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ARTICLE

Endoperoxide delivered singlet oxygen: The future of PDT, without light or oxygen

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Chemically generated singlet oxygen via cycloreversion reaction of aromatic endoperoxides, is poised to evolve into a highly promising therapeutic protocol. Singlet oxygen can also be produced endogenically, with a short half-life especially in biological media, and it acts locally, only when a threshold value is exceeded. Conserving the essence of photodynamic therapy, which is the delivery of singlet oxygen to tumors, two limiting issues of light penetration and low tumor oxygenation can be circumvented simultaneously by endoperoxide-delivered singlet oxygen. The endoperoxides are also amenable to derivatization for more specific targeting as well. In this work, pyridone-endoperoxides with mitochondria targeting triphenylphosphonium moieties were shown to target tumors and result in significant tumor suppression. The series of endoperoxides tested also confirms the importance of mitochondria targeting. In mouse tumor models, these compounds show no signs of systemic or organ level toxicity.

1. Introduction

"Photodynamic effect", first defined more than 125 years ago,[1] was later understood to be photosensitized generation of singlet oxygen through the intermediacy of an organic dye (photosensitizer). This effect has evolved into a cancer therapy modality (Photodynamic Therapy, PDT) following the clinical studies started in Roswell Park Memorial Institute in the late 1970s.[2] FDA approved the photosensitizer Photofrin® for palliative treatment of advanced esophageal cancer in 1994[3] and today, it is approved for the treatment of specific lung cancer types and Barrett's esophagus.[4] However, over the years, it has become clear that the very limited light penetration through tissues,[5] even at optimal wavelengths[6] (a.k.a., therapeutic window) will confine PDT to be a superficial treatment, most appropriate for certain skin conditions, including melanoma, and not a broadly-applicable first-line therapy.[7]

Recent research activity regarding various modifications or improvements to PDT seems to disregard light penetration issue, and the huge difference in the relevance of light penetration in a mouse model versus a potential human patient[8] considering many variances, most obviously the difference in size.

On the other hand, we previously proposed[9-10] the idea that endoperoxides can be utilized as singlet oxygen delivery agents under certain conditions, for therapeutic purposes, thus eliminating the need for external photonic excitation.[10] If the delivery[11] is selective enough, this would be a significant development transforming PDT into a special therapeutic protocol, distinct from chemotherapy, because carrier of singlet oxygen,[12,13] or the metastable "storage compound" need not to be toxic at all, and the anti-tumor activity should be only from the released singlet oxygen. Endoperoxide generated singlet oxygen was studied for its cytotoxic potential a few decades ago,[14] but the endoperoxides used in those studies were negatively charged and were not likely to be cell permeable. Also, the IC_{50} values against HepG2 cancer cells were reported to be > 5 mM. These early discouraging results were probably the reason for the lack of further interest in this line of research. Another reason is the correct, but misleading categorization that photosensitized generation of singlet oxygen is a catalytic process, whereas singlet oxygen re-lease from endoperoxides is obviously stoichiometric. This may lead to the incorrect assumption that at any rea-sonable dosage of endoperoxides, the singlet oxygen re-leased would not be sufficient to cause cellular death in tumors. However, a calculation of the threshold doses of singlet oxygen (generated by photosensitization) required for reducing the survival of cancer cells to 1/e yielded 3.6-4.7 x 10^7 singlet oxygen molecules per cell in vivo (in a radiation-induced fibrosarcoma tumor mouse model).[15] Even allowing for large error margins in the calculations, the amount is easily attainable by endoperoxide cycloreversion reactions in vivo.

Recently, in our research group, we have been focused to explore[16-19] various aspects of controlling singlet oxygen release in cell cultures and in vivo. The results displayed significant potential for therapeutic utility and suggested that targeting mitochondria may increase the efficacy of endoperoxide delivered singlet oxygen approach.

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2. Results and discussion

In order to clarify the role of mitochondria targeting *in vivo*, in this work, we targeted the synthesis (Supporting Information) of a series of 2-pyridone endoperoxides (Figure 1) with a triphenylphosphosphonium (TPP) module. The half-lives for the first order cycloreversion reactions were determined by ^{1}H NMR spectroscopy by following the changes of integrals for the well-resolved characteristic peaks of the endoperoxide and pyridone, such as the methyl substituent on the pyridone ring. As the reaction proceeds, the singlet at 1.61 ppm is replaced by another singlet at 2.11 (SI, Figure S1-S4). The half-life of endoperoxide cycloreversions are around 10 h at 37 °C, however with larger distance from the TPP unit and less steric hindrance, **Endo-py-tpp-4** decays a little faster. We then carried out MTT assays to assess cytotoxicity of these compounds using various cancer cell lines. IC_{50} values range between 30-60 μM for the longest linker (Table 1), it is clear that, if mitochondria cannot be targeted, the release of singlet oxygen is much less effective. Short chain linkers apparently do not allow transport through mitochondrial membranes as evidenced by large IC_{50} values (>200 μM). This is in agreement with the previously reported data using non-targeted endoperoxides.

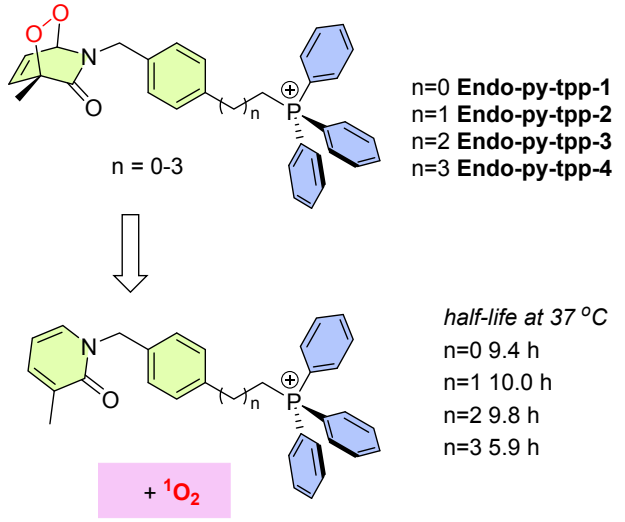

Figure 1. General structures of mitochondria targeting singlet oxygen sources synthesized and studied in this work. Half-lives for cycloreversion reactions were determined in $CDCl_3$.

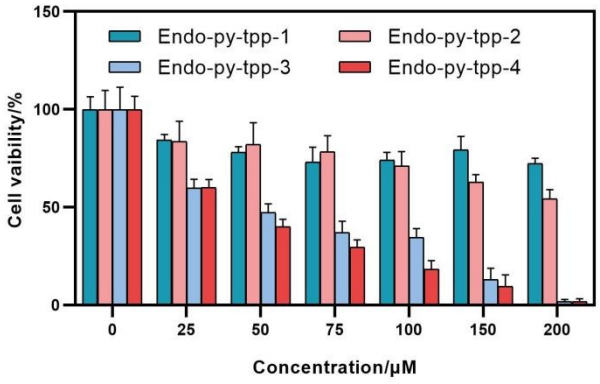

Figure 2. Cell viability of A549 cell treated with different endoperoxides at indicated concentrations.

Table 1. IC_{50} Values (μM) of Endo-py-tpp-3 and Endo-py-tpp-4.

Compound	A549	MCF-7	4T1	HeLa	HepG2
Endo-py-tpp-1	>200	>200	>200	>200	>200
Endo-py-tpp-2	>200	>200	>200	>200	>200
Endo-py-tpp-3	58.52	67.79	108.3	89.08	80.24
Endo-py-tpp-4	46.43	58.99	76.74	82.45	77.02

The compounds were tested for singlet oxygen release using the probe compound DPBF in DMF and in PBS buffer at 7.2, using singlet oxygen selective fluorescence probe SOSG. The results qualitatively corroborate ^{1}H NMR data regarding singlet oxygen release from all four compounds, **Endo-py-ttp-4** being slightly faster (Figure S9-S22). Stock solutions were prepared in DMSO, never to exceed 1% in final experiments. Fluorescence microscopy by double staining with Hoechst nuclear stain and DCFH-DA (ROS probe) showed the different outcomes due linker length difference. **Endo-py-ttp-1** and **Endo-py-ttp-2** seem not to be cell-permeable, or not to localize in mitochondria and wash out too quickly to react with the fluorescence probe. Characteristic green fluorescence, indicative of released singlet oxygen was only observed (Fig. 3a) in the cells (A549 cell line) incubated with **Endo-py-ttp-3** and **Endo-py-ttp-4**. Selective probe Si-DMA, which reports only mitochondrial singlet oxygen, shows a similar picture, again only the two longer alkyl chain endoperoxides release singlet oxygen inside the mitochondria (Fig. S23).

It is well known that apoptosis[20] is closely linked to the collapse of mitochondrial membrane potential ($\Delta\psi_m$). The cationic fluorescent probe, JC-1 (5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazoylcarbocyanine iodide), is very specific for measuring changes in $\Delta\psi_m$. In the mitochondria of normally functioning cells, JC-1 forms J-aggregates with red emission, but when membrane potential is lost, aggregates unravel, and green monomer emission is observed. Staining the cancer cells (A549) with this dye revealed loss of mitochondrial membrane potential only with **Endo-py-ttp-3** and **Endo-py-ttp-4** (Figure 3b).

Flow cytometry results of cells incubated with the endoperoxides, also display a significant increase in apoptosis in the longer linker conjugates (Figure 3c).

Additional High Content Imaging with of cells with live-dead assays using Calcein-AM and PI dyes, show larger fraction of A549 cell death under incubation with **Endo-py-ttp-4** (Figure S25). Along the same lines, we used A549 cells to generate tumor spheroids from single-cell suspensions. Similar staining procedure after incubation with the endoperoxides confirms the effectiveness of **Endo-py-ttp-4** in 3D cell cultures (Figure 4) as well.

Following these experiments, we wanted to analyze changes in the expression of critical proteins linked to apoptotic process using Western blot analysis. A549 cells were lyzed after treatment with the endoperoxides. Expression levels of

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proteins Bcl-2, caspase 3, and the amount of cleaved caspase 3 were studied. Bcl-2 is an integral outer mitochondrial membrane protein that blocks apoptotic death. Our data show that Bcl-2 expression is significantly decreased in the cells treated with **Endo-py-ttp-3** and **Endo-py-ttp-4**. Also,

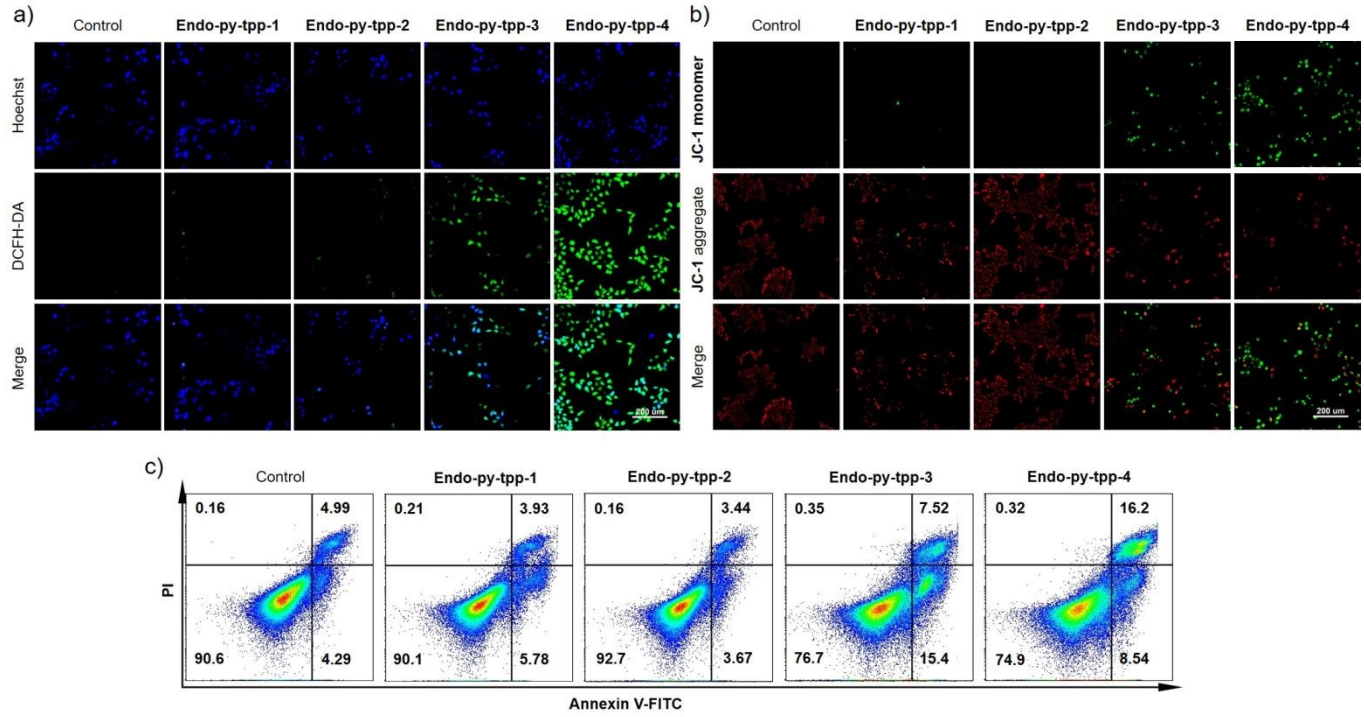

Figure 3. a) Detection of intracellular ROS after treated by different groups, 40 mM; b) JC-1 staining with different treatments. A549 cell culture was used; c) Flow cytometry analysis of apoptosis after different treatments.

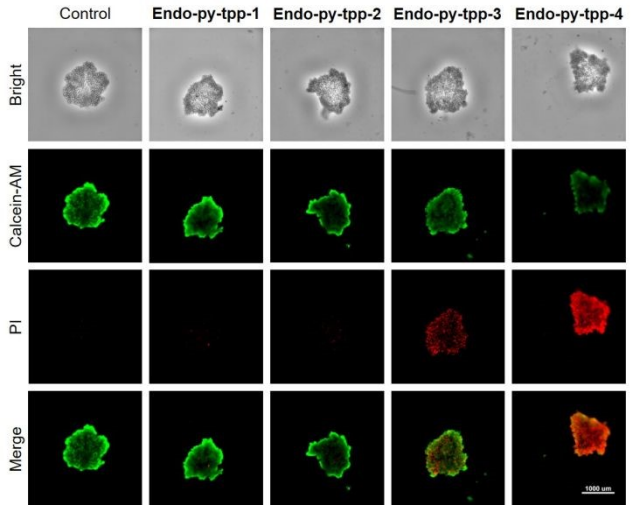

Figure 4. Calcein-AM/PI Apoptosis Detection kit was used for assessing endoperoxide-induced apoptosis in multicellular tumor spheroids.

caspase-3 levels decrease, while cleaved caspase-3 level increase significantly (Figure 5a-5d).

We also studied the effect singlet oxygen releasing pyridine endoperoxides by colony formation assays as they provide clear information about their cellular clonigenic potential. The data clearly confirms superiority of **Endo-py-ttp-4** in inhibiting colony formation of A549 cells.

Scratch tests, also known as cell migration assays were done (Figure S26). The ability of cells to migrate is essential in tumor invasion, neoangiogenesis and metastasis.[21] **Endo-py-ttp-4** is particularly effective in inhibiting cancer cell migration.

Encouraged by these results, we initiated *in vivo* experiments. Tumor model was developed by injecting 4T1 cells subcutaneously into the armpit of 5–6 week old female BALB/c mice (Figure 6a). The tumor-bearing mice of the models were raised for approximately 8 days, then divided into three groups (five mice in each group): the first group was the control group, the second group was treated with **Endo-py-tpp-1** (14 mg/kg), and the third group was treated with **Endo-py-tpp-4** (15 mg/kg). The results show that **Endo-py-tpp-4** was very effective (Figure 6b-e) in suppressing tumor growth (average tumor mass was 1.55 g in control group and 0.4 g in **Endo-py-tpp-4** group). The body weight

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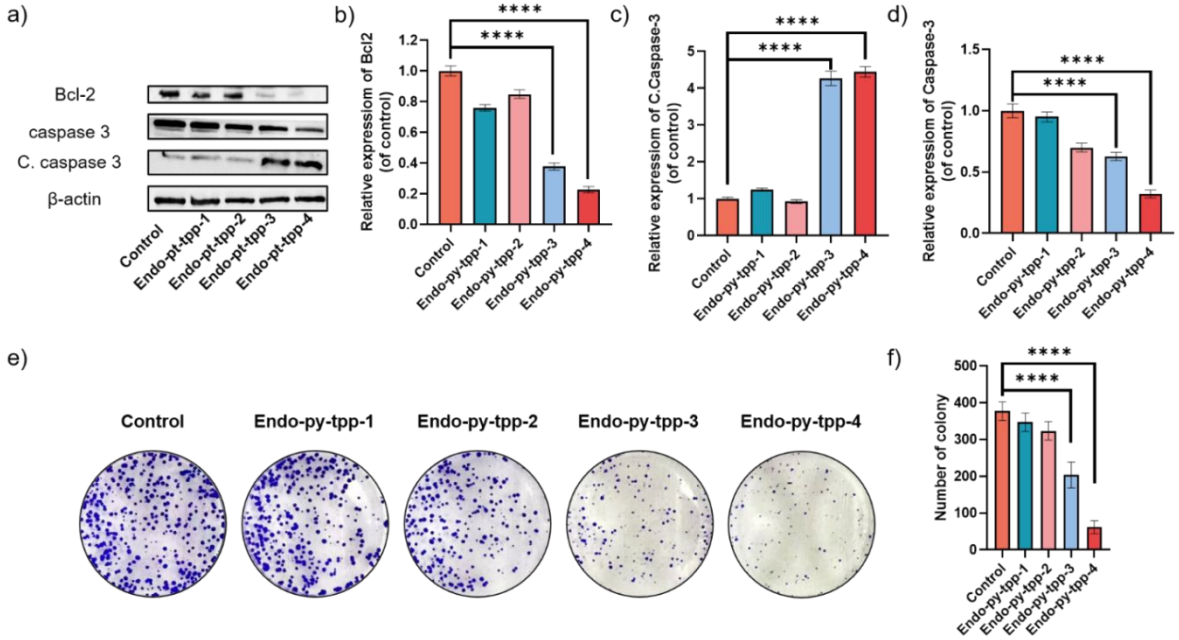

Figure 5. a) Expression levels of apoptosis related proteins, b) relative expression of Bcl-2 under different treatments, b) relative of expression of caspase-3 under different treatments, c) relative amount of cleaved caspase-3 under different treatments. e) Colony formations assay experiments, f) The number of colonies with different endoperoxide treatments. All experiments were done with A549 cells. Data shown as mean ± SD, n = 3 per treatment. ****p < 0.0001.

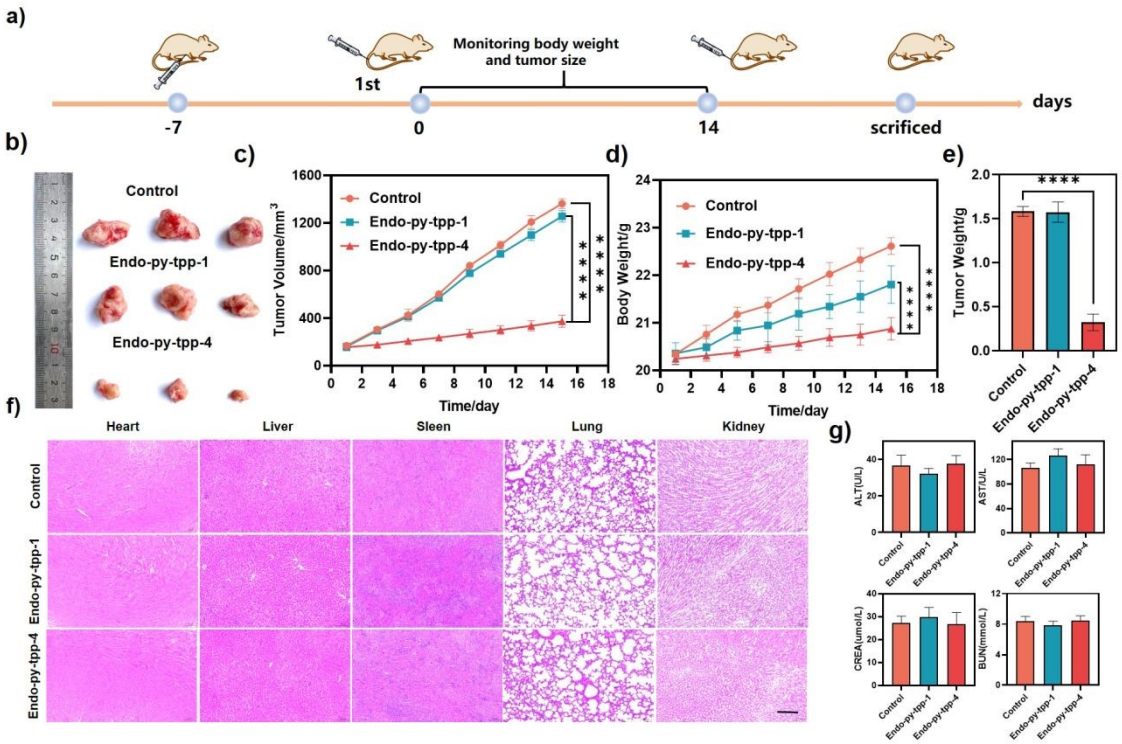

Figure 6. a) Schematic of the experimental protocol, b) Representative tumor images after different treatments. c) Inhibition of 4T1 tumor volume in different groups. d) Time evolution of mouse weight under different treatments. e) 4T1 tumor weight under different treatments. f) H&E staining of different organs in various groups of mice. Scale bar: 100 μm. g) Blood biochemistry analysis of the mice after the treatment of **Endo-py-tpp-1** and **Endo-py-tpp-4** including alanine transferase (ALT), aspartate transaminase (AST), creatinine (CREA), blood urea nitrogen (BUN). Data shown as mean ± SD, n = 3 per treatment. ****p < 0.0001.

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loss observed in mice treated with endoperoxides were within the acceptable limits (<10 %). H&E staining showed no abnormal changes in major organs (Figure 6f) and blood enzyme levels including alanine transferase (ALT), aspartate transaminase (AST), creatinine (CREA), blood urea nitrogen (BUN) (Figure 6g) confirmed systemic safety of the treatment protocol. Representative images from H&E and Ki67 staining of tumor tissues showed remarkable changes in the tumor appearance and characteristics. Treated tumor tissues show minimal Ki67 staining, which is a clear sign of inhibited mitosis (Figure S27).

Conclusion

In summary, successful targeting of mitochondria by singlet oxygen releasing pyridine endoperoxides shows significant potential in cancer therapy without any dependence on oxygen or light. This may be the way forward in transforming PDT into a more clinically accessible protocol by essentially keeping the essence of its action and cutting its dependence to external excitation, which cannot be satisfactorily provided for most tumors. In other words, as long as external excitation in the visible-Near IR region is required for any therapeutic protocol for cancer (PDT, PTT, *etc*.) the methodology is very likely to be limited to the superficial tumors at best. This realization should cause a significant change in the research direction of the PDT community at large. We will continue to explore this new path in our own research program.

Author contributions

E.U.A. directed the project and conceived the idea and designed the initial strategy. W.W. and L.W. designed the experiments, evaluated the data and wrote the first version of the manuscript. E.U.A. wrote the final version of the manuscript. R.S., X.Q. and Z.L. assisted with synthesis and characterization of the compounds.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the ESI.

Acknowledgements

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13 Following the publication of reference 12, many other works making use of the idea of combining a photosensitizer and a pyridone storage unit towards PDT

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applications appeared. Here are just two examples: (a) S. Callaghan, M. A. Filatov, E. Sitte, H. Savoie, R. W. Boyle, K. J. Flanagan, M. O. Senge, Delayed release singlet oxygen sensitizers based on pyridone-appended porphyrins *Photochem. Photobiol. Sci.*, **2017**, *16*, 1371-1374; (b) B.-R. Xie, C.-X. Li, Y. Yu, J.-Y. Zeng, M.-K. Zhang, X.-S. Wang, X. Zeng, X.-Z. Zhang. A Singlet Oxygen Reservoir Based on Poly-Pyridone and Porphyrin Nanoscale Metal–Organic Framework for Cancer Therapy, *CCS Chem*. **2021**, *3*, 1187–1202.

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Data availability

The data supporting this article have been included as part of the ESI.