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Aminosquaraines as potential photodynamic agents: Synthesis and evaluation of *in vitro* cytotoxicity

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ABSTRACT

The synthesis of several aminosquaraine cationic dyes displaying strong absorption within the so-called phototherapeutic window (650–850 nm) is described. Their cytotoxicity, under dark and illuminated conditions, was tested against several human tumor cell lines (breast, lung, cervical and hepatocellular carcinomas) and non-tumor porcine liver primary cells. All compounds showed to inhibit the growth of the tumor cells upon irradiation more than in the absence of light, in more or less extension, clearly exhibiting photodynamic activity. The photosensitizing ability against some cell lines, together with the low toxicity for the non-tumor primary PLP2 cells displayed by some of the compounds synthetized, turns them into potential candidates as photosensitizers for PDT.

Introduction

Photodynamic therapy (PDT) is a promising minimally invasive therapeutic technique that has been shown effective for cancer and other non-oncological conditions [1]. It is based on the use of a sensitizer that, after accumulating inside the abnormal cells, is excited by light of an appropriate wavelength and interacts with oxygen, leading to the production of reactive oxygen species (ROS) which induce cell death *via* oxidative stress mechanisms. Amongst those, singlet oxygen is generally considered to be the main cytotoxic agent responsible for the photodynamic effect [2]. The prerequisite of concurrence of this triad of components (photosensitizer, oxygen and light), which individually are innocuous, for the photodynamic process to take place turns PDT a particularly selective modality, with unique advantages compared to conventional tumor therapies such as surgery, chemotherapy and radiotherapy [3,4].

Photofrin®, a mixture of hematoporphyrin derivatives, was the first photosensitizer to be approved by the regulatory agencies and still the most extensively used for medical applications of PDT [5]. This so-called first generation sensitizer presents however several drawbacks, namely a lack of chemical homogeneity, prolonged coetaneous photosensitivity and a less than optimal light absorption profile ($\lambda = 630$ nm, $\varepsilon = 3200\ M^{-1}\ cm^{-1}$) [6], which entails the use of higher drug dosages, and limits the penetration depth of light into tissue.

The crucial role of the photosensitizer in the success of PDT turned the development of new sensitizers with better photophysical and photobiological properties into an active area of research in the two last decades and a lot of attention has been given to a number of different classes of compounds as potential so-called second generation sensitizers [5,7].

Squaraines, a class of functional dyes with sharp and intense visible to NIR absorption which have found wide application in the domain of photonics [8], have also attracted great interest as a new class of potential photosensitizers for PDT [9] since, in 1997, Ramaiah et al. [10] synthesized and investigated the photophysical properties, including the singlet oxygen generation ability, of some squaraines derived from phloroglucinol. As a result, a lot of work has been published on the structural variation of squaraines which, however, has been mainly focused on the variation of the nature and substitution of the heterocyclic terminal groups [11]. The derivatization of the central squaric core, on the other hand, has been poorly addressed and has been the focus of our research for some years [12–14].

Notwithstanding a considerable number of squaraine compounds has been designed and synthesized with the purpose of PDT sensitizers, the *in vitro* assessment of their photodynamic ability has been barely addressed [15–24]. The reports of *in vivo* evaluation are even scarcer [17,18,21,25–27].

In this work we synthesized several squaraine dyes substituted at the central four member ring with different amino groups and

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assessed their cytotoxicity, under dark and illuminated conditions, against several human tumor cell lines (breast, lung, cervical and hepatocellular carcinomas) and non-tumor porcine liver primary cells. These aminosquaraine dyes are envisioned to present some advantages over the common zwitterionic counterparts as their cationic character may positively influence cellular uptake taking advantage of the membrane potential, and the presence of amino groups, besides red-shifting the dye's absorption, may conveniently enhance solubility and interaction with the biological medium. To the best of our knowledge this is the first report on the *in vitro* photosensitizing ability of symmetrical aminosquaraine dyes.

Aminosquaraines **5** were synthesized from the zwitterionic dye **3** by an expeditious methodology developed earlier by some of us [12] (Scheme 1). The starting squaraine **3**, easily prepared by the condensation of two molar equivalents of 2-methylbenzothiazolium salt **2** with squaric acid, in refluxing *n*-butanol/pyridine, was methylated with methyl triflate to furnish the crucial *O*-methyl intermediate **4**. The later then undergoes nucleophilic substitution with appropriate amines to yield the triflate analogues of **5**. Each of the final compounds underwent counter-ion replacement by iodine, by treatment of a methanolic solution of the compound with excess 14% aqueous KI, in order to take benefit from the so-called external heavy atom effect on the potential enhancement of singlet oxygen generation [28].

All the synthesized dyes exhibit sharp and intense absorption ($\varepsilon > 10^4\ cm^{-1}\ M^{-1}$) in the red end of the visible spectrum, close to the bottom end of the phototherapeutic window (Table 1). In general, the amino-substituted dyes **5** display absorption at longer wavelengths than the starting non-substituted analogue **3**. The chromophore shows typical donor–acceptor characteristics with increasing bathochromic shift as the electron donating properties of the auxochrome increases. The observed bathochromic shifts ranged from 3 to 22 nm.

The cytotoxicity of the compounds synthesized was evaluated against four human tumor cell lines: breast (MCF-7), non-small cell lung (NCI-H460), cervical (HeLa) and hepatocellular (HepG2) carcinomas, and against a porcine liver primary cell culture (PLP2) established by some of us, both in the absence of light and under irradiation.

Table 1
Visible spectral data for squaraine dyes **3** and **5a–e**.

Dye	R^1	R^2	λ_{max}[a] (nm) (log ε)
3	–	–	650 (5.24)[b]
5a	H	H	648 (5.41)
5b	H	CH_2CH_2OH	657 (4.67)
5c	CH_2CH_2OH	CH_2CH_2OH	667 (5.21)
5d	H	CH_3	658 (5.23)[b]
5e	CH_3	CH_2CH_2OH	667 (5.19)

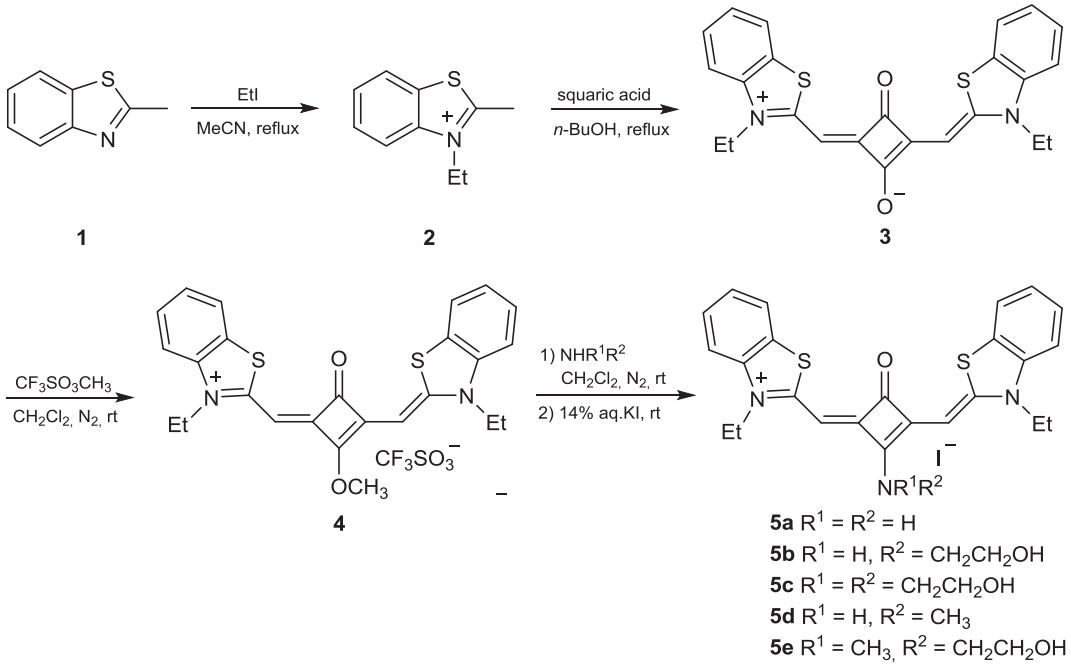
[a] Measured in $MeOH/CH_2Cl_2$ (99/1).
[b] From Ref.[12]

As a representative example, Fig. 1 shows the relative growth inhibition ability of dyes **5a–e** on the human tumor cell lines studied using 0,1 μM solutions of the dye, a concentration for which growth inhibition in the absence of light was less than 20% in all tests.

All compounds showed to inhibit the growth of the tumor cells upon irradiation more than in the dark, in more or less extension, clearly showing to display photocytotoxic effect. The more susceptible cell lines to phototoxicity were HeLa and MCF-7, for which the photodynamic treatment resulted in more significant differences in the growth inhibition of the cells in the dark and upon irradiation. Controls were set with the cells in the absence of the dyes in 3% DMSO in DMEM and in the growth medium only. The cells were independently submitted to the photodynamic treatment and maintained in the dark. Neither DMSO alone, nor light alone, nor the combination of both was found to induce toxicity on the cells.

Although the study of the exact nature of the photodynamic action mechanism of the synthesized dyes is out of the scope of this work, it is worthwhile to mention that the majority of the compounds assayed have shown singlet oxygen generation ability [14,29].

The GI_{50} values (compound concentration resulting in 50% of growth inhibition) determined for squaraine dyes **5a–e** are presented in Table 2.



Scheme 1. Synthesis of aminosquaraine dyes **5a–e**.

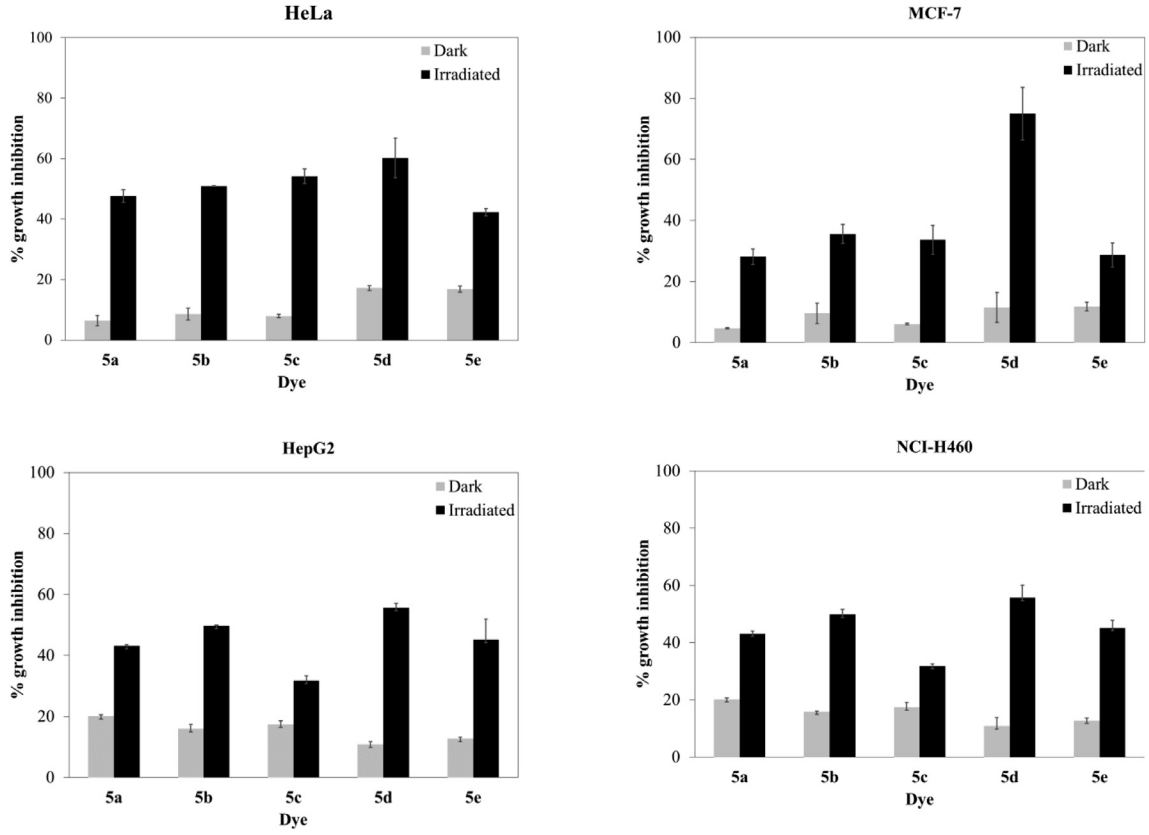


Fig. 1. Growth inhibition of MCF-7, HeLa, HepG2 and NCI-H460 cell lines upon treatment with dyes **5a**–**e** (0.1 μM) in the dark and after irradiation. Results are presented as mean values ± standard deviation (SD).

Table 2
Cytotoxicity (GI_{50} values, μM) of compounds **5a**–**e** (mean ± SD).

Compound	Condition	MCF-7	NCI-H460	HepG2	HeLa	PLP2
5a	Dark	>10	>10	4.571 ± 0.379	>10	>10
	Irradiated	0.918 ± 0.095	8.347 ± 0.363	0.665 ± 0.049	0.288 ± 0.017	4.981 ± 0.254
	t-Students test *p*-value	–	–	<0.001	–	–
5b	Dark	7.361 ± 0.764	8.820 ± 0.698	7.829 ± 0.694	>10	3.727 ± 0.579
	Irradiated	0.224 ± 0.015	0.440 ± 0.024	0.110 ± 0.011	0.097 ± 0.001	0.385 ± 0.006
	t-Students test *p*-value	<0.001	<0.001	<0.001	–	<0.001
5c	Dark	5.337 ± 0.413	0.940 ± 0.085	4.493 ± 0.391	>10	0.621 ± 0.005
	Irradiated	0.257 ± 0.026	0.404 ± 0.034	0.722 ± 0.077	0.088 ± 0.006	0.340 ± 0.017
	t-Students test *p*-value	<0.001	<0.001	<0.001	–	<0.001
5d	Dark	0.576 ± 0.043	0.507 ± 0.064	7.083 ± 0.767	2.223 ± 0.138	0.410 ± 0.050
	Irradiated	0.054 ± 0.004	0.171 ± 0.023	0.078 ± 0.005	0.071 ± 0.009	0.126 ± 0.048
	t-Students test *p*-value	<0.001	<0.001	<0.001	<0.001	0.001
5e	Dark	0.737 ± 0.039	0.767 ± 0.045	7.535 ± 0.818	2.636 ± 0.062	0.717 ± 0.115
	Irradiated	0.239 ± 0.034	0.455 ± 0.012	0.271 ± 0.014	0.273 ± 0.007	0.471 ± 0.044
	t-Students test *p*-value	<0.001	<0.001	<0.001	<0.001	0.008

Regarding the tests conducted in the absence of light, in the range of tested concentrations, squaraine **5a** only showed inhibitory activity against the HepG2 cell line, and yet with a relatively high GI_{50} value. The remaining compounds presented inhibitory activity for all the cell lines tested, though in variable extension, except dyes **5b** and **5c** which were inactive against the HeLa cell line in the range of the concentrations used.

Upon irradiation the GI_{50} values decreased, in some cases very appreciably, showing significant photodynamic effect of the dyes. For compounds **5a**–**d,** the lowest GI_{50} values were obtained consistently against the HeLa cell line, while for dye **5e** the lowest GI_{50} value was obtained against the MCF-7 cell line. The largest differences between the GI_{50} values obtained in the dark and upon irradiation, which are very relevant in terms of the usefulness of the photodynamic effect, were observed precisely for the HeLa cell line, with dyes **5a**–**c,** which were shown to be inactive against this cell line in the dark, in the range of the concentrations used. The HepG2 cell line turned out to be also very susceptible to the photodynamic treatment using dyes **5b**, **5d** and **5e**. Compound **5d** presented the lowest GI_{50} value of all, whatever the tested cell line; nevertheless, it was not the compound that presented the highest differences between the dark and irradiated assays.

The evaluation of the cytotoxicity for PLP2 (non-tumor cells) is very important since mammalian hepatocytes still represent a

mandatory step in the evaluation of toxic compounds that lead to the production of various metabolites, which are the ultimate cause of toxicity [30].

Apart from dye **5a** which, in the range of the tested concentrations, was inactive in the absence of light and presented a GI_{50} value of 4.981 ± 0.254 μM upon irradiation, all compounds showed also some cytotoxicity against PLP2 cells. Nevertheless, the GI_{50} values obtained for the PLP2 cells were consistently higher, either in the dark or under irradiation, than those obtained against the tumor cell lines. It is worth mentioning that the locoregional nature of the photodynamic effect can diminish or even surmount the importance of the cytotoxicity observed in the non-tumor cells.

In conclusion, the aminosquaraine dyes synthesized, displaying strong absorption within the phototerapeutic window, have shown to able to inhibit the growth of the human tumor cell lines tested (MCF-7, NCI-H460, HeLa, and HepG2) upon irradiation more than in the dark, in some cases very substantially, clearly showing to display photocytotoxic activity.

The differences observed for some of the dyes between the GI_{50} values obtained in the absence of light and upon irradiation, turn them into potential candidates as photosensitizers for PDT, in particular compound **5a** which showed the lowest toxicity for the non-tumor primary PLP2 cells.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.08.004.

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