{†,*‡}

[†]Departments of Radiology and Radiation Oncology, Free Radical and Radiation Biology Program, The University of Iowa, Iowa City, Iowa 52242, United States

[‡]Department of Chemistry, The University of Iowa, Iowa City, IA 52242, United States

Abstract

Metastatic melanoma is the most aggressive and lethal form of skin cancer. Emerging evidence suggests that differences in melanoma metabolism relative to non-malignant cells represent potential targets for improved therapy for melanoma. Specifically, melanoma cells exhibit increased mitochondrial electron transport chain (ETC) activity and concomitant hyperpolarized mitochondrial membrane potential relative to non-malignant cells. We have synthesized several new fluorescent lipophilic vinylpyridinium cations built from tetraarylethylene scaffolds that target mitochondria *via* attraction to the hyperpolarized mitochondrial membrane potential. Mitochondria-specific accumulation in melanoma cells relative to normal human fibroblasts was demonstrated using confocal fluorescence microscopy and resulted in disruption of oxidative metabolism leading to melanoma specific cell death *in vitro*. Thus, the pyridinium tetraarylethylene platform represents a promising new mitochondrial-targeted delivery vehicle with potential imaging and therapeutic properties.

Graphical Abstract

*Corresponding Author: Departments of Radiology, Radiation Oncology, and Chemistry, Free Radical and Radiation Biology Program and Interdisciplinary Program in Human Toxicology, The University of Iowa, B180 Medical Laboratories, 500 Newton Road, Iowa City, Iowa 52240 USA, Phone: +1 (319) 335-8017, michael-schultz@uiowa.edu.

Author Contributions

The manuscript was written through contributions of all authors.

SUPPORTING INFORMATION

The ¹³C and ¹H NMR spectra, UV-vis absorption spectra, and fluorescence emission spectra of **2**, **4**, **6**, and **9** can be found in the supporting information. In addition, the confocal microscopy of lysosomal accumulation and mitochondria accumulation can also be found in the supporting information.

Keywords

cancer; imaging; melanoma; mitochondria; targeting; tetraarylethylene; triphenylvinylpyridines; vinylpyridine

INTRODUCTION

Melanoma incidence is increasing rapidly and is now the 6th most diagnosed cancer in the United States [1]. Although recent discoveries of genetic mutations (*e.g.* BRAF^{V600E}) in melanoma have led to promising new therapies, the five-year survival has improved marginally (<20%) because melanoma almost invariably develops resistance to these treatments [1–5]. Therefore, there is a critical need to develop new therapeutic options for patients suffering from this disease.

It is well established that cancer cells, including metastatic melanoma, exhibit increased levels of oxidative phosphorylation [6–9]. This increase in electron transport chain (ETC) activity results in elevated superoxide (O₂^{•-}) production and elevated levels of reactive oxygen species (ROS) [10–12]. It is believed that in non-malignant cells, as many as 0.1% of the electrons that enter the ETC leak off and generate O₂^{•-}, which then reacts to form H₂O₂ and other organic hydroperoxides (ROOH) [10–12]. However, in cancer cells, the number of electrons that leak off the ETC and generate free radicals is significantly higher [6, 9, 12–14]. This results in chronic elevated levels of O₂^{•-}, ROS, and oxidative stress.

In addition to increased levels of oxidative stress, the increase in electron leak leads to an increase in the mitochondrial membrane potential relative to non-malignant cells [7–9]. As a result, cancer cell mitochondria exhibit a large mitochondrial inner-membrane potential (150–180 mV), which is believed to be at least 60 mV greater than non-malignant cells [15–21]. Based on this knowledge of cancer cell metabolism, we have designed and synthesized several fluorescent lipophilic cationic vinylpyridinium salts and demonstrated their selective accumulation within melanoma mitochondria *via* targeting of the hyperpolarized membrane. Mitochondrial localization of these agents produced cytotoxic effects in melanoma cells, which correlated with disruption of oxidative phosphorylation, and increases in cellular O₂^{•-}/H₂O₂ levels. The lipophilic tetraarylethylene-based vinylpyridinium salts utilized in this study also display fluorescence emission in aqueous solution and *in vitro*, and belong to a

class of aggregation-induced emission (AIE) compounds [22–26]. AIE-active compounds differ from conventional fluorophores in that their fluorescence at low concentrations becomes enhanced in the aggregated state (*e.g.*, when embedded in cell membranes) [23–26]. This fluorescence phenomenon is attributed to inhibition of aryl–ethylene bond rotations, and differentiates tetraarylethylene-based vinylpyridinium derivatives from traditional mitochondrial targeted compounds [22, 27–29]. For example, triphenylphosphonium (TPP) compounds have been used for decades as a lipophilic-cationic delivery vehicle for targeting mitochondria. However, imaging of TPP-based delivery to mitochondria requires the addition of bulky fluorescent groups [27–30]. In addition, tetraarylethylene vinylpyridinium derivatives are distinguished from traditional fluorescent and mitochondrial targeting compounds by displaying substantial synthetic flexibility and maintaining the potential for improved hydrophilicity (by increasing the number of pyridine substitutions) [22, 27–29]. Currently, few heteroaromatic tetraarylethylenes have been reported, and the biochemical properties and therapeutic potential of these compounds have not been examined [22].

Here, we present the synthesis (Scheme 1) and characterization of mitochondrial targeted triphenylvinylpyridinium (10-TPVP; 18:2-TPVP), diphenyl-divinylpyridinium (DPDVP) and phenyltrivinylpyridinium (PTVP) derivatives, together with an initial biological evaluation of their activities in metastatic melanoma cell lines (A375 and SK-Mel-3) and normal human fibroblasts (NHFs) *in vitro*. Our data suggest that lipophilic-cationic and polycationic-heteroaromatic tetraarylethylenes pass through the hydrophobic mitochondrial membrane lipid bilayer; move down the negative mitochondrial membrane voltage potential; and preferentially imbed in the hyperpolarized mitochondria membrane of melanoma cells. Imaging of the accumulation of the tetraarylethylene-based compounds demonstrated melanoma cell specific accumulation – with little accumulation in normal human fibroblasts as controls. We further find that vinylpyridine-derivative accumulation in melanoma cells disrupts the cellular oxidation state and induces increased toxicity in melanoma cells relative to non-malignant cells. Collectively, these results highlight the potential for the pyridinium tetraarylethylene platform as a new and effective therapy for metastatic melanoma.

RESULTS AND DISCUSSION

Synthesis of TPVP, DPDVP, and PTVP derivatives

The synthesis of triphenylvinylpyridine (TPVP, **1**) was accomplished by a Peterson olefination reaction between the lithiated anion of silylated diphenylmethane and 4-pyridyl phenyl ketone as recently reported [22]. The corresponding pyridinium salt **2** was easily obtained in good yield upon refluxing **1** with 1-bromodecane. In addition, construction of a TPVP derivative functionalized with linoleic acid (18:2-TPVP, **4**) was achieved by heating **1** with the alkyl bromide **3** prepared from the corresponding methyl linoleate [31]. An alternative approach was utilized for the synthesis of 1,1-diphenyl-2,2-dipyridylethylenes, which involves double Suzuki coupling of 1,1-dibromo-2,2-diarylethylenes with aryl boronic acids [32]. This approach was used to prepare compound **5** in reasonable yield [22]. Reaction of **5** with 1-bromodecane afforded the bis(pyridinium) salt (DPDVP, **6**).

Moreover, double Suzuki coupling of **7** proceeded in straightforward fashion with pyridine-4-boronic acid to yield 1-phenyl-2,2,2-tripyrindylethylene **8** from which the tripyridinium salt (PTVP, **9**) was easily prepared.

10-TPVP accumulates and fluoresces upon melanoma cell mitochondrial accumulation

Confocal microscopy was used to determine that 10-TPVP accumulates in the mitochondria of melanoma cells and fluoresces upon aggregation in mitochondrial membranes of melanoma cells relative to non-malignant cells (Figure 1). The accumulation of 10-TPVP in A375 and SK-Mel-3 cells was easily observable by confocal microscopy after incubation for 3 hours at 1 μ M (Figure 1; **Column I**). NHF cells have a basal level of 10-TPVP accumulation, supporting the hypothesis that TPVP derivatives preferentially accumulate by targeting the hyperpolarized mitochondrial membrane (Figure 1; **Column II**). Co-localization of 10-TPVP with MitoTracker Red demonstrates that 1 μ M 10-TPVP accumulates in the mitochondria of metastatic melanoma cells, but does not significantly accumulate in the mitochondria of NHFs (Figure 1; **Column III**).

Vinylpyridine derivatives disrupt melanoma mitochondrial membrane potential

JC-1 mitochondrial membrane potential assays were used to determine that TPVP derivatives are designed to accumulate in melanoma cell mitochondria and disrupt the membrane potential of melanoma cells relative to non-malignant cells (Figure 2).

Mitochondrial membrane potential is an important parameter of mitochondrial function. In healthy cells (with high mitochondrial potential), JC-1 spontaneously forms complexes known as J-aggregates that fluoresce in the red region. In unhealthy cells with a low mitochondria membrane potential or cells whose membrane integrity is disrupted with uncoupling agents (*e.g.* FCCP), JC-1 remains in the monomeric form, with fluorescence in the green region. Therefore, the ratio of J-aggregate to monomer fluorescence is an indicator of mitochondrial membrane potential and cellular health.

JC-1 fluorescence analysis reveals that 18:2-TPVP significantly decreases the ratio of JC-1 aggregates to monomers in melanoma cells relative to non-malignant cells in a concentration dependent fashion (Figure 2A). Specifically, 10 μ M 18:2-TPVP significantly decreases the fluorescence ratio by 55% in A375 and 40% in SK-Mel-3, but only decreases the fluorescence ratio in NHFs by 20%. Further results demonstrate that DPDVP also significantly decreases the mitochondria-membrane potential in A375 and SK-Mel-3 melanoma cells relative to NHFs (Figure 2B). At 10 μ M, DPDVP significantly decreases the ratio in A375 cells by 50% and SK-Mel-3 by 30%, while the NHF ratio only decreases by 10%. FCCP, a known mitochondria uncoupler, was used as positive control (Supplemental Information, Table S1). Together, these results suggest that TPVP derivatives can be designed to significantly decrease the mitochondria membrane potential of melanoma cells relative to NHFs as a function of dose.

10-TPVP increases DHE oxidation in melanoma cells relative to NHFs

Oxidation-sensitive dihydroethidium (DHE) was used to determine if mitochondrial-accumulated TPVP derivatives alter the oxidation state of melanoma cells (Figure 3).

DHE is a blue-fluorescent superoxide-sensitive probe that accumulates in the cytosol of healthy cells. However, upon reaction with superoxide, DHE intercalates with DNA and forms a red fluorescent product (2-hydroxyethidium). Therefore, the shift to red fluorescence is an indicator of the change in the redox state of the cell.

Results demonstrate that 10-TPVP significantly increases the DHE fluorescence intensity in the red-shifted channel, indicating DNA intercalation, in melanoma cells as the concentration increases. Specifically, 10 μ M 10-TPVP significantly increases DHE oxidation in A375 three-fold and SK-Mel-3 two-fold, but does not significantly alter the DHE fluorescence intensity in NHFs. Together, these results further demonstrate that TPVP derivatives can be designed to accumulate in the mitochondria and disrupt the oxidation state of melanoma cells relative to non-malignant cells.

Vinylpyridine derivatives decrease the intracellular ATP levels in melanoma cells

TPVP derivatives imbed in the mitochondria of malignant cells and increase the oxidation state relative to NHFs. Therefore, intracellular ATP levels were measured to determine the potential impact of TPVP-based treatment on cell viability (Figure 4). This plate reader based luciferase assay is a selective method for determining the number of viable cells by quantifying the ATP-dependent oxygenation of luciferin.

Results demonstrate that the TPVP derivatives PTVP, DPDVP, and 18:2-TPVP decrease melanoma cell viability in a concentration dependent manner. The concentration of A375 ATP significantly decreases at 2.5 μ M, 5.0 μ M or 10 μ M of PTVP, DPDVP, and 18:2-TPVP, relative to the untreated control. Specifically, A375 ATP levels were depleted from 1.5 mM to less than 0.8 mM after exposure to all three compounds at 10 μ M. Further results demonstrate that SK-Mel-3 intracellular ATP levels significantly decrease from 1.4 mM to less than 1.0 mM after 10 μ M of PTVP, DPDVP, or 18:2-TPVP. Together, these results suggest TPVP derivatives can be designed to accumulate in the hyperpolarized mitochondrial membrane of melanoma cells and decrease melanoma cell viability relative to normal cells.

18:2-TPVP decreases intracellular ATP in melanoma cells relative to normal human fibroblasts

Previous results demonstrate that 18:2-TPVP is the most effective compound at significantly decreasing melanoma cell viability. Therefore, intracellular ATP levels of NHF cells were measured to test the hypothesis that 18:2-TPVP can preferentially decrease melanoma cell viability relative to non-malignant cells (Figure 5).

Results demonstrate that 18:2-TPVP significantly decreases A375 intracellular ATP at all concentrations, and significantly decreases SK-Mel-3 intracellular ATP at 5.0 μ M and 10 μ M, relative to NHFs. These results support the hypothesis that TPVP derivatives can be designed to preferentially decrease melanoma cell viability relative to non-malignant cells by targeting the hyperpolarized mitochondrial membrane and disrupting oxidative metabolism.

Vinylpyridine derivatives decrease melanoma cell clonogenic survival

To further test the hypothesis that TPVP derivatives can be designed to decrease viability in melanoma cells relative to normal cells, clonogenic survival assays were performed (Figure 6).

The clonogenic cell survival assay is often used as a measure of a cells ability to proliferate indefinitely. In these data, cells were exposed to varying concentrations of TPVP derivatives for 24 hours. After exposure to cytotoxic compounds, cells are collected, counted, and reseeded at a low density. The cells undergo a series of doublings, forming cell colonies. These colonies are then counted and the resulting cell survival curve reports the dose of the agent used to produce an insult and the fraction of cells retaining their ability to reproduce.

Results demonstrate that PTVP, DPDVP, and 18:2-TPVP significantly decrease A375 (Figure 6A) and SK-Mel-3 (Figure 6B) clonogenic survival at concentrations of 5.0 μ M, 7.5 μ M, and 10 μ M, relative to the untreated control. Specifically, 18:2-TPVP decreases clonogenic survival to <50% at 5.0 μ M for both A375 and SK-Mel-3 cells, and <20% at 7.5 μ M. Together, these results support the hypothesis that TPVP derivatives can be designed to significantly decrease clonogenic survival of melanoma cells.

18:2-TPVP decreases melanoma cell viability relative to non-malignant cells

Clonogenic survival assays were performed to test the hypothesis that 18:2-TPVP is cytotoxic to melanoma cells relative to NHFs (Figure 7).

Results demonstrate a significant decrease in A375 and SK-Mel-3 clonogenic survival at concentrations of 2.5 μ M, 5.0 μ M, 7.5 μ M and 10 μ M, relative to NHFs. Specifically, 5.0 μ M 18:2-TPVP significantly decreases A375 clonogenic survival to 40% and SK-Mel-3 to 30%, but NHF clonogenic survival is only decreased to 75 %. Together, these results support the hypothesis that TPVP derivatives can be designed to selectively decrease clonogenic survival in melanoma relative to non-malignant cells.

EXPERIMENTAL PROCEDURES

Cell lines and culture conditions

Experiments were performed using metastatic BRAF^{V600E} mutant A375 (ATCC CRL-1619) human melanoma cells, metastatic BRAF^{V600E} mutant SK-Mel-3 (ATCC HTB-69) human melanoma cells isolated from a lymph node metastasis, and non-metastatic 46-year-old normal human fibroblasts (NHFs) provided by the Coriell Institute (AG13442). All cells were cultured at 21% O₂ at 37 °C in a humidified 5% CO₂ incubator. Cells were detached with 0.25% trypsin-EDTA. A375 and NHF cells were cultured in high glucose DMEM medium supplemented with 1% PenStrep and 10% FBS. SK-Mel-3 cells were cultured in high glucose McCoy's 5A medium supplemented with 1% Pen-Strep and 15% FBS. All medium components were purchased from Gibco.

Synthesis of vinylpyridine derivatives

All commercially available starting materials, reagents, and solvents were used as supplied, unless otherwise stated. Reactions were performed under argon using solvents that were dried and purified by passage through activated alumina or activated molecular sieves. Reported yields are isolated yields. Purification of all final products was accomplished by silica gel flash column chromatography. Chloroform, methanol or hexane, and ethyl acetate were used as elution solvents. Proton (^1H) and carbon (^{13}C) NMR were collected on Bruker NMR spectrometers at 300 MHz or 400 MHz for ^1H and 100 MHz or 75 MHz for ^{13}C . Chemical shifts (δ) are reported in parts-per million (ppm) relative to tetramethylsilane or residual undeuterated solvent. Melting points were recorded using a capillary melting point apparatus and are uncorrected. High-resolution mass spectra were obtained in positive ion mode using electron spray ionization (ESI) on a double-focusing magnetic sector mass spectrometer.

1-Decyl-4-(1,2,2-triphenylvinyl)pyridin-1-ium bromide (2)—1-Bromodecane (125 μL , 0.6 mmol) was added to a solution of **1** (100 mg, 0.3 mmol) in 25 mL of acetonitrile, and the reaction was heated to reflux overnight under argon. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography using chloroform/methanol 8:1 and 1:4 as eluent to give **2** (130 mg, 78%) as yellow solid. Mp $>200^\circ\text{C}$. ^1H NMR (300 MHz, acetone- d_6) δ 0.86 (t, 3H, $J = 7.2$ Hz), 1.26–1.36 (m, 16H), 4.88 (t, 2H, $J = 7.1$ Hz), 7.08–7.26 (m, 15H), 7.63 (d, 2H, $J = 6.3$ Hz), 9.40 (d, 2H, $J = 6.3$ Hz). ^{13}C NMR (100 MHz, acetone- d_6) δ 14.6, 23.5, 26.7, 29.9, 30.2, 30.3, 30.4, 32.5, 32.8, 61.1, 128.8, 128.9, 129.4, 129.5, 129.6, 129.7, 130.5, 131.9, 132.3, 132.4, 137.4, 141.9, 142.4, 142.9, 145.5, 149.3, 161.8. HRMS (ESI): calcd for $\text{C}_{35}\text{H}_{40}\text{N} [\text{M}]^+$, 474.3161; found, 474.3156.

1-((9Z,12Z)-Octadeca-9,12-dien-1-yl)-4-(1,2,2-triphenylvinyl)pyridin-1-ium bromide (4)—Compound **3** (296 mg, 0.9 mmol) was added to a solution of **1** (100 mg, 0.3 mmol) in 25 mL of acetonitrile, and the reaction was heated to reflux for 2 days under argon. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography using chloroform/methanol 9:1 and 1:6 as eluent to give **4** (123 mg, 62%) as yellowish brown solid. Mp $>200^\circ\text{C}$. ^1H NMR (300 MHz, DMSO- d_6) δ 0.89 (t, 3H, $J = 6.9$ Hz), 1.15–1.40 (m, 18H), 1.81–1.88 (m, 2H), 2.01–2.11 (m, 2H), 2.78 (t, 2H, $J = 7.0$ Hz), 4.49 (t, 2H, $J = 7.1$ Hz), 5.33–5.41 (m, 4H), 7.03–7.32 (m, 15H), 7.67 (d, 2H, $J = 6.7$ Hz), 8.83 (d, 2H, $J = 6.7$ Hz). ^{13}C NMR (75 MHz, CDCl_3) δ 16.7, 25.2, 28.3, 28.6, 29.8, 29.9, 31.7, 31.8, 31.9, 32.0, 32.3, 34.1, 34.3, 34.4, 130.6, 130.7, 130.9, 131.0, 131.5, 131.6, 131.8, 132.3, 133.6, 133.8, 133.9, 134.0, 141.9, 143.0, 143.1, 143.4, 143.8, 146.3, 152.8, 152.9, 165.8. HRMS (ESI): calcd for $\text{C}_{43}\text{H}_{52}\text{N} [\text{M}]^+$, 582.4099; found, 582.4095.

4,4'-(2,2-Diphenylethene-1,1-diyl)bis(1-decylpyridin-1-ium) bromide (6)—1-Bromodecane (500 μL , 2.4 mmol) was added to a solution of **5** (100 mg, 0.3 mmol) in 25 mL of acetonitrile, and the reaction was heated to reflux for 2 days under argon. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography using chloroform/methanol 8:1 and 1:6 as eluent to give **6** (160 mg, 69%) as yellow solid. Mp $>200^\circ\text{C}$. ^1H NMR (300 MHz, methanol- d_4) δ 0.97 (t, 6H, $J = 7.1$ Hz),

1.33–1.52 (m, 28H), 2.01–2.11 (m, 4H), 4.63 (t, 4H, $J = 7.2$ Hz), 7.23–7.44 (m, 10H), 7.88 (d, 4H, $J = 6.5$ Hz), 8.89 (d, 4H, $J = 6.5$ Hz). ^{13}C NMR (100 MHz, methanol- d_4) δ 14.2, 23.4, 26.8, 29.8, 30.1, 30.2, 30.3, 32.0, 32.7, 62.2, 128.1, 129.6, 130.7, 131.6, 132.3, 140.9, 145.3, 157.1, 158.8. HRMS (ESI): calcd for $\text{C}_{44}\text{H}_{60}\text{N}_2\text{Br}$ $[\text{M}]^+$, 695.3940; found, 695.3945.

4,4',4''-(2-Phenylethene-1,1,2-triyl)tripyridine (8)—Compound **7** (339 mg, 1.00 mmol) was dissolved in 50 mL of dioxane : water (4 : 1). The flask was charged with Na_2CO_3 (690mg, 5.00 mmol), $\text{Pd}(\text{OAc})_2$ (28 mg, 0.12 mmol), PPh_3 (130 mg, 0.50 mmol) and 4-pyridine boronic acid (615 mg, 5.00 mmol). The reaction was heated to reflux under argon overnight. After cooling, the reaction mixture was diluted with water and extracted with ethyl acetate (3×50 mL) and the combined organic fractions were dried over anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography using ethyl acetate/hexane 1:1 and 2:1 as eluent to yield **8** (187 mg, 56%) as a yellowish white solid. Mp 175–177°C. ^1H NMR (400 MHz, CDCl_3) δ 6.81–6.92 (m, 5H), 6.95 (d, 2H, $J = 7.1$ Hz), 7.12–7.25 (m, 4H), 8.34–8.42 (m, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ 122.5, 126.3, 126.5, 129.4, 129.5, 131.7, 138.8, 141.3, 144.2, 150.1, 150.3, 150.6, 150.8, 151.0, 151.7. HRMS (ESI): calcd for $\text{C}_{23}\text{H}_{18}\text{N}_3$ $[\text{M} + \text{H}]^+$, 336.1501; found, 336.1499.

4,4',4''-(2-Phenylethene-1,1,2-triyl)tris(1-decylpyridin-1-ium) bromide (9)—1-Bromodecane (1.5 mL, 7.2 mmol) was added to a solution of **8** (100 mg, 0.3 mmol) in 25 mL of acetonitrile, and the reaction was heated to reflux for 3 days under argon. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography using chloroform/methanol 8:1 and 1:4 as eluent to give **9** (708 mg, 71%) as orange solid. Mp >200°C. ^1H NMR (400 MHz, methanol- d_4) δ 0.90 (t, 9H, $J = 6.1$ Hz), 1.27–1.45 (m, 42H), 1.97–2.07 (m, 6H), 4.59–4.66 (m, 6H), 7.32–7.42 (m, 5H), 7.96–8.07 (m, 6H), 8.92–8.99 (m, 6H). ^{13}C NMR (100 MHz, methanol- d_4) δ 14.2, 23.4, 26.7, 27.0, 27.1, 28.8, 29.5, 29.8, 29.9, 30.0, 30.1, 30.2, 30.3, 30.4, 30.5, 31.9, 32.0, 32.1, 32.7, 32.8, 33.7, 34.3, 62.5, 62.8, 128.1, 130.1, 130.7, 131.2, 131.4, 131.5, 132.2, 136.6, 138.4, 145.7, 146.2, 146.3, 148.4, 154.9, 156.7. HRMS (ESI): calcd for $\text{C}_{53}\text{H}_{80}\text{N}_3\text{Br}_2$ $[\text{M}]^+$, 916.4719; found, 916.4725.

Confocal microscopy

A375, SK-Mel-3, and NHF cells were cultured at a density of 2.5×10^4 , 5×10^4 , or 7.5×10^4 (respectively) on 4-well glass chamber slide for 24 hours prior to exposure to test compounds. The cell populations were treated with 1 μM of 10-TPVP for 3 hours and 100 nM MitoTracker Red (ThermoFisher) for 30 minutes prior to fixation with 4% paraformaldehyde. Imaging was obtained at 63x oil *via* confocal microscopy using a UV laser with the 405 nm excitation wavelength. (Zeiss LSM 710; The University of Iowa Central Microscopy Research Facility).

JC-1 mitochondrial membrane potential

A375 were plated in a black, clear bottom 96-well plate at a density of 1.5×10^4 cells per well. SK-Mel-3 and NHF cells were plated in a black, clear bottom 96-well plate at a density of 2.5×10^4 cells per plate. After 24 hours, cells were treated with 2.5 μM , 5.0 μM , or 10 μM

18:2-TPVP or DPDVP for 1 hour. Cells were then washed and incubated with 2.0 μ M JC-1 (Cayman) in PBS containing 5 mM pyruvate (Gibco) for 1 hour. Control dishes were treated with 1.0 μ M FCCP (Sigma-Aldrich) 30 minutes prior to measurement. Cells were washed and analyzed in PBS using a spectrophotometer at 530 nm and 590 nm.

DHE oxidation

A375 were plated in a black, clear bottom 96-well plate at a density of 1.5×10^4 cells per well. SK-Mel-3 and NHF cells were plated in a black, clear bottom 96-well plate at a density of 2.5×10^4 cells per plate. After 24 hours, cells were treated with 2.5 μ M, 5.0 μ M, or 10 μ M 10-TPVP for 1 hour, washed, and incubated with 10 μ M DHE (Sigma-Aldrich) in PBS supplemented with 5 mM pyruvate for 30 minutes. Control dishes were treated with 10 μ M Antimycin A (Sigma-Aldrich) for 30 minutes prior to measurement in PBS. Cells were analyzed using a spectrophotometer at 590 nm.

Intracellular ATP measurements

A375 cells were plated in 60 mm culture dishes at a density of 1.5×10^5 cells per dish. SK-Mel-3 and NHF cells were plated in 60 mm culture dishes at a density of 2.5×10^5 cells per dish. After 48 hours, cells were exposed to 2.5 μ M, 5.0 μ M or 10 μ M of PTPV, DPDVP, or 18:2-TPVP for 24 hours and analyzed for intracellular ATP content using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

Clonogenic survival assays

A375 cells were plated in 60 mm culture dishes at a density of 1.5×10^5 cells per dish. SK-Mel-3 and NHF cells were plated in 60 mm culture dishes at a density of 2.5×10^5 cells per dish. After 48 hours, cells were treated with 1.0 μ M, 2.5 μ M, 5.0 μ M, 7.5 μ M or 10 μ M of PTPV, DPDVP, or 18:2-TPVP for 24 hours. After the exposure period, cells were collected, counted, and plated at either 500 (A375 and NHF) or 3000 (SK-Mel-3) cells per 60 mm dish. Following a two-week incubation, medium was removed and colonies were fixed with ethanol and stained with Coomassie Brilliant Blue. Data was normalized to each untreated control and the surviving fraction was reported.

CONCLUSIONS

Triphenylvinylpyridines (TPVPs) are a new and unique class of emissive fluorophores that maintain a high degree of synthetic flexibility, while providing lipophilic-like qualities that suggest promising potential for these compounds as anti-cancer agents. Our initial exploration of the TPVP platform in this context reveals that TPVP-based compounds can be designed to accumulate in the mitochondria of melanoma cells, relative to non-malignant cells. We further demonstrate that the synthetic flexibility of the TPVP-based platform allows their use for imaging of mitochondrial accumulation of compounds that interrupt ATP synthesis and alter the oxidation state of melanoma cells, leading to melanoma specific toxicity. These results demonstrate the potential for fluorescent-based TPVP derivatives as a visual tool to study mitochondrial biophysics and as a potential avenue for designing therapeutic agents for metastatic melanoma and other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was supported by the following grants: NRC-HQ-12-G-38-0041, K25CA172218-01A1, 1P50CA174521-01A1, 1-S10-RR025439-01.

We would like to thank and acknowledge the following University of Iowa organizations: the Free Radical and Radiation Biology Program, the Holden Comprehensive Cancer Center, the Central Microscopy Research Facility, and the Flow Cytometry Facility.

ABBREVIATIONS

TPVP	triphenylvinylpyridine
DPDVP	diphenyl-divinylpyridinium
PTVP	phenyltrivinylpyridinium
ETC	electron transport chain
AIE	aggregation-induce emission
NHF	normal human fibroblasts
ATP	adenosine triphosphate
DHE	dihydroethidium

References

1. Howlader, NNA., Krapcho, M., Garshell, J., Miller, D., Altekruse, SF., Kosary, CL., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, DR., Chen, HS., Feuer, EJ., Cronin, KA. SEER Cancer Statistics Review, 1975–2012. National Cancer Institute; 2014.
2. Tseng WW, Leong SP. Long-term survivors after immunotherapy for metastatic melanoma. *Immunol Lett.* 2011; 139(1–2):117–8. [PubMed: 21596063]
3. Ackerman A, et al. Outcomes of patients with metastatic melanoma treated with immunotherapy prior to or after BRAF inhibitors. *Cancer.* 2014; 120(11):1695–701. [PubMed: 24577748]
4. Sousa R, et al. Treatment for metastatic melanoma: a new and evolving era. *Int J Clin Pract.* 2015; 69(3):273–80. [PubMed: 25727802]
5. Tolk H, et al. Complete remission of metastatic melanoma upon BRAF inhibitor treatment - what happens after discontinuation? *Melanoma Res.* 2015; 25(4):362–6. [PubMed: 26061438]
6. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J.* 2009; 417(1):1–13. [PubMed: 19061483]
7. Simons AL, et al. Glucose deprivation-induced metabolic oxidative stress and cancer therapy. *J Cancer Res Ther.* 2009; 5(Suppl 1):S2–6. [PubMed: 20009288]
8. Aykin-Burns N, et al. Increased levels of superoxide and H₂O₂ mediate the differential susceptibility of cancer cells versus normal cells to glucose deprivation. *Biochem J.* 2009; 418(1): 29–37. [PubMed: 18937644]
9. Gius D, Spitz DR. Redox signaling in cancer biology. *Antioxid Redox Signal.* 2006; 8(7–8):1249–52. [PubMed: 16910772]

10. Ahmad IM, et al. Mitochondrial O₂^{*}- and H₂O₂ mediate glucose deprivation-induced stress in human cancer cells. *J Biol Chem*. 2005; 280(6):4254–63. [PubMed: 15561720]
11. Lin X, et al. 2-Deoxy-D-glucose-induced cytotoxicity and radiosensitization in tumor cells is mediated via disruptions in thiol metabolism. *Cancer Res*. 2003; 63(12):3413–7. [PubMed: 12810678]
12. Spitz DR, et al. Glucose deprivation-induced oxidative stress in human tumor cells. A fundamental defect in metabolism? *Ann N Y Acad Sci*. 2000; 899:349–62. [PubMed: 10863552]
13. Mueckler M. Facilitative glucose transporters. *Eur J Biochem*. 1994; 219(3):713–25. [PubMed: 8112322]
14. Adekola K, Rosen ST, Shanmugam M. Glucose transporters in cancer metabolism. *Curr Opin Oncol*. 2012; 24(6):650–4. [PubMed: 22913968]
15. Rohlena J, et al. Anticancer drugs targeting the mitochondrial electron transport chain. *Antioxid Redox Signal*. 2011; 15(12):2951–74. [PubMed: 21777145]
16. Murphy MP. Targeting lipophilic cations to mitochondria. *Biochim Biophys Acta*. 2008; 1777(7–8):1028–31. [PubMed: 18439417]
17. Murphy MP, Smith RA. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu Rev Pharmacol Toxicol*. 2007; 47:629–56. [PubMed: 17014364]
18. Murphy MP, Smith RA. Drug delivery to mitochondria: the key to mitochondrial medicine. *Adv Drug Deliv Rev*. 2000; 41(2):235–50. [PubMed: 10699318]
19. Modica-Napolitano JS, Aprille JR. Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells. *Adv Drug Deliv Rev*. 2001; 49(1–2):63–70. [PubMed: 11377803]
20. Indran IR, et al. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. *Biochim Biophys Acta*. 2011; 1807(6):735–45. [PubMed: 21453675]
21. Chen LB. Mitochondrial membrane potential in living cells. *Annu Rev Cell Biol*. 1988; 4:155–81. [PubMed: 3058159]
22. Gabr MT, Pigge FC. Synthesis and aggregation-induced emission properties of pyridine and pyridinium analogues of tetraphenylethylene. *RCS Adv*. 2015:90226–90234.
23. Tong H, et al. Fluorescent “light-up” bioprobes based on tetraphenylethylene derivatives with aggregation-induced emission characteristics. *Chem Commun (Camb)*. 2006; (35):3705–7. [PubMed: 17047818]
24. Ding D, et al. Bioprobes based on AIE fluorogens. *Acc Chem Res*. 2013; 46(11):2441–53. [PubMed: 23742638]
25. Leung CW, et al. A photostable AIE luminogen for specific mitochondrial imaging and tracking. *J Am Chem Soc*. 2013; 135(1):62–5. [PubMed: 23244346]
26. Wang Z, et al. Long-term fluorescent cellular tracing by the aggregates of AIE bioconjugates. *J Am Chem Soc*. 2013; 135(22):8238–45. [PubMed: 23668387]
27. Hu Q, et al. Mitochondria-targeted cancer therapy using a light-up probe with aggregation-induced-emission characteristics. *Angew Chem Int Ed Engl*. 2014; 53(51):14225–9. [PubMed: 25318447]
28. Yuan H, et al. Fluorescent and radiolabeled triphenylphosphonium probes for imaging mitochondria. *Chem Commun (Camb)*. 2013; 49(88):10361–3. [PubMed: 24072060]
29. Ripcke J, et al. Small-molecule targeting of the mitochondrial compartment with an endogenously cleaved reversible tag. *Chembiochem*. 2009; 10(10):1689–96. [PubMed: 19492396]
30. Trnka J, Elkalaf M, Andel M. Lipophilic triphenylphosphonium cations inhibit mitochondrial electron transport chain and induce mitochondrial proton leak. *PLoS One*. 2015; 10(4):e0121837. [PubMed: 25927600]
31. Kuklev DV, Smith WL. Chemical C₂-elongation of polyunsaturated fatty acids. *Chem Phys Lipids*. 2006; 144(2):172–7. [PubMed: 17049508]
32. Zhang GF, et al. General Synthetic Approach toward Geminal-Substituted Tetraarylethene Fluorophores with Tunable Emission Properties: X-ray Crystallography, Aggregation-Induced Emission and Piezofluorochromism. *Chemistry of Materials*. 2014; 26(15):4433–4446.

Figure 1. 10-TPVP preferentially accumulates in the mitochondria of melanoma cells relative to normal human fibroblasts (NHF's)

A375 (primary) and SK-Mel-3 (lymph node metastasis) melanoma cells and NHFs were exposed to 1 μ M 10-TPVP for 3 h (column I) prior to mitochondrial labeling with 100 nM MitoTracker Red for 30 min (column II). Mitochondrial co-localization (column III) was determined by confocal microscopy.

Figure 2. TPVP derivatives decrease the mitochondria membrane potential of melanoma cells relative to fibroblasts

A375, SK-Mel-3, and NHFs were treated with 2.5 μM, 5.0 μM, or 10 μM of (A) 18:2-TPVP or (B) DPDVP for 1 h and analyzed for mitochondria membrane potential by the JC1 method (* # significant relative to each untreated control; ± SEM; $p < 0.05$; $n = 4$ from two biological replicates).

Figure 3. 10-TPVP increases DHE oxidation in melanoma cells, but not in fibroblasts

A375, SK-Mel-3, and NHFs were treated with 2.5 μM, 5.0 μM, or 10 μM of 10-TPVP for 1 h and analyzed for alterations in the oxidation state using the DHE method. The results are normalized to the untreated control of each cell line (* # significant relative to control; ± SEM; $p < 0.05$; $n = 6$ from two biological replicates).

Figure 4. TPVP derivatives decrease intracellular ATP in melanoma cells

A375 and SK-Mel-3 with 2.5 μM, 5.0 μM or 10 μM of either: PTPV, DPDVP, or 18:2-TPVP for 24 h and analyzed for intracellular ATP content using a luciferase based assay (* # significant relative to control; ± SEM; $p < 0.05$; $n = 4$ from two biological replicates).

Figure 5. 18:2-TPVP decreases intracellular ATP in melanoma cells relative to fibroblasts
A375, SK-Mel-3, and NHFs were treated with 1 μM, 5 μM or 10 μM of 18:2-TPVP for 24 h and analyzed for intracellular ATP content using a luciferase based assay (* # significant relative to NHFs; ± SEM; $p < 0.05$; $n = 4$ from two biological replicates).

Figure 6. 18:2-TPVP, PTVP, and DPDVP decreases clonogenic survival in melanoma cells
(A) A375 and (B) SK-Mel-3 melanoma cells were treated with 1.0 μM , 2.5 μM , 5.0 μM , 7.5 μM or 10 μM of either: PTVP, DPDVP, or 18:2-TPVP for 24 h and analyzed for clonogenic survival (* # significant relative to control; $p < 0.05$; $n = 6$ from two biological replicates).

Figure 7. 18:2-TPVP significantly decreases clonogenic survival in melanoma cells relative to fibroblasts

A375, SK-Mel-3, and NHF cells were treated with 1 μM, 2.5 μM, 5 μM, 7.5 μM or 10 μM of 18:2-TPVP for 24 h and analyzed for clonogenic survival (* significant relative to NHFs; $p < 0.05$; $n = 6$ from two biological replicates).

Scheme 1.