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PERSPECTIVE

Squaraine dyes in PDT: from basic design to *in vivo* demonstration

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The design and development of novel squaraine dyes as sensitisers for photodynamic therapy (PDT) applications has grown tremendously in the last decade from the time when a squaraine dye was proposed to be a potential candidate, to-date when the use of such dyes have been demonstrated in animal models for skin cancer. This perspective article highlights the basic design, tuning of absorption, triplet excited state and two-photon absorption properties and recent developments of the squaraines as PDT sensitisers.

1. Introduction

The high selectivity in the destruction of tumor cells over normal cells has made photodynamic therapy (PDT) an attractive alternative to the traditional cancer therapies.[1] The basic principle of PDT involves the generation of highly toxic and reactive oxygen species upon excitation of a sensitiser.[2,3] Various stages involved in PDT treatment are illustrated in Fig. 1. In the first stage, the sensitiser is administered into the body. After allowing a suitable period of incubation, the sensitiser preferentially accumulates in the lipophilic compartment of the tumour cells following the receptor-mediated endocytosis mechanism (stage 2). In the third stage, sensitiser at the target tissue is irradiated with light of suitable wavelength. Light acts as a stimulus, switching on the production of cytotoxic agents such as reactive oxygen species (ROS) due to the excitation of the sensitiser. These species react with biological molecules such as proteins, amino acids, lipids, nucleotides and nucleic acids, thereby disrupting the normal functions of the cell and causing cell death (stage 4). A sensitiser in PDT can, therefore, be regarded as a 'stimuli responsive system', being inactive in the dark and active when irradiated with light of an appropriate wavelength.

Two main reaction pathways, path I and path II, are proposed to be involved in the PDT action.[4] The first pathway, called the type I mechanism, involves the generation of radical species through either hydrogen abstraction or redox processes between a sensitiser in an excited state and the biomolecule. In the type II mechanism, the sensitiser in the triplet excited state generates singlet oxygen ($^1O_2$) from ground-state molecular oxygen ($^3O_2$) through an energy transfer process.[5] It has been postulated that singlet oxygen is the most prevalent cytotoxic agent, which is responsible for the photoinactivation of tumour cells by a majority of the reported photosensitisers.

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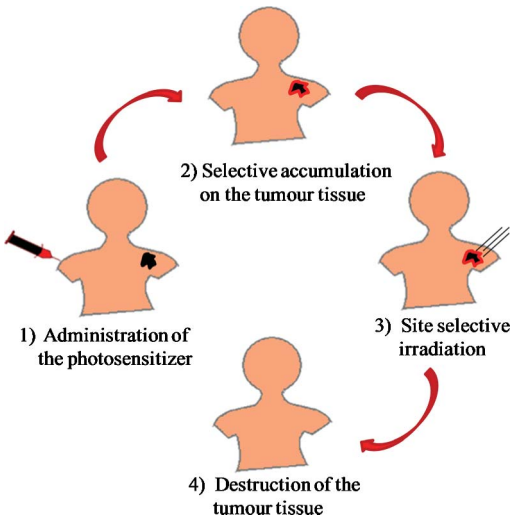

Fig. 1 Schematic representation of various stages involved in photodynamic therapy (PDT).

A desirable sensitiser should have strong absorption in the longer wavelength region, ideally in the 600–850 nm region known as the photodynamic window, wherein the tissue penetration by light is higher.[6] The sensitiser should also possess high triplet quantum yields with long lifetimes and should be able to generate reactive species such as singlet oxygen in quantitative yields. In addition, the sensitiser should have minimal dark toxicity and only be cytotoxic in the presence of light. It should also be preferentially retained by the target tissue and rapidly excreted from the body. Several compounds from simple aromatics to complex macrocycles have been proposed as sensitisers in PDT including porphyrins, phthalocyanins, chlorins, bacteriochlorins, cyanines, Rose Bengal, Methylene Blue, aminolevulinic acid and their derivatives.[5–11] One of the extensively studied sensitisers is Photofrin@, a hematoporphyrin derivative (HpD), for which first regulatory authorizations for clinical use were obtained for a variety of cancers in a number of countries. Most of the approved sensitisers have slow metabolic degradative pathways and, unfortunately, are activated by light of wavelength below 600 nm, which cannot penetrate more than a few millimeters into the skin. Due to this reason, recently, there has been great interest in the development of dyes that possess absorption in the red to near infrared region.[7–9] Among these, the squaraine dyes have attracted intense interest in recent years.

This Perspective focuses on the advances in the development of the squaraine dyes as sensitisers. Some of these aspects include the design of the squaraine dyes which show (i) improved absorption properties, (ii) improved quantum yields of triplet excited state of the sensitiser and singlet oxygen generation, as well as (iii) squaraines that exhibit two photon-absorption, and (iv) strategies to improve their cellular pharmacokinetics. A discussion on suitable carrier-based drug delivery systems as well as the studies dealing with *in vitro* and *in vivo* investigations are also covered in this Perspective including future possible directions and goals.

2. Basic design principle of squaraine dyes

Squaraines are 1,3-zwtitterionic donor–acceptor–donor (D–A–D) structures with the central acceptor four membered squaryl ring flanked by donor aromatic/heterocyclic rings on either side. They possess sharp and intense absorption bands ($\varepsilon \sim 10^5$ M^{-1} cm^{-1}), which extend in the red to near infrared region.[12] This is due to the intramolecular CT character of the S_0–S_1 electronic transition combined with an extended conjugated π-electron network present in the squaraines. The photochemical and photophysical properties make these dyes highly suitable for a number of biological applications such as metal ion sensors, long-wavelength fluorescent labels and for the detection of bio-relevant thiols.[13–19] The intense absorption in the photodynamic window (600–850 nm) makes these dyes highly suitable as sensitisers. Our group was the first to propose that appropriately substituted squaraine dyes can act as novel sensitisers in PDT applications.[20] The absorption of the sensitiser in the long wavelength region is desirable not only because of the higher tissue penetration of light in this region, but also due to the fact that the most of the

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cellular constituents compete for the absorption of light of lower wavelengths.

Substituting the aromatic rings with polyaromatic or heterocyclic rings considerably red-shifts the absorption profile of the squaraine dyes as demonstrated in the case of the several squaraine derivatives.[20–24] For example, the phloroglucinol based squaraine dye **1a** exhibited absorption in the 550–600 nm region with molecular extinction coefficients in the order 10^4 M^{-1} cm^{-1} (Fig. 2). Replacement of the phloroglucinol moiety with the benzothiazole moiety as in the case of **2**, interestingly, red-shifted the absorption maximum by *ca.* 90 nm. When the benzothiazole ring is replaced with the quinaldine moiety (**3**), the absorption maximum is further red-shifted by *ca.* 60 nm (Fig. 3). In spite of the various advantages of the squaraine dyes such as tunable absorption wavelength and high stability, the biological applications of these dyes, especially their potential use as sensitisers in PDT, have not been explored until recently.[20] This is possibly due to the fact that these dyes show low intersystem crossing efficiency, rendering them less efficient in generating reactive species required to induce cellular damage. Later studies have showed that the suitably substituted squaraine dyes possess favorable photophysical properties which enable them to act as potential sensitisers for use in PDT.[21–23]

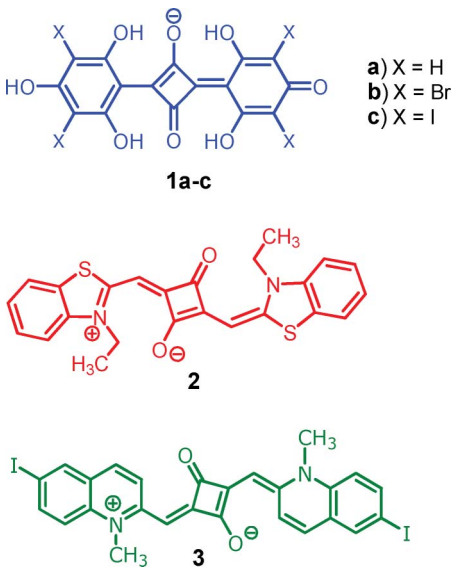

Fig. 2 Structures of squaraine dyes **1–3**.

In the type II PDT reaction, the efficiency of the sensitiser is determined by its efficiency to generate singlet oxygen. Since normal molecular oxygen ($^3O_2$) is in the triplet state, an energy transfer process to oxygen can take place only from the triplet excited state of the sensitiser. For an efficient energy transfer process, the triplet energy of the sensitiser should be ≥0.98 eV, which corresponds to the energy of the triplet molecular oxygen electronic transition ($^3\Sigma_g \rightarrow {}^1\Delta_g$).[25] This, consequently, places a limitation on the absorption maximum of the sensitiser to be <800–900 nm. Therefore, dyes based on the squaraine moiety with triplet energy above that of the triplet molecular oxygen can act as efficient type II sensitisers, provided their triplet quantum yield is sufficiently high. So, what is required for a type II PDT agent is a modification of the sensitiser to enhance its intersystem crossing (ISC) efficiency to increase the triplet quantum yield

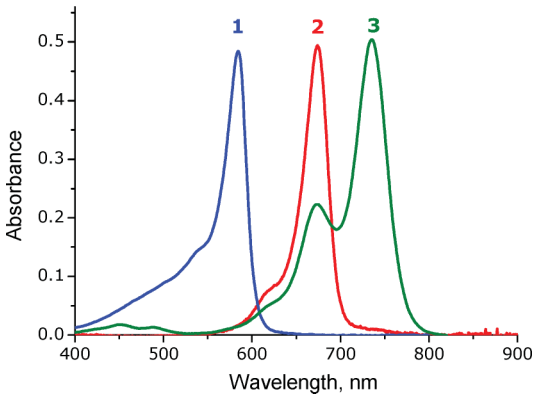

Fig. 3 Absorption spectra of representative squaraine dyes **1–3**.

and subsequently, the production of reactive oxygen species such as singlet oxygen. Efficient ISC is usually achieved by the introduction of heavy atoms, for example, bromine or iodine or heavy-metal ions into the molecular structure or in the medium, which results in spin–orbit perturbation.[26]

2.1. Squaraines as singlet oxygen generators

Squaraine dyes have inherently poor intersystem crossing efficiency and therefore, act as poor generators of singlet oxygen. The unsubstituted phloroglucinol squaraine dye **1a** has several advantages including a facile and high yielding synthetic route, simple purification method and good solubility in aqueous medium. However, the quantum yields of triplet excited state and singlet oxygen generation efficiency of **1a** were found to be insignificant (<0.1%), thereby indicating unsuitability for use as a type II sensitiser in PDT applications.

The quantum yield of photosensitised singlet oxygen generation (Φ_Δ) of **1a** could be increased significantly by halogenating the donor phloroglucinol moiety with heavier halogen atoms (Fig. 2).[20] Interestingly, upon bromination (**1b**) and iodination (**1c**), the quantum yields of the triplet excited states (Φ_T) could be increased to *ca.* 0.22 and 0.5, having lifetimes (τ_T) of 132 and 36 μs, respectively in methanol. This, in turn, resulted in significant generation of singlet oxygen quantum yields of *ca.* 0.13 and 0.47, for the bromo (**1b**) and iodo (**1c**) derivatives, respectively.

Santos *et al.*,[27] have reported a few heterocyclic squaraine dyes in which the donor moiety was varied from quinoline to benzothiazole to benzoselenazole and they observed that the efficiency of the triplet–singlet interconversion of oxygen was found to depend markedly on the nature of the terminal heteroaromatic nuclei. As expected on the basis of the 'heaviness' of the heteroatom and the consequent spin–orbit relaxation, the highest value of $\Phi_\Delta = 0.31$, was obtained for the dyes incorporating the benzoselenazole moiety (**4a**), while the dyes with benzothiazole (**4b**) and quinoline moieties (**4c**) showed lowered quantum yields of *ca.* 0.26 and 0.10, respectively, in CH_2Cl_2 (Fig. 4). By modifying the squaryl ring with an *N*-methylamine group, the authors were able to considerably enhance the quantum yields from *ca.* 0.31 (for **4a**) to 0.45 (for **5a**) for the benzoselenazole derivatives.[28] In contrast, the benzothiazole **5b** ($\Phi_\Delta = 0.04$) and quinoline **5c** ($\Phi_\Delta = 0.01$) derivatives of the ring-modified systems in CH_2Cl_2 showed lower quantum yields. Interestingly, among the modified squaraine systems, the dyes modified with *N*-methylamine group (**5a–c** and

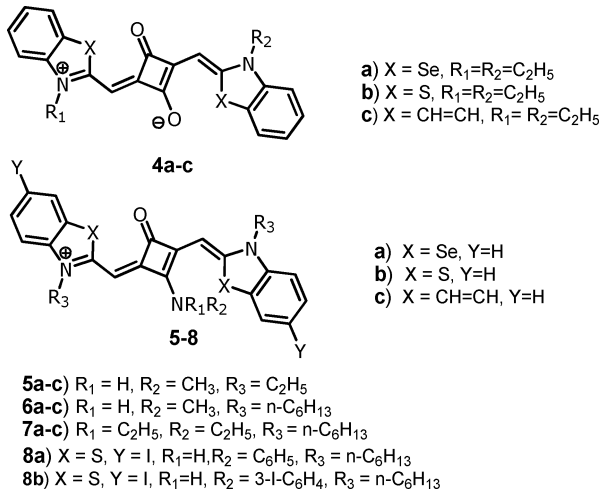

Fig. 4 Structures of squaraine dyes **4–8**.

6a–c) in the central four-membered ring exhibited higher singlet oxygen generation quantum yields than the corresponding *N*,*N*-diethylamine substituted derivatives (**7a–c**). This was attributed to the stronger intramolecular hydrogen-bonding present in the former, which inhibited the photoisomerization about the olefinic bond and led to greater structural rigidification. The resultant reduction in the non-radiative decay process caused an inherent increase in the intersystem crossing efficiency of these systems.

A similar increase in the singlet oxygen generation efficiency was observed upon increasing the length of the pendant *N*-alkyl chain within a given heterocyclic series by a maximum of *ca.* 23%. For example, the singlet oxygen quantum yields of the benzoselenazole derivatives increased from *ca.* 0.45 for **5a**, to 0.68 for the dye **6a**. The authors have also investigated the effect of iodine atoms in increasing the quantum yields of singlet oxygen for the benzothiazole based 'aminosquaraine' systems. In these systems, the quantum yield of *ca.* 0.46 for the iodobenzothiazole **8a** was further enhanced with the incorporation of more iodine atoms to *ca.* 0.66 (**8b**).[29]

Smith *et al.*, have encapsulated the squaraine dye in a mechanically interlocked structure termed as 'squaraine-rotaxane' to improve the values of singlet oxygen yield.[30,31] The authors have observed that the singlet oxygen generation efficiency of the tetraiodinated squaraine dye **9** was increased considerably when it was sterically protected by encapsulation in a rotaxane as in the case of **10** (Fig. 5). Recently, they have further beautifully demonstrated how such architectures can be used for the storage and subsequent, on-demand, release of singlet oxygen and thereby, *in vivo* chemiluminescence imaging in a living mouse.[32] The potential of various substituted 2-pyrrole-based squaraines as singlet oxygen generators have been studied by Pagani *et al.*[33,34] The dyes **11–13** shown in Fig. 6 were suitably tailored so as to shift their absorption from the red to near infrared region. The oxygen trap experiments by 1,3-diphenylisobenzofuran (DPBF) indicated that these derivatives can generate singlet oxygen, wherein **11** and **12** exhibited $\Phi_\Delta = 0.16 \pm 0.04$ and 0.33 ± 0.06, respectively in CS_2, when excited at a wavelength lower than the absorption maximum ($\lambda_{max} = 680–730$ nm, $\lambda_{exc} = 403$ nm). The halogenation of these derivatives expectedly showed significantly improved values of the singlet oxygen yields. The iodo-substituted aniline-based

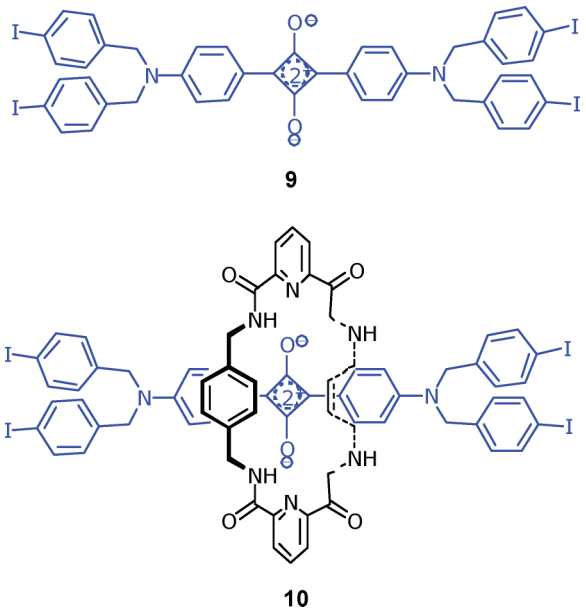

Fig. 5 Structures of squaraine dye **9** and squaraine-rotaxane **10**.

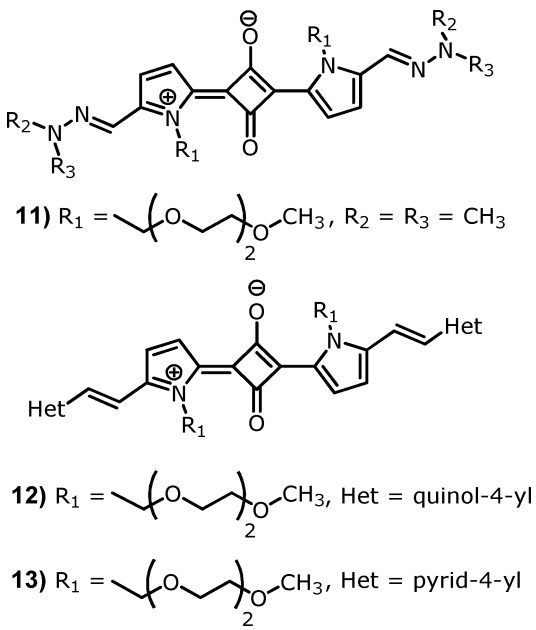

Fig. 6 Structures of squaraine dyes **11–13**.

squaraine dye **14**, synthesized by Wang *et al.*,[35] exhibited a singlet oxygen quantum yield of *ca.* 0.54 as compared to the parent non-halogenated system ($\Phi_\Delta = 0.02$, Fig. 7).

An interesting question has been raised by Ogilby and Pagani *et al.*,[36] on the squaraine-sensitised generation and deactivation of singlet oxygen of a few selected squaraines such as **15** and **16** (Fig. 7). They have proposed that these squaraines are not only poor generators of singlet oxygen, but also can independently act as effective quenchers of singlet oxygen. The authors have explained that this might be due to the intramolecular charge transfer (CT) character of the sensitiser and the intermolecular CT character of the sensitiser–oxygen encounter complex. An unusual observation in these systems is the irradiation wavelength dependent singlet oxygen generation. For example, dyes **15** and **16** showed Φ_Δ of

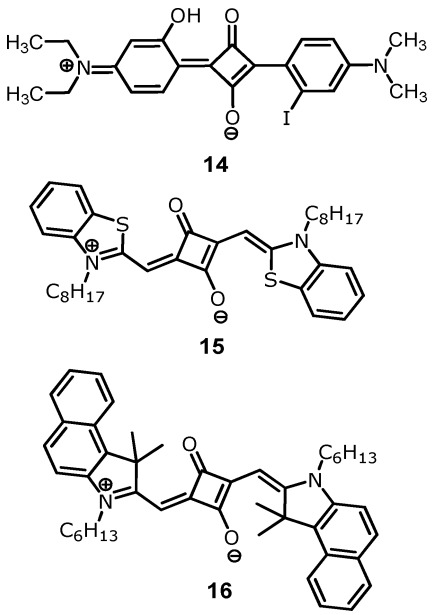

Fig. 7 Structures of squaraine dyes **14–16**.

0.05 ± 0.02 and 0.021 ± 0.008 under UV irradiation, while upon excitation with visible light, these dyes gave values of 0.012 ± 0.004 and 0.006 ± 0.001, respectively, in toluene. The authors attribute this unusual phenomenon of irradiation wavelength dependent Φ_Δ to the inner filter effect, wherein the fluorescent photons are reabsorbed to produce additional singlet oxygen precursors, rather than to the wavelength dependent population of different excited states that produce singlet oxygen with a varied efficiency.

2.2. Squaraines as two-photon absorbing agents

Recently, the focus of interest in PDT has been the development of two-photon absorbing (TPA) sensitisers due to advantages such as higher tissue penetration and minimal autofluorescene associated with the use of light of longer wavelength.[37,38] TPA is the phenomenon by which a molecule could absorb two photons simultaneously in the same quantum event. Since TPA is a way of accessing a given excited state by using photons of half the energy of the corresponding one-photon transition, it is of great importance in various applications. Pagani *et al.*,[34] have recently proposed a few π-extended heterocyclic squaraines, with absorption in the 550–760 nm region as TPA active sensitisers (Fig. 6). The TPA cross section, measured through the Z-scan method for **11** and **12** are *ca.* 9100 ± 2000 and 17400 ± 2400 GM, respectively, at 806 nm. The photosensitised singlet oxygen generation quantum yields at 806 nm, calculated by measuring the $^1O_2$ phosphorescence signal at 1270 nm, were 0.03 and 0.33 for **11** and **12**, respectively, in CS_2. The authors have pointed out that the quadratic dependence of the singlet oxygen emission signal of the two dyes is clearly indicative of the TPA nature of the singlet oxygen generation process even at an irradiation wavelength of 806 nm.

In contrast to the commonly employed strategy of heavy-atom substitution, a new approach was put forth by Webster *et al.*,[39] to enhance the quantum yields of the triplet excited state and singlet oxygen generation in the squaraine dyes. In this strategy, the oxygen atoms of the squaryl ring were replaced with sulfur atoms, which resulted in a dramatic increase in the quantum yields to near unity for the 'thiosquaraine' dye **18** (Φ_T = 0.97 ± 0.07, Φ_Δ = 1 ± 0.2) as compared to the parent dye **17** (Φ_T = < 0.01) (Fig. 8). This significant enhancement is due to the replacement of the squaryl oxygen atoms with sulphur atoms, which results in the inversion of the lowest singlet state of π-π* character to that of n-π* character. This inversion significantly reduces the singlet–triplet energy difference, and efficient intersystem crossing from the n-π* singlet state into the π–π* triplet state occurs producing near-unity triplet quantum yields (Fig. 9). The two-photon excitation of the system at 780 nm generated singlet oxygen very efficiently and its TPA nature was confirmed by the quadratic dependence of the singlet oxygen luminescence on the excitation power. The product of TPA cross section (7000 GM) and Φ_Δ, known as the merit parameter for PDT, was 7000 at 780 nm for the squaraine dye **18**, which indicated that thiosquaraines have immense potential as TPA agents in photodynamic therapy.

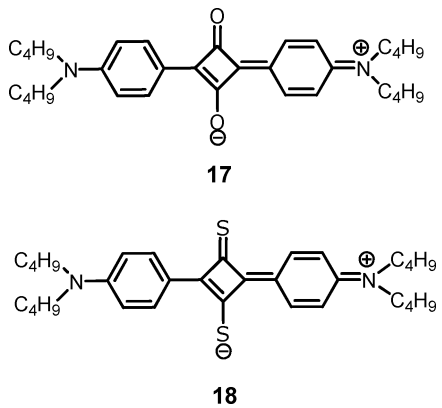

Fig. 8 Structures of squaraine dyes **17** and **18**.

2.3. Carrier systems for squaraine dyes

One of the major limitations in the use of the squaraine dyes in biological applications is the proclivity to nucleophilic attack at the squaryl ring resulting in the loss of its photophysical characteristics.[31] Aggregation of squaraine dyes in an aqueous environment also poses a serious problem during biological applications.[40] These problems can be addressed very effectively by the use of suitable carrier-based drug delivery systems, which not only improve the bioavailability of the sensitiser, but also provides necessary protection.[41] The literature on carrier systems for the squaraines in PDT are limited. Here we describe two such examples, serum albumins (bovine and human) and β-cyclodextrin (β-CD), which have been explored as potential carrier systems for the squaraine dyes.

Serum albumins such as human serum albumin (HSA) and bovine serum albumin (BSA) are transport proteins, which facilitate the disposition and transportation of various ligands to specific targets in the body.[42] It has been previously reported that sensitisers possessing high affinity for serum albumins, and having preferential binding at site II exhibited efficient photodynamic therapeutic activity.[43,44] The polyhydroxyl squaraine dye **1a** reported by our group exhibited high association (1.4–1.8 × 10^6 M^{-1}) with the serum albumins with marginal affinity to site II.[45] Interestingly, the bromo- and iodo-substituted dyes (**1b** and **1c**)

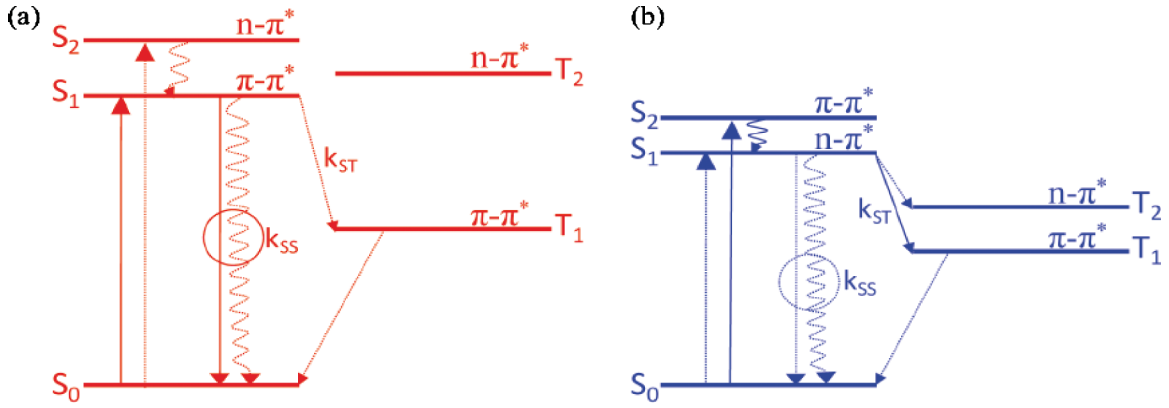

Fig. 9 Schematic of energy-level structures and the nature of transitions for the dyes **17** (a) and **18** (b). Solid-line transitions are more probable than dotted-line transitions. Reprinted with permission from ref. 39. Copyright 2010, American Chemical Society.

showed unusually very high binding preference towards the site II of serum albumins due to steric constraints at site I owing to the presence of the heavier halogen atoms (Fig. 10). The association constants of **1b** were estimated and were found to be K_{ass} = 4.9 × 10^6 M^{-1} and 6.0 × 10^6 M^{-1} for BSA and HSA, respectively. Similar observations were made with **1c** and which showed K_{ass} = 4.1 × 10^5 M^{-1} and 9.9 × 10^5 M^{-1} for BSA and HSA.[46] In view of their substantial singlet oxygen quantum yield, the significant binding affinity of the squaraine dyes **1b** and **1c** to BSA/HSA is very important especially since the serum albumins are transport proteins of the blood plasma. It could be expected that *in vivo* transportation of these sensitisers would be benefited by such an association.

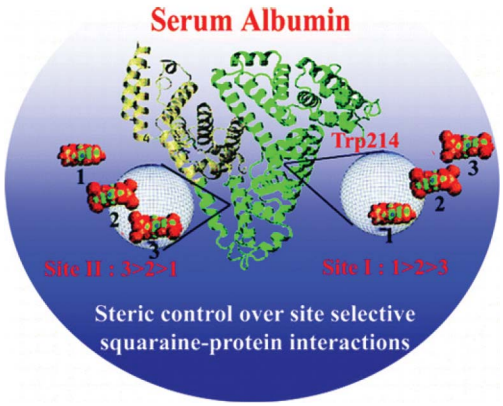

Fig. 10 Schematic representation of site selective interactions of the squaraine dyes **1a–c** with serum albumins (SA). Reprinted with permission from ref. 46. Copyright 2010, American Chemical Society.

What is particularly interesting with the use of serum albumins as carrier systems for these squaraine dyes is the enhanced triplet quantum yields and lifetimes observed for the squaraine–BSA and squaraine–HSA complexes.[46] For example, the triplet excited states of the bromo (**1b**) and iodo (**1c**) derivatives showed enhanced lifetimes of 109 and 56 μs, respectively, in the presence of HSA as compared to that in buffer (46 and 26 μs, respectively). This is due to the restricted rotational freedom of the dye molecules due to the microencapsulation and which, thereby, results in slower deactivation of the excited state *via* non-radiative processes. Significantly, the squaraine–HSA complex showed increased triplet quantum yields of Φ_T = 0.54 and 0.7, respectively, for the dyes **1b** and **1c**, as compared to their yields (Φ_T = 0.14 and 0.4, respectively) in buffer.

Apart from the use of serum albumins, cyclodextrins (CDs), cyclic oligosaccharides with hydrophobic cavities, have also been employed as suitable carrier systems for squaraine dyes. The formation of the inclusion complexes of these dyes with β-cyclodextrin (β-CD), not only improved the solubility of these systems, but also prevented their aggregation in aqueous solutions.[47,48] For the squaraine dyes whose dimensions match the hydrophobic cavity, β-CD can serve as an ideal carrier system. In particular, the phloroglucinol based dyes formed inclusion complexes with β-CD with a stoichiometry of 1 : 2 (Fig. 11).[49] The formation of this complex is highly beneficial to the dye, assuring protection from nucleophilic attack by thiols such as cysteine and glutathione present in the biological system (Fig. 11). These studies clearly indicated that β-CD can act as a suitable carrier system for dyes **1b** and **1c**.[49,50]

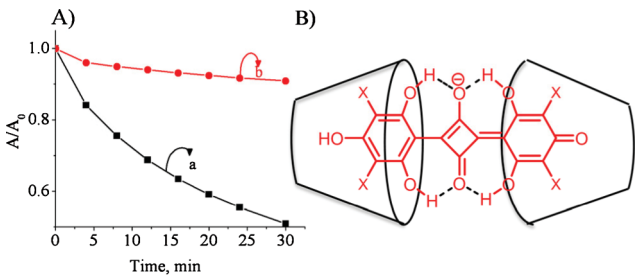

Fig. 11 (A) Change in absorbance of **1b** *vs.* time with addition of cysteine in the absence (a) and presence (b) of β-CD. (B) Proposed tentative structure of the 1 : 2 complex between **1b** and β-CD. Reprinted with permission from ref. 49. Copyright 2011, American Chemical Society.

2.4. *In vitro* studies of squaraine-PDT action

A number of squaraine dyes have been proposed as potential sensitisers in PDT, however, only a few reports describe the photosensitising action of these sensitisers in cell cultures. Our group has studied the phototoxicity of the halogenated phloroglucinol based squaraine dyes **1b** and **1c** upon photoexcitation using a 1000 W halogen lamp (337.5 kJ m^{-2}) between 400 and 800 nm in various

Table 1 Effect of pH, D_2O, SOD and catalase on the generation of SSB by the squaraine dyes **1b** and **1c**

Relative number of SSB (%)					
Compounds	pH 7.0	pH 7.8	D_2O buffer	SOD (20 μg mL^{-1})	Catalase (315 units mL^{-1})
1b	127 ± 12	105 ± 4	526 ± 28	109 ± 5	115 ± 5
1c	134 ± 9	109 ± 22	614 ± 97	100 ± 2	92 ± 6

cell lines.[51] The squaraine dyes **1b** and **1c** induced single strand breaks (SSB) in supercoiled DNA of bacteriophage PM2 when irradiated with visible light. The non-halogenated squaraine dye **1a** was inactive, indicating that the halogenation of the squaraines is essential for the generation of SSB under cell-free conditions. The dyes with bromo and iodo substitution, **1b** and **1c**, strongly inhibited the cloning efficiency of AS52 Chinese hamster ovary cells with IC_{50} values of 1–2 μM.

Further studies with L5178Y mouse lymphoma cells indicated that the reactive species produced by the dyes preferentially target cellular components other than DNA, and DNA damage was ruled out as the major mechanism in the cell inactivation by dyes **1b** and **1c** (Fig. 12).[51–55] Results further indicated that DNA damage by the dyes **1b** and **1c** was induced mainly by singlet oxygen since the *in vitro* and cellular DNA damage was significantly enhanced when the H_2O in buffer was replaced with D_2O (Table 1). The studies in presence of superoxide dismutase (SOD) and catalase showed that neither superoxide radical anion nor hydrogen peroxide was involved. Furthermore, it was postulated that reactive intermediates generated by the halogenated dyes could also be partly responsible for the modification of the cellular constituents. These reactive intermediates may be formed by the homolysis of the dye on excitation ($^3SqX \rightarrow Sq^{\bullet} + X^{\bullet}$) or through the dehalogenation of the radical anion ($SqX^{\bullet-} \rightarrow Sq^{\bullet} + X^{\bullet}$).[51] The resulting radical intermediates induce the generation of SSB and modify the cellular constituents.

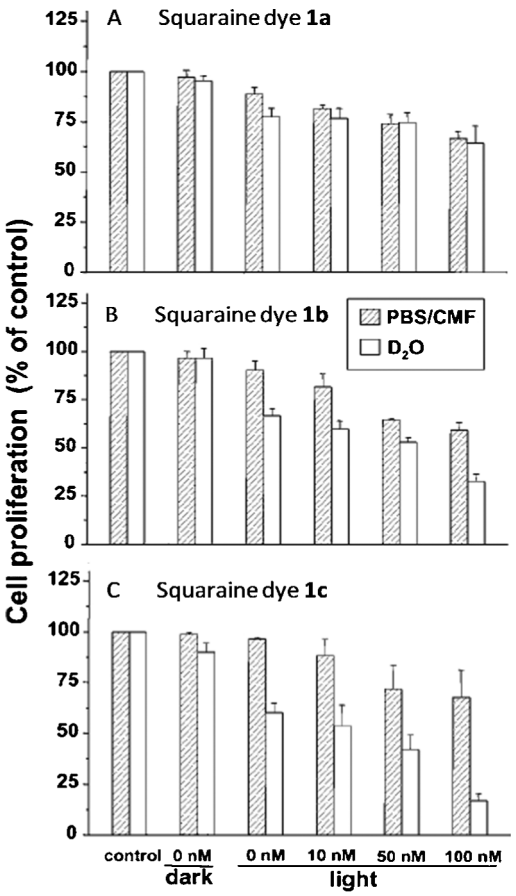

Fig. 12 Cytotoxicity of the squaraine dyes (A) **1a** (B) **1b** and (C) **1c** in L5178Y mouse lymphoma cells in PBS/CMF buffer (hatched columns) and buffer in which H_2O is replaced by D_2O (open columns). Reprinted with permission from ref. 55. Copyright 2004, American Society for Photobiology.

Interestingly, the non-halogenated dye **1a** showed non-negligible base modifications in L5178Y cells, than expected on the basis of its Φ_Δ. This unexpected photocytotoxicity of **1a** was explained as taking place from the excited singlet states reacting through the type I mechanism. The studies with mouse lymphoma L5178Y cells also indicated that squaraine dyes **1b** and **1c** induce both cytotoxic effects and DNA damage predominantly *via* singlet oxygen, whereas the photobiological activity of squaraine **1a**, could be mediated by type I reactions.

Most of the reported squaraine-based sensitisers having PDT activity are proposed to act *via* the type II mechanism through the generation of singlet oxygen. Different from this majority class are the sensitisers, which function *via* the type I mechanism, *i.e.*, through the formation of reactive oxygen species (ROS) and radicals. Xodo *et al.*,[56] have studied the photooxidation and photodynamic properties of a few benzothiazole based squaraines **19** (Fig. 13) and **20**. Air-saturated solutions of dye **20** showed significant photodegradation, when irradiated with light of wavelength >500 nm, through the reaction mechanism proposed in Fig. 14. The different photogenerated products proposed to be formed from the squaraine–oxygen encounter complex, either through photooxygenation of the enaminic bond or the production of ROS, initiate radical chain reactions that lead to type I photodynamic processes. Further studies employing limonene (which gives different oxidation products depending on the ROS involved) also indicated the involvement of type I radical chain process.

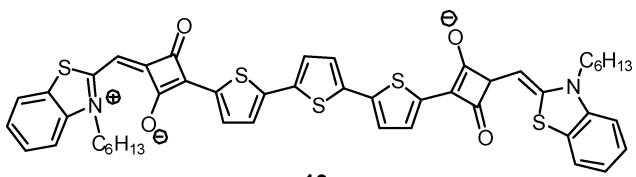

Fig. 13 Structure of squaraine dye **19**.

One of the primary features exhibited by **19** and **20** is the significant fluorescence quantum yield of these dyes in the red region of the visible spectrum. The confocal microscopic images of

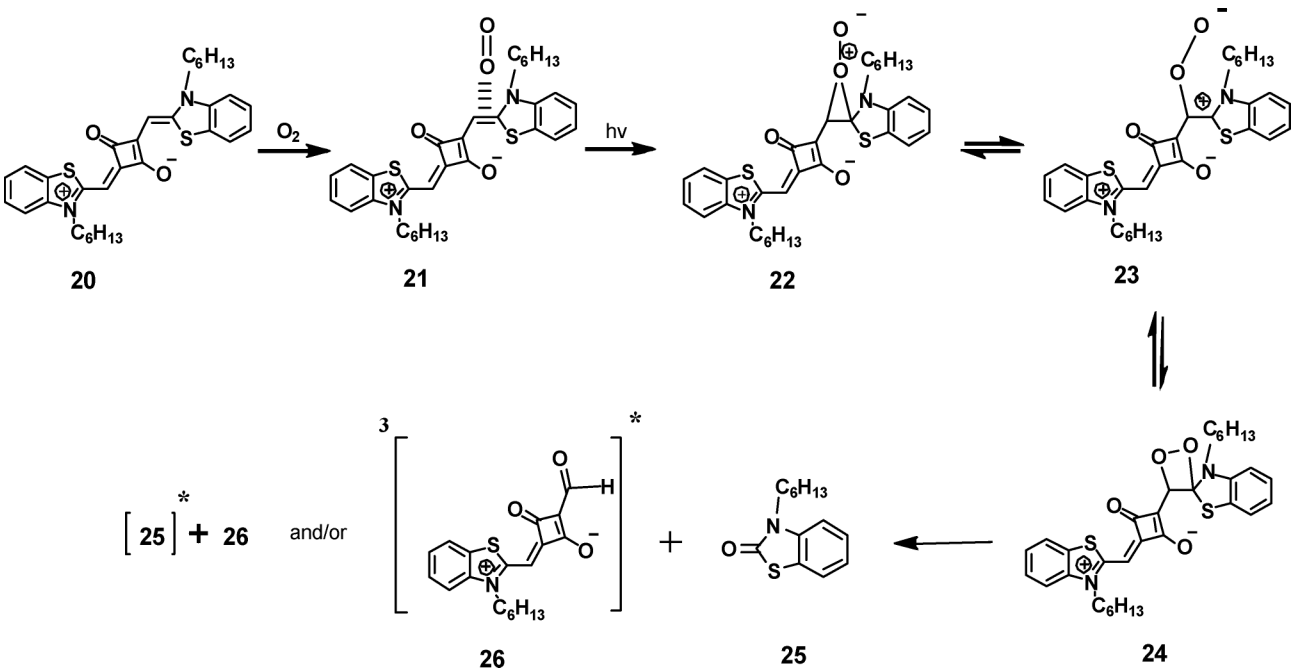

Fig. 14 Proposed mechanism for the photodegradation of squaraine **20**. Reprinted with permission from ref. 56. Copyright 2010, American Chemical Society.

cervical cancer (HeLa) cells showed that these dyes preferentially localize in the cytoplasm and associate to the organelle membranes through their hydrocarbon chains (Fig. 15). The authors indicate that due to the negligible localization at the nucleus, these dyes are less likely to cause DNA damage, mutations or carcinogenesis. The dyes **19** and **20** upon photoactivation with light of fluence 15 J cm^{-2} gave IC_{50} values of 8–16 and 0.4–2 μM, respectively, depending on the cell type. These dyes upon photoirradiation cause lipid peroxidation and predominantly results in cell membrane damage. The type I photoprocesses initiated by dyes **19** and **20** involve two pathways *i.e.* necrotic as well as apoptotic, and necrosis was proposed as the primary pathway in these dyes. Similarly, Pagani *et al.*, have studied the cellular localisation of dyes **11** and **12** in human umbilical vein derived endothelial cells, which showed that these dyes are rapidly taken up by the cells and localize in extracellular organelles including the mitochondria (Fig. 16).[34]

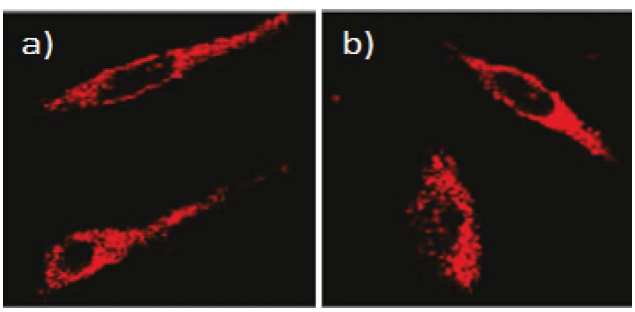

Fig. 15 Confocal laser microscopy images of HeLa cells treated for 4 h with the squaraine **20** (1 μM) and **17** (8 μM). Reprinted with permission from ref. 56. Copyright 2010, American Chemical Society.

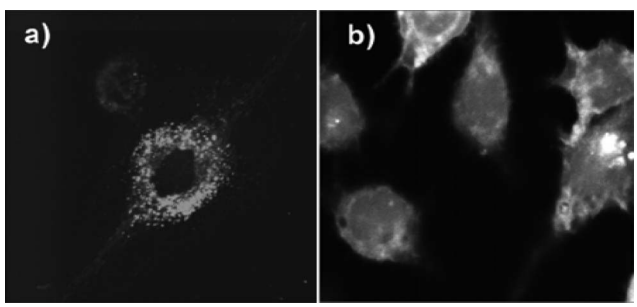

Fig. 16 Fluorescence images of cellular localisation of the squaraine dyes **11** (a) and **12** (b) in human umbilical vein derived endothelial cells (HUVEC). Reprinted with permission from ref. 38. Copyright 2008, American Chemical Society.

2.5. *In vivo* studies of squaraine-PDT action

The success of the *in vitro* studies provided the incentive for *in vivo* investigations of the squaraine dyes in the photodynamic treatment of cancer in animal models. The first of such studies was reported by us in 2008, where the polyhydroxyl squaraine dye **1c** was used for the treatment of skin cancer in mice models.[57,58] Male Swiss albino mice, induced with skin cancer, using 7,12-dimethylbenz[α]anthracene (DMBA), were treated with an intraperitoneal injection of **1c** (12.5 mg/kg body weight). The mice showed considerable reduction in tumour volume within 2 weeks after PDT treatment with a total light dose of 120 J cm^{-2} between 400 and 800 nm (Fig. 17). Interestingly, this study also showed a reversal of marker enzymes such as myeloperoxidase, β-D-glucuronidase, lactatedehydrogenase, rhodanese, hexokinase, sialic acid and caspase to near normal levels after PDT treatment with the squaraine dye **1c**.

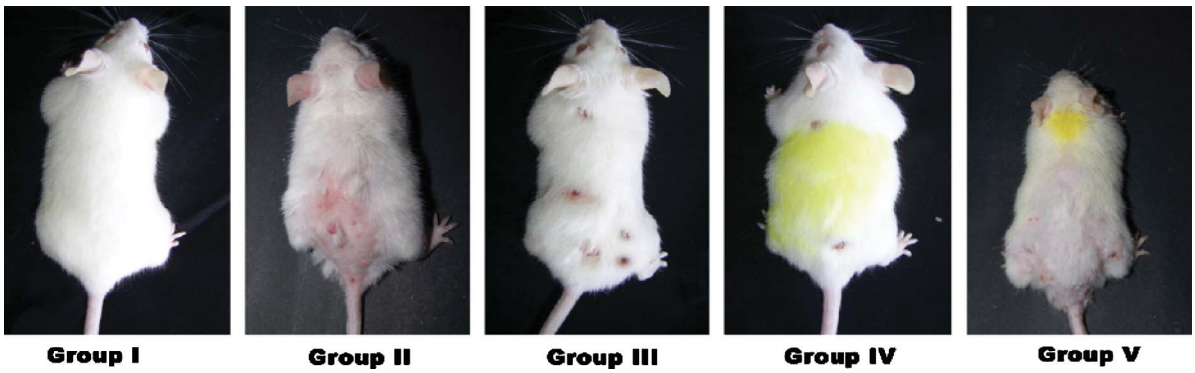

Fig. 17 Photographs of animal model groups I–IV taken after 2 weeks of PDT and group V taken after 90 days of PDT. Group I — control, group II — DMBA + croton oil, group III — DMBA + croton oil + squaraine dye **1c** (12.5 mg/kg body wt), group IV — DMBA + croton oil + squaraine dye **1c** (12.5 mg/kg body wt) + light treatment (after 2 weeks of light treatment), group V — DMBA + croton oil + squaraine dye **1c** (12.5 mg/kg body wt) + light treatment (after 90 days of light treatment). Reprinted with permission from ref. 57. Copyright 2008, Elsevier B.V.

Interestingly, it was observed that a high concentration of the dye was present in the serum within 2 h of the injection of the dye in the tumour-induced mice. After 20 h, almost all the dye was cleared from the serum. What was particularly interesting was the selective tumour targeting exhibited by squaraine dye **1c**, which does not have any tumour targeting motifs attached. The DMBA induced skin carcinogenesis caused significant elevation of oxidative stress, which further increased upon squaraine-PDT treatment and subsequently led to tumour destruction. A reduction in the caspase-3 activity was observed in tumour-induced mice in groups II and III as compared to the normal mice in group I, which was subsequently enhanced following the PDT treatment (Fig. 18).[58] These results clearly demonstrate that the squaraine-PDT treatment induces tumour destruction through the generation of significant amounts of reactive oxygen species (ROS) including singlet oxygen, and subsequent lipid peroxidative damage. An enhancement in the ROS and peroxidation products and depletion of antioxidant defences in the tumour tissues was observed after two weeks of PDT treatment. 90 days following PDT treatment, the oxidative profile of the skin samples showed almost normal level values. Further studies indicated that the photodestruction of tumours in the squaraine **1c**-PDT treatment was mediated through apoptosis.

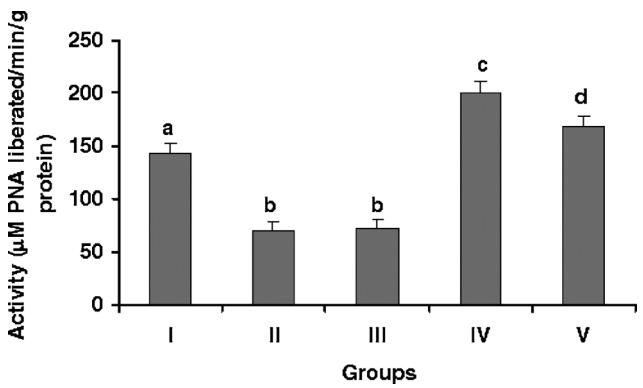

Fig. 18 Activity of caspase-3 in animal model groups I–V (for details, see Fig. 17) analysed two weeks after with and without squaraine-PDT treatment. Reprinted with permission from ref. 57. Copyright 2008, Elsevier B.V.

3. Conclusions and future perspectives

The ideal sensitiser should not only be highly selective for tumours and exhibit high potency with negligible dark toxicity but also should be broken down into simpler fractions and rapidly excreted from the body. The search for such an effective 'squaraine wonder drug' is currently an active area of photobiological research. Squaraine dyes with IC_{50} values in the nanomolar or attomolar regime as well as enhanced tumour selectivity should be easily accomplishable. Significant advances also have to be made in combining diagnosis with the squaraine-PDT treatment. Recent advances in bionanotechnology can also serve as a platform for future growth in squaraine-PDT treatment. The use of nanoparticle carrier systems for squaraines as well as the conjugation of the squaraine sensitisers to nanoparticles can, not only increase the effectiveness, but also bring in the advantages of nanotechnology to the PDT treatment. In particular, the use of nano-carrier systems can enhance the selective accumulation of the sensitisers at the tumour site by virtue of the enhanced permeation and retention (EPR) of the cancer tissues.

Though PDT treatment has been shown to be successful in treating several types of tumours, the real challenge in this area lies in the destruction of deep seated ones. This challenge needs to be addressed for PDT to grow further as an effective alternative treatment modality for cancer. Yet another, perhaps, a more serious concern is the hypoxic cellular microenvironment of tumours, which may severely compromise the PDT efficacy. Future directions in squaraine research could possibly involve such systems, which can store and deliver the reactive oxygen species to the tumour sites. The preliminary results on the use of these versatile dyes indicate that sensitisers based on squaraines and its higher homologues such as croconaines and rhodizonanes have the potential to enter clinical trials in the foreseeable future.

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