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Review

Photosensitized reactions in biology and medicine

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ABSTRACT

Photosensitized reactions are powerful tools to study cellular processes and macromolecular interactions. As therapeutic agents, photosensitizers are becoming increasingly significant in the treatment of a growing number of diseases including cancer. Moreover, chemical conjugation of photosensitizers to highly-selective receptor-specific probes, including monoclonal antibodies, promises to improve the effectiveness, selectivity and lethality of these photoactive agents. Photosensitizer-probe conjugates can site-direct photoreactions to specific cells, cell receptors and/or intracellular targets. The relatively low number of adverse effects associated with this new generation of site-directed photosensitive agents will lead to novel applications in research, photodynamic therapy and cancer chemotherapy. This chapter discusses photosensitizer agents, photoreactions, chemical conjugation to specific probes and potential applications of photosensitizer-probe conjugates.

KEYWORDS: photosensitizers, photoreaction, site-directed reactions, photodynamic therapy, photoproteolysis, chemical conjugation, conjugates

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INTRODUCTION

Photosensitizers (PS) have been used extensively in biology and medicine to elucidate cellular processes, macromolecular interactions, localize macromolecular targets and as therapeutic agents for a number of diseases [1-5]. Photosensitization occurs when a PS absorbs a photon of light and is promoted from its ground state to a singlet excited state. Subsequently, the singlet excited state PS undergoes intersystem crossing to a triplet excited state capable of reacting with a substrate either through an oxygen mediated mechanism (Type II) or radical formation (Type I) [6-9]. In a Type II mechanism, the triplet excited state PS transfers energy to molecular oxygen, thus forming reactive oxygen species (ROS) capable of further reaction. Singlet oxygen is commonly formed through this mechanism reacting with substrates in a diffusion controlled manner. When there is a transfer of an electron between an excited PS and a substrate to form a pair of radicals (PS and substrate), a Type I mechanism will dominate the photochemical process. Under aerobic conditions, electron transfer can occur between the PS and molecular oxygen, forming reactive oxygen species (ROS) that can react with other molecules. Under anaerobic conditions, electron transfer can occur directly between the PS and target molecule. Such interactions depend on the reduction potential of the PS, as well as the distance between the PS and its target.

The type of products formed upon reaction of a PS with cellular macromolecules depends on a number of factors, including the nature of the target (i.e., nucleic acids, proteins), the reactivity of the PS, the distance between PS and target, and the concentration of oxygen in the system. Both nucleic acids and proteins contain constituents that can be oxidized [10]. Photochemically induced reactions targeting these macromolecules include cleavage to smaller molecules and crosslinking to substrates [11, 12]. The conformation of the macromolecule can influence the nature of the resulting products. Products from such photoreactions can be analyzed by gel electrophoresis of nucleic acids or by combining sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (*in vitro* photoreactions) or cellular imaging (*in situ* photoreactions). Analyses of macromolecular photoreactions can be used to determine the nature of the products formed (i.e., cleavage, crosslinking), as well as demonstrating *in situ* and *in vitro* interactions between macromolecules.

In addition to elucidating macromolecular interactions in biological systems, photosensitized reactions have been exploited for their therapeutic value. Photodynamic therapy (PDT) takes advantage of the ability of certain photosensitizers to accumulate in tumor cells and, upon irradiation with visible light, selectively kill malignant cells with minimal effect on surrounding normal tissue [13, 14]. The promise of PDT as an alternative cancer therapy that carries minimal side effects and collateral damage to normal cells has spurred on the development of a number of promising PS for potential use in PDT, as well as a variety of techniques for better targeting of malignant cells. This review will discuss recent progress in the application of photosensitized reactions to study biological processes and molecular interactions, as well as in the development of new targeted and more efficient PDT.

Photosensitized reactions in biology

Photosensitized reactions have been used to study interactions and biological processes involving macromolecules, both *in vitro* and *in situ*. Common cellular targets for such photochemical reactions include nucleic acids and proteins. Both nucleic acids and proteins can be photochemically induced to undergo several reactions, including cleavage, crosslinking and the crosslinking of macromolecules to a targeted substrate. Alternatively, biological macromolecules can be modified with a PS and the resulting conjugates used to induce a site-directed photochemical reaction. This section will discuss photosensitized reactions targeting nucleic acids and proteins.

Reactions targeting nucleic acids

Photocleavage reagents, reagents that selectively cleave nucleic acids upon irradiation with light of a certain wavelength, have found utility as tools for molecular biology (DNA footprinting, structure determination) and therapeutic agents for PDT. There are three mechanisms by which PS can react with nucleic acids, (e.g., DNA), thus resulting in cleavage: 1) hydrogen atom abstraction from a deoxyribose moiety; 2) reaction of singlet oxygen with a nucleobase; and 3) electron transfer from a nucleobase to the PS [15]. Reaction at a deoxyribose residue results in direct strand breaks, whereas reaction from a nucleobase results in damage that must be further treated with pyridine to cause breakage [15] that can be analyzed by gel electrophoresis. Because guanine has a low oxidation potential, it is particularly susceptible to electron transfer and reaction with singlet oxygen. A common substrate in DNA photocleavage reactions is plasmid DNA which exists in three conformations: supercoiled form, open circular or a nicked form arising from a single strand break in the sugar-phosphate backbone, and the linear form which occurs when there are two breaks in the backbone in close proximity to one another on opposite strands [16]. The three forms can be distinguished by their apparent mobility during gel electrophoresis. This property facilitates the analyses of photoreaction products. A variety of PS reactions have been shown to produce single and double strand breaks in plasmid DNA. Wavelengths of absorption of the PS have ranged from UV (>300 nm) to visible (>400 nm) to therapeutically useful wavelengths (>600 nm). Irradiation sources have included mercury and xenon arc lamps, incadescent light sources, lasers, and more recently light emitting diodes (LED) [16].

Metal containing PS are commonly used to photocleave DNA. Zhou and collaborators have

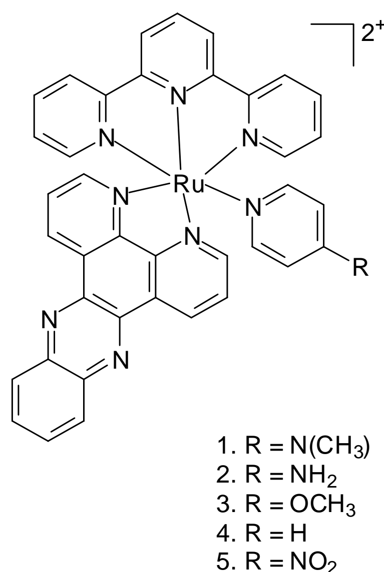

Figure 1. (Structures of $[Ru(II)(tpy)(dppz)py\text{-}R]^{2+}$ (tpy = 2,2':6",2'-terpyridine; dppz = dipyrido[3,2-a:2′,3′-c]phenazine; py-R = 4-substituted pyridine; R = $N(CH_3)_2$, NH_2, OCH_3,H,NO_2).

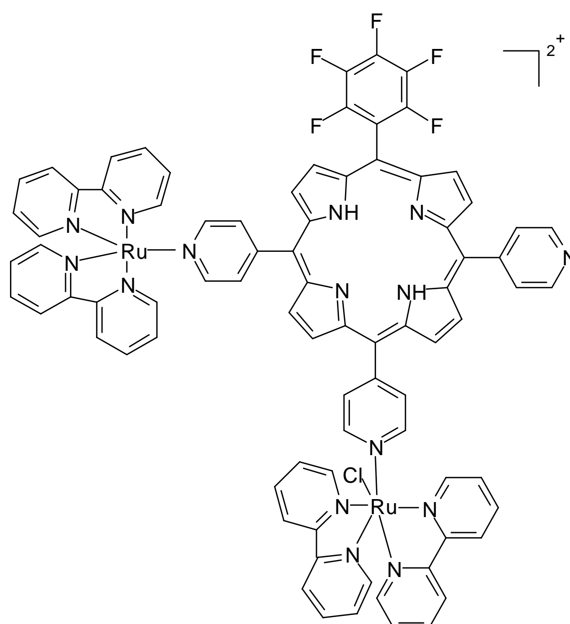

Figure 2. Complex I: meso-5-(pentafluorophenyl)-10, 15,20-tris(4-pyridyl)porphyrin.

synthesized five ruthenium (II) containing complexes to investigate substituent effects on DNA photocleavage ability, electrochemical and photophysical properties, and DNA binding [8]. The structures of the five complexes are shown in Figure 1. All metal-containing compounds exhibited high binding affinities for DNA. In addition to causing changes in the photophysical and electrochemical properties of the compounds by increasing the electron donating abilities of the pyridine substituents, they also increased their ability to generate singlet oxygen and photocleave supercoiled DNA plasmids (pBR322). When buffered solutions containing DNA and PS were irradiated with visible light (>470 nm), the order of reactivity was $N(CH_3)_2$ > NH_2 > OCH_3 >> H, NO_2. Under these conditions, H and NO_2 were largely unreactive. Supercoiled DNA was converted to the nicked form without production of linear DNA forms. Singlet oxygen quantum yields for each PS followed the order of photocleavage reactivity, with $N(CH3)_2$ having the largest quantum yield and H and NO_2 having undetectable quantum yields for singlet oxygen formation. Addition of scavengers for superoxide, hydrogen peroxide, and hydroxyl radicals (superoxide dismutase, catalase, and mannitol respectively), had no effect on the photocleavage reaction. In contrast, addition of a singlet oxygen scavenger (sodium azide), inhibited the photoreaction. Furthermore, the photocleavage was more efficient when performed in D_2O, in which singlet oxygen lifetime is increased. Taken together, these data suggest that DNA photocleavage induced by these complexes proceeds through a singlet oxygen mechanism.

Swavey and collaborators investigated the visible light induced photocleavage of plasmid DNA by a ruthenium-substituted fluorinated porphyrin under aerobic and anaerobic conditions [17, 18]. For these studies the porphyrin, meso-5-(pentafluorophenyl)-10,15,20-tris(4-pyridyl) porphyrin was synthesized (Complex I) (Figure 2). The final target compound involved coordination of the porphyrin with two $[Ru(bipy)_2Cl]^+$, where bipy is bipyridine. Buffered solutions (pH 7.2) containing pUC18 plasmid DNA and Complex I in a ratio of 100 DNA bp/metal complex were irradiated with light of wavelength >400 nm in time intervals of 2 min. After 2 minutes of irradiation, the supercoiled DNA was converted to nicked DNA and some linear DNA. Within 4 minutes most of the supercoiled DNA had reacted to form nicked and linear DNA. By 16 minutes of

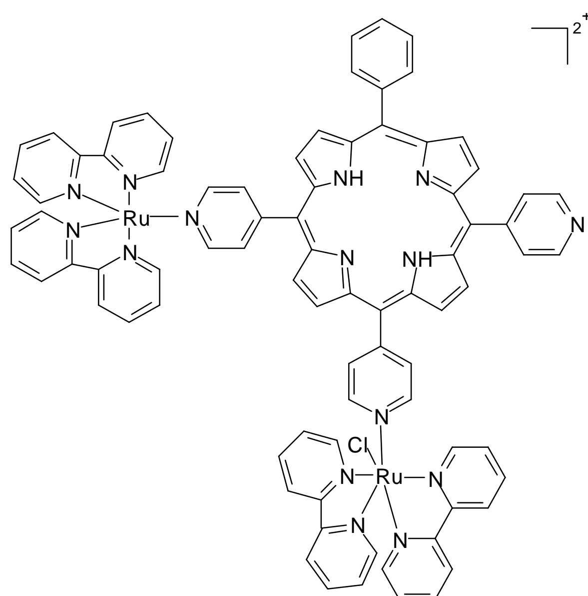

Figure 3. Analogue of Complex I.

irradiation, the supercoiled DNA plasmid was completely digested as evidenced by gel electrophoresis [17, 18].

Similar plasmid photodigestion experiments were performed under an argon atmosphere with a 10:1 plasmid DNA bp:complex ratio at 5 min intervals [18]. Under these conditions, the conversion of supercoiled DNA to nicked DNA began within 5 min of irradiation. Within 30 min, the supercoiled DNA had been fully converted to the nicked form. From these observations it was concluded that during photoreactions a type II mechanism of photocleavage dominates in the presence of oxygen while a type I mechanism dominates in its absence [18]. Additionally, when the photocleavage was performed with a phenyl substituted analogue of complex I (Figure 3) at a plasmid DNA bp:complex ratio of 5:1 (Complex II), no photocleavage was observed under anaerobic conditions but conversion of supercoiled DNA to its nicked form was complete within 30 min of irradiation under aerobic conditions. Apparently, a type I mechanism is not involved in the photocleavage reaction in complex II (plasmid DNA bp:complex ratio of 5:1), indicating a difference in the photocleavage mechanism between complexes I and II. DNA binding constants were not significantly different between complexes I and II but the electrochemical and spectroscopic properties of the two complexes were identical, indicating that the presence of fluorine atoms in complex I may account for the difference in photocleavage mechanisms.

Dipeptide conjugates of thiazole orange (TO), a fluorescent DNA intercalator were shown to photocleave plasmid DNA under visible light conditions [19]. TO was conjugated to dipeptides containing lysine and either tryptophan (TO-WK), tyrosine (TO-YK) or glycine (TO-GK) (Figure 4) [10, 19]. Lysine was the amino (N terminal) terminal peptide in each conjugate. Solutions containing TO or TO-peptide conjugate and pUC18 plasmid DNA were irradiated with visible light. Time course experiments revealed that supercoiled DNA was converted to nicked DNA in the presence of TO-WK and TO-YK but not in the presence of TO-GK, TO alone, or the lysine tryptophan dipeptide. Within 10 min of irradiation, TO-WK had completely converted the supercoiled DNA to its nicked form, and TO-YK had converted 40% of the supercoiled DNA to its nicked form [19]. Reacting TO-WK with plasmid DNA under argon gas, and adding sodium azide or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a peroxy radical scavenger), reduced the efficiency of the photocleavage reaction. In contrast, in the presence of D_2O, the above photochemical reaction was enhanced. Furthermore, plasmid DNA strand scission was direct and did not require alkaline or heating conditions as would be necessary in the case of base damage by singlet oxygen. Taken together, the data suggest conversion of singlet oxygen to the peroxy radical through a reaction with the amino acid residues tryptophan or tyrosine. Indeed both amino acids are known to react with singlet oxygen, while the amino acid glycine does not [19, 20].

In an extension of the above work, Biton and collaborators have conjugated TO and an analogue (TO2) to a triplex forming oligonucleotide (TFO) targeting MDM2 (murine double minute oncogene), an oncogene which accelerates the degradation of tumor suppressor p53 [21]. Conjugation of TO to the TFO was accomplished through an amino acid linker containing either the residues tryptophan or glycine. Triplex formation was induced by incubating the pCMV-MDM2 plasmid with the

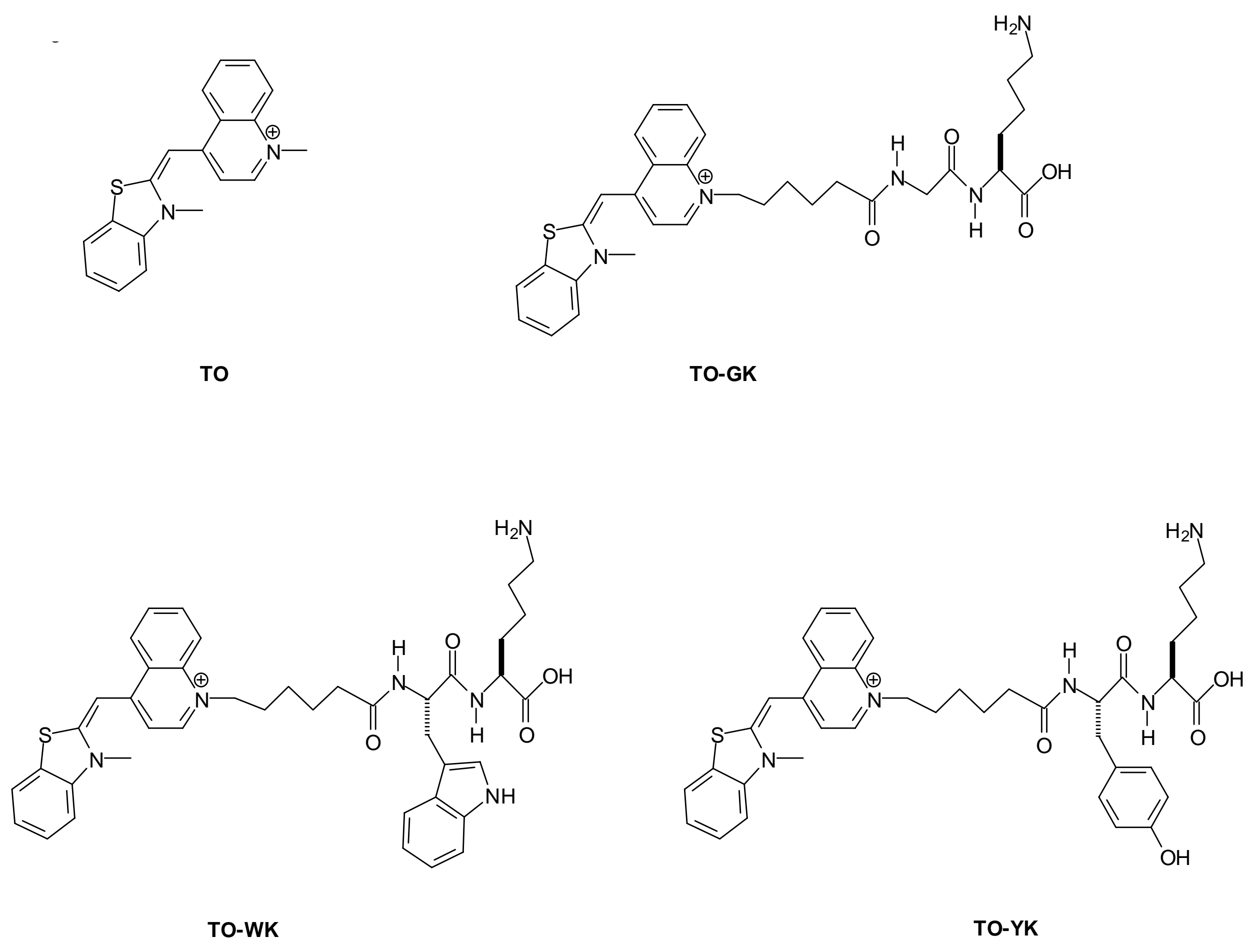

Figure 4. Dipeptides of thiazole orange (TO).

conjugate overnight at 37ºC. After incubation, the conjugate triplex was irradiated with visible light and products analyzed for the presence of nicked or linear plasmid DNA. When lysine was present in the conjugate, nicked DNA was the only product. Linear DNA was formed when tryptophan was in the conjugate. The photoreaction was oxygen dependent, with sodium azide and Trolox reducing its efficiency. Additionally, when the conjugates contained DNA/LNA as oligonucleotides, the photocleavage reaction by the tryptophan conjugate resulted in a greater formation of linear DNA, possibly because of better hybridization of this probe with the pCVM-MDM2 plasmid.

Reactions targeting proteins

A small number of amino acid residues can react directly with UVA (280-320 nm) and UVB (320-400 nm), with reactions proceeding through radicals and excited state species [20]. The UVA reactive amino acid residues are tryptophan, tyrosine, histidine, and disulfides (cystine). Alternatively, damage to proteins can occur through visible light induced reactions between reactive amino acid residues and singlet oxygen produced from the excited state of a PS. In addition to the aforementioned amino acid residues, cysteine and methionine are also susceptible to such reactions. The resulting damage may include cleavage, conformational changes, and aggregation of polypeptide chains. The development of photoproteases, photoreactive reagents that cleave proteins on exposure to visible light, is still rather new [11, 22-24]. These reagents can be used to aid in elucidating proteinaceous subcellular structures, macromolecular interactions, and as therapeutic agents for site-directed PDT.

The attractiveness of such reagents lies in the fact that their protease ability relies on their exposure to light, terminating with its removal. Furthermore, photoproteases can be designed in a manner that enables them to site-specifically target other macromolecules including proteins. This was demonstrated by the attachment of 2-bromo-4'-nitroacetophenone and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) to cysteine residues inducing protein self cleavage on exposure to UVA light [24].

One early example of site-specific photoinduced protein cleavage was demonstrated by Kumar and collaborators when they used N-(1-phenylalanine)-4-(1-pyrene) butyramide to cleave bovine serum albumin (BSA) and chicken egg lysozyme upon irradiation with UVB light [23]. Photocleavage was found to be specific in the case of each protein, cleaving BSA between leucine-346 and arginine-347, and lysozyme between tryptophan-108 and valine-109. More recently, Kumar and collaborators showed that Co(III) complexes can photochemically fragment chicken egg lysozyme [25, 26]. Figure 5 contains the structures of the Co(III) complexes. When chicken egg lysozyme was irradiated at 310 nm or higher in the presence of Co(III) complex in concentrations ranging from 0.5 to 10 mM, cleavage resulted in two photoproducts, one of molecular mass ~10.5 kDa, the other of molecular mass ~3.5 kDa. Photocleavage was found to be dependent on the concentration of Co(III), as well as the presence of Co(III) in the complex since complexes containing Ni(II), Co(II), and Gd(III) inhibited the reaction. The reaction could be performed at wavelengths up to 390 nm for two of the complexes with the same two fragments resulting, while $[Co(NH_3)_6]^{+3}$ did not cleave the protein above 310 nm. Additionally, Co(III) complexes were found to cleave apotransferrin, bovine serum albumin, and yeast enolase. Roy and collaborators, demonstrated that the oxo-bridged diiron(III) complex of L-histidine and a heterocyclic base $[Fe_2(\mu\text{-}O)(L\text{-}his)_2(B)_2]\text{-}(ClO_4)_2$, where B is 2 dipyrido [3,2-d:2',3'-f]-quinoxaline (dpq), can cleave BSA site-specifically when irradiated with 365 nm light [27]. SDS-PAGE analysis showed the formation of two photoproducts with apparent molecular masses of 45 kDa and 20 kDa respectively. Increasing concentrations of the complex resulted in increased protein photocleavage. Singlet oxygen scavengers (2,2,6,6-tetramethylpipiperidine (TEMP) and NaN_3) did not inhibit the reaction, however, hydroxyl radical scavengers (KI and ethanol) showed a significant inhibition of reaction [27].

Visible light dyes have been conjugated to proteins to site-specifically induce the cleavage of DNA and proteins [11, 22, 28, 29]. Rose Bengal (RB) is a xanthene dye that absorbs light with a λmax of absorption at 560 nm in water and can initiate visible light photoreactions in the presence or absence of oxygen [29, 30]. The reaction of a hexanoic acid derivative of RB containing an N-hydroxysuccinimide group with murine monoclonal anti-myosin antibodies or affinity purified goat polyclonal anti-mouse IgG antibodies afforded conjugates that could photochemically induce the cleavage of chicken skeletal myosin heavy chain (MHC) [11]. Figure 6 contains the structure of the N-hydroxysuccinimide- hexanoic acid (NHS) derivative of RB (RBHA-NHS). The photo-induced digestion of MHC was accomplished at 37°C using both a direct and an indirect photochemical method. In the direct method, MHC was incubated with RB-conjugated affinity purified mouse anti-myosin antibodies prior to photolysis. In the indirect method, MHC

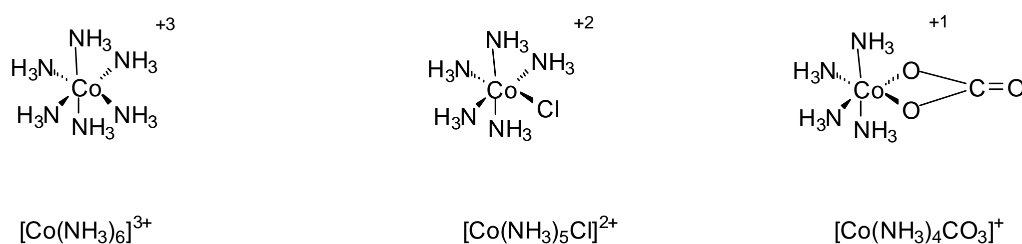

Figure 5. Co(III) complexes.

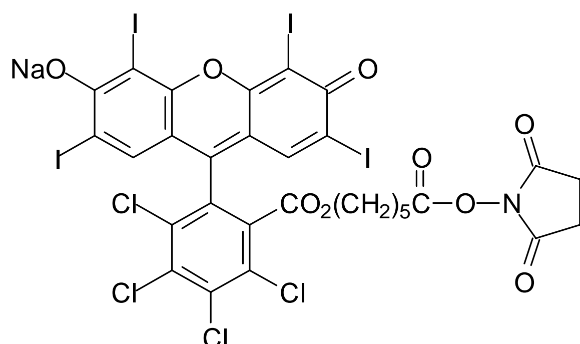

Figure 6. RB hexanoic acid-N-hydroxysuccinimide (RBHA-NHS).

was incubated with affinity purified mouse anti-myosin antibodies, followed by an additional incubation with RB-conjugated affinity purified goat anti-mouse antibodies. The resulting immuno-conjugates were then irradiated at room temperature with visible light from a 150 W halogen lamp. SDS-PAGE and immunoblot analyses confirmed that photodigestion products formed by the direct or indirect method were fragments of the intact MHC polypeptide. SDS-PAGE and immunoblot analyses revealed that MHC was not digested when similar reactions were performed with either free RB or unconjugated anti-myosin antibodies. Furthermore, reactions performed with β-galactosidase as the target protein yielded no reaction photoproducts. Moreover, the above photo-induced digestions of MHC were time and temperature dependent as an increase in digested products was observed with an increase in either temperature or time of irradiation.

Similar *in situ* photochemical reactions were used to demonstrate the site-directed disruption of the cell cytoskeleton [22]. For this purpose, two different proteins that specifically bind actin and filamentous actin (f-actin), affinity purified rabbit anti-actin antibodies and heavy meromyosin (HMM) respectively, were conjugated to RB through reaction with RBHA-NHS [22]. *In situ* photochemistry was performed on actively growing mammalian tissue culture cells (mouse embryo fibroblasts). Cells were incubated with RB-conjugated anti-actin antibodies or RB-conjugated HMM, and irradiated with visible light. After irradiation, cells were probed with fluorescently-labeled phalloidin to evidence the disruption of the actin cytoskeleton. Fluorescent microscopy revealed the disruption of the actin cytoskeleton by these RB-conjugates. *In situ* photoreaction with these RB-actin binding conjugates was specific for the actin cytoskleleton since neither free RB nor RB-conjugated antibodies directed against nuclear histones, a non-component of the actin cytoskeleton were able to disrupt the cytoskeleton. Similarly, SDS-PAGE and immunoblot analyses revealed that purified actin was photodigested *in vitro* when photochemical digestions were conducted with purified skeletal muscle actin incubated with either RB-conjugated anti-actin antibodies or RB-conjugated HMM and complexes irradiated with visible light from a 150W halogen lamp. Similar photochemical methods were used to demonstrate the *in situ* and *in vitro* association of DNA base excision repair (BER) proteins with *in vitro* assembled microtubules and with cellular microtubule networks during interphase and mitosis [31, 32].

Photodynamic therapy

Photodynamic therapy involves administering a nontoxic PS with a long wavelength of absorption to a patient, followed by irradiation of the affected area with an appropriate light source. ROS play a dual role in PDT. ROS have been associated with the onset of a number of clinical diseases, including cancer [2, 33, 34]. Malignant cells accumulate an advantageous set of genetic aberrations bestowing them with, among a number of properties, the ability to survive by boosting their cellular defenses to ever increasing ROS levels. Ideal PS for PDT have high quantum efficiencies for the formation of singlet oxygen. Therefore, the therapeutic basis of PDT is to raise cellular ROS above levels that cells can effectively combat [35]. Once cells have been overwhelmed by ROS-induced damage, cell death may occur by a number of pathways, including apoptosis. Designs for potential effective PDT agents must include delivery of the PS in a site-directed manner, as well as the potential of the PS to induce enough ROS damage to overwhelm cellular defenses and/or directly neutralize DNA damage repairing resources.

Alonso and collaborators synthesized three cationic thiol-reactive porphyrin derivatives that can be

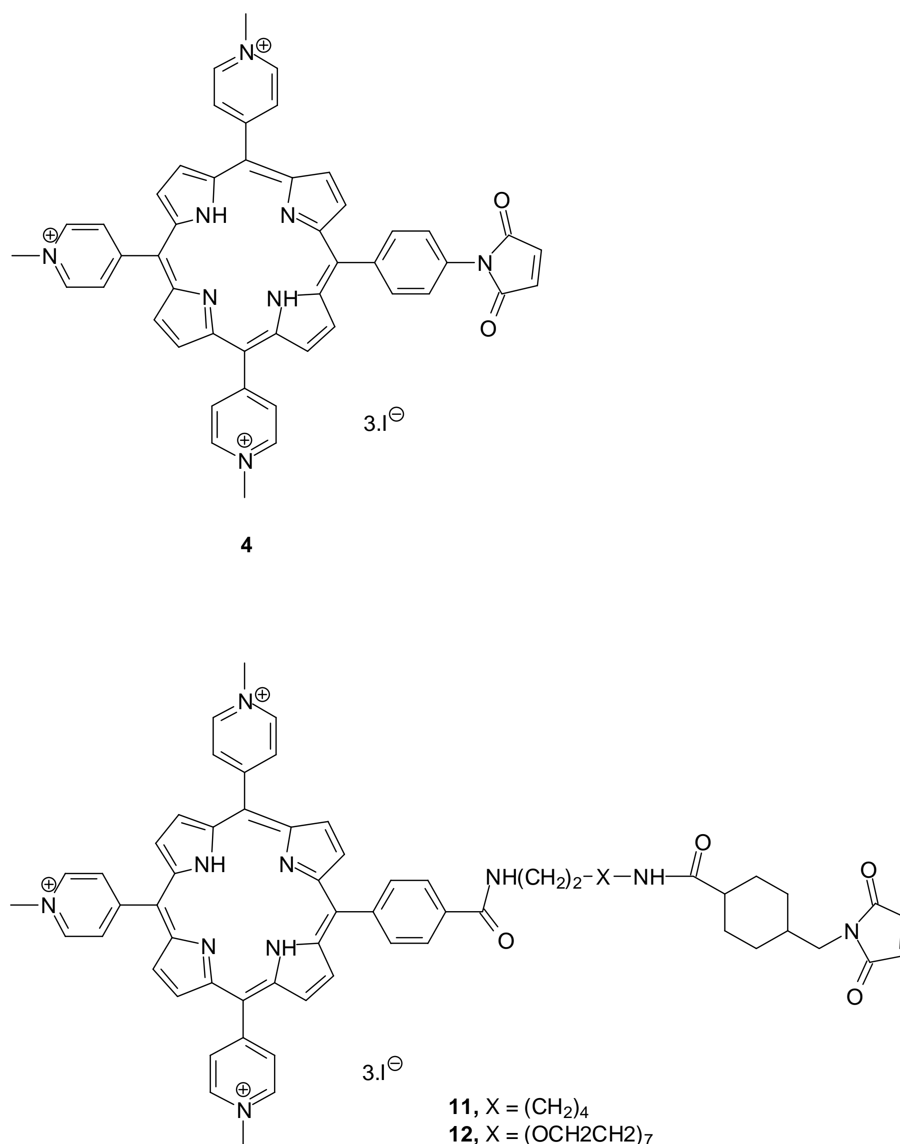

Figure 7. Cationic thiol-reactive porphyrin derivatives.

conjugated to L19 antiangiogenic antibodies [13]. The derivatives contain reactive linker arms of different lengths, thus enabling the study of the effect of chain length on SIP-PS conjugate photocytotoxicity. Figure 7 contains the structures of the porphyrin derivatives. Porphyrin PS were conjugated to SIP(L19), a recombinant antiangiogenic antibody expressed in small immunoprotein format. After the immunoreactivity of the conjugates for its specific target was confirmed, photocytotoxicty studies were performed using fibroblasts in culture expressing the antigen for the L19 antibody. Photocytotoxicity assays were performed by incubating fibroblasts with SIP-porphyrin conjugates followed by irradiation with visible light. All three compounds were photocytotoxic against fibroblasts. The PS-conjugate having the longest linker was most reactive while that having the shortest was the least reactive, suggesting that longer linker arms conferred an increased freedom for the PS to move and thereby cause damage.

An interesting approach to modulating PS ability to generate singlet oxygen and target specific sites in a cell is through the design of photodynamic

molecular beacons (PMB) [36, 37]. These constructs are an extension of killer beacons and can be used for both imaging and therapy [38]. In a PMB, a PS is linked to a singlet oxygen scavenger/ quencher through a disease specific linker [36]. While the construct remains intact, there is no photosensitization through singlet oxygen generation. However, when the construct interacts with a target protein that can cleave the linker, thus liberating the PS from the singlet oxygen quencher moiety, photosensitization can occur. Zheng and collaborators linked the porphyrin, pyropheophorbide a to a carotenoid, which can scavenge singlet oxygen, through a cleavable peptide sequence containing the caspase-3 recognition site (Pyro-peptide-CAR, PPC) (Figure 8) [36].

When PPC was incubated with caspase-3 peptide construct, cleavage was induced as evidenced by the detection of pyropheophorbide a and carotenoid fragments by HPLC. No cleavage was observed when a caspase-3 inhibitor was added to the incubation mixture. The ability of the PS to generate singlet oxygen was tested by irradiating with 523 nm light with PPC alone, caspase-3, and caspase-3 plus caspase-3 inhibitor, and measuring the NIR luminescence at 1270 nm of any singlet oxygen generated. Only when PPC was irradiated in the presence of caspase-3 was singlet oxygen formed. Irradiation of the PS with caspase-3 and caspase-3 plus inhibitor did not affect the ability of the PS to produce singlet oxygen therefore the cleavage of PPC by caspase-3 was a requirement for photosensitization. With this technique, Zheng and collaborators demonstrated that the tumor-associated protease induced cleavage of a matrix metalloproteinase-7 (MMP7) was triggered by PMB. This cleavage resulted in photosensitized production of singlet oxygen, as well as photodynamically-mediated cancer cell cytotoxicity [37].

Earlier in this review we discussed that effective PDT agents must ideally contain certain properties, including the site-directed delivery of PS and the PS potential to induce ROS damage to overcome defenses and/or directly neutralize DNA damage repairing resources. To address the use of PDT agents with potential for directly neutralizing DNA damage repair mechanisms, strategies were developed to target the murine 8-oxoguanine glycosylase (mOGG1), a DNA base excision repair enzyme, for photoinduced proteolysis. Conlon and collaborators used mOGG1 as a potential DNA repair target for site-directed photoinduced proteolysis [33]. ROS react with DNA to oxidize guanine to 8-oxoguanine which, if left unrepaired, may lead to mutations, apoptosis, and cell death. Because PDT relies on the ability of a PS to generate high levels of ROS that cells cannot effectively combat, combining standard PDT with site-directing PS to destroy or neutralize key enzymes of the DNA repair pathway may be an effective strategy for boosting the power of PDT to induce cell death and tumor

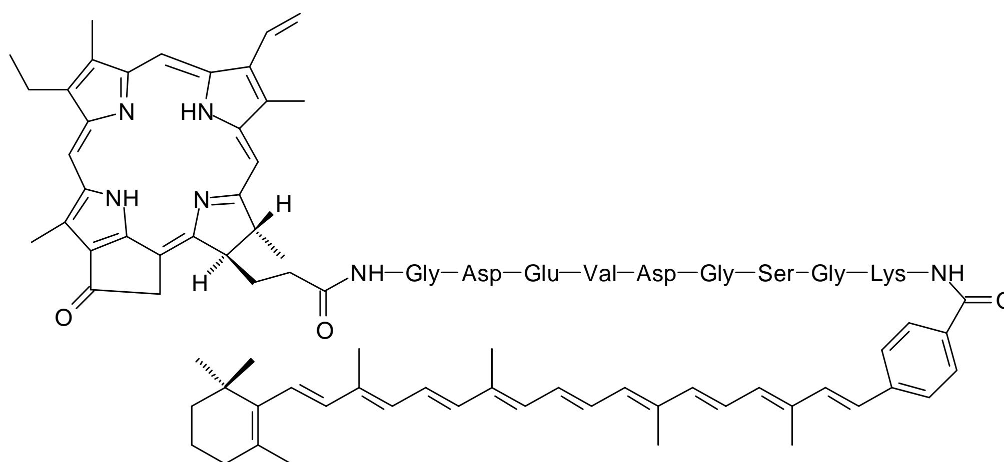

Figure 8. Pyro-peptide-CAR (PPC).

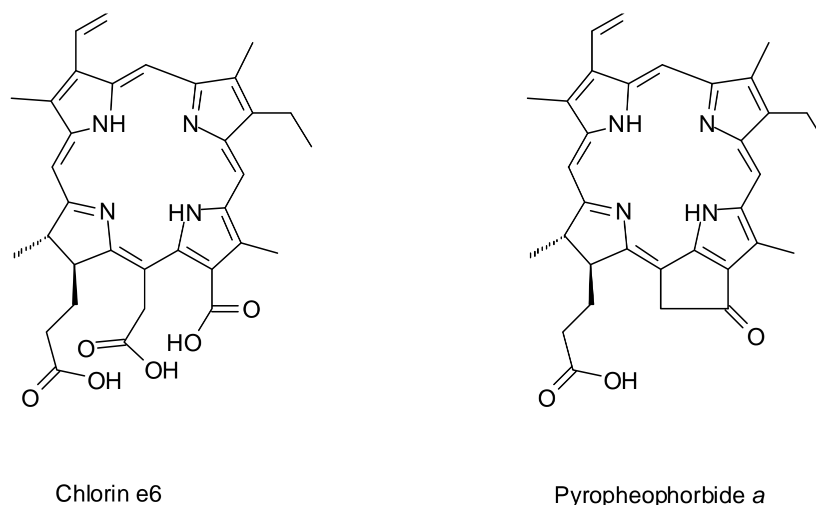

Figure 9. Pyropheophorbide a and chlorin e6.

ablation. Pyropheophorbide a and chlorin e6 were conjugated to streptavidin, rabbit anti-mOGG1 antibodies, or goat anti-rabbit IgG antibodies. Figure 9 contains structures of the porphyrins. Conlon and collaborators used visible light of wavelength greater than 600 nm to induce reactions with the porphyrins pyropheophorbide a and chlorin e6 to site-direct the photoinduced proteolysis of mOGG1. To construct the site-directed probes, carboxyl groups of the porphyrins were activated through reaction with 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC), thus forming a porphyrin ester reactive to proteins. Coupling to proteins was achieved by reacting each porphyrin activated ester with affinity purified rabbit anti-mOGG1 antibodies or goat anti-rabbit IgG antibodies. Additionally, chlorin e6 was conjugated to streptavidin. Photochemical methods were similar to those developed for RB protein conjugates where a direct and indirect method was used [11, 32]. In the case of the direct method, mOGG1 was incubated with porphyrin conjugated anti-mOGG1 antibodies, followed by irradiation with light from a 150W halogen lamp using a 600 nm wavelength cut-off filter. For the indirect method, mOGG1 was incubated with anti-mOGG1 antibodies, followed by incubation with porphyrin-conjugated goat anti-rabbit antibodies. Alternatively, mOGG1 was incubated with an appropriate dilution of biotinyated anti-mOGG1 antibodies, followed by incubation with chlorin e6 conjugated streptavidin. Irradiation was performed as for the direct method.

Both pyropheophorbide a and chlorin e6 conjugated to anti-mOGG1 antibodies photodigested mOGG1 as judged by SDS-PAGE analysis. When the photoreaction was performed indirectly using porphyrin conjugated goat anti-rabbit IgG antibodies, a greater extent of mOGG1 photodigestion was observed, possibly because of the higher local PS concentration at the reaction site afforded by porphyrin conjugated antibodies-anti-mOGG1 antibody complexes. It is also possible that direct conjugation of porphyrins to specific anti-mOGG1 antibodies reduced their binding affinity for the mOGG1 protein. When the photodigestion of mOGG1 was performed using biotinylated anti-mOGG1 antibodies and chlorin e6 conjugated streptavidin, mOGG1 was digested but not to the extent of either the direct or indirect method discussed above. There are several potential reasons for this observation. These may include 1) the biotinylation procedure was detrimental to the anti-mOGG1 antibodies, 2) streptavidin affinity for biotin could have been decreased by conjugation to the PS, 3) the increase in the local concentration of PS could be lower than that found in the indirect method and, 4) the smaller size of streptavidin as compared to that of the antibodies prevents it from getting close enough to the target mOGG1 protein. The presence of sodium azide did not alter the

outcome of the above photoreactions, possibly ruling out a singlet oxygen mechanism in the photodigestion of mOGG1 by these methods.

Selectively targeting tumor DNA repair enzymes for photoinduced proteolysis requires that PS conjugates are site-directly targeted and internalized into malignant cells. A similar site-directed approach with intracellular delivery of chemical conjugates is found in a growing number of antibody-drug conjugates (ADCs), a new class of targeted cancer chemotherapeutic agents [39, 40]. Although many ADCs are still undergoing drug phase trials, ADCs such as trastuzumab emtansine (T-DM1) site-directly targets human estrogen receptor2 (HER2)-positive breast cancer tumors delivering a cytotoxic conjugate intra-cellularly to induce tumor cell apoptosis [41, 42]. T-DM1, like PS-conjugates with cancer chemotherapeutic potential, selectively binds to and inactivates HER2 (through the selectivity of trastuzumab (T), a humanized monoclonal antibody) while the conjugate mertansine (DM1), a compound interfering with microtubules and toxic to normal cells, is, upon binding, delivered by endocytosis into HER2-positive tumor cells inducing their cell death (i.e., ADC-dependent apoptosis).

The antibody component of ADCs and PS-conjugates gives these constructs high tumor selectivity minimizing damage to normal tissues and increasing patient's drug therapy tolerability. Combining light-induced ROS production with site-directed photolytic methods targeting ROS scavengers and DNA repair enzymes for inactivation or combining site-directed light-induced disruption of microtubules together with the delivery of cytotoxins targeting microtubules or other metabolites may lead to improved PDT strategies that would have dual or multiple cytotoxic effects on tumor cells. For instance, an improved PDT based on the above strategies would be capable of producing high levels of ROS while simultaneously destroying ROS scavengers and/or DNA repair enzymes, thus on one hand overwhelming malignant cells with ROS, while on the other reducing their ability to combat effectively oxidative damage.

Other potential applications

The success demonstrated by the above strategies to site-specifically photodigest enzymes, inactivating these proteins, at wavelengths used in PDT may lead to other therapeutically relevant applications including the faster inactivation of venoms and other natural toxins by PS-conjugated antivenoms and antitoxins. A number of antibody-based antivenoms are currently in use against a variety of venomous scorpions, snakes, spiders, and marine invertebrates. There are about 3,500 known species of snakes in the world, about 11% of these are considered potentially venomous to humans. Snake venoms are complex mixtures composed mostly of proteins (glycoproteins) and low molecular weight polypeptides. A number of proteins in venoms are enzymes including proteases, nucleases, phospholipases and phosphodiesterases [43]. In most species, these proteinaceous components constitute the lethal elements of the snake's venom.

Although snakes often use their venoms as a defensive mechanism, venoms' primary purpose is to secure prey. Once delivered after a snake bite, venoms provoke complex reactions including blocking receptors and/or releasing agents that adversely affect physiological processes. Snake venoms act principally in two ways, as neurotoxins or as hemotoxic agents. Neurotoxic peptides bind to nicotinic acetylcholine receptors in neuromuscular junctions of skeletal muscles. Receptor binding in neuromuscular junctions leads to paralysis and death. Hemotoxin venoms are more pleiotropic in actions destroying erythrocytes leading to hemolysis, interfering with blood clotting, and/or causing generalized tissue and organ damage. Injury from hemotoxic agents is often painful and may lead to permanent tissue damage. The process by which hemotoxic venoms precipitate death is much slower than that caused by neurotoxin snake venoms. Certain snake venoms may demonstrate both neurotoxic and hemotoxic effects on patients. Responses to venoms are often rapid and unpredictable with regard to complications, thus an accidental snake bite on a patient is nearly always a fast evolving emergency situation that can be potentially lethal. Patients bitten by poisonous snakes are treated with combinations of supportive medications and an antivenom (antivenene, antivenin) if available. Antivenoms may be monovalent or polyvalent depending on whether they are specific against a snake's species' venom or show effectiveness against a wide range of species venoms.

Antivenoms are isolates from antisera elicited in large animal hosts (horses, sheep or goats) after repeated immunizations with relatively small doses of the venom's active component (a protein or polypeptide). Antibodies directed against the venom's active protein are then harvested from the host animal's blood and immunoglobulin fractions or fragments purified and used to treat envenomations. Anti-snake venom therapy of envenomations first involves the identification of the causative snake, usually not only the snake's species but the regional variety as well. This is followed by systemic administration of the specific antivenom to the patient. Patient recovery depends largely on the inactivation of the venom by antivenom antibodies. Inactivation of venom by antivenom may take hours or days of intensive care, a critical time for an already sickened patient. Moreover, some patients may react to the antivenom with a hypersensitivity immune reaction (anaphylaxis).

Combining the use of PS-conjugated antivenom constructs with site-directed photodigestion of venom's active components may represent a much more effective strategy than current antivenom therapies. Therapies to neutralize the effects of snake's venoms based on PS-conjugated antivenoms would have advantages including a net increase in the speed at which the active components of snake venoms are neutralized and the destruction of antivenom-venom complexes would occur without participation of complement action or the patient's own immune system. Clinically and physiologically, both the increased speed of venom neutralization and circumventing the immune system would be advantageous.

Except for irradiation conditions, chemical conjugation of PS to antivenom fractions or immunoglobulin fragments would be similar to methodologies described earlier for other protein PS-conjugates. PDT would follow the systemic administration of PS-conjugated antivenom. Irradiation of envenomated patient's blood would be accomplished by extracorporeal PDT or photopheresis [44, 45]. Photophoresis involves the systemic administration of a PS followed by *ex vivo* light irradiation of the appropriate wavelength. Extracorporeal PDT has been used effectively to target immune cells, preventing graft versus host disease (GvHD) and to treat certain skin conditions. A prototype photopheresis flow-cell for a laboratory mouse is shown in Figure 10. Photopheresis effects on lethal components of the venom by PS-conjugated antivenom would be similar to those described above for the inactivation of the enzyme mOGG1 by PS-conjugated anti-mOGG1 antibodies. An approach combining administration of specific PS-conjugated antivenom followed by extra-corporeal PDT or photopheresis would not be limited to snake venoms since such PDT methodology would be applicable to venoms from other organisms for which there is an antivenom or anti-toxin suitable for PS conjugation. PDT methodologies of this type have the potential to increase the effectiveness of therapies to treat accidental bites from venomous organisms.

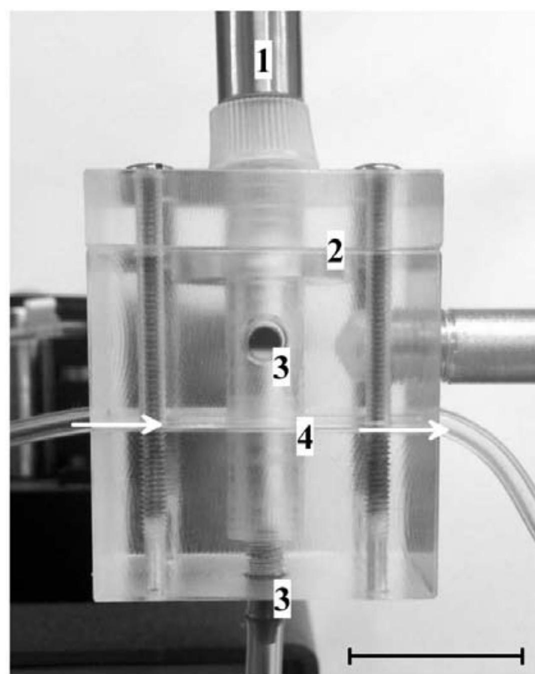

Figure 10. Photopheresis-mediated inactivation of venom by PS-conjugated antivenom. Experimental photopheresis chamber: (1) Fiber optic irradiation port. (2) Cut-off filter holder. (3) Cooling air intake and output ports. (4) Quartz photocell. Arrows point the direction of blood flow. Bar, 20 mm.

CONCLUSIONS

Photosensitized reactions are powerful tools for biology and medicine. The ability of PS-conjugates

to induce photochemical reactions on a target macromolecule either aerobically or anaerobically can lead to the generation of a variety of valuable photosensitive agents for the elucidation of a macromolecule's structure, localization and interactions with cellular components. Moreover, chemical conjugation of PS to receptor-specific monoclonal antibodies increases the selectivity and lethality of PDT agents by site-directing photoreactions to specific cell targets. In contrast to ADCs and similar cancer chemotherapeutic agents, site-specific PDT agents do not require cellular activation becoming active only on the addition of light. Upon light activation, PS conjugates would be able to inflict on malignant cells a wide range of damage leading to apoptosis and cell death. Increasing the selectivity, effectiveness and lethality of PS conjugates would lead to a new generation of photosensitive agents for research, PDT and cancer chemotherapy.

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