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COMMUNICATION

Delayed release singlet oxygen sensitizers based on pyridone-appended porphyrins

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A new type of porphyrin photosensitizer capable of generating singlet oxygen upon irradiation, storing it through binding to pyridone subunits, followed by slow release at 20–40 °C is reported. The timescale of singlet oxygen release can be varied depending on the pyridone group substitution pattern, forming endoperoxides of different stability. Modified tetra- and octa-substituted pyridone-porphyrins showed solubility in water, allowing for straightforward delivery into cells. The effect of delayed singlet oxygen formation due to endoperoxide decomposition was demonstrated on cancer cells *in vitro*.

Photodynamic therapy (PDT) relies on the generation of highly reactive singlet oxygen ($^1\text{O}_2$) through a photosensitizer (PS) excited state interaction with ground state triplet oxygen ($^3\text{O}_2$), resulting in oxidative stress and cell death.¹ One of the limitations associated with this method is skin sensitivity to strong light exposure. To minimize damage of healthy tissue during the treatment, techniques including precise delivery of the PS into diseased cells, spatiotemporal control of light treatment, special formulations and chemical activation of PS in the target cells have been developed.²

The need for prolonged irradiation of tissue to cause a significant cytotoxic effect is, in part, associated with the short lifetime of $^1\text{O}_2$ in biological media. It is susceptible to quenching by water molecules and antioxidants, causing decay back to the ground state on a timescale of a few microseconds and thus a reduced therapeutic effect.³ This near instant deactivation of singlet oxygen upon photosensitization could be overcome by using chemical sources of $^1\text{O}_2$ which slowly

release this species in target cells. Materials designed for this purpose, *e.g.*, based on inorganic peroxides combined with biodegradable polymeric carriers showed promise in tissue engineering, but suffer from poor selectivity of the release process.⁴ The only chemical process of reversible binding of singlet oxygen which can be remotely controlled relies on [4+2] cycloaddition to aromatic organic molecules. First reported by Dufraisse in 1926,⁵ it has been actively studied for almost a century and guidelines for the design of such systems have recently been formulated.⁶ Release of singlet oxygen from such adducts has been shown to induce cellular damage in malignant tissue for the deactivation of bacteria, viruses, and parasites.⁷

A combination of standard photosensitizers with endoperoxide-forming moieties could provide a significant improvement to current PDT methods. To apply this approach to conventional PDT treatments, PS bearing several endoperoxide-trapping subunits are required to ensure that significant amount of singlet oxygen is conserved in a chemically bound form within the irradiation period. Previously reported systems based on naphthalene and anthracene derivatives are not suitable as models for *in vitro* studies, due to their hydrophobic character.⁸ However, 2-pyridone derivatives are particularly well-suited for biomedical applications due to their facile synthesis and inherent polarity which enhances water solubility.⁹ Additionally, the respective endoperoxide stability is strongly affected by the substitution pattern: 2-pyridone endoperoxide possesses a half-life time of 30 min at 37 °C, while the 3-methyl-2-pyridone-endoperoxide is considerably more stable ($t_{1/2}$ ~ 8 h) (Scheme 1).^{9b} Recently, such 2-pyridones endoperoxides were demonstrated to cause cytotoxicity in hypoxic cancer tissues.¹⁰

Herein we report a new type of porphyrin photosensitizers modified with different numbers of 2-pyridone subunits (four subunits in **P1**, **P2**, **P5** and **P6**; eight subunits in **P3** and **P4**) on the periphery of the macrocycle. Upon light irradiation the system produces $^1\text{O}_2$, similar to conventional porphyrin PS. A fraction of the $^1\text{O}_2$ formed is bound to the pyridine subunits *via* a Diels-Alder-type process. Photosensitized $^1\text{O}_2$ is responsible

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for an immediate therapeutic response of the PDT agent, *i.e.* facilitates oxidation reactions in the cells. Following this initial response, a “slow” phase of the therapeutic effect occurs, whereby a retro-Diels-Alder process evolves $^1\text{O}_2$ from the trapping moieties, which can last up to several hours, depending on the substitution pattern of the trapping subunits (Scheme 1).

Scheme 1. Reversible addition of singlet oxygen to 2-pyridones and pyridone-porphyrins.

Porphyrins **P1** and **P2** bearing four pyridone subunits were prepared by reacting a 5,10,15,20-tetrakis(4-bromomethylphenyl)porphyrin precursor (**TBMPP**)[†] with 2-hydroxypyridine and 3-methyl-2-hydroxypyridine, respectively (see supporting information (SI)).¹¹ To prove the idea of delayed singlet oxygen release from the pyridone endoperoxides formed upon binding of self-sensitized $^1\text{O}_2$ to **P1** and **P2**, a Singlet Oxygen Sensor Green (SOSG) assay was applied. In SOSG a singlet oxygen responsive anthracene moiety is attached to a fluorophore and quenches its fluorescence *via* a PeT mechanism.¹² Reaction of the anthracene unit with singlet oxygen results in an increase in the probe fluorescence. Note, that such probes can overestimate the level of $^1\text{O}_2$ through self-sensitization of $^1\text{O}_2$.¹³ However, here the fluorescent response of the probe was examined without light irradiation of the sample excluding self-sensitization effects.

To determine the timescale of thermal $^1\text{O}_2$ release, **P1** and **P2** were irradiated with broadband light (400–700 nm) in THF at 0 °C for 1 h to ensure complete conversion of the pyridone moieties into the endoperoxides. After irradiation, SOSG (0.1 eq.) was added and its fluorescence was monitored over time while warming the sample to room temperature. As shown in Fig. 1A, $^1\text{O}_2$ release from **P1** took place at 20 °C and was complete within 5 h. In contrast, using **P2** under the same conditions, no detectable increase in the fluorescence was observed within 24 h. However, release of $^1\text{O}_2$ from this

system was initiated upon heating to 40 °C and was complete within 7 h at this temperature.

Figure 1. SOSG assay to detect $^1\text{O}_2$ released from endoperoxides formed by **P1** and **P2**. Fluorescence of the sensor was excited at 505 nm. A) Rise of SOSG fluorescence in a THF solution of **P1** at 20 °C (inset: 525 nm emission changes with time). B) Rise of SOSG fluorescence in solution of **P2** at 40 °C.

Porphyrins **P1** (Fig. 2)^{†,§} and **P2** were found to be insoluble in water, despite the polar nature of pyridone substituents. Hence, two approaches to increase solubility for cytotoxicity studies were attempted: i) introduction of more pyridone moieties (octasubstituted pyridone-porphyrins **P3** and **P4**) and ii) introduction of carboxyl groups in the meso-aryl substituents (**P5** and **P6**). The synthesis of the octapyridone-porphyrins involved bromination of 3,5-dimethylbenzoate **1** to yield compound **2** (Scheme 2). This was followed by reduction to the alcohol **3** with DIBAL and its oxidation to the aldehyde **4** with MnO_2 . A subsequent pyrrole condensation reaction gave the octa((4-(bromomethyl)phenyl)porphyrin **5** in 39 % yield. Treatment of **5** with excess 2-pyridone or 3-methyl-2-pyridone in the presence of NaH gave the target systems **P3** and **P4** in 58% and 65% yield, respectively.

Figure 2. Molecular structure of **P1** (thermal displacement 50%) showing the water bridges between pyridone substituents. Hydrogen bonds are indicated by dotted lines.

The second approach required selective monooxidation of dibromide **2** using bis(tetrabutylammonium)dichromate to aldehyde **6** which was used to access the tetrasubstituted pyridone-porphyrins **P5** and **P6** fashioned with carboxylic groups (Scheme 3). Condensation of **6** with pyrrole provided the brominated porphyrin **7** which was used in substitution reactions with the corresponding pyridones to yield **8** or **9**. Finally, saponification of the ester using excess KOH gave **P5** and **P6**. All of the aforementioned pyridone-substituted

porphyrins were purified by recrystallization as degradation during silica column chromatography was observed and were characterized by NMR and mass-spectrometry (see SI).

Scheme 2: Synthesis of octapyridone-porphyrins. a) NBS (2 eq.), benzoyl peroxide, CCl_4 , reflux; b) DIBAL (2 eq.), toluene, 70°C ; c) MnO_2 (10 eq.), DCM, rt; d) $\text{BF}_3\cdot\text{Et}_2\text{O}$, DCM, rt, then DDQ (0.75 eq.), rt; e) NaH (15 eq.), THF, reflux.

Scheme 3: Synthesis of tetrapyrindone-porphyrins with carboxylic acid functionalities. a) $(\text{Bu}_4\text{N})_2\text{Cr}_2\text{O}_7$ (0.66 eq.), CHCl_3 , 70°C ; b) $\text{BF}_3\cdot\text{Et}_2\text{O}$, DCM, rt, then DDQ (0.75 eq.), rt; c) NaH (15 eq.), THF, reflux; d) KOH (30 eq.), THF, reflux.

In order to ascertain whether the presence of pyridone versus methylpyridone units on the porphyrin core resulted in measurable differences, in terms of the compound's ability to cause photosensitized damage to cells, human adenocarcinoma cells (HT-29) were incubated with **P3**, **P4**, **P5** and **P6**. After 1 h the cells were washed, fresh medium was added, and the cells were irradiated with broadband visible light ($400\text{--}700\text{ nm}$, $20\text{ J}\cdot\text{cm}^{-2}$). On completion of irradiation cells were returned to the incubator for 24 h, after which cell viability was determined using the MTT assay.¹⁴

A comparison of the photocytotoxicity results for **P5** and **P6**, which bear four pyridone/methyl pyridone units revealed little

difference (Fig. 3). However, **P3** and **P4**, carrying eight pyridone/methyl pyridone units showed clear differences with 80% cell inactivation for **P4** versus 23% for **P3** at the highest concentration used ($200\text{ }\mu\text{M}$). Taking the structural similarities of **P3** and **P4** into account this result may be explained by enhanced cell killing due to slow $^1\text{O}_2$ release post irradiation.

Figure 3. HT29 cell viabilities after 1 h incubation with porphyrins **P3-6** with and without light irradiation ($400\text{--}700\text{ nm}$, $20\text{ J}\cdot\text{cm}^{-2}$).

Conclusions

In summary, we have synthesized and studied porphyrin photosensitizers bearing endoperoxide-forming 2-pyridone substituents on the periphery of the macrocycle. Photosensitization of oxygen by the porphyrin yields $^1\text{O}_2$, a fraction of which is stored due to [4+2] cycloaddition with the 2-pyridone subunits. The effect of the substitution pattern on the stability of endoperoxide gives access to systems which either release $^1\text{O}_2$ at room temperature or are completely stable and require moderate heating to trigger endoperoxide decomposition.

Further synthetic modifications of the sensitizers gave water-soluble derivatives, which can be used *in vitro* and *in vivo*. The differences observed in the cytotoxicities of the systems studied illustrate a viable concept for delayed singlet oxygen release from endoperoxide moieties formed upon irradiation of tissue/cell-localized sensitizers.

While this study gives proof-of-concept for using a photo- and chemodynamic approach for $^1\text{O}_2$ production, storage, and release, further studies and optimizations are needed to achieve a significant improvement over conventional PDT. In addition to uptake and intracellular localization studies these include a significant shortening of the irradiation time to provide a cytotoxic effect and require materials with a larger number of oxygen traps located in the proximity of the PS (such as polymeric endoperoxide carriers) which are currently under development.

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Notes and references

‡ CCDC 1559133, 1559134 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

§ The crystal structure of **P1** exhibits a rare packing arrangement where porphyrin layers are separated by channels of water molecules (see S.I.). This is aided by an extensive hydrogen-bonded water bridge between two pyridone oxygen atoms with a donor-acceptor distance of 2.739–2.771(6) Å.

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