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The squaraine derivatives as potential photosensitizers in photodynamic therapy of cancer

A. T. Syniugina[1,2], O. M. Malanchuk[1], S. V. Chernii[1], A. V. Bdzhola[1], K. P. Horbatok[2], A.R. Syniugin[1], S. M. Yarmoluk[1]

[1] Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03143

[2] Taras Shevchenko National University of Kyiv,
Ave. Glushkova 4-g, Kyiv, Ukraine, 03022
asyniugina@gmail.com

Aim. One of the potential methods for treating cancer is photodynamic therapy (PDT), which involves the targeted destruction of cancer cells through the activation of photosensitizers (PS) using light irradiation. The aim of the study was to investigate the photodynamic properties of the squaraine dyes with hydrophilic and hydrophobic substituents. **Methods.** Fluorescence spectroscopy, UV-VIS spectroscopy, laser scanning confocal microscopy, cytotoxicity assay. **Results.** It was shown that all studied dyes have maximum absorption in the far-red region, which makes them suitable for PDT. It has been investigated that the squarains can form complexes with proteins, as indicated by changes in their fluorescence. The samples demonstrated minimal dark toxicity but exhibited cytotoxicity after irradiation. The cytotoxic effect of dyes with hydrophilic groups was reduced by binding to BSA. Meanwhile, a dye with hydrophobic substituents shows a photodynamic effect in the presence of albumin. All studied dyes can penetrate through the cellular membrane, stain the cell components in the cytoplasm, and do not accumulate in nuclei. **Conclusions.** All dyes showed low dark toxicity. However, cytotoxicity increased after irradiation with light of a wavelength of 670 nm. The binding of the dyes with hydrophilic substitutes to albumin negatively impacted their photosensitizing properties. At the same time, the dye with the highest tendency to aggregate exhibited the most significant cytotoxic effect.

K e y w o r d s: squaraine dyes, fluorescence spectroscopy, absorptions spectroscopy, fluorescence microscopy

Introduction

Photodynamic therapy (PDT) is one of the promising cancer treatment methods. The advantage of using PDT is the selective destruction of cancer cells by activating photosensiti-

zers (PS) upon light irradiation [1, 2]. The nature and properties of PS play an essential role in the effectiveness of PDT. The most critical requirements for PS are strong absorption in far-red NIR region, non-toxicity without light, and selectivity for tumors. The main principle of the activation of PS is the absorption of a photon and the transition to an excited singlet state under the action of light. Additionally, the molecule of PS may convert to the triplet state (T_1) by the intersystem crossover mechanism (ISC). Due to the formation of the triplet state, PS can form free radicals (type I reaction) or react with oxygen molecules to form singlet oxygen (type II reaction) [3]. Reactive oxygen species and free radicals lead to oxidative stress and various oxidizing substrates, that results in the cell death. Moreover, PDT can induce apoptosis, necrosis, or autophagy [4–6]. These cell death mechanisms were reported to be activated simultaneously and to depend on the nature of PS. Several studies have demonstrated the PDT-induced neutrophil migration into the treated tumor due to the activation of anti-tumor immunity [7, 8].

Currently, the most widely employed photosensitizers are the porphyrin derivatives [1]. The possibility of using organic dyes as potential PS for PDT has also been reported. Far-red fluorescent dyes are the compounds absorbing and emitting light in the "therapeutic window" (600–800 nm) of biological tissues. These dyes are potentially non-invasive, high-resolution, and rapid biological materials. Squaraine (SQ) dyes are a class of organic dyes derived from the aromatic squaric acid core and containing electron-donating aromatic rings at the diametrically opposite sides of the four-member ring. These dyes are widely used due to their high molar extinction coefficient, absorption and fluorescent emission in the visible and far-red regions, photocytotoxicity, and ability to accumulate in tumor cells selectively [10]. They can be used as probes and markers in biology and medicine, as far-red probes for the serum albumins detection [10–13], as photosensitizers for the PDT of cancer [14, 15]. Several scientific reviews have described the use of squaraine dyes as photosensitizers over the past decade [16–18]. One disadvantage of dyes with a polymethine chain is their photoisomerization, which leads to an insufficient population of the triplet state [19]. To avoid it, the chemical structure is modified, in particular, heavy atoms are introduced. Heavy atoms strengthen the spin-orbital interaction and intersystem crossing. Atoms of halogens [20], sulfur [21], and selenium [22] were introduced into their molecules to increase the phototoxicity. Lima *et al.* investigated the influence of various substituents on the four-membered ring in their potential use as photosensitizers [23]. It is also noted that the main problem with using squaraine dyes is their low water solubility. Introducing polar substituents can increase water solubility, reduce aggregation, and affect cell permeability and albumin binding. Many works related to the ability of dyes to bind non-covalently to serum albumins, which can be one of the means of transport and distribution of these molecules *in vivo* [24]. The interaction of most squaraine dyes with albumins increases their fluorescence due to the destruction of non-fluorescence aggregates. However, in some works it is noted that binding to albumin can have both negative [25]

and positive [26] effects of photosensitizers' phototoxic properties.

In our previous work [27], we reported the spectral-luminescent properties of N-substituents benzoindoliumsquaraine dyes and their interaction with albumins of different structures. All studied dyes in complexes with serum albumins demonstrated an increase in fluorescence emission intensity compared to the free state in an aqueous solution. Here we examined the squaraine dyes bearing with alkyl, sulfonatoalkyl and carboxyl groups as PS for PDT (Fig. 1). These dyes' dark cytotoxicity and PDT effect against the human breast adenocarcinoma cells were evaluated.

Materials and Methods

Preparation of the solutions

The 2 mM stock solutions were prepared by dissolving the dyes in DMSO. The stock solutions of proteins (HSA, BSA, BLG, LYS, and OVA) were prepared by dissolving in 0.05M TRIS-HCl buffer (pH 7.9) in a concentration 0.2 mg/ml. The protein concentrations in stock solutions were 3 μM for BSA, HSA and 4.5 μM for OVA, BLG, and LYS. The free dyes' working solution was prepared by diluting the dye stock solution in 0.05 M TRIS-HCl buffers (pH 7.9). Working solutions of the dyes and proteins were prepared by adding the dye stock solution to the protein stock solution. The concentrations of dye in working solutions amounted from 5 to 10 μM. All working solutions were prepared immediately before the experiments.

Absorption and fluorescence measurements

The absorption spectra of studied dyes were recorded on a SHIMADZU UV-VIS-NIR spectrophotometer UV-3600. The spectra were recorded in 350–850 nm regions. The fluorescence emission spectra were acquired using the fluorescent spectrophotometer Cary Eclipse (Varian, Australia). All spectral measurements were performed in quartz cuvettes (1 × 1 cm) at room temperature. The quantum yield values of the dyes in free state and in the presence of BSA were determined using Nile Blue (NB) solution in EtOH as the reference (quantum yield value 0.27).

In vitro singlet oxygen detection

The DPBF (1,3-diphenylisobenzofuran) was chosen as a singlet oxygen sensor. All tests

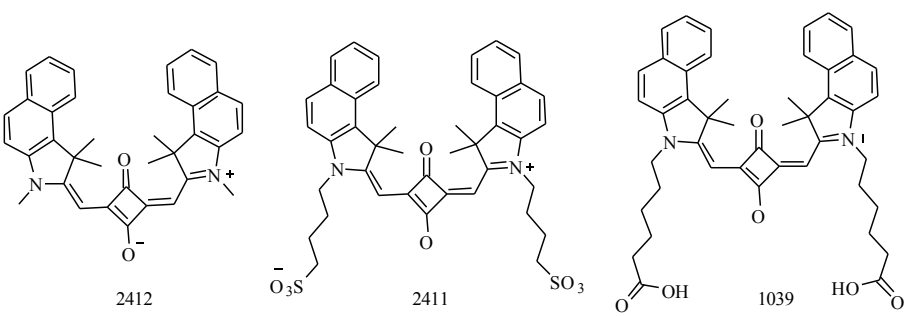

Fig. 1. Chemical structures of studied SQ dyes

were carried out in different concentrations of aqueous buffer and ethanol. First, a fresh solution of DPBF (5mM) in ethanol was added to the solvent mixture in a quartz cuvette in the dark with stirring. Then 5 μM of the dye were added and optical density at the absorption maximum of DPBF (412nm) was measured. The solution was irradiated by a 670 nm laser (180 mW/cm^2) at various time intervals. After each irradiation, the absorbance of DPBF solution was evaluated using a UV- spectrophotometer.

Cytotoxicity assay

The human breast adenocarcinoma cell line MCF-7 was cultivated in DMEM culture medium (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, USA), 2 mM glutamine, 50 U/ml penicillin and 0.25 μg/ml of streptomycin (Lonza). The cells were cultured in the atmosphere of 5 % carbon dioxide (CO_2) at 37 °C. Cells were uniformly seeded in each well of two 96 well plates (8000 cells per well) and grown up for 24 h in complete DMEM with 10 % FBS. Then the culture media were removed and replaced with the studied dyes in serum-free medium at different concentrations in serum-free medium or with the addition of BSA (5 %) to the culture medium in several tests. After one hour of incubation, the plates (with and without serum in the media) were irradiated with 670 nm (180 mW/cm^2) for 1min per well. Another plate was kept in the dark to evaluate dark cytotoxicity. All plates were overnight incubated, then the media were removed and MTT reagent (5.0 mg/mL, 10 μL), prepared in the serum-free medium, was added to the wells at dark and followed by incubation for 4 h. After that, the solution was removed and 100 μL of DMSO were added to dissolve the formazan crystals. The optical density (OD) was recorded at 570 nm by a microplate reader after 15 min incubation at RT. As a negative control sample, the background controls were set up. The cell viability was calculated by the following equation: Cell viability (%) = $(OD_{dye} - OD_{blank}) / (OD_{control} - - OD_{blank}) \times 100$ %.

Statistics

All experiments were carried out minimum in four replicates. The data analysis was performed using the GraphPad Prism 8 Software (GraphPad Software Inc., USA). Paired Student's t-test was performed. The differences with p-values < 0.05 were considered to be significant.

Confocal imaging

For confocal analysis, MCF-7 cells were placed in 12-well cultural plates containing circular sterile glass coverslips and incubated for 48 h at 37 °C in the presence of 5 % CO_2. After reaching 80 % confluence, the growth medium was removed and the cells were washed with PBS for three times. Next, the cells were incubated with the studied dyes at a concentration of 0.1 μM in FluoroBrite DMEM (Gibco, USA) without FBS for 15 min at 37 °C in the presence of 5 % CO_2. After incubation, the cells were washed with PBS, fixed in 10 % neutral buffered formalin (Sigma-Aldrich, USA) for 15 min at 22 °C and washed with PBS again. Then, Hoechst 33342 at a concentration of 1μM was added for 15 min and rewashed with PBS. Finally, glass coverslips were embedded into Mowiol mounting medium containig 0.1 % DABCO. Analysis was performed using Zeiss confocal microscope.

Results and Discussion

Spectral-luminescent properties of squaraine dyes

The absorbance of studied dyes in MeOH, 0.05M Tris·HCl buffer solution, DMSO and in the presence of BSA was measured. The obtained results are summarized in Table 1. The dyes show absorption maximum in the far-red spectrum region. All compounds are characterized by a narrow absorption band around 660–678 nm in organic solution. The spectra of squaraine dyes in methanol are suggested to belong to their monomeric form. DMSO produces a 15 nm bathochromic shift compared to MeOH for all dyes (Fig. 2). The behavior in an aqueous solution is slightly different. Dye 1039 is characterized by a slight decrease in the extinction coefficient and the appearance of a minor peak at 611 nm. The aggregation of dye 2411 in the buffer was much less intensive than in dye 1039. At the same time, the shape of the dye 2412 absorption spectrum broadens (520–750 nm) with a new peak in the short wavelength regions and a low extinction coefficient value, which probably indicates the dye aggregation in water. In our previous work, we showed that no evidence of the aggregate destruction was observed after a change in concentration of dye 2412 [27].

However, the destruction of the aggregates was observed after the dissolving of dye 2412 in the BSA solution. In the presence of albumin, there was a redistribution between the absorption maxima of the aggregate form of the dye (618 nm) and the monomers (676 nm). Additionally, in the complexes with BSA, all three dyes demonstrated an increase in the quantum yield compared to the free dye in an aqueous buffer and shifted the absorption and fluorescence maxima at 16 nm to the long-wavelength spectral region (Table 1), which could point to the binding of the dyes to protein. For dyes 1039 and 2411 in the BSA solution, the original curve shape observed in methanol regained with a notable bathochromic shift of the absorption non-covalent binding of dyes to serum albumin.

The fluorescence intensity changes for the studied dyes in a free state and of structurally different proteins are shown in Fig. 3. Dye 2412 possesses weak emissions in the aqueous buffer. All dyes showed a significantly higher fluorescence intensity in the presence of BSA and approximately the same fluorescence response in the presence of HSA and BLG. Dyes 1039 and 2411 showed a noticeably lower fluorescence intensity in the OVA solution, and no increase in the presence of lysozyme. Since all three dyes showed the highest affinity for BSA, it was chosen for further cytotoxicity studies.

Phodynamic Effect of Dyes

The capability for ROS generation was evaluated using DPBF (1,3-diphenylisobenzofuran). As a standard we used methylene blue (MB) at the same concentration as the studied dyes. The solution with each dye (5 μM) and DPBF was irradiated with a laser (wavelength of 670 nm; 180 mW/cm^2). Time- dependent losses of DPBF in the presence of MB, 2412, 1039 and 2411 are represented in Fig. 4. Dye 1039 showed the fastest bleaching of DPBA compared to the other two dyes.

At the same time, an influence of aggregation on the photodynamic effect was tested. Since

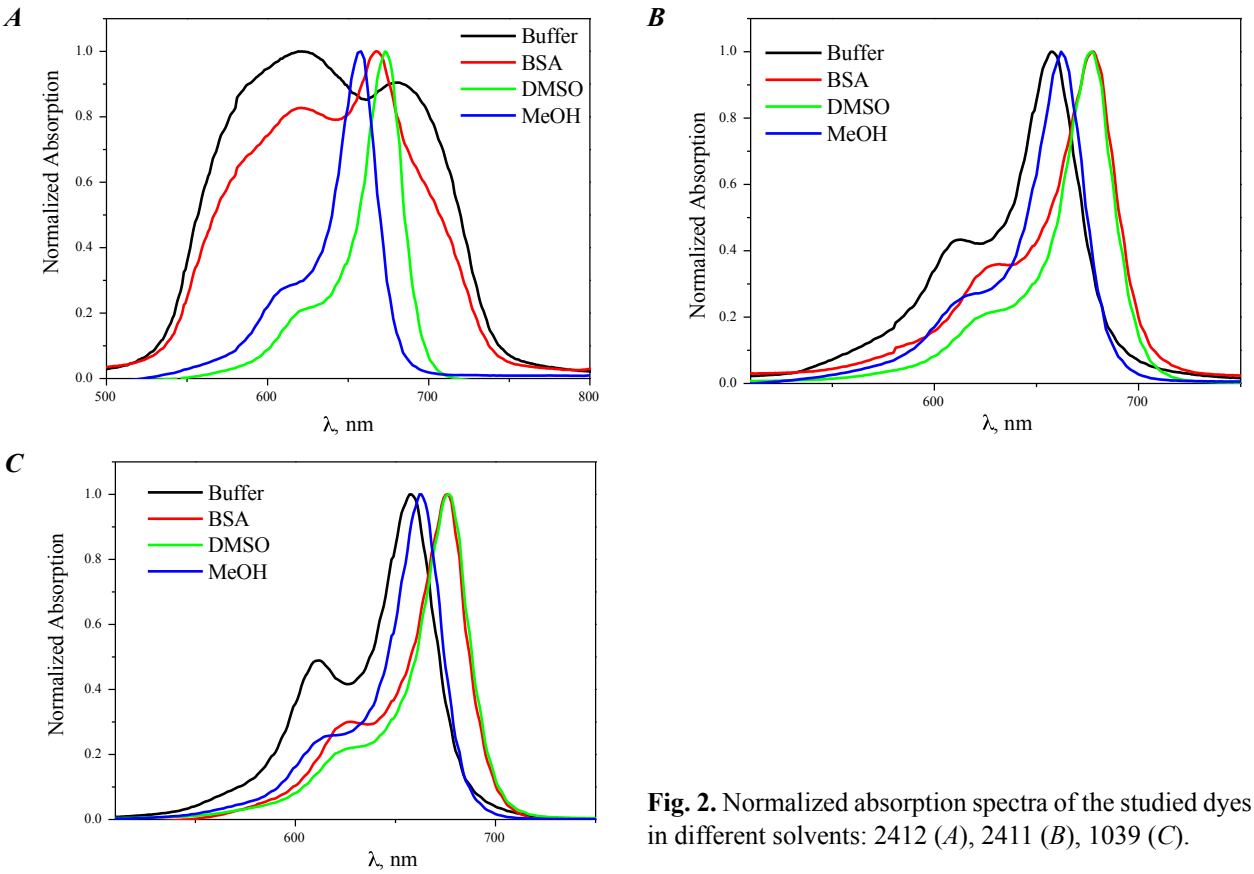

Fig. 2. Normalized absorption spectra of the studied dyes in different solvents: 2412 (*A*), 2411 (*B*), 1039 (*C*).

Table 1. **Optical properties of the dyes (5 μM) in different solvents.**

compound	solvent	l_{abs} (nm)	l_{em} (nm)	ε_{max} $10^5 M^{-1}cm^{-1}$	QY
dye 2412	buffer	619/676	690	-----	
	BSA	667	671	0.19	-----
	DMSO	673	686	0.86	0.54
	MeOH	658	668	0.63	
dye 2411	buffer	657	675	0.56	0.08
	BSA	678	692	0.67	0.67
	DMSO	677	689	0.92	
	MeOH	662	674	0.87	
dye 1039	buffer	658	672	0.86	
	BSA	676	690	1.99	0.07
	DMSO	677	694	3.11	0.50
	MeOH	663	680	2.32	

(λ_{abs}(λ_{em}) — maximum wavelength of absorption (emission) spectrum; ε — molar extinction coefficient at λ_{abs}., QY — fluorescence quantum yield values).

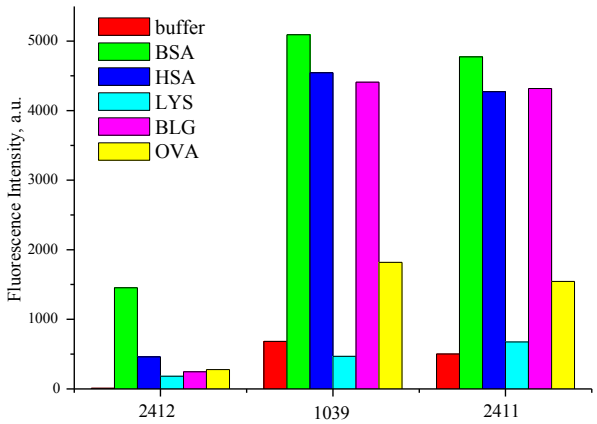

Fig. 3. Fluorescence intensity of SQ dyes in buffer and the presence of structurally different proteins.

dye 2412 showed the most significant tendency to aggregation, the PDT effect was evaluated in an environment with different water content. The rate of formation of ROS in the medium of water: ethanol in ratios 50/50 and 75/25, respectively, was investigated. As can be seen from Fig. 3B, the rate of degradation of DPBF increases with the rise in the aggregation forms of dye 2412 formed in the water fraction. We suppose aggregates facilitate intersystem crossover (ISC) between the singlet excited state $S_1$to the T_1 states that lead to ROS growth [28].

Toxicity of squaraine dyes towards human breast adenocarcinoma cells

The viability of MCF7 cells incubated in the presence of increasing concentrations of studied dyes was estimated with MTT test at 24 h of treatment. The toxic effects of dyes were observed in the 0.10–10 μM concentration range. Since the spectra-luminescence studies showed that these dyes have an excellent tendency to bind to albumins, we compared the PDT therapeutic efficiency of dyes in the serum free medium and in the presence of BSA (5 %). As shown in Fig. 5, the studied dyes demonstrated low dark toxicity, and the cell viability remained above 85 % in the presented concentration range. At the same time, after the laser irradiation for 1 min, the cell viability in the presence of dye 2412 decreased

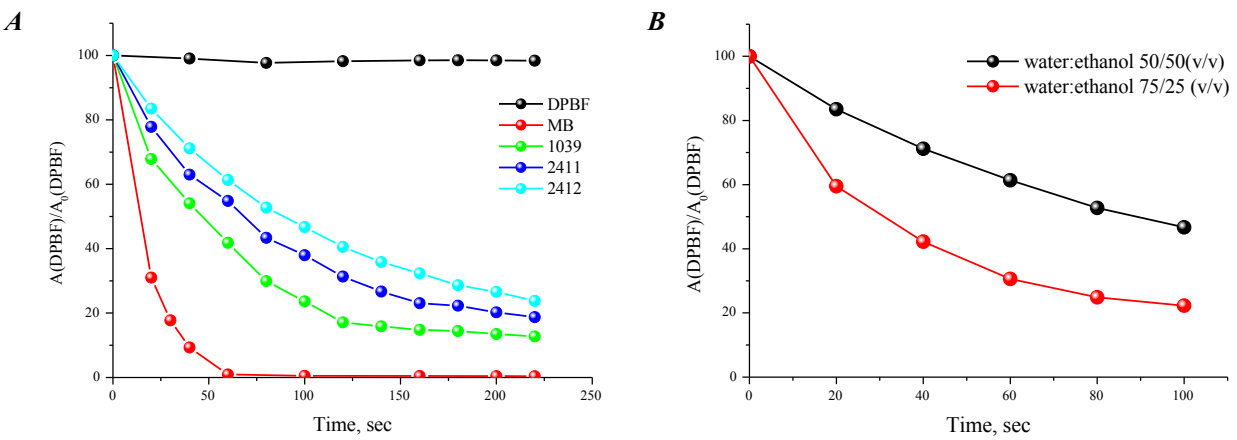

Fig. 4. Comparative analysis of singlet oxygen generation ability of studied dyes and MB as a standard reference (*A*). Degradation rates of DPBF in the presence of dye 2412 in water:ethanol (v/v) mixture (*B*). $A_{(DPBF)}$ — absorbance of DPBF after irradiation at time t, $A_{0(DPBF)}$ — absorbance of DPBF before irradiation.

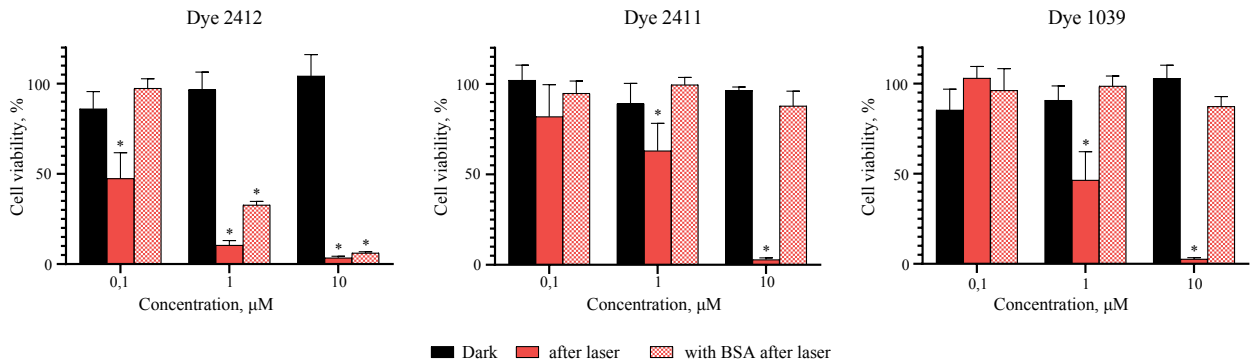

Fig. 5. The cytotoxicity of different concentrations of the squaraine dyes without and with exposure to 670 nm laser for 1 min and in the presence of BSA; *p < 0.05 compared to dark toxicity of the respective concentration of dye.

by almost 40 % at a concentration of 0.1 μM (and nearly 90 % at a concentration of 10 μM. These results confirm the effect of aggregates on the generation of ROS, whereas for dye 1039 a drop of more than 50 % cell viability was observed at a concentration of 1 μM and for 2411 at a concentration of about 10 μM. It is interesting that the laser irradiation practically did not affect the viability of cells in the presence of BSA for dyes 1039 and 2411, whereas for dye 2412 the therapeutic effect was achieved, but with greater cell viability. We assume that the lack of therapeutic effect of dyes 2411 and 1039 is possible due to their negatively charged side groups forming non-covalent interactions with the positively charged groups of BSA. The oxygen diffusion during binding the molecule to the protein is complicated. As a result, the production of singlet oxygen decreases. Besides, in the free state the dye molecules can be close to the cellular structures, which leads to their destruction with type I. At the same time the protein shell sterically limits the mechanism of electron transfer, which leads to the quenching of excited molecules by albumin subunits. Dye 2412 does not give such stable complexes, so its effect as a sensitizer remains partially the same as in the absence of albumin.

The fluorescence microscopy study.

The MCF-7 cell line was used to study the ability of dyes to penetrate the cell membrane and their applicability as the probes for fluorescence imaging of cells. The blue fluorescent dye Hoechst binding to nuclear DNA was used for the co-staining (Fig. 6). We have found that the studied dyes are able to penetrate through the cellular membrane and stain the cell components, but not the nucleus.

Conclusions

All of the synthesized dyes exhibited maximum absorption in the “therapeutic window” area, which makes them suitable for the *in vivo* bioimaging and PDT. It was demonstrated that all of the studied dyes had low dark toxicity but exhibited toxicity after being irradiated with red light. Dyes 2411 and 1039 demonstrated low phototoxicity in the presence of

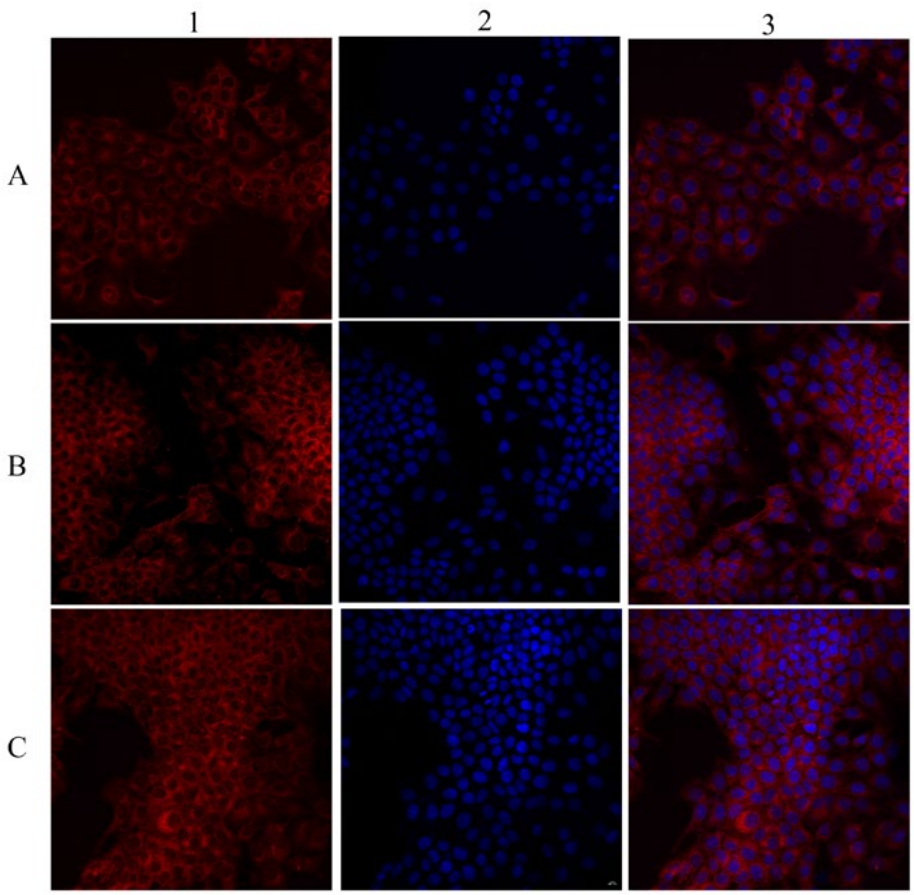

Fig. 6. 2412 (1A), 2411 (1B) and 1039 (1C) staining of MCF7 cells at a concentration of 0.1 μm (1). Hoechst was used to visualize the nuclear (2), and their merged image (3). Scale bars — 40 μm.

albumin, which limits their use as photosensitizers but makes them good candidates for bioimaging. Dye 1039 also has potential as a fluorescent label due to its free carboxylic groups.

Dye 2412 had weak intrinsic fluorescence, but its fluorescence increased significantly upon binding to proteins. It showed good phototoxicity due to the aggregates formed in the aqueous environment, and binding to albumin increased fluorescence but slightly reduced phototoxicity. Overall, these findings suggest that the synthesized dyes have potential for use in biomedical applications.

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Похідні сквараїну як потенційні фотосенсибілізатору фотодинамічній терапії раку

А. Т. Синюгіна, О. М. Маланчук, С. В. Черній, А. В. Бджола, К. П. Горбаток, А.Р. Синюгін, С. М. Ярмолюк

Мета. Фотодинамічна терапія (ФДТ) є однією із перспективних методів лікування раку. Перевагою використання ФДТ є вибіркове знищення ракових клітин шляхом активації фотосенсибілізаторів (ФС) при опроміненні. Метою дослідження є вивчення фотодинамічних властивостей сквараїнових барвників із N-замісниками. **Методи.** Флуоресцентна спектроскопія, UV-VIS спектроскопія, лазерна скануюча конфокальна мікроскопія, аналіз цитотоксичності. **Результати.** Досліджено спектрально-люмінесцентні властивості досліджуваних барвників у метанолі, ДМСО, водному розчині та в присутності альбуміну. Усі досліджувані барвники в комплексах із сироватковими альбумінами продемонстрували підвищення інтенсивності флуоресценції. Максимуми спектрів поглинання розташовані в області 657–677 нм. Максимуми випромінювання флуоресценції знаходяться в діапазоні 670–690 нм, що робить барвники придатними для фотодинамічної терапії. Ефективність досліджуваних барвників як фотосенсибілізаторів для фотодинамічної терапії (ФДТ) оцінювали *in vitro*. Барвники характеризуються незначною темновою токсичністю, але виявляють цитотоксичність після опромінення. Зв'язування з БСА зменшувало цитотоксичну дію барвників з гідрофільними групами. Водночас барвник з гідрофобною структурою зберігав властивості ФДТ як у водному середовищі, так і в присутності альбуміну. Лінія клітин раку молочної залози (MCF-7) була використана для вивчення здатності досліджуваних барвників проникати крізь клітинну мембрану. Було встановлено, що досліджувані барвники можуть проникати крізь клітинну мембрану, неспецифічно фарбувати клітинні компоненти в цитоплазмі та не накопичуватися в ядрах, про що показало спільне фарбування з Hoechst. **Висновки.** Усі синтезовані барвники продемонстрували поглинання в зоні «терапевтичного вікна», що робить їх максимально придатними для біовізуалізації *in vivo* та ФДТ. Показано, що всі досліджувані барвники мали низьку темнову токсичність, але проявили токсичність після промінення червоним світлом. Було продемонстровано, що зв›язок з альбуміном і агрегація барвників може мати як негативний, так і позитивний вплив на фототоксичну властивість фотосенсибилизаторів в залежності від їх будови.

Ключові слова: сквараїнові барвники, флуоресцентна спектроскопія, спектроскопія поглинання, флуоресцентна мікроскопія.

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