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Enabling next-generation antibody-based photodynamic therapeutics

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Herein we present a significant step towards next-generation antibody-based photodynamic therapeutics. Site-selective modification of a clinically relevant monoclonal antibody, with a serum-stable linker bearing a strained alkyne, allows for the controlled Cu-free "click" assembly of an *in vitro* active antibody-based PDT agent using a water soluble azide porpyhrin.

Targeted photodynamic therapy (PDT) directed by macromolecules is based on the conjugation of a non-toxic photosensitizer to a biomolecule that has an ability to discriminate between different cell types.[1] The use of monoclonal antibodies for the targeted delivery of photosensitizers was first proposed by Mew and co-workers in the 1980s,[2] with minor advancements achieved throughout the next few decades. However, in recent years, there has been somewhat of a re-birth in the area, particularly due to developments in antibody targeting (*i.e.* to distinguish between cancerous and non-cancerous cells) and the FDA-approval of several antibody-drug conjugates (ADCs).[1,3] Conceptually, by attaching photosensitizers to cancer cell directed antibodies and injecting them into suitable cancer patients that have a cancer which overexpresses an appropriate antibody receptor, there will be selective delivery of the photosensitizers to cancerous tissue. Irradiation of the tumour will then activate the photosensitizers and induce the formation of reactive oxygen species (ROS), which will effect cell death. The destruction of cells by this method has been shown to destroy tumours by various mechanisms of action, however, they are all mediated by ROS generation.[4] This provides an important additional level of selectivity over conventional antibody-drug conjugates, *i.e.* the drug is only activated upon irradiation. Thus, targeted PDT has great potential to eliminate highly undesirable off-target toxicity, which is a major issue in existing chemotherapy and results in a low therapeutic ceiling for ADCs where the active cytotoxic drugs are delivered off-site due to issues of imperfect antibody targeting and poor drug-linker stability in serum.[1,3,5]

Whilst the concept of targeted PDT using monoclonal antibodies is undoubtedly very attractive, the delivery of a suitable platform to realise the true potential of this concept has lagged behind.[1] For targeted PDT, conjugation to antibodies is typically achieved through multiple lysine modification. Lysine modification is suboptimal as it generates heterogeneous products, which can result in batch-to-batch variability and poor pharmacokinetics.[6] Cysteine modification, following inter-chain disulfide reduction is another viable strategy, however this results in the permanent loss of structural disulfide bonds, which may reduce the stability of the antibody conjugate *in vivo*.[6] Moreover, cysteine modification is often carried out by reaction with classical maleimide compounds. Whilst this reaction is reliable, it has been widely reported (and unequivocally proven) that the formed succinimide conjugate is unstable in serum.[5,7a] This is due to the propensity of this motif to undergo retro-conjugate addition. This subsequently leads to undesirable transfer of the attached cargo onto blood thiols, particularly human serum albumin, thus resulting in off-target delivery.[5,7a]

Recently, we have described a site-selective method for the insertion of a dibromomaleimide into the disulfide bond of a fragment antigen-binding (Fab) arm of trastuzumab, a clinically approved antibody for the treatment of breast cancer.[8] Whilst this study provided proof of concept for site-selective porphyrin attachment to an antibody fragment, several shortcomings have come to light: (i) use of a Fab fragment with a single disulfide bond limits porphyrin attachment to one; (ii) dithiomaleimides (without hydrolysis) have been shown to be unstable in blood serum;[7b] (iii) access to the Fab fragment of trastuzumab through bacterial expression is not facile; and (iv) a copper-based "click" procedure necessitates prior porphyrin metalation (*e.g.* Zn) to prevent loss of both therapeutic singlet oxygen generation and fluorescent properties caused by copper insertion into the

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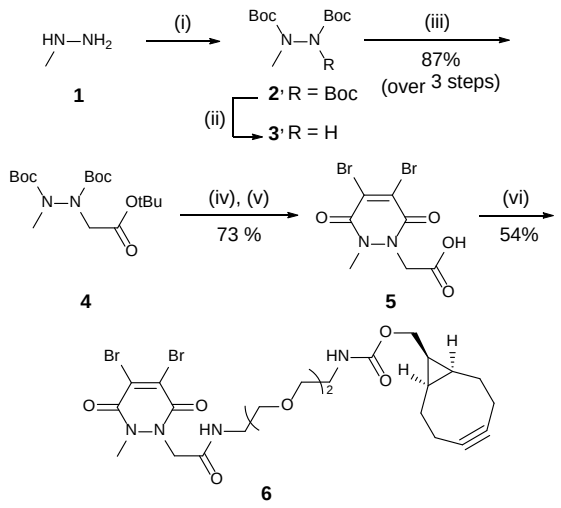
central cavity – this results in a blue shift of porphyrin light absorption, which is clinically undesirable.[9] Herein, we overcome all of the issues by providing a copper-free "click" method for the site-selective decoration of an antibody with serum stable linkers and an improved porphyrin loading of four.

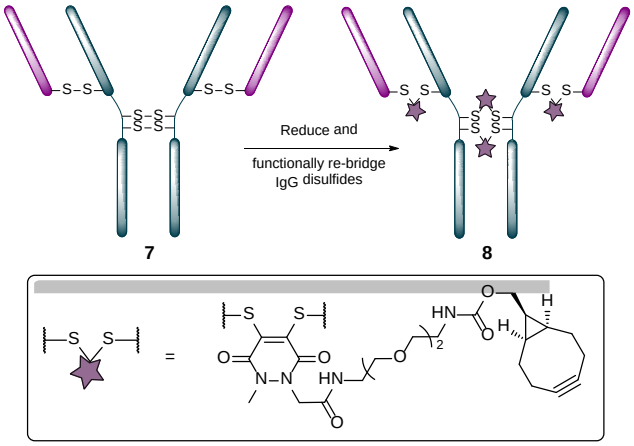
Initially we needed to select a suitable biomolecule that would allow for higher porphyrin loading, as well as still using a technology that allowed for site-selective modification. To this this end, we choose to use IgG trastuzumab in view of its clinical validation alone, as the antibody component of FDA-approved ADC Kadcyla™ and as it contains four disulfides for site-selective functionalisation.[7b,10,11a] Following this, we considered what disulfide linker technology should be applied for modification of the IgG. Chudasama, Caddick and co-workers have recently shown dibromopyrdazinediones to be viable candidates for disulfide stapling and that the resulting bisthioether is stable in blood plasma mimicking conditions.[11] As such, we chose this entity for functional disulfide bridging. Finally, to make our new platform modular and amenable to the use of copper-free "click" chemistry, a strained alkyne moiety was to be appended to the dibromopyrdazinedione, to allow for a copper-free strain-promoted alkyne-azide cycloaddition (SPAAC) reaction with a porphyrin azide.



Scheme 1 Synthesis of dibromopyrdazinedione-strained alkyne **6**. *Reagents and Conditions*: (i) Boc_2O, NEt_3, DMAP, CH_2Cl_2, 21 °C, 16 h; (ii) $Mg(ClO_4)_2$, MeCN, 50 °C, 1 h; (iii) *tert*-butyl bromoacetate, Cs_2CO_3, DMF, 21 °C, 16 h; (iv) TFA, CH_2Cl_2, 21 °C, 30 min; (v) dibromomaleic anhydride, AcOH, 21 °C for 16 h, then reflux, 2 h; (vi) PyBOP, DIPEA, CH_2Cl_2, 21 °C, 16 h.

Our study began with the synthesis of a suitable dibromopyrdazinedione-strained alkyne (Scheme 1). Initially, inexpensive methylhydrazine **1** was fully Boc protected to give hydrazine **2** in >99% yield. Following a procedure by Stafford *et al.*, hydrazine **2** was selectively mono-deprotected by mild cleavage of one of the Boc groups, using $Mg(ClO_4)_2$ catalysis, to yield mono-protected hydrazine **3**,[12] which was then alkylated to give *tert*-butyl ester **4** in 87% yield over the three steps. Hydrazine-ester **4** was then deprotected and condensed with dibromomaleic anhydride to afford acid-bearing pyridazinedione **5** in 73% yield. Finally, PyBOP-mediated coupling between commercially available BCN–PEG_2–NH_2 and acid **5** afforded bicyclononyne-based **Me**thyl-**Str**ained **A**lkyne PD **6** (Mestra PD) in 54% yield.

We next appraised whether dibromopyrdazinedione-strained alkyne **6** was amenable for the functional re-bridging of the disulfides of trastuzumab **7**. To do this, TCEP was added to a solution of trastuzumab in borate buffered saline (pH 8.0), containing an excess of Mestra PD **6**, and the mixture incubated at 4 °C for 16 h. Gratifyingly, analysis by SDS-PAGE revealed formation of Mestra-trastuzumab conjugate **8**. The number of pyrdazinediones per antibody was appraised by UV-Vis and was found to be four, which is consistent with complete re-bridging of the four accessible disulfides of trastuzumab.[11a]



Scheme 2 Insertion of dibromopyrdazinedione-strained alkyne **6** into the four disulfide bonds of trastuzumab **7** to form conjugate **8**.

Following on from the successful synthesis of conjugate **8**, we set about synthesising water soluble porphyrin azide **9**. This porphyrin is ideal as a therapeutic agent as the cationic tripyridinium motif provides excellent hydrophilicity without the toxicity associated with sulfonate derivatives.[8] The azide PEG chain also allows for facile "click" conjugation whilst also reducing steric hindrance between the porphyrin and antibody conjugate. In comparison to its previously employed zinc metallated counterpart, free base derivative **9** has more favourable photophysical properties; most significantly, a large (*ca.* 40 nm) red shift in the highest wavelength Q-band. This increase in absorption at longer wavelengths allows for enhanced tissue penetration by light, and thus, increased clinical relevance. Synthesis of porphyrin azide **9** was carried out according to the procedure described by Bryden *et al.*,[8] excluding the zinc metalation step.

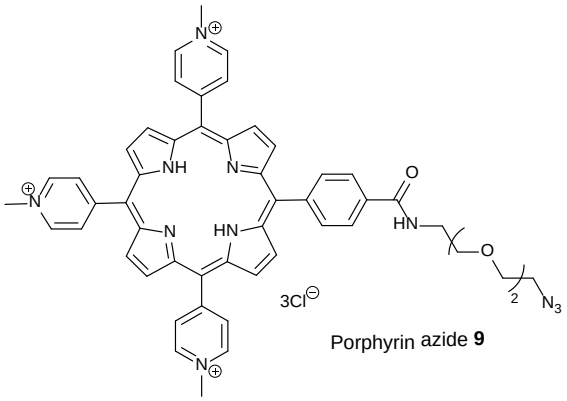


Figure 1 Structure of water soluble porphyrin azide **9**.

Following the successful synthesis of azide **9**, it was reacted with Mestra-trastuzumab conjugate **8** using SPAAC chemistry. The optimised conditions, incubation of conjugate **8** with 5 equivalents of the porphyrin azide for 4 h at 37 °C, effected complete conversion to conjugate **10** in near quantitative yield and in high purity. Porphyrin loading was confirmed by UV-Vis and optimisation of the reaction conditions was carried out on the Fab arm of trastuzumab (see ESI for details).

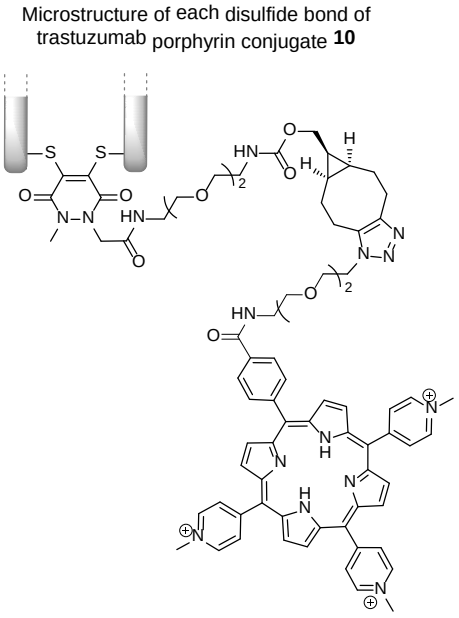


Figure 2 Structure of trastuzumab porphyrin conjugate **10**.

With trastuzumab-porphyrin conjugate **10** in hand we next appraised its efficacy *in vitro*, with trastuzumab only as a control, using appropriate HER2+ and HER2- cell lines (*i.e.* BT-474 and MDA-MB-468, respectively). To do this, experiments were carried out as described in the ESI. In these experiments, broad spectrum light (20 J cm^{-2}) was used to ensure sufficient activation of the photosensitizer from the QTH lamp used. Clinically, light in the red region of the spectrum would more typically be used, where the weaker absorption of the porphyrins in this region is compensated for by using biomedical lasers with light fluences which are orders of magnitude greater than those used here. The present study was thus designed to assess photodynamic potential *in vitro*, and further studies using red light would clearly be needed before moving to a clinical setting.

Under the tested conditions, it can unambiguously be seen that conjugate **10** exhibits remarkable abilities to eradicate HER2+ cells (*ca.* 90% kill), while at the same concentration leaving HER2- cells unaffected (Figure 3). In both cell lines, conjugate **10** displayed minimal dark toxicity at the highest antibody concentration used (3 µM) and no dark toxicity at the concentration required to kill 90% of cells on irradiation (625 nM).

Control experiments using trastuzumab alone or unconjugated porphyrin **9** (Full data in ESI) showed no cytotoxicity for the antibody and minimal cytotoxicity (< 80%) for the unconjugated porphyrin with both cell lines at the concentration where 90% of cells were killed by the conjugate (625 nM).

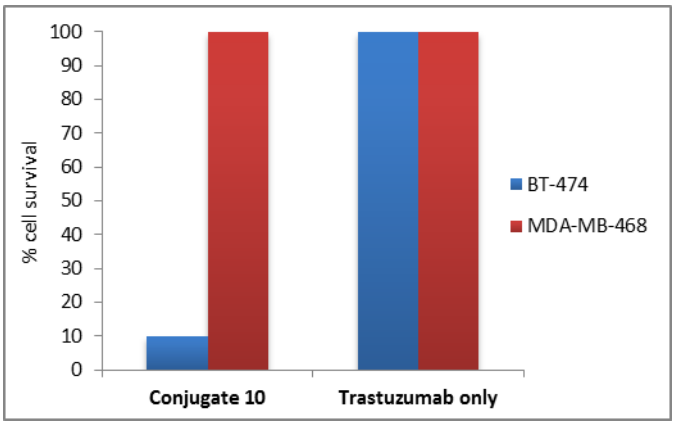


Figure 3 *In vitro* appraisal of trastuzumab porphyrin conjugate **10** and trastuzumab alone in broad spectrum light at an antibody concentration of 625 nM (Full cytotoxicity data in ESI).

Conclusions

In conclusion, we have provided an important step towards delivering on next-generation antibody-based porphyrin conjugates. In this manuscript we site-selectively modify a full antibody with four pyrdazinedione linkers (one per native disulfide), each bearing a strained alkyne handle, in a controlled manner. Following this, facile SPAAC chemistry was performed to provide a defined multi-porphyrin antibody conjugate, which was shown to be active *in vitro*.

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