Bioorganic & Medicinal Chemistry 25 (2017) 3803–3814

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis, spectroscopic characterization and biological evaluation of unsymmetrical aminosquarylium cyanine dyes

Sofia Friães [a,b], Amélia M. Silva [b,c,*], Renato E. Boto [d], Diana Ferreira [e], José R. Fernandes [f], Eliana B. Souto [g,h], Paulo Almeida [d], Luis F. Vieira Ferreira [e], Lucinda V. Reis [a,*]

[a] Department of Chemistry and CQ-VR, UTAD, Quinta de Prados, 5001-801 Vila Real, Portugal
[b] Centre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Quinta de Prados, 5001-801 Vila Real, Portugal
[c] Department of Biology and Environment, University of Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, 5001-801 Vila Real, Portugal
[d] CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6201-506 Covilhã, Portugal
[e] CQFM-Centro de Química-Física Molecular and IN-Institute of Nanoscience and Nanotechnology, Instituto Superior Técnico, Universidade Técnica de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
[f] INESC-TEC Porto and Department of Physics, University of Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, 5000-801 Vila Real, Portugal
[g] Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra (FFUC), Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal
[h] REQUIMTE/LAQV, Group of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra, 3000-548 Coimbra, Portugal

ARTICLE INFO

Article history:
Received 20 February 2017
Revised 7 May 2017
Accepted 10 May 2017
Available online 11 May 2017

Keywords:
Unsymmetrical aminosquarylium cyanine dyes
Singlet oxygen
Caco-2 cells
HepG2 cells
Cell viability
Photosensitizers

ABSTRACT

New unsymmetrical aminosquarylium cyanine dyes were synthesized and their potential as photosensitizers evaluated. New dyes, derived from benzothiazole and quinoline, were prepared by nucleophilic substitution of the corresponding *O*-methylated, the key intermediate that was obtained by methylation with $CF_3SO_3CH_3$ of the related zwitterionic unsymmetrical dye, with ammonia and methylamine, respectively. All three news dyes herein described displayed intense and narrow bands in the Vis/NIR region (693–714 nm) and their singlet oxygen formation quantum yields ranged from 0.03 to 0.05. *In vitro* toxicity, in Caco-2 and HepG2 cells, indicated that dark toxicity was absent for concentrations up to 5 μM (for the less active dye) or up to 1 μM (for the two more active dyes). The three dyes present potential as photosensitizers, differing in irradiation conditions and period of incubation in the presence of irradiated dye. The less active dye needs a longer irradiation period to exhibit phototoxicity which is only evident after longer period of contact with cells (24 h). However, the remaining two more active dyes produce higher phototoxicity, even at shorter incubation periods (1 h), with shorter irradiation time (7 min). Although in different extents, these dyes show promising *in vitro* results as photosensitizers.

1. Introduction

Squaraine dyes, also called squaraines, can be regarded as derivatives of cyanine dyes as they result from the introduction of a squaraine ring in the cyanine polymethine chain.[1] These dyes resulting from the condensation of one molar equivalent of squaric acid with two molar equivalents of aromatic or heterocyclic methylene bases,[2] exhibit special properties such as high photoconductivity, good photochemical stability, sharp and intense absorption in the visible and near infrared regions, high quantum yield and high molar absorption coefficient.[3–6] These properties make squaraine dyes very attractive for several photonic applications, such as optical recording media, organic solar cells and xerographic photoreceptors,[7,8] as well as for biological and biomedical applications, such as fluorescent markers and labels[9] and sensitizers for photodynamic therapy (PDT), also known as photosentizers.[5]

PDT is a promising treatment modality for several type of diseases, such as cancer, which requires the delivery of a photosensitising drug into the target tissue and/or cells, followed by exposure of the target region with light of appropriate wavelengths which gives rise to a cascade of photochemical and biochemical processes leading to destruction of the abnormal cells.[10] The use of PDT in cancer therapy is particularly interesting, mainly for skin cancer, because the photosensitizer (PS) can selectivity be applied through the malignant tissue. The light, used to activate the PS to induce

* Corresponding authors at: Department of Biology and Environment, School of Life and Environmental Sciences, University of Trás-os-Montes e Alto Douro (UTAD), Quinta de Prados, 5001-801 Vila Real, Portugal (A.M. Silva). Department of Chemistry and CQ-VR, School of Life and Environmental Sciences, University of Trás-os-Montes e Alto Douro (UTAD), Quinta de Prados, 5001-801 Vila Real, Portugal (L.V. Reis).

E-mail addresses: amsilva@utad.pt (A.M. Silva), lucinda.reis@utad.pt (L.V. Reis).

http://dx.doi.org/10.1016/j.bmc.2017.05.022
0968-0896/

the generation of reactive oxygen species (ROS), is then directly focused on the lesion, enabling the destruction of tumour tissue while preserving the healthy one.[11,12] Also, new technologies that ensure the targeted delivery of PS to the tumour tissue, followed by appropriate irradiation, seem to ensure enhanced selectivity and induce less side effects to the patients.[12,13] The effectiveness of PDT depends however on several factors, such as the PS location, irradiation conditions and tissue oxygenation.[14] These factors, altogether, determine the mechanism of cell death that will be induced, *i.e.* apoptosis, necrosis or autophagy.[14–16] However, it is commonly accepted that singlet oxygen is the main cytotoxic species responsible for the biological effects developed during PDT treatment resulting mainly in apoptosis.[5,17] Another important feature of PDT is that the light sources used for irradiation are non-ionizing which also reduces toxicity to tissues that did not incorporate the PS.[12]

The light source should present spectral characteristics that overlap with the maximum absorption wavelength of the used PS, in order to activate the PS and generate enough ROS to produce a cytotoxic effect.[18] For irradiation in PDT, lasers (pulsed or continuous) and incoherent light, such as intense pulsed light (IPL) or light-emitting diode (LED), have been used.[19] LED light sources have become increasingly popular, also for application in PDT, since they have a low cost, can be displayed in arrays that allow the irradiation of large areas and can emit a narrow spectrum of light that matches the absorption spectra of the PS used.[20] On the other hand, LEDs emit light with low-intensity without generating heat, thus no cooling system is required, and irradiation does not cause pain or other discomfort. It has been therefore accepted that this irradiation system consists in a simple alternative to more complicated and expensive PDT units.[21]

Ideally, PS for PDT should meet several characteristics, amongst which an inherent ability to produce reactive singlet oxygen and strong absorption ($>10^5$ M^{-1} cm^{-1}) at wavelengths between 600–850 nm (within the so called "phototherapeutic window"). Indeed, at wavelengths far into the red or near infrared regions, the scattering of light is minimal and penetration into tissues can achieve 5–6 mm depth.[5,22]

During the past decades, many significant advances have been made in the field of PS design. However, many of the sensitizer drawbacks used in the therapeutic procedure have not yet been surpassed. Thus, researchers are constantly seeking to design, synthesize, purify and to characterize new compounds that can be used as PS.[23]

As described in the literature, simple squarylium cyanine dyes, derived from benzothiazole, benzoselenazole and quinoline, have the ability to generate singlet oxygen and have a strong absorption in the "phototherapeutic window".[3,24] However, the potential application of these dyes as sensitizers for PDT has been poorly explored.[3,4,6,24] The aim of this work was the synthesis and photochemical characterization of new unsymmetrical aminosquarylium cyanine dyes derived from benzothiazole and quinoline, as well as to evaluate their phototherapeutic potential *in vitro*. Unsymmetrical aminosquarylium cyanine dyes were chosen for this work because these dyes have in their structure a unit of benzothiazole ring capable of increasing production of singlet oxygen and a moiety of quinoline that shift the maximum absorption wavelength to NIR region. To the best of our knowledge, this class of dyes is not mentioned in the literature.

In this work, we also report the dark toxicity of newly synthesized and characterized dyes, as well as their phototoxicity using HepG2 (human hepatocellular carcinoma cell line) and Caco-2 cells (human colorectal adenocarcinoma cell line) as *in vitro* models. These two cell lines were chosen as cell models by the following reasons: i) they are adherent epithelial cells, facilitating the exposure to the dyes and the washing steps preventing cell number loss which is relevant for cell viability assessment; ii) these cell models may permit data extrapolation concerning to liver toxicity (upon dye administration), and potential use of PDT to treat colon cancer.

2. Results and discussion

2.1. Chemistry

The new unsymmetrical aminosquarylium cyanine dyes **11** and **12**, having a benzothiazole and quinoline moieties, were synthesized by a multistep procedure as illustrated in Scheme 1. In the first stage, dibutyl squarate **2**, obtained by refluxing squaric acid (3,4-dihydroxycyclobut-3-en-1,2-dione) (**1**) in *n*-BuOH[25] was reacted with 3-hexyl-2-methylbenzothiazolium iodide (**4**), which was obtained by alkylation of the 2-methybenzothiazole (**3**) with an excess of 1-iodohexane, to obtain the monosubstituted intermediate **5**. Hydrolysis of this intermediate by a 40% sodium hydroxide aqueous solution and protonation of the resulting sodium salt with 2 M hydrochloride acid led to the key intermediate monosubstituted squaric acid **6**.[26] The zwitterionic unsymmetrical squarylium cyanine dye **9** was synthesized by base catalyzed condensation of **6** with the quinoline moiety quaternary salt **8**, in refluxing *n*-BuOH/pyridine. The methylation of the latter with the strong methylating agent $CF_3SO_3CH_3$ (3 eq.), in dry dichloromethane, provided the *O*-methyl derivative **10** in good yield (89%).

The desired unsymmetrical aminosquarylium cyanine dyes **11** and **12** were easily obtained by nucleophilic substitution of the methoxyl group in the four-membered ring of **10** with ammonia or methylamine, respectively. Finally, we proceeded with the counter-ion exchange, namely trifluoromethanesulfonate anion by iodide anion, upon treatment of a methanolic solution of each dye with 14% aqueous KI, with the purpose to favor the singlet to triplet state intersystem crossing, that is due to the well-known heavy atom effect.[6,27]

The synthesized squarylium cyanine dyes **9**–**12** showed narrow and strong absorption ($\varepsilon > 1–2 \times 10^5$ cm^{-1} M^{-1}) within the so-called "phototherapeutic window", in the visible and near-infrared region (λ_{max} 666–714 nm), and because of this property, they offer photosensitizing activity useful for PDT (Table 1). The zwitterionic compound **9** has almost the same λ_{max} as the symmetrical quinoline analogue reported in the literature.[3] The substituted dyes **10**–**12** displayed a shift to the blue regarding the corresponding non-substituted dye **9**, and the most pronounced effect was observed for *O*-methylated derivative **10**. This strong shift has been mentioned in the literature[28] for symmetrical dyes possessing the benzothiazole and quinoline moieties.

In order to create a more suitable LED system for irradiating the cells, Vis spectra of dyes **9**, **11** and **12**, were also recorded in DMEM (Dulbescco's Modified Eagle's Medium), the base medium used in cell culture (Fig. 1). In the latter solvent, spectra of all compounds displayed a significant hypsocromic shift and broadening of absorption spectra probably due to formation of aggregates.[29] These spectral changes do not invalidate their use in PDT, since the dyes maintain a high absorption in the so-called phototherapeutic window. The shift to the Vis absorption range implies that precautions must be taken when handling these dyes under ambient light.

The IR spectra of **9**–**12** do not show any evidence of carbonyl absorptions at approximately 1700 cm^{-1}, nor exhibit a strong absorption band in the region of 1600 cm^{-1}, as reported in the literature[30,31] for symmetrical squarylium and *N*-alkylaminosquarylium dyes possessing the indolenine moiety. The IR spectra for the unsymmetrical aminosquarylium dyes **11** and **12** show a weak band around 1630 cm^{-1} which is similar to that reported in the literature for symmetrical aminosquarylium