αβ3-Isoform specific erbium complexes highly specific for bladder cancer imaging and photodynamic therapy†

Yan Zhou,‡ Chi-Fai Chan,§ Daniel W. J. Kwong,* Ga-Lai Law,*‡ Steven Cobb,§ Wai-Kwok Wong* and Ka-Leung Wong*‡

We have synthesized a bifunctional erbium–porphyrin tumor imaging and PDT agent (Er–R3) that is capable of killing bladder cancer cells via its selective binding to the integrin αβ3 isoform overexpressed on the cell membrane.

Photodynamic therapy (PDT) is an emerging novel cancer treatment modality suitable for repeated applications in treating diverse tumors. Since the treatment is highly localized, its systemic side-effects are relatively minimal with little adverse impact on the quality of life of the patients.1 Despite these advantages and the approval of a number of photo-sensitizers by the US Food and Drug Administration in the 1980s as a treatment option for localized (i.e., non-metastatic) cancers as well as for pre-cancerous lesions on skin and in the mouth, clinical use of PDT is still rather limited.2 This is due partly to technological constraints and partly to a lack of recognition of PDT as a medical specialty.3 Recently, with significant advances in light delivery and imaging technology, we begin to see a renaissance of PDT research and clinical translation.4–7 Nevertheless, conventional PDT drugs still suffer from several drawbacks: (i) it is only able to treat those lesions where light can penetrate, i.e., a few millimeters under the skin or from the irradiated tissue surface; (ii) some currently used PDT drugs render patients very sensitive to light and special precautions against light exposure must be taken until the drugs are cleared from the body in several days or even weeks; (iii) adverse in vitro/in vivo reactions occur due to the variation in physiological conditions and notched distribution of cytotoxic singlet oxygen; and (iv) non-specific cytotoxic activity may inflict damage to the normal cells during the PDT treatment.

Porphyran-based compounds, the first-generation PDT drugs, have been investigated continuously since their first clinical approval in an effort to overcome these drawbacks.8–10 Recently, several porphyrin derivatives have been developed to absorb strongly in the near-infrared (NIR) region (λ > 800 nm), where the tissue-penetration depth is much higher, via multi-photon femtosecond laser excitation with pinpoint accurate targeting to reduce collateral photodamage.11,12 In addition to the precise targeting using multi-photon laser, cancer selectivity can be incorporated into the PDT agents by taking advantages of some specific characteristics of the cancer cells. One recent example is a bifunctional gadolinium–porphyrin derivative which binds specifically to the anionic membrane of the cancer cells and then exerts its PDT action after NIR-excited imaging.13

Our design of a new generation bifunctional tumor-imaging and PDT agent is based on porphyrin–lanthanide complexes, with specific functional groups which allow them to localize selectively on particular tumor types, together with responsive imaging via NIR emission from the lanthanide.14,15 In this work, the tumor selective binding of the porphyrin–erbium complexes, Er–Rn (n = 1–3), is achieved through conjugation with bladder cancer-specific as well as integrin αβ3 isoform-specific peptides. The bladder cancer-specific peptide sequences, R1–R3 (molecular structures of R1–R3 are shown in Scheme S2, ESI†), are obtained from a combinatorial chemistry approach, with R3 further reported to be αβ3 integrin-specific as well.16 An increased integrin αβ3 expression has been observed in the neovascularization of bladder cancer, particularly in invasive carcinoma.16–18 Our results show that Er–R3 is able to interrupt bladder tumor growth significantly with specific lysosome localization indicated by responsive emission from Er. To increase the water solubility of the Er complexes over our previously reported analogues,13,14 a hydrophilic peptide R(RK was conjugated to the relatively more hydrophobic bladder cancer-specific peptide sequence R2 (–cGRLKEKKc–), affording the amphiphilic R3 peptide with an improved cell membrane permeability. The absorption coefficients and emission quantum yields of Er–R1, Er–R2 and Er–R3 are similar. The results of the photophysical measurement of Ln–Rn are shown in Table 1. Er exhibits stronger singlet oxygen quantum efficiency than Yb due to the fact that energy...
transfers from porphyrin to Yb for f-f emission is much better than that to Er for f-f emission, resulting in more excitation energy being channeled to singlet oxygen production.

In Fig. 1, the photophysical properties of Er or Yb porphyrin-based complexes are similar. However, the in vitro subcellular localization uptake and cytotoxicity (light and dark) are different due to the conjugated peptides. First of all, the subcellular localizations of Er-R1 and Yb-R1 complexes (n = 1, 2 and 3) in bladder cancer 5637 and T24 cells, cervical cancer HeLa cells and normal lung MRC5 cells (Fig. 2 and Fig. S22, ESI†) are different (dosed concentration = 5 μM; incubation time = 6 hours). The in vitro fluorescence intensity of the three Er complexes is higher than those of their Yb counterparts. This is due to an efficient energy transfer from the porphyrin to the Yb3+ ion which emits in the near-infrared region. In the bladder cancer 5637 and T24 cells, red porphyrin emission from Er-R1 is found only on the cell membrane, while the red emissions from Er-R2 and Er-R3 are found inside the cells. Their Yb analogues also showed the same subcellular localization pattern, i.e., the porphyrin emission from Yb-R1 is found on the cell membrane while Yb-R2 and Yb-R3 are found within the cells. Co-localization experiments using green LysoTracker were conducted. The red emissions from Er-R2, Er-R3, Yb-R2 and Yb-R3 were observed to overlap well with the green fluorescence from the LysoTracker in the 5637 and T24 cells (Fig. S22, ESI†). No such overlap was seen with Er-R1 and Yb-R1. These observations clearly indicate that the Er-R2, Er-R3, Yb-R2 and Yb-R3 complexes are mostly localized in the lysosomes of the 5637 and T24 cells. Er-R1 and Yb-R1, however, are localized in the 5637 and T24 cell membrane. This is consistent with the reported specificity of the conjugated R1 peptide towards integrin αvβ3 over-expressed on the bladder cancer cell membrane.

To confirm that the peptide sequences R1, R2 and R3 recognize bladder cancer specifically, in vitro imaging of Er-Rn and Yb-Rn (n = 1, 2 and 3) was performed using the non-bladder cancer cells, HeLa and MRC-5, under identical experimental conditions. No red emission was detected in HeLa or MRC-5 cells, thus showing very limited uptake of these porphyrin–lanthanide complexes. As the porphyrin complexes Er-Rn and Yb-Rn (n = 1, 2 and 3) will not bind to HeLa and MRC-5 cells, only the green emission from the LysoTracker is observed in the fluorescence staining experiment (Fig. S22, ESI†).

To confirm that the selective uptake of Er-Rn and Yb-Rn (n = 1, 2 and 3) complexes by bladder cancer cells was due to the recognition of the αvβ3 integrin on the 5637 and T24 cell surface, cellular uptake of these complexes functionalized with different peptides R1, R2 and R3 was studied by flow cytometry on the 5637, T24, HeLa and MRC-5 cells. Both R1 and R2 peptides were shown to exhibit specific binding towards bladder cancer through screening using the one-bead one-compound (OBOC) combinatorial peptide library technology,19 with R1 further reported to bind to the αvβ3 integrin on the T24 cell. The bladder cancer-specific binding of R1 was further demonstrated in vivo on a xenograft mouse model.20 R1 is designed as an amphiphilic peptide modified from R2 by the addition of a hydrophilic peptide RrRK to its N-terminal to enhance its cell permeability. The results from the flow cytometric cell uptake experiments are shown in Fig. S23 (ESI†). From Fig. S23 (ESI†),

Table 1 Summary of photophysical properties of Ln-Rn (Ln = Yb, Er; n = 1, 2, 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption (λmax) [nm] log(ε [dm^3 mol^-1 cm^-1])</th>
<th>Emission (λem) [nm] (Φ = ε)</th>
<th>Φem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yb-R1</td>
<td>425(5.37), 554(4.09)</td>
<td>656, 712(0.012), 975(29.86 μs)</td>
<td>Not found</td>
</tr>
<tr>
<td>Yb-R2</td>
<td>425(5.34), 554(4.16)</td>
<td>656, 712(0.013), 975(30.08 μs)</td>
<td>Not found</td>
</tr>
<tr>
<td>Yb-R3</td>
<td>425(5.27), 554(4.04)</td>
<td>656, 712(0.013), 975(99.97 μs)</td>
<td>Not found</td>
</tr>
<tr>
<td>Er-R1</td>
<td>426(5.32), 554(4.05)</td>
<td>654, 715(0.014), 1531</td>
<td>0.11</td>
</tr>
<tr>
<td>Er-R2</td>
<td>426(5.50), 554(4.53)</td>
<td>654, 715(0.014), 1531</td>
<td>0.12</td>
</tr>
<tr>
<td>Er-R3</td>
<td>426(5.36), 554(4.24)</td>
<td>654, 715(0.015), 1531</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Absorption and emission were measured in water (3% DMSO) at room temperature. b The emission quantum yield standard used was tetraphenylporphyrin (H2TPP) in anhydrous DCM (Φem = 0.120 at 298 K). c Lifetime was measured in water (3% DMSO) at room temperature. d The singlet oxygen quantum yield measured was referenced to tetraphenylporphyrin (H2TPP) in anhydrous DCM (Φem = 0.62 at 298 K).
no significant uptake of the Er–R2 and Yb–Rn complexes into T24 cells, in vitro PDT in various cell lines was carried out. A clinically approved conventional PDT agent, aminolevulinic acid (ALA), which exhibits no specific tumor selectivity, was used for comparison as well.21 An ideal PDT photosensitizer should have a low dark cytotoxicity and high photo-cytotoxicity under a light dose of 10 J cm−2.

These two contrasting properties can be summarized in terms of a photodynamic therapeutic index, PTI, which is defined as the ratio of the dark IC50 over light IC50 of the PDT agent. The phototoxicity of Er–Rn and Yb–Rn complexes towards T24, HeLa and MRC-5 cells was measured in dark and under photo-irradiation (550 nm long-pass filter, 6 mW cm−2, 28 min) using MTT assay. The results are shown in Fig. 3. These complexes exhibited high photo-cytotoxicity under a light dose of 10 J cm−2. Furthermore, their photo-cytotoxicity increased with increasing concentrations of the Er–Rn and Yb–Rn complexes. The IC50 of Er–Rn and Yb–Rn complexes to the T24 cells is 8–10 fold lower than those towards the HeLa and MRC-5 cells, thus demonstrating their selective PDT activities towards bladder cancer. Due to the amphiphilic character of the R3 peptide in Er–Rn and Yb–Rn, the cellular uptake of these complexes is higher than those of Er–R1, Er–R2, Yb–R1 and Yb–R2, thus resulting in their higher photo-cytotoxicity. In comparison, the ALA-PDT activity towards the T24 cells is 4–8 fold lower than those of the Er–Rn and Yb–Rn complexes. As for the HeLa and MRC-5 cells, ALA showed photocytotoxicity either comparable to or lower than those of the Er–Rn and Yb–Rn complexes. Among all of the Er–Rn and Yb–Rn complexes, Er–R3 shows the highest PTI of ca. 34, followed by Er–R2 > Er–R1. A similar trend is seen with the Yb–Rn complexes: Yb–R3 > Yb–R2 > Yb–R1. The dark cytotoxicity of all these complexes is very low (with their dark IC50 of over 1000 μM).

Based on these results, Er–R3 is the most promising candidate as a new generation PDT agent to selectively kill bladder cancer. Since a peptide specific for a particular cancer or stem cell type can be identified from a phage-displayed random peptide,22–24 our new generation erbium porphyrin complex can be extended not only to targeted imaging and destruction of any cancer type but also to tracking stem cell migration.

In conclusion, we present a multi-modal lanthanide–porphyrin PDT agent that is capable of killing tumor cells via 1O2 produced from a porphyrin moiety, affording fluorescence imaging simultaneously. Er–Rn is synthesized, and it shows high selectivity for bladder cancer cells by specifically targeting the integrin αvβ3 isofrom with strong NIR emission and 1O2 generation. The selective uptake of our complexes by cancer cells is confirmed by flow cytometry and in vitro imaging, and they are able to significantly interrupt the growth of bladder cancer cells via specific binding to the “integrin αvβ3 isofrom”.

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