

# Mono- and tri-cationic porphyrin–monoclonal antibody conjugates: photodynamic activity and mechanism of action

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## Introduction

Photodynamic therapy (PDT) is now an established clinical cancer treatment.<sup>1</sup> PDT utilizes the combination of a photosensitizing compound, light and oxygen to generate reactive oxygen species (ROS) *in situ*, thus promoting destruction of localized neoplastic lesions.<sup>2,3</sup> As a consequence of the short lifetime of ROS, the oxidative damage is limited to a spherical area of no more than 10 nm surrounding the photosensitizer that generated it; this small area, combined with the ability to direct light with high precision via fibre optics, gives PDT a high degree of spatial control. PDT is employed in most of the developed countries of the world with good effect; however, the selectivity of commercial PDT agents, such as Photofrin<sup>®</sup> (Axcan Pharma Inc., Quebec, Canada), Foscan<sup>®</sup> (Biolumitec Pharma Ltd, Dublin, Ireland) and Verteporfin<sup>®</sup> (QLT Inc., Vancouver, Canada), for tumour tissue remains an area where there is considerable room for improvement. Typically, tumour to peritumoural tissue ratios are signi-

## Summary

Two cationic porphyrins bearing an isothiocyanate group for conjugation to monoclonal antibodies have been synthesized. The two porphyrins conjugated efficiently to three monoclonal antibodies (anti-CD104, anti-CD146 and anti-CD326), which recognize antigens commonly over-expressed on a range of tumour cells. *In vitro*, all conjugates retained the phototoxicity of the porphyrin and the immunoreactivity of the antibody. Mechanistic studies showed that conjugates formed from the mono- and tri-cationic porphyrin and anti-CD104 antibody mediated apoptosis following irradiation with non-thermal red light of  $630 \pm 15$  nm wavelength. *In vivo* antibody conjugates caused suppression of human LoVo tumour growth in immunodeficient NIH III mice, similar to the commercial photodynamic therapy (PDT) agent Photofrin<sup>®</sup>, but at administered photosensitizer doses that were more than two orders of magnitude lower. Positron emission tomography (PET) following PDT showed a large, early increase in uptake of <sup>18</sup>fluorodeoxyglucose (FDG) by tumours treated with the anti-CD104 conjugates. This effect was not observed with Photofrin<sup>®</sup> or with conjugates formed from the same photosensitizers conjugated to an irrelevant antibody.

**Keywords:** bioconjugation; head and neck cancer; monoclonal antibody; photodynamic therapy

ficantly below 10 : 1 at the time of treatment.<sup>4</sup> Lack of selectivity requires the administration of higher doses of photosensitizer to achieve the desired clinical effect, which can lead to generalized photosensitivity for the patient and a reduced safety margin when treating tumours in sensitive areas, such as the brain.

It is unsurprising that relatively low tumour to peritumoural tissue ratios are achieved, as a result of the fact that the photosensitizer is usually not targeted in any specific manner. In the case of cancer, accumulation at the desired site simply relies on the poorly developed and permeable neovasculature together with a lack of sufficient lymphatic drainage,<sup>5</sup> leading to the enhanced penetration and retention effect. Recently, there has been great interest in targeting photodynamic sensitizers more accurately, using a variety of strategies.<sup>6</sup> One way in which accurate delivery of photosensitizers can be achieved is by conjugation to antibodies that recognize tumour-associated antigens.<sup>7</sup> Such compounds allow the direct targeting of the photosensitizer to the correct cell type, as well as

quicker clearance of the conjugate from the blood stream, resulting in a reduced risk of photosensitisation to normal tissues. The easiest way to achieve a stable and active antibody conjugate is to link the photosensitizers covalently to the antibody in a defined manner.<sup>8</sup>

Over the past decade we have developed a highly efficient method for conjugating photodynamic sensitizers to proteins based on mono-isothiocyanato (NCS) porphyrins.<sup>9–12</sup> Advantages of this method, compared with other methods of conjugation, such as carbodiimide mediated coupling of carboxy-bearing photosensitizers and the use of activated esters, include the following.

- (1) No opportunity for protein cross-linking exists, as only one amine-reactive NCS group is presented on each photosensitizer molecule. This contrasts with naturally occurring porphyrins such as haem-atoporphyrin (Hp), and chlorin *e*<sub>6</sub>, which bear multiple carboxyl groups.
- (2) The NCS group, unlike the carboxyl groups of Hp and chlorin *e*<sub>6</sub>, which require activating agents, reacts spontaneously with amino groups of lysine residues by an addition rather than substitution reaction. This bypasses the need to separate the conjugate from by-products generated by the activating agent.
- (3) Generic synthetic routes to NCS porphyrins allow control of factors such as solubility and charge, by incorporation of suitable groups early in the synthesis. Such factors allow minimization of non-covalent binding of photosensitizer to the protein, as well as the potential targeting of photosensitizer to specific intracellular sites following internalization.

A large number of variations of this general class of compounds using different NCS porphyrin/antibody combinations have been screened, and now a set of conjugates which are highly selective for antigens over-expressed on a range of tumours, including colorectal and lung, are reported. The antigens chosen were CD104 ( $\beta$ 4 integrin), CD146 (melanoma cell adhesion molecule) and CD326 (epithelial cell adhesion molecule). Previous studies on conjugates of porphyrins with internalizing versus non-internalizing antibodies have shown the internalizing conjugates to be more powerful PDT agents,<sup>11</sup> so for this reason antibodies have been selected that correspond to antigens that are known to internalize following ligand binding. Two well-established human cell lines originating from colorectal and lung tumours (LoVo and CORL23, respectively), which show differential expression of these cell antigens, were used to demonstrate proof of concept. Lung tumours are routinely treated with PDT and there are ongoing clinical trials investigating efficacy in colorectal malignancies.<sup>1</sup> Specific immunoconjugates show enhanced photodynamic activity *in vitro*, against LoVo and CORL23 cells, and suppress the growth of tumours that over-express

antigens corresponding to the relevant conjugates *in vivo*.

## Materials and methods

### *Synthetic procedures*

Synthesis of isothiocyanato porphyrins was achieved using procedures similar to those previously reported.<sup>10</sup>

### *Cell lines and antibodies*

The LoVo human colon adenocarcinoma and CORL23 human large cell lung carcinoma (ECACC) cell lines were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) and RPMI, respectively. All medium was supplemented with 10% [volume/volume (v/v)] fetal calf serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 µg/ml penicillin (Invitrogen, Paisley, UK). Cells were maintained at 37° in a humidified incubator with a 5% CO<sub>2</sub> atmosphere.

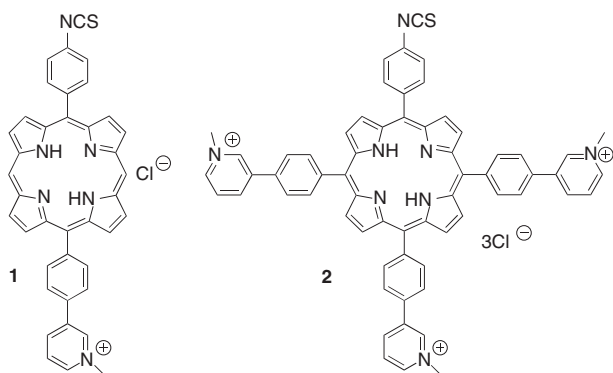
The mouse anti-human antibodies, anti-CD104, anti-CD146 and anti-CD326, were all purchased from AbD Serotec (Kidlington, Oxford, UK). Anti-CD104 binds to a 205-kDa glycoprotein involved in cell–cell adhesion that is up-regulated in colorectal and bladder carcinomas. Anti-CD146 (Muc-18) binds a 113-kDa glycoprotein that has been reported to be expressed on metastatic tumours. Anti-CD326 (EpCAM) recognizes a 40 kDa cell–cell adhesion molecule up-regulated on many carcinomas, particularly colorectal.

### *Conjugation to monoclonal antibodies*

Conjugation was carried out as described previously.<sup>11</sup> Briefly, the antibody and porphyrin 1 or 2 (Fig. 1) were agitated gently at room temperature for 1 hr, while being protected from light. Conjugates were purified using Sephadex G25 columns (Amersham, Buckinghamshire, UK) and eluted with phosphate-buffered saline (PBS), pH 7.4. The degree of labelling (the number of moles of porphyrin conjugated per mole of antibody) was calculated using spectroscopic methods.<sup>12</sup> Immunoconjugates were centrifuged at 13 000 g for 10 min to remove any traces of aggregates, and then the supernatant was filtered through a 0.2-µm syringe filter prior to storage at 4° or, for long-term storage, at –20°.

### *Flow cytometry*

Cells were removed from culture vessels with 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS. After washing in PBS, cells were counted and re-suspended in PBS/0.25% w/v bovine serum albumin (BSA) and 2 × 10<sup>5</sup> cells were added to each Falcon 2054 fluorescence-activated



**Figure 1.** Structures of 5-(4-isothiocyanatophenyl)-15-(4-(3-*N*-methylpyridinium)phenyl) porphyrin (porphyrin 1) and 5-(4-isothiocyanatophenyl)-10,15,20-tri(4-(3-*N*-methylpyridinium)phenyl) porphyrin (porphyrin 2).

cell sorter (FACS) tube (BD Biosciences, Oxford, UK). The cells were labelled with 50  $\mu$ l (5  $\mu$ g/ml) of the test antibody, either conjugated or unconjugated, for 1 hr at 4°. After washing with PBS/BSA, cells were labelled with 5  $\mu$ l (10  $\mu$ g/ml) of rabbit anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) (AbD Serotec) for 1 hr at 4°. A further wash with PBS/BSA was performed before analysis of cells in a FACS Calibur flow cytometer running CELLQUEST PRO v3.3 (BD Biosciences, Oxford, UK).

#### *In vitro cytotoxicity*

Cells were removed from culture vessels with 5 mM EDTA in PBS, washed and counted, and  $1 \times 10^5$  cells were added to 5-ml polypropylene tubes. Antibody immunconjugates and unconjugated porphyrin were diluted in serum-free medium, added to the cells, and incubated at 37° for 6 hr. Cells were washed three times with medium to remove unbound immunconjugate or porphyrin, re-suspended in complete medium and plated out in duplicate in two 96-well plates ( $2.5 \times 10^4$  cells/well). One plate acted as the dark control (i.e. no irradiation) while the other was irradiated with 15 J/cm<sup>2</sup> of cooled and filtered red light (630 nm) delivered by a Paterson light system (Paterson Lamp BL1000A, bandpass 630  $\pm$  15 nm filter; Phototherapeutics Ltd; Altricham, UK). The plates were incubated at 37° overnight before a commercial MTS assay (Promega, Southampton, UK) was performed. The percentage of cell survival was calculated in proportion to the number of cells incubated without photosensitizer. All assays were performed in quadruplicate.

#### *Assessment of mitochondrial membrane potential with JC-1*

The MitoProbe JC-1 assay kit (Molecular Probes, Invitrogen, Paisley, UK) was used according to the manufacturer's instructions. Briefly, LoVo or CORL23 cells

( $1 \times 10^6$ /ml) were re-suspended in PBS to which freshly reconstituted JC-1 dye solution was added to give a final concentration of 2  $\mu$ M. Cells were incubated for 15 min, washed to remove excess dye and analysed by flow cytometry. JC-1 is taken up by cells and will accumulate in the mitochondria of healthy, functioning cells where it fluoresces (peak emission 590 nm) in the FL-2 channel of a flow cytometer; however, if the mitochondrial membrane potential is disrupted, JC-1 'leaks' into the cytoplasm and breaks down into the monomeric form that fluoresces (peak emission 529 nm) in the FL-1 channel.

#### *Assessment of apoptosis*

The proportion of cells undergoing apoptosis (annexin positive) or necrosis (annexin and propidium iodide dual positive) was determined using a standard flow cytometry Annexin-V-FITC binding assay according to the manufacturer's instructions (AbD Serotec).

Caspase 3 activation was measured using a polyclonal rabbit anti-human caspase 3 antibody (1 : 1000 dilution, AHP476; AbD Serotec) that binds both the 32-kDa procaspase and the active (17-kDa) moiety, detected with a horseradish peroxidase (HRP)-conjugated sheep anti-rabbit secondary antibody and ECL Western Blotting Detection Reagents (Amersham). Cell death was assessed by both methods following incubation with immunconjugates and red-light illumination.

#### *In vivo tumour response*

Immunodeficient NIH III mice bearing two LoVo tumours (diameter 5–6 mm; one in each leg) were injected with Photofrin® (5 mg/kg or 8.3  $\mu$ mol/kg using the molecular weight for monomeric hematoporphyrin; Photofrin® is a mixture of haematorporphyrin oligomers) or conjugate (10 nmol/kg) 24 hr prior to irradiation: one tumour per mouse was irradiated while the second served as an unirradiated control. The light source was a 1000 W mercury-xenon lamp with two glass filters (Corion LL590 and LS700; Newport Corporation, Stratford, CT) to give light between 590 and 650 nm. The light was fed into a fibre-optic cable to permit irradiation in a laminar flow hood. The fluence at the delivery end of the cable was 26 mW. The total dose given to each tumour was 200 J/cm<sup>2</sup>. Tumour volume was calculated by measuring the diameter of the tumours with a calliper.<sup>13,14</sup>

#### *PET investigation of PDT tumour response*

Mice were imaged 24 hr, 48 hr or 1 week after PDT. Images were acquired using the LabPet™ Small Animal scanner (University of Sherbrooke, Sherbrooke, QC, Canada), with a resolution of 1.35 mm full width at half-maximum peak height transaxial.<sup>15</sup> The animal was

anaesthetized under isoflurane anesthesia (1% at 2 l/min oxygen flow) and 37 MBq of  $^{18}\text{F}$  fluorodeoxyglucose (FDG) was administered via the caudal vein in 200–300  $\mu\text{l}$  of physiological saline. To obtain a more accurate measure of the injected FDG dose, the radioactivity in each animal was quantified in the well radioactivity counter immediately after injection of the radiotracer. The mouse was then revived and replaced in its cage until the scan. This procedure reduced the time spent by the animal under anaesthesia. Imaging was initiated 60 min after intravenous administration and lasted for 20 min. The animal was anaesthetized immediately before imaging and was placed supine on the bed of the camera and kept warm with a warm air flow system. A single bed position was used with both the radial and axial double sampling modes. The images were reconstructed using 10, 15 and 20 iterations of the maximum likelihood expectation maximization (ML-EM) algorithm that models detector response.<sup>16</sup> Overall, 31 images in  $128 \times 128$  pixel format (voxel size of  $0.5 \times 0.5 \times 1.193 \text{ mm}^3$ ) were obtained, covering 37 mm axially. Images were then used to quantify FDG uptake in the regions of interest (treated and untreated tumours). After imaging, the mice were sacrificed.

Images were analysed with the SHERBROOKE LAB-TEP software (University of Sherbrooke, QC, Canada). To quantify tumour  $^{18}\text{F}$ FDG uptake we first selected all the slides on which the tumours were visible. We drew a region of interest (ROI) surrounding the tumour on each of these slices, revealing the partial activity. The activities of each ROI were then summed to obtain the total radioactivity content of the tumour. The results were then expressed as the %ID/ $\text{mm}^3$  of tumour by taking into account the correction for  $^{18}\text{F}$ FDG decay and the counting efficiency of the scanner.

## Results

### Photosensitizer synthesis

Previous studies of porphyrin-based isothiocyanato porphyrins have demonstrated that the combination of cationic charge with a single isothiocyanate group is optimal for bioconjugation.<sup>10,17</sup> Amphiphilicity has also been shown to be important for good PDT activity.<sup>4</sup> We have identified two structures that combine these physico-chemical characteristics (Fig. 1).

### Antibody labelling with porphyrin photosensitizers

A series of *in vitro* experiments were initially undertaken to verify the integrity of a series of immunoconjugates utilizing different antibodies. Firstly, the degree of porphyrin labelling was assessed spectroscopically and, after optimization of the conjugation procedure, an average of

1:1 photosensitizer per antibody was achieved, which was slightly lower than previous results using 35A7, FSP77 and 17-1A antibodies (average of 1.8).<sup>11</sup>

Having demonstrated that several different antibodies could successfully be conjugated to both porphyrin derivatives, flow cytometry was used to assess whether this process affected antibody binding. Figure 2 clearly demonstrates that conjugation did not significantly affect antibody reactivity, neither reducing binding as a result of steric hindrance caused by conjugation to amino acid residues in, or close to, the complementarity-determining regions, nor increasing non-specific interactions resulting from changes in hydrophobicity. As expected, all three antibodies bound to CORL23 and only anti-CD104 and anti-CD326 bound to the LoVo cells, reflecting the absence of CD146 on LoVo cells.

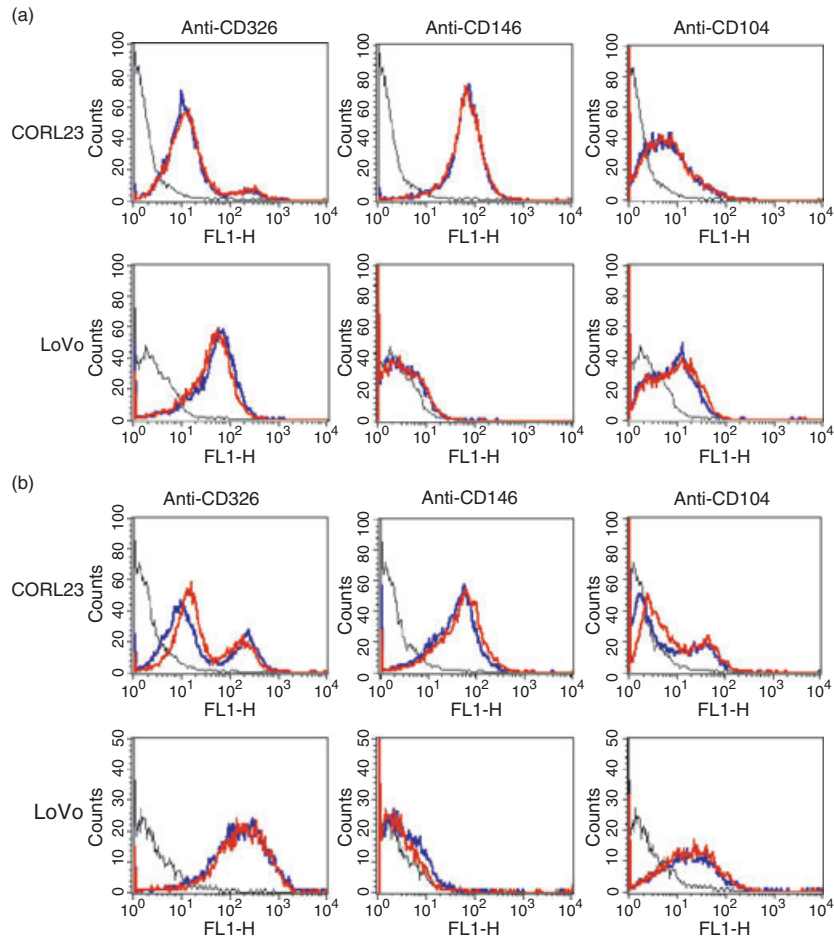
### Photocytotoxicity *in vitro*

Phototoxicity of conjugates and a non-conjugated ‘control’ porphyrin was assessed as previously reported.<sup>17</sup> Because of the reactive nature of the isothiocyanate group the ‘control’ porphyrins were prepared by reacting porphyrin 1 and porphyrin 2 (Fig. 1) with an excess of propylamine. The resulting product represented the photosensitizer with an identical thiourea linkage to the conjugates, but with a short propyl chain in place of the antibody. Table 1 shows the  $\text{LD}_{50}$  values (dose of porphyrin needed to kill 50% cells) for the six immunoconjugates, compared with the corresponding porphyrin ‘control’. Although it was clear that the immunoconjugates had approximately 10-fold lower  $\text{LD}_{50}$  compared with their respective ‘control’ porphyrins, a non-specific PDT effect was observed. This was shown by the relatively similar  $\text{LD}_{50}$  for the anti-CD146 conjugates against LoVo; a cell line that does not express the CD146 antigen.

The *in vitro* data shown in Table 1 were obtained with a 6-hr incubation period, in order to allow time for internalization of antigens and any bound antibody. It was thought likely that the extended length of time (for similar experiments using no conjugated photosensitizers a 1-hr incubation is normal) permitted the non-specific enhancement of phototoxicity seen with the anti-CD146/LoVo combination. It should be noted, however, that with both photosensitizers the minimum enhancement of photocytotoxicity, as compared with porphyrin alone, was obtained with the anti-CD146 conjugates.

### Anti-CD104-porphyrin mode of cell death

The mechanism of cell death is highly relevant to the treatment of some cancers, as the promotion of apoptotic, rather than necrotic, mechanisms can reduce scarring and lead to regeneration of functional tissue. An early stage of apoptosis is the ‘flipping’ of phosphatidyl serine (PS)



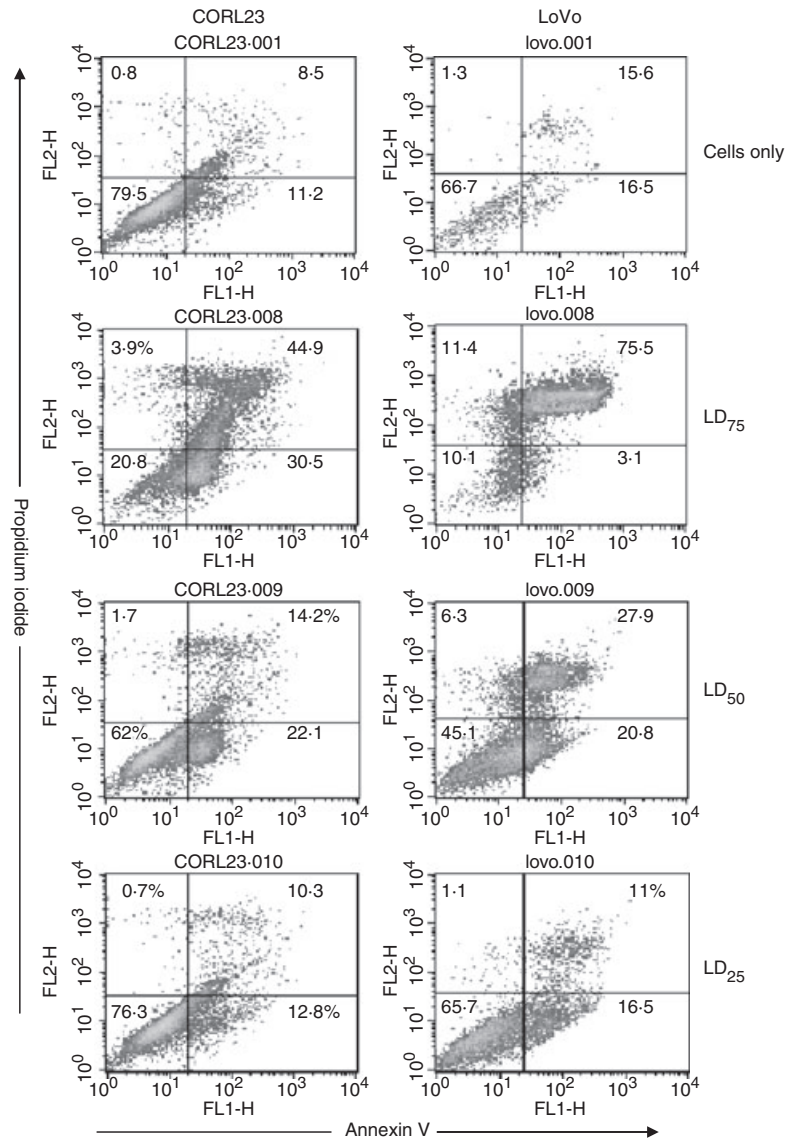
**Figure 2.** Conjugated and unconjugated anti-CD326, anti-CD146 and anti-CD104 were analysed by flow cytometry for binding to CORL23 and LoVo cells [black line, negative control; blue line, unconjugated antibody; red line, immunoconjugates with porphyrin 1 (a) or porphyrin 2 (b)].

from the inner to the outer side of the plasma membrane, which is detectable with Annexin V-FITC. To investigate the possible mechanisms of cell death, cells were incubated with anti-CD104-porphyrin 1 immunoconjugates for 6 hr before irradiation with 15 J/cm<sup>2</sup> light. Cell death was then assessed for Annexin-V/propidium iodide binding 15 min post-irradiation (Fig. 3). At the LD<sub>25</sub> concentration the cells behaved similarly; however, from LD<sub>50</sub> and above the LoVo cells were more sensitive to the effects of PDT, with a greater percentage in the upper right (apoptotic and necrotic) quadrant. At LD<sub>75</sub> LoVo cells appeared markedly more sensitive to killing, with the vast majority of cells in the upper right quadrant compared with 45% of CORL23. The latter cell type also showed 30% in the lower right (apoptotic) quadrant at the highest drug dose, whereas the LoVo cells had ‘progressed’ to necrosis. A similar trend was seen with the other immunoconjugates, with LoVo showing higher levels of necrotic cell death; surprisingly, the anti-CD146 conjugate also showed high levels of non-specific toxicity (data not shown).

**Table 1.** Immunoconjugate LD<sub>50</sub> concentrations from *in vitro* cytotoxicity assay

	LoVo (µM)	CORL23 (µM)
Porphyrin 1	2	3
Anti-CD326-porphyrin 1	0.57	0.4
Anti-CD146-porphyrin 1	1.05	0.72
Anti-CD104-porphyrin 1	0.68	0.68
Porphyrin 2	6	3.5
Anti-CD326-porphyrin 2	0.43	0.4
Anti-CD146-porphyrin 2	0.76	0.5
Anti-CD104-porphyrin 2	0.48	0.25

It has also been shown previously that photosensitizers that localize to the mitochondria are very rapid inducers of apoptosis, as compared with those that localize in the lysosomes or the plasma membrane.<sup>18</sup> After irradiation, mitochondrially localized photosensitizers cause immediate release of cytochrome C and associated disruption of the mitochondrial membrane

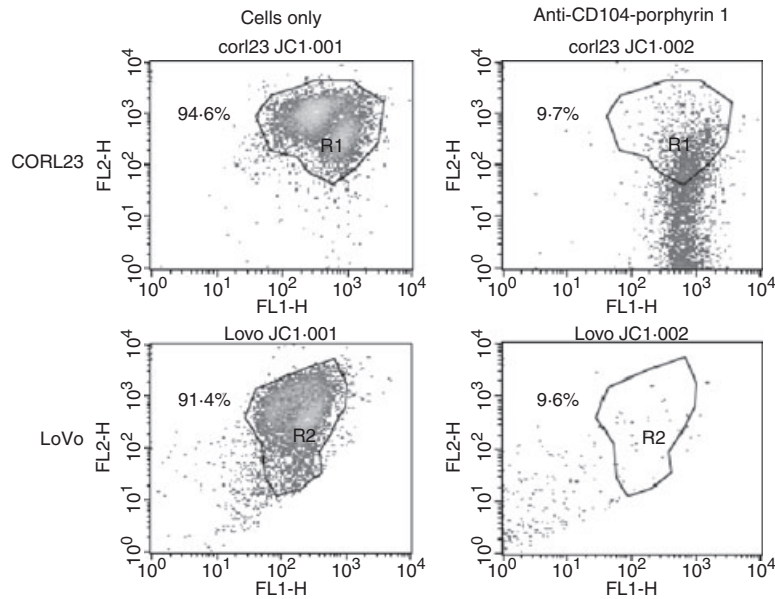


**Figure 3.** CORL23 and LoVo cells were treated with LD<sub>25</sub>, LD<sub>50</sub> and LD<sub>75</sub> concentrations of anti-CD104-porphyrin 1 and incubated for 6 hr prior to irradiation at 15 J/cm<sup>2</sup>. Cells in the lower left quadrant are viable, those in the lower right quadrant are apoptotic and those in the upper right quadrant are necrotic; 10 000 events were analysed for each sample.

potential. These events are quickly followed by activation of various caspases, particularly caspase-3. Here we have shown that the mitochondrial membrane potential, as measured by JC-1 dye staining, is significantly disrupted by both porphyrin derivatives, with the effect being most pronounced for the anti-CD104-porphyrin 1 reagent (Fig. 4). Once again, LoVo cells appeared to be far more susceptible to photodynamic-induced damage, as shown by the almost complete destruction of the cells, when assayed by flow cytometry. Such a clear difference in susceptibility was not observed in the MTS assay (Table 1), probably as a result of the fact that the latter assay is assessing a more general measure of cell

metabolism, rather than the specific function of the mitochondria.

Furthermore, when the cells were assayed for procaspase 3 conversion to active caspase 3, with associated commitment to apoptotic cell death, it was clear that, although this occurred in both cell types, once again it was considerably faster in LoVo cells. Figure 5 shows procaspase 3 activation in LoVo and CORL23 cells following incubation with an LD<sub>75</sub> concentration of anti-CD104-porphyrin 1 for 6 hr and irradiation with 15 J/cm<sup>2</sup> light. Cell lysates were prepared immediately (0 min) and at 15 min, 1.5 hr and 3 hr post-irradiation and were analysed by western blotting. Loss of procaspase 3



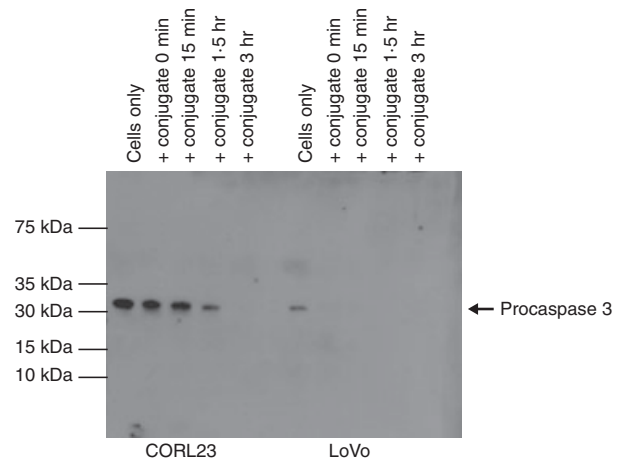
**Figure 4.** Cells were incubated with LD<sub>75</sub> concentrations of anti-CD104-porphyrin 1 for 6 hr prior to irradiation at 15 J/cm<sup>2</sup>. Cells were then labelled with 2 μM JC-1 dye for 15 min and then analysed by flow cytometry; 10 000 events were analysed for each sample. At least 90% of cells showed a decrease in red fluorescence, indicating a disruption of mitochondrial membrane potential.

demonstrates activation, which was completed ‘immediately’ following irradiation in LoVo cells, whereas more than 1.5 hr was required to obtain a similar effect with the same immunoconjugate against CORL23.

***In vivo* PDT studies in a LoVo xenograft tumour model**

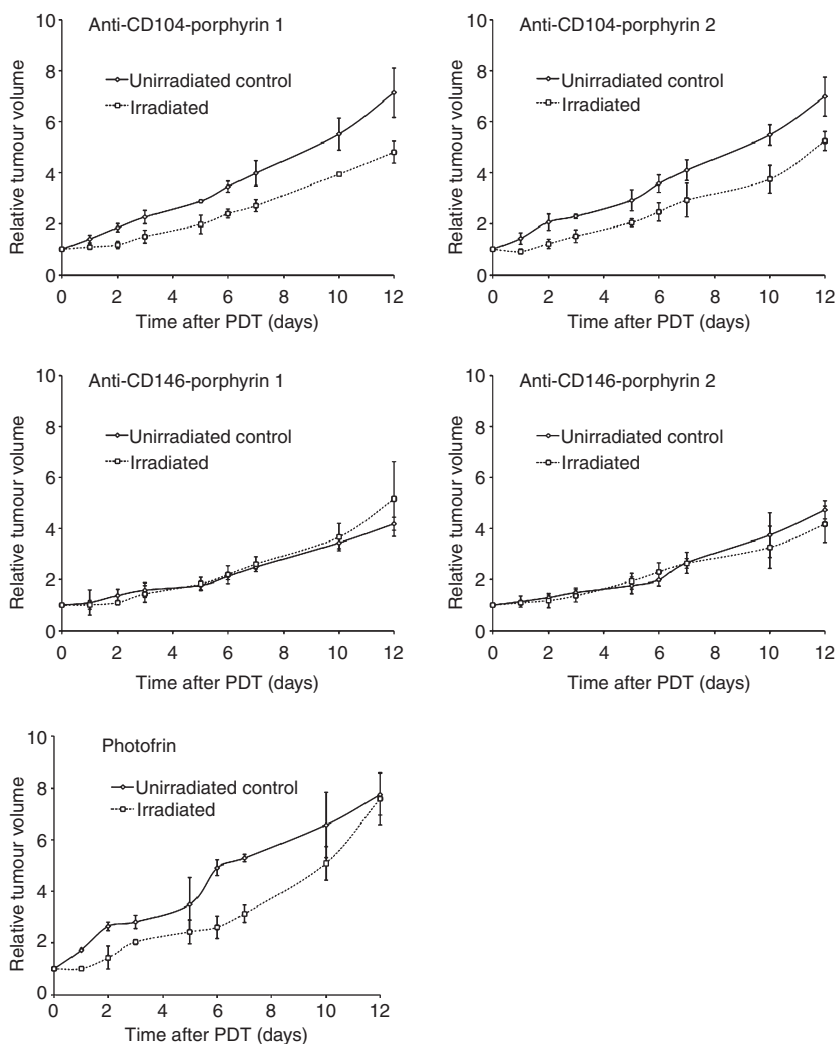
The above *in vitro* investigations suggested that photosensitizer–antibody conjugates could be prepared which maintained both the immunoreactivity of the antibody and the phototoxicity of the photosensitizer. Mechanistic studies also suggested a significant apoptotic contribution to photodynamic cell death. The major goal of any antibody–toxin conjugate is, however, the successful delivery of the cytotoxin efficiently to the target tissue, with a subsequent enhanced therapeutic effect. In order to demonstrate this for the conjugates described here, an *in vivo* model system was designed which allowed tumours of one of the cell types used for our *in vitro* studies to be grown into solid tumour masses on NIH III immunodeficient mice. In order to allow direct ‘benchmarking’ against a commercially available clinical PDT agent, Photofrin<sup>®</sup> was included in these studies.

Photodynamic treatment was conducted by intravenous injection of immunoconjugates, or Photofrin<sup>®</sup>, into mice bearing two tumours, one on each hind leg. One tumour was then irradiated, while the second was protected from light and acted as a control. Doses of immunoconjugates (10 nmol/kg) were based on injected



**Figure 5.** Cells ( $1 \times 10^5$ ) were lysed in reducing sample buffer, over a range of times, following 6 hr of incubation with anti-CD104-porphyrin 1 and then irradiation at 15 J/cm<sup>2</sup>. Following sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting with an anti-caspase 3 polyclonal rabbit anti-human immunoglobulin G (IgG) (1 : 1000) antibody, loss of the 32-kDa procaspase 3 was observed; however, the 17-kDa active form was not detectable. Caspase 3 activation was far more rapid in LoVo than the CORL23 cells.

photosensitizer concentrations, which were determined spectroscopically. Photofrin<sup>®</sup> was administered at a standard dose (5 mg/kg; equivalent to 8.3 μmol/kg) used for PDT treatment of tumour-bearing mice. Two relevant



**Figure 6.** Immunodeficient NIH III mice bearing two LoVo tumours (diameter 2–3 mm; one in each leg) were injected with Photofrin<sup>®</sup> (5 mg/kg) or conjugate (10 nmol/kg) 24 hr prior to irradiation: one tumour per mouse was irradiated while the second served as an unirradiated control. Tumour size was measured by calliper and growth was followed to 12 days post-irradiation. Tumour size for three mice per point was averaged and the standard error is shown.

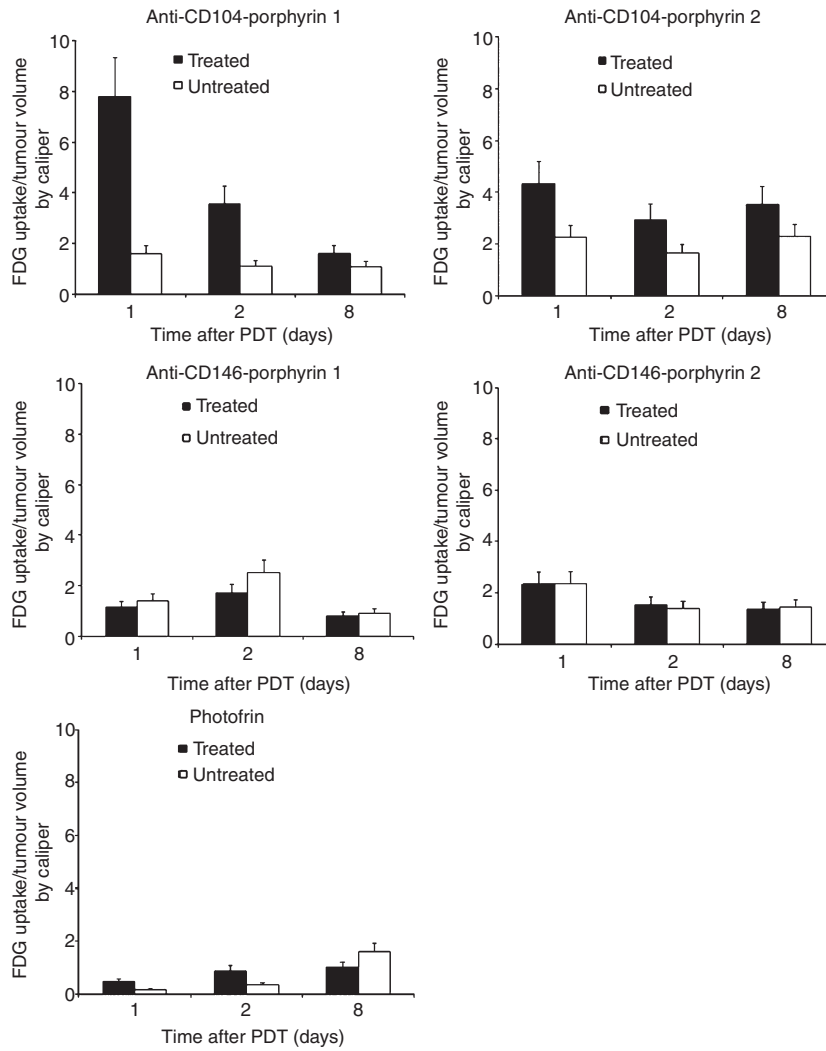
(anti-CD104-porphyrin 1 and anti-CD104-porphyrin-2) conjugates and two irrelevant (anti-CD146-porphyrin 1 and anti-CD146-porphyrin 2) conjugates (to act as controls) were investigated, and compared with Photofrin<sup>®</sup>. Figure 6 shows the growth of PDT-treated tumours for anti-CD104-porphyrin 1, anti-CD104-porphyrin 2, anti-CD146-porphyrin 1, anti-CD146-porphyrin 2, and Photofrin<sup>®</sup>, relative to non-irradiated control tumours on the same animal.

Clearly, PDT using both relevant conjugates and Photofrin<sup>®</sup> inhibited tumour growth at shorter time-points post-irradiation; however, by day 12 the Photofrin<sup>®</sup>-treated tumour matched the control, while for both anti-CD104 conjugates treated tumours were still significantly smaller relative to untreated controls. The irrelevant antibody (anti-CD146) conjugates gave no significant growth inhibition at any time-point, demonstrating that the effect

seen with relevant conjugates is attributable to the binding of anti-CD104 with antigens on the LoVo tumours.

The tumour inhibitory effects of conjugates are remarkable when it is considered that the effective administered dose of porphyrin 1 and porphyrin 2 bound to anti-CD104 is between 2 and 3 orders of magnitude less than the dose of Photofrin<sup>®</sup> used. Comparing these results with those for Photofrin<sup>®</sup> and the irrelevant conjugates (anti-CD146-porphyrin 1 and 2), it can be seen that the longer term growth inhibition (day 12) was not found when treating with a clinically relevant PDT drug, nor when porphyrin 1 or porphyrin 2 was bound to an antibody that cannot react with its complementary cell surface antigen. We believe that this demonstrates that the enhancement of photodynamic activity is directly linked to the ability of the anti-CD104 antibody to deliver photosensitizers efficiently to the target tissue.





**Figure 7.** Mice were imaged 24 hr, 48 hr or 1 week after photodynamic therapy (PDT). Images were acquired using the LabPet™ Small Animal scanner, with a resolution of 1.35 mm full width at half -maximum peak height transaxial.<sup>14</sup> The animal was anaesthetized under isoflurane anaesthesia (1% at 2 l/min oxygen flow) and 37 MBq of <sup>18</sup>F fluorodeoxyglucose (FDG) was administered via the caudal vein in 200–300 µl of physiological saline.

Finally, in order to gain a preliminary insight into possible mechanisms of tumour growth inhibition resulting from photodynamic treatment, PET was used to follow the energetic state of the tumour mass. In order to achieve this, PDT procedures were repeated as for the tumour growth inhibition experiments described above, but at time-points (1, 2 and 8 days) post PDT, animals were infused with FDG. PET images of the animals were then acquired and the uptake of FDG in the tumour volume quantified. As is clearly shown in Fig. 7 the anti-CD104-porphyrin immunoconjugates gave markedly increased levels of FDG uptake, especially at day 1 post-irradiation, compared with the irrelevant (anti-CD146) reagents or Photofrin®. No significant differences were observed between the red-light treated and untreated tumours with either irrelevant immunoconjugate or Photofrin®.

## Discussion

The results presented here clearly demonstrate that targeting of photodynamic sensitizers, by conjugation to monoclonal antibodies, can result in significant tumour response *in vivo* (Fig. 6). This is similar to the results obtained for the clinically relevant photosensitizer Photofrin® at shorter time periods post-therapy, but by day 12 only the anti-CD104 conjugates gave a significant difference between treated and untreated tumour volumes. The fact that these enhanced photodynamic results were obtained at an administered dose more than two orders of magnitude less than that for Photofrin® makes this even more remarkable. The ability to retard tumour growth to the same extent as, or to a greater extent than, the most widely used clinical PDT agent, Photofrin®, at significantly reduced administered doses of photosensitizer

suggests that this strategy could address one of the major drawbacks of PDT, that of generalized photosensitivity. The fact that no significant inhibition of tumour growth was observed with conjugates of the same photosensitizers with an irrelevant antibody confirms that the *in vivo* effects for the relevant conjugates are attributable to antibody/antigen interactions.

The mechanism of action of the immunoconjugates, demonstrated using anti-CD104-porphyrin 1 for consistency throughout the investigations, points to a predominantly apoptotic mechanism *in vitro*, as shown by annexin V/propidium iodide (Fig. 3) and caspase 3 (Fig. 5) assays, which are linked to depolarization of the mitochondrial membrane (Fig. 4). Interestingly, these results suggest an energy-dependent mechanism of cell death, which may account for the much greater uptake of FDG detected *in vivo* 24 hr post PDT (Fig. 7). Photofrin®-mediated PDT, which operates by a predominantly necrotic mechanism, following destruction of tumour vasculature, shows negligible increase in FDG uptake relative to the control tumour. An alternative but not mutually exclusive hypothesis is that the relevant conjugates induce an important acute inflammatory response following PDT. The energy consumed by the neutrophils involved in this response could account for the increased FDG uptake in the tumour volume and would tend to mask any reduction in FDG uptake by the tumour cells, as a result of cell death.

In summary, we have shown that two cationic porphyrin-based photosensitizers, bearing a single isothiocyanate group, can be conjugated efficiently to three monoclonal antibodies. Immunogenicity and photodynamic activity are retained for all conjugates. The relevant antibody conjugates induce cell death primarily by apoptosis when activated with red light, and result in improved tumour growth inhibition, relative to Photofrin®, at significantly lower administered photosensitizer doses. Relevant conjugates induce a large increase in uptake of FDG relative to control tumours 24 hr post PDT, an effect not observed for Photofrin®; this suggests that the improved tumour inhibition for the relevant conjugates may be linked to differences in the mechanism of photodynamic damage. Future studies with higher concentrations of these immunoconjugates, coupled with an investigation of any non-specific localization, are now needed to confirm the full potential of these derivatives and similar compounds.

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## Disclosures

The authors have no conflicts of interest to disclose.

## References

- Brown SB, Brown EA, Walker I. The present and future role of photodynamic therapy in cancer treatment. *Lancet Oncol* 2004; **5**:497–508.
- Pandey RK, Zengh G. Chapter 43. Porphyrins as photosensitizers in photodynamic therapy. In: Kadish KM, Smith KM, Guillard R, eds. *The Porphyrin Handbook. Volume 6: Applications: Past, Present and Future*. New York: Academic Press, 2000:157–225.
- Josefsen LV, Boyle RW. Photodynamic therapy and the development of metal-based photosensitizers. *Met Based Drugs* 2008; **1**–24.
- Boyle R, Dolphin D. Structure and biodistribution relationships of photodynamic sensitizers. *Photochem Photobiol* 1996; **64**:469–85.
- Pass HI. Photodynamic Therapy in Oncology: Mechanisms and Clinical Use. *J Natl Cancer Inst* 1993; **85**:443–56.
- Hudson R, Boyle RW. Strategies for Selective Delivery of Photodynamic Sensitizers to Biological Targets. *J Porphyr Phthalocyanines* 2004; **8**:954–75.
- van Dongen GAMS, Visser GWM, Vrouenraets MB. Photosensitizer-antibody conjugates for detection and therapy of cancer. *Adv Drug Deliv Rev* 2004; **56**:31–52.
- Hermanson GT. *Bioconjugate Techniques*, 2nd Edn. London, Amsterdam, San Diego: Academic Press, 2008.
- Clarke OJ, Boyle RW. Isothiocyanatoporphyrins, useful intermediates for the conjugation of porphyrins with biomolecules and solid supports. *Chem Commun* 1999; **2231**–2.
- Sutton JM, Clarke OJ, Fernandez N, Boyle RW. Porphyrin, Chlorin and Bacteriochlorin Isothiocyanates: Useful Reagents for the Synthesis of Photoactive Bioconjugates. *Bioconjug Chem* 2002; **13**:249–63.
- Hudson R, Carcenac M, Smith K, Madden L, Clarke OJ, Pelegrin A, Greenman J, Boyle RW. The Development and Characterisation of Porphyrin Isothiocyanate-Monoclonal Antibody Conjugates for Photoimmunotherapy. *Br J Cancer* 2005; **92**:1442–9.
- Staneloudi C, Smith KA, Hudson R, Malatesti N, Savoie H, Boyle RW, Greenman J. Development and characterization of novel photosensitizer: scFv: porphyrin conjugates for use in photodynamic therapy of cancer. *Immunology* 2007; **120**:512–7.
- Peng Q, Warloe T, Moan J, Godal A, Apricena F, Giercksky KE, Nesland JM. Antitumour effect of 5-aminolevulinic acid-mediated photodynamic therapy can be enhanced by the use of a low dose of photofrin in human tumour xenografts. *Cancer Res* 2001; **61**:5824–32.
- Thong PS, Watt F, Ren MQ, Tan PH, Soo KC, Olivo M. Hypericin-photodynamic therapy (PDT) using an alternative treatment regime suitable for multi-fraction PDT. *J Photochem Photobiol B* 2006; **82**:1–8.
- Bergeron M, Cadorette J, Beaudoin J-F *et al*. Performance Evaluation of the LabPET (TM) APD-based digital PET scanner. *IEEE Nucl Sci Symp Conf Rec* 2007; **M24**:4185–91.
- Andersen A, Kak A. Simultaneous algebraic reconstruction technique (SART): a superior implementation of the art algorithm. *Ultrason Imaging* 1984; **6**:81–94.
- Malatesti N, Smith K, Greenman J, Boyle RW. Synthesis and *in vitro* investigation of cationic 5,15-diphenyl porphyrin-monoconjugated antibody conjugates as targeted photodynamic sensitizers. *Int J Oncol* 2006; **28**:1561–9.
- MacDonald IJ, Dougherty TJ. Basic principles of photodynamic therapy. *J Porphyr Phthalocyanines* 2001; **5**:105–29.