Using enzymatic reactions to enhance the photodynamic therapy effect of porphyrin dityrosine phosphates†

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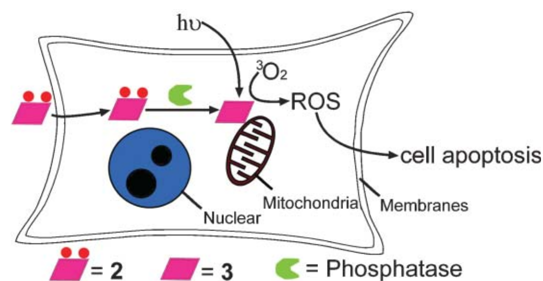
This paper reports the synthesis and photodynamic therapy (PDT) effect of a porphyrin derivative containing tyrosine phosphate, which promises a new, useful approach to develop PDT agents.

Photodynamic therapy (PDT) uses light to irradiate a photosensitizer (PS) to form intracellular reactive oxygen species (ROS) that kill cells.[1–3] It is an attractive minimal-invasive treatment protocol for cancers and several neovascular diseases because of the preferential distribution and retention of photosensitizers in neoplastic tissues and the localized phototoxic effect upon irradiation.[4] Furthermore, unlike common cancer therapies such as surgery, radiation therapy, and chemotherapy, which are more or less immunosuppressive, PDT can stimulate the immune response against cancers.[5] The essential element in the development of PDT is the photosensitizer that absorbs the appropriate wavelength of light at the site of the photodynamic reaction to produce singlet oxygen ($^1O_2$, presumably the most important ROS) and give the desired therapeutic outcome. Among a large number of photosensitizers synthesized and studied, only a few compounds received regulatory approval for clinical use—for example, Photofrin®, a complex of porphyrin oligomers, licensed for treatment of cancers in lung, stomach, cervix, bladder, and esophagus; Foscan®, the *m*-tetra(3-hydroxyphenyl)chlorin (*m*-THPC), approved against head and neck cancers.[6] These photosensitizers still have drawbacks, such as dark cytotoxicity and post-treatment skin sensitivity, due to their insufficient selectivity.[7] Hence, it is necessary to optimize PDT agents and explore the structure–activity relationship (SAR) of photosensitizers carefully and thoroughly *via* chemically modifying porphyrin derivatives.[8]

Recently, several new strategies have emerged to improve the performance of PDT agents, including conjugating them with oligonucleotides, monoclonal antibodies, epidermal growth factors, carrier proteins, carbohydrates, or hydrophilic polymers for selective delivery of the agents into tumor tissues.[9] For example, conjugates of poly-*S*-lysine or poly-arginine with porphyrins have achieved enhanced cellular uptake and significantly improved efficacy in PDT experiments.[9] Some of these conjugates even exhibited the ability to photo-inactivate methicillin-resistant *Staphylococcus aureus* (MRSA).[9,10] These poly-*S*-lysine or poly-arginine peptide conjugated porphyrins still suffer from disadvantages such as the synthetic difficulties of polypeptides, high cost, and undesired aggregation. Besides the conjugates of polycationic peptides with porphyrins, other cationic porphyrins[3] also have been investigated as photosensitizers due to their binding affinity with nucleic acids and their ability to photocleave DNA selectively, to inhibit telomerases, or to serve as vehicles for oligonucleotide delivery to tumors.[11,12] The photooxidation of the counter ions of the cationic porphyrins, however, leads to the ion-pairs formation, causes an extensive aggregation of porphyrins, and decreases PDT effiects.[11]

Despite a variety of approaches to derivatize porphyrins and porphyrin-related compounds, the lack of specific target(s) of porphyrin-based photosensitizers[2] and the dark cytotoxicity still remain a major challenge for PDT.[2] To address these important issues, we designed and made a new porphyrin derivative (protoporphyrin dityrosine phosphate (PpIX-DTP), **2**) as a substrate of phosphatase to serve as a potential agent to specifically target cancer cells because the over-expression of phosphatases is closely associated with the development of cancers.[13] Presumably, the over-expressed phosphatases in cancer cells would dephosphorylate the substrate (*e.g.*, **2**), and the more hydrophobic product (*e.g.*, **3**) would form and build up inside the cells to cause photo damages to cancer cells upon irradiation by light (Scheme 1). The result in this work shows that **2** indeed enters cancer cells and accumulates in mitochondria.† As shown in Fig. 1A, **2** kills 50% of HeLa cells at 5.1 J cm^{-2}, and **1** obviously requires more than 45 J cm^{-2} to reach the same effect. This preliminary result, together with the absence of a PDT effect for a porphyrin bisphosphonate (**5**), suggests that this new enzymatic



Scheme 1 Conversion of the precursor (**2**) by enzyme(s) in a cell to yield the photosensitizer (**3**) that induces cell death under the irradiation of light. The red dots represent the phosphate groups.

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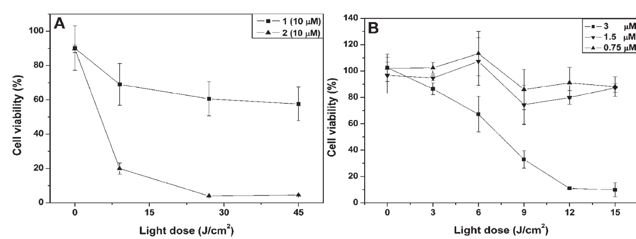


Fig. 1 (A) PDT effect of **1** & **2** on HeLa cell at a concentration of 10 μM; (B) PDT effect of **2** on HeLa cell at different concentrations.

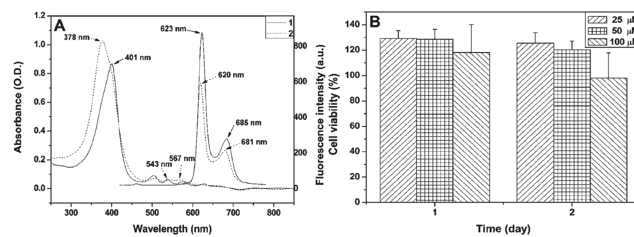
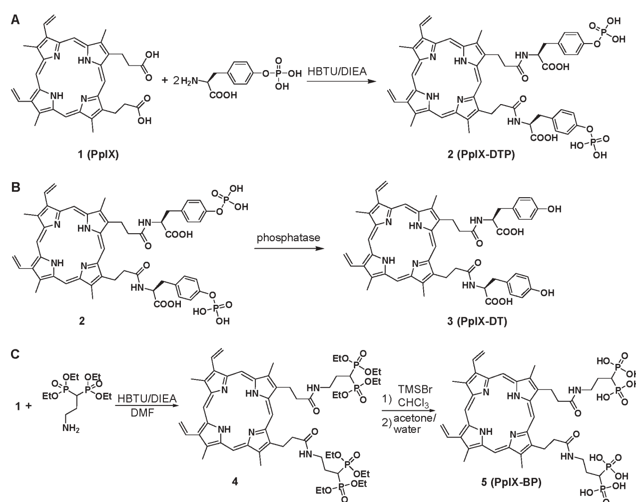


Fig. 2 (A) Absorption and emission spectra of **1** & **2** in water; (B) MTT assay of **2** on HeLa cell.

approach offers an effective alternative to create potential PDT therapeutics for the management of cancers.

Partially based on our previous work related to porphyrins[14] and dephosphorylation,[15] we designed **2** to compare its PDT effect with that of protoporphyrin (PpIX, **1**, Scheme 2). We chose protoporphyrin as the parent compound for reasons other than its light absorption characteristics: (i) **1** has already received approval for the PDT treatment of actinic keratosis and basal cell carcinoma;[16] (ii) the derivatives of **1** could be biodegradable after PDT, thus minimizing dark toxicity; and (iii) the tendency of **1** to form aggregate readily[17] should increase the retention of **3** inside the cells to enhance the PDT effect after intracellular dephosphorylation of **2**. Because many diseases (*e.g.*, cancer, diabetes, Alzheimer's disease, and multiple sclerosis) are associated with the abnormal activities of phosphatases and/or kinases, especially metastatic cancer cells over-expressing tyrosine phosphatases,[13] the most useful and important feature of **2** would be the ability to form the hydrophobic protoporphyrin dityrosine (PpIX-DT, **3**) as the PDT agent according to the level of the expression of phosphatases. In other words, once **2** is taken up by the HeLa cells, the intracellular enzymatic reaction changes **2** to the more hydrophobic metabolite **3** (Scheme 2B), which promises a better cellular retention due to a better affinity with cell membranes and lipidic mitochondria, and thus killing the cells under the irradiation with light.

Scheme 2 shows the synthesis of the new phosphate conjugates of protoporphyrin: **2** and **5**. Starting from **1** and *o*-phosphotyrosine and using HBTU as the coupling agent and DIEA as a base,



Scheme 2 (A) Synthesis of **2**; (B) dephosphorylation of **2** by phosphatases to give **3**; (C) synthesis of **5**. HBTU: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. DIEA: diisopropylethylamine.

we obtained **2** in 46% yield after purification. We also prepared **4** by using HBTU and DIEA to couple **1** with three equiv. of tetraethyl-3-amino-propane-1,1-bisphosphonate. The treatment of **4** with trimethylsilane bromide affords **5** in 40% yield, which acts as a control compound for **2**.

Soluble well in water, **2** gives typical spectral signatures of a porphyrin—a Soret band at 378 nm with a high extinction coefficient and four Q bands at 508, 543, 567 and 606 nm in absorption, as well as emission peaks at 620 and 681 nm (Fig. 2A). The presence of the tyrosine phosphate groups leads to slight blue shifts in both UV-vis absorption and fluorescence spectra compared with those of **1**. The insignificant shifts (3 nm) permit us to investigate the PDT effects of **1** and **2** using light of same range of wavelengths. As shown in the *in vitro* experiment,† the phosphate groups of **2** can be cleaved by a tyrosine phosphatase and thereafter the more hydrophobic product protoporphyrin dityrosine (PpIX-DT, **3**) is formed. To evaluate the dark cytotoxicity and the phototoxicity of **2**, we used the MTT reduction assay[18] to test the dark cell viability in the presence of **2**. As shown in Fig. 2B, at the concentration from 25 μM and 100 μM, 118% and 98% HeLa cells survive at 100 μM for day 1 and day 2, respectively. This result confirms that **2** has a low dark cytotoxicity.

As shown in the phase contrast and fluorescence images of HeLa cells before and after irradiation (Fig. 3), **2** exhibited appreciable phototoxicity for HeLa cells when a green light (510–560 nm) irradiated the HeLa cells for a short period of time (2 min) after 24 h of pre-incubation of the cells with **2** at 25 μM. Without the irradiation of light, **2** mainly localizes on the

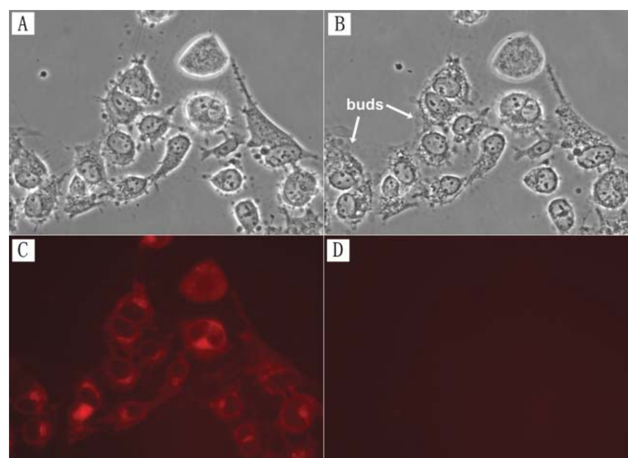


Fig. 3 HeLa cells incubated with compound **2** at concentration of 25 μM. The optical images (A, B) and fluorescence images (C, D) of HeLa cells (A, C) before irradiation and (B, D) after 2 min green light irradiation.

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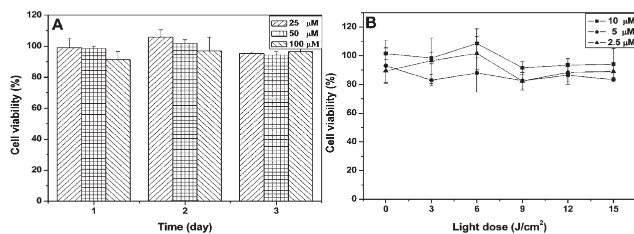

Fig. 4 (A) MTT assay and (B) PDT effect of **5** on HeLa cell.

mitochondria,[19] and a small portion of **2** appears on plasma membrane, nuclear membrane, and the membranes of other cytosomes (Fig. 3C). After being exposed to light, the cells exhibit significant morphological changes by sprouting out multiple small buds around their surfaces (Fig. 3B) and show symptoms of photo damage to the membranes and cell death—**2** leaks out of the cells, thus obscuring the fluorescence image of cells (Fig. 3D). This observation also agrees with the established PDT effect: the photo damage happens at specific subcellular sites, most notably mitochondria,† and ultimately leads to cell death by apoptosis that typically exhibits the "sprouting".[20] Based on the images in Fig. 3, we can conclude that the photo damage of the HeLa cells in the presence of 25 μM of **2** likely results in both apoptosis and necrosis of the cells.

Since mitochondria are the primary target of **2**, if the concentration of **2** was lowered, the cells should mainly undergo apoptosis upon the irradiation of light. Therefore, we examined the photodynamic effect of **2** at 10 μM, using **1** as a control. As shown in Fig. 1A, **2** displays a stronger PDT effect than that of **1**. The PDT experiment with different concentrations of **2** indicates the actual 50% inhibition concentration (IC_{50}) of **2** on the HeLa cells for PDT (7.5 J cm^{-2} at 3 μM). This result clearly establishes that **2** acts as a better PDT agent than **1** does.

We also did two control experiments to support our hypothesis that the enzymatic dephosphorylation contributes to the PDT effect of compound **2**. First, we used another new protoporphyrin-based derivative that contains bisphosphonate groups (PpIX-BP, **5**, Scheme 2) as the control because phosphatase can't cleave the bisphosphonate groups on **5**. This hydrophilic compound, **5**, shows the low dark cytotoxicity to HeLa cells (*e.g.*, 99%, 106% and 98% cells survive at 100 μM at day 1, 2 and 3, respectively, Fig. 4A). Furthermore, **5** shows no PDT effect on the HeLa cells from 2.5 μM to 10 μM (Fig. 4B), likely because **5** is too hydrophilic to build up adequate intracellular concentrations. This result excludes the possibility that the enhanced PDT effect of **2** arises from the hydrophilic phosphate groups. Second, we assayed the cytotoxicity of **3** with and without irradiation of light. Our result indicates that **3** has higher dark cytotoxicity (IC_{50} = 8.5 μM at day 1) for HeLa cells than that of **2** or **5** (possibly due to its phenol groups being oxidized to toxic quinones[19]). The IC_{50} of **3** in HeLa cells for PDT (8.7 J cm^{-2} at 3 μM) is close to that of **2**, suggesting that the PDT effect of **2** arises from its dephosphorylation metabolite **3**.

In summary, we have demonstrated that an enzymatic reaction successfully renders tyrosine phosphate modified protoporphyrin (**2**) able to exhibit the PDT effect without compromising the biocompatibility of **2**. Although the difference in dark cytotoxicity of **2** and **3** remains to be elucidated in more detail, it likely originates from the lower concentration of **3** built up in the cells in the case for **2**. In this work, we utilized only one type of enzyme for generating the PDT agent, it is conceivable that a substrate of two or more enzymes could afford improved specificity for targeting tumors. We are currently exploring the use of other enzymes to convert porphyrin derivatives into PDT agents.

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