Targeting tumor acidic microenvironment, a cis-aconitate linked pyropheophorbide a dimer 3 was designed and prepared. The observed fluorescence quenching of 3 verified our FRET based molecular design. Acid-dependent cleavage in aqueous solution, singlet oxygen generation, and cytotoxicity against HepG2 cell lines of dimer 3 were investigated. The dimer demonstrated different levels of fluorescence recovery when incubated in acidic aqueous environment as well as effective phototoxicity against HepG2 cells.

Keywords: Pyropheophorbide a, dimer, FRET, cis-aconitic anhydride, phototoxicity, PDT.

Димер пирофеофорбида a для направленного действия в кислой микросреде опухоли: альтернативная стратегия одновременной флуресцентной визуализации опухоли и ФДТ

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цис-Аконитат-связанный димер пирофеофорбида a (3), структура которого была разработана на основании резонансного переноса энергии флуресценции, был синтезирован целенаправленно для использования в качестве фотосенсибилизатора в кислой микросреде опухоли. Для полученного димера было исследовано кислотно-зависимое расщепление в водном растворе, способность к генерации синглетного кислорода и цитотоксичность в отношении клеточных линий HepG2. Димер показал различные уровни восстановления флуресценции при инкубации в кислой водной среде, а также эффективную фототоксичность в отношении клеток HepG2.

Ключевые слова: Пирофеофорбид a, димер, резонансный перенос энергии флуресценции, цис-аконит ангирид, фототоксичность, ФДТ.

In recent years, chlorin based photodynamic therapy (PDT) has drawn attentions worldwide, several candidates have shown their potential in treating various malignancies and other diseases in clinical trials, such as NPe6,[1-3] HPPH[4-5] and Verteporfin.[6-7] Other than treating effect, these chlorins may also serve as imaging agents for photodiagnosis, owing to the ability to emit near-infrared (NIR) fluorescence upon light treatment.[8-11] However, the drawback of in vivo low tumor selective accumulation of nature chlorins resulted in insufficient signal contrast between...
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tumor and surrounding tissue, and further limited its clinical application. Therefore, many researchers have paid their attention to tumor microenvironment, especially the representative acidic extracellular fluid. Based on this, various chlorin loaded nanoparticles with acid-sensitive linkers including \( \text{cis-Aconitate} \) were designed to optimize the fluorescence diagnosis and PDT efficacy of photosensitizers.\[^{12-16}\] Although some of them did improve the specificity to malignancy by combining the advantage of reversible fluorescence resonance energy transfer (FRET) and the enhanced permeability and retention (EPR) effect, none of the above drug delivery systems has been approved by FDA because of some intrinsic defects such as poor stability and repeatability. On the other hand, to our knowledge, easily obtained acid-sensitive chlorin dimers had been neglected in this field. Therefore, based on the theory of FRET, a chlorin based dimer \( \text{3} \) utilizing acid-cleavable \( \text{cis-Aconitate} \) as a linker whose acidity-sensitivity had been verified in the above chlorin loaded nanoparticles was designed and synthesized, to investigate the feasibility for tumor imaging and treating upon light irradiation.

In this study, the starting material Phaeophorbid \( \text{a} \) was prepared from \( \text{Spirulina} \) power produced in Cheng-Hai Lake in Yunnan Province of China, referring to Smith’s method.\[^{17}\] Phaeophorbid \( \text{a} \) was obtained by refluxing Phaeoiborbid \( \text{a} \) in pyridine and ethylenediamine was employed to link Pyropheophorbide \( \text{a} \) with \( \text{cis-Aconitic} \) moieties. Firstly, Boc-ethylenediamine was efficiently introduced at the 173-carboxylic acid of Pyropheophorbide \( \text{a} \) with HBTU as coupling reagent, then following deprotection of Boc group with TFA/DCM gave functionalized monomer \( \text{2} \) (Scheme 1). Next, the free carboxylic acid of \( \text{cis-Aconitic anhydride} \) was converted to active ester with equivalent HBTU, and the result mixture was dropwise added to a solution of alcalized \( \text{2} \) in DMF. Whereafter, concurrent nucleophilic attack of free amino groups to the carbonyl carbons of both active ester and anhydride produced desired dimer \( \text{3} \) in a yield of 31 %. Together with the overlap between absorption and emission spectra of \( \text{2} \), the appropriate distance between the two chromophores made FRET possible for this synthetic dimer.

The target product \( \text{3} \) was characterized by the two sets of chlorin macrocyclic signals in \(^1\text{H NMR}, \) along with HRMS and typically absorption properties in MeOH (longer wavelength and enhanced intensity). On account of strong hydrophobicity, week absorption as well as minimal emission of both Pyropheophorbid \( \text{a} \) and dimer \( \text{3} \) in sole phosphate buffer (PB) were detected (Figure 1). However, the spectral intensity was successfully recovered upon addition of 1 % Cremophor, a commonly used co-solvent. Differing from the precursor Pyropheophorbid \( \text{a} \), expected fluorescence quenching of dimer \( \text{3} \) was observed in both MeOH and PB due to the FRET effect, which suggested week background signal in neutral environment.

To further verify the sensitivity of \( \text{3} \) to week acidity, PB solutions (containing 1 % Cremophor) with \( pH = 7.4, \)
Figure 1. Absorption (left, 5 μM) and emission spectra (right, 1 μM, excited at 410 nm) of Pyropheophorbide $a$ and 3 in different solvent.

Figure 2. Fluorescence spectra recorded at different time intervals of dimer 3 in PB with 1% Cremophor (A: $pH = 7.4$, B: $pH = 6.5$, C: $pH = 5.5$), and the ratio of fluorescence intensity at time $t$ to that at time $t_0$ (D).

6.5 or 5.5 were adopted to simulate the microenvironment of normal tissue, tumor tissue and intracellular lysosome, respectively. Dimer 3 was dissolved in the above PB solution (1 μM), and the fluorescence spectra were recorded at different time intervals. It turned out that the fluorescence intensity increased tardily in neutral environment ($pH = 7.4$), indicating relative stability under this condition. But the signals observed in PB ($pH = 6.5$ and 5.5) dramatically improved in the wake of enhanced acidity, reaching to nearly 15 and 20 folds 24 h later compared to the initial
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Figure 3. The singlet oxygen quantum yields in DMF (A), dark toxicity (B) and phototoxicity (C) of Pyropheophorbide $a$, 2 and 3.

Table 1. Photo and dark toxicity of chlorins against HepG2 cells evaluated by MTT assay. Data represents the mean ± standard deviation (SD) of three independent experiments.

| Compounds          | Dark IC$_{50}$ (µM) | Photo IC$_{50}$ (µM) |
|--------------------|---------------------|----------------------|
| Pyropheophorbide $a$ | $>50$               | 0.53 ± 0.01          |
| 2                  | 1.50 ± 0.02         | 0.27 ± 0.01          |
| 3                  | 18.91 ± 0.11        | 1.37 ± 0.13          |

Since singlet oxygen has been considered as the main cytotoxic substance in PDT process,[18-19] its generation was determined as we previously described.[20] Compared with Pyropheophorbide $a$, dimer 3 and chlorin 2 demonstrated undifferentiated singlet oxygen generation in DMF (Figure 3A), suggesting that photosensitivity of 3 was retained in spite of fluorescence quenching. Meanwhile, the in vitro dark and phototoxicity against HepG2 cell lines were assessed by MTT assay. As shown in Figure 3 and summarized in Table 1, the dimer showed slightly weaker phototoxicity than Pyropheophorbide $a$, which might be ascribed to lower cellular uptake of 3 on account of its high molecular weight. What was noteworthy was that the hydrolysis product 2 had both stronger dark and phototoxicity than the precursor 3. It was very likely that the production of 2 was unavoidable during the incubation of HepG2 cell with 3, so we suspected that the potent 2 could contribute to the efficacy of dimer 3 to some extent, and this contribution may increase under in vivo tumor acidic microenvironment, where more 2 would be produced. Unfortunately, in vivo study was infeasible due to the poor water solubility of 3, so further optimization of this model in our lab aimed at better hydrophily and also longer absorption wavelength.

In summary, cis-Aconitate linked Pyropheophorbide $a$ dimer 3 demonstrated arresting sensitivity to acidity, and the cleavage-driven fluorescence recovery provided impressive signal contrast of different aqueous environment. Taking into account of efficient singlet oxygen production and in vitro phototoxicity at the same time, our work may provide an ideal model to design chlorin based dimers for simultaneous imaging and treating of superficial solid tumor.

Experimental

All reactions were carried out under nitrogen in air-free solvents and with protection from direct light and monitored by TLC on gel F254 plates. Silica gel (200–300 mesh) was used for column chromatography. $^1$H NMR and $^{13}$C NMR spectra were measured at Bruker Avance 400 MHz spectrometer. Chemical shifts (d) are given in ppm relative to tetramethylsilane (TMS, 0 ppm). HR-MS were obtained on an LTQ Orbitrap XL high resolution mass spectrometer (Thermo Fisher Scientific). Absorption and emission data (Figure 2D). Supported by the mass spectrometry (ESI) of the hydrolysis product (found $m/z = 577.33$, compound 2), this fluorescence recovery should be attributed to the acidity driven cleavage of the linker, and this energetic response to acidity brought about remarkable signal contrast of neutral ($pH = 7.4$) and acidulous ($pH = 6.5$ and 5.5) environment. At this point, dimer 3 had showed its potential for tumor imaging and even diagnosing.

In summary, cis-Aconitate linked Pyropheophorbide $a$ dimer 3 demonstrated arresting sensitivity to acidity, and the cleavage-driven fluorescence recovery provided impressive signal contrast of different aqueous environment. Taking into account of efficient singlet oxygen production and in vitro phototoxicity at the same time, our work may provide an ideal model to design chlorin based dimers for simultaneous imaging and treating of superficial solid tumor.

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m), 1.78 (3H, d, J = 7.6 Hz), –1.77 (2H, s).

Preparation of Pyropheophorbide a. 600 mg Pyropheophorbide a was dissolved in 35 mL pyridine in a 100 mL round-bottom flask, the solution was heated to reflux under nitrogen atmosphere for 10 h. Then the mixture was concentrated under vacuum to remove the solvent. The residue was purified on a silica gel column eluting with dichloromethane/methanol = 50:1, 25:1.

To a solution of 155 mg Pyropheophorbide a in 5 mL DMF in a 25 mL round-bottom flask was added 16.7 mg phenol and 3 mL TFA, the mixture was stirred for 2 h at room temperature under nitrogen atmosphere. The solution was heated to reflux under nitrogen atmosphere for 1 h, the mixture was stirred for 2 h at room temperature under nitrogen atmosphere then a solution of 137 μL Boc-ethylenediamine-Pyropheophorbide a in 5 mL dichloromethane/methanol = 50:1, 15:1 as the eluent. Yield: 60 mg (31 %).

HRMS (ESI) m/z for C_{33}H_{35}N_{4}O_{3} [M+H]^+ calc. 533.26, found 533.34. 1H NMR (400 MHz, CDCl3) δ ppm: 9.15 (1H, s), 8.75 (1H, s). 5.17 (1H, d, J = 11.5 Hz), 5.21 (1H, d, J = 19.7 Hz), 5.01 (1H, t, J = 5.2 Hz), 4.93 (1H, d, J = 19.7 Hz), 4.44 (1H, m), 4.18 (1H, m), 3.38 (2H, m), 3.30 (3H, s), 3.18 (2H, m), 3.08 (5H, s), 2.88 (3H, s), 2.62 (1H, m), 2.30 (2H, m), 2.05 (1H, m), 1.73 (3H, d, J = 7.2 Hz), 1.47 (3H, m), 1.19 (9H, s), –1.79 (2H, s).

Synthesis of 17'-Boc-ethylenediamine-Pyropheophorbide a I. To a solution of 155 mg Pyropheophorbide a in 5 mL DMF in a 25 mL round-bottom flask was added 165 mg HBTU and 75 μL Et3N in 5 mL acetonitrile, the mixture was stirred for 2 h at room temperature under nitrogen atmosphere. The solution was heated to reflux under nitrogen atmosphere for 10 min and the light intensity at the treatment site was 1.4 J/cm². The surviving fraction of cells was immediately evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) spectrophotometric method. Each experiment was repeated three times. Phototoxicity was evaluated with different concentrations (range from 0 to 50 μM) separately following a similar procedure for dark toxicity. After 24 h incubation with PSs, the cells were exposed to the LED light of 660 nm wavelength for 10 min and the light intensity at the treatment site was 1.4 J/cm². The surviving fraction of cells was also evaluated by MTT assay 2 h after treatment. Each experiment was repeated three times.

The authors gratefully acknowledge the support of the State Key Development Program for Basic Research of China (Grant No. 2009CB918501).

References
1. Kato H., Furukawa K., Sato M., Okunaka T., Kusunoki Y., Kawahara M., Fukuoka M., Miyazawa T., Yana T., Matsui K., Shiraishi T., Horinouchi H. Lung Cancer 2003, 42, 103–111.
2. Spikes J.D., Bommer J.C. J. Photochem. Photobiol., B: Biol. 2003, 77, 135–143.
3. Wong T.-W., Azaiwa K., Shehedin I., Wushur C., Kato H. J. Pharm. Sci. 2003, 92, 136–142.
4. Bellnier D.A., Greco W.R., Loewen G.M., Nava H., Oseroff A.R., Pandeck R.K., Tsuchida T., Dougherty T.J. Cancer Res. 2003, 63, 1806–1813.
5. Bellnier D.A., Greco W.R., Loewen G.M., Oseroff A.R., Dougherty T.J. Cancer Chemother. Pharmacol. 2006, 57, 40–45.
6. Treatment of Age-related Macular Degeneration with Photodynamic Therapy (TAP) Study Group Arch. Ophthalmol. 1999, 117(10), 1329–1345.
7. Brown D.M., Kaiser P.K., Michels M., Soubrane G., Heier J.S., Kim R.Y., Sy J.P., Schneider S. New Engl. J. Med. 2006, 353, 1432–1444.
8. Song X., Liang C., Gong H., Chen Q., Wang C., Liu Z. J. Photochem. Photobiol., B: Biol. 2012, 115, 3932–3941.
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11. Ling D., Park W., Park S.-j., Lu Y., Kim K.S., Hackett M.J., Kim B.H., Yim H., Jeon Y.S., Na K., Hyeon T. J. Am. Chem. Soc. 2014, 136, 5647–5655.
12. Battogtokh G., Ko Y.T. J. Mater. Chem. B 2015, 3, 9349–9359.
13. Hou W., Zhao X., Qian X., Pan F., Zhang C., Yang Y., de la Fuente J.M., Cui D. Nanoscale 2016, 8, 104–116.
14. Xia F., Hou W., Zhang C., Zhi X., Cheng J., de la Fuente J.M., Song J., Cui D. Acta Biomater. 2018, 68, 308–319.
15. Yao X., Chen X., He C., Chen L., Chen X. J. Mater. Chem. B 2015, 3, 4707–4714.
16. Wang L., Zhu X., Xie C., Ding N., Weng X., Lu W., Wei X., Li C. Chem. Commun. (Camb) 2012, 48, 11677–9.
17. Hargus J.A., Fronczek F.R., Vicente M.G.H., Smith K.M. Photochem. Photobiol. 2007, 83, 1006–1015.
18. Dąbrowski J.M., Krzykawska M., Arnaut L.G., Pereira M.M., Monteiro C.J.P., Simões S., Urbainska K., Stochel G. ChemMedChem 2011, 6, 1715–1726.
19. Ye Y., Wang L.-X., Zhang D.-P., Yan Y.-J., Chen Z.-L. J. Innov. Opt. Heal. Sci. 2015, 08(01), 1540001.
20. Cao L., Guo X., Wang L., Wang S., Li Y., Zhao W. New J. Chem. 2017, 41, 14279–14287.

Received 15.03.2019
Accepted 24.04.2019