Published in final edited form as:

Photochem Photobiol. 2009 ; 85(5): 1225–1232. doi:10.1111/j.1751-1097.2009.00575.x.

PHOTOCYTOTOXICITY OF THE FLUORESCENT NON- STEROIDAL ANDROGEN RECEPTOR LIGAND TDPQ

P.J. Bilski^{1,3}, B. Risek², C.F. Chignell¹, and W.T. Schrader^{2,*}

¹Laboratory of Pharmacology, National Institute of Environmental Health Sciences, NIH, 111 T.W. Alexander Drive, Research Triangle Park, NC 27709

²Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NIH, 111 T.W. Alexander Drive, Research Triangle Park, NC 27709

Abstract

1,2,3,4-tetrahydro-2,2-dimethyl-6-(trifluoromethyl)-8-pyridono[5,6-*g*]quinoline (TDPQ), a selective non-steroidal ligand of the androgen receptor (AR), is a fluorescent compound we found to be photocytotoxic. We have characterized its spectral properties in comparison to the structural precursor carbostyryl 151 (C151) and to its racemic structural isomer ETPQ. The absorption maximum in CH₃CN of either TDPQ or ETPQ is 400 nm whereas that of C151 is 350 nm. The fluorescence lifetimes (τ) and quantum yields (ϕ_f) in CH₃CN are typical for fluorescent dyes: TDPQ (4.2ns, 0.8) and ETPQ (4.6ns, 0.76). C151 showed lower τ and ϕ_f of 0.2ns, and 0.02. Thus, from the spectral point of view, TDPQ can function as a fluorescent label in the (sub)micromolar concentration range. While the fluorescence maxima of the compounds were solvent insensitive, the ϕ_f for ETPQ decreased in aqueous solutions regardless of the presence of albumin or DNA. The ϕ_f of TDPQ was less affected. The quantum yield of singlet oxygen (1O_2) photosensitization (ϕ_{so}) by TDPQ and ETPQ was about 7% in CH₃CN, which is sufficient to induce photocytotoxicity. We detected TDPQ fluorescence in human breast tumor cells using confocal microscopy. Using AR-negative MDA-MB-231 and AR-positive MDA-MB-453 cells, we confirmed that TDPQ was photocytotoxic in the AR-positive breast cancer cells. The combination of the well-defined AR activity with the photocytotoxic potential makes TDPQ a promising candidate for the selective targeting of AR-positive malignant cells during photodynamic therapy.

Keywords

Androgen receptor; AR ligand; breast cancer cells; carbostyryl 151; ETPQ; fluorescence; fluorescence lifetime; nuclear receptor; photocytotoxicity; photosensitization; phosphorescence; singlet oxygen; TDPQ; quantum yield

Introduction

The quinoline derivative 1,2,3,4-tetrahydro-2,2-dimethyl-6-(trifluoromethyl)-8-pyridono[5,6-*g*]quinoline (TDPQ) shown in Scheme 1 has been reported as a potent androgen receptor (AR) antagonist (1), despite the fact that it hardly resembles the steroidal structure of physiological androgenic ligands (2). TDPQ was developed from a parent quinoline chromophore (3) that often introduces fluorescence to its derivatives. One such derivative was found to be a fluorescent marker for proteins (4). Another quinoline

*To whom correspondence should be addressed: William T. Schrader, National Institute of Environmental Health Sciences, 111 T.W. Alexander Drive, Research Triangle Park, NC, 27709, schrader@niehs.nih.gov, Tel. (919) 541-3433.

³NIH Contract to SRA-International.

derivative showing close resemblance to TDPQ is carbostyryl 151 (C151, Scheme 1). Carbostyryls (also referred to as 1-aza coumarins) are a class of compounds showing a wide range of interesting biological and pharmacological activities (5), including beta-adrenergic (6–12) and neuronal mediation (13). Although the biological properties of carbostyryls may manifest themselves in a different manner from TDPQ, C151 is a fluorescent molecule with an identical aromatic core and $-\text{CF}_3$ moiety at the same position (see formulas Scheme 1). We employed it in our study to better understand the spectral properties of TDPQ.

In addition to being strongly fluorescent, some quinoline derivatives may also possess photochemical activity. We have recently found that TDPQ can photosensitize the production of singlet oxygen ($^1\text{O}_2$) (14), a member of the reactive oxygen species (ROS) class of compounds. Similar photosensitization results were found using the TDPQ structural isomer ETPQ. When human prostate tumor cells were exposed to TDPQ and light, AR-targeted photodamage and cell killing occurred (15). $^1\text{O}_2$ is a strong oxidant that reacts with numerous bio-molecules such as indoles (16), B6 vitamins (17), DNA (18,19), and proteins (20,21). When targeted to selected malignant tissue, $^1\text{O}_2$ becomes an essential oxidant in photodynamic therapy (PDT).

PDT relies on killing cells upon light activation of drugs called photosensitizers (22) that, in the presence of molecular oxygen, produce $^1\text{O}_2$ and other ROS (23,24). While PDT is already used in clinical practice to treat some skin disorders (25) and malignancies (24,26) it is also an area of ongoing research (27,28) with promising new applications. In particular, certain types of malignancies such as pancreatic and prostate cancers are resistant to conventional chemotherapies (29), but may be more sensitive to $^1\text{O}_2$ and PDT (29,30). However, the photosensitizer's distribution often lacks tissue specificity, so that nearby healthy organs may be harmed during PDT treatment (31). Specific targeting using AR-mediated pathways may improve tissue selectivity, because only AR-positive cells would be affected. Thus, AR could serve as a preferred target because its activation by ligands is essential for growth regulation of certain cancers.

In this paper we have characterized the spectral and photochemical properties of both TDPQ and ETPQ ligands in comparison to the structural precursor C151. We report that light activation of TDPQ induces cell death in AR-positive human breast cancer cells as compared to AR-negative cells. In addition, because both compounds are also highly fluorescent, they can potentially be used for biological fluorescence imaging.

Materials and Methods

Acetonitrile, sulfuric acid, sodium dodecyl sulfate (SDS), DMSO, C151, perinaphthenone, Ludox colloidal silica, calf thymus DNA type I, calf thymus histone type II A, and bovine albumin were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA) and were used as received. Quinine sulfate (QS, Aldrich, Milwaukee, WI) was purified by crystallizing three times. Deuterium oxide was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). TDPQ and ETPQ were synthesized at GlaxoSmithKline (RTP, NC) and were kindly provided by Dr Tim Willson. ETPQ is a racemic mixture of two stereo-isomers and was used without enantiomeric enrichment. The extinction coefficients in DMSO at 390 nm were measured at concentrations up to 0.1 mM yielding perfectly linear plots from which the molar decadic extinction coefficient values were extracted as $\epsilon(\text{ETPQ}) = (1.840 \pm 0.008) \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon(\text{TDPQ}) = (1.819 \pm 0.005) \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The stock solutions (1 mM) were prepared in DMSO and were kept at -30°C . De-ionized water was used to prepare aqueous solutions. All experiments were performed at room temperature, if not indicated otherwise.

Singlet oxygen measurements

Singlet oxygen phosphorescence spectra were measured, as described previously (17), using a steady-state $^1\text{O}_2$ laser spectrometer featuring an optimized optical system as in our pulse $^1\text{O}_2$ spectrophotometer (32). Briefly, the apparatus utilized a germanium diode (Model 403 HS, Applied Detector Corporation, Fresno, CA), a light chopper with a monochromator in conjunction with an efficient optical system (32), and a digital lock-in amplifier (Stanford Research, Sunnyvale, CA) for signal detection. Samples were excited from a 500W Hg lamp operating at 300W through a 366 nm interference filter in combination with a KG3 heat-removing glass filter. The $^1\text{O}_2$ phosphorescence spectra were recorded over the range of 1200–1350nm during one ca. 30 s scan and were normalized to the same number of absorbed photons at the excitation wavelength. Perinaphthenone was used as a standard (33) for the $^1\text{O}_2$ quantum yield calculations.

Absorption spectra

Absorption spectra were acquired using an HP Diode Array Spectrophotometer model 8452A (Hewlett Packard Co., Palo Alto, CA). The relative number of absorbed photons at the excitation wavelength was calculated using the Lambert-Beer law. The samples were prepared in a suprasil fluorescence cuvette (0.5 cm path length), and were air-equilibrated or purged with either oxygen or nitrogen gases, if required.

Fluorescence spectra

Fluorescence spectra were acquired using a FluoroLog 3 spectrofluorometer (Horiba Jobin Yvon, Edison, NY). The fluorescence spectra were corrected and normalized to the same number of absorbed photons at the excitation wavelength. Fluorescence quantum yields (ϕ_f) were calculated using QS as a fluorescence standard (34). Fluorescence spectra were integrated over wavelength and the integrals were used for ϕ_f calculations. Fluorescence lifetime measurements were made using a FluoroLog-TCSPC unit (Horiba Jobin Yvon, Edison, NY). A pulse diode (1 MHz) emitting at 372 nm was used to excite the samples, while a colloidal silica (Ludox) solution was used for light scattering to measure the diode emission profile. The lifetimes were calculated using a simulation program supplied with the FluoroLog-TCSPC instrument.

Cell culture experiments

The experiments involving immunocytochemical detection of AR, cellular localization of TDPQ and ETPQ and cell culture irradiation conditions were performed as previously described (15) except that human breast cancer cells MDA-MB-231 and MDA-MB-453 were used in the present study.

A) Immunocytochemical detection of AR—Human breast cancer cells MDA-MB-231 (AR-negative) and MDA-MB-453 (AR-positive) were grown in IMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, and were seeded at a density of 100,000 cells/ cm^2 on a cover glass in 8-well chambers (Lab-Tek® II Chamber Coverglass™, Nalge Nunc Intl., Rochester, NY). After overnight incubation, the cell medium was replaced with fresh IMEM without or with 100 nM TDPQ or ETPQ. The cells were incubated for 1 hr and subjected to AR analysis by immunocytochemistry. Briefly, AR was detected in formalin-fixed and Triton X-100 permeabilized cells using PG-21 primary antibody (Abcam, Inc., Cambridge, MA) at a concentration of 3 $\mu\text{g}/\text{ml}$ in 3% blocking solution. After several washes in PBS, cells were incubated with a secondary FITC-conjugated goat anti-rabbit antibody (10 $\mu\text{g}/\text{ml}$ in blocking solution; Abcam, Inc., Cambridge, MA). Specimens were examined using a fluorescence microscope equipped with FITC filter sets.

B) Detection of TDPQ and ETPQ fluorescence in cells—For cellular detection of TDPQ and ETPQ by fluorescence microscopy, human breast cancer cells MDA-MB-231 and MDA-MB-453 were grown as described. Cells were incubated for 1 hr in absence or presence of 100 nM TDPQ or ETPQ respectively. Due to the strong fluorescent properties of TDPQ and ETPQ, their cellular distribution and localization were directly monitored by conventional fluorescence microscopy using appropriate excitation (HQ405/20 nm) and emission filters (HQ430 nm long pass; Chroma Technology Corp., Rockingham, VT). For a higher resolution of intracellular fluorescence, we have applied a laser-scanning confocal microscope (LSCM, 510 NLO; Carl Zeiss, Inc., Thornwood, NY) in combination with two-photon absorption (TPA) fluorescence. TPA fluorescence was excited by a Ti:sapphire ultrafast tunable laser source (Mai Tai Laser 720–960, Spectra Physics Lasers, Inc., Mountain View, CA) set at 810 nm wavelength. The fluorescent signal was collected using an oil immersion objective (Plan Apochromat 63×/1.4; Carl Zeiss, Inc., Thornwood, NY) and HQ430 nm long-pass emission filter. Images were acquired and analyzed using Axiovision software (Carl Zeiss, Inc., Thornwood, NY).

C) Cell irradiation studies—Human breast cancer cells MDA-MB-231 and MDA-MB-453 were grown as described. Cells were incubated for 1 hr in absence or presence of 300 nM TDPQ or ETPQ respectively. Cells were irradiated with 12 J/mm² at 405 nm using a mercury light source (HBO 100; Osram GmbH, Munich, Germany) in combination with an appropriate excitation filter (HQ405/20 nm; Chroma Technology Corp., Rockingham, VT) and incubated for 6 hr. The medium was replaced with fresh cell culture medium containing two nuclear dyes, propidium iodide (PI) and Hoechst 33342 (Invitrogen, Carlsbad, CA). PI was used to detect compromised cells that have lost cell membrane integrity and as a consequence accumulated PI bound to DNA in the nuclei. Routinely, PI (30 μM) was used in combination with Hoechst 33342 (10 μM) to differentiate between compromised (PI-positive, red stained nuclei) and healthy cells (Hoechst 33342-positive, blue stained nuclei). Cells were stained with nuclear dyes for 30 min and examined by fluorescence microscopy using appropriate dual-band filter sets (Chroma Technology Corp., Rockingham, VT) suitable for excitation and emission of PI (BP 546/12; LP 590) and Hoechst 33342 (BP 365/12; LP 397). Images were captured using a monochrome digital camera (AxioCam MRm, Zeiss Inc., Oberkochen, Germany). Routinely, all cell culture experiments were repeated at least two or three times.

RESULTS AND DISCUSSION

Spectral Characterization in Solution

Non-steroidal androgen receptor ligands TDPQ and ETPQ are strongly fluorescent compounds that can be visualized in cells by fluorescence microscopy at concentrations in the nanomolar range[†]. Here, we formally characterized their spectral properties in different environments in comparison to the structural precursor C151 (Figs. 1–3, Table 1).

The absorption spectra of TDPQ and ETPQ are practically identical in DMSO[‡] and CH₃CN, while in aqueous solutions the ETPQ absorption differs, which we believe is caused by aggregation (*vide infra*). In contrast, the C151 absorption spectrum has a different shape, lower absorption and is blue-shifted in both CH₃CN and water. This indicates that the identical aromatic core in the C151 and AR ligands does not ensure similar absorption properties. This is in contrast to their fluorescence spectra, which, except for intensity, are similar.

[†]Unpublished work of WTS and BR.

[‡]In DMSO, the molar extinction coefficients were practically the same (see Materials and Methods).

The shape and position of the fluorescence spectra of TDPQ and ETPQ are comparable to that of C151, especially in aqueous solution[§] (Figs. 1–3). This finding indicates that fluorescence is determined to a larger degree by the identical aromatic cores. The fluorescence intensity, on the other hand, was strongly affected by the different substitutions. Thus, C151 turned out to be less fluorescent: the ϕ_f for the C151 fluorescence is about 40 times lower compared to the AR ligands (Fig. 1, Table 1). In contrast, the ϕ_f values for both TDPQ and ETPQ in acetonitrile are higher than that of the QS fluorescence standard (Table 1). This observation shows that the additional non-aromatic ring with a nitrogen atom that is present in the TDPQ/ETPQ molecules, but not in the C151 (Scheme 1), causes a strong spectral intensity alteration. The heterocyclic ring also is essential for androgenic bioactivity (3).

Because androgenic activity requires ligand interaction with the respective AR in cell nuclei, we examined how the DNA and nuclear protein environments may affect ligand fluorescence. We initially used histone IIA to model the nuclear protein environment (not shown). Because the results were similar to albumin, we used albumin for our spectral characterizations. The presence of albumin or DNA had little effect on the TDPQ fluorescence in aqueous solutions (Fig. 3, Table 1). The same would be true for ETPQ but this expectation was overshadowed by the dominant influence of the aqueous solvent. In contrast, the fluorescence of C151 was the highest in the presence of DNA (Table 1) suggesting some degree of intercalation.

The ETPQ ϕ_f decreased strongly in aqueous solutions, regardless of the presence of DNA or albumin (Figs. 1–3, Table 1). This decrease is caused by the susceptibility of ETPQ to aggregation in aqueous media. Aggregation is evidenced by low ϕ_f values (Table 1) and a strong scattering of the excitation light in the fluorescence spectra (Figs. 1–5). In addition, the absorption spectrum of freshly prepared aqueous ETPQ solution is red-shifted and broader (Fig. 1D). The spectral intensity decreased and the spectrum broadened even more with time due to progressive aggregation (not shown).

ETPQ aggregates can be de-aggregated in micellar SDS solution (Fig. 3). This treatment restored the fluorescence by about half (as referenced to CH₃CN), and increased one component of the observed two-component fluorescence lifetime (τ) (Table 1). A micellar SDS environment had little effect upon the absorption spectra of the three compounds, whereas the fluorescence was determined by the aqueous phase and micellar interior (Fig. 2, Table 1).

We can speculate about the reasons for the different behavior of ETPQ vs. TDPQ in aqueous solutions. Despite its structural similarity to TDPQ, ETPQ appears to form dimers and higher aggregates much more extensively in water than TDPQ. It seems that the ETPQ ethyl group (Scheme 1) can be directed outwards, permitting the formation of a tighter hydrophobic sandwich complex between the ETPQ molecules, whereas the two adjacent twin methyl groups of TDPQ may hinder the formation of an analogous TDPQ complex. The racemic nature of ETPQ may also play a role in the formation of hydrophobic dimers that apparently seed aggregation.

We measured the τ for TDPQ and ETPQ in all the media studied (Table 1). In most cases, the fluorescence decays are best approximated by bi-exponential modes. The τ of the dominant component, constituting more than 65% of the fluorescence signal, had a duration of around 5 ns (Table 1). The τ of the shorter-lived component was less than 1 ns, with the

[§]The large spectral shift between the absorption and fluorescence spectra of C151 in aqueous solutions suggests that the excited singlet state is polar in nature. This state is stabilized less in CH₃CN resulting in a smaller Stokes shift.

exception of values measured in micellar SDS solution where the τ were higher (Table 1). The τ of C151 was mostly mono-exponential with lifetimes of around 3–4 ns in aqueous media, but the value decreased to 0.2 ns in CH₃CN, reflecting very low fluorescence (Table 1). The ETPQ τ values were generally comparable to those of TDPQ (Table 1).

The much lower ϕ_f of ETPQ in aqueous media may be a disadvantage for fluorescence detection in biological staining applications. Conversely, the ϕ_f and τ for TDPQ were consistently high and insensitive to the environmental polarity, suggesting potential usefulness for biological staining. Both the ϕ_f and τ are typical of these values found for other efficient fluorescent dyes. One potential drawback is that both ligands were slowly photo-bleached upon irradiation by the 405 nm light typically used for the *in vitro* excitation (not shown). While this may put some restrictions on their possible use for biological staining, it does not affect their photocytotoxic potential that requires short irradiation (*vide infra*).

Singlet Oxygen Production

We found that both TDPQ and ETPQ ligands are moderate ¹O₂ photosensitizers in CH₃CN, which can result in phototoxicity. Fig. 4 shows the spectra of ¹O₂ phosphorescence photo-generated by TDPQ and the spectrum of a perinaphthenone standard for comparison. A very similar spectrum yielding almost identical Φ_{so} was observed for ETPQ in CH₃CN. On the other hand, ¹O₂ production was not detected in D₂O by either ETPQ or TDPQ (baseline, Fig. 4). This result shows that photosensitization by TDPQ is very sensitive to the medium, and that ¹O₂ is produced in aprotic and relatively nonpolar CH₃CN, but not in water.

The above observations have repercussions for cell culture experiments, because the production site of ¹O₂ in the cell will affect its reactivity and subsequent toxicity. A simple measure for ¹O₂ reactivity is its lifetime, which in turn is very sensitive to the physicochemical environment, whereas other ¹O₂ properties are less affected (35). The ¹O₂ lifetime will limit the diffusion distance, which is extremely short in a cell (36) due to an abundance of ¹O₂ quenchers (37). Because of this, the photocytotoxicity *via* ¹O₂ must occur mainly in nonpolar cellular regions where ¹O₂ can be produced efficiently by TDPQ irradiation. In the aqueous cellular milieu where TDPQ is also present, other, if any, non-¹O₂ mechanism(s) may operate.

Fluorescence in cells

We investigated the fluorescence of TDPQ and ETPQ in AR-negative MDA-MB-231 and AR-positive MDA-MB-453 human breast cancer cells. Intracellular fluorescence distribution was monitored using either conventional fluorescence microscopy or 3D confocal imaging whereas morphology of the intact cells in the presence of TDPQ or ETPQ was routinely assessed by phase contrast microscopy. Cell morphologies of MDA-MB-231 and MDA-MB-453 cell lines with 100 nM TDPQ are illustrated in Fig. 5A, B and Fig. 6A, B. In contrast to MDA-MB-231 cells, which displayed epithelial morphology with well discernible nuclei (Fig. 5A, 6A), MDA-MB-453 cells appeared spherical with a large nuclear/cytoplasmic ratio (Fig. 5B, 6B). Shortly after incubation, we observed a significant increase in intracellular TDPQ fluorescence in both cell lines. LSCM and optical sectioning on the z plane revealed that TDPQ fluorescence was localized diffusely over the cytoplasm (Fig. 5C, D). Curiously, the nuclei of all the stained cells displayed little or no fluorescence, regardless of the presence or absence of AR. This observation was unexpected since TDPQ is a small (MW=296) and highly hydrophobic molecule that should readily permeate the cells and their organelles. Similar fluorescence results were obtained with ETPQ (data not shown).

We are unaware of a specific mechanism that could exclude a small free compound such as TDPQ or ETPQ from cell nuclei. We assume that TDPQ is present in the nucleus but becomes non-fluorescent there: the compelling evidence for the nuclear presence is the ability of TDPQ to translocate AR into the nucleus (see Fig. 6B). Loss of fluorescence due to quenching of TDPQ fluorescence by free DNA and proteins were excluded (*vide supra*). However, it is common for some planar fluorescent compounds to lose their fluorescence in the nuclear environment. For example, fluorophores such as hypericin (38), photofrin (39), and certain 8-hydroxyquinoline derivatives similar to TDPQ (40) do not fluoresce in nuclei, although the reason for the lack of nuclear signal was not examined in those reports. A similar observation about nuclear fluorescence was made for rose bengal (41), although its fluorescence was observed previously from the nuclear membrane (42). This is because rose bengal is known to fluoresce stronger in the hydrophobic environment (43) where it may also aggregate (44,45) due to potential specific interaction with ionic cellular components.

We believe that the lack of fluorescence in our case is due to specific interaction/s between TDPQ (or ETPQ) and highly organized nuclear components. Tentative evidence for such a possibility is provided by our own experiments with the ETPQ aggregates in which fluorescence is strongly quenched in sandwich dimers (*vide supra*). A similar situation may occur in the nuclei where planar TDPQ molecules are intercalated in the vicinity of moieties with fluorescence quenching potential. Additionally, the exciting and emitting light can be attenuated through scattering and possibly a small degree of absorption by lipid bilayers and other nuclear structures. All these interactions may contribute to weak fluorescence that is simply below the detection limit in the presence of the background of strong cytoplasmic fluorescence of TDPQ.

Although the observations above preclude the use of TDPQ or ETPQ for AR imaging, they may amplify their phototoxic potential. This is because chemically inactive fluorescence energy may be converted into forms other than heat such as intersystem crossing and/or chemical cross-linking. Inter-system crossing populates the TDPQ triplet state that is the $^1\text{O}_2$ precursor. The absence of AR-imaging potential for TDPQ is compensated for by our discovery of the compound's AR-specific photocytotoxicity (15) that can provide selective (photo)intervention into cellular processes *via* nuclear receptor interactions.

Photocytotoxicity

We have recently reported that AR is required for TDPQ and ETPQ photocytotoxicity in human prostate tumor cells (15). Here we extend these observations to AR-negative MDA-MB-231 and AR-positive MDA-MB-453 human breast cancer cells as shown in Fig. 6. AR was not detectable by indirect immunofluorescence in MDA-MB-231 cells (Fig. 6A), but it was readily detectable in MDA-MB-453 cells (Fig. 6B). The translocation of the androgen receptors to the nucleus was studied extensively using numerous other AR ligands, and TDPQ's ability to do the same was reported previously for the human prostate cancer cells (15).

Next we tested the photocytotoxicity in these breast tumor cells. As predicted from our earlier work (15), there is a noticeable difference in the killing efficacy of TDPQ between these two cell lines (Fig. 6C and 6D). No cell death was observed when cells were irradiated in absence of TDPQ or ETPQ (data not shown). AR-positive MDA-MB-453 cells were much more sensitive to cell death than the MDA-MB-231 cells following excitation of the AR ligand TDPQ by visible light at 405 nm. Similar phototoxicity results were obtained with ETPQ (data not shown). Cells were spared from photoinduced death in the regions outside the irradiation zone outlined by the circle (Fig. 6C and 6D). Because most of the AR/TDPQ or AR/ETPQ complexes are associated within nuclei, this finding indicates that photochemically-initiated reactions in the nucleus must be involved in the death response.

Our observations based on the PI staining indicate the loss of plasma membrane integrity of the exposed cells, which can be due to necrotic or apoptotic events. In the present article we did not investigate the nature of cell death. However, such investigations were previously performed for the AR positive human prostate cancer cells (15). In that work, we demonstrated that cells died predominantly from apoptosis. Because all the experimental conditions for the breast cancer cells were like those we used for the prostate cell lines (15), we hypothesize that the cellular events observed in the AR positive human breast cancer cells can also be driven by apoptosis. ROS-induced apoptosis is an important feature of the PDT mechanism (27,29), and indeed in our earlier report we demonstrated that the TDPQ-induced cell death involved oxidative DNA damage in the prostate cancer cells (15).

In conclusion, our fluorescence measurements show that TDPQ and to some extent ETPQ have attractive attributes for their use as fluorescent dyes for biological fluorescence imaging. However, their application as specific fluorescent sensors for intracellular AR imaging will remain limited due to the lack of fluorescence detection in cell nuclei in association with AR.

More importantly, our data demonstrate that certain AR ligands can be used in combination with light to act as photosensitizers. This methodology provides a novel approach for PDT by specific targeting and ablation of AR-dependent cells such as those from certain human breast tumors.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, NIEHS. The authors also thank Mr. B. Karriker for assistance during fluorescence measurements and Mr. J.M. Reece for confocal microscopy. This paper is dedicated to the memory of Dr. Colin F. Chignell.

Abbreviations

AR	androgen receptor; carbostyryl 151 (C151), 7-hydroxy-4-(trifluoromethyl)-2(1H)-quinolinone
ETPQ	4-ethyl-1,2,3,4-tetrahydro-6-(trifluoromethyl)-8-pyridino[5,6-g]quinoline
PDT	photodynamic therapy
SDS	sodium dodecyl sulfate
ROS	reactive oxygen species
TDPQ	1,2,3,4-tetrahydro-2,2-dimethyl-6-(trifluoromethyl)-8-pyridono [5,6-g]quinoline
QS	quinine sulfate

References

1. Hamann LG, Mani NS, Davis RL, Wang XN, Marschke KB, Jones TK. Discovery of a potent, orally active, nonsteroidal androgen receptor agonist: 4-ethyl-1,2,3,4-tetrahydro-6-(trifluoromethyl)-8-pyridono[5,6-g]quinoline (LG121071). *Journal of Medicinal Chemistry*. 1999; 42:210–212. [PubMed: 9925725]
2. Ojasoo T, Raynaud JP. Unique Steroid Congeners for Receptor Studies. *Cancer Research*. 1978; 38:4186–4198. [PubMed: 359134]
3. Hamann LG, Higuchi RI, Zhi L, Edwards JP, Wang XN, Marschke KB, Kong JW, Farmer LJ, Jones TK. Synthesis and biological activity of a novel series of nonsteroidal, peripherally selective androgen receptor antagonists derived from 1,2-dihydropyridino[5,6-g]quinolines. *Journal of Medicinal Chemistry*. 1998; 41:623–639. [PubMed: 9484511]

4. Chakrabarty T, Xiao M, Cooke R, Selvin PR. Holding two heads together: Stability of the myosin II rod measured by resonance energy transfer between the heads. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:6011–6016. [PubMed: 11972024]
5. Kulkarni MV, Kulkarni GM, Lin CH, Sun CM. Recent advances in coumarins and 1-azacoumarins as versatile biodynamic agents. *Current Medicinal Chemistry*. 2006; 13:2795–2818. [PubMed: 17073630]
6. Deyrup MD, Nowicki ST, Richards GJ, Otero DH, Harrison JK, Baker SP. Structure-affinity profile of 8-hydroxycarbostryl-based agonists that dissociate slowly from the beta 2-adrenoceptor. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 1999; 359:168–177.
7. Deyrup MD, Greco PG, Otero DH, Dennis DM, Gelband CH, Baker SP. Irreversible binding of a carbostryl-based agonist and antagonist to the beta-adrenoceptor in DDT1 MF-2 cells and rat aorta. *British Journal of Pharmacology*. 1998; 124:165–175. [PubMed: 9630356]
8. Standifer KM, Pitha J, Baker SP. Carbostryl-Based Beta-Adrenergic Agonists - Evidence for Long Lasting or Apparent Irreversible Receptor-Binding and Activation of Adenylate-Cyclase Activity *Invitro*. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 1989; 339:129–137.
9. Milecki J, Baker SP, Standifer KM, Ishizu T, Chida Y, Kusiak JW, Pitha J. Carbostryl Derivatives Having Potent Beta-Adrenergic Agonist Properties. *Journal of Medicinal Chemistry*. 1987; 30:1563–1566. [PubMed: 2887658]
10. Kaiser C, Dandridge PA, Garvey E, Flaim KE, Zeid RL, Hieble JP. Dopamine Receptor Agonist Activity of Some 5-(2-Aminoethyl)Carbostryl Derivatives. *Journal of Medicinal Chemistry*. 1985; 28:1803–1810. [PubMed: 4068005]
11. Tominaga M, Tone H, Nakagawa K, Takada K, Hoshino Y, Watanabe K. Syntheses and Beta-Adrenergic Blocking Activities of Carbostryl Derivatives. *Chemical & Pharmaceutical Bulletin*. 1981; 29:2166–2181.
12. Shridhar DR, Sastry CVR, Mehrotra AK, Nagarajan R, Lal B. Synthesis and Biological-Activity of Some Substituted “3-[2-(Heteroaryl)Vinyl]Carbostryls and Carbostryl-3-(2'-Heteroarylidene) Carbohydrazides. *Indian Journal of Chemistry Section B-Organic Chemistry Including Medicinal Chemistry*. 1980; 19:59–61.
13. Kalkhambkar RG, Kulkarni GM, Shivkumar H, Rao RN. Synthesis of novel triheterocyclic thiazoles as anti-inflammatory and analgesic agents. *European Journal of Medicinal Chemistry*. 2007; 42:1272–1276. [PubMed: 17337096]
14. Bilski P, Risek B, Chignell CF, Schrader WT. Photocytotoxicity and photochemistry of the selective non-steroidal androgen receptor modulator TDPQ. *Free Radical Biology and Medicine*. 2007; 43:S126–S126.
15. Risek B, Bilski P, Rice AB, Schrader WT. Androgen receptor-mediated apoptosis is regulated by photoactivatable androgen receptor ligands. *Molecular Endocrinology*. 2008; 22:2099–2115. [PubMed: 18562628]
16. Matuszak Z, Bilska MA, Reszka KJ, Chignell CF, Bilski P. Interaction of singlet molecular oxygen with melatonin and related indoles. *Photochemistry and Photobiology*. 2003; 78:449–455. [PubMed: 14653575]
17. Bilski P, Li MY, Ehrenshaft M, Daub ME, Chignell CF. Vitamin B-6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. *Photochemistry and Photobiology*. 2000; 71:129–134. [PubMed: 10687384]
18. Cadet J, Douki T, Pouget JP, Ravanat JL. Singlet oxygen DNA damage products: Formation and measurement. *Singlet Oxygen, Uv-a, and Ozone*. 2000; 319:143–153.
19. Cadet J, Ravanat JL, Martinez GR, Medeiros MHG, Di Mascio P. Singlet oxygen oxidation of isolated and cellular DNA: Product formation and mechanistic insights. *Photochemistry and Photobiology*. 2006; 82:1219–1225. [PubMed: 16808595]
20. Davies MJ. Reactive species formed on proteins exposed to singlet oxygen. *Photochemical & Photobiological Sciences*. 2004; 3:17–25. [PubMed: 14743273]
21. Davies MJ. The oxidative environment and protein damage. *Biochimica Et Biophysica Acta-Proteins and Proteomics*. 2005; 1703:93–109.

22. Juzeniene A, Peng Q, Moana J. Milestones in the development of photodynamic therapy and fluorescence diagnosis. *Photochemical & Photobiological Sciences*. 2007; 6:1234–1245. [PubMed: 18046478]
23. Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumour immunity. *Nature Reviews Cancer*. 2006; 6:535–545.
24. Dolmans D, Fukumura D, Jain RK. Photodynamic therapy for cancer. *Nature Reviews Cancer*. 2003; 3:380–387.
25. Garcia-Zuazaga J, Cooper KD, Baron ED. Photodynamic therapy in dermatology: current concepts in the treatment of skin cancer. *Expert Review of Anticancer Therapy*. 2005; 5:791–800. [PubMed: 16221049]
26. Kurwa HA, Barlow RJ. The role of photodynamic therapy in dermatology. *Clinical and Experimental Dermatology*. 1999; 24:143–148. [PubMed: 10354165]
27. Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochimica Et Biophysica Acta-Reviews on Cancer*. 2007; 1776:86–107.
28. Chen J, Jarvi M, Lo PC, Stefflova K, Wilson BC, Zheng G. Using the singlet oxygen scavenging property of carotenoid in photodynamic molecular beacons to minimize photodamage to non-targeted cells. *Photochemical & Photobiological Sciences*. 2007; 6:1311–1317. [PubMed: 18046487]
29. Lebedeva IV, Washington I, Sarkar D, Clark JA, Fine RL, Dent P, Curiel DT, Turro NJ, Fisher PB. Strategy for reversing resistance to a single anticancer agent in human prostate and pancreatic carcinomas. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:3484–3489. [PubMed: 17360670]
30. Moore CM I, Hoh M, Bown SG, Emberton M. Does photodynamic therapy have the necessary attributes to become a future treatment for organ-confined prostate cancer? *Bju International*. 2005; 96:754–758. [PubMed: 16153193]
31. Huang Z, Chen Q, Dole KC, Barqawi AB, Chen YK, Blanc DQ, Wilson BC, Hetzel FW. The effect of Tookad-mediated photodynamic ablation of the prostate gland on adjacent tissues - in vivo study in a canine model. *Photochemical & Photobiological Sciences*. 2007; 6:1318–1324. [PubMed: 18046488]
32. Bilski P, Chignell CF. Optimization of a pulse laser spectrometer for the measurement of the kinetics of singlet oxygen $O_2(\Delta(1)(g))$ decay in solution. *Journal of Biochemical and Biophysical Methods*. 1996; 33:73–80. [PubMed: 8951528]
33. Schmidt R, Tanielian C, Dunsbach R, Wolff C. Phenalenone, a universal reference compound for the determination of quantum yields of singlet oxygen $O_2(1-\Delta-G)$ sensitization. *Journal of Photochemistry and Photobiology a-Chemistry*. 1994; 79:11–17.
34. Melhuish WH. Quantum Efficiencies of Fluorescence of Organic Substances - Effect of Solvent and Concentration of Fluorescent Solute. *Journal of Physical Chemistry*. 1961; 65:229.
35. Bilski P, Holt RN, Chignell CF. Properties of singlet molecular oxygen $O_2((1)\Delta(g))$ in binary solvent mixtures of different polarity and proticity. *Journal of Photochemistry and Photobiology a-Chemistry*. 1997; 109:243–249.
36. Redmond RW I, Kochevar E. Spatially resolved cellular responses to singlet oxygen. *Photochemistry and Photobiology*. 2006; 82:1178–1186. [PubMed: 16740059]
37. Gorman AA, Rodgers MAJ. Current Perspectives of Singlet Oxygen Detection in Biological Environments. *Journal of Photochemistry and Photobiology B-Biology*. 1992; 14:159–176.
38. Wielgus AR, Chignell CF, Miller DS, Van Houten B, Meyer J, Hu DN, Roberts JE. Phototoxicity in human retinal pigment epithelial cells promoted by hypericin, a component of St. John's wort. *Photochemistry and Photobiology*. 2007; 83:706–713. [PubMed: 17576381]
39. Saczko J, Mazurkiewicz M, Chwilowska A, Kulbacka J, Kramer G, Lugowski M, Sniatura M, Banas T. Intracellular distribution of Photofrin (R) in malignant and normal endothelial cell lines. *Folia Biologica*. 2007; 53:7–12. [PubMed: 17328837]
40. Farruggia G, Iotti S, Prodi L, Montalti M, Zaccheroni N, Savage PB, Trapani V, Sale P, Wolf FL. 8-Hydroxyquinoline derivatives as fluorescent sensors for magnesium in living cells. *Journal of the American Chemical Society*. 2006; 128:344–350. [PubMed: 16390164]

41. He YY, Council SE, Feng L, Bonini MG, Chignell CF. Spatial distribution of protein damage by singlet oxygen in keratinocytes. *Photochemistry and Photobiology*. 2008; 84:69–74. [PubMed: 18173704]
42. Bilski P, Kukielczak BM, Chignell CF. Photoproduction and direct spectral detection of singlet molecular oxygen ($O_2(^1\Delta_g)$) in keratinocytes stained with rose bengal. *Photochemistry and Photobiology*. 1998; 68:675–678. [PubMed: 9825697]
43. Bilski P, Dabestani R, Chignell CF. Influence of Cationic Surfactant on the Photoprocesses of Eosin and Rose-Bengal in Aqueous-Solution. *Journal of Physical Chemistry*. 1991; 95:5784–5791.
44. Bilski P, Holt RN, Chignell CF. Premicellar aggregates of Rose Bengal with cationic and zwitterionic surfactants. *Journal of Photochemistry and Photobiology a-Chemistry*. 1997; 110:67–74.
45. Bilski P, Dabestani R, Chignell CF. Photoprocesses of Eosin and Rose-Bengal Ion-Pairs with Cationic Surfactant in Nonpolar-Solvents - Application in Photosensitization Studies. *Journal of Photochemistry and Photobiology a-Chemistry*. 1994; 79:121–130.

Figure 1.

Fluorescence (A, C) and absorption (B, D) spectra of TDPQ (100 μ M), ETPQ (100 μ M), C151 (992 μ M) in CH₃CN (A, B) and water (C, D), and QS (200 μ M) in 1N H₂SO₄ (A, B). The fluorescence spectrum of C151 in CH₃CN has been multiplied 10 $\times$ for clarity of presentation on the same axes (A). The fluorescence spectra (measured in a 0.5cm path length fluorescence suprasil cell) were corrected and normalized to the same number of absorbed photons. Fluorescence excitation wavelength (λ_{ext}) shown by scattered light is also indicated. TDPQ, solid black line; ETPQ; dashed red line; C151, solid blue line; QS, dotted green line

Figure 2. Fluorescence (A) and absorption (B) spectra of TDPQ (100 μ M), ETPQ (100 μ M), and C151 (992 μ M) in SDS micelles (5 mM). Complete description and symbols are as shown in Fig. 1.

Figure 3.

Fluorescence (A, C) and absorption (B, D) spectra of TDPQ (100 μ M), ETPQ (100 μ M), and C151 (992 μ M) in H₂O in the presence of calf thymus DNA (0.5 mg/ml) (A, B), and bovine albumin (0.5 mg/ml) (C, D). Complete description and symbols are as shown in Fig. 1.

Figure 4.

$^1\text{O}_2$ phosphorescence spectra photosensitized at 366 nm by TDPQ and perinaphthenone in CH_3CN and by TDPQ in D_2O (baseline). The spectrum produced by TDPQ in CH_3CN was multiplied 10 $\times$ for clarity of presentation on the same axes. The quantum yields for $^1\text{O}_2$ produced by both TDPQ and ETPQ are also indicated. TDPQ, solid black line; perinaphthenone, solid blue line.

Figure 5. Phase contrast (A, B) and TDPQ confocal fluorescence images (C, D) of AR-negative MDA-MB-231 (left panel) and AR-positive MDA-MB-453 (right panel) human breast cancer cells. Cells were incubated with 100 nM TDPQ for 1 hr at 37 C and subjected to detection of TDPQ fluorescence by using two-photon absorption LSCM. Scale bar, 20 μ m.

Figure 6. Detection of AR and TDPQ/light-induced cell death in AR-negative MDA-MB-231 (left panels) and AR-positive MDA-MB-453 (right panels) human breast cancer cells. A, B) Immunocytochemical detection of AR in formalin-fixed, Triton X-100 permeabilized MDA-MB-231 and MDA-MB-453 cells. The cells were incubated with 100 nM TDPQ for 1 hr and subjected to AR immunocytochemistry using PG-21 primary antibody. C, D) Effect of TDPQ and light irradiation on MDA-MB-231 and MDA-MB-453 cell death. Cells were incubated with 300 nM TDPQ for 1 hr and irradiated with 12 J/mm² at 405 nm. Six-hours later, nuclear dyes (PI and Hoechst 33342) were added and images were captured using conventional fluorescence microscopy equipped with appropriate excitation and emission filters for PI (red) or Hoechst 33342 (blue), respectively. Nuclei of all cells are stained blue, while only nuclei of compromised cells undergoing death are stained red. Circles indicate the size of irradiated area. No cell death was observed in non-irradiated cells outside the circled regions. Scale bar for A-B, 20 μ m; for C, D, 400 μ m.

Scheme 1.

TDPQ: 1,2,3,4-tetrahydro-2,2-dimethyl-6-(trifluoromethyl)-8-pyridono[5,6-*g*]quinoline

ETPQ: 4-ethyl-1,2,3,4-tetrahydro-6-(trifluoromethyl)-8-pyridino[5,6-*g*]quinoline

C151: 7-hydroxy-4-(trifluoromethyl)-2(1H)-quinolinone (Carbostyryl 151)

*The asterisk in the ETPQ structure denotes an optically active carbon center.

Table 1

Spectral properties of TDPQ, ETPQ and C151. Absorption maxima ($A_{\max}$) and their amplitudes (Abs); fluorescence maxima ($F_{\max}$), fluorescence lifetimes (τ) with corresponding contributions (C1, C2), and fluorescence quantum yields (ϕ_f) for TDPQ (100 μ M), ETPQ (100 μ M) and C151 (992 μ M) in different solvents.

Compound	$A_{\max}$ (nm)	Abs	$F_{\max}$ (nm)	τ_1 (ns) [C1]	τ_2 (ns) [C2]	ϕ_f
CH₃CN						
TDPQ	382	0.84	460	1.76 [13%]	4.21 [87%]	0.798
ETPQ	382	1.04	462	2.07 [21%]	4.60 [79%]	0.758
C151	338	0.79	391	0.21 [100%]		0.020
H₂O						
TDPQ	382	0.45	494		5.0 [100%]	0.734
ETPQ	396, 422	0.51, 0.45	491	0.3 [36%]	5.0 [64%]	0.067
C151	336	1.45	494	0.02 [8%]	3.5 [92%]	0.101
QS	348	0.77	452	8.14 [11%]	21.1 [89%]	0.546
H₂O containing SDS (5 mM)						
TDPQ	390	0.54	484	2.52 [23%]	5.23 [77%]	0.609
ETPQ	394	0.59	484	2.27 [15%]	5.39 [85%]	0.368
C151	338	1.22	494	2.51 [8%]	4.00 [92%]	0.123
H₂O containing DNA (calf thymus, 0.5mg/mL)						
TDPQ	382	0.52	496		5.1 [100%]	0.639
ETPQ	398, 422	0.51, 0.46	492	0.3 [33%]	5.0 [67%]	0.072
C151	350	0.98	491		3.8 [100%]	0.160
H₂O containing albumin (bovine, 0.5mg/mL)						
TDPQ	382	0.51	494		5.2 [100%]	0.769
ETPQ	396, 422	0.59, 0.48	486	0.3 [21%]	5.8 [79%]	0.101
C151	344	1.26	494		4.3 [100%]	0.129

* Quantum yields in acetonitrile were not corrected for refractive indices (very small and often negligible adjustment). Fluorescence spectra were normalized to the same number of absorbed photons using the Lambert-Beer law. All solutions, except QS, contained 1% v/v of DMSO used to prepare stock solutions of TDPQ, ETPQ and C151. Time-resolved fluorescence lifetimes were measured using a NanoLed pulse diode emitting at 372nm. Quinine (hi)sulfate (QS, 200 μ M, 1N H₂SO₄) was used as a fluorescence standard for ϕ_f calculations.