(54) Title: NOVEL PHOTOIMMUNOCONJUGATES FOR USE IN PHOTODYNAMIC THERAPY
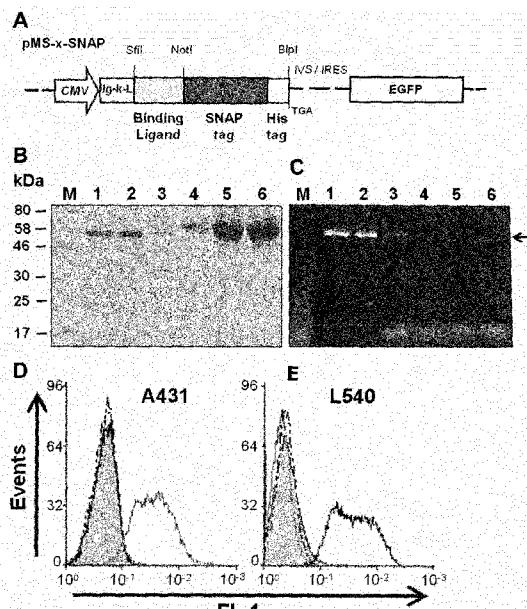
Fig. 1

(57) Abstract: A compound comprising : •a photosensitizer covalently
coupled to • a protein selected from the group consisting of antibodies or
their derivatives or fragments thereof, synthetic peptides such as scFv,
mimotopes •which bind CD antigens, cytokine receptors, interleukin recept-
ors, hormone receptors, growth factor receptors, more particularly tyrosine
kinase growth factor receptor of the ErbB family, wherein •the photosensit-
izer is coupled to the binding protein via 06-alkylguanine-DNA alkyltrans-
ferase (hAGTm), a modified human DNA repair protein.

— *with sequence listing part of description (Rule 5.2(a))*

Novel Photoimmunoconjugates for Use in Photodynamic Therapy

Field of the invention

Photodynamic therapy (PDT) is a promising and minimally invasive approach for

5   the treatment of cancer. Following the introduction of improved photosensitizers
and clinical application protocols, several FDA-approved PDT drugs have become
available and others are in various stages of preclinical and clinical develop-
ment[1]. The photosensitizing agent can exert its effect when activated by non-
hazardous light directly, by becoming cytotoxic, or indirectly, by initiating the *in*

10   *situ* production of toxic free radicals or reactive oxygen species (ROS). These
processes cause damage to cells and ultimately induce cell death by apoptosis or
necrosis[2]. The site of cellular damage depends on the photosensitizer type, the
incubation period and the mode of delivery. Hydrophobic photosensitizers tend to
damage cell membranes, whereas cationic photosensitizers accumulate within

15   membrane vesicles such as mitochondria and cause local damage[3].

One of the greatest challenges in PDT is the lack of targeting specificity. Photo-
sensitizers damage healthy tissue as well as tumor tissue after activation by
light, and this can result in prolonged skin photosensitivity[4]. To increase the
specificity of PDT, photosensitizers have been conjugated to tumor-specific mon-

20   oclonal antibodies or single chain antibody fragments (scFv), resulting in so-
called photoimmunoconjugates that deliver the photosensitizer directly to the
tumor tissue. This approach is known as photoimmunotherapy (PIT)[5]. Standard
coupling reactions are unsuitable for the conjugation of photosensitizers and an-
tibodies because there is no reliable way to ensure that antibody-photosensitizer

25   conjugates are produced in the optimal stoichiometric ratio[6]. Furthermore, the
chemical properties of the photosensitizer (e.g. hydrophobicity and the number
and arrangement of charged groups) can alter the pharmacokinetic properties
and biodistribution of the antibody, finally causing non-specific binding and inter-
nalization behavior. Random conjugation can also induce the self-quenching of

30   photosensitizer-excited states, thus reducing photodynamic activity[5]. More con-
trolled conjugation reactions are therefore required to overcome these limita-
tions.

One of the main drawbacks of PDT is the non-selective effect of activated photo-sensitizers, which tend to damage healthy as well as tumor cells. Targeted therapy using antibodies has revolutionized cancer treatment and several antibodies that bind to tumor cell antigens have achieved blockbuster status. The efficacy of

5 therapeutic antibodies can be improved by their covalent conjugation to additional effector molecules (e.g. radio nuclides, drugs or toxins)[7], as this achieves selective delivery and should reduce the systemic toxicity traditionally associated with small molecule drugs[8]. The same principle can be applied to photosensitizers. Effector molecules are generally conjugated to antibodies using either the

10 reduced sulfhydryl groups of cysteine residues or amino groups in lysine side chains. However, both methods yield heterogeneous products, comprising a mixture of conjugated antibodies with the effector attached at different sites, and a variable number of effectors attached to each antibody resulting in a range of molar rations and very different pharmacokinetic, efficacy and safety profiles.

15 Hamblett and colleagues[9] have studied the toxicity, pharmacokinetic properties and therapeutic efficacy of heterogeneous antibody-drug conjugates by purifying three antibody fractions containing two, four and eight conjugated molecules of monomethyl-auristatin E (MMAE). The fraction with eight MMAE groups was poorly tolerated and rapidly cleared compared to the other fractions, and demon-

20 strated the lowest efficacy. This suggests that the key design parameter for antibody-drug conjugates is the number of drug molecules attached to the antibody. However, even purified antibodies carrying the same number of drug molecules still constitute a complex mixture because of the many alternative attachment sites. For example, there are approximately 40 lysine residues in a typical anti-

25 body, potentially resulting in more than one million different conjugated antibody species. Similarly, there are between one and eight cysteine residues, typically generating approximately 100 different conjugated variants. Each version of the antibody-drug conjugant typically displays a unique and unpredictable pharmacokinetic profile[9].

30 Summary of the invention

Cancer cells can be killed by photosensitizing agents that induce toxic effects when exposed to non-hazardous light, but this also causes significant damage to surrounding healthy cells. The specificity of photodynamic therapy can be in-

creased by conjugating photosensitizing agents to antibodies and antibody frag-
ments that bind specifically to tumor-associated cell surface antigens. However,
standard conjugation reactions produce heterogeneous products whose targeting
specificity and spectroscopic properties can be compromised.

5   In this invention, an antibody fragment (scFv-425) has been used that binds to
the epidermal growth factor receptor (EGFR) as a model to investigate the use of
SNAP-tag fusions as an improved conjugation strategy. The scFv-425-SNAP-tag
fusion protein allowed the specific conjugation of a photosensitizer, such as
chlorin e6, modified with O(6)-benzylguanine, generating a homogeneous prod-
10   uct that was delivered specifically to EGFR$^+$ cancer cells and resulted in signifi-
cant, tumor cell-specific cytotoxicity. The impact of our results on the develop-
ment of photodynamic therapy is discussed.

The present invention provides a compound comprising a photosensitizer cova-
lently coupled to a binding structure selected from the group consisting of anti-
15   bodies or their derivatives or fragments thereof, synthetic peptides such as scFv,
mimotopes, which binding structure binds to CD antigens, cytokine receptors,
interleukin receptors, hormone receptors, growth factor receptors, more particu-
larly tyrosine kinase growth factor receptor of the ErbB family, wherein the pho-
tosensitizer is coupled to the internalizing receptor binding protein via the modi-
20   fied human DNA repair protein O6-alkylguanine-DNA alkyltransferase (hAGTm).

In one embodiment of the present invention the epidermal growth factor recep-
tor binding protein is a scFv antibody fragment, in particular the scFv antibody
fragment of the Seq ID No 1, encoded by the polynucleotide sequence of Seq ID
No 2.

25   In another embodiment of the invention the compound of the invention compris-
es or has the amino acid sequence Seq ID No 3, encoded by the polynucleotide
sequence of Seq ID No 4.

In yet another embodiment of the compound of the invention the photosensitizer
is coupled at the active site of the O6-alkylguanine-DNA alkyltransferase.

30   In the compound of the invention the photosensitizer is selected from the group
consisting of porphyrins, chlorophylls and dyes having photosensitizing power.

Subject matter of the invention is also a compound devoid of a photosensitizer. The compound comprises a binding protein selected from the group consisting of antibodies or their derivatives or fragments thereof, synthetic peptides such as scFv, mimotopes, which binding protein binds CD antigens, cytokine receptors,

5   interleukin receptors, hormone receptors, growth factor receptors, more particularly tyrosine kinase growth factor receptor of the ErbB family, which is covalently coupled to a modified human DNA repair protein called O6-alkylguanine-DNA alkyltransferase (hAGTm)

In particular the binding protein is an scFv antibody fragment, in particular the

10   scFv antibody fragment of the Seq ID No 1 and/or Seq ID No 3. This compound can be encoded by a polynucleotide having the sequence of Seq ID NO 2 and/or Seq ID No 4. The specific embodiments bind to tyrosine kinase growth factor receptor of the ErbB family.

A specific embodiment of the compound is encoded by the polynucleotide of the

15   nucleotide sequence of Seq ID No 5.

Another subject matter of the invention is a method for manufacturing the compound of the invention comprising the step of fusing O6-alkylguanine-DNA alkyltransferase (hAGTm) with a binding protein selected from the group consisting of antibodies or their derivatives or fragments thereof, synthetic peptides

20   such as scFv, mimotopes, which binding protein binds CD antigens, cytokine receptors, interleukin receptors, hormone receptors, growth factor receptors, more particularly tyrosine kinase growth factor receptor of the ErbB family. In particular, the scFv-425 DNA sequence is inserted into the SifI and NotI-digested site of eukaryotic expression vector pMS-SNAP providing an N-terminal binding ligand

25   (scFv-425) and a C-terminal SNAP-tag sequence.

In particular, a His_6 tag is also fused to the protein. The fused protein can be expressed in human cells in particular in embryonic kidney cell line such as HEK-293T cells (ATCC: CRL-11268) and purified using an affinity resin for the tag for example a Ni-NTA modified resin.

30   Also subject matter of the present invention is a porphyrin derivative of the formula
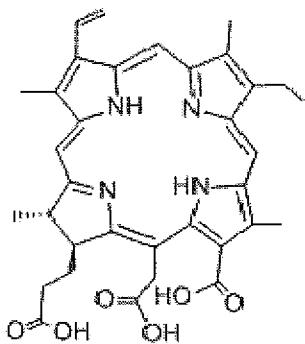
Chlorin e6 ($C_{34}H_{36}N_4O_6$)

5   wherein the carboxyl groups of the porphyrin photosensitizer, such as chlorin e6,
     are at least partially reacted to an activated ester or by a coupling agent, fol-
     lowed by reacting with O6-benzylguanine, O2-benzylcytosine or a coenzyme A
     (CoA).

     In the method of the invention O6-benzylguanine, O2-benzylcytosine or a coen-
10   zyme A (CoA) a coupled to a linker molecule, such as PEG-24-NH$_2$ and/or the
     activated ester is formed by succinimides, such as NHS, or the coupling agent
     selected from the group consisting of a carbodiimide, such as EDC, EDAC and
     DCC.

     Subject matter of the invention is also a medicament comprising the compounds
15   of the invention and a pharmaceutically acceptable adjuvant for improving or
     render   possible   the   pharmaceutical   effect   associated   with   the
     photoimmunotherapy.

     The invention is also providing a use of the compounds of the invention for treat-
     ing cancer by photoimmunotherapy.

20   The skilled person knows that the term "comprising" can be replaced by "consist-
     ing" without introducing new subject-matter extending beyond the matter dis-
     closed herein.

6

The effects of the compound of the invention are demonstrated and more de-
tailed described in the following by means of specific examples. The epidermal
growth factor receptor (EGFR, erbB1, HER1), one of four member of the ErbB
family of tyrosine kinase growth factor receptors, is overexpressed in approxi-
5   mately 30% of epithelial cancers and has thus become an attractive target for
cancer immunotherapy[10]. The recombinant anti-EGFR antibody fragment scFv-
425 binds to EGFR on the surface of cancer cells and induces receptor internali-
zation efficiently[11]. scFv-425 is used as a model for the development of a new
conjugation strategy to improve the specificity and efficacy of PIT. To achieve
10 these aims the SNAP-tag technology has been used which is based on a 20-kD a
modified human DNA repair protein called O6-alkylguanine-DNA alkyltransferase
(hAGTm) that was initially developed for the site-specific labeling of antibodies
with optically-active moleculess[11]. The SNAP-tag allows efficient, covalent cou-
pling to any substrate modified with the acceptor group O(6)-benzylguanine
15 (BG). The SNAP-tag reacts with para-substituted BG derivatives by transferring
the substituted benzyl group to its active site via a nucleophilic substitution reac-
tion and releasing free guanine[11].

According to the invention a scFv-425-SNAP-tag fusion protein was designed and
synthesised. A BG-modified chlorin e6 (Ce6) photosensitizer was delivered to
20 EGFR+ cancer cells. The construct also included a linker region and 24 polyeth-
ylene glycol (PEG) chains to increase the distance between the photosensitizer
and the protein. The BG-modified Ce6 was conjugated specifically and covalently
to the scFv-425-SNAP-tag fusion protein with no detrimental impact on the bind-
ing and internalization activities of the antibody. Ce6 was delivered specifically to
25 four EGFR+ carcinoma cell lines (A431, MDA-MB-231, MDA-MB-468 and SiHa)
and resulted in significant, tumor cell-specific cytotoxicity.

Detailed description of the invention

The present invention is further exemplary described in greater detail using Ce6
as photosensitizer and tyrosine kinase growth factor receptor of the ErbB family
30 as the binding.

Legends of the Figures

Figure 1: Construction, expression and binding of the SNAP-tag fusion proteins.
(a) Schematic diagram of the bicistronic eukaryotic expression cassettes for the
recombinant SNAP-tag fusion protein. The pMS-scFV-425-SNAP vector encodes
5   binding ligand (scFv-425) which is under the transcriptional control of the CMV
promoter, and joined in-frame to the SNAP-tag. An immunoglobulin κ leader se-
quence (Ig-κ-L) facilitates protein secretion, and a TGA stop codon is placed
immediately after the C-terminal His_6-tag. The expression cassettes for the con-
trol vector were the same as the PMS-scFv-425-SNAP but contain scFv-Ki4 in-
10   stead of scFv-425 as a binding ligand. (b) Purification fractions of scFv-425-SNAP
protein were separated by SDS-PAGE, and then stained with coomassie brilliant
blue, (c) scFv-425-SNAP was incubated with BG-Vista green, proteins were visu-
alized with UV light. M: Protein marker, 1: 3μl of eluted scFv-425-SNAP with
250mM Imidazol, 2: 1.5μl of eluted scFv-425-SNAP with 250mM Imidazol, 3:
15   10μl of eluted scFv-425-SNAP with 40mM Imidazol, 4: eluted protein with 10mM
Imidazol, 5: Flow through, 6: HEK-293T cells supernatant. Binding analysis of
scFv-425-SNAP and scFv-Ki4-SNAP were assessed by flow cytometry using
$EGFR^+$ A431 (d) and $EGFR^-$ L540 cells (e). Filled gray curves represent untreated
cells. Cells were incubated with 0.5 μg/ml of the purified fusion protein scFv-425-
20   SNAP (light gray curve) and Ki4-SNAP (black curve). As a secondary antibody a
Penta-His Alexa Fluor 488 Conjugate (dilution 1/500) (Qiagen) was used. To ex-
clude nonspecific staining of the anti-His Alexa Fluor 488 detection antibody,
omission of the His-tagged fusion protein served as control (dotted black
curves).

25   Figure 2: Analysis of Ce6 photosensitizer by mass spectrometry before and after
modification with benzylguanine (BG). (a) ESI mass spectrum of Ce6, BG-PEG24-
NH_2, and BG-PEG24-Ce6. The top panel represents Ce6 (597.215 Da), the middle
panel represents BG-PEG24-NH_2 (1398.761 Da) and the bottom panel represents
BG-PEG24-Ce6 (1979.004 Da). (b) Coupling of BG-PEG24-Ce6 to scFv-425-
30   SNAP. M, protein marker; 1, scFv-425-SNAP incubated with a 1.5-fold molar ex-
cess of BG-VistaGreen; 2, scFv-425-SNAP blocked with a 3-fold molar excess of
bromothenylpteridine (BTP), incubated with BG-Ce6 for 2 h, and finally mixed
with BG-VistaGreen; 3, scFv-425-SNAP incubated with a 1.5-fold molar excess
of BG-Ce6 for 2 h, then a 1.5-fold molar excess of BG-VistaGreen. Coupled pro-

teins were separated by SDS-PAGE and visualized with the CRi Maestro Imaging System. The different dye spectra were unmixed using Maestro software and the corresponding gel was stained with Coomassie Brilliant Blue (c).

Figure 3: The binding activities of scFv-425-SNAP-VistaGreen and scFv-425-SNAP-Ce6, which specifically recognize EGFR$^+$ cells. Flow cytometry analysis was carried out after incubating 4×10^5 cells with each fusion protein for 20 min at 37°C in PBS. (a) The scFv-425-SNAP-VistaGreen (light gray curve) was tested against A431, MDA-MB-468, MDA-MB-231, SiHa, L540, and CHO-K1 cells (filled gray curve). As a control, scFv-Ki4-SNAP was labeled with BG-VistaGreen (black curve) and its binding activity was tested against A431, L540 and CHO-K1 cells (filled gray curves). (b) The binding efficiency of scFv-425-SNAP-Ce6 (light gray curve) was tested against A431, MDA-MB-468, MDA-MB-231, SiHa, L540 and CHO-K1 cells (filled gray curves). As a control, scFv-Ki4-SNAP labeled with BG-Ce6 (black curve) was tested against A431, L540 and CHO-K1 cells (filled gray curves).

Figure 4: Internalization of fusion proteins analyzed by confocal microscopy. Confocal images were obtained for the EGFR$^+$ cell lines A431, MDA-MB-468, MDA-MB-231 and SiHa, and for the EGFR$^-$ cell lines L540 and CHO-K1 incubated with 0.5 µg scFv-425-SNAP-Ce6 for 30 min at 4°C (a) or for 60 min at 37°C (b). (1) Ce6 fluorescence signal; (2) transmitted light; (3) overlay of fluorescence signal and transmitted light.

Figure 5: Evaluation of photodynamic therapeutic efficiency. Cell proliferation and apoptosis assays were carried out using the scFv-425-SNAP-Ce6. The cytotoxicity of scFv-425-BG-Ce6 was determined against cell lines A431 (■), MDA-MB-468 (▲), MDA-MB-231 (♦), SiHa (●) and CHO-K1 (▼) using the XTT assay on (a) irradiated cells and (b) non-irradiated cells. The cytotoxicity scFv-Ki4-SNAP-Ce6 against A431 cells (x) was tested as a control. The same cells were treated with different concentrations of BG-Ce6 and cell viability was analyzed with (c) and without (d) light activation. (e) Apoptosis was evaluated using the Apo-ONE™ Homogeneous Caspase-3/7 Assay, with 50 nM BG-Ce6, 200 nM scFv-SNAP-Ce6 and 200 nM scFv-Ki4-SNAP-Ce6. (f) The generation of reactive oxygen species by illuminating photosensitized A431 cells, detected using the dichlorofluorescein derivative carboxy-H2DCFDA.

Photodynamic therapy (PDT) is a minimally invasive treatment that uses non-toxic photosensitizers and harmless visible light in combination with oxygen to produce cytotoxic reactive oxygen species that kill malignant cells by apoptosis and/or necrosis[12]. Many different photosensitizers have been developed, but Ce6

5 has been chosen as a model because it has been evaluated extensively in PDT studies and also has advantageous physical and chemical properties. Ce6 has an absorption maximum at 664 nm, which is a good compromise between photon efficacy and cell penetration[13], and the presence of carboxyl groups allows further functionalization[5].

10 The use of SNAP-tag technology of the present invention provides a unique conjugation site on the antibody, allowing the production of a homogeneous conjugate preparation. The construct of the invention in which the coding sequence of an scFv antibody that binds specifically to EGFR was genetically fused to the hAGT cassette, endows the antibody with a SNAP-tag and therefore allows site-

15 specific conjugation BG-modified substrates, in particular Ce6. This conjugation method can be applied to any antibody–photosensitizer combination as long as the antibody carries the SNAP-tag and the substrate is modified with a BG group.

The conjugation reaction was efficient, allowing the preparation of homogeneous samples of scFv-425-SNAP-Ce6 and scFv-Ki4-SNAP-Ce6. These preparations

20 were tested for their ability to kill tumor cells specifically. It has been found that scFv-425-SNAP-Ce6 selectively killed EGFR$^+$ cells in four human tumor-derived cell lines representing epidermal, breast and cervical carcinomas (A431, MDA-MB-231, MDA-MB468 and SiHa) after exposure to light. The phototoxicity of scFv-425-SNAP-Ce6 was dependent on the presence of EGFR and light, and tox-

25 icity was most potent in A431 and MDA-MB468 cells, which express the largest amount of the receptor ($1–1.3 \times 10^6$ receptors/cell)[14,15]. The other cells lines expressed less EGFR (1.3×10^5 receptors/cell for MDA-MB-231 and 2×10^4–2×10^5 receptors/cell for SiHa)[15,16], and the toxicity of scFv-425-SNAP-Ce6 was concomitantly reduced, although not to the point where the fusion protein would be ther-

30 apeutically ineffective. This means that scFv-425-SNAP-Ce6 can target a wide range of EGFR$^+$ cells not only those with the highest expression levels. No toxicity was observed when EGFR$^-$ cells (CHO-K1) were exposed to scFv-425-SNAP-Ce6.

It has been previously shown that scFv-425-SNAP accumulates directly in mouse kidneys after injection, and is subsequently detected in the bladder, indicating clearance by renal filtration[10]. Despite the rapid clearance, the accumulation and retention of scFv-425-SNAP in tumor tissue was evidently sufficient to yield very

5   high tumor to background ratio 10 h post-injection.

Expression, purification and functional analysis of scFv/SNAP-tag fusion proteins

The coding sequences for the EGFR-specific scFv-425 antibody fragment[10] and a control fragment (scFv-Ki4)[17] that binds to a different antigen (CD30) were transferred to the pMS-SNAP bicistronic vector to generate the complete scFv-

10   425-SNAP and scFv-Ki4-SNAP cassettes, as shown in (Fig. 1a). The constructs were introduced into HEK-293T cells by transfection and stably transformed cells were identified by selection on zeocin and by monitoring green fluorescent pro-tein (GFP) activity. The fusion proteins were isolated from the culture superna-tant to a final purity of ~90% by affinity chromatography (using the C-terminal

15   His_6 tag) and the final yield was 18 mg/L of protein in the supernatant (Fig. 1b).

The activity of the SNAP-tag was confirmed in each of the fusion proteins by mix-ing the unprocessed culture supernatant, the flow through fraction and the eluate from the chromatography step with BG-modified Vista Green (Fig. 1c). The bind-ing activity of the scFv-425-SNAP protein was confirmed by flow cytometry using

20   one target cell line expressing EGFR (A431), and one control cell line lacking this antigen but expressing CD30 (L540). Binding was detected with a secondary an-ti-His_5 Alexa 488 antibody. Flow cytometry data confirmed the rapid and efficient binding of scFv-425-SNAP specifically to $EGFR^+$ target cells (Fig. 1d), whereas scFv-Ki4/SNAP bound only to the $CD30^+$ L540 cells (Fig. 1e).

25   Modification of the photosensitizer chlorin e6 with benzylguanine

The photosensitizer chlorin e6 (Ce6) was modified successfully using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), the sodium salt of hydroxysulfosuccinimide (sulfo-NHS) and a BG-PEG24-NH_2 linker. Ce6 carbox-yl groups were modified to BG groups, and the efficiency of the reaction was de-

30   termined by HPLC (data not shown). The high purity of BG-PEG24-Ce6 was con-firmed by mass spectrometry. The accurate masses of Ce6, BG-PEG24-NH_2 and BG-PEG24-Ce6 were detected on a Micromass QTOFII mass spectrometer, which

confirmed that purified BG-PEG24-Ce6 had the same mass as the theoretical mass calculated for coupled Ce6 and BG-PEG24-NH2 (Fig. 2a)

Protein labeling with BG-modified fluorophores and Ce6

The functionality of the SNAP-tag was tested by coupling to BG-modified fluorescent dye, which revealed a labeling efficiency of 85–90% after a 2-h incubation at room temperature (data not shown). The reaction was repeated using BG-modified Ce6. The photosensitizer reacted solely with the active SNAP-tag in the fusion proteins and the reaction could be irreversibly blocked with the bromothenylpteridine (BTP), as shown by post-incubation with a 1.5-fold molar excess of BG-Vista Green. Analysis with the CRi Maestro imaging system showed no fluorescence associated with the previously blocked fusion protein (Fig. 2b,c).

Flow cytometry and confocal microscopy

To determine the activity of labeled scFv-425-SNAP fusion proteins, flow cytometry analysis was carried out using proteins that had been labeled with either BG-Vista Green or BG-Ce6. All the labeled proteins showed a strong fluorescence signal on the corresponding target cell line (A431, MDA-MB-231, MDA-MB-468 and SiHa) but not on control cells (L540 and CHO-K1) after a 30-min incubation on ice. As expected, labeled scFv-Ki4-SNAP showed a strong fluorescence signal on L540 but not on A431 and CHO-K1 cells (Fig. 3).

Confocal microscopy revealed strong, specific and homogeneous membrane staining on A431, MDA-MB-231, MDA-MB468 and SiHa cells incubated with scFv-425-SNAP-Ce6 (Fig. 4a). The labeled fusion protein was specifically and efficiently taken up into A431, MDA-MB-231, MDA-MB468 and SiHa cells after a 30-min incubation at 37°C but not at 4°C (Fig. 4b). In contrast, no signal was detected when the EGFR⁻ cell lines L540 and CHO-K1 were incubated with scFv-425-SNAP-Ce6 under the same conditions (Fig. 4a,b).

Photocytotoxicity of scFv-425-SNAP-Ce6

The concentration-dependent cytotoxic effects of scFv-425-SNAP-Ce6 and unconjugated BG-Ce6 were evaluated using an XTT-based colorimetric cell proliferation assay with the four EGFR⁺ cell lines and CHO-K1 as a negative controls. The viability of A431, MDA-MB-231, MDA-MB-468 and SiHa cells treated with scFv-425-

SNAP-Ce6 was reduced significantly, in a concentration-dependent manner, after a 24-h incubation followed the light activation. The IC_{50} values were 48 nM (A431), 200 nM (MDA-MB-231), 38 nM (MDA-MB-468) and 218 nM (SiHa). CHO-K1 cells remained unaffected even when exposed to 800 nM of the conjugated

5   fusion proteins, and the control construct scFv-Ki4-SNAP-Ce6 had a negligible effect in both A431 and CHO-K1 cells. In contrast, unconjugated Ce6 was toxic towards all the cell lines, with IC_{50} values of 16 nM (A431), 22 nM (MDA-MB-231), 22 nM (MDA-MB-468), 26 nM (SiHa) and 18 nM (CHO-K1). These data are shown in (Fig. 5a,c).

10 Both the conjugated and unconjugated forms of Ce6 were toxic only after light activation, as confirmed by carrying out parallel experiments without the light activation step. No significant reduction in viability was observed in any of the cell lines (Fig. 5b,d).

To determine whether scFv-425-SNAP-Ce6 selectively induced programmed cell

15 death in target cells by triggering the apoptotic pathway, the activity of caspase-3 and caspase-7 has been analyzed in A431, MDA-MB-231, MDA-MB468, SiHa and CHO-K1 cells 24 h after light activation. Both scFv-425-SNAP-Ce6 (200 nM) and unconjugated Ce6 (50 nM) increased the levels of caspase-3 and caspase-7, whereas no significant increase was observed in A431 cells treated with 200 nM

20 scFv-Ki4-SNAP-Ce6 (Fig. 5e).

The production of ROS in photoactivated A431 cells was investigated by measuring the 485/535-nm fluorescence of DCF, produced by the oxidation and deacetylation of 6-carboxy-20,70-dichlorodihydrofluoresceindiacetatedi-(acetoxy-methyl)ester (H_2DCFDA). It has been found that a burst of ROS synthesis follows

25 light activation in the presence of 200 nM of the conjugated Ce6 and 50 nM of the unconjugated Ce6, but there was only a small increase in ROS levels in non-irradiated cells, barely above the background level observed in cells that were not treated with the photosensitizer (Fig. 5f).

METHODS

Cell culture

All cell lines were of human origin, including the EGFR$^+$ A431, MDA-MB-231, MDA-MB468 and SiHa cells, and the EGFR$^-$ L540, CHO-K1 and HEK-293T cells.

5 A431, L540, CHO-K1 and HEK-293T cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. MDA-MB-231, MDA-MB468 and SiHa cells were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. All cells were incubated at 37°C in a 5% CO_2 atmosphere. All

10 media and additives were obtained from Invitrogen, Darmstadt, Germany.

Protein expression and purification.

The sequence for each scFv was inserted into an expression cassette providing an N-terminal binding ligand (scFv-425 or scFv-Ki4) and a C-terminal O6-alkylguanine-DNA alkyltransferase (SNAP-tag) sequence. The TGA stop codon is

15 generated immediately after His$_6$ tag sequence. His$_6$-tagged fusion proteins were purified from cell-free supernatants by Ni-NTA metal affinity chromatography. Larger volumes were purified on an Äkta FLPC system with a 5-mL Ni-NTA Superflow cartridge (Qiagen, Hilden, Germany) after equilibration with 4x buffer (200 mM NaH_2PO_4, 1.2 M NaCl, 40 mM imidazole, pH 8). Bound His-tagged pro-

20 teins were eluted in 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8). After elution, proteins were dialyzed at 4°C overnight against phosphate-buffered saline (PBS) containing 1 mM dithioerythritol (Carl Roth GmBH, Karlsruhe, Germany). Ectoine cryopreservative was added to a final concentration of 50 mM, and aliquots were stored at −20°C.

25 Modification of Ce6 with benzylguanine

The carboxyl groups of Ce6 (Porphyrin Products, Logan, UT), were modified with benzylguanine by mixing 2 mg Ce6 in dimethylformamide for 30 min at room temperature with a five-fold molar excess of EDC and sulfo-NHS (Sigma-Aldrich, St Louis, MO). The activated mixture was then mixed with a four-fold molar ex-

30 cess of the benzylguanine linker BG-PEG24-NH$_2$ (Covalys Biosciences AG, Witterswil, Switzerland) in the dark at room temperature overnight. The modified

Ce6 was purified by HPLC using a Shimadzu Prominence HPLC system, and a 2.5μm (4.6 x 50 mm) Water XBridge™ OSTC$_{18}$ column (Waters, Milford, MA) at a flow rate was 1 mL/min. Separations were carried out using a 20-min gradient from 100% 0.1 M TEAA to 100% acetonitrile, monitored at 280 and 410 nm. The masses of Ce6, BG-PEG24-NH2 and BG-PEG24-Ce6 were confirmed using a Micromass QTOFII mass spectrometer with an electrospray ion source Advion Nanomate (Advion, Ithaca, NY, USA) 7μl sample volume, 1.4 kV. Accurate masses were derived from mass spectra in the range 300–2500 m/z using the MaxEnt3™ algorithm (Micromass) in the range of 400–2000 Da.

Protein labeling

The purified SNAP-tag fusion proteins were conjugated with BG-modified dyes (Covalys Biosciences AG, Witterswil, Switzerland) or BG-modified Ce6 by incubation in the dark with a 1.5–3-fold molar excess of dye for 2 h at room temperature. Residual dye was removed by gel filtration chromatography using zeba spin desalting columns, 7K MWCO (Thermo Fisher Scientific, Rockford, IL). Coupling efficiency was determined photometrically using the extinction coefficients of the corresponding dyes and the theoretical extinction coefficient of the fusion proteins. Labeled proteins were visualized after separation by SDS-PAGE with either a UV transilluminator Gel Doc XR gel documentation (Bio-Rad Laboratories, München, Germany) or a CRi Maestro imaging system (CRi, Woburn, MA, USA) using the blue and yellow filter sets.

Flow cytometry

The binding efficiency of the labeled and unlabeled fusion proteins was determined by flow cytometry using a FACSCalibur (Becton & Dickinson, Heidelberg, Germany) and CellQuest software. EGFR$^+$ cell lines A431, MDA-MB-231, MDA-MB468 and SiHa were used to test the binding efficiency of scFv-425-SNAP, and EGFR$^-$ cell lines L540 and CHO-K1 were used as negative controls. The control fusion protein scFv-Ki4-SNAP recognizes the antigen CD30 and should therefore bind to L540 cells but not to the other cell lines. Approximately 4×10^5 cells were incubated in 200 μL PBS containing 0.5 μg of labeled protein for 20 min on ice. The cells were then washed twice with 1.8 mL PBS in a conventional cell washer and analyzed by flow cytometry.

Confocal microscopy

Images were visualized with a TCS SP5 confocal microscope (LEICA Microsystem, Wetzlar, Germany). Cells were prepared as described above for flow cytometry. Binding efficiency was determined by incubating cells with the labeled fusion pro-
teins for 30 min on ice. Internalization was monitored by incubating cells with the labeled fusion proteins for 30 min at 37°C.

Phototoxicity of scFv-425-SNAP-Ce6

Aliquots of A431, MDA-MB-231, MDA-MB468, SiHa and CHO-K1 cells (2×10^4) cul-
tured as described above were washed twice in PBS and then treated with in-
creasing concentrations of either Ce6, scFv-425-SNAP-Ce6 or Ki4-scFv/SNAP-Ce6 followed by incubation for 3 h at 37°C. Control cultures were incubated with 500 µg/ml zeocin instead of the photosensitizer. The cells were then irradiated with 24 J/cm² broadband visible/near infrared light using Hydrosun type 505, 7-mm water cuvette and orange filter OG590, spectrum in the range 580-1400 nm
(Hydrosun Medizintechnik GmbH, Müllheim, Germany) and incubated for a fur-
ther 24 h at 37°C in a 5% CO_2 atmosphere.

Cell viability was determined using the XTT cell proliferation kit II (Roche, Mann-
heim Germany), 24 h after light activation. Cells were incubated with 2,3-bis(2-
methoxy-4-nitro-5sulphonyl)-5[(phenyl-amino)carbonyl]-2H-tetrazolium hydrox-
ide reagent (1mg/ml), and incubated for 2 h at 37°C. Reduction of XTT to formazan by viable tumor cells was monitored colorimetrically at an absorbance wavelength of 450 nm and a reference wavelength of 630 nm using an ELISA plate reader Elisareader ELx808 (Bio-TEK, Bad Friedrichsahll, Germany).

Caspase-3/7 activity in cell lysates was determined using the Apo-ONE Caspase-
3/7 assay (Promega, Mannheim, Germany) 24 h after light activation. Briefly, 100 µl of Apo-ONE reagent was added to the cells, and they were incubated for 6 h before fluorescence readings were taken with an ELISA plate reader Elisareader ELx808 (Bio-TEK, Bad Friedrichsahll, Germany) using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The concentration of ROS was
determined by measuring the 485/535 nm fluorescence ratio of H2DCFDA (Invi-
trogen, Darmstadt, Germany). Briefly, 2×10^4 cells were incubated in the pres-
ence of 50 nM Ce6 or 200 nM scFv-425-SNAP-Ce6 and 10 µM H2DCFDA for 30

min in PBS containing 1% FCS. The cells were washed twice with warm PBS containing 2.5% FCS, cultured for 2 h in RPMI-160 medium and illuminated as described above. Fluorescence readings were taken directly after illumination. A blank probe (cells and medium) reading was used as the background and sub-
5   tracted from all the sample readings.

Data Analysis

Statistical analysis and curve fitting were performed with GraphPad Prism software (GraphPad, San Siego, CA). Data are presented as the mean $\pm$ MES. Student's t test and two-way analysis of variance were used to assess the signifi-
10   cance of independent experiments. The criterion $p < 0.05$ was used to determine statistical significance.

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Claims

1. A compound comprising
   - a photosensitizer covalently coupled to
   - a protein selected from the group consisting of
        antibodies or their derivatives or fragments thereof, synthetic peptides such as scFv, mimotopes
   - which bind CD antigens, cytokine receptors, interleukin receptors, hormone receptors, growth factor receptors, more particularly tyrosine kinase growth factor receptor of the ErbB family, wherein
   - the photosensitizer is coupled to the binding protein via O6-alkylguanine-DNA alkyltransferase (hAGTm), a modified human DNA repair protein.

2. The compound of claim 1, specifically targeting an internalizing and disease-specific cell surface receptor.

3. The compound of claim 1 or 2, wherein the tyrosine kinase growth factor receptor binding protein is an scFv antibody fragment, in particular the scFv antibody fragment of the Seq ID No 1, encoded by the polynucleotide sequence of Seq ID NO 2.

4. The compound of claim 1 to 3 with the amino acid sequence Seq ID No 3, encoded by the polynucleotide sequence of Seq ID No 4.

5. The compound of claim 1 to 4, wherein the photosensitizer is coupled at the active site of the O6-alkylguanine-DNA alkyltransferase.

6. The compound of claim 1 to 4 wherein the photosensitizer is selected from the group consisting of porphyrins, chlorophylls and dyes.

7. A compound comprising
   a binding protein selected from the group consisting of antibodies or their derivatives or fragments thereof, synthetic peptides such as scFv, mimotopes, which binding protein binds CD antigens, cytokine receptors, interleukin receptors, hormone receptors, growth factor receptors, more particularly tyrosine kinase growth factor receptor of the ErbB family, which is covalently coupled to a modified human DNA repair protein called O6-alkylguanine-DNA alkyltransferase (hAGTm).

8. The compound of claim 7 wherein the binding protein is a scFv antibody fragment, in particular the scFv antibody fragment of the Seq ID No 1 and/or Seq ID No 3.

9. A polynucleotide sequence encoding the compound of claim 7 in particular comprising Seq ID NO 2 and/or Seq ID No 4.

10. The polynucleotide of the nucleotide sequence of Seq ID No 5 encoding the compound of claim 1.

11. A method for manufacturing the compound of claim 7 comprising the step of fusing O6-alkylguanine-DNA alkyltransferase (hAGTm) with a binding protein selected from the group consisting of antibodies or their derivatives or fragments thereof, synthetic peptides such as scFv, mimotopes, which binding protein binds CD antigens, cytokine receptors, interleukin receptors, hormone receptors, growth factor receptors, more particularly tyrosine kinase growth factor receptor of the ErbB family.

12. The method of claim 11 wherein the scFv-425 DNA sequence is inserted into the SifI and NotI-digested site of eukaryotic expression vector pMS-SNAP providing an N-terminal binding ligand (scFv-425) and a C-terminal SNAP-tag sequence.

13. The method of claim 11 or 12 wherein the scFv-425-SNAP fusion protein is expressed in human embryonic kidney cell line in particular HEK-293T cells (ATCC: CRL-11268).

14. The method of any one of the claims 11 to 13 wherein the scFv-425-SNAP fusion protein is purified from cell-free supernatant by an affinity chromatograpy, in particular a Ni-NTA affinity chromatography.

15. A porphyrin derivative of the formula
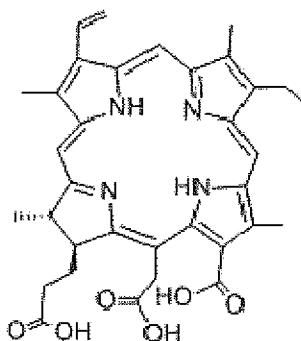
coupled to the compound of claim 7.

16. A method of manufacturing of the compound of claim 15, wherein the carboxyl groups of the porphyrin photosensitizer, such as chlorin e6, are

at least partially reacted to an activated ester or by a coupling agent, followed by reacting with O6-benzylguanine, O2-benzylcytosine or a coenzyme A (CoA).

17. The method of claim 16, wherein O6-benzylguanine, O2-benzylcytosine or a coenzyme A (CoA) a coupled to a linker molecule, such as PEG-24-NH_2 and/or the activated ester is formed by succinimides, such as NHS, or the coupling agent selected from the group consisting of a carbodiimide, such as EDC, EDAC and DCC.

18. Medicament comprising the compound of at least one of the claims 1 to 8 and a pharmaceutically acceptable adjuvant for improving or render possible the pharmaceutical effect associated with the photoimmunotherapy.

19. Use of a compound of at least one of the claims 1 to 8 for treating cancer by photoimmunotherapy.
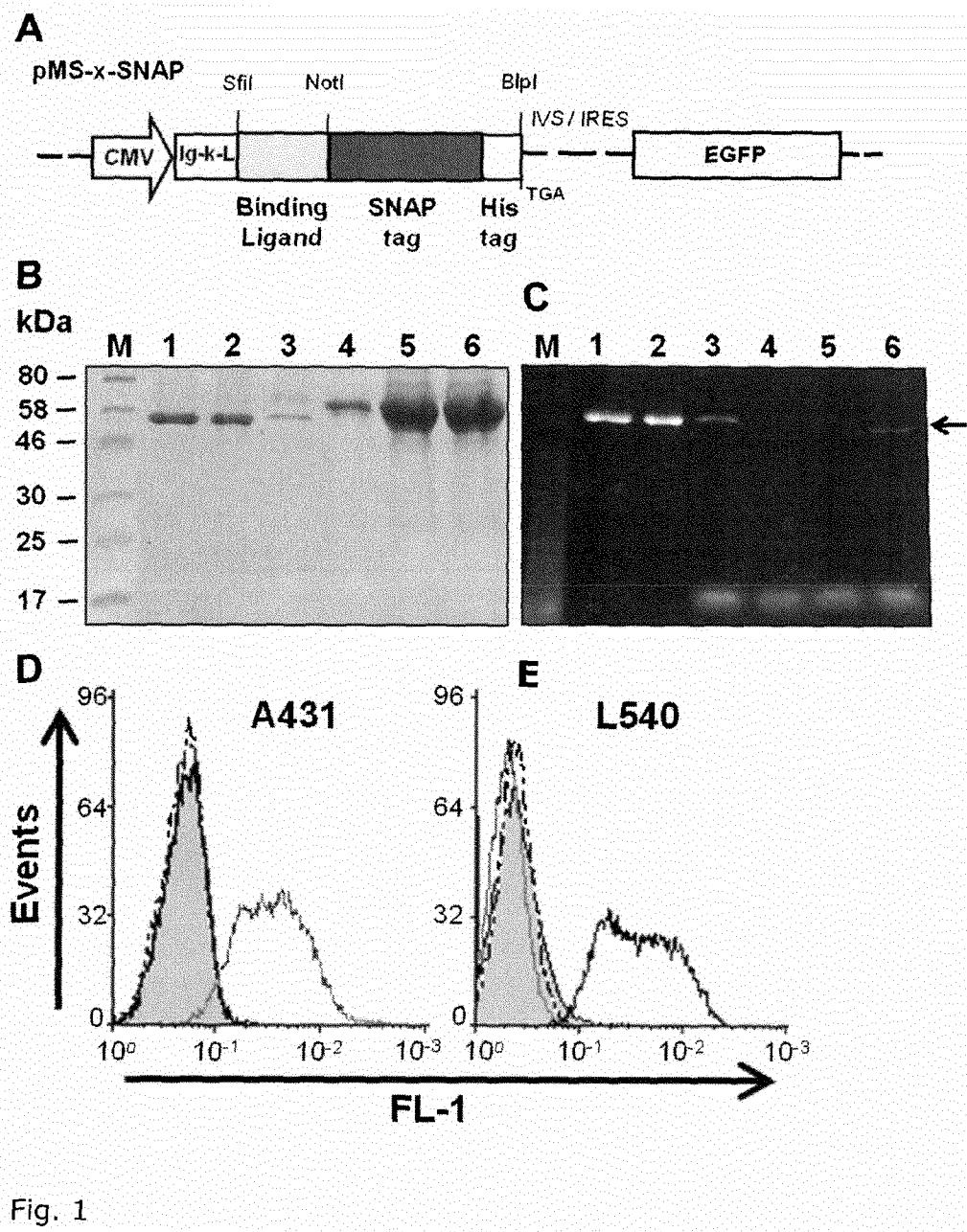
Fig. 1
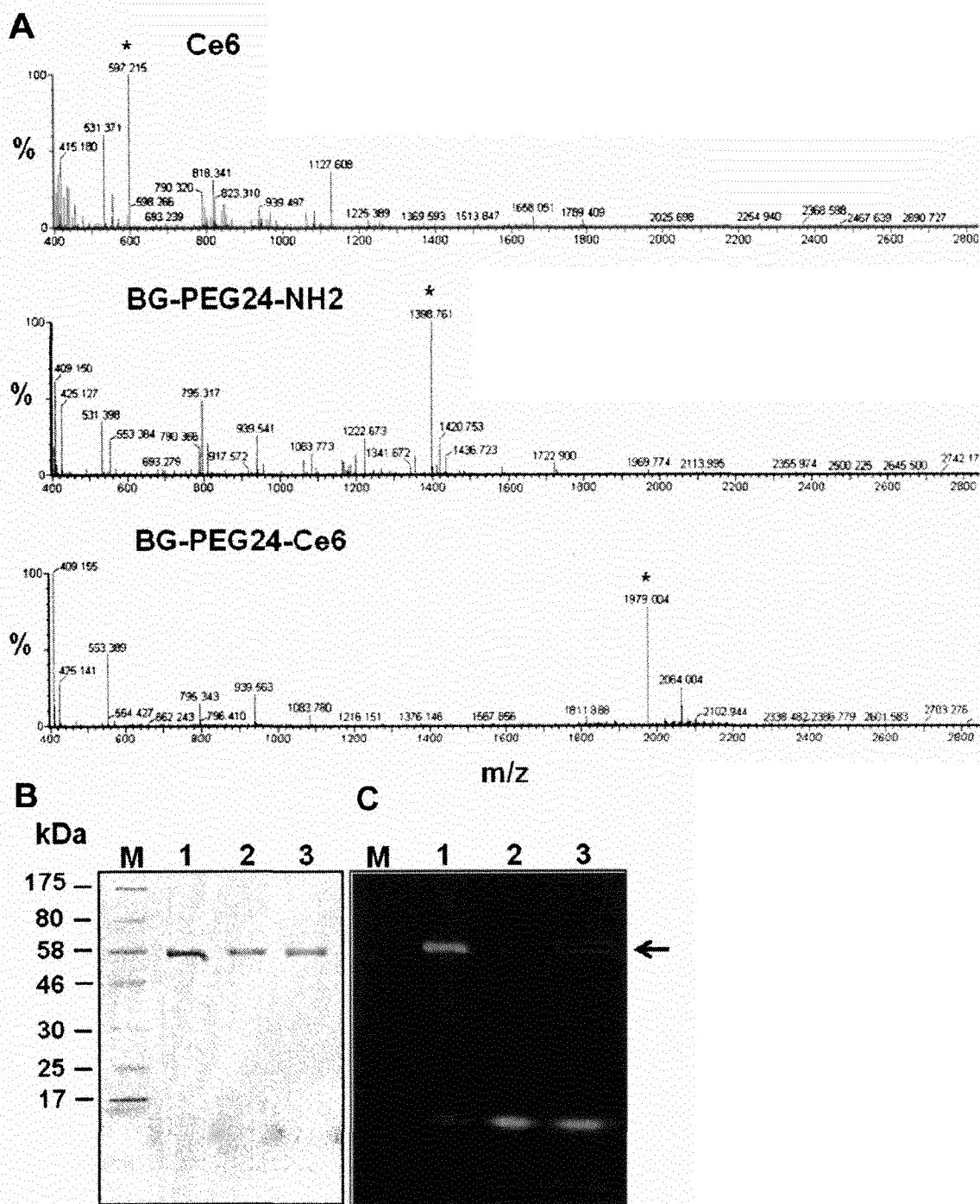
Fig. 2
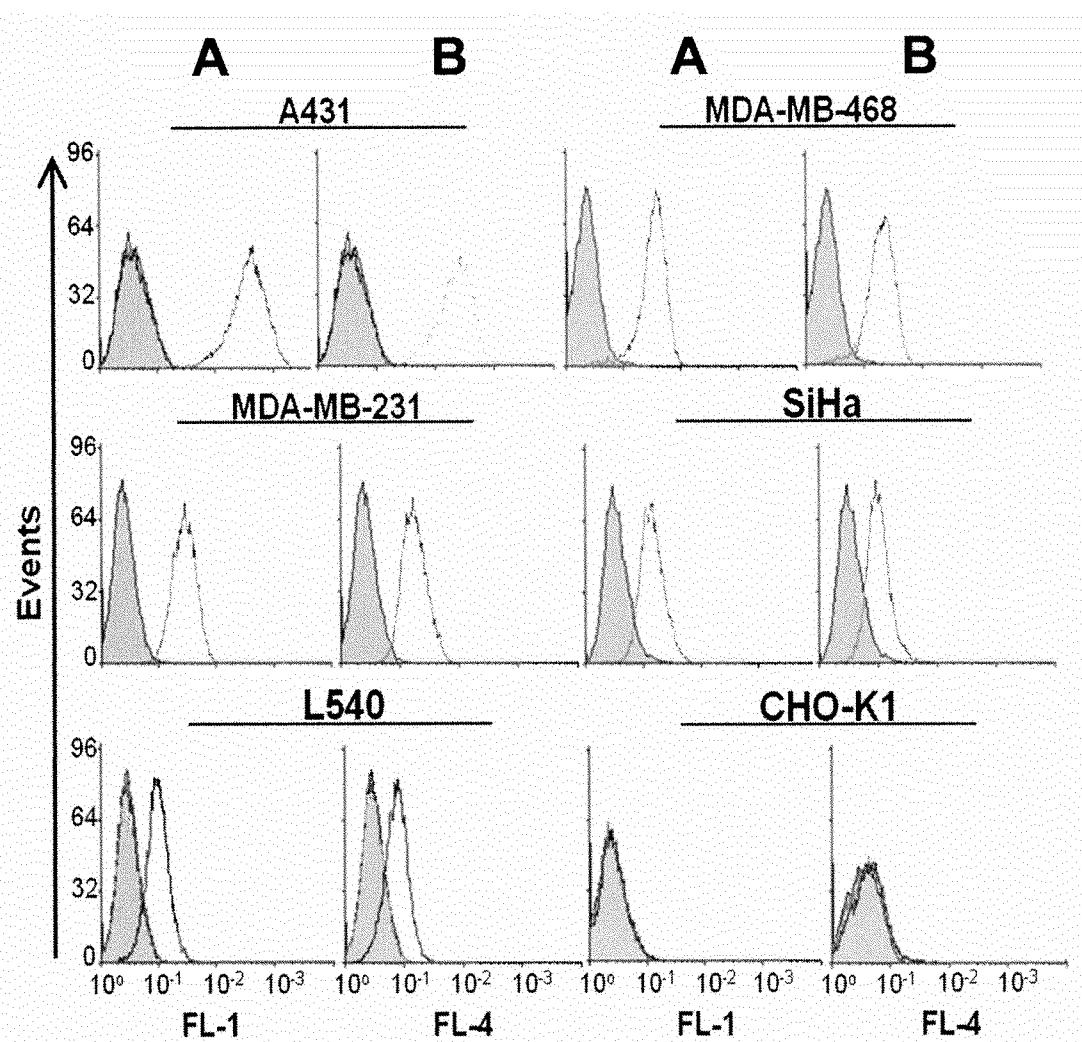
Fig. 3
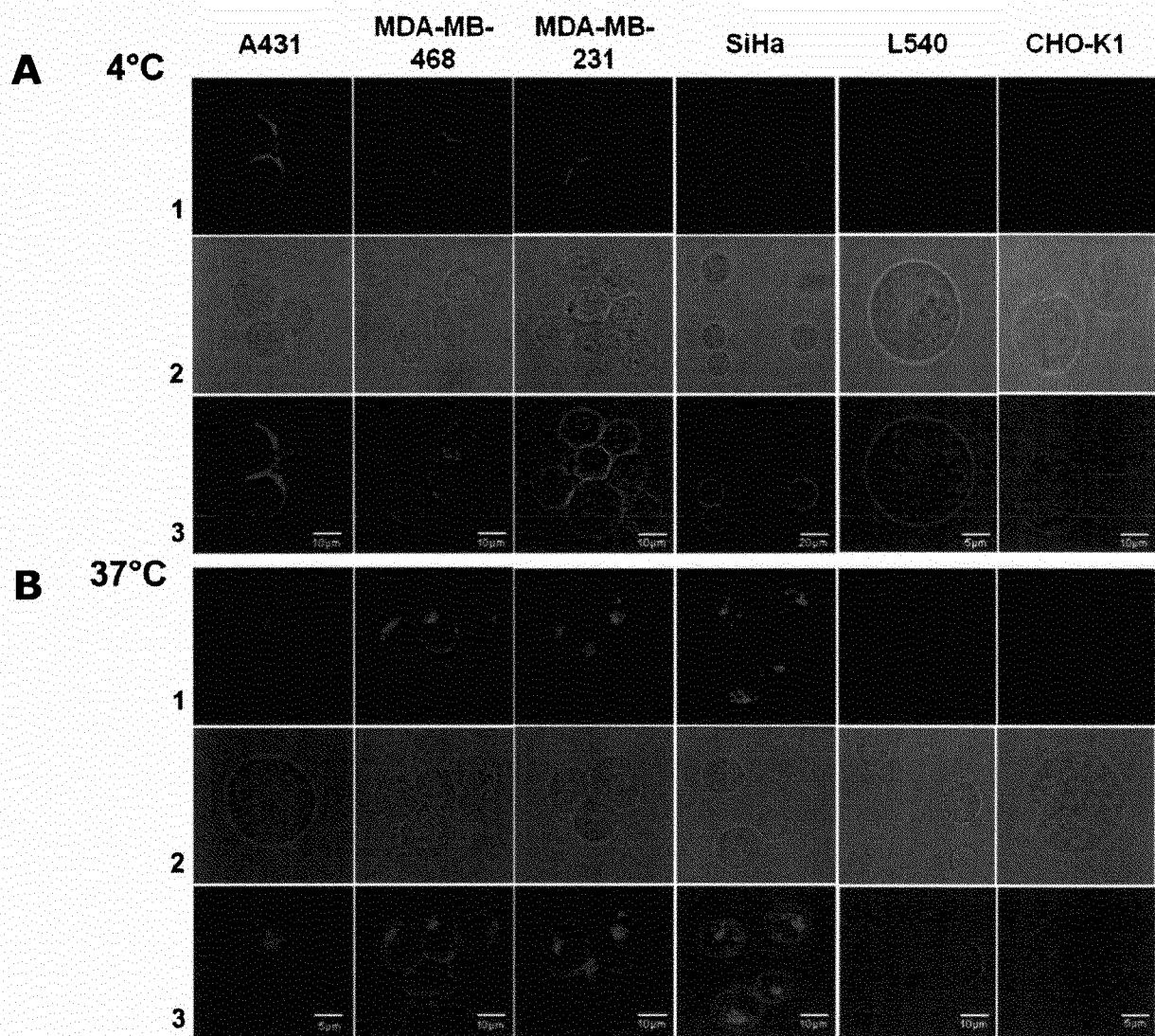
Fig. 4
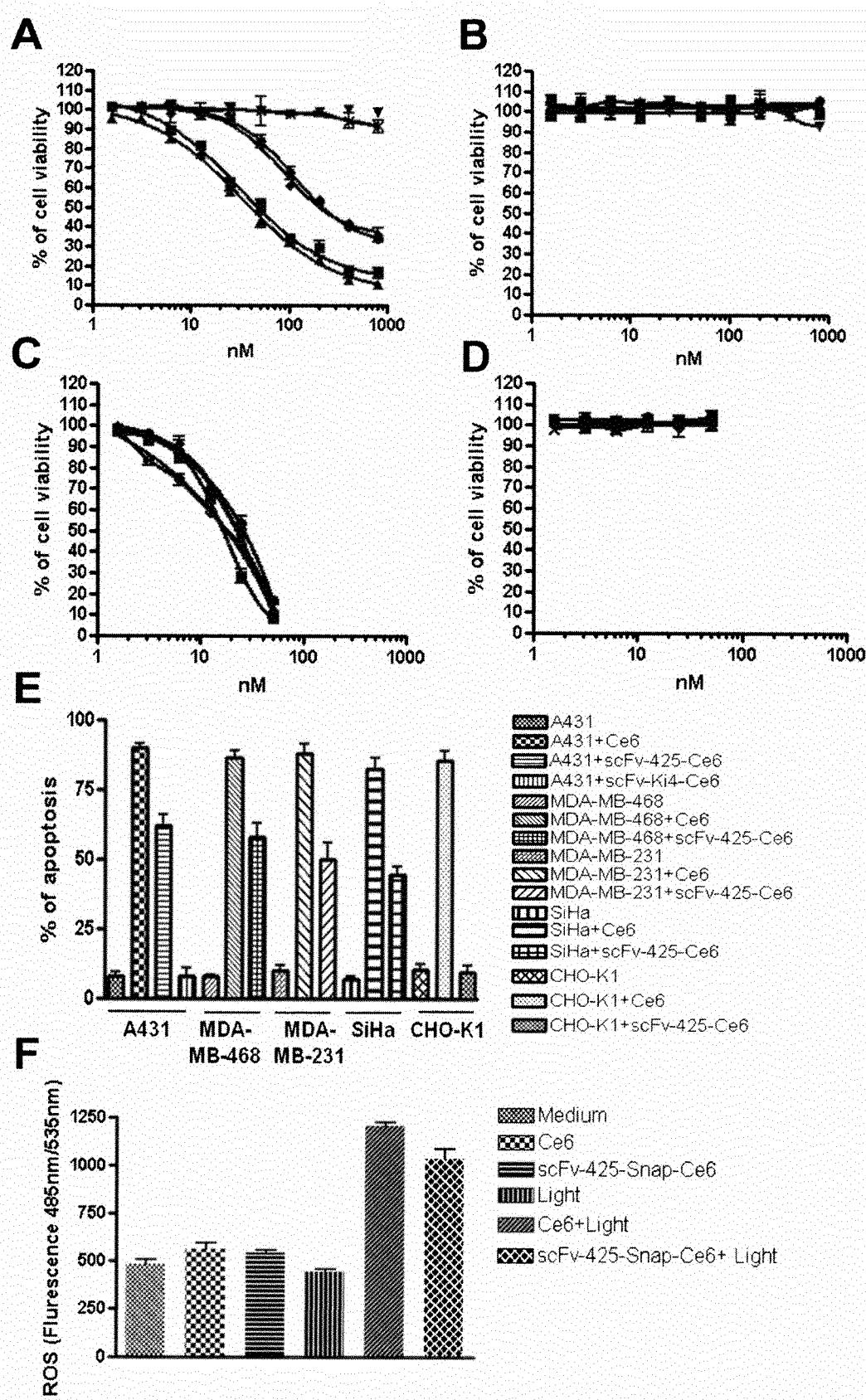
Fig. 5

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48   A61K41/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2009/013359 A2 (FRAUNHOFER GES FORSCHUNG [DE]; RWTH AACHEN [DE]; RIBBERT MARKUS [DE];) 29 January 2009 (2009-01-29) Page 59 lines 18, 19; page 65 lines 1-20; Figures 4-16, 98 and 112. Seq ID 14. -----	1-3,5-9, 11,12, 14,17 4,8,10, 13,15, 16,18,19
Y	NELA MALATESTI ET AL: "Synthesis and in vitro investigation of cationic 5,15-diphenyl porphyrin-monoclonal antibody conjugates as targeted photodynamic sensitisers.", INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 28, no. 6, 1 June 2006 (2006-06-01), pages 1561-1569, XP055003794, ISSN: 1019-6439 Abstract, page 1566 Figure 1, page 1568 paragraphs 3-5 ----- -/--	15,16, 18,19

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 2012	24/10/2012

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Bettio, Andrea

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C(Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GRONEMEYER T ET AL: "DIRECTED EVOLUTION OF O<6>-ALKYLGUANINE-DNA ALKYLTRANSFERASE FOR APPLICATIONS IN PROTEIN LABELING", PROTEIN ENGINEERING, DESIGN AND SELECTION, OXFORD JOURNAL, LONDON, GB, vol. 19, no. 7, 1 January 2006 (2006-01-01), pages 309-316, XP009079596, ISSN: 1741-0126, DOI: 10.1093/PROTEIN/GZL014 Page 324 figure 3, lines 5-7. -----	13
Y	WO 95/25167 A1 (MERCK PATENT GMBH [DE]; KETTLEBOROUGH A CATHRINE [GB]; BENDIG MARY M []) 21 September 1995 (1995-09-21) Claim 4, Seq ID 20 -----	4,8
Y	US 6 936 469 B2 (DEJONG GARY [CA] ET AL) 30 August 2005 (2005-08-30) SEQ ID 13 -----	10
A	KEPPLER A ET AL: "Labeling of fusion proteins with synthetic fluorophores in live cells", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 101, no. 27, 6 July 2004 (2004-07-06) , pages 9955-9959, XP002462236, ISSN: 0027-8424, DOI: 10.1073/PNAS.0401923101 the whole document -----	1-19
A	BARTH STEFAN ET AL: "Ki-4(scFv)-ETA', a new recombinant anti-CD30 immunotoxin with highly specific cytotoxic activity against disseminated Hodgkin tumors in SCID mice", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 95, no. 12, 15 June 2000 (2000-06-15) , pages 3909-3914, XP002245489, ISSN: 0006-4971 the whole document -----	1-19
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