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Synthesis, photochemical and *in vitro* cytotoxic evaluation of benzoselenazole-based aminosquaraines

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Squaraine dyes have recently attracted interest as potential second generation photosensitizers for photodynamic therapy. Several cationic aminosquaraine dyes bearing benzoselenazole terminal nuclei were synthesized and their cytotoxic activity was tested against four different human tumor cell lines – breast (MCF-7), non-small cell lung (NCI-H460), cervical (HeLa) and hepatocellular (HepG2) carcinomas – and against a non-tumor porcine liver primary cell line (PLP2), both in the absence of light and under irradiation. All dyes, which displayed strong absorption within the phototherapeutic window, were found to exhibit photodynamic activity and were shown to be, in most cases, more cytotoxic, both in the dark and upon irradiation, than their benzothiazole analogues.

Introduction

Squaraines are a class of dyes with a resonance-stabilized zwitterionic structure, containing an electron-deficient central four-membered ring derived from squaric acid (3,4-dihydroxy-1,2-dioxocyclobut-3-ene) connected to two electron-donating groups, and can be considered donor–acceptor–donor charge transfer chromophores.[1] Although being zwitterionic in nature, because of their polymethine-like structure squaraines have been sometimes classified as cyanines.[2]

Squaraine dyes usually display sharp and intense absorption ($\varepsilon > 10^5$ L mol^{-1} cm^{-1}) in the red and near infrared regions of the electromagnetic spectrum and good photochemical stability.[3] The absorption and emission properties associated with the intramolecular charge transfer (ICT) transitions in these dyes make them highly suitable for photosensitization purposes.[4] For these reasons, along with the development of versatile routes of synthesis, squaraines have attracted a lot of attention and have found wide technologic applications, mostly in the domain of photonics.[5,6] Lately, there is a rapidly increasing interest regarding this class of dyes for biomedical applications, namely as sensitizers for Photodynamic Therapy (PDT).[7,8]

PDT is an emerging therapeutic procedure, minimally invasive, for cancer and several non-oncological conditions,[9] which has already been granted regulatory approval in a number of countries worldwide.[10] Briefly, it is based on the use of a sensitizer that, following cellular uptake, is excited by light of an appropriate wavelength to an activated state that directly or indirectly interacts with ground-state oxygen giving rise to the production of reactive oxygen species (ROS), which ultimately trigger cell death.[11] It is generally accepted that among the different ROS that can be generated in these processes, singlet oxygen is the chief cytotoxic species responsible for the photodynamic effect.[12] PDT is a very selective therapeutic modality, with several advantages compared to conventional tumor therapies;[13,14] for the photodynamic event to occur the coexistence of sensitizer, oxygen and light is mandatory, which individually are innocuous.

Photofrin®, a mixture of hematoporphyrin derivatives, was the first photosensitizer to receive regulatory approval in diverse countries and it is the most used PDT sensitizer for cancer therapy.[15] Photofrin® has several disadvantages, including chemical inhomogeneity, prolonged skin photosensitivity and weak absorption of light in the phototherapeutic window (600–800 nm).[16] These drawbacks triggered the search for new molecules closer to an ideal sensitizer.

The interest in squaraine dyes as potential sensitizers for PDT emerged in 1997 when Ramaiah *et al.* studied the singlet

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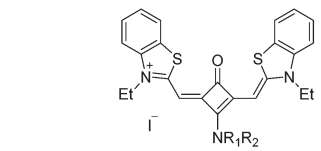
oxygen generation ability of some halogenated squaraines derived from phloroglucinol.[17] Since then a considerable number of different squaraines have been designed and synthesized envisioning their use as PDT photosensitizers.[7,18] The structural modification of the squaraine skeleton has been focused essentially on the variation of the nature and substitution of the (hetero)aromatic terminal groups.[19] Contrastingly, the functionalization of the squaric core, which has been the center of our research in squaraine chemistry,[20–23] has received much less attention. Even though a significant number of different squaraine compounds have been tailored to be used as PDT photosensitizers, both the *in vitro* and *in vivo* assessment of their photodynamic action have been poorly explored.[24–37]

Aminosquaraines, a particular class of cationic squaraine dyes bearing an amino group replacing one of the oxygen atoms of the central squaric ring, have previously shown to be able to produce singlet oxygen in variable quantum yields depending on the nature of the amino substituents and the degree of halogenation of both the terminal nuclei and the amino group.[21,23,38,39] The ability of aminosquaraines to generate singlet oxygen is generally much superior to that of the non-functionalized zwiterionic analogues[39] and is thought to arise from a higher degree of rigidification of the dye's structure due to the presence of a bulkier group and to possible intramolecular hydrogen bonding. In fact, rigidification can diminish non-radiative decay by photoisomerization and, ultimately, enhance the singlet oxygen quantum yield. Besides increasing singlet oxygen production, the amino group bathochromically shifts the dye's absorption deeper inside the phototherapeutic window, where light scattering and absorption by endogenous biological molecules is weaker, and may impart other advantages to the dye, namely to potentially increase cellular uptake, benefiting from the cell's membrane potential, and to favor interaction with the biological medium.

Recently, we assessed the photocytotoxicity of several symmetrical aminosquaraine dyes derived from benzothiazole (Fig. 1) and found that they exhibited photodynamic activity within the phototherapeutic window against several human cancer cell lines.[40] Once the substitution of sulfur by a heavier atom of selenium in aminosquaraines was shown to improve singlet oxygen formation through the internal heavy atom effect,[23,39] in this work we have replicated the aforementioned study with aminosquaraine analogues derived from benzoselenazole.



$R_1 = R_2 = H$; $R_1 = H$, $R_2 = CH_2CH_2OH$; $R_1 = R_2 = CH_2CH_2OH$;
$R_1 = H$, $R_2 = CH_3$; $R_1 = CH_3$, $R_2 = CH_2CH_2OH$

Fig. 1 Benzothiazole-derived aminosquaraines previously studied.[40]

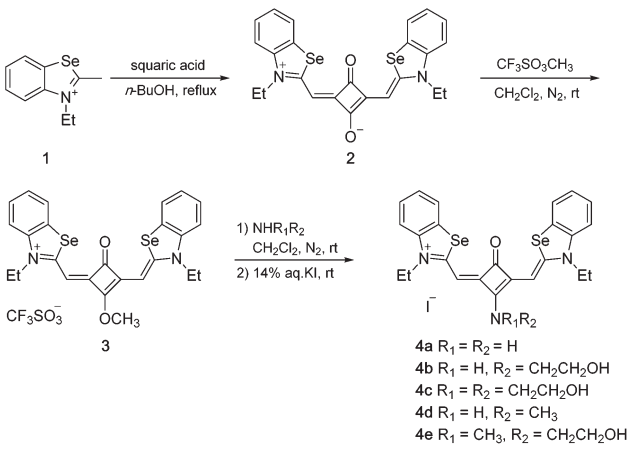
Results and discussion

Molecular design

In the strategy of designing suitable aminosquaraines the choice of the substituents was driven by the attempt of modulating three important features: (i) the molecule's rigidity, which may influence the capacity of singlet oxygen generation; (ii) the dye's absorption, determined by the electron-donating ability of the auxochrome; and (iii) the capacity for hydrogen bonding, which, besides influencing the molecule's rigidity, may increase its compatibility with the biological medium, and provide a potential means for bonding the dye to other substrates. Thus, substituent groups capable of generating secondary or tertiary amines, some of which bearing hydroxyethyl arms able to provide a certain degree of spatial flexibility to the hydroxyl group, were chosen.

Synthesis of dyes

Aminosquaraines **4a–e** were synthesized following a synthetic route developed previously by some of us[20] (Scheme 1). Squaraine dye **2**, readily prepared by condensation of 2 molar equivalents of benzoselenazolium salt **1** with one equivalent of squaric acid in refluxing *n*-butanol/pyridine (52% yield), was methylated with methyl trifluoromethanessulfonate to produce the crucial *O*-methyl ether intermediate **3** (53% yield). Subsequent reaction of the latter with different appropriate amines furnished the triflate analogues of **4a–e** through nucleophilic displacement of the methoxy group. Finally, treatment with excess 14% aqueous KI in MeOH resulted in the desired aminosquaraines bearing the iodide counter-ion in reasonable yields (**4a**: 36%; **4b**: 49%; **4c**: 22%; **4d**: 46%; **4e**: 60%). The different nucleophilicities of the substituting amines most probably play a role in the variability observed in the obtained yields. Moreover, some of the amines are in fact bidentate nucleophiles, which, despite nitrogen being a better nucleophile than oxygen, may contribute to the formation of undesirable by-products therefore decreasing the reaction yields.



Scheme 1 Synthesis of aminosquaraine dyes **4a–e**.

This counter-ion exchange was carried out to potentially enhance the production of singlet oxygen through the so-called external heavy atom effect.[41]

UV-Visible absorption spectroscopy

All the dyes synthesized exhibited sharp and intense absorption ($\varepsilon > 10^5$ M^{-1} cm^{-1}) close to the near-infrared region, within the phototherapeutic window (Table 1). Substitution of one of the oxygen atoms of the squaric ring of the core of the starting zwitterionic squaraine **2** by an amine group shifts the absorption of the resulting aminosquaraine dye **4** to longer wavelengths, except for **4a** which exhibits the same value of λ_{max}. The observed bathochromic shifts, ranging from 10 to 20 nm, depend on the nature of the amino substituent, increasing as the electron donating properties of the auxochrome increases.

Singlet oxygen quantum yields

Dye **4a** displays the highest quantum yield when compared to the other squaraine dyes (Φ_Δ = 0.29) (Table 1). As previously reported[23] the amino group in the squaric ring enhances the singlet oxygen generation efficiency when compared with the unsubstituted zwitterionic dye **2**. The results for Φ_Δ are strongly dependent on the substituents of the amine group: in general, the highest Φ_Δ values occur for the smallest substituents, and the lowest Φ_Δ values were determined for the hydroxyethylamino and the dihydroxyethylamino substituents (dyes **4e** and **4c**). This is related with the ICT mechanism, which provides an additional non-radiative deactivation mechanism, therefore decreasing the singlet oxygen formation quantum yields. Smaller substituents in the amine group decrease the relative importance of the ICT mechanism, therefore decreasing the importance of this non-radiative de-excitation pathway (which competes with the intersystem crossing process of deactivation of the first singlet excited state), and, in this way, it reinforces the Φ_Δ values.

An interesting case of acidic activation of phthalocyanines as photosensitizers was reported.[42] The amino moieties of an amine-modified phthalocyanine quench the singlet excited state of the compound by a photoinduced electron transfer mechanism (PET). Therefore, in acidic media, by quaternization of the amine group, the PET mechanism is prevented and both ϕ_F and the generation of singlet oxygen are increased.[42] In our squaraines, however, all molecules bear the amine group which is not quaternized and probably the dominant mechanism is the rotation of the amino relative to the squaraine body, ruled by the steric hindrance of the amine substituents.

As expected, the substitution of the triflate ion by iodine in the final aminosquaraines effectively enhanced the singlet oxygen generation ability of the dyes, increasing Φ_Δ, in some cases significantly.

Table 1 Visible spectra data and singlet oxygen quantum yields for squaraine dyes **2** and **4a–e**

Dye	R^1	R^2	λ_{max}[a] (nm) ($\log \varepsilon$)	Φ_Δ[b]
2	—	—	665 (5.49)[c]	0.10[d]
4a	H	H	665 (5.32)	0.29 (0.13)[d]
4b	H	$(CH_2)_2OH$	675 (5.31)	0.19 (0.17)[e]
4c	$(CH_2)_2OH$	$(CH_2)_2OH$	684 (5.40)	0.16 (0.08)[e]
4d	H	CH_3	676 (5.17)	0.22 (0.13)[d]
4e	CH_3	$(CH_2)_2OH$	685 (5.17)	0.10 (0.04)

[a] Measured in $MeOH/CH_2Cl_2$ (99/1). [b] Measured in $CHCl_3$. Values in parentheses correspond to Φ_Δ values of the analogues of **4a–e** bearing the triflate counter-ion. [c] From ref. 20. [d] From ref. 39. [e] From ref. 23.

Biological activity

The cytotoxicity of the aminosquaraine dyes **4a–e** was assessed against four selected human tumor cell lines: breast (MCF-7), non-small cell lung (NCI-H460), cervical (HeLa) and hepatocellular (HepG2) carcinomas, both in the dark and under irradiation. The (photo)cytotoxicity of the dyes was also evaluated against a non-tumor porcine liver primary cell culture (PLP2).

All dyes were found to exhibit photodynamic activity, inhibiting cellular growth upon exposure to light in greater extension than in the absence of it. Fig. 2 shows the growth inhibition displayed by 0.1 μM solutions of dyes **4a–e** against the human tumor cell lines studied, in the dark and upon irradiation. This concentration was selected as a representative example to allow a direct comparison with the benzothiazole-derived analogues previously assessed against the same cell lines.[40] The solubility of the synthesised compounds in water is low and for that reason the biological assays were performed in solutions containing 3% DMSO. The lack of toxicity of both DMSO and light, individually and combined, was confirmed by carrying out appropriate controls under dark and illuminated conditions in the absence of dyes.

In general, the growth inhibition of HepG2 and NCI-H460 cells seemed to be somewhat more independent of the nature of the sensitizer, ranging from 49.1 to 71.8% and from 27.4 to 53.7%, respectively, than the growth inhibition of the HeLa and MCF-7 cells for which the inhibitory action was more diverse, going from 31.4 to 64.8% and from 27.7 to 83.7%, respectively. In most cases, the difference between the growth inhibition promoted by the tested dyes in the dark and upon irradiation is smaller than that exhibited by the parent benzothiazole-based squaraines previously studied.[40] An exception is the inhibitory activity of compound **4e** against HeLa and MCF-7 cell lines, for which that difference is superior.

The squaraine dyes prepared in this work were shown to be, for the most part, more (photo)cytotoxic than their benzothiazole analogues, presenting much lower GI_{50} values (Table 2), in some cases considerably (up to 90 times), except for dye **4d** for which the values are similar.

Contrary to the aminosquaraines derived from benzothiazole, all dyes evaluated herein showed inhibitory activity in the dark within the range of the tested concentrations, which is also indicative of their higher cytotoxicity. The greater ability of dyes **4a–e** for singlet oxygen generation[23,39] is most likely to play a role in the difference of photocytotoxicity observed

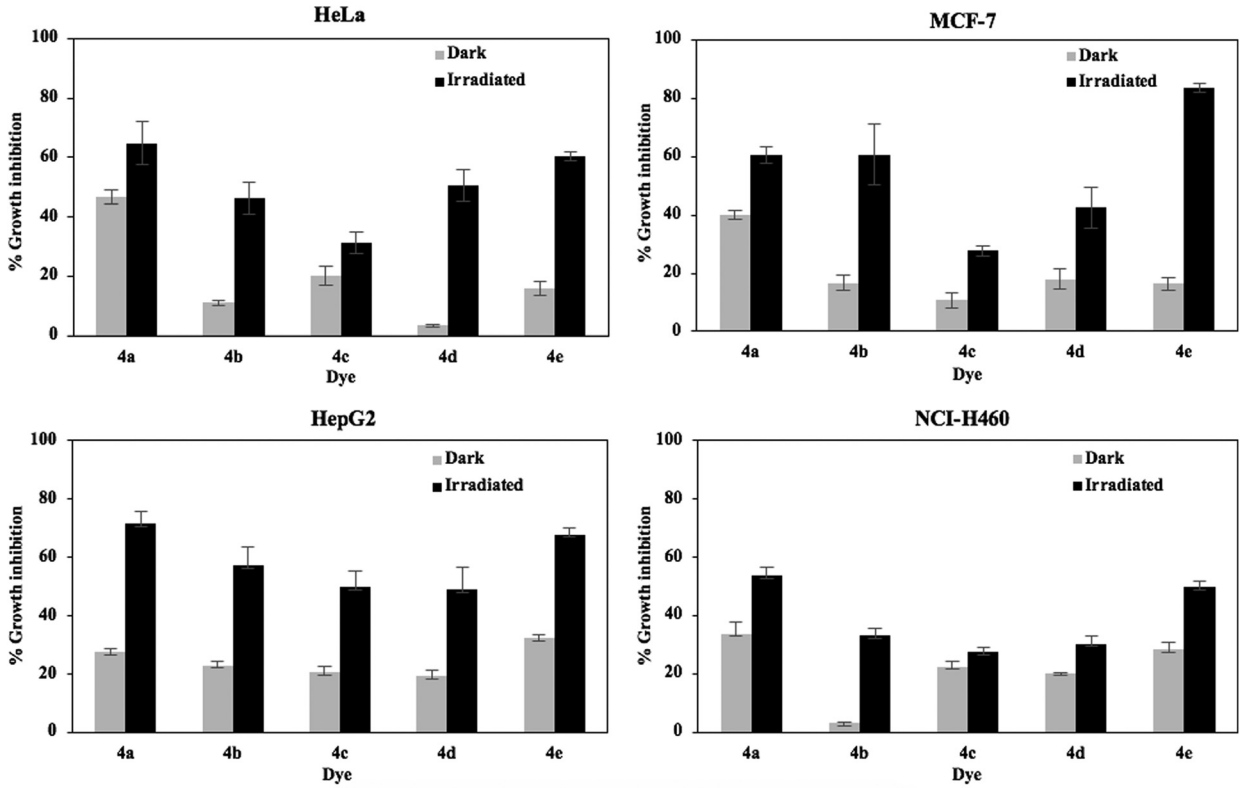


Fig. 2 Growth inhibition of HeLa, MCF-7, HepG2 and NCI-H460 cell lines upon treatment with squaraines **4a–e** (0.1 μM) in the dark and after irradiation. Results are presented as mean values ± standard deviation (SD).

Table 2 Cytotoxicity (GI_{50} values, nM) of squaraines **4a–e** (mean ± SD)

Compound	Condition	HeLa	MCF-7	HepG2	NCI-H460	PLP2
4a	Dark	178 ± 15	628 ± 61	1289 ± 67	1736 ± 132	846 ± 67
	Irradiated	51 ± 9	39 ± 4	48 ± 4	92 ± 5	86 ± 10
	t-Student's test *p*-value	<0.001	<0.001	<0.001	<0.001	<0.001
4b	Dark	957 ± 81	396 ± 34	691 ± 24	5297 ± 407	1283 ± 48
	Irradiated	155 ± 8	78 ± 8	72± 8	327 ± 23	227 ± 31
	t-Student's test *p*-value	<0.001	<0.001	<0.001	<0.001	<0.001
4c	Dark	3823 ± 357	408 ± 24	991 ± 89	795 ± 22	689 ± 18
	Irradiated	308 ± 9	254 ± 21	124 ± 7	448 ± 38	371 ± 21
	t-Student's test *p*-value	<0.001	<0.001	<0.001	<0.001	<0.001
4d	Dark	4808 ± 435	497 ± 48	847 ± 46	564 ± 32	475 ± 46
	Irradiated	125 ± 6	167 ± 16	132 ± 8	355 ± 29	315 ± 15
	t-Student's test *p*-value	<0.001	<0.001	<0.001	<0.001	<0.001
4e	Dark	736 ± 31	362 ± 7	499 ± 46	413 ± 48	318 ± 40
	Irradiated	79 ± 1	52 ± 1	36 ± 1	108 ± 10	75 ± 4
	t-Student's test *p*-value	<0.001	<0.001	<0.001	<0.001	<0.001

amongst the two group of dyes. Compounds **4a** and **4e** displayed the lowest GI_{50} values amongst all the dyes tested, which were identical for all the cell lines. However, the GI_{50} values for the PLP2 cell line were also very small and identical to those obtained for the tumor cell lines. Comparing the GI_{50} values obtained in the absence of light and under irradiation, the most promising compounds seem to be **4c** and **4d**, for the HeLa cell line, and **4b** for the NCI-H460 cell line, the GI_{50} values determined in the dark being from 12 to 27 times higher than those under illumination. It is worthwhile to note that, as observed for the benzothiazole analogues of **4a–e**, the GI_{50} values found for the NCI-H460 cell line were only somewhat higher than those found for the non-tumor PLP2 cell line, whatever the dye used. Notwithstanding that squaraines **4a–e** also present (photo)cytotoxicity for the PLP2 cells, the locoregional nature of the photodynamic event may circumvent, at least partially, the undesirable toxicity observed for the non-tumor cells.

Experimental

Chemistry

All reagents were obtained commercially and used as received. Solvents were of analytical grade. Anhydrous solvents were dried[43] and freshly distilled. All reactions were monitored using TLC using 0.20 mm aluminum-backed silica-gel plates (Macherey-Nagel SIL G UV_{254}). Melting points were measured in a melting-point apparatus equipped with a binocular microscope (Rotoquímica) and are uncorrected. IR spectra were recorded on a Unicam Research Series FT-IR spectrophotometer; ν_{max} in cm^{-1}. Vis spectra were performed on a PerkinElmer Lambda 25 instrument; λ_{max} in nm. ^1H and ^{13}C NMR spectra were recorded on a Brücker ARX 400 spectrometer; δ in ppm relative to residual solvent signals, J in Hz. High resolution electrospray ionization time-of-flight mass spectra (HRMS ESI-TOF) were determined on a VG AutoSpec M spectrometer. Benzothiazolium salt **1**,[44] squaraine dye **2** and the corresponding *O*-methyl derivative **3**[20] and the aminosquaraine dyes **4a–d**[20,23,39] were prepared as previously described.

Synthesis of 3-ethyl-2-{3-(3-ethyl-3*H*-benzoselenazol-2-ylidenemethyl)-2-[(2-hidroxyethyl)methylamino]-4-oxocyclobut-2-enylidenemethyl}benzoselenazol-3-ium iodide (4e)

To a solution of *O*-methylsquaraine dye **3** (0.15 g, 2.2 mmol) in anhydrous CH_2Cl_2 (75 mL), under a N_2 atmosphere, was added an excess of 2-(methylamino)ethanol (0.044 mL, 0.054 mmol). The reaction mixture was stirred at r.t. for 3 h and the resulting solid was collected by filtration under reduced pressure and washed thoroughly with water and Et_2O. The obtained solid was dissolved in MeOH and to this solution was added an approximately equal volume of 14% aqueous KI. After about 1 h, the precipitated dye was collected by filtration under reduced pressure, washed with water and Et_2O. Yield: 60%. M.p. 284 °C (dec.). UV/Vis (MeOH/CH_2Cl_2, 99/1) λ_{max} (log ε): 685 nm (5.17). IR (KBr) ν_{max}: 762 (m), 1006 (m), 1030 (w), 1057 (w), 1111 (s), 1160 (m), 1221 (s), 1309 (m), 1352 (m), 1415 (s), 1455 (m), 1514 (w), 1613 (m), 1709 (m), 2889 (w), 2995 (w), 3469 (m). ^1H-NMR (400.13 MHz, DMSO-d_6) δ: 1.28 (6H, brs, NCH_2CH_3), 3.43 (3H, s, NCH_3), 3.67 (2H, brs, NCH_2CH_2OH or NCH_2CH_2OH), 3.73 (2H, brs, NCH_2CH_2OH or NCH_2CH_2OH), 4.35 (4H, brs, NCH_2CH_3), 5.16 (1H, brs, NCH_2CH_2OH), 6.17 (2H, s, CH=C), 7.26 (2H, brs, ArH), 7.45 (2H, brs, ArH), 7.59 (2H, J = 7.7, d, ArH), 8.01 (2H, J = 7.0, d, ArH). ^{13}C-NMR (100.62 MHz, DMSO-d_6) δ: 12.1, 12.2, 41.9, 42.1, 55.7, 58.4, 91.0, 91.9, 114.4, 124.5, 124.9, 127.6, 129.0, 141.7, 155.4, 156.1, 162.9, 163.0, 163.8, 175.2. HRMS (ESI-TOF) m/z: 586.05247 ($[M - I]^+$, calc. for $C_{27}H_{28}N_3O_2Se_2$: 586.05114).

Photochemistry

Singlet oxygen formation quantum yields. The singlet oxygen measurement set-up was assembled in our laboratory. As an excitation source a N_2 laser (PTI model 2000, *ca.* 600 ps FWHM, ~1.0 mJ per pulse) was used, the excitation wavelength being 337 nm. The detector was an InGaAs CCD (model i-Dus from Andor) working at low temperature (−60 °C) coupled to a fixed spectrograph (model Shamrock 163i from Andor).

The Φ_Δ values were obtained by comparing the total area of the emission spectra for the reference and for the sample under study in the same solvent, with the same optical density at the excitation wavelength. Phenazine (standard) was used at OD = 0.60 in $CHCl_3$.[45]

Biology

Fetal bovine serum (FBS), L-glutamine, Hank's Balanced Salt Solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U mL^{-1} and 100 mg mL^{-1}, respectively), RPMI-1640 and DMEM were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), Trypan Blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma Chemical Co. (Saint Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Preparation of the compound solutions. Stock solutions of each squaraine dye (0.2 μM, 2 μM, 10 μM, 20 μM and 200 μM) were prepared by dissolving the solid in 3% DMSO in DMEM and kept at −20 °C. Prior to the assays, appropriate dilutions were prepared with the same solvent to obtain solutions with final concentrations of 0.01 μM, 0.1 μM, 0.5 μM, 1 μM and 10 μM.

Photodynamic treatment. The cytotoxicity of the squaraine dyes was tested in the dark and under irradiation. For irradiation of the cells a halogen/tungsten lamp (24 V and 250 W, Osram, Portugal) was used with a fluence rate of 23–24 μW cm^{-2} (measured with an ILT 1400-A radiometer equipped with a SEL033 detector, ILT, USA). The cells were irradiated continuously for 30 min. A 3% aqueous solution of $K_2Cr_2O_7$ was placed between the lamp and the cells as a liquid cut off filter to remove light of wavelength shorter than ~500 nm. The temperature to which the cells were exposed was carefully monitored to guarantee cell viability.

Evaluation of cytotoxicity in human tumor cell lines. Four human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) from DSMZ (Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells per well for MCF-7 and NCI-H460, and 1.0×10^4 cells per well for HeLa and HepG2) in 96-well plates, allowed to attach for 24 h and then treated with different concentrations of each squaraine dye. Controls were set with the cells in the absence of the dyes in 3% DMSO in DMEM and in the growth medium only. After the incubation step (24 h), the cells were submitted to the photodynamic treatment for 30 minutes. Controls in the absence of light were performed on every test. Neither DMSO alone, nor light alone, nor the combination of both induced toxicity in the cells. Then, the

growth medium was changed and the cells were incubated for a further 24 h. Following this incubation period, the adherent cells were fixed by adding cold 10% TCA (100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and subsequently dried. SRB solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilized with 10 mM Tris (200 μL) and the absorbance was measured at 540 nm in an ELX800 Microplate Reader (Bio-Tek Instruments, Inc, Winooski, USA).[47] The results were expressed in GI_{50} values. Ellipticine was used as standard.

Evaluation of cytotoxicity in a porcine liver primary cell culture. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's Balanced Salt Solution containing 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin and divided into 1 × 1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two or three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0 × 10^4 cells per well, and cultivated in DMEM medium with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.[46] The PLP2 cells were incubated with the squaraine dyes and submitted to the two independent processes – dark and irradiation – as described earlier for the human tumor cell lines. The SRB assay was performed according to the procedure previously described. The results were expressed in GI_{50} values. Ellipticine was used as standard.

Statistical analysis

For all the experiments, three solutions were prepared from each compound's concentration, and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation. The results were analysed using a Student's *t*-test to determine the significant difference among the different samples, with $\alpha = 0.05$. This treatment was carried out using the SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

Conclusions

All aminosquaraine dyes **4a–e**, displaying strong absorption in the range 665–685 nm, were found to exhibit photodynamic activity against the human tumor cell lines tested (MCF-7, NCI-H460, HeLa, and HepG2), inhibiting cellular growth upon exposure to light in greater extension than in the absence of it, mostly with GI_{50} values less than 5 μM.

In general, the synthesized dyes were shown to be more (photo)cytotoxic than their benzothiazole analogues. Although the singlet oxygen generation ability of dyes **4a–e** is superior to that of the benzothiazole-derived analogues, the difference between the growth inhibition promoted by the tested dyes in the dark and upon irradiation is smaller than that observed for the parent benzothiazole-based aminosquaraines.

Contrary to the aminosquaraines derived from benzothiazole, all dyes evaluated herein showed inhibitory activity in the dark within the range of the tested concentrations, which is also indicative of their higher cytotoxicity.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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