The invention provides a compound comprising a photosensitising agent coupled to a carrier molecule with a minimum coupling ratio of 3:1 wherein the carrier molecule has a binding specificity for a target cell. There is also provided a process of conjugation comprising the use of a first and second aprotic solvent and uses of the conjugated compounds.
The invention relates to photodynamic therapy (PDT) for the selective destruction of malignant, diseased, or infected cells or infective agents without causing damage to normal cells. This may lead to a more effective clinical treatment.

Current treatment of disease is predominantly non-targeted. Drugs are administered systemically or orally which expose many other tissues as well as the tissues which are diseased. In cancer therapy, chemotherapeutic drugs are specific for cells which are growing and dividing rapidly as they work mainly by a mechanism which interferes with DNA replication [1] (For details of all references, see later references section). Other cells can take up the drug and also become intoxicated, such as rapidly dividing bone marrow stem cells, resulting in immunosuppression and sickness. In infectious diseases, the anti-bacterial drug is introduced into the blood (orally or by injection) and interferes with a particular bacterial metabolic pathway. Exposure of other tissues to the drug can result in side effects as well as the major problem of drug resistance. Virally-infected cells are difficult to treat as their metabolism is practically identical to uninfected human cells.

It is widely hypothesised that advances in medicine may be in the tailoring of drugs to the disease. This means, inter alia, delivering the therapeutic to the correct target tissue or organism, rather than the non-selective hit and miss approach of most of the conventional drugs used today. This will result in lower doses administered, lower side effects and toxicities and overall better responses. Advances in genomics will one day mean that drugs can be tailored to the individual, as breast cancer in one individual may differ from breast cancer in another individual.

There are many drugs used clinically today that are very good at destroying or treating the diseased cells, once the drug has accumulated in the correct tissue. Therefore the problem is with the specific targeting of drugs rather than their mechanism of action. Examples of this include targeted ionising radiation as opposed to external beam radiotherapy [2], targeted chemotherapy drugs (e.g.
methotrexate or doxorubicin) as opposed to free drugs [3] and toxins [4]. PDT is a particularly good example as it is already well established in many treatments, but it is becoming apparent that a better therapeutic result, and in consequence greatly expanded clinical applications, would come from pre-targeting the sensitizing drag to the correct tissues in addition to targeting the light source, which is not accurate at the cell level [5,6].

Targeting drugs or other effectors to the desired cells is a well-established area. One of the main approaches to targeting is to use antibodies or cell-specific ligands as the targeting element of a multifunctional molecule [7,8]. A good design for such a multifunctional molecule would be one which is highly specific for diseased cells, able to carry many drugs with high capacity without compromising their function, and able to deposit the drag in the sub-cellular compartment which would primarily be affected.

Antibodies have naturally evolved to act as the first line of defence in the mammalian immune system. They are complex glycoproteins which have exquisite specificity and tremendous diversity. This diversity comes about from programmed gene shuffling and targeted mutagenesis, resulting in probably a trillion different antibody sequences [9]. This diversity means that antibodies can bind to practically any target molecule which is usually protein in nature. It is now possible to mimic antibody selection and production in vitro, selecting for recombinant human antibodies against virtually any desired target [10].

That antigenic selectivity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better et al (1988) Science 240, 1041); Fv molecules (Skerra et al (1988) Science 240, 1038); single-chain Fv (ScFv) molecules where the VH and VL partner domains are linked via a flexible oligopeptide (Bird et al (1988) Science 242, 423; Huston et al (1988) Proc. Natl. Acad. Sci USA 85, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward et al. (1989) Nature 341, 544). A general review of the techniques involved in the synthesis of
antibody fragments which retain their selective binding sites is to be found in Holliger and Hudson, *Nature Biotechnology* (2005) 23, 1126-36

A significant number of biotechnological drugs are in development are based on antibody targeting [7,11,12]. The most popular *in vitro* selection technique is antibody phage display, where antibodies are displayed and manipulated on the surface of viruses [10].

The display of proteins and polypeptides on the surface of bacteriophage (phage), fused to one of the phage coat proteins, provides a powerful tool for the selection of selective ligands. This 'phage display' technique was originally used by Smith in 1985 (*Science* 228, 1315-7) to create large libraries of peptides for the purpose of selecting those with high affinity for a particular antigen. More recently, the method has been employed to present antibodies at the surface of phage in order to identify ligands having desired properties (McCafferty et al., *Nature*, 1990, 552-554).


There are many therapeutic antibodies being developed for a range of diseases, primarily cancer, autoimmune diseases and prevention of allograft rejection. Table 1 lists some of these major antibodies.
Antibodies can bind with a high degree of specificity to target cells expressing the appropriate receptor. The affinity of an antibody is a measure of how well an antibody binds to the target (antigen). It is usually described by an equilibrium dissociation constant (Kd). For antibodies that need to be internalised, the association rate is more important as the dissociation rate is not applicable if the antibody is taken into the cell. A variety of technology exists to select and manipulate antibodies which have desired structural and binding properties [13].
As with all biological molecules, the size of the antibody affects its pharmacokinetics in vivo [14,15]. Larger molecules persist longer in the circulation due to slow clearance (large glycoproteins are cleared through specific uptake by the liver). For whole antibodies (molecular weight 150 KDa) which recognise a cancer cell antigen in an experimental mouse model system, 30-40% can be taken up by the tumour, but because they persist longer in the circulation, it takes 1-2 days for a tumour: blood ratio of more than one to be reached. Typical tumour: blood ratios are 5-10 by about day 3 [16]. With smaller fragments of antibodies, which have been produced by in vitro techniques and recombinant DNA technology, the clearance from the circulation is faster (molecules smaller than about 50 KDa are excreted through the kidneys). Single-chain Fvs (about 30 KDa) are artificial binding molecules derived from whole antibodies, but contain the minimal part required to recognise antigen [17]. Again in mouse model systems, scFvs can deliver 1-2 % of the injected dose, but with tumour: blood ratios better than 20:1, with some tumour: organ ratios even better [18]. As scFvs have only been developed over the last 10 years, there are not many examples in late clinical trials [19]. From clinical trials of whole antibodies, the amount actually delivered to tumours is about 1 % of that seen in mouse models, but with similar tumour:organ ratios [20]. If another molecule is attached to the antibody, then the new size and chemico-physical properties determines the altered pharmacokinetics. Additionally, properties such as net charge and hydrophilicity can affect the targeting kinetics [21].

Some cell surface antigens are static or very slowly internalise when bound by a ligand such as an antibody. There are some which have a function that requires internalisation, such as cell signalling or uptake of metals and lipids. Antibodies can be used to deliver agents intracellularly through such antigens. These agents can be therapeutic- repairing or destroying diseased cells. Examples include gene delivery [22] the intracellular delivery of toxins (e.g. Pseudomonas exotoxin [4], enzymes (e.g. ribonuclease [23], deoxyribonuclease [24] and drugs (e.g. methotrexate [3]. Some of these agents need targeting to particular sub-cellular organelles in order to exert their effects [24]. Advances in cell biology have uncovered intracellular targeting 'codes'- these are amino acid sequences which
direct intracellular proteins to certain sub-cellular compartments. There are specific sequences which have been discovered that target various proteins to the nucleus, endoplasmic reticulum, golgi, lysosomes and mitochondria ([25], Table 2). These are being developed as add-ons to improve drug action by localising therapeutic proteins to the target compartment.

Table 2 Peptide sequences which could be used for sub-cellular localisation

<table>
<thead>
<tr>
<th>Name of Sequence</th>
<th>Function</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV 40 large T nuclear localisation</td>
<td>Targets polypeptides to the nucleus</td>
<td>KKKKRPR</td>
</tr>
</tbody>
</table>
| Human SRY                               | Targets polypeptides to the nucleus     | KRPMNADFVRSRDQR
               |                                         | RK                  |
| ATP-binding protein N-terminal peptide containing mitochondria targeting | Targets polypeptides to the mitochondria | MLVHLFRVIRGGPFPGRRLPPLRFQTFSAVR
               |                                         | YSDGYRSSLRLRAVHLPSQLWA |
| Lysosomal membrane targeting            | Targets polypeptides to the lysosomes   | TMGY or TMLI        |
| Endoplasmic reticulum (ER) Retention Signal | Allows proteins to traffic back to the ER | KDEL                |
| Influenza Haemaglutinin HA2             | Disrupts membrane                       | GLFGAIAGFIENGWEG
               |                                         | MIDGWYG             |
| Polio virus vp1                         | Disrupts membrane                       | GIEDLISEVAQGALTLVP  |
| Human defensin                          | Disrupts membrane                       | ACYCRIPTACIAGERRYGTICIYQGRLWAFCC |
| Sendai virus fusion protein F1           | Disrupts membrane                       | FFHAVIGTIALVGATSAQITAGIALAEAR |
There has been much research into targetable therapeutic drugs where novel effector functions have been linked to antibodies or other targeting ligands. Some of these need to be internalised to successfully deliver a toxic agent. Many of these have shown good results in vitro and in vivo in animal models, but have been disappointing in the clinic. Immunotoxins have shown problems such as immune reactions and liver/kidney toxicity [26]. There have been developments with new 'humanised' immunotoxins based on enzymes such as ribonuclease [23] and deoxyribonuclease [24]. These potentially have lower side effects are more tolerable, but still do not have a bystander killing effect. Chemotherapy drugs tend to be much less active when linked to proteins as they do not get released effectively, thus requiring selectively cleavable chemical linkers. Radioimmunotherapy tends to irradiate other tissues en route to the tumour, giving bone marrow and liver toxicity. Photosensitising (PS) drugs are particularly attractive agents to link to proteins as the cytotoxic elements are the singlet oxygen and other reactive oxygen species generated from them and not the PS drugs themselves [5,6].

Although antibodies are the first choice when it comes to considering ligands for targeting or detection, there exist many alternative ligands, some of which have been exploited through phage (or other) display/selection techniques. These include natural ligands for receptors (e.g. interleukin-6 (IL-6) [27] and tissue necrosis factor (TNF) [8], peptides (e.g. neuropeptides [28]) immunoglobulin-like domains (such as fibronectin (fh3) domains [29], single immunoglobulin domains [30]), anticalins [31], ankyrin repeats [32], etc. Many of these can be engineered and optimised to improve their biological and therapeutic properties [33].

Photodynamic Therapy (PDT) is a minimally invasive treatment for a range of conditions where diseased cells and tissues need to be removed [6,34,35]. Unlike ionising radiation, it can be administered repeatedly at the same site. Its use in cancer treatment is attractive because the use of conventional modalities such as chemotherapy, radiotherapy or surgery do not preclude the use of PDT and vice versa. PDT is also finding other applications where specific cell populations must be destroyed, such as blood vessels (in age-related macular degeneration (AMD


or in cancer), the treatment of immune disorders [37], cardiovascular disease [38], and microbial infections [39,40].

PDT is a two-step or binary process starting with the administration of the PS drug, by intravenous injection, or topical application for skin cancer. The physicochemical nature of the drug causes it to be preferentially taken up by cancer cells or other target cells [41]. Once a favourable tumour (or other target) : normal organ ratio is obtained, the second step is the activation of the PS drug with a specific dose of light, at a particular wavelength. The photosensitizer, in its ground or singlet state absorbs a photon of light at a specific wavelength. This results in a short-lived excited singlet state. This can be converted by intersystem crossing to a longer-lived triplet state. It is this form of the sensitizer which carries out various cytotoxic actions.

The main classes of reactions are photooxidation by radicals (type I reaction), photooxidation by singlet oxygen (type II reaction), and photoreaction not involving oxygen (type III reaction). The triplet state form of the sensitizer causes the conversion of molecular oxygen found in the cellular environment into reactive oxygen species (ROS) primarily singlet oxygen (\(^{1}\text{O}_2\)) via a Type II reaction. If an activated photosensitizer interacts with cellular components, a Type I reaction occurs where electrons or protons are abstracted forming radicals such as hydroxyl radicals (\(\text{OH}^{*}\) and superoxide (\(\text{O}_2^{*-}\)). These molecular species cause damage to cellular components such as DNA, proteins and lipids [42]. A Type III mechanism has also been proposed where the triplet state photosensitier interacts with free radicals to cause cellular damage. The site of cellular damage depends upon the type of photosensitizer, length of incubation, type of cells and mode of delivery. Hydrophobic photosensitizers tend to damage cell membranes [42], whereas cationic photosensitizers localise within membrane vesicles such as mitochondria and cause damage there [43].

The light activation of ROS is highly cytotoxic. In fact some natural processes in the immune system utilise ROS as a way of destroying unwanted cells. These species have a short lifetime (<0.04 ms) and act in a short radius (<0.04 mm) from
their point of origin. The destruction of cells leads to a necrotic-like area of tissue which eventually sloughs away or is resorbed. The remaining tissue heals naturally, usually without scarring. There is no tissue heating and connective tissue such as collagen and elastin are unaffected. This results in less risk to the underlying structures compared to thermal laser techniques, surgery or external beam radiotherapy. More detailed research has shown that PDT induces apoptosis (non-inflammatory cell death), and the resulting necrosis (inflammatory cell lysis) seen is due to the mass of dying cells which are not cleared away by the immune system [44]. Research on a number of PS drugs including silicon phthalocyanines has shown that PDT induces apoptosis-programmed cell death [45]. Apoptosis is the highly orchestrated and evolutionary conserved form of cell death in which cells neatly commit suicide by chopping themselves into membrane-packaged pieces [46]. These apoptotic bodies are marked for phagocytosis by the immune system. Usually, too much apoptosis in a small area Overloads’ the immune system and the area eventually becomes necrotic, with inflammatory consequences.

PDT is a cold photochemical reaction, i.e. the laser light used is not ionising and delivers low levels of thermal energy, and the PS drugs have very low systemic toxicity. The combination of PS drug and light result in low morbidity and minimal functional disturbance and offers many advantages in the treatment of diseases. There is growing evidence that PDT response rates and durability of responses are as good as or even superior to standard locoregional therapies [35,36].

Generally PS drugs are administered systemically, with some topical applications for skin lesions. When the PS drug has accumulated in the target tissue, with ratios typically 2:5:1 compared with normal surrounding tissues (except in the brain where the ratio can be up to 50:1), low power light of a particular wavelength is directed onto the tumour (or the eye in AMD treatment [37]). Because human tissue can transmit light most effectively in the red region of the visible light spectrum, PS drugs which can absorb red light (630 nm or above) can be activated up to a depth of about 1 cm. After treatment direct sunlight must be avoided until
systemically administered PS drugs clear from the body, otherwise they may have skin photosensitivity, resulting in skin burn.

The newer generation of PS drugs have longer activation wavelengths thus allowing deeper tissue penetration by red light, higher quantum yield and better pharmacokinetics in terms of tumour selectivity and residual skin photosensitivity. These classes of PS drugs include the phthalocyanines, chlorins, texaphyrins and purpurins. The synthetic chlorin, Foscan™ is a very potent PS drug with a wavelength of activation of 652 nm, quantum yield of 0.43 and skin photosensitivity of about 2 weeks. There have been many clinical trials for a variety of cancers, with good results [35,36]. There are other PS drugs which have been developed and are in trials which can absorb at >700 nm, such as meta-tetrahydrophenyl bacteriochlorin (m-THPBC). A palladium-bacteriopheophorbide photosensitizer (TOOKAD) has been developed which shows promise in the treatment of prostate cancer with favourable, deep red absorption properties (763 nm absorption peak) [47].

Preclinical studies have shown that fractionating the light dose results in enhanced PDT effects, allowing oxygen to replenish the system prior to the next round of activation. This has been observed for the Miravant drug MV6401 in an orthotopic breast cancer model [48]. The literature describes other ways to enhance non-targeted PDT effects such as the use of vitamin analogues administered separately to modulate the free radical pathway [49], the coupling of photosensitizers (e.g. m-THPC) to polyethylene glycol carriers to increase their half-life [50], the use of polymeric micelle carriers [51] and the use of adjuvants to potentiate the immune response after PDT treatment [52].

The PDT treatment scheme is attractive to the clinician in that superficial diseases can usually be treated with local anaesthesia and sedation [35]. The generally low toxicity (with the possible exception of skin photosensitivity) limits the need for other medication. Topical treatments do not require sterile conditions and can be given in an outpatient clinic.
Photofrin™ (porfimer sodium), 5-aminolaevulinic acid (ALA) and Visudyne™ (verteporfm, BPD-MA benzoporphyrin derivative mono acid) are three PS drugs which have regulatory approval. A promising, potent second generation PS drug, Foscan™ (temoporfm; meta-tetrahydroxyphenyl chlorin) has recently been approved in Europe, as has meiJryl-5-aminolevullmate. Porfimer sodium, the first PS drug to be approved, is licensed for use in bladder, stomach, oesophagus, cervix and lung cancer. Its performance is moderate due to poor light absorption characteristics in the red end of the spectrum (activated at 630 nm), meaning it can only penetrate about 5mm into tissues, and limited selectivity for target tumour tissue. It also persists in the body for weeks, leading to skin photosensitivity. However it has been effective in the treatment of bladder, stomach, oesophagus, cervix and lung cancers [35,36]. ALA is applied topically in the treatment of skin lesions and is converted endogenously to protoporophyrin IX, a naturally-occurring PS molecule. This can be activated at many wavelengths and its depth of effect is less than 2 mm. Verteporfm also performs well in age-related macular degeneration AMD [37,53], without the issues of tissue penetration found in tumour applications. The TAP and VP Clinical trials showed that PDT with verteporfm was more effective at recovering vision loss associated with AMD compared to the placebo control [53].

PDT can achieve control rates similar to conventional techniques with lower morbidity rates, simplicity of use and improved functional and cosmetic outcome. PDT has mainly been used where conventional approaches have failed or are unsuitable. These include pre-malignant dysplastic lesions and non-invasive cancers which are commonly found in the mucosa of aerodigestive and urinary tracts (e.g. oral cavity, oesophagus and bladder). Current treatments for cancer at this stage are not very successful and good responses here would prevent larger solid tumours or metastatic spreads occurring. Treatment for Barrett's oesophagus usually means oesophagectomy, which requires general anaesthesia, has a risk of morbidity and loss of function and disfigurement. PDT is being seen as an attractive option because of the large area which can be treated superficially with less risk. Photofrin™, ALA and Foscan™ have produced good responses in these
types of cancers in clinical trials (Table 3). Breast cancer chest wall recurrences have been successfully treated with Foscan™ [54] and Photofrin™ [55]

<table>
<thead>
<tr>
<th>Disease</th>
<th>Photosensitiser</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barrett’s mucosal cancer</td>
<td>Porfimer sodium</td>
<td>75% conversion to normal epithelium and tumours eliminated</td>
</tr>
<tr>
<td>Barrett’s oesophagus cancer</td>
<td>Systemic ALA</td>
<td>High-grade dysplasia eradicated in all patients</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>Hematoporphyrin derivative</td>
<td>74% complete response, 30% alive after 5 years</td>
</tr>
<tr>
<td>Basal cell cancer of skin</td>
<td>Topical ALA</td>
<td>90% complete response</td>
</tr>
<tr>
<td>Oral cancer</td>
<td>Dihematoporphyrin ether</td>
<td>87% complete response over 5-53 months</td>
</tr>
<tr>
<td>Chest wall recurrence in breast cancer</td>
<td>Dihematoporphyrin ether</td>
<td>20% complete response, 45% partial response</td>
</tr>
</tbody>
</table>

Due to the easy light accessibility, treatment of cutaneous disease such as skin cancer has produced good results with systemic and topical PS drugs (Table 3). Head, neck and oral lesions have also produced good results and are well suited due to the good cosmetic outcome of the treatment (Table 3). Treatment of other cancers are being tested as advances are being made in laser and light delivery technology. Endoscopes can be used to deliver the activating light dose to any hollow structure such as the oesophagus and bronchial cavity, thus expanding the treatment range to gastrointestinal and lung cancers (Table 3) with minimal surgery. Large areas such as the pleura and peritoneum can be treated, where radiotherapy would not be able to give a high enough curative dose. PDT has great promise in the treatment of these surface serosal cancers, in combination with debulking surgery. Light can be delivered to these large surfaces in a short time, through hollow cavities. The limited depth of activity would be an advantage as critical underlying organs would be spared (Table 3). Adjuvant therapy is also an
option being investigated, where the solid tumour is surgically removed and any remaining tumour cells are destroyed by one round of PDT in the cavity formed.

Although surface cancers may be the most amenable to PDT, solid tumours may be able to undergo interstitial treatment, where the PS drug is administered systemically or by intra-tumour injection, followed by the insertion of laser fibres through needles equally spread throughout the tumour. This can result in necrosis of very large tumours [56,57] (Table 3).

Therefore, there are several advantages of PDT therapy. It offers non-invasive, low toxicity treatments which can be targeted by the light activation. The target cells cannot develop resistance to the cytotoxic species (ROS). Following treatment, little tissue scarring exists. However, PS drugs are not very selective for the target cells with target: blood ratios typically in single figures at best. In many situations this lack of selectivity leads to unacceptable damage to proximal normal tissues e.g. Photofrin™ [58, 59] in oesophageal cancers [60, 61], bladder cancer [62]. Because PS drugs "piggy-back" on blood proteins, they persist longer in the circulation than is desired, leaving the patient photosensitive for 2 weeks in the best of cases.

Unlike standard chemotherapeutics, photosensitiser drugs can still be active and functional while attached to carriers as the cytotoxic effect is a secondary effect resulting from light activation. This makes them amenable to specific drug delivery mechanisms involving conjugation to targeting molecules. Currently, the preferred approach to link photosensitizer drugs to targetable elements is the direct conjugation of derivatized photosensitizer drugs to whole monoclonal antibodies. Whole antibodies have a molecular weight of 150 KDa, resulting in very large photo-immunoconjugates with unfavourable pharmacokinetics, such as poor tumour: organ ratios (2:1) [63,64] which take a long time to achieve. Current literature suggest that photosensitizer drugs linked to residues on a monoclonal antibody can have a detrimental effect on each other, with quenching effects occurring due to poor spectroscopic properties [65]. In addition to this, it has been shown that poor, and unreliable, loading of photosensitizer onto the antibody is
seen with ratios of 4:1 being typical before the antibody aggregates or loses function [63-69].

Coupling of photosensitisers has been tried using various strategies with various monoclonal antibodies. For example PPa has been coupled to anti-Her 2 monoclonal antibodies. In order to achieve good sensitiser:antibody coupling ratios (in the region of 10:1) the antibody had to be made more soluble by attaching chains of polyethylene glycol [68]. This PEGylation would have a detrimental effect on the conjugate pharmacokinetics resulting in poorer tumour/blood ratios. Furthermore, non-covalent binding of photosensitiser to antibody was also seen here. Such non-covalent binding has been a feature of most reported attempts to produce antibody-photosensitiser conjugates, and represents a major problem in reliably producing well characterised conjugates. In a further study, a porphyrin sensitiser was used with monoclonal antibodies 17.1A₅ FSP77 and 35A7 using a isothiocyanate coupling method resulting in sensitiser:antibody ratio no better than 2.8:1 [67]. Another example was verteporfin (benzoporphyrin derivative, BPD) with monoclonal antibody C225 (anti-EGFR). Here, coupling ratios of greater than 11:1 resulted in poor immunoreactivity and solubility [69]. The best ratios were about 7:1. These examples serve to illustrate the problems of producing well characterised conjugates with high photosensitiser:antibody ratios, and suggest that the use of fragments which are one third to one sixth smaller than whole antibodies would be even less successful given the solubility and loading problems seen with the larger protein species.

The work on PS drugs attached to monoclonal antibodies has shown that if too many PS molecules are attached to an individual monoclonal antibody the hydrophobicity can be affected and an adverse effect on the pharmacokinetics may result. Given the problems with whole monoclonal antibodies, it is widely believed that small fragments (such as a scFv, 30KDa) would have very unfavourable coupling efficiencies, resulting in only one or two photosensitisers being coupled. Birchler et al [70] attempted to produce an effective ScFv -
photosensitiser conjugate but were only able to couple a single photo sensitiser through a site-specific cysteine residue to a scFv.

Other groups have tried to circumvent these problems by attempting to link PS drugs to designated 'carriers' such as branched carbohydrate [71] or polyethylene glycol chains [72] and poly-lysine [73] chains. These approaches all require additional conjugation steps as the ligand-carriers cannot be made entirely recombinantly. Using such polymers may also have problems such as proteolytic instability in vivo. It is known that when photosensitizers are attached in this way, they self-quench, destroying their photophysical properties and rely on degradation in lysozymes to 'de-quench' before they can become active photosensitizers [71]. Therefore, higher coupling ratios can be achieved, up to 10:1, but only with lower phototoxicity and lower singlet oxygen yield than the free photo sensitiser. Studies by Roder et al. [71] showed that the photosensitisising activity of pheophorbides when covalently linked in large numbers around the periphery of a dendrimer were dramatically reduced. This is a result of energy transfer processes mainly Forster energy transfer from dye to dye. Forster transfer is distance dependant and drops off rapidly with distance. The interaction of dye molecules leads to changes in the absorption spectrum, reduced fluorescence lifetimes and singlet oxygen quantum yields. Fusion proteins combining an antibody fragment with a protein carrier molecule have also been described by our group [74].

Glickman et al [75,76] describe monoclonal antibody targeted PDT against the VEGF vasculature target for ocular disease. This uses standard coupling conditions with no description of antibody: photosensitizer ratios. However Hasan et al [77] discloses a two-solvent system to improve upon the photosensitizer: antibody coupling ratios. Here, using very high concentrations of organic solvents (typically 40-60%) mixed with aqueous buffers, ratios of up to 11:1 have been reported. However, the high concentrations of solvent used are unlikely to be tolerated by all antibodies. No mention is made of using fragments, but given their greater sensitivity to organic solvents, they would not be expected to viable in this method. Also in Hasan et al [77], the large number of coupled
photosensitizers are self-quenching, hence this system relies upon internalisation and lysozomal degradation to release phototoxic molecules. Photo-immunoconjugates bound to the cell surface are not expected to be exposed to degradation enzymes like those found in intracellular lysozomes. This may exclude the targeting of low/non-internalizing antigens such as CEA and matrix/stromal antigens.

By linking novel or established PS drugs to small, targetable carrier proteins, it is possible to deliver a highly specific dose of PS drug to a target tissue, which can later be activated by light. These carrier-PS drug conjugates have advantages over existing targeted and non-targeted PDT approaches in that a greater amount of PS drug can accumulate in the target tissue, with tissue to blood/normal organ ratios of 20:1 or better, in shorter time intervals. Additionally, these agents could have advantages over other targetable strategies with little or no immunogenicity and lower side effects. Smaller ligands have been used to deliver photosensitizers such as insulin [78], transferrin [79,80], albumin [81], annexins [82], toxins [83], estrogen [84], rhodamine derivatives [85], folate [86] and growth factors such as EGF [87] and VEGF [88]. None of these examples or anything else in the current literature proposes that such ligands can be engineered by protein evolution or rational mutagenesis to improve or enhance the photosensitizers attached to them.

Summary of the Invention

The present invention provides a method for coupling photo-sensitisers to biological targeting proteins such as antibody fragments (e.g. scFvs) using previously unknown and optimised coupling conditions to ensure that the carrier remains functional and soluble. The conjugates preferably possess a high and consistent molar ratio of covalently attached photosensitisers without non-covalent binding. The invention also provides engineered recombinant antibody-photosensitiser conjugates with optimised photophysical and photodynamic properties, and methods to produce them. Furthermore the invention provides ways of coupling other 'non-photosensitising' molecules which enhance the photo-physical and photodynamic properties of the overall conjugate.
We describe compounds made by a process which produces very effective, potent targeted photodynamic therapy conjugates based on small recombinant antibody fragments, chemically coupled to photosensitising molecules and other modulating molecules. The use of such modulating molecules can alter the mechanism of reactive oxygen species generation resulting in more type I species (free radicals and superoxides) than type II ROS. This has important implications for PDT because when targeting non- or low-internalizing antigens such as matrix proteins, none or little of the photosensitizer is internalised, meaning that species which can damage surface cell membranes more effectively will be more potent than the type II singlet oxygen generators. Furthermore, targeting non-internalizing antigens may be preferable in some cases, particularly if the cancer cells have developed some form of drug resistance to reactive oxygen species, for example in the up-regulation of ROS scavenging enzymes (e.g. superoxide dismutase).

The biological nature of antibodies requires that they be maintained in mostly aqueous buffers in order to retain function and integrity. However, photosensitizers tend to be hydrophobic in nature and are poorly soluble in the buffer conditions normally used for antibodies. Coupling a photosensitizer to an antibody under aqueous conditions will result in poor photosensitizer: antibody ratios and in solvents will result in damaged antibody proteins. We describe a method utilizing a combination of organic solvents at low concentration.

We have developed a robust conjugation protocol which can efficiently couple a number of photosensitisers to antibody fragments whilst minimising non-covalent binding.

The hydrophobic and highly adsorptive nature of most photosensitisers and the water-soluble nature of antibodies and other biomolecules has made conjugation chemistry difficult and more importantly rendered almost impossible the removal of unconjugated photosensitiser impurities from such conjugations. These problems were overcome, surprisingly, by using (a) very pure monofunctional photosensitisers which enable us to use relatively small coupling ratios between
antibody and photosensitiser, and (b) using a combination of 2 aprotic solvents with an aqueous component which can be water, phosphate buffered saline (PBS) or any other approximately neutral buffering solution known in the art.

In a first aspect of the invention there is provided a process of making a compound comprising a photosensitising agent coupled to a carrier molecule comprising the steps of:

(i) providing a photosensitising agent;
(ii) providing a carrier molecule;
(iii) conjugating the photosensitizing agent and the carrier molecule in the presence of a first and a second polar aprotic solvent and an aqueous buffer.

Preferably, the compound comprises a ratio of photosensitising agent to carrier molecule of at least 3:1. More preferably, the functional and physical properties of the photosensitising agent and the carrier molecule are substantially unaltered after coupling.

Appropriate polar aprotic solvents from which the first and second polar aprotic solvent are selected from (but are not limited to) the group consisting of: dimethyl sulfoxide (DMSO); acetonitrile; N,N-dimethylformamide (DMF); HMPA; dioxane; tetrahydrofuran (THF); carbon disulfide; glyme and diglyme; 2-butaneone (MEK); sulpholane; nitromethane; N-methylpyrrolidone; pyridine; and acetone. Other polar aprotic solvents which may be used are well known to those skilled in the art. The total amount of both polar aprotic solvents relative to the aqueous mixture should be about 50% by volume. The relative amounts of the 2 polar aprotic solvents to each other can vary from 1 to 49% : 49% to 1

Preferably, the first and second aprotic solvent are selected from the group consisting of: DMSO; DMF; and acetonitrile. More preferably, the first and second aprotic solvent are DMF and acetonitrile.
Even more preferably, the ratio of aqueous buffer to first aprotic solvent to second aprotic solvent is approximately 50% : 1 to 49% : 49 to 1%.

Even more preferably, the aprotic solvent mixture is 92% PBS : 2% DMSO : 6% acetonitrile and the step of conjugating the photosensitizing agent and the carrier molecule is conducted at a temperature between 0°C and 5°C. The combination of solvents keeps the whole reaction homogeneous especially at these low temperatures and by carrying out the coupling for approximately only 30 min, we are able to achieve high coupling ratios and very low degrees of non-covalent binding.

The invention further provides a process wherein the carrier molecule is an antibody fragment and/or a derivative thereof. Preferably, the antibody fragment and/or derivative is a single-chain antibody, and may conveniently be an ScFv. The carrier molecule is preferably humanised or human.

Using the above protocol, photosensitisers with carboxylic acid groups derivatised to form active esters may be coupled efficiently and with high molar ratio to antibody fragments via surface-accessible lysine residues. Pyropheophorbide a (PPA) is a photosensitiser derived from natural products, and apart from excellent photophysics which makes it an ideal photosensitiser, it possesses a single propionic acid side chain. The PPA propionic acid function may be readily converted to the corresponding N-hydroxysuccinimide ester (NHS) or ‘active ester’ and purified through a combination of chromatography and recrystallisation to obtain very pure derivatives ready for conjugation, and thereafter coupled efficiently to antibody fragments.

Preferably, the photosensitising agent is a monofunctional photosensitiser. More preferably, the photosensitising group is at least one selected from the group (but not limited to) consisting of: haematoporphyrins, Photofrin™, naturally-occurring porphyrins, chlorins and bacteriochlorins, pheophorbides like pyropheophorbide a and its derivatives like Photochlor, chlorins, chlorin e6, mono-1-aspartyl derivative of chlorin e6, di-1-aspartyl derivative of chlorin e6, tin (IV) chlorin eβ, the
palladium derivatives of naturally occurring bacteriochlorophylls like TOOKAD (Pd-bacteriopheophorbide), synthetic chlorins and bacteriochlorins like meta-tetrahydroxyphenyl chlorin (Foscan) and bacteriochlorin, benzoporphyrin derivatives, monobenzoporphyrin derivatives like verteporfin, phthalocyanines, sulphonated aluminium phthalocyanines (disulphonated and tetrasulphonated), sulphonated aluminium naphthalocyanines and derivatives, purpurins like purpurin-18, tin and zinc derivatives of octaethylporphyrin, tin etiopurpurin, verdins, porphycenes, synthetic porphyrins, chlorins and bacteriochlorins, like the meso-triethynylporphyrins (WO2004/046151) both metal free and metallated, core-modified porphyrins (WO2004/076461), expanded porphyrins (texaphyrins) like motexafin lutetium and motexafin gadolinium.

Non-porphyrinic compounds can also be used as photosensitisers and include but are not limited to, phenothiazinium derivatives like methylene blue, toluidine blue, cyanines such as merocyanine-540, acridine dyes, BODIPY dyes and aza-BODIPY derivatives, hypericin, halogenated squarine dyes and halogenated xanthene dyes like eosin and rose Bengal.

Other suitable photosensitisers for conjugation to antibody fragments will readily occur to those skilled in the art. However, the presence of multiple reactive functionalities on the photosensitiser can lead to a number of problems. It is difficult to obtain sufficiently pure material to control the stoichiometry of the conjugation reaction and as a consequence reactions are carried out using large excesses of the reactive photosensitiser resulting in increased non-covalent binding. Intramolecular antibody cross-linking can also occur during conjugation resulting in low coupling yields and increased aggregate formation.

Our work with antibody fragments has shown that by controlling the stoichiometry of the photosensitiser during the conjugation and having lysine residues sufficiently spaced apart geometrically can lead to photoimmunoconjugates with high photosensitiser loadings and excellent PDT activity.
Conveniently, the process further comprises the following step performed after step (iii):

(iv) coupling a modulating agent to the carrier molecule, wherein the modulating agent is capable of modulating the function of the photosensitising agent.

As well as coupling photosensitisers to ligands, it is also possible, using similar coupling chemistries to couple other molecules to the ligands in such a way that they modify the photophysical or photodynamic properties of the overall photo-immunoconjugate. These alternative molecules can be coupled to same residue type as the photosensitisers (i.e. before or after photosensitiser coupling) at stoichiometric ratios to allow both types of molecules to be coupled/accommodated or on different residue types (e.g. photosensitiser coupled onto lysines and subsequently modifying chemical coupled to aspartate/glutamate residues).

Photodynamic modulators may serve to alter the types and amounts of reactive oxygen species generated upon light illumination of the photosensitiser. For example photosensitisers which generate a more type II reaction (i.e. singlet oxygen) can be modulated to generate more type I reaction with high concentrations of superoxide and hydroxide radicals. This could have major implications on the PDT potency or therapeutic outcome. For example a photo-immunoconjugate targeting a non-internalising tumour antigen may be more potent if it generated a predominantly type I reaction at the surface of the cell, causing membrane damage and being less susceptible to anti-oxidant responses such as superoxide dismutase (which is generated intracellularly).

Preferably, the modulating agent is selected from the group consisting of: benzoic acid; benzoic acid derivatives containing an azide group like 4-azidotetrafluorophenylbenzoic acid and other aromatic or heteroaromatic groups containing an azide moiety (N₃) including polyfluorobenzenes, naphthalenes, napthaquinones, anthracenes, anthraquinones, phenanthrenes, tetracenes,
naphthacenediones, pyridines, quinolines, isoquinolines, indoles, isoindoles, pyrroles, imidazoles, pyrazoles, pyrazines, benzimidazoles, benzofurans, dibenzofurans, carbazoles, acridens acidones, and phenanthridines, xanthenes, xanthiones, flavones and coumarins. Aromatic and heteroaromatic sulfenates derived from the aromatic/heteroaromatic groups above. Other specific modulating agents include vitamin E analogues like Trolox, butyl hydroxyl toluene, propyl gallate, deoxycholic acid and ursodeoxycholic acid. One example of a chemical modifier which can be coupled to a ligand alongside the photosensitising agent is the succinimidyl ester of benzoic acid (BA).

This has been shown to result in more potent PDT cell killing in vitro when co-coupled with PPa to an anti-CEA scFv compared to the scFv coupled with PPa alone.

Preferably, the process further comprises the following step performed after step (iii) or (iv):

(v) combining the compound with a pharmaceutically-acceptable carrier to form a pharmaceutical formulation.

The process of the invention may also include the optional step of coupling a visualising agent to the conjugate. Alternatively the photosensitising agent forming part of the conjugate may also be used as a visualising agent.

The use of recombinant antibodies in immuno-assays or diagnostics is a well studied area. The exquisite specificity, high affinity and versatility of antibodies and antibody fragments make them ideal binding molecules as part of a detection system. For example, in medical imaging, antibodies have been linked to optically-active compounds such as fluorescent dyes and used to detect pre-cancerous and cancerous lesions, measuring treatment response and early detection of recurrences [95] and in vitro, transmissible spongiform encephalopathies (prion diseases) have been detected with fluorescently labelled antibodies [96].
Clinically useful tumour imaging requires detection of small lesions. The benefits of detection can then be realised by early action. One of the problems associated with conventional imaging techniques is poor tumour to background contrast. Various strategies have been developed to increase the localization of targeting molecules in tumours and to reduce their uptake by normal tissue, thus improving tumour: tissue ratio. These approaches include developing small tumour specific peptide molecules with favourable pharmacokinetics [97], improved labelling techniques [98], using pre-targeting strategies, modifying tumour delivery and up-regulating of tumour marker expression. In addition, several new dyes have been developed [99]. Far-red fluorochromes have been synthesized that have many properties desirable for in vivo imaging. Far-red fluorochromes absorb and emit at wavelengths at which blood and tissue are relatively transparent, have high quantum yields, and have good solubility even at higher molar ratios of fluorochrome to antibody. Small antibody species such as single-chain Fv fragments possess pharmacokinetics which can result in good contrast ratios, but clear rapidly resulting in low absolute levels of reporter groups in the target tissue. Higher fluorescent yields can compensate for this lower deposition increasing the sensitivity of detection.

Other applications of imaging include the development of research tools. Antibodies labelled with dyes have been invaluable in visualising cell biological processes such as receptor trafficking [100]. Increased fluorescent yields would enable the detection and monitoring of low abundance molecules. The usual method for visualising labelled cells is immunofluorescent microscopy where multiply-labelled molecules can be simultaneously monitored using a range of specific antibodies possessing different and non-overlapping fluorescence emission spectra.

As described above, the coupling of dye molecules to antibody fragments or other appropriate ligands using our novel coupling conditions results in higher loading ratios. This can translate directly into enhanced photophysics. As well as higher singlet oxygen generation for improved PDT, superior photophysics can manifest
as increased fluorescence. Antibody fragment photo-immunoconjugates with appropriate dye molecules can make more effective diagnostic reagents due to their favourable pharmacokinetics and enhanced fluorescence. Rapid clearance and low non-specific tissue binding will lead to very high contrast ratios and high fluorescence will allow more sensitive detection of the output signal. The use of antibody fragments, constructed, selected or engineered to contain favourably-spaced functional groups for coupling (e.g. lysine amino groups) as described above can lead to dyes with more favourable fluorescence yields due to reduced quenching and mis-interactions. This will have applications primarily in medical imaging, but can also be used to make more sensitive reagents for diagnostic kits or cellular imaging and by coupling fluorescent dyes and photosensitisers to the same antibody fragments a bifunctional agent can be produced, allowing both tumour imaging and phototherapy.

In a second aspect of the invention there is provided a compound comprising a photosensitising agent coupled to a carrier molecule obtainable by the process of the invention.

In a third aspect of the invention there is provided a compound comprising a photosensitising agent coupled to a carrier molecule with a minimum coupling ratio of 3:1 wherein the carrier molecule binds selectively to a target cell.

Preferably the carrier molecule has an upper size limit of 3:1 when compared to the photosensitiser, typically an upper limit of 30kDa. An example of such a carrier is an ScFv.

Advantageously the functional and physical properties of the photosensitising agent and the carrier molecule are substantially unaltered in the coupled form in comparison to the properties when in an uncoupled form.

Preferably, the carrier molecule is selected from the group consisting of: an antibody fragment and/or a derivative thereof, or a non-immunogenic peptide ligand.
Conveniently the antibody fragment and/or derivative thereof is a single-chain antibody fragment, in particular an ScFv.

Alternatively the carrier molecule is humanised or human.

Conveniently the photosensitising agent is a monofunctional photosensitiser. Preferably the photosensitising agent is at least one selected from the group consisting of: haematoporphyrins, Photofrin™, naturally occurring porphyrins, chlorins and bacteriochlorins, pheophorbides like pyropheophorbide a and its derivatives like Photocholor, chlorins (e.g. chlorin e6), mono-1-aspartyl derivative of chlorin e6, di-1-aspartyl derivative of chlorin e6, tin (FV) chlorin e6, the palladium derivatives of naturally occurring bacteriochlorophylls like TOOKAD (Pd-bacteriopheophorbide), synthetic chlorins and bacteriochlorins like meta-tetrahydroxyphenyl chlorin (Foscan) and bacteriochlorin, benzoporphyrin derivatives, monobenzoporphyrin derivatives like verteporfin, phthalocyanines, sulphonated aluminium phthalocyanines (disulphonated and tetrasulphonated), sulphonated aluminium naphthalocyanines and derivatives, purpurins like purpurin-18, tin and zinc derivatives of octaethylpurpurin, tin etiopurpurin, verdins, porphycenes, synthetic porphyrins, chlorins and bacteriochlorins, like the meso-triethynylporphyrins, metal-free and metallated core-modified porphyrins, expanded porphyrins (texaphyrins) like motexafin lutetium and motexafin gadolinium and non-porphyrinic compounds such as phenothiazinium derivatives like methylene blue, toluidine blue, cyanines such as merocyanine-540, acridine dyes, BODIPY dyes and aza-BODIPY derivatives, hypericin, halogenated squarine dyes and halogenated xanthene dyes like eosin and rose Bengal.

Conveniently the photosensitising agent is coupled to the carrier molecule at an amino acid residue or a sugar molecule on the carrier molecule.

Preferably the amino acid residue is at least one selected from the group consisting of: lysine; cysteine; tyrosine; serine; glutamate; aspartate; and arginine. Alternatively, the sugar molecule is selected from at least one of the group
consisting of: sugars comprising an hydroxyl group; sugars comprising an aldehyde group; sugars comprising an amino group; and sugars comprising a carboxylic acid group.

Although coupling photosensitisers to lysine residues is generally straightforward, the above conjugation methodology can also apply to the coupling of photosensitisers to antibody fragments via other amino acid residues or sugar molecules attached to the protein by N- or O-linked glycosylation using different functional groups on the photosensitiser moieties. Table 4 lists these residues and the other possible coupling chemistries which can be used with this coupling method.

### Table 4 Functional groups for coupling photosensitizers onto antibodies

<table>
<thead>
<tr>
<th>Residue(s)</th>
<th>Functional group</th>
<th>Coupling chemistry</th>
<th>Resulting bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>Amine</td>
<td>Active-ester</td>
<td>Amide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isothiocyanate</td>
<td>Isothiourea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isocyanates</td>
<td>Isourea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acyl azides</td>
<td>Amide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulphonyl chloride</td>
<td>Sulphonamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbonyl, reduce.</td>
<td>Schiff Base, 2° amine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epoxide</td>
<td>2° Amine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbonates</td>
<td>Carbamate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorobenzene deriv.</td>
<td>Arylamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imidoesters</td>
<td>Amidine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbodiimides</td>
<td>Amide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anhydrides</td>
<td>Amide</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Thiol</td>
<td>Haloacetyl</td>
<td>Thioether</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maleimides</td>
<td>Thioether</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acryloyl</td>
<td>Thioether</td>
</tr>
</tbody>
</table>
Antibody fragments vary in amino acid sequence and the number and spacing of functional groups to couple photosensitizers to. The most common frequently used functional group for conjugation is the primary amine found at the N-terminus and on lysine residues, as described above. We have found, surprisingly, that a major determinant of the effectiveness of a particular photosensitiser-antibody fragment is

<table>
<thead>
<tr>
<th>Tyrosine, serine</th>
<th>Hydroxyl</th>
<th>Diazonium</th>
<th>Mannich</th>
<th>Active-ester</th>
<th>Active Alkylation</th>
<th>Isocyanates</th>
<th>Diazo</th>
<th>2°amine</th>
<th>Ester</th>
<th>Ether</th>
<th>Carbamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate, aspartate</td>
<td>Carboxylic acid</td>
<td>Diazoaalkyl</td>
<td>Carbodiimides</td>
<td>Acylimidazole</td>
<td>Ester</td>
<td>Amide, Ester, Thioester</td>
<td>Amide, Ester, Thioester</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>Guanidinyl</td>
<td>Dicarbonyl</td>
<td>Schiff base</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugars</td>
<td>Hydroxyl (e.g. glucose)</td>
<td>Acylation</td>
<td>Alkylation</td>
<td>Oxidative cleavage to the aldehyde</td>
<td>Ester</td>
<td>Ether</td>
<td>Schiff base, mild redn. to the 2° amine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugars</td>
<td>Aldehyde (e.g. mannose)</td>
<td>Reductive amination</td>
<td>Schiff base, 2° amine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugars</td>
<td>Amino (e.g. b-D-mannosamine)</td>
<td>See reactions for lysine</td>
<td>See reactions for lysine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugars</td>
<td>Carboxylic acid (e.g. sialic acid)</td>
<td>See reactions for glutamate</td>
<td>See reactions for glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
conjugate is the spatial separation of the residues to which photosensitiser molecules are attached. These residues must be distinct and topologically separated on the surface of the antibody for effective coupling and optimal photophysics of the resulting conjugate.

Generally, proteins fold to form a hydrophobic core at the centre of the molecule with a hydrophilic surface to enable solubility in physiological solvents. Basic residues such as lysines and arginines, acidic residues such as glutamates and aspartates, polar residues such as serines (and sometimes tyrosines), cysteines, glutamines and asparagines are commonly found on the surface of proteins. In many examples these residues are involved in maintaining the structure and function of that protein.

In the example of antibody fragments such as single-chain Fv each domain is made up of a variable heavy (VH) and variable light (VL) domain. These can be one of any family of VH and VL domains. An alignment of the families of VH and VL genes (Figure 1) shows that generally, many residues can be tolerated at any position. In the case of the antigen binding loops (complementarity determining regions-CDRs), these sequences are specific to the ability of that antibody to recognise its cognate antigen. These can be manipulated to alter the specificity or affinity of the antibody but for no other reasons. The major part of the domain sequence is the framework region. Figure 1 indicates which areas tend to be present at the surface of the antibody and which areas tend to be interior as part of the core. Given the high degree of structural and sequence homology between antibodies, these regions can generally be applied to all antibody sequences. The surface framework regions tend to contain the charged or polar residues, evenly spaced out (i.e. no particular requirement at any particular position).

Taking lysine residues as one example. These are commonly found at the surface of antibody domains. In the case of members of the germline human VH1 family, there are 5-6 lysine residues, only one or two of which are close to each other. A definition of a residue being close to another can be one that is adjacent in the
primary sequence hence adjacent in the 3-dimensional structure. Alternatively, a residue may be separated according to the primary sequence, but adjacent in space due to the structure of the fold of the antibody domain. A directly adjacent residue can be defined as 3-4 angstroms apart in space.

We have found that the coupling of photosensitisers onto lysine residues which are directly adjacent will result in photophysical quenching and poorer photodynamic effects (such as increased aggregation and poorer solubility of photo-immuno conjugates). Coupling is more effective when lysine residues are further separated, preferably two amino acids apart (3.5 to 7.5 angstroms), more preferably three amino acids apart (9 to 12 angstroms), more preferably four amino acids apart (10-15 nm), even more preferably five amino acids apart (15-20 nm), yet even more preferably six amino acids apart (20-25 nm). Antibodies should be chosen, selected or engineered to possess these properties. The more lysine residues an antibody possess, with more optimal separation, the better that antibody will be at forming effective and potent photo-immuno conjugates with optimal photophysical and photodynamic effects.

Methods of determining whether amino acid residues for photosensitiser coupling are close or adjacent to one another are well known in the art. Clustal sequence alignment (using web resources such as http://www.ebi.ac.uk/clustalw/ European bioinformatics Institute) is a well established tool for comparing primary amino-acid sequence. Furthermore, in the absence of full 3 dimensional structural data for an antibody fragment, it is possible to use well-established techniques such as homology modelling using known structures (for example that of a murine scFv [89] to deduce probable structure of the antibody fragment, and thereby to identify whether residues for coupling are close or adjacent in space. The high degree of homology exhibited by antibodies and antibody fragments means these techniques can be applied with a high degree of confidence. Web resources for homology modelling are available, such as the Expert Bioinformatics Analysis System from the Swiss Institute of Bioinformatics (http://us.expasy.org) which also provides the free desktop modelling programme SwissPDB Viewer.
An example of such a favourable distribution of lysine residues on a scFv is shown in Figure 2 (a scFv derived from human VH1-VK3). If the distribution of lysine residues is less favourable for conjugation and optimal photophysics, the antibody fragment may be altered using standard molecular biological techniques, such as site directed mutagenesis to remove poorly spaced (too closely positioned) or introduce well-spaced residues.

The above concept can also apply to the spacing and coupling to other amino acid residues other than lysine or to sugar molecules attached to the protein by N- or O-linked glycosylation. Table 4 lists these residues and the possible coupling chemistries which can be used.

The above concept can also be applied to non-antibody based ligands. Examples of ligands which can be used to target photosensitisers which can also be influenced by amino acid spacing are listed in Table 5.

**Table 5**

<table>
<thead>
<tr>
<th>Type</th>
<th>Ligand name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin-based</td>
<td>Domain antibodies</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Single chain Fvs</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Fab fragment</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Fn3 domains</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Protein L</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>T cell receptors</td>
<td>92</td>
</tr>
<tr>
<td>Non-immunoglobulin</td>
<td>Peptides</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Ankyrin repeats</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Anticalin</td>
<td>31</td>
</tr>
</tbody>
</table>
This will lead to coupled photosensitizers retaining their photophysical properties and therefore good photodynamic therapy function. There are many examples of antibodies where many of the lysine residues are adjacent in primary sequence or in 3-dimensional space. By molecular modelling and site-directed mutagenesis, we are able to engineer the position of these lysine residues, adding additional ones if there are too few, removing adjacent residues or increasing the distance between others.

This leads to antibody fragments which are more amenable to photosensitizer coupling, capable of achieving higher loading (increased photosensitizer: antibody ratios) and more potent PDT effects. One indirect measurement of enhanced photophysics is increased fluorescence.

Advantageously the compound further comprises a modulating agent wherein the modulating agent capable of modulating the function of the photosensitising agent coupled to the carrier molecule. Preferably the modulating agent is selected from the group of benzoic acid, benzoic acid derivatives containing an azide group like 4-azidotetrafluorophenylbenzoic acid and other aromatic or heteroaromatic groups containing an azide moiety (N₃) including polyfluorobenzenes, naphthalenes, naphthaquinones, anthracenes, anthraquinones, phenanthrenes, tetracenes, naphthacenediones, pyridines, quinolines, isoquinolines, indoles, isoindoles, pyrroles, imidazoles, pyrazoles, pyrazines, benzimidazoles, benzofurans, dibenzofurans, carbazoles, acridens acridones, and phenanthridines, xanthenes, xanthones, flavones and coumarins. Aromatic and heteroaromatic sulfenates derived from the aromatic/heteroaromatic groups above. Other specific modulating agents include vitamin E analogues like Trolox, butyl hydroxyl toluene, propyl gallate, deoxycholic acid and ursodeoxycholic acid.

Conveniently, the compound further comprises a visualising agent, for example a fluorescent or luminescent dyes (see above).

Preferred examples of conjugates are:
(a) wherein the carrier molecule is an ScFv and the photosensitising agent is Pyropheophorbide a.

(b) wherein the carrier molecule is an ScFv and the photosensitising agent is benzoporphyrin derivative mono acid (Verteporfin, Visudyne™)

(c) wherein the carrier molecule is an ScFv and the photosensitising agent is palladium-bacteriopheophorbide (TOOKAD™).

(d) wherein the carrier molecule is an ScFv and the photosensitising agent is mono-1-aspartyl derivative of chlorin e6.

(e) wherein the carrier molecule is an ScFv and the photosensitising agent is meta-tetrahydroxyphenyl chlorin (Foscan™)

(f) wherein the carrier molecule is an ScFv and the photosensitising agent is tin etiopurpurin (rostaporfin).

In a fourth aspect of the invention there is provided a use of the compound in the treatment and/or prevention of a disease requiring the destruction of a target cell.

There is also provided the use of the compound in the manufacture of a medicament for the diagnosis, treatment and/or prevention of a disease requiring the destruction of a target cell.

Preferably, the disease to be treated is selected from the group consisting of: cancer; age-related macular degeneration; immune disorders; cardiovascular disease; and microbial infections including viral, bacterial or fungal infections, prion diseases such as BSE and oral/dental diseases such as gingivitis.

Most preferably the disease to be treated is cancer of the colon, lung, breast, Head and neck, prostate, skin, stomach/gastrointestinal, bladder and precancerous lesions such as Barrett's oesophagus.
Conveniently the diagnosis of diseases is conducted by visualisation of either the photosensitizing agent or an optional visualisation agent such as a fluorescent or luminescent dye.

Advantageously the compound or composition is administered to a patient prior to light exposure.

In fifth aspect of the invention there is provided a composition comprising the compound of the invention and a pharmaceutically acceptable carrier, excipient or diluent

**Meanings of terms used**

The term "antibody fragment " shall be taken to refer to any one of an antibody, an antibody fragment, or antibody derivative. It is intended to embrace wildtype antibodies (i.e. a molecule comprising four polypeptide chains), synthetic antibodies, recombinant antibodies or antibody hybrids, such as, but not limited to, a single-chain modified antibody molecule produced by phage-display of immunoglobulin light and/or heavy chain variable and/or constant regions, or other immunointeractive molecule capable of binding to an antigen in an immunoassay format that is known to those skilled in the art.

The term "antibody derivative" refers to any modified antibody molecule that is capable of binding to an antigen in an immunoassay format that is known to those skilled in the art, such as a fragment of an antibody (e.g. Fab or Fv fragment), or a modified antibody molecule that is modified by the addition of one or more amino acids or other molecules to facilitate coupling the antibodies to another peptide or polypeptide, to a large carrier protein or to a solid support (e.g. the amino acids tyrosine, lysine, glutamic acid, aspartic acid, cysteine and derivatives thereof, \( \text{NH}_2\)-acetyl groups or COOH-terminal amido groups, amongst others).
The term "ScFv molecule" refers to any molecules wherein the VH and VL partner domains are linked via a flexible oligopeptide.

The terms "nucleotide sequence" or "nucleic acid" or "polynucleotide" or "oligonucleotide" are used interchangeably and refer to a heteropolymer of nucleotides or the sequence of these nucleotides. These phrases also refer to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent the sense or the antisense strand, to peptide nucleic acid (PNA) or to any DNA-like or RNA-like material. hi the sequences herein A is adenine, C is cytosine, T is thymine, G is guanine and N is A, C, G or T (U). It is contemplated that where the polynucleotide is RNA, the T (thymine) in the sequences provided herein is substituted with U (uracil). Generally, nucleic acid segments provided by this invention may be assembled from fragments of the genome and short oligonucleotide linkers, or from a series of oligonucleotides, or from individual nucleotides, to provide a synthetic nucleic acid which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon, or a eukaryotic gene.

The terms "polypeptide" or "peptide" or "amino acid sequence" refer to an oligopeptide, peptide, polypeptide or protein sequence or fragment thereof and to naturally occurring or synthetic molecules. A polypeptide "fragment," "portion," or "segment" is a stretch of amino acid residues of at least about 5 amino acids, preferably at least about 7 amino acids, more preferably at least about 9 amino acids and most preferably at least about 17 or more amino acids. To be active, any polypeptide must have sufficient length to display biological and/or immunological activity.

The terms "purified" or "substantially purified" as used herein denotes that the indicated nucleic acid or polypeptide is present in the substantial absence of other biological macromolecules, e.g., polynucleotides, proteins, and the like. In one embodiment, the polynucleotide or polypeptide is purified such that it constitutes at least 95% by weight, more preferably at least 99% by weight, of the indicated biological macromolecules present (but water, buffers, and other small molecules,
especially molecules having a molecular weight of less than 1000 daltons, can be present).

The term "isolated" as used herein refers to a nucleic acid or polypeptide separated from at least one other component (e.g., nucleic acid or polypeptide) present with the nucleic acid or polypeptide in its natural source. In one embodiment, the nucleic acid or polypeptide is found in the presence of (if anything) only a solvent, buffer, ion, or other component normally present in a solution of the same. The terms "isolated" and "purified" do not encompass nucleic acids or polypeptides present in their natural source.

The term "recombinant," when used herein to refer to a polypeptide or protein, means that a polypeptide or protein is derived from recombinant (e.g., microbial, insect, or mammalian) expression systems. "Microbial" refers to recombinant polypeptides or proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant microbial" defines a polypeptide or protein essentially free of native endogenous substances and unaccompanied by associated native glycosylation. Polypeptides or proteins expressed in most bacterial cultures, e.g., *E. coli*, will be free of glycosylation modifications; polypeptides or proteins expressed in yeast will have a glycosylation pattern in general different from those expressed in mammalian cells.

The term "expression vector" refers to a plasmid or phage or virus or vector, for expressing a polypeptide from a DNA (RNA) sequence. An expression vehicle can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters and often enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an amino terminal methionine residue. This
residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

The terms "selective binding" and "binding selectivity" indicates that the variable regions of the antibodies of the invention recognise and bind polypeptides of the invention exclusively (i.e., able to distinguish the polypeptide of the invention from other similar polypeptides despite sequence identity, homology, or similarity found in the family of polypeptides), but may also interact with other proteins (for example, S. aureus protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and in particular, in the constant region of the molecule. Screening assays to determine binding selectivity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see Harlow et al. (Eds), Antibodies A Laboratory Manual; Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y. (1988), Chapter 6. Antibodies that recognise and bind fragments of the polypeptides of the invention are also contemplated, provided that the antibodies are first and foremost selective for, as defined above, full-length polypeptides of the invention. As with antibodies that are selective for full length polypeptides of the invention, antibodies of the invention that recognise fragments are those which can distinguish polypeptides from the same family of polypeptides despite inherent sequence identity, homology, or similarity found in the family of proteins.

The term "binding affinity" includes the meaning of the strength of binding between an antibody molecule and an antigen.

The term "coupling ratio" means the number of molecules of photosensitising agent coupled to one carrier molecule.

The term "carrier molecule" includes the meaning of any agent to which the photosensitising agent is coupled, in particular, the carrier molecule may be a small compound including but not limited to antibody fragments and non-immunogenic peptides.
The term "monofunctional photosensitizer" or "monofunctional phosensitising agent" means a photosensitizer like PPa which contains a single propionic acid side chain which can be activated and coupled or by the use of chemistry known in the art a sensitizer can be modified through protection/deprotection chemistry to possess a group that can be activated/coupled.

The term "aprotic solvent" means a solvent that has no OH groups and therefore cannot donate a hydrogen bond.

**Preferred Embodiments**

Examples embodying certain preferred aspects of the invention will now be described with reference to the following figures in which:

**Figure 1** - Alignment of human immunoglobin variable genes, with lysine residues highlighted in bold. Certain families such as human VH-I contain more and favourably separated lysine residues making scFvs (or other antibody formats) derived from them more effective for photosensitizer coupling. FR=framework CDR=complementarity determining regions.

**Figure 2** - Structural representation of a scFv from the human VH1-VK3 family with naturally occurring lysine residues highlighted in black. The lysine residues are favourably placed for efficient photosensitizer coupling and good photophysics.

**Figure 3** - Cloning of a scFv into a pET expression system

**Figure 4** - Purification of C6.5 by immobilised metal affinity chromatography (IMAC) using nickel chloride-charged resin. C6.5 was eluted from the column using 250 mM imidazole Lane 4 shows C6 eluted from the column and concentrated 5-fold using a spin column.
**Figure 5** - Preparation of PPa succinimidyl ester

**Figure 6** - Absorbance profile of C6.5 scFv conjugated to PPa (bold line) and free PPa (thin line), in PBS buffer. The absorbance peaks are used to determine the PPa: scFv ratio as described in the examples.

**Figure 7** - Absorbance profile of MFE-23 scFv conjugated to PPa and PPa/benzoic acid (Ba) and free PPa, in PBS buffer. The absorbance peaks are used to determine the PPa: scFv ratio as described in the examples.

**Figure 8** - Preparation of PBI succinimidyl ester

**Figure 9** - Absorbance profile of HuBC-I scFv conjugated to PPa. The absorbance peaks are used to determine the PPa: scFv ratio as described in the examples. The poor structure of HuBC-I results in poor absorbance properties of the scFv-PPa conjugate compared to C6.5 scFv

**Figure 10** - Absorbance profile of C6.5 scFv coupled to Pyropheophorbide-a and Chlorin e6 photosensitisers

**Figure 11** - Fluorescence profiles of various concentrations of C6.5 scFv-PPa conjugate and free PPa measured in PBS buffer. Free PPA does not fluoresce significantly in aqueous buffers, but when conjugated to an scFv retains good photophysical properties.

**Figure 12** - Fluorescence profiles of various concentrations of C6.5 scFv-PPa, MFE-23 scFv-PPa and NFE-23 scFv-PPa/Ba (benzoic acid) conjugates measured in PBS buffer. Free PPA does not fluoresce significantly in aqueous buffers (Fig. 7), but when conjugated to an scFv retains good photophysical properties. The C6.5 scFv is better at retaining fluorescence (hence photophysics including singlet oxygen generation) than the MFE-23 scFv.

**Figure 13** - CEA antigen ELISA of MFE-23 scFv, MFE-23 scFv-PPa and MFE-
A small decrease in binding affinity is observed upon coupling.

**Figure 14** - In vitro PDT cell killing of C6.5 scFv-PPa on antigen-positive cells (SKOV-3) and antigen negative cells (LS174T).

**Figure 15** - In vitro PDT cell killing of C6.5 scFv-PPa on antigen-positive cells (LS174T) and antigen negative cells (SKOV-3).

**Figure 16** - In vivo tumour: blood ratios of C6.5 scFv compare to C6.5-PPa conjugate after 24 hr in a SKOV-3 human tumour xenograft model (upper) and percentage tumour uptake after 24 hr (lower).

**Figure 17** - In vivo pharmacokinetics (blood clearance profiles) of scFvs and scFv-Ppa conjugates in nude mice.

**Figure 18** - In vivo PDT therapy of tumour-bearing nude mice results in necrosis of a human SKOV-3 xenografted tumour. Left panel, C6.5 alone plus light, right panel, C6.5-PPa plus light.

**Figure 19** - Alignment of an optimal 'PDT' scFv such as C6.5 (a VH1-VK3 scFv) with HuBC-I reveals changes which can be made by mutagenesis. These 6 changes are made which can result in a HuBC-I scFv (BC-I-mut) with more favourable photosensitiser coupling properties. These changes are K13Q, Q43K, T87K, R152K, R180K and G210K.

**Figure 20** - SKOV3 cells labelled with C6.5scFV-PPa allows the sensitive visualization of the Her-2 receptor which is seen to effectively internalised.

**Figure 21** - Emission spectrum of PPa from SKOV-3 cells.

**Figure 22** - Absorbance spectra of PPa and scFv-PPa photo-immunon conjugates (A) PPa (14mg/ml) in PBS/1.9% DMSO [1] and 100% DMSO [2]. (B) 50mg/ml of C6.5-PPa (C) 10mg/ml each of MFE-PPa from frozen material [1] and fresh
material [2]. (D) A panel of alternative scFv-PPa photo-immunoconjugates all at 10 mg/ml, D1.3 [I], Fl [2], GP6 [3], and HuBC-I [4].

**Figure 23** - In vitro cytotoxicity of C6.5-PPa and MFE-PPa photo-immunoconjugates

(A) LoVo (●), SK0V3 (○) exposed to free PPa (B) C6.5-PPa exposed to SKOV3 cells (●) and LoVo cells (○) (C) MFE scFv (fresh material)-PPa exposed to LoVo cells (»), MFE scFv (frozen material)-PPa exposed to LoVo cells (○), MFE scFv (fresh material)-PPa exposed to SKOV3 cells (T)

**Figure 24** - Immunofluorescent microscopy of C6.5-PPa photo-immunoconjugate

Antigen negative KB cells (A-D) or antigen positive SKOV3 cells (E-J) were incubated with free PPa or C6.5-PPa photo-immunoconjugate for 1 hour. Images and emission spectra were recorded.

**Figure 25** - *in vivo* analyses of C6.5-PPa PICs

(a) Blood pharmacokinetics—the fraction remaining in the blood over a period of 24 hr was measured for antibodies, PPa and photo-immunoconjugates. Whole IgG (○), free PPa (●), C6.5-PPa (Δ), MFE-PPa (T), free C6.5 scFv (α), free MFE scFv (■)

(b) Biodistribution of the C6.5 scFv-PPa PIC in tumor-bearing nude mice at 8 hr (black bars) and 24 hr (grey bars). The tumor: blood ratio at 24 hr was chosen as a good value to perform the therapy study.

(c) Two sets of SKOV3 tumor-bearing nude mice were treated with PBS-saline (●) and 40 mg C6.5-PPa photo-immunoconjugate (○) followed by laser illumination. The tumor growth progress was recorded for the following 25 days. Significant growth delay was seen (p=0.0075).
**Figure 26** - Amino acid alignment of scFvs

The variable heavy-linker-variable light domains are shown with lysine residues highlighted in bold to illustrate the variability in number and position which may influence PDT coupling efficiency and photo-immunoconjugate potency.

**Figure 27** - Preparation of Verteporfin (Visudyne™) succinimidyl ester

**Figure 28** - Absorbance profiles of various concentrations of C6.5 scFv-Verteporfm (Visudyne™) conjugate and free Verteporfin (Visudyne™) measured in PBS buffer.

**Figure 29** - In vitro PDT cell killing of C6.5 scFv-Verteporfm (Visudyne™) conjugate and free Verteporfin (Visudyne™) on antigen-negative cells (KB) and antigen-positive cells (SKOV-3). Percentage (%) cell survival was determined for:

- C6.5 scFv-Verteporfm (Visudyne™) conjugate on SKOV3 cells (•);
- free Verteporfin (Visudyne™) on SKOV3 cells (o);
- C6.5 scFv-Verteporfin (Visudyne™) conjugate on KB cells (▼);
- free Verteporfin (Visudyne™) on KB cells (Δ).

**Materials**

All chemicals were purchased from Sigma-Aldrich UK unless stated. PPa was from Frontier Scientific, UK, C6.5 scFv was a gift from Prof. Marks (University of California, San Francisco, USA), MFE-23 scFv was a gift from Dr Chester (Royal Free Hospital, University College London, UK), HuBC-I scFv was a gift from Antisoma Research Ltd (London, UK). Molecular biology reagents and bacteria were from Stratagene, Human cell lines were from the ECACC, UK, Chromatography media was from Amersham Biosciences, UK, Mice were from Harlan, UK, Light sources were from Phototherapeutics, UK and High Powered Devices, New Jersey, USA.
Example 1 - Preparation of an anti-Her 2 scFv

1. A chosen, well characterised, anti-cancer scFv for example c6.5 (anti-Her2) was PCR amplified and cloned as an Nco I/Not I fragment into the bacterial expression vector (e.g. pET20b, Novagen) to create pETC6.5scFv (Fig. 3).

2. The vector pETc6.5scFv was transformed into E. coli BL21(DE3) (Novagen) by the calcium chloride method (Sambrook et al, 1990) and plated onto 2TY agar plates containing 100 mg/ml ampicillin (Sambrook et al, 1990). Single colony transformants were picked and re-streaked onto fresh 2TY Agar plates containing ampicillin.

3. A single colony was picked and grown in 5 ml of 2TY media containing 100 mg/ml ampicillin at 30 °C, in a shaking incubator (250rpm) for 8-16 hr. This culture was then used to inoculate a culture of 500 ml 2TY media containing 100 mg/ml ampicillin and grown under similar conditions for a further 3-16 hr.

4. The culture supernatant was harvested and concentrated using an Amicon ultrafiltration stirred cell with a 30 KDa cut-off membrane to a final volume of 10 ml. Alternatively, the bacterial periplasm can be prepared using the sucrose osmotic shock method in a volume of 10 ml.

5. The concentrated supernatant or periplasmic extract was dialysed for 16 hr against 5 L of phosphate-buffered saline (PBS) containing 0.5 M NaCl and 2 mM MgCl₂. This was then applied to a copper (II) or nickel (II)-charged chelating sepharose column (Amersham-Pharmacia Biotech) and purified by immobilised metal affinity chromatography (IMAC) for example as described in [14]. The recombinant protein eluted in an imidazole gradient at between 40 and 150 mM imidazole (Fig. 4).

6. The eluted fusion protein is further purified by gel filtration on a superdex-75 column (Amersham-Pharmacia Biotech) equilibrated in PBS. The resulting protein is called c6.5 scFv.
Example 2 - Preparation of PPa succinimidyl ester (Fig. 5)

1. To a light protected solution of the pyropheophorbide \( a \) (50 mg, 0.094 mmol) in a mixture of dry DCM/THF (9:1) N-hydroxysuccinimide (12.9 mg, 0.11 mmol) was added followed by dicyclohexylcarbodiimide (DCC) (23.2 mg, 0.11 mmol).

2. After stirring for 12 h, the precipitated dicyclohexylurea was filtered off and the solvents removed. The crude product was taken up in a small volume of chloroform and precipitated by the addition of hexane. The precipitate was collected, washed well with hexane and the resulting crude product purified by column chromatography on silica gel eluting with 2% hexane in ethyl acetate (\( R_f \) 0.66).

3. The isolated product was recrystallised from DCM/hexane to give pure pyropheophorbide a succinimidyl ester (1) in 70% yield. MS (FAB\(^+\)) 631 (M\(^+\), 100%)

4. A stock solution of C6.5 scFv at 500\( \mu \)g/ml was defrosted at room temperature and 200\( \mu \)l added to 706\( \mu \)l of PBS. Acetonitrile (60\( \mu \)l) was added to the solution. The solution was stirred on ice until cool.

Example 3 - Conjugation of C6.5 scFv to PPa - solvent system 1

1. Pyropheophorbide \( a \) succinimidyl ester made up in 100% DMSO was then added (34\( \mu \)l) from a stock solution of 1.58mM to the C6.5 scFv with continuous stirring (to give 16 equivalents of PPa). The mixture was kept on ice and in the dark, with stirring for 30 mins, after which time the solution was placed in dialysis tubing and dialysed against 5L of PBS at 4\( ^0 \)C overnight in the dark.

2. Each sample of the conjugate was placed in a quartz cuvette and an absorbance profile was run against a blank containing PBS (Fig. 6). The absorbance value at 410nm was measured and the concentration of PS in g/ml was determined by comparing to a standard curve of PPa.

3. For example, if the concentration of PPa found in the coupling reactions was 0.0000159g/ml. The number of molecules of PPa in 0.0000159g/ml was
1.4x10^{16}. The number of molecules of Cβ in 100 µg/ml was 2x10^{15}. The ratio therefore of PPa:C6.5 was 8:1.

**Example 4 - Conjugation of MFE-23 (anti-CEA) scFv to PPa - solvent system 2**

1. A stock solution of MFE-23 at 500 µg/ml was defrosted at room temperature and 200 µl added to 706 µl of PBS. Acetonitrile (60 µl) was added to the solution. The solution was stirred on ice until cool.

2. Pyropheophorbide α succinimidyl ester was then added (34 µl) from a stock solution of DMSO 1.58 mM to the MFE-23 with continuous stirring (to give 16 equivalents of PPa). The mixture was kept on ice and in the dark, with stirring for 30 mins, after which time the solution was placed in dialysis tubing and dialysed against 5 L of PBS at 4°C overnight in the dark.

3. Each sample of the conjugate was placed in a quartz cuvette and an absorbance profile run against a blank containing PBS (Fig. 7). The absorbance value at 410 nm was measured and the concentration of PS in g/ml determined by comparing to a standard curve of PPa. For example, if the concentration of PPa found in the coupling reactions was 0.0000129 g/ml. The number of molecules of PPa in 0.0000129 g/ml was 1.4x10^{16}. The number of molecules of MFE in 100 µg/ml was 2x10^{15}. The ratio therefore of PPa:MFE-23 was 6:1.

**Example 5 - Preparation of PBl succinimidyl ester (Fig. 8)**

1. To a light-protected solution of the benzoic acid derivative of PBl (20 mg, 0.01136 mmol) in anhydrous THF, N-hydroxysuccinimide (2 mg, 0.017 mmol) was added followed by dicyclohexylcarbodiimide (DCC) (3.5 mg, 0.017 mmol). After stirring for 12 h, the precipitated dicyclohexylurea was filtered off and the solvents removed. The resulting crude product was purified by column chromatography on silica gel eluting with THF (Rf 0.79) to give the desired compound (2) as dark-green solid in 65% yield. MS (FAB^+) 1860 (M+2, 80%).
Example 6 - Conjugation of C6.5 scFV to PBl - solvent system 1

1. A stock solution of C6.5 at 500 µg/ml was defrosted at room temperature and 200 µl added to 706 µl of PBS. Acetonitrile (60 µl) was added to the solution. The solution was stirred on ice until cool.

2. PBl (see [94]) made up in 100% DMSO was then added (34 µl) from a stock solution of 1.58 mM to the C6.5 with continuous stirring (to give 16 equivalents of PBl). The mixture was kept on ice and in the dark, with stirring for 30 mins, after which time the solution was placed in dialysis tubing and dialysed against 5 L of PBS at 4°C overnight in the dark.

3. Analysis of conjugate. Each sample of the conjugate was placed in a quartz cuvette and an absorbance profile was run against a blank containing PBS. The absorbance value at 460 nm was measured and the concentration of PS in g/ml was determined by comparing to a standard curve of PBl.

Example 7 - Coupling of BA modulator to a scFv or scFv-PPa conjugate

1. A stock solution of MFE-23 at 500 µg/ml was defrosted at room temperature and 200 µl added to 690.8 µl of PBS. Acetonitrile (60 µl) was added to the solution.

2. The solution was stirred and 15.2 ul of 0.491 µM solution of the benzoyl succinimidyl ester (prepared by the reaction of benzoic acid with N-hydroxsuccinimide and DCC in dry dichloromethane), dissolved in DMSO was added (to give 16 equivalents of benzoic acid). The solution was stirred at room temperature for 30 minutes, after which time, the flask was cooled on ice with continuous stirring.

3. Pyropheophorbide a succinimidyl ester made up in 100% DMSO was then added (34 µl) from a stock solution of 1.58 mM (to give 16 equivalents of PPa). The mixture was kept on ice and in the dark, with stirring for 30 mins, after which time the solution was placed in dialysis tubing and dialysed against 5 L of PBS at 4°C overnight in the dark.

4. Each sample of the conjugate was placed in a quartz cuvette and an absorbance profile run against a blank containing PBS (Fig. 7). The
absorbance value at 410nm was measured and the concentration of PS in g/ml determined by comparing to a standard curve of PPa. For example, if the concentration of PPa found in the coupling reactions was 0.0000129 g/ml. The number of molecules of PPa in 0.0000129 g/ml was 1.4 x 10^6. The number of molecules of MFE in 100 ug/ml was 2 x 10^15. The ratio therefore of PPa:MFE-23 was 6:1

Example 8 - Conjugation of HuBC-I scFv to PPa (a poor scFv for PDT)

1. To a light protected solution of the pyropheophorbide a (50 mg, 0.094 mmol) in a mixture of dry DCM/THF (9:1) N-hydroxysuccinimide (12.9 mg, 0.11 mmol) was added followed by dicyclohexylcarbodiimide (DCC) (23.2 mg, 0.11 mmol).

2. After stirring for 12 h, the precipitated dicyclohexylurea was filtered off and the solvents removed. The crude product was taken up in a small volume of chloroform and precipitated by the addition of hexane. The precipitate was collected, washed well with hexane and the resulting crude product purified by column chromatography on silica gel eluting with 2% hexane in ethyl acetate (Rf 0.66).

3. The isolated product was recrystallised from DCM/hexane to give pure pyropheophorbide a succinimidyl ester (1) in 70% yield. MS (FAB+) 631 (M+ 100%)

4. A stock solution of HuBC-I scFv at 500 µg/ml was defrosted at room temperature and 200 µl added to 706 µl of PBS. Acetonitrile (60 µl) was added to the solution. The solution was stirred on ice until cool.

5. Pyropheophorbide a succinimidyl ester made up in 100% DMSO was then added (34 µl) from a stock solution of 1.58 mM to the HuBC-I scFv with continuous stirring (to give 16 equivalents of PPa). The mixture was kept on ice and in the dark, with stirring for 30 mins, after which time the solution was placed in dialysis tubing and dialysed against 5 L of PBS at 4°C overnight in the dark.

6. Each sample of the conjugate was placed in a quartz cuvette and an absorbance profile was run against a blank containing PBS (Fig. 9). The
absorbance value at 410nm was measured and the concentration of PS in g/ml was determined by comparing to a standard curve of PPa.

7. The low absorbance peak at 410 nm means that it was not possible to determine the degree of PPA coupling.

Example 9 - Conjugation of C6.5 scFv to Chlorin (e6)

1. To a light-protected solution of chlorin e6 (0.00184 mmol) in anhydrous DMF equimolar amounts of both N-hydroxysuccinimide and dicyclohexyl carbodiimide were added and the mixture stirred for 12h under argon.

2. The resulting mixture was briefly cooled in ice-water and then filtered to remove the dicyclohexyl urea by-product and evaporated to dryness to give the chlorin e6 succinimidyl ester as a dark-green-solid.

3. A stock solution of C6.5 scFv at 500µg/ml was defrosted at room temperature and 200µl added to 706µl of PBS. Acetonitrile (60µl) was added to the solution. The solution was stirred on ice until cool.

4. Chlorin e6 succinimidyl ester made up in 100% DMSO was then added (34µl) from a stock solution of 1.58mM to the C6.5 scFv with continuous stirring (to give 16 equivalents of Ce6). The mixture was kept on ice and in the dark, with stirring for 30 mins, after which time the solution was placed in dialysis tubing and dialysed against 5L of PBS at 4°C overnight in the dark.

5. Each sample of the conjugate was placed in a quartz cuvette and an absorbance profile was run against a blank containing PBS (Fig. 10). The absorbance value at 410nm was measured and the concentration of PS in g/ml was determined by comparing to a standard curve of Ce6.

6. For example, if the concentration of Ce6 found in the coupling reactions was 0.000034 g/ml. The number of molecules of Ce6 in 0.000034 g/ml was 3.43x10^{16}. The number of molecules of C6 in 100 µg/ml was 2x10^{15}. The ratio therefore of Ce6:C6.5 was 9:1.
Example 10 - Conjugation of C6.5 scFv to a hydrazine derivative of PPa

1. Preparation of the Hydrazide Derivative of PPa. The propionic acid side chain of pyropheophorbide a was converted to the acyl chloride by standard literature procedures (oxally chloride in DCM). The acid chloride was obtained as a sticky green residue and used without further purification.

2. A solution of the acid chloride in anhydrous DCM was added drop-wise to an excess of 98% hydrazine in anhydrous DCM, the reaction was monitored by TLC and was over in less than 1h. The excess solvent and reagent was evaporated and the residue purified by chromatography. A stock solution of the PPa hydrazide was then made up in DMSO.

3. A scFv, e.g. C6.5 was engineered to possess carbohydrate chains as follows: Site-directed mutagenesis was used to incorporate N-linked glycosylation sites across the surface of the scFv, at positions which are all well-separated, according to the concept already described for Lysine residue spacing. This clone was placed in an expression vector suitable for a host cell which can carry out glycosylation (e.g. pPIC vector for expression in Pichia pastoris yeast).

4. The scFv was expressed and purified according to the manufacturer's instructions, using NTA-Nickel chromatography.

5. The derivatized PPa was coupled to the aldehyde residues on the glycosylated scFv. Coupling to the aldehyde residues proceed rapidly in buffered environments with the formation of a hydrazone linkage.

Example 11 - Photophysical characterisation of a scFV-PPa conjugate

1. Serial dilutions (halving concentrations) of the conjugates were made in PBS and the fluorescence was measured at an excitation wavelength of 410nm and an emission wavelength of 680nm.

2. These were compared to free PPa in PBS. Examples are shown for c6.5 scFv-PPa (Fig. 11) and MFE scFv-PPA (+/- benzoic acid) (Fig. 12)
**Example 12 - Biochemical characterisation of a scFV-PPa and scFv-PPa/BA conjugate**

1. *In vitro* binding characteristics of the anti-CEA scFv-PPa molecule was carried out by ELISA (Lane, 1990) or by BIACore surface plasmon resonance using published methods Lipschultz *et. al.* 'Experimental Design For Analysis of Complex Kinetics Using Surface Plasmon Resonance' Methods (2000) 20, 3180, compared to the unmodified scFv. Cell binding of the scFv-PPa/BA can also be compared to the unmodified proteins can be determined by Fluorescently Activated Cell Sorting (FACS), Confocal fluorescence microscopy.

2. As an example, a 96-well ELISA plate was coated with 1µg/ml carcinoembryonic antigen (CEA) in PBS and incubated overnight at 4°C. The next day the plate was washed three times in PBS-0.1% tween and three times PBS.

3. The ELISA plate was then incubated in blocking buffer (10% Marvel™ in PBS-0.1% tween) for 60min at 37°C.

4. The blocking buffer was removed from the wells and 50µl of conjugate or unconjugated MFE diluted in blocking buffer to give halving dilutions of MFE from 100-1.9x10⁴ µg/ml was added to each well. The plate was incubated as above and the wells washed as described above.

5. Primary antibody (50µl, rabbit anti-MFE; diluted in blocking buffer at 1:40 000) was added into each well. The plate was incubated and washed as described above.

6. Secondary antibody (50µl, anti-rabbit horse-radish peroxidase conjugate; diluted in blocking buffer at 1:10 000) was added to each well. Plates were incubated and washed as above. BM blue (50µl) was added to each well and incubated at room temperature, in the dark, until a blue colour developed.

7. The reaction was stopped by adding 50 µl of 0.5M hydrochloric acid. Samples were then read at 460nm (Fig. 13).
Example 13 - *In vitro* cytotoxicity qf a c6.5 scFv-PPa conjugate

1. *In vitro* cell cytotoxicity was measured as followed: The target cells (in this example LoVo and LS17T) were maintained at 37°C, 5% CO₂ in media (DMEM) supplemented with 10% foetal calf serum and 5 nM penicillin/streptomycin in a 75cm² flask. For SkoV3 cells, the medium used was McCoy's 5A medium supplemented with 15% FBS, 5 mM penicillin/streptomycin.

2. When 70-80% confluent, the cells were washed in PBS and 5 ml trypsin added. The flask was incubated at 37°C 5% CO₂ for 15 mins or until the cells had detached from the flask. The cells were then placed in a 50ml Falcon tube and the trypsin deactivated by adding 15ml DMEM or McCoy's medium.

3. Cells (20µl) were taken out of the tube and placed on a haemocytometer for counting. The remaining cells were harvested at 1800g for 10 min at room temperature and the pellet gently resuspended in 1ml of DMEM or McCoy's medium. The cells were thoroughly resuspended and a further 19ml of DMEM or McCoy's medium added. The cells were diluted in DMEM or McCoy's medium accordingly to give 2x10⁶ cells/ml. Cells (50µl) were then added to each well of a 96 well plate and incubated overnight at 37°C and 5% CO₂.

4. The following procedure was carried out with subdued lighting: The next day, the conjugate was diluted in PBS to give C6.5 concentrations equivalent to 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml. Cells were washed once with PBS and 50µl of the conjugate added to wells in quadruplicate. Control wells were also included (wells with conjugate added but not exposed to light, and wells with neither conjugate added nor exposed to light). It was ascertained in previous experiments that laser light alone had no affect on the cell viability, so no 'light alone' controls are included unless the light source, or energy dose of the light is changed.

5. Cells were incubated in the conjugate or free PS (concentration varies) for 30min at 37°C, 5% CO₂ and then washed 3 times with PBS. PBS (50µl)
was added to each well and quadruplicate wells exposed to laser light for 2 min (energy dose = 4.2J; energy density = 1.4J/cm²).

6. The PBS was removed from each well and 100µl of DMEM or McCoy's medium added. The plates were loosely wrapped in foil, but covered adequately so that no ambient light could enter. The plates were then incubated as above for 48 hours, after which time a cell kill assay was carried out.

7. Cell kills assays were carried out using the Cytotox-96 kits (according to the Promega protocol). Cells were washed 3 times with PBS and 50µl of cell lysis solution added. Plates were incubated for 60 minutes at 37°C in the dark. After this time, 50µl of substrate solution was added (which indicates the amount of lactate dehydrogenase in cells). This was incubated at room temperature for 30min, after which time, 50µl of stop solution (0.5M acetic acid) was added. The cell suspensions were removed from the wells and placed in a fresh microtitre plate. The absorbance was then measured at 490nm in a microtitre plate reader.

5 Cell kills were determined and expressed as a percentage of controls (Fig. 14).

20 Example 14 - In vitro cytotoxicity of a MFESCv scFv-PPa/BA conjugate

1. *In vitro* cell cytotoxicity was be measured as followed: The target cells (in this example, LoVo, LS17T or Skov3) were maintained at 37°C, 5% CO₂ in media (DMEM) supplemented with 10% foetal calf serum and 5mM penicillin/streptomycin in a 75cm² flask. For Skov3 cells, the medium used was McCoy's 5A medium supplemented with 15% FBS, 5 mM penicillin/streptomycin.

2. When 70-80% confluent, the cells were washed in PBS and 5 ml trypsin added. The flask was incubated at 37°C, 5% CO₂ for 15 mins or until the cells detached from the flask. The cells were then placed in a 50ml Falcon tube and the trypsin deactivated by adding 15ml DMEM or McCoy's medium.
3. Cells (20µl) were taken out of the tube and placed on a haemocytometer for counting. The remaining cells were harvested at 1800g for 10 min at room temperature and the pellet gently resuspended in 1ml of DMEM or McCoy's medium. The cells were thoroughly resuspended and a further 19ml of DMEM or McCoy's added. The cells were diluted in DMEM or McCoy's medium accordingly to give 2x10^6 cells/ml. Cells (50µl) are then added to each well of a 96 well plate and incubated overnight at 37°C and 5% CO₂.

4. The following procedure is carried out in subdued lighting: The next day, the conjugate was diluted in PBS to give MFE concentrations equivalent to 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78µg/ml. Cells were washed once with PBS and 50µl of the conjugate added to wells in quadruplicate. Control wells were also included (wells with conjugate added but not exposed to light, and wells with neither conjugate added nor exposed to light). It was ascertained in previous experiments that laser light alone has no affect on the cell viability, so no 'light alone' controls are included unless the light source, or energy dose of the light is changed.

5. Cells were incubated in the conjugate or free PS (concentration varies) for 30min at 37°C, 5% CO₂ and then washed 3 times with PBS. PBS (50µl) was added to each well and quadruplicate wells were exposed to laser light for 2 min (energy dose = 4.2J; energy density = 1.4J/cm²).

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7. Cell kills assays were carried out using the Cytotox-96 kits (according to the Promega protocol). Cells were washed 3 times with PBS and 50µl of cell lysis solution added. Plates were incubated for 60min at 37°C in the dark. After this time, 50µl of substrate solution was added (which indicates the amount of lactate dehydrogenase in cells). This was incubated at room temperature for 30min, after which time, 50µl of stop solution (0.5M acetic acid) was added. The cell suspensions were removed from the wells
and placed in a fresh microtitre plate. The absorbance was then measured at 490nm in a microtitre plate reader.

8. Cell kills were determined and expressed as a percentage of controls (Fig 15).

Example 15 - *In vivo targeting of a scFv-PPa conjugate*

1. *In vivo* tumour eradication can be demonstrated as follows: Approx 1 x 10^7 SKOV-3 cells was injected s.c. into the flank of a nude BALB/C mouse and tumours are allowed to establish for 4-6 weeks.

2. Ten-50 µg of 125-Iodine radiolabeled (using Iodogen method, Pierce Chemical Co.) scFv-PPa was injected i.v. into the tail vein of tumour-bearing mice and allowed to accumulate in the tumour over a period of 1-48 hrs.

3. Groups of three or more mice from each time point analysed were culled under terminal anaesthesia, dissected and the tumour, blood and various organs were analysed for uptake of the scFv-PPa. Control experiments with PPa alone and scFv are carried out.

4. As an example, the tumour targeting of c6.5-PPa is shown compared to the scFv and PPa alone in Fig. 16. The blood circulation time of the hydrophobic photosensitiser was seen to decrease after attaching to a hydrophilic scFv (Fig. 17).

Example 16 - *In vivophotodynamic therapy of a scFv-PPa conjugate*

1. *In vivo* tumour eradication can be demonstrated as follows: Approx 1 x 10^7 SKOV-3 cells are injected s.c. into the flank of a nude BALB/C mouse and tumours are allowed to establish for 4-6 weeks.

2. Fifty-200 µg of scFv-PPa is injected i.v. into the tail vein of tumour-bearing mice and allowed to accumulate in the tumour over a period of 12-24 hrs.

3. At a time when the tumour: normal organ ratio is high (5: 1 or better e.g. 16 hr), light is irradiated onto the tumours at 2.4 W per cm^2.
4. The size of the tumours is measured using callipers and compared to mice treated with saline only. The tumours were observed for PDT-induced necrosis (Fig 18).

5. **Example 17 - Engineering a scFv (e.g. HuBC-I) to have optimised functional groups for photosensitizer coupling**

1. A scFv which has shown in practice to display very poor photophysical properties (such as fluorescence, singlet oxygen generation and in vitro photo-cytotoxicity) is analysed at the primary structure and tertiary structure level. This can be done by amino acid alignment to a scFv which is known to be a good one for coupling to photosensitizers or examination of the three-dimensional structure.

2. Residues which are going to be used to couple with activated photosensitizers are identified, for example lysine residues.

3. Ones which are adjacent to each other, either in the primary sequence or topologically from a three-dimensional model (or actual structure) are manipulated by site directed mutagenesis. The alteration can be the introduction of a optimally spaced lysine residue, the removal of a lysine which is too close to another or the replacement of an unwanted lysine with another similar but non-conjugatable residue (such as arginine or glutamine).

4. In this example, the anti-fibronectin scFv HuBC-I was aligned to c6.5 and lysine positions identified. Six changes were identified (Fig. 10) which converted the lysine positioning to look more like that found in c6.5 (Fig. 19).

5. Each possible change (6 in all in this example) identified is made in the antibody fragment as a single mutation in the antibody gene. Mutagenesis was done using the Stratagene Quick Change™ system.

6. Each mutant antibody from (4) is tested to see whether any of the antibody properties have been altered or destroyed upon mutagenesis. Expression of the antibody protein in the host cell (e.g. E.coli), purification, antigen binding (by ELISA and BIACore surface plasmon resonance), cell binding
(by ELISA, FACS and immunomicroscopy), stability assays (temperature, urea-induced unfolding and serum stability) are all carried out.

7. Mutations which do not significantly alter the stability and function of the antibody are retained, ones which are detrimental are discarded.

8. All the mutations are combined into one antibody gene, forming a protein which has newly positioned lysine residues for optimised photosensitizer coupling.

9. This antibody is used as in Examples 1-11 to make a antibody-photosensitizer conjugate.

10. Example 18 - Antimicrobial targeting with a scFv-photosensitizer conjugate

1. A well-characterised anti-microbial antibody is cloned, expressed and purified using the same techniques as described in Example 1 (above).

2. Photosensitisers are attached as described in Examples 2-5 (above).

3. Anti-bacterial cell killing. Initially a quick method to screen a number of photosensitiser conjugates against a number of bacterial species was carried out. An overnight culture of the bacteria was harvested by centrifugation and resuspended in PBS. The bacterial culture (1mL) was spread onto an agar plate and allowed to dry for 30 minutes.

4. After this time, 5 ul of the photosensitizer was placed onto the spread bacteria and exposed to light from a laser diode (35mW, 675nm) for 2 minutes (energy density = 1.4 J/cm²). The plates were incubated overnight at 37 °C.

5. The next day, a lawn of bacteria should have grown on the plate except for where the photosensitiser conjugate and light was applied. If bacterial growth was found to occur here, the corresponding photosensitiser conjugate was not investigated further. Those photosensitiser conjugate/bacterial combinations that were found to be successful were further analysed as follows (modified method from [93, 94]):

6. An overnight culture of bacteria was harvested and resuspended in PBS. An aliquot (100ul) of the bacteria was taken and added to wells of a 24-well plate. Then, 100ul of serially diluted photosensitiser conjugate was
added to each well in triplicate. The suspensions were stirred for a specific length of time (usually between 1 and 30 minutes) after which time the bacteria were harvested by centrifugation and washed 4 times with PBS or 0.15M NaCl. Bacterial pellets were resuspended in PBS or 0.15M NaCl and placed into a 24-well plate. Wells were then exposed to light from a laser diode (energy density = 1.4 J/cm²). The entire suspension was removed from each well and serially diluted in 2TY broth. An aliquot (25ul) was removed from each dilution and placed on one half of an agar plate. The suspension was then spread across one half of the agar plate and the plates incubated overnight at 37°C.

7. The following day, the number of colonies present on the plates was counted (i.e. plates that had between 20-200 colonies). Bacterial cell survival was then calculated by comparing to colonies from suspensions that had no photosensitiser or light treatment.

Example 19 - Cellular imaging of SKOV3 cells with C6.5 scFv-PPa

1. Round coverslips were washed in ethanol and rinsed in PBS. Coverslips were then placed in 12-well tissue culture plates.

2. SKOV3 cells were trypsinised and washed with PBS. The cell pellet was resuspended in McCoy's media and cells were seeded onto the coverslips at 2x10⁵ cells/ml. The cells were incubated overnight at 37°C and 5% CO₂.

3. The coverslips with the adherent cells were rinsed carefully in PBS and either C6-PPa or PBS was added to the wells. The cells were incubated at 37°C and 5% CO₂ for 30 minutes after which time they were washed carefully with PBS.

4. The cells were fixed onto the coverslips by incubating in 2 ml 4% paraformaldehyde for 60 minutes at room temperature. After this time, the coverslips were washed with PBS and inverted cell-side down onto glass slides. The edges of the coverslips were then sealed using nail varnish.
5. Fluorescence imaging was then performed using an Ar+ laser (418nm) as excitation source, using a Leica laser scanning confocal microscope, images are shown in figure 20 and 24, and the emission spectrum is shown in figure 21 and 24.

Figs 24a & 24e respectively show the HER-2-negative and HER-2-positive cell lines incubated with the same amount of free PPa with corresponding respective emission spectra in Figs 24b & 24f. The images and emission spectra show that the KB cells take up the PPa just over 2-fold better than the SKOV3 cells. Figs 24c and 24g shows the HER2-negative and -positive cell lines incubated with C6-PPa (equivalent amount of PPa to Fig 24a, b, e, f) and the corresponding emission spectra are shown in Figs 24d & 24h.

The C6.5 scFv clearly has influenced the targeting of the PPa with very weak fluorescence, not associated with the emission wavelength of PPa, being observed in the KB cells. However strong PPa-based fluorescence is seen in the SKOV3 cell line. The transmission overlays (Fig. 24i & 24j) show more clearly that the PPa is spread throughout the cell with punctate, endosomal-like staining.

Example 20 - Fl, GP6 and D1.3 antibody conjugates compared to C6.5

Fl and GP6 (anti-human placental alkaline phosphatase) and D1.3 (31) were expressed in pHEN2. The expression and purification of all scFvs was the same as described above for C6.5.

Coupling of pyropheophorhide-a photosensitizer to scFvs.

Pyropheophorbide-a succinimidyl ester was synthesised for coupling to the scFv as follows. To a light protected solution of the pyropheophorbide-a (50 mg, 0.094 mmol) in a mixture of dry DCM/THF (9:1) N-hydroxysuccinimide (12.9 mg, 0.11 mmol) was added followed by dicyclohexylcarbodiimide (DCC) (23.2 mg, 0.11 mmol).
After stirring for 12 h (at room temperature), the precipitated dicyclohexylurea was filtered off and the solvents removed. The crude product was taken up in a small volume of chloroform and precipitated by the addition of hexane. The precipitate was collected, washed well with hexane and the resulting crude product purified by column chromatography on silica gel eluting with 2% hexane in ethyl acetate (Rf 0.66). The isolated product was re-crystallised from DCM/hexane to give pure succinimidyl ester in 70% yield.

The pyropheophorbide-a succinimidyl ester was resuspended in 100% DMSO and added at a concentration of 52.8 mM to 3.3 mM MFE-23, C6.5 or HuBCl in PBS containing 6% acetonitrile and with continuous stirring at 4°C for 30 min. The photoimmunoconjugates (PICs) were then dialysed against PBS with one buffer change. For comparison of C6.5, Fl, GP6 and Dl.3, the concentrations of all the scFvs were adjusted so as to be the same as GP6 which gave the poorest expression of all the scFvs. The scFvs were coupled to PPa at a concentration of 0.3 mM. There was no precipitation of the protein before coupling and the scFv-PPa conjugate remained soluble at concentrations of 0.5 mg/ml or below.

SDS-PAGE was carried out as described for C6.5 above and stained with coomassie blue. Non-stained gels were transferred using a semi-dry blotting apparatus (Biorad) onto nitrocellulose and gently dried.

Fluorescence was visualised by exciting the PPa on the blot on a short wavelength UV-transilluminator. As an example of a calculation to determine the Ppa: scFv ratio, the absorbance of 65 mg/ml PPa give 1 AU at 670 nm. Thus 0.2 AU is equal to 13 mg/ml PPa which is equal to 2.4 x 10^-5 M PPa (MW=535). This was found coupled to a scFv at a concentration of 50 mg/ml (see Fig.1B), which is equal to 1.7 x 10^-6 M (MW=30,000). The ratio works out to be 14.1:1, which becomes 9.9:1 after correcting for 30% non-covalent binding.
Results

The absorbance profile of free PPa in 100% DMSO and PBS/1.9% DMSO is shown in Fig 22A. Both show the characteristic peaks around 400nm (Soret peak), minor peaks between 500-630nm and the Q-band at 670nm. Fig. 22B shows the profile for the C6.5 scFv coupled to PPa. The peaks remain sharp and similar to that of free PPa. The absorbance at 670nm was used to determine the concentration of PPa and used to calculate the PPa:scFv ratio which was 11.92 ± 1 (mean of 5 independent coupling reactions. This gives an effective ratio approximately 8:1 after correction for a small amount (30%) of non-covalent binding. The profile of PPa when attached to the MFE scFv is shown in Fig. 22C.

Four other scFvs were coupled to PPa in order to understand those factors important in obtaining good coupling ratios (Fig. 22D). D1.3 scFv-PPa gives close to the 'ideal' absorbance pattern exemplified by the C6.5 scFv, F1 scFv-PPa is slightly less effective.

However, GP6 scFv-PPa and HuBC-I scFv-PPa show poor profiles with broadened peaks, indicating possible aggregation. The ratio of PPa:scFv for all scFv coupling experiments are shown in Table 6.

Table 6 PPa: scFv coupling ratios
Effective PPa: scFv coupling ratios determined by comparison to a PPa standard curve and correcting for 30% non-covalent binding.

<table>
<thead>
<tr>
<th>scFv</th>
<th>PPa:scFv ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6.5</td>
<td>8.3</td>
</tr>
<tr>
<td>MFE-23 (fresh)</td>
<td>6.0</td>
</tr>
<tr>
<td>MFE-23 (frozen)</td>
<td>3.0</td>
</tr>
<tr>
<td>D1.3</td>
<td>6.1</td>
</tr>
<tr>
<td>F1</td>
<td>5.1</td>
</tr>
<tr>
<td>GP6</td>
<td>3.1</td>
</tr>
<tr>
<td>HuBC-I</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Sequence alignment (Fig. 26) of the scFvs used in this study revealed that C6.5, which gives reproducible coupling and good singlet oxygen yields has more lysines which are predicted to be spatially separated compared with scFvs which make poorer PICs (HuBC-I, GP6 and Fl).

*Example 21 - Preparation of Verteporfin (Visudyne™) succinimidyl ester (Fig. 27)*

Verteporfin was obtained as described in Scherrer *et al.* (1986) *J. Org. Chem.* 51: 1094-1100.

1. The Verteporfin succinimidyl ester (Figure 27; Compound 'b') was prepared as described for PPa. To a light-protected solution of verteporfin (6mg) in dry THF (5ml), N-hydroxysuccinimde (3mg) was added followed by dicyclohexylcarbodiimide (DCC, 6mg).
2. The reaction mixture was stirred for 12h at room temperature under nitrogen at which point all starting material had been consumed.
3. The solvent was evaporated and the crude product purified by column chromatography on silica gel, loaded as a solution in DCM and eluting with ethyl acetate (Rf 0.74) to give pure verteporfin succinimidyl ester in 75% yield. MS (FAB+) 832 (M+).
4. A stock solution of C6.5 scFv at 500µg/ml was defrosted at room temperature and 200µl added to 706µl of PBS. Acetonitrile (60µl) was added to the solution. The solution was stirred on ice until cool.

*Example 22 - Conjugation of c6.5 scFv to Verteporfin (Visudyne™) — solvent system 1*

1. Verteporfin (Visudyne™) succinimidyl ester made up in 100% DMSO was then added (34µl) from a stock solution of 1.58mM to the C6.5 scFv with continuous stirring (to give 16 equivalents of Verteporfin (Visudyne™)). The mixture was kept on ice and in the dark, with stirring
for 30 mins, after which time the solution was placed in dialysis tubing and
dialysed against 5 L of PBS at 4°C overnight in the dark.

2. Each sample of the conjugate was placed in a quartz cuvette and an
absorbance profile was run against a blank containing PBS. The
absorbance value at 410 nm was measured and the concentration of PS in
g/ml was determined by comparing to a standard curve of Verteporfin
(Visudyne™).

Example 23 - Conjugation of MFE-23 (anti-CEA) scFv to Verteporfin
(Visudyne™) - solvent system 2

1. A stock solution of MFE-23 at 500 µg/ml was defrosted at room
temperature and 200 µl added to 706 µl of PBS. Acetonitrile (60 µl) was
added to the solution. The solution was stirred on ice until cool.

2. Verteporfin (Visudyne™) succinimidyl ester was then added (34 µl) from a
stock solution of DMSO 1.58 mM to the MFE-23 with continuous stirring
(to give 16 equivalents of Verteporfin (Visudyne™)). The mixture was
kept on ice and in the dark, with stirring for 30 mins, after which time the
solution was placed in dialysis tubing and dialysed against 5 L of PBS at
4°C overnight in the dark.

3. Each sample of the conjugate was placed in a quartz cuvette and an
absorbance profile run against a blank containing PBS. The absorbance
value at 410 nm was measured and the concentration of PS in g/ml determined by comparing to a standard curve of Verteporfin (Visudyne™).
The ratio therefore of Verteporfin (Visudyne™): MFE-23 was between 8:1
and 10:1.

Example 24 - Photophysical characterisation of a scFV-Verteporfin
(Visudyne™) conjugate (Fig. 28)

1. Serial dilutions (halving concentrations) of the conjugates were made in
PBS and the absorbance was measured at an excitation wavelength of
690 nm and an emission wavelength of 680 nm.
2. These were compared to free Verteporfin (Visudyne™) in PBS (Figure 28).

Example 25 - *In vitro* cytotoxicity of a c6.5 scFv-Verteporfin (Visudyne™) conjugate (Fig. 29)

1. *In vitro* cell cytotoxicity was be measured as followed: The target cells (in this example, SKOV3 cells were used as antigen-positive cells and KB cells were used as antigen-negative cells) were maintained at 37°C, 5% CO₂ in media (DMEM) supplemented with 10% foetal calf serum and 5 mM penicillin/streptomycin in a 75cm² flask. For SKOV3 cells, the medium used was McCoy's 5A medium supplemented with 15 % FBS, 5 mM penicillin/streptomycin.

2. When 70-80% confluent, the cells were washed in PBS and 5 ml trypsin added. The flask was incubated at 37°C, 5% CO₂ for 15 mins or until the cells had detached from the flask. The cells were then placed in a 50ml Falcon tube and the trypsin deactivated by adding 15ml DMEM or McCoy's medium.

3. Cells (20µl) were taken out of the tube and placed on a haemocytometer for counting. The remaining cells were harvested at 1800g for 10 min at room temperature and the pellet gently resuspended in Iml of DMEM or McCoy's medium. The cells were thoroughly resuspended and a further 19ml of DMEM or McCoy's medium added. The cells were diluted in DMEM or McCoy's medium accordingly to give 2x10⁶ cells/ml. Cells (50µl) were then added to each well of a 96 well plate and incubated overnight at 37°C and 5% CO₂.

4. The following procedure was carried out in subdued lighting: The next day, the conjugate was diluted in PBS to give C6.5 concentrations equivalent to 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml. Cells were washed once with PBS and 50µl of the conjugate added to wells in quadruplicate. Control wells were also included (wells with conjugate added but not exposed to light, and wells with neither conjugate added nor exposed to light). It was ascertained in previous experiments that laser
light alone had no affect on the cell viability, so no 'light alone' controls are included unless the light source, or energy dose of the light is changed.

6. Cells were incubated in the conjugate or free PS (concentration varies) for 30 min at 37°C, 5% CO₂ and then washed 3 times with PBS. PBS (50µl) was added to each well and quadruplicate wells exposed to laser light for 2 min (energy dose = 4.2J; energy density = 1.4J/cm²).

7. The PBS was removed from each well and 100µl of DMEM or McCoy's medium added. The plates were loosely wrapped in foil, but covered adequately so that no ambient light could enter. The plates were then incubated as above for 48 hours, after which time a cell kill assay was carried out.

8. Cell kills assays were carried out using the Cytotox-96 kits (according to the Promega protocol). Cells were washed 3 times with PBS and 50µl of cell lysis solution added. Plates were incubated for 60 minutes at 37°C in the dark. After this time, 50µl of substrate solution was added (which indicates the amount of lactate dehydrogenase in cells). This was incubated at room temperature for 30 min, after which time, 50µl of stop solution (0.5M acetic acid) was added. The cell suspensions were removed from the wells and placed in a fresh microtitre plate. The absorbance was then measured at 690nm in a microtitre plate reader.

9. Cell kills were determined and expressed as a percentage of controls (Fig. 29).

Results: The IC50s are as follows:

C6.5-Verteporfm(Visudyne™) conjugate on SKOV3 cells = 2.2 µM;
C6.5-Verteporfm(Visudyne™) conjugate on KB cells = 28.1 µM;
Verteporfm(Visudyne™) on SKOV3 cells = 15.3 µM;
Verteporfm(Visudyne™) on KB cells = 10 µM.

Thus, when targeted using the C6.5 scFv, Verteporfm(Visudyne™) is 7-fold more potent and 13-fold more specific.
Example 26 - Pharmaceutical formulations and administration.

A further aspect of the invention provides a pharmaceutical formulation comprising a compound according to the first aspect of the invention in admixture with a pharmaceutically or veterinarily acceptable adjuvant, diluent or carrier.

Preferably, the formulation is a unit dosage containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of the active ingredient.

The compounds of the invention will normally be administered orally or by any parenteral route, in the form of a pharmaceutical formulation comprising the active ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form. Depending upon the disorder and patient to be treated, as well as the route of administration, the compositions may be administered at varying doses.

In human therapy, the compounds of the invention can be administered alone but will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the compounds of the invention can be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications. The compounds of invention may also be administered via intracavernosal injection.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), sucrose, gelatin and acacia.
Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The compounds of the invention can also be administered parenterally, for example, intravenously, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.
For oral and parenteral administration to human patients, the daily dosage level of the compounds of the invention will usually be from 1 mg/kg to 30 mg/kg. Thus, for example, the tablets or capsules of the compound of the invention may contain a dose of active compound for administration singly or two or more at a time, as appropriate. The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention.

The compounds of the invention can also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoro-ethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A3 or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA3), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active compound, e.g. using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

Aerosol or dry powder formulations are preferably arranged so that each metered dose or "puff" delivers an appropriate dose of a compound of the invention for delivery to the patient. It will be appreciated that the overall daily dose with an aerosol will vary from patient to patient, and may be administered in a single dose or, more usually, in divided doses throughout the day.

Alternatively, the compounds of the invention can be administered in the form of a suppository or pessary, or they may be applied topically in the form of a lotion,
solution, cream, ointment or dusting powder. The compounds of the invention may also be transdermally administered, for example, by the use of a skin patch. They may also be administered by the ocular route, particularly for treating diseases of the eye.

For ophthalmic use, the compounds of the invention can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

For application topically to the skin, the compounds of the invention can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Generally, in humans, oral or topical administration of the compounds of the invention is the preferred route, being the most convenient. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug absorption after oral administration, the drug may be administered parenterally, e.g. sublingually or buccally.
For veterinary use, a compound of the invention is administered as a suitably acceptable formulation in accordance with normal veterinary practice and the veterinary surgeon will determine the dosing regimen and route of administration which will be most appropriate for a particular animal.
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Boehm MK, Corper AL, Wan T, Sohi MK, Sutton BJ, Thornton JD, Keep


CLAIMS

1. A process of making a compound comprising a photosensitising agent coupled to a carrier molecule comprising the steps of:

   (i) providing a photosensitising agent;
   (ii) providing a carrier molecule;
   (iii) conjugating the photosensitizing agent and the carrier molecule in the presence of a first and a second polar aprotic solvent and an aqueous buffer.

2. The process according to Claim 1 wherein the compound comprises a ratio of photosensitising agent to carrier molecule of at least 3:1.

3. The process according to Claim 1 or 2 wherein the functional and physical properties of the photosensitising agent and the carrier molecule are substantially unaltered after coupling.

4. The process according to any one of Claims 1 to 3 wherein the first and second polar aprotic solvent are selected from the group consisting of: dimethyl sulfoxide (DMSO); acetonitrile; N,N-dimethylformamide (DMF); HMPA; dioxane; tetrahydrofuran (THF); carbon disulfide; glyme and diglyme; 2-butanone (MEK); sulfolane; nitromethane; N-methylpyrrolidone; pyridine; and acetone.

5. The process according to Claim 4 wherein the first and second aprotic solvent are selected from the group consisting of: DMSO; DMF; and acetonitrile.

6. The process according to Claim 5 wherein the first and second aprotic solvent are DMF and acetonitrile.

7. The process according to any one of Claims 1 to 6 wherein the ratio of
aqueous buffer to first aprotic solvent to second aprotic solvent is approximately 50% : 1 to 49% : 49 to 1%.

8. The process according to Claim 7 wherein the ratio is 92% PBS : 2% DMSO : 6% acetonitrile.

9. The process according to any one of Claims 1 to 8 wherein the step of conjugating the photosensitizing agent and the carrier molecule is conducted at a temperature between 0°C and 5°C.

10. The process according to any one of Claims 1 to 9 wherein the step of conjugating the photosensitizing agent and the carrier molecule is conducted for approximately 30 minutes.

11. The process according to any one of Claims 1 to 10 wherein the carrier molecule is an antibody fragment and/or a derivative thereof.

12. The process according to Claim 11 wherein the antibody fragment and/or derivative is a single-chain antibody.

13. The process according to Claim 12 wherein the single-chain antibody is an ScFv.

14. The process according to any one of Claims 1 to 13 wherein the carrier molecule is humanised or human.

15. The process according to any one of Claims 1 to 14 wherein the photosensitising agent is a monofunctional photosensitiser.

16. The process according to Claim 15 wherein the photosensitising agent is at least one selected from the group consisting of: haematoporphyrins; naturally-occurring porphyrins; naturally-occurring chlorines; naturally-occurring bacteriochlorins;
pheophorbides; pyropheophorbide a and derivatives thereof; photochlor; chlorines; chlorin e6; mono-1-aspartyl derivative of chlorin e6; di-1-aspartyl derivative of chlorin e6; tin (IV) chlorin e6; palladium derivatives of naturally-occurring bacteriochlorophylls; palladium derivatives of palladium-bacteriopheophorbide; synthetic chlorines; synthetic bacteriochlorins; meta-tetrahydroxyphenyl chlorin and bacteriochlorin; benzoporphyrin derivatives; monobenzoporphyrin derivatives; verteporfin; phthalocyanines; sulphonated aluminium phthalocyanines (disulphonated and tetrasulphonated); sulphonated aluminium naphthalocyanines and derivatives thereof; purpurins; purpurin-18; tin and zinc derivatives of octaethylpurpurin; tin etiopurpurin; verdins; porphycenes; synthetic porphyrins; synthetic chlorines; synthetic bacteriochlorins; metal-free meso-triethylporphyrins; metallated meso-triethylporphyrins; core-modified porphyrins; expanded porphyrins (texaphyrins); motexafin lutetium; motexafin gadolinium; non-porphyrinic compounds; phenothiazinium derivatives; methylene blue, toluidine blue; cyanines; merocyanine-540; acridine dyes; BODIPY dyes; aza-BODIPY derivatives; hypericin; halogenated squarine dyes; halogenated xanthene dyes; eosin; rose Bengal.

17. The process according to any one of Claims 1 to 16 further comprising the following step performed after step (iii):

(iv) coupling a modulating agent to the carrier molecule, wherein the modulating agent is capable of modulating the function of the photosensitising agent.

18. The process according to Claim 17 wherein the modulating agent is selected from the group consisting of: benzoic acid; benzoic acid containing an azide group; 4-azidotetrafluorophenylbenzoic acid; benzoic acid containing an aromatic group having an azide moiety; benzoic acid containing a heteroaromatic group having an azide
moiety; vitamin E analogues; Trolox; butyl hydroxyl toluene; propyl gallate; deoxycholic acid; ursodeoxycholic acid.

19. The process according to Claim 18 wherein the aromatic group or heteroaromatic group is selected from the group consisting of: polyfluorobenzenes, naphthalenes, napthaquinones, anthracenes, anthraquinones, phenanthrenes, tetracenes, naphthacenediones, pyridines, quinolines, isoquinolines, indoles, isoindoles, pyroles, imidazoles, pyrazoles, pyrazines, benzimidazoles, benzofurans, dibenzofurans, carbazoles, acridiens acridones, and phenanthridines, xanthines, xanthones, flavones, coumarins, and sulfenates thereof.

20. The process according to any one of Claims 1 to 19 further comprising the following step performed after step (iii) or (iv):

(v) combining the compound with a pharmaceutically-acceptable carrier to form a pharmaceutical formulation.

21. The process according to any one of Claims 1 to 20 wherein the distance between photosensitizing agents coupled to the carrier molecule is between 3.5 angstroms and 25nm.

22. The process according to claim 21 wherein the distance between photosensitizing agents coupled to the carrier molecule is between 20 and 25 nm.

23. The process according to any one of Claims 1 to 22 further comprising the following step performed before step (v) of

(vi) coupling a visualising agent to the carrier molecule, photosensitising agent or conjugate thereof.
24. The process according to claim 23 wherein the visualising agent is a fluorescent or luminescent dye.

25. A compound comprising a photosensitising agent coupled to a carrier molecule obtainable by the process of any one of Claims 1 to 24.

26. A compound comprising a photosensitising agent coupled to a carrier molecule with a minimum coupling ratio of 3:1 wherein the carrier molecule binds selectively a target cell.

27. The compound according to Claim 25 or 26 wherein the functional and physical properties of the photosensitising agent and the carrier molecule are substantially unaltered in the coupled form in comparison to the properties when in an uncoupled form.

28. The compound according to any one of Claims 25 to 27 wherein the carrier molecule is selected from the group consisting of: an antibody fragment and/or a derivative thereof, or a non-immunogenic peptide ligand.

29. The compound according to Claim 28 wherein the antibody fragment and/or derivative thereof is a single-chain antibody fragment.

30. The compound according to Claim 29 wherein the single-chain antibody is an ScFv.

31. The compound according to any one of Claims 25 to 30 wherein the carrier molecule is humanised or human.

32. The compound according to any one of Claims 25 to 31 wherein the photosensitising agent is a monofunctional photosensitiser.
33. The compound according to Claim 32 wherein the photosensitising agent is at least one selected from the group consisting of: haematoporphyrins; naturally-occurring porphyrins; naturally-occurring chlorines; naturally-occurring bacteriochlorins; pheophorbides; pyropheophorbide a and derivatives thereof; photochlor; chlorines; chlorin e6; mono-1-aspartyl derivative of chlorin e6; di-1-aspartyl derivative of chlorin e6; tin (IV) chlorin e6; palladium derivatives of naturally-occurring bacteriochlorophylls; palladium derivatives of palladium-bacteriochlorophorbid; synthetic chlorines; synthetic bacteriochlorins; meta-tetrahydroxyphenyl chlorin and bacteriochlorin; benzoporphyrin derivatives; monobenzoporphyrin derivatives; verteporfim; phthalocyanines; sulphonated aluminium phthalocyanines (disulphonated and tetraslphonated); sulphonated aluminium naphthalocyanines and derivatives thereof; purpurins; purpurin-18; tin and zinc derivatives of octaethylpurpurin; tin etiopurpurin; verdins; porphycenes; synthetic porphyrins; synthetic chlorines; synthetic bacteriochlorins; metal-free meso-triethylporphyrins; metallated meso-triethylporphyrins; core-modified porphyrins; expanded porphyrins (texaphyrins); motexafin lutetium; motexafin gadolinium; non-porphyrinic compounds; phenothiazinium derivatives; methylene blue, toluidine blue; cyanines; merocyanine-540; acridine dyes; BODIPY dyes; aza-BODIPY derivatives; hypericin; halogenated squarine dyes; halogenated xanthene dyes; eosin; rose Bengal.

34. The compound according to any one of Claims 25 to 33 wherein the photosensitising agent is coupled to the carrier molecule at an amino acid residue or a sugar molecule on the carrier molecule.

35. The compound according to Claim 34 wherein the amino acid residue is selected from the group consisting of: lysine; cysteine; tyrosine; serine; glutamate; aspartate; and arginine.
36. The compound according to Claim 35 wherein the sugar molecule is selected from the group consisting of: sugars comprising an hydroxyl group; sugars comprising an aldehyde group; sugars comprising an amino group; and sugars comprising a carboxylic acid group.

37. The compound according to any one of Claims 25 to 36 wherein the distance between photosensitizing agents coupled to the carrier molecule is between 3.5 angstroms and 25nm.

38. The compound according to claim 37 wherein the distance between photosensitizing agents coupled to the carrier molecule is between 20 and 25 nm.

39. The compound according to any one of Claims 25 to 38 further comprising a modulating agent capable of modulating the function of the photosensitising agent.

40. The compound according to Claim 39 wherein the modulating agent is selected from the group consisting of: benzoic acid; benzoic acid containing an azide group; 4-azidotetrafluorophenylbenzoic acid; benzoic acid containing an aromatic group having an azide moiety; benzoic acid containing a heteroaromatic group having an azide moiety; vitamin E analogues; Trolox; butyl hydroxyl toluene; propyl gallate; deoxycholic acid; ursodeoxycholic acid.

41. The compound according to Claim 40 wherein the aromatic group or heteroaromatic group is selected from the group consisting of: polyfluorobenzenes, naphthalines, napthaquinones, anthracenes, anthraquinones, phenanthrenes, tetracenes, naphthacenediones, pyridines, quinolines, isoquinolines, indoles, isoindoles, pyrroles, imidazoles, pyrazoles, pyrazines, benzimidazoles, benzofurans, dibenzofurans, carbazoles, acridiens acridones, and phenanthridines, xanthines, xanthones, flavones, coumarins, and sulfenates thereof.
42. The compound according to any one of Claims 25 to 41 further comprises a visualising agent.

43. The compound according to claim 42 wherein the visualising agent is a fluorescent or luminescent dye.

44. The compound according to any one of Claims 25 to 43 wherein the carrier molecule is an ScFv and the photosensitising agent is Pyropheophorbide a.

45. The compound according to any one of Claims 25 to 43 wherein the carrier molecule is an ScFv and the photosensitising agent is benzoporphyrin derivative mono acid (Verteporfin)

46. The compound according to any one of Claims 25 to 43 wherein the carrier molecule is an ScFv and the photosensitising agent is palladium-bacteriopheophorbide.

47. The compound according to any one of Claims 25 to 43 wherein the carrier molecule is an ScFv and the photosensitising agent is mono-1-aspartyl derivative of chlorin e6.

48. The compound according to any one of Claims 25 to 43 wherein the carrier molecule is an ScFv and the photosensitising agent is meta-tetrahydroxyphenyl chlorine.

49. The compound according to any one of Claims 25 to 43 wherein the carrier molecule is an ScFv and the photosensitising agent is tin etiopurpurin (rostaporfin).

50. Use of the compound as defined in any one of Claims 25 to 49 in the diagnosis and/or treatment and/or prevention of a disease requiring the
destruction of a target cell.

51. Use of the compound as defined in any one of Claims 25 to 49 in the manufacture of a medicament for the treatment and/or prevention of a disease requiring the destruction of a target cell.

52. The use according to Claim 50 or 51 wherein the disease to be treated is selected from the group consisting of: cancer; age-related macular degeneration; immune disorders; cardiovascular disease; and microbial infections including viral, bacterial or fungal infections, prion diseases such as BSE, and oral/dental diseases such as gingivitis.

53. The use according to Claim 52 wherein the disease to be treated is cancer of the colon, lung, breast, Head and neck, prostate, skin, stomach/gastrointestinal, bladder and precancerous lesions such as Barretts oesophagus.

54. The use according to any one of claims 50 to 53 wherein diagnosis of disease is conducted by visualisation of either the photosensitising agent or an optional visualisation agent.

55. The use according to any one of Claims 50 to 54 wherein the compound is administered to a patient prior to light exposure.

56. A pharmaceutical composition comprising the compound of any one of Claims 25 to 49 and a pharmaceutically-acceptable carrier, excipient or diluent.
Figure 1 (con't)
Figure 1 (con’t)
VH5 1-2 5-51 SIY---HSGSTYYNPSLKS RVTISVDTSKNQFSKLSSLSSVTAAADTAVYYCAR
1-2 5-a IIYP--GSDTTRYSPSFQG QVTISADKSIISTAYLQWSSLKASKDTAMYCAR
VH6 3-5 6-01 RIDP--SDSYTNYPSFQG HVTISADKSIISTAYLQWSSLKASKDTAMYCAR
VH7 1-2 7-4.1 RTYYR-SKWYNDYAVSVKS RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR
WINT--NTGNPTYAQGFTG RFVFSLDTSTAYLQICSLKAEDTAVYYCAR

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**Figure 1 (con’t)**
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Figure 1 (con’t)
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**Figure 1 (con’t)**
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CDR3
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**Figure 1 (cont')**
Figure 3
M = Marker
1 = flow through
2 = 10 mM imidazole
3 = 40 mM imidazole
4 = 250 mM imidazole elution (concentrated 5-fold)

Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10

Absorption profile of C6.5 coupled to C6 or PPa

Wavelength (nm)

300 350 400 450 500 550 600 650 700 750 800

0 0.2 0.4 0.6 0.8 1 1.2

C6.5-Ce6

C6.5-PPa

Figure 10
Figure 11
Figure 12
Figure 13
Figure 14
Figure 15
Figure 16
Figure 17
BC-1 EVQLVQSGADV-**KKPGASVKVSCKASGYTFNYVMHWVRQAPGQGLEWLGYINPYNDGTQ
C6.5 QVQLLQSGAEV-**KKPGESLKSCKGSGYFTSYWIAWVRQMPK**KGLEYMGILYPGDSDTK
BC-1-mut EVQLVQSGADV-**KQPGASVKVSCKASGYTFNYVMHWVRQAPGK**GLEWLGYINPYNDGTQ

BC-1 YNERFKGRVMTGTDTSISTAYMELSRLTSDDTAVYYCAR-EVYG----------NYIWGN
C6.5 YSPSFQGQVTISVDKSVSTAYIWQSSLKPSDSAAYFCAR-HDVGYCSSSNCAK**WPEYFQH
BC-1-mut YNERFKGRVMTGTDTSISTAYMELSRLKSDDTAVYYCAR-EVYG----------NYIWGN

BC-1 WCGTLVSVSSGGGSGGSGGSALEIVLTQS-PGTLSLSPGERATLSCSASSS-**ISSN
C6.5 WCGTLVTVSSGGGSGGSGGSGGSGGQSVLTQP-P-SVSAAPGQKVTS CSCGSSSNI**GN
BC-1-mut WCGTLVSVSSGGGSGGSGGSALEIVLTQS-PGTLSLSPGEKATLSCSASSS-**ISSN

BC-1 YLHWYQQKPGQAPRLIYR----TSNLASGIPDRFSGS--SGTDFTLTISRLEPEDFAV
C6.5 YVSYWQQLPGTAPKLIIYG----HTNRPAVGPDRFSGS--KS**GTSASLAISGFRSDEAD
BC-1-mut YLHWYQQKPGQAPKLIIYR----TSNLASGIPDRFSGS--KSGTDFTLTISRLEPEDFAV

BC-1 YYCQGSS--IPFTFGQGKLEIN
C6.5 YYCAAWDSLSGWVFGGSTKLTVL
BC-1-mut YYCQGSS--IPFTFGQGKLEIN

Figure 19
Figure 21
Figure 22
Figure 23
Figure 25
### Variable-Heavy domain

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### Variable-Light domain

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### Figure 26
Verteporfin-mixture of isomers

Figure 27
Figure 28
Cell Killing of C6 scFv-Verteporfin (VP) conjugates on SKOV3 (+ve) & KB (-ve cells)

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<td>10</td>
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IC-50s

- C6-VP on SKOV3 = 2.2 uM
- Free VP on SKOV3 = 15.3 uM
- C6-VP on KB cells = 28.1 uM
- Free VP on KB cells = 10 uM

Therefore C6-VP is approx 7-times more potent on targeted cells and the specificity compared to a non-targeted cell is approx 13-fold greater.

Figure 29