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A novel strategy for targeting photodynamic therapy. Molecular combo of photodynamic agent zinc(II) phthalocyanine and small molecule target-based anticancer drug erlotinib†

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In this study, two phthalocyanine–erlotinib conjugates linked by an oligoethylene glycol chain have been synthesised and fully characterised. Having erlotinib as the targeting moiety, the two conjugates exhibited high specific affinity to HepG2 cancer cells and tumour tissues, therefore leading to high photodynamic activity.

Photodynamic therapy (PDT) is an increasingly valuable therapeutic modality for a range of tumours and non-malignant diseases. In the PDT process, the combined action of a photosensitizer (PS), appropriate light and molecular oxygen is used to generate cytotoxic reactive oxygen species (ROS) and cause destruction of tumours.[1] Compared with conventional therapies, PDT holds the promise of dual selectivity, which could be fulfilled by restricting the illumination to a specific region and preferential tumour uptake of the PS.[2] To date, most of the first- and second-generation PSs studied for PDT have exhibited only a slight preference for malignant cells, which brings about significant skin photosensitivity and high uptake by healthy cells and tissues.[2] In recent years, much effort has been devoted to the development of third-generation PSs for targeting photodynamic therapy.[3] There are several approaches to establish this kind of PS. One is making use of nanoparticles modified with target moieties as delivery vehicles for PSs.[4] Another is conjugation of second-generation PSs to biomacromolecules with targeting function such as antibody,[5] lipoprotein,[6] peptide,[7] transferrin,[8] aptamer[9] *etc.* Promising results and decades of progress have been achieved by these two ways, but they also faced some challenges. For the former, the complex control procedure over the size, shape, stability, drug loading and releasing capacity of nanoparticles makes its accuracy, a basic demand for drug administration, much lower than PSs possessing targeting properties inherently.[10] For the latter, the complicated structure and low stability of biomacromolecules always induce a difficult synthesis and purification process, more importantly, their biological activities are often changed, even lost during the process of modification.[11]

Small molecule target-based cancer therapy that aims to specific affinity to cancer cells by modulating the aberrant molecular pathways underlying tumour growth and progression has achieved tremendous success in recent decades.[12] Gefitinib and erlotinib can target the ATP binding domain of tyrosine kinase in EGFR (epidermal growth factor receptor); overexpressed tumours have shown specific affinity to tumour cells, thereby leading to targeting cancer therapy.[13] It is envisioned that PSs covalently binding with such small molecule target-based anticancer drugs may combine the high therapeutic efficiency of PSs and excellent specificity of small molecule target-based anticancer drugs.

In this communication, we choose erlotinib as the target moiety to conjugate with a zinc(II) phthalocyanine core through an oligoethylene glycol spacer which can improve the amphiphilicity and biocompatibility of the conjugate. The developed phthalocyanine–erlotinib conjugates display targeting photodynamic activities against HepG2 cancer cells and specific affinity to tumour tissues in nude mice. To the best of our knowledge, there are few reports on the small molecule target-based anticancer drugs conjugated with PDT agents.

Synthesis of the phthalocyanine–erlotinib conjugates **3a–3b** is shown in Scheme 1. Firstly, azide (oligoethylene glycol) underwent 1,3-dipolar cycloaddition with erlotinib in the presence of sodium ascorbate and $CuSO_4{\cdot}5H_2O$ to afford **1a–1b** (54–63%). Treatment of these two compounds with 3-nitrophthalonitrile gave phthalonitriles **2a–2b** in 64–72% yield, which then underwent statistical condensation with unsubstituted phthalonitrile using DBU (1,8-diazabicyclo-[5.4.0]undec-7-ene) as a base to afford "3+1" products **3a–3b** (23–25%). For comparison, phthalocyanine **4** without the erlotinib moiety was synthesised as a reference compound according to the previous procedure (Scheme S1, ESI†).[14]

Because of the large-conjugated structure, phthalocyanine derivatives always tend to aggregate. The aggregated phthalocyanines

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† Electronic supplementary information (ESI) available: Experimental details, synthesis of phthalocyanine **4**, UV-Vis spectra of **3a–4** in DMF and cell culture medium, cytotoxic effects of **3a–4** toward HepG2 cells, *in vitro* ROS generation for **3a–4**, confocal fluorescence images of HepG cells after incubation with **3a–4**, and ^1H, $^{13}C\{^1H\}$ NMR, and HRMS spectra of all the new compounds. See DOI: 10.1039/c3cc45487h

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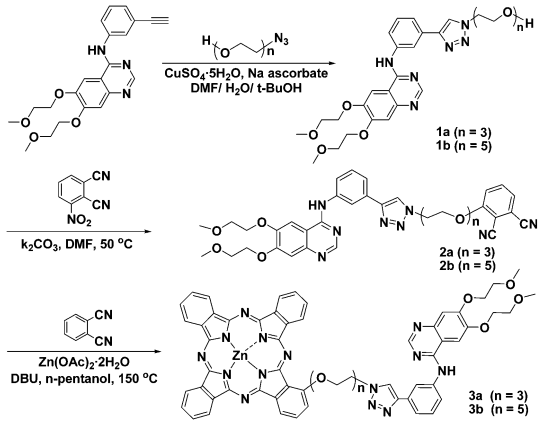

Scheme 1 Synthesis of phthalocyanine–erlotinib conjugates.

exhibit no photocytotoxicity.[15] The absorption spectra of phthalocyanines **3a** and **3b** showed a strong and sharp Q-band at 678 nm suggesting that they were dissolved well and almost did not aggregate in DMF (Fig. S1, ESI†). To account for the *in vitro* photodynamic activities of compounds **3a** and **3b**, their absorption spectra in the culture medium (RPMI 1640) were also recorded (Fig. S2, ESI†), which are very similar to those of **3a** and **3b** in DMF. Their singlet oxygen quantum yields (Φ_Δ) and fluorescence quantum yields (Φ_F) in DMF were also determined and are listed in Table 1. Both **3a** and **3b** display comparative Φ_Δ and Φ_F to the reference compound **4**. This indicates that the photophysical properties of the phthalocyanines did not change obviously after conjugation with erlotinib.

To evaluate the photosensitizing efficiency of these conjugates, the HepG2 cells which overexpress EGFR were selected and a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cell viability assay was employed. The cytotoxicity of these compounds toward HepG2 cells is shown in Fig. S3 (ESI†) and the IC_{50} values are summarized in Table 2. It can be seen that all these phthalocyanines do not show obvious dark cytotoxicity up to 50 μM. However, upon illumination with light at 670 nm, they exhibited considerable cytotoxicity. The IC_{50} values of **3a** and **3b** were determined to be 0.01 and 0.04 μM, respectively, with the light dose of 1.5 J cm^{-2}. The values are roughly equivalent to that of phthalocyanine **4**. The high photodynamic activities agree well with their very low aggregation in the culture medium. The phototoxicity of erlotinib was also investigated under the same conditions, but no photocytotoxicity was observed up to 0.5 μM.

The excitation of the photosensitizer results in the generation of ROS, which is thought to be the main mediator of cellular death induced by PDT. It can mediate cellular effects such as lipid peroxidation and vascular effects, leading to direct or indirect cytotoxic effects on the treated cells. Generally, the higher the ROS, the higher the photocytotoxicity. Here, the ROS generation efficiency of all the phthalocyanines against HepG2 cells was also investigated (Fig. S4, ESI†). It follows the order **3a** > **4** > **3b** which is in accordance with their Φ_Δ in DMF and *in vitro* phototoxicity. The results reveal that the oligoethylene glycol chain and the erlotinib moiety may have an effect on the ROS generation efficiency and photodynamic activity of phthalocyanines to a certain extent.

The subcellular localisation of **3a**, **3b** and **4** was studied using an Olympus FV 1000 Confocal laser scanning microscope. The cells were first incubated with the phthalocyanines in the culture medium for 24 h, and then stained with LysoTracker DND 26 (for 60 min) or MitoTracker Green FM (for 30 min), which are specific dyes for lysosomes and mitochondria, respectively. As shown in Fig. S5a (ESI†), the fluorescence caused by the LysoTracker (excited at 488 nm, monitored at 510–570 nm) or the MitoTracker (excited at 488 nm, monitored at 510–570 nm) can superimpose with the fluorescence caused by **3a** (excited at 633 nm, monitored at 650–750 nm). The very similar fluorescence intensity line profiles (Fig. S5b, ESI†) of **3a** and LysoTracker or MitoTracker traced along the white lines in Fig. S5a (ESI†) also indicate that **3a** can be localised in the lysosomes and mitochondria of the cells. The very similar subcellular localisations of **3b** and compound **4** were observed (Fig. S6 and S7, ESI†). The results showed that these three PSs had no organelle specificity and were distributed throughout the cytoplasm. This may induce the all-around destruction of cancer cells after PDT treatment with these PSs.

To assess the specificity of the conjugates to the cancer cells, we mixed the HELF cells (human embryo lung fibroblasts, a cell line with low expression of EGFR) and HepG2 cells which have huge morphological differences from each other in a cell culture dish. After incubation with phthalocyanines for 24 h, the fluorescence caused by **3a**, **3b** or **4** (all excited at 633 nm, monitored at 650–750 nm) in these two cell lines was recorded using a confocal laser scanning microscope (Fig. 1a). The fluorescence of **3a** and **3b** in HepG2 cancer cells is obviously brighter than in HELF (approximately 3 fold). However, there is no obvious difference between HELF and HepG2 cells for reference **4** (Fig. 1b). The results indicate that **3a** and **3b** can successfully target the cancer cells with high expression of EGFR. The fluorescence ratio of **3a** among the two cell lines is equivalent to that of **3b** indicating that the oligoethylene glycol chain length did not influence the target ability of erlotinib.

Table 1 Photophysical/photochemical data of phthalocyanines in DMF

Compounds	λ_{max}/nm (log ε)	λ_{em}[a]/nm	Φ_F[b]	Φ_Δ[c]
3a	678 (5.25)	685	0.27	0.66
3b	678 (5.28)	686	0.27	0.57
4	677 (5.41)	684	0.26	0.63

[a] Excited at 610 nm. [b] Using unsubstituted zinc(II) phthalocyanine (ZnPc) in DMF as the reference (Φ_F = 0.28). [c] Using ZnPc in DMF as the reference (Φ_Δ = 0.56).

Table 2 IC_{50} values for phthalocyanines **3a**, **3b** and **4** against HepG2 cancer cells with the light dose of 1.5 J cm^{-2}

Compounds	IC_{50} (μM)
3a	0.01
3b	0.04
4	0.03
Erlotinib	*N*[a]

[a] Noncytotoxic up to 0.5 μM.

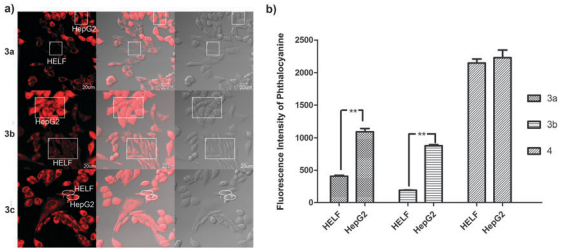

Fig. 1 Confocal fluorescence images of mixed HepG2 and HELF cells after incubation with **3a**, **3b** and **4** for 24 h (all at 10 μM); (b) comparison of relative intracellular average fluorescence intensity of phthalocyanines in HepG2 and HELF cells (measured in the ROIs). Data are expressed as means ± SD. Statistical significance **($P < 0.01$).

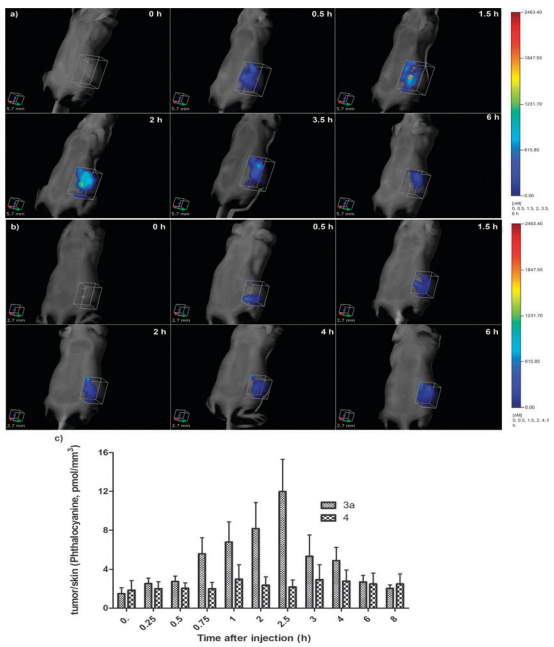

Fig. 2 *In vivo* FMT of **3a** and **4**. (a) Injected with **3a**, (b) injected with **4**, (c) comparison of the average value of the tumour/skin biodistribution ratio of **3a** and **4**. Values are means ± SD. Statistical significance **($P < 0.01$), *($P < 0.05$).

To further confirm the specificity of the conjugates to tumour tissues, the *in vivo* fluorescence imaging was also performed by *in vivo* fluorescence molecular tomography (FMT) using the FMT™ 2500 system (PerkinElmer Inc.) at 680/700 nm excitation/emission wavelength. **3a** and **4** were injected into nude mice bearing A431 tumour through the tail vein. As shown in Fig. 2, **3a** exhibited quick accumulation in tumour tissues in 2.5 hours and then gradually decreased (Fig. 2a). However, compound **4** in tumour tissues is rare and no obvious accumulation appeared along with time (Fig. 2b). The total amount of **3a** in the tumour is visibly higher than that of **4** (Fig. 2c) and the tumour/skin ratio of **3a** is actually about 5-fold higher than that of **4** at 2.5 hours after administration, which confirms that conjugate **3a** has a high selective affinity to tumour over normal tissues.

In summary, we have synthesised and characterised two zinc(II) phthalocyanine–erlotinib conjugates and evaluated their *in vitro* photodynamic activities and selective affinity toward HepG2 cancer cells and A431 tumour tissues. The conjugates contain both photodynamic and targeting anticancer therapy agents which are covalently linked and function in a cooperative manner. The introduction of the erlotinib moiety can enhance the specificity of phthalocyanine units to HepG2 cancer cells and A431 tumour tissues. The IC_{50} value of the conjugates is as low as 0.01 μM toward the HepG2 cells, which is equivalent to that of the reference compound **4** without the erlotinib derivative. The tumour/skin ratio of the conjugate is actually about 5-fold higher than that of reference **4**. The overall results show that the conjugates are highly promising antitumour agents for dual targeting and photodynamic therapy. This may provide a novel targeting strategy for PDT, as well as other cancer therapy modalities.

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Notes and references

1 (*a*) D. E. J. G. J. Dolmans, D. Fukumura and R. K. Jain, *Nat. Rev. Cancer*, 2003, **3**, 380; (*b*) A. P. Castano, P. Mroz and M. R. Hamblin, *Nat. Rev. Cancer*, 2006, **6**, 535.
2 (*a*) N. Rousset, L. Bourre and S. Thibaud, *Sensitizers in Photodynamic Therapy*, ed. T. Patrice, The Royal Society of Chemistry, London, 2003, vol. 2, p. 59; (*b*) N. Brasseur, *Sensitizers for Photodynamic Therapy: phthalocyanines*, ed. T. Patrice, The Royal Society of Chemistry, London, 2003, vol. 2, p. 105.
3 (*a*) J. F. Lovell, T. W. B. Liu, J. Chen and G. Zheng, *Chem. Rev.*, 2010, **110**, 2839; (*b*) A. M. Bugaj, *Photochem. Photobiol. Sci.*, 2011, **10**, 1097.
4 (*a*) S. Y. Park, H. J. Baik, Y. T. Oh, K. T. Oh, Y. S. Youn and E. S. Lee, *Angew. Chem., Int. Ed.*, 2011, **50**, 1644; (*b*) G. Obaid, I. Chambrier, M. J. Cook and D. A. Russell, *Angew. Chem., Int. Ed.*, 2012, **51**, 6158; (*c*) M. Yin, Z. Li, Z. Liu, J. Ren, X. Yang and X. Qu, *Chem. Commun.*, 2012, **48**, 6556.
5 (*a*) M. B. Vrouenraets, G. W. M. Visser, M. Stigter, H. Oppelaar, G. B. Snow and G. A. M. S. van Dongen, *Int. J. Cancer*, 2002, **98**, 793; (*b*) M. B. Vrouenraets, G. W. M. Visser, M. Stigter, H. Oppelaar, G. B. Snow and G. A. M. S. van Dongen, *Cancer Res.*, 2001, **61**, 1970.
6 (*a*) U. Schmidt-Erfurth, H. Diddens, R. Birngruber and T. Hasan, *Br. J. cancer*, 1997, **75**, 54; (*b*) P. Urizzi, C. M. Allen, R. Langlois, R. Ouellet, C. La Madeleine and J. E. Van Lier, *J. Porphyrins Phthalocyanines*, 2001, **5**, 154.
7 (*a*) L. Tirand, C. Frochot, R. Vanderesse, N. Thomas, E. Trinquet, S. Pinel, M.-L. Viriot, F. Guillemin and M. Barberi-Heyob, *J. Controlled Release*, 2006, **111**, 153; (*b*) M. Sibrian-Vazquez, T. J. Jensen and M. G. H. Vicente, *J. Med. Chem.*, 2008, **51**, 2915.
8 (*a*) M. R. Hamblin and E. L. Newman, *J. Photochem. Photobiol., B*, 1994, **26**, 45; (*b*) P. G. Cavanaugh, *Breast Cancer Res. Treat.*, 2002, **72**, 117.
9 (*a*) Y. Gao, G. Qiao, L. Zhuo, N. Li, Y. Liu and B. Tang, *Chem. Commun.*, 2011, **47**, 5316; (*b*) P. Mallikaratchy, Z. Tang and W. Tan, *ChemMedChem*, 2008, **3**, 425.
10 K. Cho, X. Wang, S. Nie and D. M. Shin, *Clin. Cancer Res.*, 2008, **14**, 1310.
11 S. Verma, G. M. Watt, Z. Mai and T. Hasan, *Photochem. Photobiol.*, 2007, **83**, 996.
12 (*a*) A. Zelent, K. Petrie, Z. Chen, R. Lotan, M. Lübbert, M. S. Tallman, R. Ohno, L. Degos and S. Waxman, *Cancer Res.*, 2005, **65**, 1117; (*b*) K. Imai and A. Takaoka, *Nat. Rev. Cancer*, 2006, **6**, 714.
13 (*a*) M. A. Fabian, W. H. Biggs, D. K. Treiber, C. E. Atteridge, M. D. Azimioara, M. G. Benedetti, T. A. Carter, P. Ciceri, P. T. Edeen and M. Floyd, *Nat. Biotechnol.*, 2005, **23**, 329; (*b*) J. Zhang, P. L. Yang and N. S. Gray, *Nat. Rev. Cancer*, 2009, **9**, 28.
14 J. T. Lau, P.-C. Lo, W.-P. Fong and D. K. Ng, *J. Med. Chem.*, 2012, **55**, 5446.
15 A. W. Snow, in *The Porphyrin Handbook*, ed. K. M. Kadish, K. M. Smith and R. Guilard, Academic Press, San Diego, 2003, ch. 109, vol. 17.

