

CONJUGATE OF A SINGLE DOMAIN ANTIBODY, A SAPONIN AND AN EFFECTOR MOLECULE,  
PHARMACEUTICAL COMPOSITION COMPRISING THE SAME, THERAPEUTIC USE OF SAID  
PHARMACEUTICAL COMPOSITION

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## TECHNOLOGICAL FIELD

The invention relates to a conjugate for transferring an effector molecule from outside a cell into said cell, the conjugate comprising at least one effector molecule to be transferred into the cell, at least one  
10 saponin of the mono-desmosidic triterpene glycoside type or the bi-desmosidic triterpene glycoside type, and at least one single-domain antibody (sdAb), covalently bound to each other, wherein the sdAb is capable of binding to a cell-surface molecule of said cell. The invention also relates to a pharmaceutical composition comprising the conjugate of the invention. Furthermore, the invention relates to the pharmaceutical composition of the invention, for use as a medicament. In addition, the invention relates  
15 to a pharmaceutical composition of the invention, for use in the treatment or the prophylaxis of any one or more of: a cancer, an auto-immune disease such as rheumatoid arthritis, an enzyme deficiency, a disease related to an enzyme deficiency, a gene defect, a disease relating to a gene defect, an infection such as a viral infection, hypercholesterolemia, primary hyperoxaluria, haemophilia A, haemophilia B, alpha-1 antitrypsin related liver disease, acute hepatic porphyria, an amyloidosis and transthyretin-mediated amyloidosis. The invention also relates to an *in vitro* or *ex vivo* method for transferring the conjugate from outside a cell to inside said cell or for transferring the effector molecule comprised by  
20 the conjugate of the invention from outside a cell to inside said cell, preferably to the cytosol of said cell.

## 25 BACKGROUND

Molecules with a therapeutic biological activity are in many occasions in theory suitable for application as an effective therapeutic drug for the treatment of a disease such as a cancer in human patients in need thereof. A typical example are small-molecule biologically active moieties. However, many if not  
30 all potential drug-like molecules and therapeutics currently used in the clinic suffer from at least one of a plethora of shortcomings and drawbacks. When administered to a human body, therapeutically active molecules may exert off-target effects, in addition to the desired biological activity which is directed to the treatment of a disease or health problem. Such off-target effects are undesired and bear a risk for induction of health- or even life-threatening side effects of the administered molecule. It is the occurrence  
35 of such adverse events that cause many drug-like compounds and therapeutic moieties to fail phase III clinical trials or even phase IV clinical trials (post-authorisation surveillance). Therefore, there is a strong desire to provide drug molecules such as small-molecule therapeutics, wherein the therapeutic effect of the drug molecule should, *e.g.*, (1) be highly specific for a biological factor or biological process driving the disease, (2) be sufficiently safe, (3) be sufficiently efficacious, (4) be sufficiently directed to the diseased cell with little to no off-target activity on non-diseased cells, (5) have a sufficiently timely mode  
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of action (e.g. the administered drug molecule should reach the targeted site in the human patient within a certain time frame and should remain at the targeted site for a certain time frame ), and/or (6) have sufficiently long lasting therapeutic activity in the patient's body, amongst others. Unfortunately, to date, 'ideal' therapeutics with many or even all of the beneficial characteristics here above outlined, are not available to the patients, despite already long-lasting and intensive research and despite the impressive progress made in several areas of the individually addressed encountered difficulties and drawbacks.

Chemotherapy is one of the most important therapeutic options for cancer treatment. However, it is often associated with a small therapeutic window because it has no specificity towards cancer cells over dividing cells in healthy tissue. The invention of monoclonal antibodies offered the possibility of exploiting their specific binding properties as a mechanism for the targeted delivery of cytotoxic agents to cancer cells, while sparing normal cells. This can be achieved by chemical conjugation of cytotoxic effectors (also known as payloads or warheads) to antibodies, to create antibody-drug conjugates (ADCs). Typically, very potent payloads such as emtansine (DM1) are used which have a limited therapeutic index (a ratio that compares toxic dose to efficacious dose) in their unconjugated forms. The conjugation of DM1 to trastuzumab (ado-trastuzumab emtansine), also known as Kadcycla, enhances the tolerable dose of DM1 at least two-fold in monkeys. In the past few decades tremendous efforts and investments have been made to develop therapeutic ADCs. However, it remains challenging to bring ADCs into the clinic, despite promising preclinical data. The first ADC approved for clinical use was gemtuzumab ozogamicin (Mylotarg, CD33 targeted, Pfizer/Wyeth) for relapsed acute myelogenous leukemia (AML) in 2000. Mylotarg was however, withdrawn from the market at the request of the Federal Drug Administration (FDA) due to a number of concerns including its safety profile. Patients treated with Mylotarg were more often found to die than patients treated with conventional chemotherapy. Mylotarg was admitted to the market again in 2017 with a lower recommended dose, a different schedule in combination with chemotherapy or on its own, and a new patient population. To date, only five ADCs have been approved for clinical use, and meanwhile clinical development of approximately fifty-five ADCs has been halted. However, interest remains high and approximately eighty ADCs are still in clinical development in nearly six-hundred clinical trials at present.

Despite the potential to use toxic payloads that are normally not tolerated by patients, a low therapeutic index is a major problem accounting for the discontinuance of many ADCs in clinical development, which can be caused by several mechanisms such as off-target toxicity on normal cells, development of resistance against the cytotoxic agents and premature release of drugs in the circulation. A systematic review by the FDA of ADCs found that the toxicity profiles of most ADCs could be categorized according to the payload used, but not the antibody used, suggesting that toxicity is mostly determined by premature release of the payload. Of the approximately fifty-five ADCs that were discontinued, it is estimated that at least twenty-three were due to a poor therapeutic index. For example, development of a trastuzumab tesirine conjugate (ADCT-502, HER-2 targeted, ADC therapeutics) was recently discontinued due to a low therapeutic index, possibly due to an on-target, off-tissue effect in pulmonary tissue which expresses considerable levels of HER2. In addition, several ADCs in phase 3 trials have been discontinued due to missing primary endpoint. For example, phase 3 trials of a depatuxizumab mafodotin conjugate (ABT-414, EGFR targeted, AbbVie) tested in patients with newly

diagnosed glioblastoma, and a mirvetuximab soravtansine conjugate (IMGN853, folate receptor alpha (FR $\alpha$ ) targeted, ImmunoGen) tested in patients with platinum-resistant ovarian cancer, were recently stopped, showing no survival benefit. It is important to note that the clinically usable dose of some ADCs may not be sufficient for its full anticancer activity. For example, ado-trastuzumab emtansine has an MTD of 3.6 mg/kg in humans. In preclinical models of breast cancer, ado-trastuzumab emtansine induced tumor regression at dose levels at or above 3 mg/kg, but more potent efficacy was observed at 15 mg/kg. This suggests that at the clinically administered dose, ado-trastuzumab emtansine may not exert its maximal potential anti-tumor effect.

ADCs are mainly composed of an antibody, a cytotoxic moiety such as a payload, and a linker. Several novel strategies have been proposed and carried out in the design and development of new ADCs to overcome the existing problems, targeting each of the components of ADCs. For example, by identification and validation of adequate antigenic targets for the antibody component, by selecting antigens which have high expression levels in tumor and little or no expression in normal tissues, antigens which are present on the cell surface to be accessible to the circulating ADCs, and antigens which allows internalizing of ADCs into the cell after binding; and alternative mechanisms of activity; design and optimize linkers which enhance the solubility and the drug-to-antibody ratio (DAR) of ADCs and overcome resistance induced by proteins that can transport the chemotherapeutic agent out of the cells; enhance the DAR ratio by inclusion of more payloads, select and optimize antibodies to improve antibody homogeneity and developability. In addition to the technological development of ADCs, new clinical and translational strategies are also being deployed to maximize the therapeutic index, such as, change dosing schedules through fractionated dosing; perform biodistribution studies; include biomarkers to optimize patient selection, to capture response signals early and monitor the duration and depth of response, and to inform combination studies.

An example of ADCs with clinical potential are those ADCs such as brentuximab vedotin, inotuzumab ozogamicin, moxetumomab pasudotox, and polatuzumab vedotin, which are evaluated as a treatment option for lymphoid malignancies and multiple myeloma. Polatuzumab vedotin, binding to CD79b on (malignant) B-cells, and pinatuzumab vedotin, binding to CD22, are tested in clinical trials wherein the ADCs each were combined with co-administered rituximab, a monoclonal antibody binding to CD20 and not provided with a payload [B. Yu and D. Liu, *Antibody-drug conjugates in clinical trials for lymphoid malignancies and multiple myeloma; Journal of Hematology & Oncology* (2019) 12:94]. Combinations of monoclonal antibodies such as these examples are yet a further approach and attempt to arrive at the 'magic bullet' which combines many or even all of the aforementioned desired characteristics of ADCs.

Meanwhile in the past few decades, nucleic acid-based therapeutics are under development. Therapeutic nucleic acids can be based on deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), Anti-sense oligonucleotides (ASOs, AONs), and short interfering RNAs (siRNAs), MicroRNAs, and DNA and RNA aptamers, for approaches such as gene therapy, RNA interference (RNAi). Many of them share the same fundamental basis of action by inhibition of either DNA or RNA expression, thereby preventing expression of disease-related abnormal proteins. The largest number of clinical trials is being carried out in the field of gene therapy, with almost 2600 ongoing or completed clinical trials worldwide but with

only about 4% entering phase 3. This is followed by clinical trials with ASOs. Similarly to ADCs, despite the large number of techniques being explored, therapeutic nucleic acids share two major issues during clinical development: delivery into cells and off-target effects. For instance, ASOs such as peptide nucleic acid (PNA), phosphoramidate morpholino oligomer (PMO), locked nucleic acid (LNA) and bridged nucleic acid (BNA), are being investigated as an attractive strategy to inhibit specifically target genes and especially those genes that are difficult to target with small molecules inhibitors or neutralizing antibodies. Currently, the efficacy of different ASOs is being studied in many neurodegenerative diseases such as Huntington's disease, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis and also in several cancer stages. The application of ASOs as potential therapeutic agents requires safe and effective methods for their delivery to the cytoplasm and/or nucleus of the target cells and tissues. Although the clinical relevance of ASOs has been demonstrated, inefficient cellular uptake, both *in vitro* and *in vivo*, limit the efficacy of ASOs and has been a barrier to therapeutic development. Cellular uptake can be < 2% of the dose resulting in too low ASO concentration at the active site for an effective and sustained outcome. This consequently requires an increase of the administered dose which induces off-target effects. Most common side-effects are activation of the complement cascade, the inhibition of the clotting cascade and toll-like receptor mediated stimulation of the immune system.

Chemotherapeutics are most commonly small molecules, however, their efficacy is hampered by the severe off-target side toxicity, as well as their poor solubility, rapid clearance and limited tumor exposure. Scaffold-small-molecule drug conjugates such as polymer-drug conjugates (PDCs) are macromolecular constructs with pharmacological activity, which comprises one or more molecules of a small-molecule drug bound to a carrier scaffold (e.g. polyethylene glycol (PEG)).

Such conjugate principle has attracted much attention and has been under investigation for several decades. The majority of conjugates of small-molecule drugs under pre-clinical or clinical development are for oncological indications. However, up-to-date only one drug not related to cancer has been approved (Movantik, a PEG oligomer conjugate of opioid antagonist naloxone, AstraZeneca) for opioid-induced constipation in patients with chronic pain in 2014, which is a non-oncology indication. Translating application of drug-scaffold conjugates into treatment of human subjects provides little clinical success so far. For example, PK1 (N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer doxorubicin; development by Pharmacia, Pfizer) showed great anti-cancer activity in both solid tumors and leukemia in murine models, and was under clinical investigation for oncological indications. Despite that it demonstrated significant reduction of nonspecific toxicity and improved pharmacokinetics in man, improvements in anticancer efficacy turned out to be marginal in patients, and as a consequence further development of PK1 was discontinued.

The failure of scaffold-small-molecule drug conjugates is at least partially attributed to its poor accumulation at the tumor site. For example, while in murine models PK1 showed 45–250 times higher accumulation in the tumor than in healthy tissues (liver, kidney, lung, spleen, and heart), accumulation in tumor was only observed in a small subset of patients in the clinical trial.

A potential solution to the aforementioned problems is application of nanoparticle systems for drug delivery such as liposomes. Liposomes are sphere-shaped vesicles consisting of one or more

phospholipid bilayers, which are spontaneously formed when phospholipids are dispersed in water. The amphiphilicity characteristics of the phospholipids provide it with the properties of self-assembly, emulsifying and wetting characteristics, and these properties can be employed in the design of new drugs and new drug delivery systems. Drug encapsulation in a liposomal delivery system may convey several advantages over a direct administration of the drug, such as an improvement of and control over pharmacokinetics and pharmacodynamics, tissue targeting property, decreased toxicity and enhanced drug activity. An example of such success is liposome-encapsulated form of a small molecule chemotherapy agent doxorubicin (Doxil: a pegylated liposome-encapsulated form of doxorubicin; Myocet: a non-pegylated liposomal doxorubicin), which have been approved for clinical use.

Therefore, a solution still needs to be found that allows for drug therapies such as anti-tumor therapies, applicable for non-systemic use when desired, wherein the drug has for example an acceptable safety profile, little off-target activity, sufficient efficacy, sufficiently low clearance rate from the patient's body, sufficiently wide therapeutic window, *etc.*

In European patent EP1623715B1, a composition comprising a pharmacologically active agent coupled to a target-cell specific binding molecule, combined with a saponin, has been described. The pharmacologically active agent is for example a toxin.

## SUMMARY

For an embodiment of the present invention, it is a first goal to provide an improved biologically active compound or composition comprising such improved biologically active compound.

It is one of several objectives of embodiments of the current invention to provide a solution to the problem of non-specificity, encountered when administering (small-molecule) therapeutically active compounds to a human patient in need thereof. It is one of several objectives of embodiments of the current invention to provide a solution to the problem of drugs with non-optimal specificity for a biological factor or biological process driving a disease. It is one of several objectives of embodiments of the current invention to provide a solution to the problem of insufficient safety characteristics of current drugs, when administered to human patients in need thereof. It is one of several objectives of embodiments of the current invention to provide a solution to the problem of current drugs being less efficacious than desired, when administered to human patients in need thereof. It is one of several objectives of embodiments of the current invention to provide a solution to the problem of current drugs being not sufficiently directed to the diseased cell with little to no off-target activity on non-diseased cells, when administered to human patients in need thereof. It is one of several objectives of embodiments of the current invention to provide a solution to the problem that current drugs do not have a sufficiently timely mode of action (*e.g.* the administered drug molecule should reach the targeted site in the human patient within a certain time frame and should remain at the targeted site for a certain time frame), when administered to human patients in need thereof. It is one of several objectives of embodiments of the current invention to provide a solution to the problem that current drugs have not sufficiently long lasting therapeutic activity in the patient's body, when administered to human patients in need thereof.

At least one of the above objectives of embodiments of the invention is achieved by providing an antibody-drug conjugate (ADC) or an antibody-oligonucleotide (AOC) such as an antibody-BNA covalent complex, of the invention, comprising a cell-targeting moiety which is a single-domain antibody (sdAb) such as a  $V_{HH}$ , and at least one saponin and at least one effector moiety such as a proteinaceous toxin and/or an oligonucleotide such as a BNA, the ADC provided with a covalently linked saponin and/or the AOC provided with a covalently linked saponin also suitable for use as a medicament, according to the invention.

The present invention will be described with respect to particular embodiments but the invention is not limited thereto but only by the claims. The embodiments of the invention described herein can operate in combination and cooperation, unless specified otherwise.

A first aspect of the invention relates to a conjugate for transferring an effector molecule from outside a cell into said cell, the conjugate comprising at least one effector molecule to be transferred into the cell, at least one single-domain antibody (sdAb) and at least one saponin, covalently bound to each other, directly or via at least one linker, wherein the at least one saponin is a mono-desmosidic triterpene glycoside or is a bi-desmosidic triterpene glycoside, and wherein the sdAb is capable of binding to a cell-surface molecule of said cell. If the conjugate comprises more than one sdAb, these sdAb's either bind to the same cell-surface molecule present on the same cell, that is to say to the same molecule or to different copies of the same type of cell-surface molecule, or these sdAb's bind to a first cell-surface molecule and to a second cell-surface molecule which is present at the same cell as the first cell-surface molecule. The cell-surface molecule(s) is/are typically (a) cell-surface receptor.

A second aspect of the invention relates to a pharmaceutical composition comprising the conjugate of the invention, and optionally a pharmaceutically acceptable excipient and/or pharmaceutically acceptable diluent.

A third aspect of the invention relates to a pharmaceutical composition of the invention, for use as a medicament.

A fourth aspect of the invention relates to a pharmaceutical composition of the invention, for use in the treatment or the prophylaxis of any one or more of: a cancer, an auto-immune disease such as rheumatoid arthritis, an enzyme deficiency, a disease related to an enzyme deficiency, a gene defect, a disease relating to a gene defect, an infection such as a viral infection, hypercholesterolemia, primary hyperoxaluria, haemophilia A, haemophilia B, alpha-1 antitrypsin related liver disease, acute hepatic porphyria, an amyloidosis and transthyretin-mediated amyloidosis.

A fifth aspect of the invention relates to an *in vitro* or *ex vivo* method for transferring the effector molecule of the invention (the effector molecule comprised by the conjugate of the invention) from outside a cell to inside said cell, preferably to the cytosol of said cell, comprising the steps of:

- a) providing a cell which expresses on its cell surface the binding site for the at least one sdAb comprised by the conjugate of the invention, said binding site preferably being present on a cell-surface molecule of the cell, as described herein, said cell preferably being selected from a liver cell, an aberrant cell such as a virally infected cell, an auto-immune cell, a cell comprising a gene defect, a cell comprising an enzyme deficiency and a tumor cell;

- b) providing the conjugate of the invention, said conjugate comprising the effector molecule to be transferred into the cell provided in step a); and
- c) contacting the cell of step a) *in vitro* or *ex vivo* with the conjugate of step b), therewith effecting the transfer of said conjugate comprising the effector molecule from outside the cell to inside said cell, and by effecting the transfer of said conjugate effecting the transfer of the effector molecule from outside the cell to inside said cell, preferably into the cytosol of said cell.

A sixth aspect of the invention relates to an *in vitro* or *ex vivo* method for transferring the conjugate of the invention from outside a cell to inside said cell, comprising the steps of:

- a) providing a cell which expresses on its cell surface the binding site for the at least one sdAb comprised by the conjugate of the invention, said binding site preferably being present on a cell-surface molecule of the cell, as described herein, said cell preferably being selected from a liver cell, an aberrant cell such as a virally infected cell, an auto-immune cell, a cell comprising a gene defect, a cell comprising an enzyme deficiency and a tumor cell;
- b) providing the conjugate of any one of the invention; and
- c) contacting the cell of step a) *in vitro* or *ex vivo* with the conjugate of step b), therewith effecting the transfer of the conjugate from outside the cell to inside said cell.

An aspect of the invention relates to a kit of parts, comprising the conjugate of the invention or the pharmaceutical composition of the invention, and instructions for use of said conjugate or said pharmaceutical composition in the use for treatment or prophylaxis of any one or more of: a cancer, an auto-immune disease such as rheumatoid arthritis, an enzyme deficiency, a disease related to an enzyme deficiency, a gene defect, a disease relating to a gene defect, an infection such as a viral infection, hypercholesterolemia, primary hyperoxaluria, haemophilia A, haemophilia B, alpha-1 antitrypsin related liver disease, acute hepatic porphyria, an amyloidosis and transthyretin-mediated amyloidosis, or instructions for application of the *in vitro* or *ex vivo* methods according to the invention.

An aspect of the invention relates to a conjugate such as an ADC or an AOCs, or to a semi-finished ADC conjugate or a semi-finished AOC conjugate, comprising a cell-surface molecule targeting molecule such as an sdAb of the invention and comprising at least one effector moiety of the invention and/or comprising at least one saponin of the invention, of Structure C:

$$A(-S)_b(-E)_c$$

(Structure C)

wherein A is the cell-surface molecule targeting molecule such as the sdAb;

S is the saponin;

E is the effector moiety;

b = 0 – 64, preferably 0, 1, 2, 3, 4, 8, 16, 32, 64 or any whole number (or fraction) therein between;

c = 0 – 8, preferably 0, 1, 2, 3, 4, 6, 8 or any whole number (or fraction) therein between,

wherein S is coupled to A and/or to E, E is coupled to A and/or to S, preferably S is coupled to A and E is coupled to A.

## DEFINITIONS

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The term "proteinaceous" has its regular scientific meaning and here refers to a molecule that is protein-like, meaning that the molecule possesses, to some degree, the physicochemical properties characteristic of a protein, is of protein, relating to protein, containing protein, pertaining to protein, consisting of protein, resembling protein, or being a protein. The term "proteinaceous" as used in for example 'proteinaceous molecule' refers to the presence of at least a part of the molecule that resembles or is a protein, wherein 'protein' is to be understood to include a chain of amino-acid residues at least two residues long, thus including a peptide, a polypeptide and a protein and an assembly of proteins or protein domains. In the proteinaceous molecule, the at least two amino-acid residues are for example bound via (an) amide bond(s), such as (a) peptide bond(s). In the proteinaceous molecule, the amino-acid residues are natural amino-acid residues and/or artificial amino-acid residues such as modified natural amino-acid residues. In a preferred embodiment, a proteinaceous molecule is a molecule comprising at least two amino-acid residues, preferably between two and about 2.000 amino-acid residues. In one embodiment, a proteinaceous molecule is a molecule comprising from 2 to 20 (typical for a peptide) amino acids. In one embodiment, a proteinaceous molecule is a molecule comprising from 21 to 1.000 (typical for a polypeptide, a protein, a protein domain, such as an antibody, a Fab, an scFv, a ligand for a receptor such as EGF) amino acids. Preferably, the amino-acid residues are (typically) bound via (a) peptide bond(s). According to the invention, said amino-acid residues are or comprise (modified) (non-)natural amino acid residues.

The term "effector molecule", or "effector moiety" when referring to the effector molecule as part of *e.g.* a covalent conjugate, has its regular scientific meaning and here refers to a molecule that can selectively bind to for example any one or more of the target molecules: a protein, a peptide, a carbohydrate, a saccharide such as a glycan, a (phospho)lipid, a nucleic acid such as DNA, RNA, an enzyme, and regulates the biological activity of such one or more target molecule(s). In the conjugate of the invention the effector moiety for example exerts its effect in the cytosol and/or in the cell nucleus, and/or is delivered intracellularly in the endosome and/or lysosome and/or is active after exiting or escaping the endosomal-lysosomal pathway. The effector molecule is for example a molecule selected from any one or more of a small molecule such as a drug molecule, a toxin such as a protein toxin, an oligonucleotide such as a BNA, a xeno nucleic acid or an siRNA, an enzyme, a peptide, a protein, or an active fragment or active domain thereof, or any combination thereof. Thus, for example, an effector molecule or an effector moiety is a molecule or moiety selected from any one or more of a small molecule such as a drug molecule, a toxin such as a protein toxin, an oligonucleotide such as a BNA, a xeno nucleic acid or an siRNA, an enzyme, a peptide, a protein, or any combination thereof, that can selectively bind to any one or more of the target molecules: a protein, a peptide, a carbohydrate, a saccharide such as a glycan, a (phospho)lipid, a nucleic acid such as DNA, RNA, an enzyme, and that upon binding to the target molecule regulates the biological activity of such one or more target

molecule(s). For example, an effector moiety is a toxin or an active toxic fragment thereof or an active toxic derivative or an active toxic domain thereof. Typically, an effector molecule can exert a biological effect inside a cell such as a mammalian cell such as a human cell, such as in the cytosol of said cell. An effector molecule or moiety of the invention is thus any substance that affects the metabolism of a cell by interaction with an intracellular effector molecule target, wherein this effector molecule target is any molecule or structure inside cells excluding the lumen of compartments and vesicles of the endocytic and recycling pathway but including the membranes of these compartments and vesicles. Said structures inside cells thus include the nucleus, mitochondria, chloroplasts, endoplasmic reticulum, Golgi apparatus, other transport vesicles, the inner part of the plasma membrane and the cytosol. Typical effector molecules are thus drug molecules, an enzyme, plasmid DNA, toxins such as toxins comprised by antibody-drug conjugates (ADCs), oligonucleotides such as siRNA, BNA, nucleic acids comprised by an antibody-oligonucleotide conjugate (AOC). For example, an effector molecule is a molecule which can act as a ligand that can increase or decrease (intracellular) enzyme activity, gene expression, or cell signalling. Typically, an effector moiety comprised by the conjugate exerts its therapeutic (for example toxic, enzymatic, inhibitory, gene silencing, *etc.*) effect in the cytosol and/or in the cell nucleus. Typically, the effector moiety is delivered intracellularly in the endosome and/or in the lysosome, and typically the effector moiety is active after exiting or escaping the endosomal-lysosomal pathway.

The term "saponin" has its regular scientific meaning and here refers to a group of amphipatic glycosides which comprise one or more hydrophilic glycone moieties combined with a lipophilic aglycone core which is a sapogenin. The saponin may be naturally occurring or synthetic (*i.e.* non-naturally occurring). The term "saponin" includes naturally-occurring saponins, functional derivatives of naturally-occurring saponins as well as saponins synthesized *de novo* through chemical and/or biotechnological synthesis routes. Saponin has a triterpene backbone, which is a pentacyclic C30 terpene skeleton, also referred to as sapogenin or aglycone. Within the conjugate of the invention saponin is not considered an effector molecule nor an effector moiety in the conjugates according to the invention. Thus, in the conjugates comprising a saponin and an effector moiety, the effector moiety is a different molecule than the conjugated saponin.

The term "saponin derivative" (also known as "modified saponin") has its regular scientific meaning and here refers to a compound corresponding to a naturally-occurring saponin which has been derivatised by one or more chemical modifications, such as the oxidation of a functional group, the reduction of a functional group and/or the formation of a covalent bond with another molecule (also referred to as "conjugation" or as "covalent conjugation"). Preferred modifications include derivatisation of an aldehyde group of the aglycone core; of a carboxyl group of a saccharide chain or of an acetoxy group of a saccharide chain. Typically, the saponin derivative does not have a natural counterpart, *i.e.* the saponin derivative is not produced naturally by *e.g.* plants or trees. The term "saponin derivative" includes derivatives obtained by derivatisation of naturally-occurring saponins as well as derivatives synthesized *de novo* through chemical and/or biotechnological synthesis routes resulting in a compound corresponding to a naturally-occurring saponin which has been derivatised by one or more chemical modifications. A saponin derivative in the context of the invention should be understood as a saponin

functional derivative. "Functional" in the context of the saponin derivative is understood as the capacity or activity of the saponin or the saponin derivative to enhance the endosomal escape of an effector molecule which is contacted with a cell together with the saponin or the saponin derivative.

5 The term "aglycone core structure" has its regular scientific meaning and here refers to the aglycone core of a saponin without the one or two carbohydrate antenna or saccharide chains (glycans) bound thereto. For example, quillaic acid is the aglycone core structure for SO1861, QS-7 and QS21. Typically, the glycans of a saponin are mono-saccharides or oligo-saccharides, such as linear or branched glycans.

10 The term "saccharide chain" has its regular scientific meaning and here refers to any of a glycan, a carbohydrate antenna, a single saccharide moiety (mono-saccharide) or a chain comprising multiple saccharide moieties (oligosaccharide, polysaccharide). The saccharide chain can consist of only saccharide moieties or may also comprise further moieties such as any one of 4E-Methoxycinnamic acid, 4Z-Methoxycinnamic acid, and 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid), such as for example present in QS-21.

15 The term "Api/Xyl-" or "Api- or Xyl-" in the context of the name of a saccharide chain has its regular scientific meaning and here refers to the saccharide chain either comprising an apiose (Api) moiety, or comprising a xylose (Xyl) moiety.

20 The term "saponin on which the modified saponin is based" has its regular scientific meaning and here refers to a saponin that has been modified in order to provide the modified saponin. Typically, the saponin on which the modified saponin is based is a naturally occurring saponin, which is subjected to chemical modification for the provision of the modified saponin.

25 The term "modified saponin based on a saponin" has its regular scientific meaning and here refers to a saponin that has been subjected to a chemical modification step such that the modified saponin is provided, wherein the saponin from which the modified saponin has been made is typically a naturally occurring saponin.

30 The term "oligonucleotide" has its regular scientific meaning and here refers to amongst others any natural or synthetic string of nucleic acids encompassing DNA, modified DNA, RNA, mRNA, modified RNA, synthetic nucleic acids, presented as a single-stranded molecule or a double-stranded molecule, such as a BNA, an antisense oligonucleotide (ASO, AON), a short or small interfering RNA (siRNA; silencing RNA), an anti-sense DNA, anti-sense RNA, *etc.* The term "oligonucleotide" here also refers to a string of two or more nucleotides, *i.e.*, an oligonucleotide is a short oligomer composed of ribonucleotides or deoxyribonucleotides. Examples are RNA and DNA, and any modified RNA or DNA, such as a string of nucleic acids comprising a nucleotide analogue such as a bridged nucleic acid (BNA), also known as locked nucleic acid (LNA) or a 2'-O,4'-C-aminoethylene or a 2'-O,4'-C-aminomethylene bridged nucleic acid (BNA<sup>NC</sup>), wherein the nucleotide is a ribonucleotide or a deoxyribonucleotide.

35 The term "antibody-drug conjugate" or "ADC" has its regular scientific meaning and here refers to any conjugate of an antibody such as an IgG, a Fab, an scFv, an immunoglobulin, an immunoglobulin fragment, one or multiple V<sub>H</sub> domains, single-domain antibodies, a V<sub>HH</sub>, a camelid V<sub>H</sub>, *etc.*, and any molecule that can exert a therapeutic effect when contacted with cells of a subject such as a human

patient, such as an active pharmaceutical ingredient, a toxin, an oligonucleotide, an enzyme, a small molecule drug compound, *etc.*

The term “antibody-oligonucleotide conjugate” or “AOC” has its regular scientific meaning and here refers to any conjugate of an antibody such as an IgG, a Fab, an scFv, an immunoglobulin, an immunoglobulin fragment, one or multiple V<sub>H</sub> domains, single-domain antibodies, a V<sub>HH</sub>, a camelid V<sub>H</sub>, *etc.*, and any oligonucleotide molecule that can exert a therapeutic effect when contacted with cells of a subject such as a human patient, such as an oligonucleotide selected from a natural or synthetic string of nucleic acids encompassing DNA, modified DNA, RNA, mRNA, modified RNA, synthetic nucleic acids, presented as a single-stranded molecule or a double-stranded molecule, such as a BNA, an antisense oligonucleotide (ASO, AON), a short or small interfering RNA (siRNA; silencing RNA), an anti-sense DNA, anti-sense RNA, *etc.*

The term “bridged nucleic acid”, or “BNA” in short, or “locked nucleic acid” or “LNA” in short or 2'-O,4'-C-aminoethylene or 2'-O,4'-C-aminomethylene bridged nucleic acid (BNA<sup>NC</sup>), has its regular scientific meaning and here refers to a modified RNA nucleotide. A BNA is also referred to as ‘constrained RNA molecule’ or ‘inaccessible RNA molecule’. A BNA monomer can contain a five-membered, six-membered or even a seven-membered bridged structure with a “fixed” C<sub>3</sub>'-endo sugar puckering. The bridge is synthetically incorporated at the 2', 4'-position of the ribose to afford a 2', 4'-BNA monomer. A BNA monomer can be incorporated into an oligonucleotide polymeric structure using standard phosphoramidite chemistry known in the art. A BNA is a structurally rigid oligonucleotide with increased binding affinity and stability.

The term “single domain antibody”, or “sdAb”, in short, has its regular scientific meaning and here refers to an antibody fragment consisting of a single monomeric variable antibody domain. In the conjugates of the invention, more than one sdAb can be present, which sdAb's can be the same (multivalent and mono-specific) or can be different (multivalent and/or for example multi-paratope, bi-paratope, multi-specific, bi-specific). In addition, for example the more than two sdAb's are for example a combination of mono-specific and multivalent sdAb's and at least one further sdAb that binds to a different epitope (*e.g.* multispecific or biparatope).

The term ‘S’ as used such as in an antibody-saponin conjugate comprising a linker, represents ‘stable linker’ which remains intact in the endosome and in the lysosome of mammalian cells, such as human cells, such as a human tumor cell, thus under slightly acid conditions (pH < 6.6, such as pH 4.0 – 5.5).

The term ‘L’ as used such as in an antibody-saponin conjugate comprising a linker, represents ‘labile linker’ which is cleaved under slightly acid conditions (pH < 6.6, such as pH 4.0 – 5.5) in the endosome and in the lysosome of mammalian cells, such as human cells, such as a human tumor cell.

The terms first, second, third and the like in the description and in the claims, are used for distinguishing between for example similar elements, compositions, constituents in a composition, or separate method steps, and not necessarily for describing a sequential or chronological order. The terms are interchangeable under appropriate circumstances and the embodiments of the invention can operate in other sequences than described or illustrated herein, unless specified otherwise.

The embodiments of the invention described herein can operate in combination and cooperation, unless specified otherwise.

Furthermore, the various embodiments, although referred to as "preferred" or "e.g." or "for example" or "in particular" and the like are to be construed as exemplary manners in which the invention may be implemented rather than as limiting the scope of the invention.

The term "comprising", used in the claims, should not be interpreted as being restricted to for example the elements or the method steps or the constituents of a compositions listed thereafter; it does not exclude other elements or method steps or constituents in a certain composition. It needs to be interpreted as specifying the presence of the stated features, integers, (method) steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or components, or groups thereof. Thus, the scope of the expression "a method comprising steps A and B" should not be limited to a method consisting only of steps A and B, rather with respect to the present invention, the only enumerated steps of the method are A and B, and further the claim should be interpreted as including equivalents of those method steps. Thus, the scope of the expression "a composition comprising components A and B" should not be limited to a composition consisting only of components A and B, rather with respect to the present invention, the only enumerated components of the composition are A and B, and further the claim should be interpreted as including equivalents of those components.

In addition, reference to an element or a component by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element or component are present, unless the context clearly requires that there is one and only one of the elements or components. The indefinite article "a" or "an" thus usually means "at least one".

The term "Saponinum album" has its normal meaning and here refers to a mixture of saponins produced by Merck KGaA (Darmstadt, Germany) containing saponins from *Gypsophila paniculata* and *Gypsophila arostii*, containing SA1657 and mainly SA1641.

The term "Quillaja saponin" has its normal meaning and here refers to the saponin fraction of Quillaja saponaria and thus the source for all other QS saponins, mainly containing QS-18 and QS-21.

"QS-21" or "QS21" has its regular scientific meaning and here refers to a mixture of QS-21 A-apio (~63%), QS-21 A-xylo (~32%), QS-21 B-apio (~3.3%), and QS-21 B-xylo (~1.7%).

Similarly, "QS-21A" has its regular scientific meaning and here refers to a mixture of QS-21 A-apio (~65%) and QS-21 A-xylo (~35%).

Similarly, "QS-21B" has its regular scientific meaning and here refers to a mixture of QS-21 B-apio (~65%) and QS-21 B-xylo (~35%).

The term "Quil-A" refers to a commercially available semi-purified extract from Quillaja saponaria and contains variable quantities of more than 50 distinct saponins, many of which incorporate the triterpene-trisaccharide substructure Gal-(1→2)-[Xyl-(1→3)]-GlcA- at the C-3β-OH group found in QS-7, QS-17, QS18, and QS-21. The saponins found in Quil-A are listed in van Setten (1995), Table 2 [Dirk C. van Setten, Gerrit van de Werken, Gijsbert Zomer and Gideon F. A. Kersten, *Glycosyl Compositions and Structural Characteristics of the Potential Immuno-adjuvant Active Saponins in the Quillaja saponaria Molina Extract Quil A*, RAPID COMMUNICATIONS IN MASS SPECTROMETRY,

VOL. 9,660-666 (1995)]. Quil-A and also Quillaja saponin are fractions of saponins from Quillaja saponaria and both contain a large variety of different saponins with largely overlapping content. The two fractions differ in their specific composition as the two fractions are gained by different purification procedures.

5           The term "QS1861" and the term "QS1862" refer to QS-7 and QS-7 api. QS1861 has a molecular mass of 1861 Dalton, QS1862 has a molecular mass of 1862 Dalton. QS1862 is described in Fleck *et al.* (2019) in Table 1, row no. 28 [Juliane Deise Fleck, Andresa Heemann Betti, Francini Pereira da Silva, Eduardo Artur Troian, Cristina Olivaro, Fernando Ferreira and Simone Gasparin Verza, *Saponins from Quillaja saponaria and Quillaja brasiliensis: Particular Chemical Characteristics and Biological Activities*,  
10 *Molecules* 2019, 24, 171; doi:10.3390/molecules24010171]. The described structure is the api-variant QS1862 of QS-7. The molecular mass is 1862 Dalton as this mass is the formal mass including proton at the glucuronic acid. At neutral pH, the molecule is deprotonated. When measuring in mass spectrometry in negative ion mode, the measured mass is 1861 Dalton.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1A (Fig. 1A):** *The targeted 2-component approach (1 target).* SO1861 and toxin (e.g. ribosomal inactivating protein) are each, separately, conjugated to a V<sub>HH</sub> or antibody (mAb) for delivery and internalization into target cells. 1) mAb-toxin and V<sub>HH</sub>-SO1861 bind to the cell surface receptor, 2) receptor-mediated endocytosis of both conjugates occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endolysosomal escape, 4) release of toxin  
25 into cytoplasm occurs and 5) toxin induces cell death.

**Figure 1B (Fig. 1B):** *The targeted 2-component approach (2 targets).* SO1861 and toxin (ribosomal inactivating protein) are each, separately, conjugated to a V<sub>HH</sub> or antibody (mAb) for delivery and internalization into target cells. 1) mAb-toxin and V<sub>HH</sub>-SO1861 bind to their corresponding cell surface receptor, 2) receptor-mediated endocytosis of both conjugates occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endolysosomal escape, 4) release of toxin into cytoplasm occurs and 5) toxin induces cell death.

**Figure 1C (Fig. 1C):** *The targeted 2-component approach (2 targets).* SO1861 and toxin (ribosomal inactivating protein) are each, separately, conjugated to a V<sub>HH</sub> for delivery and internalization into target cells. 1) V<sub>HH</sub>-toxin and V<sub>HH</sub>-SO1861 bind to their corresponding cell surface receptor, 2) receptor-mediated endocytosis of both conjugates occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endolysosomal escape, 4) release of toxin into cytoplasm occurs and 5) toxin induces cell death.

**Figure 1D (Fig. 1D):** *The targeted 2-component approach (2 targets).* SO1861 and toxin (ribosomal inactivating protein) are each, separately, conjugated to a  $V_{HH}$  or mAb for delivery and internalization into target cells. 1)  $V_{HH}$ -toxin and mAb-SO1861 bind to their corresponding cell surface receptor, 2) receptor-mediated endocytosis of both conjugates occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endolysosomal escape, 4) release of toxin into cytoplasm occurs and 5) toxin induces cell death.

**Figure 1E (Fig. 1E):** *The targeted 2-component approach (1 target).* SO1861 and toxin (ribosomal inactivating protein) are each, separately, conjugated to a  $V_{HH}$  or antibody (mAb) for delivery and internalization into target cells. 1)  $V_{HH}$ -toxin and mAb-SO1861 bind to the cell surface receptor, 2) receptor-mediated endocytosis of both conjugates occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endolysosomal escape, 4) release of toxin into cytoplasm occurs and 5) toxin induces cell death.

**Figure 1F (FIG. 1F):** Cartoon displaying an exemplifying molecule and conjugate of the present invention. Shown is an IgG antibody covalently conjugated with four saponin molecules 'S', bound to the light chains of the antibody, and with four effector molecules 'E' that are covalently bound to the constant domains of the heavy chain of the antibody.

**Figure 1G (FIG. 1G):** Cartoon displaying an exemplifying molecule and conjugate of the present invention. Shown is an IgG antibody covalently conjugated with four trivalent linkers, wherein each linker is covalently bound to a saponin and is covalently bound to an effector molecule. The trivalent linkers are covalently bound to the antibody light chains.

**Figure 1H (FIG. 1H):** Cartoon displaying an exemplifying molecule and conjugate of the present invention. Shown is a single domain antibody covalently conjugated with two trivalent linkers, wherein each linker is covalently bound to a saponin and is covalently bound to an effector molecule.

**Figure 1I (FIG. 1I):** Cartoon displaying an exemplifying molecule and conjugate of the present invention. Shown is a single domain antibody covalently conjugated with a trivalent linker, wherein the trivalent linker is covalently bound to a saponin and is covalently bound to an effector molecule.

**Figure 1J (FIG. 1J): (S)<sub>n</sub> – (L)(E) concept:**  $mAb-(SO1861)^n(protein\ toxin)^m$ . Both, SO1861 covalently linked at the cysteine residues (Cys) and protein toxin (ribosomal inactivating protein) at the lysine residues are conjugated to the same antibody (mAb) for delivery and internalization into the target cells. 1)  $mAb-(Cys-L-SO1861)^4(Lys-protein\ toxin)^2$  bind to its corresponding cell surface receptor, 2) receptor-mediated endocytosis the conjugate occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endolysosomal escape, 4) release of toxin into cytoplasm occurs and 5) toxin induces cell death

**Figure 1K (FIG. 1K): (S)<sub>n</sub> – (L)(E) concept:**  $mAb-(SO1861)^n(antisense\ BNA\ oligo)^m$ . Both, SO1861, bound to the cysteine residues (Cys) and the antisense BNA oligonucleotide bound to the lysine residues are conjugated to the same antibody (mAb) for delivery and internalization into the target cells. 1)  $mAb-(Cys-SO1861)^4(Lys-BNAoligo)^2$  binds to its corresponding cell surface receptor, 2)

receptor-mediated endocytosis of both conjugates occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endosomal, lysosomal and endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endosomal, lysosomal and endolysosomal escape, 4) release of BNA oligo into cytoplasm occurs and 5) target gene silencing is induced.

**Figure 1L (FIG. 1L): (S)<sub>n</sub> – (L)(E) concept:** *mAb-(SO1861-scaffold-antisense BNA oligo)<sup>n</sup>*. the (SO1861-trifunctional linker-BNAoligo)<sup>n</sup> is conjugated to an antibody (mAb) for delivery and internalization into the target cells. The antibody is for example an IgG, or an sdAb such as a V<sub>HH</sub>. 1) mAb-(SO1861-trifunctional linker-BNAoligo)<sup>4</sup> binds to its corresponding cell surface receptor, 2) receptor-mediated endocytosis of both conjugates occurs (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex), 3) at low endolysosomal pH and appropriate concentration, SO1861 becomes active to enable endolysosomal escape, 4) release of BNA oligo into cytoplasm occurs and 5) target gene silencing is induced. The term “scaffold” in the context of the conjugates of the invention is to be understood as an oligomeric molecule or polymeric molecule bearing one or multiple chemical groups for covalent binding to one or multiple further molecule(s) such as saponin molecules and/or effector molecules such as a protein toxin or an oligonucleotide, and bearing at least one chemical group for covalent coupling to a protein such as an antibody, such as an IgG or an sdAb.

**Figure 2 (Fig. 2):** Cell killing (MTS) assay) with the combination treatment according to the invention of V<sub>HH</sub>(HER2)-SO1861 (DAR1) + 50 pM trastuzumab-saporin (DAR4) or 10 pM CD71mab-saporin (DAR4) on SK-BR-3 cells (HER2<sup>++</sup>/CD71<sup>+</sup>) (A) and MD-MB-468 cells (HER2<sup>-</sup>/CD71<sup>+</sup>) (B). The legend to Fig. 2B, depicted next to the graphs, also applies to Fig. 2A.

**Figure 3 (Fig. 3):** Cell killing (MTS) assay) with the combination treatment according to the invention of Trastuzumab-saporin (DAR4) or CD71-saporin (DAR4) + 900 nM HER2V<sub>HH</sub>-SO1861 (DAR1) on SK-BR-3 cells (HER2<sup>++</sup>/CD71<sup>+</sup>) (A) and MD-MB-468 cells (HER2<sup>-</sup>/CD71<sup>+</sup>) (B). The legend to Fig. 3B, depicted next to the graphs, also applies to Fig. 3A.

**Figure 4 (Fig. 4):** Cell killing (MTS) assay) with the combination treatment according to the invention of V<sub>HH</sub>(HER2)-SO1861 (DAR1) + 50 pM CD71V<sub>HH</sub>-dianthin (DAR1) on SK-BR-3 cells (HER2<sup>++</sup>/CD71<sup>+</sup>) (A) and MD-MB-468 cells (HER2<sup>-</sup>/CD71<sup>+</sup>) (B). The legend to Fig. 4B, depicted next to the graphs, also applies to Fig. 4A.

**Figure 5 (Fig. 5):** Cell killing (MTS) assay) with the combination treatment according to the invention of CD71V<sub>HH</sub>-dianthin (DAR1) + 900 nM HER2V<sub>HH</sub>-SO1861 (DAR1) on SK-BR-3 cells (HER2<sup>++</sup>/CD71<sup>+</sup>) (A) and MD-MB-468 cells (HER2<sup>-</sup>/CD71<sup>+</sup>) (B). The legend to Fig. 5B, depicted next to the graphs, also applies to Fig. 5A.

**Figure 6 (Fig. 6):** Cell killing (MTS) assay) with the combination treatment according to the invention of CD71V<sub>HH</sub>-dianthin (DAR1) + cetuximab-SO1861 (DAR4) or HER2V<sub>HH</sub>-dianthin (DAR1) + cetuximab-SO1861 (DAR4) or EGFRV<sub>HH</sub>-dianthin (DAR1) + cetuximab-SO1861 (DAR4) on A431 cells (EGFR<sup>+</sup>/HER<sup>+</sup>/CD71<sup>+</sup>) (A) and A2058 cells (EGFR<sup>-</sup>/HER<sup>+</sup>/CD71<sup>+</sup>) (B).

**Figure 7 (Fig. 7):** Cell killing (MTS) assay) with the combination treatment according to the invention of CD71V<sub>HH</sub>-dianthin (DAR1) + 77 nM cetuximab-SO1861 (DAR4) or HER2V<sub>HH</sub>-dianthin

(DAR1) + 77 nM cetuximab-SO1861 (DAR4) or EGFRV<sub>HH</sub>-dianthin (DAR1) + 77 nM cetuximab-SO1861 (DAR4) on SK-BR-3 cells (HER2<sup>++</sup>/EGFR<sup>+</sup>/CD71<sup>+</sup>) (A) and MDA-MB-468 cells (HER2<sup>-</sup>/EGFR<sup>++</sup>/CD71<sup>+</sup>) (B).

**Figure 8. (FIG. 8): Tumor targeted protein toxin delivery results in tumor volume reduction and tumor growth inhibition in vivo, in tumor bearing mice.** A) Dose escalation (intraperitoneal, i.p.) of cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2</sup> in A431 tumor bearing mice reveals tumor volume reduction at day 26, compared to the control. B, C) Dose escalation (intraperitoneal, i.p. (B) or intravenous i.v. (C)) of cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2</sup> in A431 tumor bearing mice reveals tumor growth reduction, compared to the controls. The term "Cys-L-" in the context of the covalent conjugates of the invention is to be understood as a covalent bond to a cysteine side chain, wherein the bond is labile and cleavable under the acidic conditions as present in the endosome and lysosome of mammalian cells such as a human cell, such as a human tumor cell. The term "Lys-S-" in the context of the covalent conjugates of the invention is to be understood as a covalent bond to a lysine side chain, wherein the bond is stable and not cleavable under the acidic conditions as present in the endosome and lysosome of mammalian cells such as a human cell, such as a human tumor cell.

**Figure 9. (FIG. 9): Tumor targeted antisense BNA oligonucleotide delivery and gene silencing in tumor bearing mice.** 30 mg/kg cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-HSP27BNA)<sup>1,8</sup> in A431 tumor bearing mice reveals induced efficient tumor targeted gene silencing, compared to the controls.

**Figure 10. (FIG. 10): Tumor targeted antisense BNA oligo nucleotide delivery and gene silencing in tumor bearing mice.** 30 mg/kg cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> in A431 tumor bearing mice reveals induced efficient tumor targeted gene silencing, compared to the controls.

**Figure 11 (FIG. 11): HER2 or EGFR targeted protein toxin delivery and cell killing in cancer cells (tumor cells).** A, B) Trastuzumab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-dianthin)<sup>1,7</sup> or Trastuzumab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-S-dianthin)<sup>1,7</sup> treatment and controls on SK-BR-3 cells (HER2<sup>++</sup>) and MDA-MB-468 cells (HER2<sup>-</sup>). REMARK: the legend for Figure 11B also relates to the graphs in Figure 11A. C, D) Cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2,0</sup> or Cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2,0</sup> treatment and controls on A431 cells (EGFR<sup>++</sup>) and A2058 cells (EGFR<sup>-</sup>). REMARK: the legend for Figure 11D also relates to the graphs in Figure 11C. Remark: For target receptor expression data of each cell line (determined by FACS analysis) see Table A2.

**Figure 12 (FIG. 12): EGFR targeted antisense BNA oligo delivery and gene silencing in cancer cells, according to the invention.** A,B) Cetuximab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-HSP27BNA)<sup>3,8</sup> treatment and controls on A431 cells (EGFR<sup>++</sup>) and A2058 cells (EGFR<sup>-</sup>). REMARK: the legend for Figure 12B also relates to the graphs in Figure 12A. Remark: For target receptor expression data of each cell line (determined by FACS analysis) see Table A2.

**Figure 13 (FIG. 13): HER2 targeted antisense BNA oligo delivery and gene silencing in cancer cells.** Trastuzumab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-HSP27BNA)<sup>3,5</sup> treatment and controls on SK-BR-3 cells (HER2<sup>++</sup>). Remark: For target receptor expression data of each cell line (determined by FACS analysis) see Table A2.

**Figure 14 (FIG. 14):** *EGFR targeted antisense BNA oligo delivery and gene silencing in cancer cells.* A,B) Cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> treatment and controls on A431 cells (EGFR<sup>++</sup>) and A2058 cells (EGFR<sup>-</sup>). Remark: For target receptor expression data of each cell line (determined by FACS analysis) see Table A2.

5 **Figure 15. (FIG. 15):** *1-target 2-component (EGFR high expression).* EGFR targeted cell killing in A431 (EGFR<sup>+++</sup>) and CaSKi (EGFR<sup>++</sup>), by a therapeutic combination. A, B) cetuximab-SO1861 titration in combination with a fixed concentration of 10 pM cetuximab-saporin shows that a 100-400 fold reduced concentration of conjugated SO1861 is required, versus unconjugated SO1861, to induce cell killing by cetuximab-saporin. C, D), Cetuximab-saporin titration in combination with 278 nM cetuximab-SO1861 kills cells in contrast to 300 nM unconjugated SO1861 + cetuximab-saporin. 1500 nM unconjugated SO1861 + cetuximab-saporin is more efficient compared to the therapeutic combination, since both cetuximab conjugates compete for the same EGFR receptor. Only simultaneous targeted delivery of both cetuximab conjugates leads to efficient cell-killing, in contrast to monotherapy with either conjugate alone. REMARK: the legend displayed in Fig. 15B also relates to the graphs in Fig. 15A.  
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15 REMARK: the legend displayed in Fig. 15D also relates to the graphs in Fig. 15C.

**Figure 16 (FIG. 16):** *1-target 2-component (EGFR no/low expression).* EGFR targeted cell killing in HeLa (EGFR<sup>+</sup>) and A2058 (EGFR<sup>-</sup>) cells, by a therapeutic combination. A, B) cetuximab-SO1861 titration in combination with a fixed concentration of 10 pM cetuximab-saporin does not induce cell killing by cetuximab-saporin. C, D), Cetuximab-saporin titration in combination with 278 nM cetuximab-SO1861 cannot induce cell killing. Low EGFR receptor expression is prohibitive for sufficient SO1861 to be delivered via antibody-mediated delivery, while 1500 nM of unconjugated SO1861 induces cell killing. REMARK: the legend displayed in Fig. 16B also relates to the graphs in Fig. 16A. REMARK: the legend displayed in Fig. 16D also relates to the graphs in Fig. 16C.  
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**Figure 17 (FIG. 17):** *1-target 2-component (HER2 high expression).* HER2 targeted cell killing in SKBR3 (HER2<sup>+++</sup>) cells by a therapeutic combination. A) Trastuzumab-SO1861 titration in combination with a fixed concentration of 673 pM trastuzumab-saporin shows that a 1000 fold reduced concentration of conjugated SO1861 is required, versus unconjugated SO1861, to induce cell killing by trastuzumab-saporin. B), Trastuzumab-saporin titration in combination with 9,4 nM Trastuzumab-SO1861 kills cells in contrast to 10 nM unconjugated SO1861 + trastuzumab-saporin. 1075 nM unconjugated SO1861 + trastuzumab-saporin is more efficient compared to the therapeutic combination, since both trastuzumab conjugates compete for the same HER2 receptor. Only simultaneous targeted delivery of both trastuzumab conjugates leads to efficient cell-killing, in contrast to monotherapy with either conjugate alone. SPT001 is SO1861.  
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**Figure 18 (FIG. 18):** *1-target 2-component (HER2 no/low expression).* HER2 targeted cell killing in JIMT1 (HER2<sup>+</sup>) and A431 (HER2<sup>+/+</sup>) cells, by a therapeutic combination. A, B) trastuzumab-SO1861 titration in combination with a fixed concentration of 50 pM trastuzumab-saporin does not induce cell killing by trastuzumab-saporin. C, D), Trastuzumab-saporin titration in combination with 10 nM trastuzumab-SO1861 can not induce cell killing. Low HER2 receptor expression is prohibitive for sufficient SO1861 to be delivered via antibody-mediated delivery, while 1500 nM of unconjugated SO1861 induces efficient cell killing. REMARK: the legend displayed in Fig. 18B also relates to the  
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graphs in Fig. 18A. REMARK: the legend displayed in Fig. 18D also relates to the graphs in Fig. 18C. SPT001 is SO1861.

**Figure 19 (FIG. 19): 2-target 2-component (EGFR high expression and HER2 low expression).** EGFR/HER2 targeted cell killing in A431 (EGFR<sup>+++</sup>/HER2<sup>+/-</sup>) and Caski (EGFR<sup>++</sup>/HER2<sup>+/-</sup>) cells by a therapeutic combination. A, B) Cetuximab-SO1861 titration in combination with a fixed concentration of 50 pM trastuzumab-saporin shows a 100 fold reduced concentration of conjugated SO1861 is required, versus unconjugated SO1861, to induce cell killing by trastuzumab-saporin. C, D), Trastuzumab-saporin titration in combination with 278 nM cetuximab-SO1861 kills cells in contrast to 300 nM unconjugated SO1861 + trastuzumab-saporin. 1500 nM unconjugated SO1861 + trastuzumab-saporin has comparable cell killing efficiency compared to the therapeutic combination, 278 nM cetuximab-SO1861 + trastuzumab-saporin, since both conjugates do not compete for the same receptor. Only simultaneous targeted delivery of both conjugates leads to efficient cell-killing, in contrast to monotherapy with either conjugate alone. REMARK: the legend displayed in Fig. 19B also relates to the graphs in Fig. 19A. REMARK: the legend displayed in Fig. 19D also relates to the graphs in Fig. 19C. SPT001 is SO1861.

**Figure 20. (FIG. 20): 2-target 2-component (EGFR low expression and HER2 no/low expression).** EGFR/HER2 targeted cell killing in HeLa (EGFR<sup>+</sup>/HER2<sup>+/-</sup>) and A2058 (EGFR<sup>+</sup>/HER2<sup>+/-</sup>) cells by a therapeutic combination. A, B) Cetuximab-SO1861 titration in combination with a fixed concentration of 50 pM trastuzumab-saporin does not induce cell killing by trastuzumab-saporin. C, D), Trastuzumab-saporin titration in combination with 278 nM cetuximab-SO1861 does not potentiate cell killing, while 1500 nM of unconjugated SO1861 induces efficient cell killing. Low EGFR receptor expression is prohibitive for sufficient SO1861 to be delivered via antibody-mediated delivery. REMARK: the legend displayed in Fig. 20B also relates to the graphs in Fig. 20A. SPT001 is SO1861.

**Figure 21. (FIG. 21): 2-target 2-component (HER2 high expression and EGFR low expression).** HER2 targeted cell killing in SKBR3 (HER2<sup>+++</sup>/EGFR<sup>+/-</sup>) cells by a therapeutic combination. A) Trastuzumab-SO1861 titration in combination with a fixed concentration of 1.5 pM EGFdianthin shows that a 400 fold reduced concentration of conjugated SO1861 is required, versus unconjugated SO1861, to induce cell killing by EGFdianthin. B), EGFdianthin titration in combination with 9,4 nM trastuzumab-SO1861 can kill cells in contrast to 10 nM unconjugated SO1861 + EGFdianthin. 1075 nM unconjugated SO1861 + EGFdianthin has comparable cell killing efficiency compared to the therapeutic combination, 9,4 nM trastuzumab-SO1861 + EGFdianthin, since both conjugates do not compete for the same receptor. Only simultaneous targeted delivery of both conjugates leads to efficient cell-killing, in contrast to monotherapy with either conjugate alone. SPT001 is SO1861.

**Figure 22. (FIG. 22): 2-target 2-component (HER2 low expression and EGFR low or high expression).** HER2 targeted cell killing in JIMT1 (HER2<sup>+</sup>) and A431 (HER2<sup>+/-</sup>) cells, by a therapeutic combination according to the invention. A, B) trastuzumab-SO1861 titration in combination with a fixed concentration of 5 pM cetuximab-saporin does not induce cell killing by cetuximab-saporin. C, D), Cetuximab-saporin titration in combination with 10 nM trastuzumab-SO1861 can not induce cell killing. Low HER2 receptor expression is prohibitive for sufficient SO1861 to be delivered via antibody-mediated delivery, while 1500 nM of unconjugated SO1861 induces efficient cell killing. Even a high EGFR receptor expression level (D) for delivery of cetuximab-saporin does not change its potency in the

presence of trastuzumab-SO1861, indicating that the bottleneck for cell-killing activity is a too low HER2 expression level, leading to insufficient SO1861 inside target cells to switch on endosomal escape. REMARK: the legend displayed in Fig. 22B also relates to the graphs in Fig. 22A. REMARK: the legend displayed in Fig. 22D also relates to the graphs in Fig. 22C. SPT001 is SO1861.

5 **Figure 23. (FIG. 23):** 2-target 2-component versus T-DM1. Cells with high EGFR expression and low HER2 expression (A431) are efficiently killed with the therapeutic combination, however T-DM1 is not effective at such low toxin concentrations. T-DM1 is Trastuzumab-emtansine (Kadcyla®), carrying ~3.5 DM1 toxin molecules per antibody.

10 **Figure 24A-E. (FIG. 24A-E):** displays the relative cell viability when trastuzumab (Fig. 24A), cetuximab (Fig. 24B) or T-DM1 (Fig. 24C), free toxins saporin (Fig. 24D) and dianthin (Fig. 24D), saporin coupled to a non-cell binding IgG (Fig. 24D), and saporin coupled to a non-cell binding IgG combined with free saporin SO1861 (Fig. 24E) are contacted with the indicated cell lines SK-BR-3, JIMT-1, MDA-MB-468, A431, CaSki, HeLa, A2058, BT-474. REMARK: the legend displayed in Fig. 24C also relates to the curves in Fig. 24A and 24B.

15 **Figure 25. (FIG. 25):** *1T2C in vivo activity.* The 1T2C combination of 50 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup> + 25 mg/kg cetuximab-(-L-HSP27BNA)<sup>4</sup> in A431 tumor bearing mice reveals strong tumor targeted gene silencing, compared to the controls.

20 **Figure 26. (FIG. 26):** *1T2C in vivo activity.* The 1T2C combination of 40 mg/kg trastuzumab-(Cys-L-SO1861)<sup>4</sup> + 0.02/0.03 mg/kg trastuzumab-saporin in a PDX tumor mouse model (high HER2 expression) shows effective tumor growth inhibition.

**Figure 27. (FIG. 27):** *The 2T2 component system tested in A431 tumor bearing mice model reveals tumor regression.*

**Figure 28. (FIG. 28):** *The 2T2 component system tested in A431 tumor bearing mice model reveals tumor regression and eradication.*

25 **Figure 29 (FIG. 29):** *2-target 2-component.* EGFR/HER2 targeted cell killing in A431 cells (EGFR<sup>++</sup>/HER2<sup>+/-</sup>) (A, C) and CaSki cells (EGFR<sup>++</sup>/HER2<sup>+/-</sup>) (B, D) by a therapeutic combination according to the invention. A, B) Cetuximab-(Cys-L-SO1861)<sup>3,7</sup> titration + fixed concentration 50 pM trastuzumab-saporin and controls on A431 cells. C, D) Trastuzumab-saporin titration + fixed concentration of 75 nM cetuximab-(Cys-L-SO1861)<sup>3,7</sup> and controls on Caski cells. The legends and the y-axes are the same for A, B, and the legends and y-axes are the same for figures C and D.

30 **Figure 30. (FIG. 30):** *2-target 2-component.* EGFR/HER2 targeted cell killing in HeLa cells (EGFR<sup>+/+</sup>/HER2<sup>+/-</sup>) (A, C) and A2058 cells (EGFR<sup>-</sup>/HER2<sup>+/-</sup>) (B, D) by a therapeutic combination according to the invention. A, B) Cetuximab-(Cys-L-SO1861)<sup>3,7</sup> titration + fixed concentration 50 pM trastuzumab-saporin and controls on HeLa cells. C, D) Trastuzumab-saporin titration + fixed concentration of 75nM cetuximab-(Cys-L-SO1861)<sup>3,7</sup> and controls on A2058 cells. The legends and y-axes are the same for Fig. 30A and Fig. 30B.

**Figure 31. (FIG. 31):** *2-target 2-component.* HER2/EGFR targeted cell killing in SKBR3 cells (HER2<sup>++</sup>/EGFR<sup>+/-</sup>) (A, B) by a therapeutic combination according to the invention. A) Trastuzumab-(Cys-L-SO1861)<sup>4</sup> titration + fixed concentration 1.5 pM EGFdianthin and controls on SKBR3 cells. B)

EGFdianthin titration + fixed concentration of 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and controls on SKBR3 cells.

**Figure 32. (FIG. 32): 2-target 2-component.** HER2/EGFR targeted cell killing in JIMT-1 cells (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) (A, C) and MDA-MB-468 cells (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) (B, D) by a therapeutic combination according to the invention. A, B) Trastuzumab-(Cys-L-SO1861)<sup>4</sup> titration + fixed concentration 1.5 pM EGFdianthin and controls on JIMT-1 cells. C, D) EGFdianthin titration + fixed concentration of 2.5nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and controls on MDA-MB-468 cells. The legends and the y-axes are the same for C and D.

**Figure 33. (FIG. 33): 2-target 2-component.** HER2/EGFR targeted cell killing in SKBR3 cells (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) (A, B) by a therapeutic combination according to the invention. A) Trastuzumab-(Cys-L-SO1861)<sup>4</sup> titration + fixed concentration 10 pM cetuximab-saporin and controls on SKBR3 cells. B) Cetuximab-saporin titration + fixed concentration of 2.5nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and controls on SKBR3 cells.

**Figure 34. (FIG. 34): 2-target 2-component.** HER2/EGFR targeted cell killing in JIMT-1 cells (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) (A, C) and MDA-MB-468 cells (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) (B, D) by a therapeutic combination according to the invention. A, B) Trastuzumab-(Cys-L-SO1861)<sup>4</sup> titration + fixed concentration 10 pM cetuximab-saporin and controls on JIMT-1 cells. C, D) Cetuximab-saporin titration + fixed concentration of 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and controls on MDA-MB-468 cells. The legends and the y-axes are the same for A and B, and the legends and the y-axes are the same for C and D.

**Figure 35.** Cell killing assay with SO1861 + VHH-toxin

**Figure 36.** Cell killing assay with SO1861 + VHH-toxin

**Figure 37.** Cell killing (MTS) assay with bivalentVHHEGFR-dianthin + SO1861-EMCH, bivalentVHHEGFR-dianthin + 76.9 nM cetuximab-SO1861 (DAR4), cetuximab-saporin + SO1861-EMCH or cetuximab-saporin + cetuximab-SO1861 (DAR4) on A) MDA-MB-468 cells (EGFR<sup>+/+</sup>), B) A431 cells (EGFR<sup>+/+</sup>) and C) A2058 cells (EGFR<sup>+/+</sup>/HER<sup>+/+</sup>)

**Figure 38** Cell killing (MTS) assay with SO1861 + 1 or 5 pM bivalentVHHEGFR-dianthin or cetuximab-SO1861 (DAR4) + 1 or 5 pM bivalentVHHEGFR-dianthin A) MDA-MB-468 cells (EGFR<sup>+/+</sup>) and B) A2058 cells (EGFR<sup>+/+</sup>)

**Figure 39:** Cell killing (MTS) assay with the combination treatment according to the invention of cetuximab-SO1861 (DAR4) + 50 pM bivalent VHHEGFR-dianthin (recombinant fusion protein) or trastuzumab-SO1861 (DAR4) + 50 pM bivalent VHHEGFR-dianthin (recombinant fusion protein) on A431 cells (EGFR<sup>+/+</sup>/HER<sup>+/+</sup>), A2058 cells (EGFR<sup>+/+</sup>/HER<sup>+/+</sup>), SK-BR-3 cells (EGFR<sup>+/+</sup>/HER2<sup>+/+</sup>) and MDA-MB-468 cells (EGFR<sup>+/+</sup>/HER2<sup>+/+</sup>).

**Figure 40:** synthesis of VHH-L-BNA.

**Figure 41:** synthesis of Trifunctional linker-(L-SO1861)-(L-BNA)-(VHH), intermediate 2, 3.

**Figure 42:** synthesis of Trifunctional linker-(L-SO1861)-(L-BNA)-(VHH), intermediate 4.

**Figure 43:** synthesis of Trifunctional linker-(dendron-(L-SO1861)<sub>4</sub>)-(L-BNA)-(VHH), intermediate 5, 6

**Figure 44:** synthesis of Trifunctional linker-(dendron-(L-SO1861)<sub>4</sub>)-(L-BNA)-(VHH)

**Figure 45:** VHH-EGFR-dianthin (conjugate) was titrated alone or on a fixed concentration of 4000 nM SO1861-EMCH or 76.9 nM cetuximab-SO1861 (DAR4) and targeted protein toxin (dianthin) mediated

cell killing was determined on: A) A431 (EGFR<sup>++</sup>) cells, and B) A2058 (EGFR<sup>-</sup>) cells. "SPT001" is saponin SO1861.

## 5 DETAILED DESCRIPTION

In order for a bioactive molecule (*e.g.* an effector molecule) to work, the molecule must be able to engage with its target, *e.g.* in the blood serum, on the outside of the cell surface or inside a cell or an organelle. The active moiety of almost all protein-based targeted toxins, *e.g.*, must enter the cytosol of the target cell to mediate its target modulatory effect. In many constellations the toxin remains ineffective since (1) the targeting moiety is poorly internalized and remains bound to the outside of the cells, (2) is recycled back to the cell surface after internalization or (3) transported to the endolysosomes where it is degraded. Although these fundamental issues are known for decades and more than 500 targeted toxins have been investigated in the past decades, the problems have not been solved yet and only a couple of antibody-targeted protein toxin have been admitted to the market, albeit with warning labels for severe toxicity. Moxetumomab pasudotox-tdfk (LUMOXITI®, AstraZeneca Pharmaceuticals LP), has been approved for relapsed or refractory hairy cell leukemia by the FDA to date. Other of such approved ADCs are Elzonris, Ontak.

To overcome these problems, many strategies have been described including approaches to redirect the toxins to endogenous cellular membrane transport complexes of the biosynthetic pathway in the endoplasmic reticulum and techniques to disrupt or weaken the membrane integrity of endosomes, *i.e.* the compartments of the endocytic pathway in a cell, and thus facilitating the endosomal escape. This comprises the use of lysosomotropic amines, carboxylic ionophores, calcium channel antagonists, various cell-penetrating peptides of viral, bacterial, plant, animal, human and synthetic origin, other organic molecules and light-induced techniques. Although the efficacy of the targeted toxins was typically augmented in cell culture hundred- or thousand-fold, in exceptional cases more than million-fold, the requirement to co-administer endosomal escape enhancers with other substances harbors new problems including additional side effects, loss of target specificity, difficulties to determine the therapeutic window and cell type-dependent variations.

All strategies, including physicochemical techniques, require enhancer molecules that interact more or less directly with membranes and comprise essentially small chemical molecules, secondary metabolites, peptides and proteins. A common feature of all these substances is that they are *per se* not target cell-specific and distribute with other kinetics than the targeted toxins. This is one major drawback of the current approaches.

It is a first goal of the present invention to provide improved ADCs and AOCs with an increased therapeutic window, and to provide improved ADCs and AOCs for delivery of an effective amount or dose of an effector molecule, when for example the delivery from outside a target cell into said cell, is considered, or more in particular when the delivery of the effector molecule in the cytosol of said target cell is considered. It is a second goal of the present invention to provide an improved method of treatment of a (human) patient suffering from a disease to be treated with a conjugate comprising an

effector molecule and a ligand, such as EGF, a cytokine, Her2 targeting V<sub>HH</sub> or IgG, EGFR targeting IgG, *etc.*, or an active domain, active derivative or an active fragment thereof, preferably an sdAb or multiple sdAb's, such as one or more V<sub>HH</sub>'s, for *e.g.* a target tumor cell, *i.e.* to improve the therapeutic window of the ADC or the AOC comprising the effector molecule to be delivered in the cytosol of *e.g.* target tumor cells. The multiple sdAb's either can bind to the same type of cell-surface molecule, such as the same copy of the cell-surface molecule or to different copies of the cell-surface molecule present on the same cell, or the multiple sdAb's such as two sdAb's can bind to a first cell-surface molecule and to a different second cell-surface molecule present on the same cell. The multiple sdAb's can be multivalent for the same binding site on a cell-surface molecule, multiparatopic such as biparatopic, and/or multi-specific such as bi-specific for a first cell-surface molecule and a second cell-surface molecule present at the same cell. The multiple sdAb's such as two sdAb's (a first sdAb and a second sdAb) are selected for their binding capacity towards the same or different binding sites present at a single cell selected for binding of the conjugate of the invention. Binding sites for the at least two sdAb's are present at the same cell. Typically, binding sites are epitopes in cell-surface receptors, such as tumor-cell specific cell-surface receptors.

It is an objective of the current invention to provide a conjugate which is a combination of an effector-molecule activity enhancing molecule and an ADC or an AOC, for use in therapy such as anti-cancer therapy. By provision of such conjugate of the invention, the therapeutic window of the effector molecule, which is part of the conjugate, such as an ADC or an AOC, is effectively widened.

At least one of the above objectives is achieved by providing improved ADCs and improved AOCs, which are conjugates further comprising an effector-molecule activity enhancing molecule.

The present invention will be described with respect to particular embodiments but the invention is not limited thereto but only by the claims. While the invention has been described in terms of several embodiments, it is contemplated that alternatives, modifications, permutations and equivalents thereof will become apparent to one having ordinary skill in the art upon reading the specification and upon study of the drawings and graphs. The invention is not limited in any way to the illustrated embodiments. Changes can be made without departing from the scope which is defined by the appended claims.

The inventors established that the therapeutic window of a conjugate such as an antibody drug conjugate or an antibody-oligonucleotide conjugate, increases when administered to a tumor-bearing mammal (mouse) to whom the conjugate is administered, when said conjugate comprises at least one covalently bound saponin (see for example Figures 8-14 in the Examples section for a series of *in vitro* tumor cell- and *in vivo* tumor model examples of the effect of saponin covalently bound to an effector molecule and an antibody). The saponin is conjugated with an antibody and an effector molecule such as protein toxins and an oligonucleotide such as a BNA. The inventors were the first who established and determined that conjugating a saponin of the invention with a ligand for binding to a cell-surface molecule, such as an antibody such as a full-length intact IgG, or such as an sdAb such as a V<sub>HH</sub>, provides a conjugate for cell-specific delivery of the saponin at the cell surface of a target cell exposing the cell-surface molecule at its cell surface, and subsequent delivery of the saponin inside the cell, such as the cell endosome, endolysosome, lysosome and ultimately in the cell cytosol. Examples of such

cell-targeting saponin conjugates are for example provided in Figures 2-5 for saponin-V<sub>HH</sub> conjugates and Figures 5-7, 15-23, and 25-34, as outlined in the Examples section here below. Saponin is conjugated to ligands such as EGF, Her2 targeting V<sub>HH</sub> or IgG, EGFR targeting IgG, EGFR binding V<sub>HH</sub> 7D12 with amino-acid sequence as depicted as SEQ ID NO: 75, EGFR binding V<sub>HH</sub> 9G8 with amino acid sequence as depicted as SEQ ID NO: 76, EGFR binding covalently linked tandem of biparatopic V<sub>HH</sub>'s 7D12-9G8 with amino-acid sequence as depicted as SEQ ID NO: 73, *etc.*

The conjugate of the invention, comprising an sdAb or a full-length antibody or a different immunoglobulin (Ig) format, as the cell-surface molecule binding molecule in the conjugate of the invention, has at least one glycoside such as a saponin of the invention bound thereto, preferably, and in all exemplary examples, covalently, more preferably via a (cleavable) linker. The saponin augments the therapeutic efficacy of the effector moiety bound to the cell-surface molecule targeting molecule (antibody, sdAb), without wishing to be bound by any theory, likely by enhancing the endosomal escape of the effector moiety into the cytosol where the activity of the effector moiety is desired. This way, already at a lower dose of the effector molecule than the conventional dose of the ADC or the AOC, *i.e.* a lower dose of the conjugate of the invention, therapeutic effect is established under influence of the presence of the conjugate comprising the saponin(s) thereby bringing the saponin(s) near, at and/or inside the targeted cell. The targeted cell is for example a diseased cell such as a tumor cell or an auto-immune cell or a B-cell disease related B-cell, *etc.* The effector moiety is for example a toxin as part of an ADC or an oligonucleotide such as an antisense BNA as part of an AOC according to the invention.

A first aspect of the invention relates to a conjugate for transferring an effector molecule from outside a cell into said cell, the conjugate comprising at least one effector molecule to be transferred into the cell, at least one single-domain antibody (sdAb) and at least one saponin, covalently bound to each other, directly or via at least one linker, wherein the at least one saponin is a mono-desmosidic triterpene glycoside or is a bi-desmosidic triterpene glycoside, and wherein the sdAb is capable of binding to a cell-surface molecule of said cell.

The inventors disclose here that covalently coupling saponins such as saponins in the water-soluble fraction of *Quillaja saponaria*, QS-21, SA1641, SO1861, to the cell-surface molecule targeting molecule, *e.g.* an antibody, an sdAb, such as via a tri-functional linker, *e.g.* the tri-functional linker of Structure A (displayed here below), or via an oligomeric or polymeric structure of a scaffold comprising covalently bound saponins, results in improved cell toxicity exerted by the effector moiety such as a toxin, comprised by the conjugate of the invention, under influence of the covalently coupled saponin in the conjugate.

Thus, an aspect of the invention relates to a conjugate comprising an endosomal escape enhancing molecule, *i.e.* a saponin, an effector moiety and a binding molecule, *e.g.* an sdAb, thus a conjugate wherein the glycoside molecule and the effector molecule are for example bound to one and the same binding molecule in the endosomal escape enhancing conjugate, and wherein the endosomal escape enhancing conjugate is able to specifically bind to a target cell-specific surface molecule or structure, thereby inducing receptor-mediated endocytosis of the complex of the conjugate and the target cell-specific surface molecule (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex). Preferably, the combination of the saponin and the effector moiety

in the endosomal escape enhancing conjugate enables augmentation of endosomal escape of said effector moiety by said saponin. By doing so, the conjugate preferably improves the effect of the effector molecule compared to an ADC comprising the binding molecule and the effector moiety without the saponin.

5 To explain the invention in more detail, the process of cellular uptake of substances and the used terminology in this invention is described first. The uptake of extracellular substances into a cell by vesicle budding is called endocytosis. Said vesicle budding can be characterized by (1) receptor-dependent ligand uptake mediated by the cytosolic protein clathrin, (2) lipid-raft uptake mediated by the cholesterol-binding protein caveolin, (3) unspecific fluid uptake (pinocytosis), or (4) unspecific particle  
10 uptake (phagocytosis). All types of endocytosis run into the following cellular processes of vesicle transport and substance sorting called the endocytic pathways. The endocytic pathways are complex and not fully understood. Earlier, it was thought that organelles are formed *de novo* and mature into the next organelle along the endocytic pathway. Nowadays, it is hypothesized that the endocytic pathways involve stable compartments that are connected by vesicular traffic. A compartment is a complex,  
15 multifunctional membrane organelle that is specialized for a particular set of essential functions for the cell. Vesicles are considered to be transient organelles, simpler in composition, and are defined as membrane-enclosed containers that form *de novo* by budding from a preexisting compartment. In contrast to compartments, vesicles can undergo maturation, which is a physiologically irreversible series of biochemical changes. Early endosomes and late endosomes represent stable compartments in the  
20 endocytic pathway while primary endocytic vesicles, phagosomes, multivesicular bodies (also called endosome carrier vesicles), secretory granules, and even lysosomes represent vesicles. The endocytic vesicle, which arises at the plasma membrane, most prominently from clathrin-coated pits, first fuses with the early endosome, which is a major sorting compartment of approximately pH 6.5. A large part of the cargo and membranes internalized are recycled back to the plasma membrane through recycling  
25 vesicles (recycling pathway). Components that should be degraded are transported to the acidic late endosome (pH lower than 6) via multivesicular bodies. Lysosomes are vesicles that can store mature lysosomal enzymes and deliver them to a late endosomal compartment when needed. The resulting organelle is called the hybrid organelle or endolysosome. Lysosomes bud off the hybrid organelle in a process referred to as lysosome reformation. Late endosomes, lysosomes, and hybrid organelles are  
30 extremely dynamic organelles, and distinction between them is often difficult. Degradation of the endocytosed molecules occurs inside the endolysosomes. *Endosomal escape* is the active or passive release of a substance from the inner lumen of any kind of compartment or vesicle from the endocytic pathway, preferably from clathrin-mediated endocytosis, or recycling pathway into the cytosol. Endosomal escape thus includes but is not limited to release from endosomes, endolysosomes or  
35 lysosomes, including their intermediate and hybrid organelles. After entering the cytosol, said substance might move to other cell units such as the nucleus. Glycoside molecules (saponins) in the context of the invention are compounds that are able to enhance the effect of an effector molecule, in particular by facilitating the endosomal escape. The glycoside molecules interact with the membranes of compartments and vesicles of the endocytic and recycling pathway and make them leaky for said  
40 effector molecules resulting in augmented endosomal escape.

With the term “improving an effect of an effector molecule” is meant that a saponin increases the functional efficacy of the effector molecule (*e.g.* the therapeutic index of a toxin or a drug; the metabolic efficacy of a modifier in biotechnological processes; the transfection efficacy of genes in cell culture research experiments), preferably by enabling or improving its target engagement. Acceleration, prolongation, or enhancement of antigen-specific immune responses are preferably not included. Therapeutic efficacy includes but is not limited to a stronger therapeutic effect with lower dosing and/or less side effects. “Improving an effect of an effector molecule” can also mean that an effector molecule, which could not be used because of lack of effect (and was *e.g.* not known as being an effector molecule), becomes effective when used in combination with the present invention. Any other effect, which is beneficial or desired and can be attributed to the combination of effector moiety and saponin in one conjugate, as provided by the invention, is considered to be “an improved effect”. In the context of the invention, a saponin of the invention is an “enhancer” of the functional efficacy of an effector molecule in the conjugate of the invention.

One major drawback of targeted toxin enhancement by glycosides, such as for instance saponins, up to the present invention is that the targeted toxins are internalized by receptor-mediated endocytosis (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex) while glycosides passively diffuse through the plasma membrane and reach the endosomal membranes presumably via interaction with cholesterol. In principal, glycosides can enter any cell, also non-target cells (off-target cells), resulting in inefficient enhancer availability in the target cells for effective release of the targeted toxin and possible side effects in non-target cells. One major problem is that entry of the targeted toxin and the glycosides proceed with different kinetics and that these kinetics are different from cell (line) to cell (line) and from tissue to tissue, so that the correct time difference for the application of the two substances (ADC, free saponin) can widely vary from tumor (cell (line)) to tumor (cell (line)). Moreover, in living organisms, liberation, absorption, distribution, metabolism and excretion of these substances is also different. Furthermore, the  $\alpha$ -specific uptake of glycosides by non-targeted cells may induce unwanted effects in these cells. This can, *e.g.*, be cytosolic delivery of compounds that should have been delivered to the lysosomes, disturbed antigen presentation, *etc.* Non-targeted administration of the glycoside and the targeted drug may also be problematic in drug development and may hinder or at least postpone marketing authorization by the relevant authorities (*e.g.* FDA or EMA). With targeted toxin or targeted drug in the context of the present invention is meant that a toxin or drug is specifically targeted to a membrane bound molecule on a target cell, *e.g.* a toxin or drug bound to a ligand of a membrane receptor or bound to an antibody that specifically recognizes a structure on the cell membrane of a target cell.

It is thus very useful to direct the glycoside via the same route as the effector molecule, *e.g.*, via a targeting ligand to the target cell in order for the enhancer to be available at effective concentration inside the acidic compartments of the endocytic pathway of the target cell and in order to exhibit a synergistic effect with the toxin. The present invention, therefore, provides novel approaches to redirect both the effector molecule and the endosomal escape enhancer (*i.e.* a saponin of the invention) via a targeting ligand (binding molecule) to the acidic compartments of the endocytic pathway of the target cell.

The inventors established that an effector molecule which is part of the conjugate comprising the sdAb is delivered inside a cell with high efficiency under influence of a saponin which is also comprised by the conjugate, when the effect of the effector molecule inside the cell is considered. Surprisingly, despite the relative small size of an sdAb such as a  $V_{HH}$ , binding of the conjugate comprising such sdAb to the cell surface receptor is still occurring when both an effector molecule and a saponin are comprised by the conjugate comprising the sdAb such as a  $V_{HH}$ . The binding of a saponin and the binding of an effector molecule together, to the sdAb such as a  $V_{HH}$ , does not result in *e.g.* steric hindrance when the capacity of the  $V_{HH}$  to bind to the cell surface molecule is considered. That is to say, contacting *e.g.* tumor cells with a sub-optimal dose of *e.g.* an ADC does not result in intracellular effector molecule activity (the target cell is not efficiently killed upon biological activity of the effector molecule), in the absence of the saponin covalently coupled to said ADC. However, when the target tumor cell is contacted with the conjugate of the invention comprising the effector molecule and comprising the saponin, and further comprising the target-cell binding sdAb, efficient tumor cell killing is achieved.

By targeting a single cell-surface molecule with the conjugate of the invention, the delivery of the saponin and the effector moiety bound to the cell-surface molecule targeting antibody such as an sdAb in the conjugate of the invention, at and inside the cytosol of the targeted cell, exposing the cell-surface molecule on the cell surface, is improved and more specific, compared to for example contacting the cell with only a regular ADC lacking the saponin of the invention, thus without the presence of the cell-targeted saponin (conjugate of the invention). An aberrant cell selected for targeting by the cell-surface molecule targeting sdAb of the conjugate, ideally bears the epitope on the cell-surface molecule to which the cell-surface molecule targeting molecule can bind, to a high extent (*i.e.* relatively higher expression of the targeted cell-surface molecule on the targeted cell such as for example a tumor cell or an auto-immune cell, than the expression on a non-targeted cell such as for example a healthy cell) and/or expose the epitope in the targeted cell-surface molecule for binding of the cell-surface molecule targeting sdAb of the conjugate, specifically, when (neighboring) healthy cells in a patient are considered. Preferably, the cell-surface molecule targeted by the cell-surface molecule targeting sdAb of the conjugate of the invention is relatively highly and/or specifically expressed on the targeted (diseased, tumor) cell compared to healthy cells. An embodiment is the conjugate of the invention, wherein the target cell-surface molecule for the cell-surface molecule targeting sdAb of the conjugate such as a tumor-cell receptor, is expressed specifically or to a relatively higher extent when compared to expression of the cell-surface molecule on the surface of a healthy (neighboring) cell. Thus, the epitope on the targeted cell-surface molecule is ideally unique to the targeted diseased cells, and is at least specifically present and exposed at the surface of the targeted cells. Binding of the conjugate of the invention to the epitope on the cell-surface molecule on a targeted cell is followed by endocytosis of the complex of the conjugate and the target cell-surface molecule (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex). Since the conjugate only can enter the target cell through binding interaction with a cell-surface molecules specifically expressed to a sufficient extent or uniquely expressed on the targeted cell when compared to healthy cells that should not be targeted, accumulation of a therapeutically active amount of effector moiety and saponin comprised by the conjugate, inside the target cells is only possible and occurring if expression levels of the targeted

cell-surface molecule is above a certain minimal expression threshold. At the same time, the fact that the effector moiety bound to the cell-surface molecule targeting sdAb of the conjugate is only capable of exerting its intracellular (*e.g.* cytotoxic or gene silencing) activity in the presence of very same conjugate bearing the covalently bound saponin, also provides a safeguard against negative and undesired side effects of the effector moiety towards *e.g.* healthy cells and healthy tissue not meant to be targeted and affected by the effector moiety, when compared to exposure of cells to an ADC without the covalently bound saponin(s). That is to say, sufficiently low expression or even absence of exposed cell-surface molecule, to which a conjugate could bind, does ideally not allow entrance into (non-targeted) healthy cells of the conjugate to amounts that would result in endosomal escape of the effector moiety under influence of the saponin comprised by the conjugate. Since the ADC with coupled saponin or the AOC with covalently coupled saponin according to the invention can be used at lower dose compared to when the ADC or AOC without coupled saponin was applied in the therapeutic regimen, entrance of ADC with coupled saponin or entrance of AOC with coupled saponin in healthy cells to low extent already bears a lower risk for occurrence of unwanted side effects when for example the targeting and killing of target diseased cells such as tumor cells and auto-immune cells is considered.

Inclusion of an sdAb in the conjugate has thus manifold advantages compared to inclusion of an antibody such as an IgG, or of a binding fragment or binding domain thereof. Importantly, since sdAbs do not comprise the Fc tail present in IgGs, risk for off-target side effects due to binding of the conjugate to Fc receptors on cells such as endothelial cells of a host to whom the conjugate is administered, is absent. Thus, the risk profile of the conjugate of the invention is improved compared to IgG-based ADCs and AOCs, or compared to ADCs or AOCs comprising an Fc tail. In addition, since the conjugate of the invention cannot be bound by Fc receptors, the conjugate is already effective at a dose which is lower than the dose required for reaching the same effector molecule activity with full-length antibody-based ADCs and AOCs, due to less or no undesired capturing of the conjugate by cell-surface receptors, different from the aimed target cell-surface molecule. Furthermore, due to the relatively small size of sdAbs compared to *e.g.* Fab, scFv, IgG, tissue penetration is improved, which is beneficial for reaching the target cells once the conjugate is administered to a patient in need of therapy. All these advantages of the application of an sdAb in the conjugate of the invention, when compared to the application of larger antibodies or fragments thereof, such as IgGs comprising an Fc tail, in similar ADCs or AOCs, result in an improved therapeutic window for the effector molecule, when comprised by the conjugate of the invention. For example, an ADC based on a sdAb may achieve improved target cell killing in case of a targeted tumor cell when the effector molecule is for example a toxin, at the same dose at which an ADC based on an IgG and comprising the same effector molecule, is not or only sub-optimally effective. Thanks to the aspects of the invention, it is now possible to treat patients with a lower dose of effector molecule as part of a conjugate comprising the sdAb, *i.e.* the conjugate of the invention, therewith reaching the same or improved effector molecule mediated effect in the target cells, compared to a higher dose required when an antibody-based ADC or AOC would be used, which comprises the same effector molecule. Administering such conjugate of the invention at lower dose lowers the risk for the patient for occurrence of side effects, *e.g.* by non-specific entrance of non-targeted, healthy cells. This is for example important when the cell-surface molecule that is targeted by the sdAb comprised by the

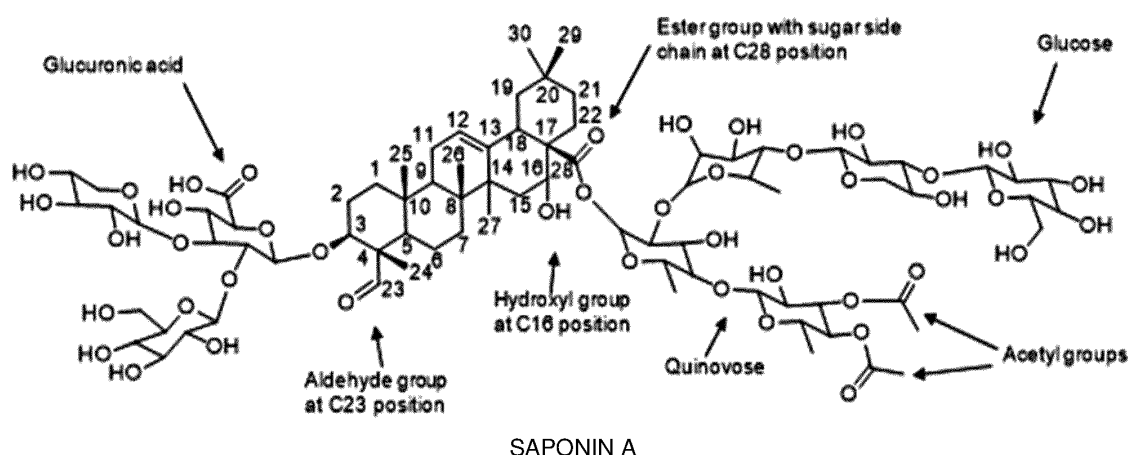
conjugate is expressed to a higher extent on target (tumor) cells, but is not uniquely expressed on such target cells. A lower dose of the conjugate lowers the risk for binding of the conjugate to such low expressors, such as non-tumor healthy cells.

The inventors also found that the therapeutic window of the conjugate of the invention is widened  
5 due to the incorporation of covalently bound saponin in the conjugate of the invention. That is to say, when the ADC or the AOC provided with a saponin (*i.e.* the conjugate of the invention) is contacted with target cells, upon binding of the sdAb to its binding partner at the surface of the target cell, the saponin that is comprised by the conjugate of the invention is also brought in close proximity, *i.e.* at the surface of the target cell, together with the effector molecule of the conjugate. When target cells that bear the  
10 cell-surface molecule, *i.e.* the target for the sdAb comprised by the conjugate, are contacted with the conjugate of the invention, both the effective dose of the effector molecule and the effective dose of the saponin is lower than when the target cells are contacted with an ADC or AOC in the absence of saponin or in the presence of free (untargeted) saponin. The presence of the targeted saponin as part of the conjugate of the invention potentiates the activity of the effector molecule in the target cells, such that  
15 the therapeutic window of the conjugate, and therewith the therapeutic window of the effector molecule is widened. Sufficient effector molecule efficiency is achieved at lower dose when target cells are contacted with the conjugate of the invention. The similar effect is found by the inventors when an ADC or an AOC is contacted with the target cells in the presence of saponin or a functional derivative thereof, when effector-molecule potentiating activity of the saponin is considered, however, at a 100-fold to 1000-  
20 fold higher concentration of the free saponin (functional derivative) compared to the effective dose established when the conjugate of the invention is applied, which now comprises both the effector molecule and the effector molecule activity enhancing saponin, together with the sdAb for targeted binding of the conjugate to the target (tumor)m cell. Thus, providing the saponin or the functional derivative thereof with a binding molecule (*i.e.* the sdAb comprised by the conjugate of the invention) and also providing the very same conjugate with an effector molecule (*i.e.* the effector molecule  
25 comprised by the conjugate of the invention) results in an improved effector-molecule activity potentiating effect, when the conjugate of the invention is contacted with the target cell that expresses the cell-surface molecule on its surface, *i.e.* the binding target for the sdAb. Targeted saponin is already effective at lower dose than free saponin, in delivery of the effector molecule inside the target cell, and  
30 in delivery from the endosome or lysosome of said cell into the cytosol, where the effector molecule should bind its target binding partner and should exerts its biological activity (*e.g.* cell killing in case of the target cell being a tumor cell and the effector molecule being *e.g.* a toxin), however the present inventors have found that the combination of saponin, targeting moiety (sdAb) and effector molecule (*e.g.* toxin, AON) in a single molecule is even more effective.

35 Hence, the inventors provide a pharmaceutical composition comprising the conjugate comprising the saponin (functional derivative), the effector molecule and the sdAb for targeted delivery of the conjugate at target cells, which pharmaceutical composition has an improved therapeutic window, less risk for inducing side effects when an effective dose of the effector molecule comprised by the conjugate is administered to a patient in need of effector molecule based therapy, and improved effector molecule  
40 activity due to improved delivery of the conjugate inside target cells under influence of the targeted

saponin as part of the conjugate of the invention, more specifically inside the cytosol of such target cells, when compared to current ADCs based on full-length antibodies or Fc comprising constructs thereof, which are not provided with a covalently linked saponin. It is part of the invention that such conjugates of the invention are administered to patients in need of effector molecule based therapy together with a dose of free saponin (functional derivative), although the application of the conjugate alone is preferred.

An example of a saponin suitable for application in the conjugate of the invention is a mono-desmosidic or bi-desmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with the aldehyde group in position C-23 and optionally comprising a glucuronic acid group in a carbohydrate substituent at the C-3 $\beta$ -OH group of the saponin, preferably a bi-desmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with the aldehyde group in position C-23 and comprising a glucuronic acid group in a carbohydrate substituent at the C-3 $\beta$ -OH group of the saponin. An exemplary saponin according to the invention comprises one, several, or all of the features of the saponin depicted as SAPONIN A and illustrated by the following structure:



This group of saponins has demonstrated endosomal escape enhancing activity towards an effector moiety when the saponin and the effector moiety were present in the endosome of a cell. Typically, the saponins suitable for application in the conjugates are saponins with a triterpene backbone wherein the structure of the triterpene backbone is a pentacyclic C30 terpene skeleton (also referred to as sapogenin or aglycone). Table A1 lists saponins suitable for synthesizing a conjugate comprising at least one sdAb such as VHH and comprising at least one saponin, such as 1-16 saponin moieties, 1-8 such as 2, 4 or 8 saponin moieties, wherein the saponin is for example SO1861 or QS-21, preferably SO1861.

An embodiment is the conjugate of the invention, comprising at least one sdAb which is any one or more of: a V<sub>H</sub> domain derived from a heavy chain of an antibody, preferably of immunoglobulin G origin, preferably of human origin; a V<sub>L</sub> domain derived from a light chain of an antibody, preferably of immunoglobulin G origin, preferably of human origin; a V<sub>HH</sub> domain such as derived from a heavy-chain only antibody (HCAb) such as from *Camelidae* origin or *Ig-NAR* origin such as a variable heavy chain

new antigen receptor ( $V_{\text{NAR}}$ ) domain, preferably the HCAb is from *Camelidae* origin; and preferably the at least one sdAb is a  $V_{\text{HH}}$  domain derived from an HCAb from *Camelidae* origin (camelid  $V_{\text{H}}$ ) such as derived from an HCAb from camel, lama, alpaca, dromedary, vicuna, guanaco and Bactrian camel.

5 In particular,  $V_{\text{HH}}$  domains are suitable for application in the conjugate of the invention. Such  $V_{\text{HH}}$  domains are commonly renowned for their high stability, *i.e.* resistance to unfolding, for their capability to bind to a binding partner without the requirement of the presence of a second V domain, such as present in *e.g.* IgG and required for the IgG to bind to its binding partner via the two V domains, for their ease of production by techniques known in the art (camelid immunization, phage display techniques, *etc.*), for their capability of penetrating tissue to a higher extent than seen for full-length IgGs, which is beneficial  
10 when target (tumor) cells are located inside or as part of such (organ) tissue.

An embodiment is the conjugate of the invention, comprising at least two sdAbs with a single first sdAb covalently bound to one of the at least one effector molecule and/or to one of the at least one saponin, or with two or more sdAbs of which at least one sdAb is bound to the at least one effector molecule and/or of which at least one sdAb is bound to the at least one saponin, or with all of the at  
15 least two sdAbs each bound separately to either an effector molecule of the at least one effector molecule or to a saponin of the at least one saponin, or both.

An embodiment is the conjugate of the invention, wherein the at least one sdAb comprises at least two sdAbs, which are the same sdAbs, preferably two – eight sdAbs, more preferably two – four sdAbs.

20 An embodiment is the conjugate of the invention, comprising at least two sdAbs which are biparatopic, preferably comprising two sdAbs which are biparatopic.

An embodiment is the conjugate of the invention, comprising one – eight sdAbs, capable of binding to a same binding site on a cell-surface molecule, wherein the at least one effector molecule and/or the at least one saponin is/are bound to a single first sdAb of the one – eight sdAbs or wherein the at least one effector molecule and/or the at least one saponin is/are bound to two or more of the  
25 sdAbs, if present, wherein the at least one effector molecule and the at least one saponin are bound to the same sdAb or are bound to different sdAbs, wherein preferably each of the at least one effector molecule is bound to a separate sdAb and/or each of the at least one saponin is bound to a separate sdAb, wherein an effector molecule and a saponin are bound to the same sdAb or are bound to separate sdAbs.

30 An embodiment is a conjugate of the invention, comprising at least two sdAbs which are biparatopic, preferably comprising two sdAbs which are biparatopic. An example of such a biparatopic tandem of sdAbs is the biparatopic tandem of sdAbs with amino-acid sequence as depicted as SEQ ID NO: 74. The bivalent tandem of sdAb having amino-acid sequence of SEQ ID NO: 74 binds to EGFR. A first sdAb, 7D12 with amino-acid sequence as depicted as SEQ ID NO: 75, binds to a first epitope on  
35 the EGFR and a second sdAb, 9G8, with amino-acid sequence as depicted as SEQ ID NO: 76, binds to a second epitope on the EGFR. Of course, the sdAb 7D12 or the sdAb 9G8 are also suitable for application in a conjugate of the invention which comprises a single sdAb, or which comprises at least one further sdAb different from 7D12 and 9G8, which at least one further sdAb binds to EGFR or to a further cell-surface molecule present on the same cell surface as at which the EGFR is exposed. For  
40 providing a conjugate of the invention, an effector molecule is covalently linked to the one or multiple

sdAb's, such as a protein toxin such as dianthin (see example of 7D12-9G8-dianthin, in the Examples section, with amino-acid sequence as outlined in SEQ ID NO: 73), and at least one copy of a saponin such as QS-21 or SO1861, is covalently linked to the sdAb's, for example via linker and/or via an oligomeric molecule or polymeric molecule which oligomeric molecule or polymeric molecule is covalently linked to one or multiple saponin molecules and to the sdAb's.

Providing a conjugate of the invention which comprises a (linear) string of multiple sdAbs covalently linked to each other, can provide the benefit of the capacity of the conjugate to bind with higher avidity to the target cell, which can result in improved uptake (endocytosis) of the conjugate by the target cell (binding of conjugates to the receptor is followed by internalization of the conjugate/receptor complex).

Synchronization is the missing link between a successful delivery strategy for mice and its application in humans, when the application of the endosomal escape enhancing effect of saponin towards effector molecules is considered. Indeed, the inventors established in a series of *in vivo* mouse tumor models that separately administering to the mice a dose of free saponin and a dose of *e.g.* ADC without coupled saponin, did not result in any desired anti-tumor activity such as delayed tumor growth, tumor regression, diminished and slower tumor growth, compared to control animals not treated with the ADC in the presence of free saponin. See also the Examples 4-18, here below. The free saponin was administered using various routes of administration and using various time points of administering the free saponin compared to the moment of administering the ADC (administering free saponin before, during and after administering the ADC). The ADC tested in *in vivo* tumor models was cetuximab-dianthin (with free SO1861), or trastuzumab-saporin (with free SO1861). Varying the dose of free saponin did not provide for an efficacious anti-tumor activity. The ADCs referred to were administered at a dose that in itself did not inflict any beneficial anti-tumor effect on the tumor-bearing animals. Surprisingly, the inventors now established that beneficial anti-tumor activity in various *in vitro* mammalian cell-based bioassays using human tumor cells and/or in various *in vivo* animal tumor models can be achieved by treating the cells or animals with conjugates according to the invention. The conjugates optionally comprising a scaffold according to the invention (see below; a covalent saponin conjugate comprising an oligomeric or polymeric structure with one or multiple saponin moieties covalently bound thereto). The scaffold for example being a tri-functional linker with a covalently bound saponin (*e.g.* SO1861, QS-21) via a cleavable or non-cleavable linkage, and/or with a covalently bound effector moiety (*e.g.* dianthin, gene-silencing antisense BNA (HSP27) via a non-cleavable bond or a cleavable bond, the scaffold linked with a covalently bond to the cell-surface molecule targeting molecule of the conjugate such as a monoclonal antibody such as cetuximab, trastuzumab, OKT-9, or the scaffold being a dendron, such as a dendron, for example G4-dendron, to which for example four moieties can bind such as four saponin molecules, or a dendron for binding for example two saponins and two effector molecules, the dendron comprising a chemical group for (covalent) coupling to the cell-surface molecule targeting antibody such as an sdAb, of the conjugate. Reference is made to the further embodiments and the Examples section, exemplifying several of these scaffolds according to the invention, showing *in vivo* and/or *in vitro* anti-tumor cell activity when cell toxicity exerted by *e.g.* a proteinaceous toxin is considered or when gene silencing in the tumor cell is considered.

Without wishing to be bound by any theory, in view of the failures observed when treatment of tumor-bearing animals with an ADC together with free saponin is considered, it is preferred to synchronize the presence of both, the at least one saponin, and the effector moiety, preferably a toxin or an oligonucleotide, in compartments or vesicles of the endocytic pathway of the target cell, *e.g.* a tumor cell or an auto-immune cell. With ADC and free saponin, synchronizing the presence of the molecules in the late endosomes, in order to obtain the synergistic effects *in vivo* was not beneficially obtainable according to attempts of the inventors. In one aspect, the invention preferably solves at least the following problem with respect to combining the effector moiety and the saponin(s) in a single conjugate molecule: without wishing to be bound by any theory the only reasonable chemical group within, *e.g.*, the saponins that can be used for (covalent), in particular single and cleavable, retainable coupling is required for the endosomal escape activity. Known restrictions are most likely the reason why saponins have not been used in combination with pharmaceutically active substances in clinical investigations other than the application of saponins in vaccination regimes wherein the use of an immune-potentiating adjuvant substance was implied, although the striking endosomal escape enhancer effect of, *e.g.*, saponins of the invention and exemplified herein, is known for more than 10 years. For example providing a conjugate of the invention with a covalently bound saponin, for example in the context of a scaffold carrying several saponins, solves these difficulties, at least in part. Surprisingly, the saponins previously applied for their immune-potentiating activity in the vaccination context involving saponins as adjuvant component, are now also suitably for (covalent) coupling to the cell-surface molecule targeting antibody, such as an sdAb, comprised by the conjugate of the invention, for anti-tumor activity *in vitro* and *in vivo*.

An embodiment is the conjugate of the invention, wherein the at least one sdAb is a single sdAb or are at least two, preferably two sdAbs, wherein the sdAb(s) is/are capable of binding to a cell-surface molecule of the cell such as HIVgp41 or wherein the sdAb(s) is/are capable of binding to a cell-surface receptor of the cell, such as a tumor-cell surface receptor of the cell, preferably a tumor-cell specific receptor, more preferably to a receptor selected from any one or more of: CD71, CA125, EpCAM(17-1A), CD52, CEA, CD44v6, FAP, EGF-IR, integrin, syndecan-1, vascular integrin alpha-V beta-3, HER2, EGFR, CD20, CD22, Folate receptor 1, CD146, CD56, CD19, CD138, CD27L receptor, prostate specific membrane antigen (PSMA), CanAg, integrin-alphaV, CA6, CD33, mesothelin, Cripto, CD3, CD30, CD239, CD70, CD123, CD352, DLL3, CD25, ephrinA4, MUC-1, Trop2, CEACAM5, CEACAM6, HER3, CD74, PTK7, Notch3, FGF2, C4.4A, FLT3, CD38, FGFR3, CD7, PD-L1, CTLA-4, CD52, PDGFRA, VEGFR1, VEGFR2, c-Met (HGFR), EGFR1, RANKL, ADAMTS5, CD16, CXCR7 (ACKR3), glucocorticoid-induced TNFR-related protein (GITR), most preferably selected from: HER2, c-Met, VEGFR2, CXCR7, CD71, EGFR and EGFR1.

It is part of the invention that the sdAb comprised by the conjugate of the invention has binding specificity for a cell-surface molecule that is specifically expressed on the target cell. 'Specifically expressed' should here be understood as the unique expression of the cell-surface molecule on the target cell only, wherein *e.g.* healthy cells that should not bind the conjugate, are not targeted due to the absence of cell-surface exposure of the targeted molecule, or should here be understood as the upregulated or relatively high expression of the target cell-surface molecule on the target cells,

compared to lower expression of the cell-surface molecule on *e.g.* healthy cells that should not or at least to a much lower extent, bind the conjugate. These listed cell receptors are such cell-surface molecules that are sufficiently specific for the cells that are the target of the conjugate, and are therewith preferred candidates for binding by the conjugate. It will be appreciated that the higher the specificity of a certain cell-surface molecule, when expression of the cell-surface molecule on the target cell is compared to the expression on other cells not meant to be targeted by the conjugate of the invention, the better the therapeutic window is, when the activity of the effector molecule inside the cells is considered. For example, suitable targets for targeting by the conjugate are amongst other tumor cell specific receptors, HER2, EGFR, such as EGFR1, and CD71.

5 An embodiment is the conjugate of the invention, wherein the at least one sdAb is a single sdAb or are at least two, preferably two, wherein the sdAb(s) is/are selected from: an anti-CD71 sdAb, an anti-HER2 sdAb, an anti-CD20 sdAb, an anti-CA125 sdAb, an anti-EpCAM (17-1A) sdAb, an anti-EGFR sdAb, an anti-CD30 sdAb, an anti-CD33 sdAb, an anti-vascular integrin alpha-v beta-3 sdAb, an anti-CD52 sdAb, an anti-CD22 sdAb, an anti-CEA sdAb, an anti-CD44v6 sdAb, an anti-FAP sdAb, an anti-CD19 sdAb, an anti-CanAg sdAb, an anti-CD56 sdAb, an anti-CD38 sdAb, an anti-CA6 sdAb, an anti-IGF-1R sdAb, an anti-integrin sdAb, an anti-syndecan-1 sdAb, an anti-CD79b, an anti-c-Met sdAb, an anti-EGFR1 sdAb, an anti-VEGFR2 sdAb, an anti-CXCR7 sdAb, an anti-HIVgp41, wherein the sdAb(s) is/are preferably V<sub>HH</sub>(s), more preferably camelid V<sub>H</sub>(s).

10 An embodiment is the conjugate of the invention, wherein the at least one sdAb comprises an sdAb that is capable of binding to HER2, CD71, HIVgp41 and/or EGFR, wherein said sdAb is preferably a V<sub>HH</sub>, more preferably a camelid V<sub>H</sub>.

An embodiment is the conjugate of the invention, wherein the at least one sdAb comprises an sdAb for binding to HER2 selected from: sdAb produced by clone 11A4, clone 18C3, clone 22G12, clone Q17 or clone Q17-C-tag; or comprises an sdAb for binding to EGFR and produced by clone anti-EGFR Q86-C-tag; or comprises an sdAb for binding to CD71 and produced by clone anti-CD71 Q52-C-tag; or comprises an sdAb for binding to HIVgp41 and produced by clone anti-HIVgp41 Q8C-tag; or comprises an sdAb encoded by a cDNA of any one of the SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 and 31; or comprises any one of the sdAbs with an amino-acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36-72, or comprises the biparatopic tandem of V<sub>HH</sub> domains binding to EGFR, consisting of V<sub>HH</sub> 7D12 and V<sub>HH</sub> 9G8 with the amino-acid sequence as depicted as SEQ ID NO: 74, or comprises V<sub>HH</sub> 7D12 with the amino-acid sequence as depicted as SEQ ID NO: 75 and/or V<sub>HH</sub> 9G8 with the amino-acid sequence as depicted as SEQ ID NO: 76, wherein optionally the conjugate further comprises a further sdAb, different from the at least one sdAb, the further sdAb for binding to albumin, such as any one or more of the further sdAbs with an amino-acid sequence of SEQ ID NO: 33, 34 and 35, preferably the further sdAb is a V<sub>HH</sub>, more preferably a camelid V<sub>H</sub>. An embodiment is the conjugate of the invention, wherein the conjugate comprises an sdAb for binding to HER2 selected from: sdAb produced by clone 11A4, clone 18C3, clone 22G12, clone Q17, clone Q17-C-tag; or an sdAb for binding to EGFR and produced by clone anti-EGFR Q86-C-tag; or an sdAb for binding to CD71 and produced by clone anti-CD71 Q52-C-tag; or an sdAb for binding to HIVgp41 and produced by clone anti-HIVgp41 Q8C-tag; or an sdAb encoded by a cDNA of any one of the SEQ ID

NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 and 31; or any one of the sdAbs with an amino-acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36-72, or the biparatopic tandem of sdAbs 7D12 and 9G8 with an amino-acid sequence of SEQ ID NO: 74 or sdAb 7D12 with an amino-acid sequence of SEQ ID NO: 75 or sdAb 9G8 with an amino-acid sequence of SEQ ID NO: 76, wherein optionally the conjugate further comprises an sdAb for binding to albumin, such as any one or more of sdAbs with an amino-acid sequence of SEQ ID NO: 33, 34 and 35. For example, the conjugate of a saponin, an effector moiety and at least one V<sub>HH</sub> comprises the tandem of biparatopic sdAbs with the amino-acid sequence of SEQ ID NO: 74, or comprises one or more copies of 7D12 and/or one or more copies of 9G8.

V<sub>HHS</sub> suitable for incorporation in the conjugate of the invention are for example found in the single domain antibody database (Wilton, E.E. *et al.* (2018)), in patent applications US20160251440 (anti-CD123, anti-CEACAM), US9683045 (anti-c-Met), US20090252681 (anti-EGFR, anti-IGF-1R), US9969805 (anti-HER2), US20190023796A1 (anti-HER3), and in Kijanka *et al.* (2013), for anti-HER2 and in Mercier *et al.* (2019) for anti-HER2. The amino-acid sequences and/or the cDNA sequences of a series of suitable V<sub>HHS</sub> is also provided here below for anti-HER2, anti-HER3, anti-CD123, anti-CEACAM, anti-c-Met, anti-EGFR, anti-IGF-1R, anti-PD-L1, anti-CTLA-4, anti-CD19, anti-HER1 and anti-VGFR2, as SEQ ID NOs 1-32 and 36-72 and 74 (tandem of V<sub>HH</sub>'s 7D12 and 9G8) and V<sub>HH</sub> 7D12 with the amino-acid sequence as depicted as SEQ ID NO: 75 and/or V<sub>HH</sub> 9G8 with the amino-acid sequence as depicted as SEQ ID NO: 76, in view of their ability to bind to tumor-cell specific receptors. In particular, a V<sub>HH</sub> capable of binding to a binding site on any of the tumor-cell specific receptors HER2, VEGFR, EGFR and CD71 is suitable for incorporation in the conjugate of the invention. In particular, a V<sub>HH</sub> capable of binding to a binding site on tumor-cell specific receptor EGFR is suitable for incorporation in the conjugate of the invention, such as V<sub>HH</sub> 7D12 with the amino-acid sequence as depicted as SEQ ID NO: 75 and V<sub>HH</sub> 9G8 with the amino-acid sequence as depicted as SEQ ID NO: 76. The inventors revealed that an ADC comprising a V<sub>HH</sub> that targets any one of such receptors is effective in delivery of the effector molecule bound to the sdAb. See for example the Examples section, and Figures 4-7.

An embodiment is the conjugate of the invention, wherein the effector molecule comprises or consists of at least one of a small molecule such as a drug molecule, a toxin such as a protein toxin, an oligonucleotide such as a BNA, a xeno nucleic acid or an siRNA, an enzyme, a peptide, a protein, or any combination thereof.

An embodiment is the conjugate according to the invention, wherein the effector molecule is a pharmaceutically active substance, such as a toxin such as a proteinaceous toxin, a drug, a polypeptide or a polynucleotide. A pharmaceutically active substance in this invention is an effector molecule that is used to achieve a beneficial outcome in an organism, preferably a vertebrate, more preferably a human being. Benefits include diagnosis, prognosis, treatment, cure and prevention of diseases and/or symptoms. The pharmaceutically active substance may also lead to undesired harmful side effects. In this case, pros and cons must be weighed to decide whether the pharmaceutically active substance is suitable in the particular case. If the effect of the pharmaceutically active substance inside a cell is predominantly beneficial for the organism as a whole, *e.g.* a human patient, the cell is called a target cell. If the effect inside a cell is predominantly harmful for the organism as a whole, the cell is called an

off-target cell. In artificial systems such as cell cultures and bioreactors, target cells and off-target cells depend on the purpose and are defined by the user. Examples of effector molecules are a drug, a toxin, a polypeptide (such as an enzyme), and a polynucleotide, including polypeptides and polynucleotides that comprise non-natural amino acids or nucleic acids. Effector molecules include, amongst others:

5 DNA: single stranded DNA (*e.g.* DNA for adenine phosphoribosyltransferase); linear doubled stranded DNA; circular double stranded DNA (*e.g.* plasmids); RNA: -mRNA (*e.g.* TAL effector molecule nucleases), tRNA, rRNA, siRNA, miRNA, asRNA, LNA and BNA; Protein and peptides; toxins (*e.g.* saporin, dianthin, gelonin, (de)bougainin, agrostin, ricin (toxin A chain); pokeweed antiviral protein, apoptin, diphtheria toxin, pseudomonas exotoxin) metabolic enzymes (argininosuccinate lyase,

10 argininosuccinate synthetase), enzymes of the coagulation cascade, repairing enzymes; enzymes for cell signalling; cell cycle regulation factors; gene regulating factors (transcription factors such as NF- $\kappa$ B or gene repressors such as methionine repressor). A toxin, as used in this invention, is defined as a pharmaceutically active substance that is able to kill or inactivate a cell. Preferably, a targeted toxin is a toxin that is only, or at least predominantly, toxic for target cells but not for off-target cells. The net effect

15 of the targeted toxin is preferably beneficial for the organism as a whole.

An embodiment is the conjugate of the invention, wherein the at least one effector molecule is selected from any one or more of a vector, a gene, a cell suicide inducing transgene, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), anti-sense oligonucleotide (ASO, AON), short interfering RNA (siRNA), anti-microRNA (anti-miRNA), DNA aptamer, RNA aptamer, mRNA, mini-circle DNA, peptide

20 nucleic acid (PNA), phosphoramidate morpholino oligomer (PMO), locked nucleic acid (LNA), bridged nucleic acid (BNA), 2'-deoxy-2'-fluoroarabino nucleic acid (FANA), 2'-O-methoxyethyl-RNA (MOE), 3'-fluoro hexitol nucleic acid (FHNA), a plasmid, glycol nucleic acid (GNA) and threose nucleic acid (TNA), or a derivative thereof, more preferably a BNA, for example a BNA for silencing HSP27 protein expression or a BNA for silencing apolipoprotein B expression.

25 An embodiment is the conjugate of the invention, wherein the at least one effector molecule is an oligonucleotide selected from any one or more of a(n): short interfering RNA (siRNA), short hairpin RNA (shRNA), anti-hairpin-shaped microRNA (miRNA), single-stranded RNA, aptamer RNA, double-stranded RNA (dsRNA), anti-microRNA (anti-miRNA, anti-miR), antisense oligonucleotide (ASO), mRNA, DNA, antisense DNA, locked nucleic acid (LNA), bridged nucleic acid (BNA), 2'-O,4'-aminoethylene bridged nucleic Acid (BNA<sup>NC</sup>), BNA-based siRNA, and BNA-based antisense oligonucleotide (BNA-AON).

30

An embodiment is the conjugate of the invention, wherein the at least one effector molecule is an oligonucleotide selected from any one of an anti-miRNA, a BNA-AON or an siRNA, such as BNA-based siRNA, preferably selected from chemically modified siRNA, metabolically stable siRNA and

35 chemically modified, metabolically stable siRNA.

An embodiment is the conjugate of the invention, wherein the at least one effector molecule is an oligonucleotide that is capable of silencing a gene, when present in a cell comprising such gene, wherein the gene is any one of genes: apolipoprotein B (apoB), transthyretin (TTR), proprotein convertase subtilisin/kexin type 9 (PCSK9), delta-aminolevulinate synthase 1 (ALAS1), antithrombin 3

40 (AT3), glycolate oxidase (GO), complement component C5 (CC5), X gene of hepatitis B virus (HBV), S

gene of HBV, alpha-1 antitrypsin (AAT) and lactate dehydrogenase (LDH), and/or is capable of targeting an aberrant miRNA when present in a cell comprising such aberrant miRNA.

An embodiment is the conjugate of the invention, wherein the effector molecule is an oligonucleotide that is capable of targeting an mRNA, when present in a cell comprising such mRNA, wherein the mRNA is involved in expression of any one of proteins: apoB, TTR, PCSK9, ALAS1, AT3, GO, CC5, expression product of X gene of HBV, expression product of S gene of HBV, AAT and LDH, or is capable of antagonizing or restore an miRNA function such as inhibiting an oncogenic miRNA (onco-miR) or suppression of expression of an onco-miR, when present in a cell comprising such an miRNA.

The inventors show that a tumor-cell targeting monoclonal antibody provided with covalently coupled antisense BNA such as BNA(HSP27) and provided with covalently coupled saponin of the invention, that is contacted with tumor cells, both the BNA and the saponin coupled to the antibody (*e.g.* cetuximab) via a cleavable bond, is capable of silencing HSP27 *in vivo* in tumors, compared to control and compared to the AOC bearing the BNA only and not the saponin (SO1861, Quil-A). Administering an ADC-saponin conjugate of the invention or an antibody-oligonucleotide conjugate-saponin conjugate of the invention (AOC-saponin), such as an antibody-BNA-saponin conjugate, thus endows the ADC-saponin or AOC-saponin with anti-tumor cell activity not seen with only the ADC or only the AOC, which do not have the covalently saponins bound to the monoclonal antibody, at the same dose. Noteworthy, the AOC and the separate monoclonal antibody with covalently coupled saponin as a combination of two separate conjugates, increase HSP27 expression in tumor cells, when administered to tumor-bearing mice separately in separate groups of mice, compared to a control group (vehicle administered, only). Only administration of the AOC-saponin conjugate of the invention comprising the effector moiety of the invention, displays reduced HSP27 expression when compared to controls. The antisense BNA (HSP27) was a BNA with oligonucleic acid sequence according to Zhang *et al.* (2011) [Y Zhang, Z Qu, S Kim, V Shi, B Liao1, P Kraft, R Bandaru, Y Wu, LM Greenberger and ID Horak, *Down-modulation of cancer targets using locked nucleic acid (LNA)-based antisense oligonucleotides without transfection, Gene Therapy (2011) 18, 326–333*]. Noteworthy, to the best of the knowledge of the inventors, BNA is designed for application as a free nucleic acid. The inventors are now the first to demonstrate that the antisense BNA can be covalently coupled through a (non-)cleavable linker with a ligand or an antibody, in a way that gene-silencing activity is retained *in vitro* and more importantly *in vivo* in the tumor cells of a tumor-bearing animal. This approach of providing BNA based AOCs opens new ways to administer targeted BNA to human (cancer) patients in need thereof.

An embodiment is the conjugate of the invention, wherein the at least one effector molecule comprises or consists of at least one proteinaceous molecule, preferably selected from any one or more of a peptide, a protein, an enzyme and a protein toxin. The inventors found that very effective tumor cell killing is achieved when sdAbs are selected that bind any of HER2, VEGFR, CD71, which sdAb is combined in the conjugate with a toxin such as a protein toxin, such as dianthin or saporin, and which sdAb is combined with the saponin. Examples demonstrating the high efficacy of certain conjugates comprising an sdAb and an effector molecule are provided in the Examples section and are displayed in Figures 4-7.

An embodiment is the conjugate of the invention, wherein the at least one effector molecule comprises or consists of at least one of: urease and Cre-recombinase, a proteinaceous toxin, a ribosome-inactivating protein, a protein toxin, a bacterial toxin, a plant toxin, more preferably selected from any one or more of a viral toxin such as apoptin; a bacterial toxin such as Shiga toxin, Shiga-like toxin, *Pseudomonas aeruginosa* exotoxin (PE) or exotoxin A of PE, full-length or truncated diphtheria toxin (DT), cholera toxin; a fungal toxin such as alpha-sarcin; a plant toxin including ribosome-inactivating proteins and the A chain of type 2 ribosome-inactivating proteins such as dianthin *e.g.* dianthin-30 or dianthin-32, saporin *e.g.* saporin-S3 or saporin-S6, bouganin or de-immunized derivative debouganin of bouganin, shiga-like toxin A, pokeweed antiviral protein, ricin, ricin A chain, modeccin, modeccin A chain, abrin, abrin A chain, volkensin, volkensin A chain, viscumin, viscumin A chain; or an animal or human toxin such as frog RNase, or granzyme B or human angiogenin, or any toxic fragment or toxic derivative thereof; preferably the protein toxin is dianthin and/or saporin.

An embodiment is the conjugate of the invention, wherein the at least one effector molecule comprises or consists of at least one payload.

An embodiment is the conjugate of the invention, wherein the at least one effector molecule comprises or consists of at least one of: a toxin targeting ribosomes, a toxin targeting elongation factors, a toxin targeting tubulin, a toxin targeting DNA and a toxin targeting RNA, more preferably any one or more of emtansine, pasudotox, maytansinoid derivative DM1, maytansinoid derivative DM4, monomethyl auristatin E (MMAE, vedotin), monomethyl auristatin F (MMAF, mafodotin), a Calicheamicin, N-Acetyl-γ-calicheamicin, a pyrrolobenzodiazepine (PBD) dimer, a benzodiazepine, a CC-1065 analogue, a duocarmycin, Doxorubicin, paclitaxel, docetaxel, cisplatin, cyclophosphamide, etoposide, docetaxel, 5-fluorouracyl (5-FU), mitoxantrone, a tubulysin, an indolinobenzodiazepine, AZ13599185, a cryptophycin, rhizoxin, methotrexate, an anthracycline, a camptothecin analogue, SN-38, DX-8951f, exatecan mesylate, truncated form of *Pseudomonas aeruginosa* exotoxin (PE38), a Duocarmycin derivative, an amanitin, α-amanitin, a spliceostatin, a thailanstatin, ozogamicin, tesirine, Amberstatin269 and soravtansine, or a derivative thereof.

An effector moiety useful in the present invention preferably relies on late endosomal escape for exerting its effect. Some effector molecules, such as, *e.g.*, a *pseudomonas* exotoxin, are rerouted to other organelles prior to the "late endosomal stage" and, thus, would normally not benefit from incorporation in the conjugate according to the present invention. However, such toxin may be adapted for use with the present invention, *e.g.*, by deleting the signal peptide responsible for rerouting. In particular toxins that are highly toxic and would require only one molecule to escape the endosomes to kill a cell maybe modified to be less potent. It is preferred to use a toxin that kills a cell if at least 2, more preferably at least 5, more preferably at least 10, more preferably at least 20, more preferably at least 50, most preferably at least 100 toxin molecules escape the endosome (and enter the cytosol). It is further preferred that a conjugate of the invention comprises a covalently conjugated functionalized scaffold, *i.e.* a scaffold such as an oligomeric or polymeric scaffold or a tri-functional linker, comprising covalently bound effector moiety or moieties for targeting the scaffold comprising the bound effector moiety/moieties at a target cell such as a tumor cell or an auto-immune cell. Further, in order to reduce

off-target toxicity, cell membrane non-permeable small molecule toxins are preferred effector molecules over cell membrane permeable toxins.

Preferably, the effector moiety comprised by the conjugate of the invention, which effect is enhanced by the saponins comprised by the conjugate, detaches from the conjugate, *e.g.* detaches from the antibody, such as an sdAb, present in the conjugate as the cell-surface molecule targeting moiety of the conjugate, when endocytosed. This can be achieved by a cleavable bond that breaks, *e.g.* under acidic, reductive, enzymatic or light-induced conditions.

An embodiment is the conjugate of the invention, wherein the conjugate comprises an antibody-drug conjugate (ADC), such as an ADC comprising at least one sdAb derived from: gemtuzumab ozogamicin, brentuximab vedotin, trastuzumab emtansine, inotuzumab ozogamicin, moxetumomab pasudotox and polatuzumab vedotin, and/or comprising at least one effector molecule which is a toxin present in any one or more of: gemtuzumab ozogamicin, brentuximab vedotin, trastuzumab emtansine, inotuzumab ozogamicin, moxetumomab pasudotox and polatuzumab vedotin, and/or selected from dianthin and saporin. It will be appreciated that when an sdAb is derived from such a human antibody, the V<sub>H</sub> domain of such a human antibody may require some improvements with regard to domain stability ('camelization' of the human V<sub>H</sub> domain), known in the art.

An embodiment is the conjugate of the invention, wherein the at least one saponin comprises an aglycone core structure selected from (Group C):

2alpha-hydroxy oleanolic acid;  
16alpha-hydroxy oleanolic acid;  
hederagenin (23-hydroxy oleanolic acid);  
16alpha,23-dihydroxy oleanolic acid;  
gypsogenin;  
quillaic acid;  
protoaescigenin-21(2-methylbut-2-enoate)-22-acetate;  
23-oxo-barringtonenol C-21,22-bis(2-methylbut-2-enoate);  
23-oxo-barringtonenol C-21(2-methylbut-2-enoate)-16,22-diacetate;  
digitogenin;  
3,16,28-trihydroxy oleanan-12-en;  
gypsogenic acid; or  
a derivative thereof,

preferably, the at least one saponin comprises an aglycone core structure selected from quillaic acid and gypsogenin, more preferably the at least one saponin comprises aglycone core structure quillaic acid.

Without wishing to be bound by any theory, presence of an aldehyde group (or derivative thereof) in the aglycone core structure of the saponin (here, also referred to as 'aglycone') is beneficial for the capacity of the saponin to stimulate and/or potentiate the endosomal escape of the effector molecule comprised by the conjugate of the invention, when such a saponin co-localizes in a cell, in the endosome of said cell, with these effector molecules, as part of the conjugate of the invention or when in free form inside the endosome (*e.g.* split off from the conjugate once the conjugate is delivered inside

the target cell endosome or lysosome). Therefore, the conjugates of the invention comprising saponin which has an aglycone with an aldehyde group is preferred. In quillaic acid and in gypsogenin the aldehyde group is at the C<sub>23</sub> atom of the aglycone.

An embodiment is the conjugate of the invention, wherein the at least one saponin comprises  
 5 one or both of: a first saccharide chain bound to the C<sub>3</sub> atom or to the C<sub>28</sub> atom of the aglycone core structure of the at least one saponin, preferably bound to the C<sub>3</sub> atom, and a second saccharide chain bound to the C<sub>28</sub> atom of the aglycone core structure of the at least one saponin, and preferably the at least one saponin comprises the first and the second saccharide chain. Thus, when the saponin  
 10 comprised by the conjugate of the invention bears two glycans (saccharide chains), the first saccharide chain is bound at position C<sub>3</sub> of the aglycone core structure and the second saccharide chain is bound at position C<sub>28</sub> of the aglycone core structure of the saponin.

An embodiment is the conjugate of the invention, wherein

- the at least one saponin comprises the first saccharide chain selected from (Group A):

GlcA-,  
 15 Glc-,  
 Gal-,  
 Rha-(1→2)-Ara-,  
 Gal-(1→2)-[Xyl-(1→3)]-GlcA-,  
 Glc-(1→2)-[Glc-(1→4)]-GlcA-,  
 20 Glc-(1→2)-Ara-(1→3)-[Gal-(1→2)]-GlcA-,  
 Xyl-(1→2)-Ara-(1→3)-[Gal-(1→2)]-GlcA-,  
 Glc-(1→3)-Gal-(1→2)-[Xyl-(1→3)]-Glc-(1→4)-Gal-,  
 Rha-(1→2)-Gal-(1→3)-[Glc-(1→2)]-GlcA-,  
 Ara-(1→4)-Rha-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 25 Ara-(1→4)-Fuc-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 Ara-(1→4)-Rha-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 Ara-(1→4)-Fuc-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 Ara-(1→4)-Rha-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 Ara-(1→4)-Fuc-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 30 Ara-(1→4)-Rha-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-,  
 Ara-(1→4)-Fuc-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-,  
 Xyl-(1→4)-Rha-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 Xyl-(1→4)-Fuc-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 Xyl-(1→4)-Rha-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 35 Xyl-(1→4)-Fuc-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 Xyl-(1→4)-Rha-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 Xyl-(1→4)-Fuc-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 Xyl-(1→4)-Rha-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-,  
 Xyl-(1→4)-Fuc-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-, and  
 40 any derivative thereof,

and/or

- the at least one saponin comprises the second saccharide chain selected from (Group B):

Glc-,

Gal-,

5 Rha-(1→2)-[Xyl-(1→4)]-Rha-,

Rha-(1→2)-[Ara-(1→3)-Xyl-(1→4)]-Rha-,

Ara-,

Xyl-,

Xyl-(1→4)-Rha-(1→2)-[R1-(→4)]-Fuc- wherein R1 is 4E-Methoxycinnamic acid,

10 Xyl-(1→4)-Rha-(1→2)-[R2-(→4)]-Fuc- wherein R2 is 4Z-Methoxycinnamic acid,

Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-4-OAc-Fuc-,

Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-3,4-di-OAc-Fuc-,

Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R3-(→4)]-3-OAc-Fuc- wherein R3 is 4E-Methoxycinnamic acid,

Glc-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-4-OAc-Fuc-,

15 Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-4-OAc-Fuc-,

(Ara- or Xyl-)(1→3)-(Ara- or Xyl-)(1→4)-(Rha- or Fuc-)(1→2)-[4-OAc-(Rha- or Fuc-)(1→4)]-(Rha- or Fuc-),

Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Qui-(1→4)]-Fuc-,

Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-Fuc-,

20 Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-Fuc-,

Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-Fuc-,

Ara/Xyl-(1→4)-Rha/Fuc-(1→4)-[Glc/Gal-(1→2)]-Fuc-,

Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R4-(→4)]-Fuc- wherein R4 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),

25 Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R5-(→4)]-Fuc- wherein R5 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),

Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,

Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,

6-OAc-Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3-OAc-Rha-(1→3)]-Fuc-,

30 Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3-OAc-Rha-(1→3)]-Fuc-,

Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Qui-(1→4)]-Fuc-,

Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-[Qui-(1→4)]-Fuc-,

Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Xyl-(1→3)-4-OAc-Qui-(1→4)]-Fuc-,

Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3,4-di-OAc-Qui-(1→4)]-Fuc-,

35 Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-Fuc-,

6-OAc-Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-Fuc-,

Glc-(1→3)-[Xyl-(1→3)-Xyl-(1→4)]-Rha-(1→2)-Fuc-,

Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Xyl-(1→3)-4-OAc-Qui-(1→4)]-Fuc-,

Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,

40 Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,

- Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R6-(→4)]-Fuc- wherein R6 is 5-O-[5-O-Rha-(1→2)-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R7-(→4)]-Fuc- wherein R7 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 5    Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R8-(→4)]-Fuc- wherein R8 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R9-(→4)]-Fuc- wherein R9 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R10-(→4)]-Fuc- wherein R10 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 10    Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R11-(→3)]-Fuc- wherein R11 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R12-(→3)]-Fuc- wherein R12 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)  
 15    Glc-(1→3)-[Glc-(1→6)]-Gal-, and  
 any derivative thereof.

Thus, when the saponin comprised by the conjugate of the invention bears two glycans (saccharide chains), the first saccharide chain is bound at position C<sub>3</sub> of the aglycone core structure of the saponin and the second saccharide chain is bound at position C<sub>28</sub> of the aglycone core structure of the saponin.

- 20    Preferably, the saponin has an aldehyde group in the aglycone.

An embodiment is the conjugate of the invention, wherein the at least one saponin comprises the first saccharide chain and comprises the second saccharide chain according to Group A and Group B, respectively, wherein the first saccharide chain comprises more than one saccharide moiety and the second saccharide chain comprises more than one saccharide moiety, and wherein the aglycone core structure preferably is quillaic acid or gypsogenin, more preferably quillaic acid, wherein one, two or  
 25    three, preferably one or two, of:

- i. an aldehyde group in the aglycone core structure has been derivatised,
- ii. a carboxyl group of a glucuronic acid moiety in the first saccharide chain has been derivatised, and
- 30    iii. at least one acetoxyl (Me(CO)O-) group in the second saccharide chain has been derivatised.

An embodiment is the conjugate of the invention, wherein the at least one saponin comprises:

- i. an aglycone core structure comprising an aldehyde group which has been derivatised by:
  - reduction to an alcohol;
  - transformation into a hydrazone bond through reaction with N-ε-maleimidocaproic acid hydrazide (EMCH) wherein the maleimide group of the EMCH is optionally derivatised by formation of a thioether bond with mercaptoethanol;
  - 35    - transformation into a hydrazone bond through reaction with N-[β-maleimidopropionic acid] hydrazide (BMPH) wherein the maleimide group of the BMPH is optionally derivatised by formation of a thioether bond with mercaptoethanol; or

- transformation into a hydrazone bond through reaction with N-[ $\kappa$ -maleimidoundecanoic acid] hydrazide (KMUH) wherein the maleimide group of the KMUH is optionally derivatised by formation of a thioether bond with mercaptoethanol;

- ii. a first saccharide chain comprising a carboxyl group, preferably a carboxyl group of a glucuronic acid moiety, which has been derivatised by transformation into an amide bond through reaction with 2-amino-2-methyl-1,3-propanediol (AMPD) or *N*-(2-aminoethyl)maleimide (AEM);
- iii. a second saccharide chain comprising an acetoxy group (Me(CO)O-) which has been derivatised by transformation into a hydroxyl group (HO-) by deacetylation; or
- iv. any combination of two or three derivatisations i., ii. and/or iii., preferably any combination of two derivatisations i., ii. and/or iii.

An embodiment is the conjugate of the invention, wherein the at least one saponin is any one or more of: *Quillaja* bark saponin, dipsacoside B, saikosaponin A, saikosaponin D, macranthoidin A, esculentoside A, phytolaccagenin, aescinate, AS6.2, NP-005236, AMA-1, AMR, alpha-Hederin, NP-012672, NP-017777, NP-017778, NP-017774, NP-018110, NP-017772, NP-018109, NP-017888, NP-017889, NP-018108, SA1641, AE X55, NP-017674, NP-017810, AG1, NP-003881, NP-017676, NP-017677, NP-017706, NP-017705, NP-017773, NP-017775, SA1657, AG2, SO1861, GE1741, SO1542, SO1584, SO1658, SO1674, SO1832, SO1904, SO1862, QS-7, QS1861, QS-7 api, QS1862, QS-17, QS-18, QS-21 A-apio, QS-21 A-xylo, QS-21 B-apio, QS-21 B-xylo, beta-Aescin, Aescin Ia, Teaseed saponin I, Teaseedsaponin J, Assamsaponin F, Digitonin, Primula acid 1 and AS64R, or a derivative thereof, or a stereoisomer thereof, and/or any combinations thereof, preferably any one or more of QS-21 or a QS-21 derivative, SO1861 or a SO1861 derivative, SA1641 or a SA1641 derivative and GE1741 or a GE1741 derivative, more preferably a QS-21 derivative or a SO1861 derivative, most preferably a SO1861 derivative, such as a saponin derivative according to the invention.

An embodiment is the conjugate of the invention, wherein the at least one saponin is any one or more of: SO1861, SA1657, GE1741, SA1641, QS-21, QS-21A, QS-21 A-api, QS-21 A-xyl, QS-21B, QS-21 B-api, QS-21 B-xyl, QS-7-xyl, QS-7-api, QS-17-api, QS-17-xyl, QS1861, QS1862, *Quillaja* saponin, Saponinum album, QS-18, Quil-A, Gyp1, gypsoside A, AG1, AG2, SO1542, SO1584, SO1658, SO1674, SO1832, SO1862, SO1904, or a derivative thereof, or a stereoisomer thereof, and/or any combinations thereof, preferably the saponin derivative is a SO1861 derivative and/or a GE1741 derivative and/or a SA1641 derivative and/or a QS-21 derivative, more preferably the saponin derivative is a SO1861 derivative or a QS21 derivative, most preferably, the saponin (functional) derivative is a SO1861 derivative according to the invention.

Preferred is the saponin-comprising conjugate of the invention, wherein the at least one saponin is selected from: 3-O-beta-D-galactopyranosyl-(1 $\rightarrow$ 2)-[beta-D-xylopyranosyl-(1 $\rightarrow$ 3)]-beta-D-glucuronopyranosyl quillaic acid 28-O-beta-D-glucopyranosyl-(1 $\rightarrow$ 3)-beta-D-xylopyranosyl-(1 $\rightarrow$ 4)- alpha-L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[beta-D-xylopyranosyl-(1 $\rightarrow$ 3)]-4OAc-beta-D-quinovopyranosyl-(1 $\rightarrow$ 4)]-beta-D-fucopyranoside, *Quillaja* bark saponin, dipsacoside B, saikosaponin A, saikosaponin D, macranthoidin A, esculentoside A, phytolaccagenin, aescinate, AS6.2, NP-005236, AMA-1, AMR, alpha-Hederin, NP-012672, NP-017777, NP-017778, NP-017774, NP-018110, NP-

017772, NP-018109, NP-017888, NP-017889, NP-018108, SA1641, AE X55, NP-017674, NP-017810, AG1, NP-003881, NP-017676, NP-017677, NP-017706, NP-017705, NP-017773, NP-017775, SA1657, AG2, SO1861, SO1862, SO1904, GE1741, SO1542, SO1584, SO1658, SO1674, SO1832, QS-7, QS1861, QS-7 api, QS1862, QS-17, QS-18, QS-21 A-apio, QS-21 A-xylo, QS-21 B-apio, QS-21 B-xylo, beta-Aescin, Aescin Ia, Teaseed saponin I, Teaseedsaponin J, Assamsaponin F, Digitonin, Primula acid 1 and AS64R, preferably the at least one saponin is selected from: QS-21, GE1741, SA1641 and SO1861, and/or a functional derivative thereof, the functional derivative lacking an aldehyde group in the aglycone core structure of the at least one saponin, and/or without a glucuronic acid moiety comprising a carboxyl group in a first saccharide chain of the at least one saponin, more preferably QS-21, SO1861, most preferably, SO1861. Typically, the saponin is a saponin selected from Table A1, known for the endosomal escape enhancing activity towards an effector molecule when contacted with a selected cell together with the effector molecule (such as part as an effector moiety of an ADC or an AOC).

An embodiment is the saponin-comprising conjugate of the invention, wherein the at least one saponin is selected from: SO1861, SA1657, GE1741, SA1641, QS-21, QS-21A, QS-21 A-api, QS-21 A-xyl, QS-21B, QS-21 B-api, QS-21 B-xyl, QS-7-xyl, QS-7-api, QS-17-api, QS-17-xyl, QS1861, QS1862, Quillaja saponin, Saponinum album, QS-18, Quil-A, Gyp1, gypsoside A, AG1, AG2, SO1542, SO1584, SO1658, SO1674, SO1832, SO1862, SO1904, and preferably the at least one saponin is selected from: SO1861, GE1741, SA1641 and QS-21, preferably a functional derivative thereof without an aldehyde group in the aglycone core structure of the at least one saponin, and/or without a glucuronic acid moiety comprising a carboxyl group in a first saccharide chain of the at least one saponin. Typically, the at least one saponin comprised by the sdAb-saponin conjugate is selected from Table A1. SO1861 is preferred.

Such saponins of the triterpene glycoside type are capable of enhancing the endosomal escape of the effector molecules comprised by the conjugate, and that are present in the endosome (or lysosome) of a cell, when the saponin as part of the conjugate or in free form co-localizes with such effector molecule inside the cell. The inventors established that the endosomal escape enhancing activity of these saponins is about 100 to 1000 times more potent when the saponin is contacted with a cell as part of the conjugate of the invention. The free saponin is capable of stimulating the delivery of effector molecules in the cytosol of cells, when such cells are contacted with the effector molecules as part of a certain cell-targeting conjugate such as an ADC or an AOC, and the saponin, at 100-1000 times higher saponin concentration, compared to the concentration of the same saponin which is comprised by the conjugate of the invention, required to achieve the same extent of delivery of the effector molecule from outside the target cell to inside the endosome and finally in the cytosol of said cell. Saponins which display such endosomal escape enhancing activity are listed in Table A1, as well as saponins with high structural similarity with saponins for which the ability to potentiate the cytosolic delivery of the effector molecules comprised by the conjugate has been established. When the saponin is part of the conjugate of the invention, the targeted delivery of the saponin upon binding of the sdAb of the conjugate, to the targeted cell-surface binding site on the target cell, on said cell, and after endocytosis, into the endosome of said cell, is thus about 100 to 1000 times more effective compared to contacting the same

cell with free, untargeted saponin (derivative) which is not provided with a binding molecule such as an antibody or an sdAb for binding to cell-surface molecule of the target cell.

The small size of an sdAb of the conjugates of the invention, compared to *e.g.* IgG type of antibodies, or fragments thereof such as Fab, scFv, contributes to efficient uptake by the target cell that exposes the binding site for binding of the sdAb comprised by the conjugate, *e.g.* uptake by endocytosis. Typically, the sdAbs in the conjugates of the invention are capable of binding to a cell-surface receptor of a target cell, such as a tumor cell specific cell-surface receptor. This way, the conjugate of the invention is particularly suitable for endocytosis into *e.g.* tumor cells expressing the cell-surface receptor.

An embodiment is the conjugate of the invention, wherein the at least one sdAb comprises an sdAb for binding to a cell-surface molecule of the cell wherein the cell is an aberrant cell such as a tumor cell, an auto-immune cell, an infected cell such as a virally infected cell, or a cell comprising a gene defect or an enzyme defect.

An embodiment is the conjugate of the invention, wherein the at least one sdAb comprises an sdAb for binding to a cell-surface molecule of the cell, the sdAb derived from or based on any one or more of immunoglobulins: an anti-CD71 antibody such as IgG type OKT-9, an anti-HER2 antibody such as trastuzumab (Herceptin), pertuzumab, an anti-CD20 antibody such as rituximab, ofatumumab, tositumomab, obinutuzumab ibritumomab, an anti-CA125 antibody such as oregovomab, an anti-EpCAM (17-1A) antibody such as edrecolomab, an anti-EGFR antibody such as cetuximab, matuzumab, panitumumab, nimotuzumab, an anti-CD30 antibody such as brentuximab, an anti-CD33 antibody such as gemtuzumab, huMy9-6, an anti-vascular integrin alpha-v beta-3 antibody such as etaracizumab, an anti-CD52 antibody such as alemtuzumab, an anti-CD22 antibody such as epratuzumab, pinatuzumab, binding fragment (Fv) of anti-CD22 antibody moxetumomab, humanized monoclonal antibody inotuzumab, an anti-CEA antibody such as labetuzumab, an anti-CD44v6 antibody such as bivatzumab, an anti-FAP antibody such as sibrotuzumab, an anti-CD19 antibody such as huB4, an anti-CanAg antibody such as huC242, an anti-CD56 antibody such as huN901, an anti-CD38 antibody such as daratumumab, OKT-10 anti-CD38 monoclonal antibody, an anti-CA6 antibody such as DS6, an anti-IGF-1R antibody such as cixutumumab, 3B7, an anti-integrin antibody such as CNTO 95, an anti-syndecan-1 antibody such as B-B4, an anti-CD79b such as polatuzumab, an anti-HIVgp41 antibody, preferably any one of an anti-HIVgp41 antibody, an anti-CD71 antibody, an anti-HER2 antibody and an anti-EGFR antibody, more preferably any one of: trastuzumab, pertuzumab, cetuximab, matuzumab, an anti-CD71 antibody, OKT-9, most preferably trastuzumab, cetuximab, the anti-CD71 antibody OKT-9.

These cell-surface molecules are typically present on tumor cells with tumor cell specificity, at least to a certain extent. Tumor cell specificity makes these receptors suitable targets for the conjugates of the invention, and therefore the sdAb in the conjugate is capable of binding to such a cell-surface receptor. Since the saponins comprised by the conjugate of the invention are capable of stimulating the release and delivery of the effector molecules comprised by the conjugate of the invention, in the cytosol of cells, such as the (tumor) cells targeted by the sdAb comprised by the conjugate of the invention, it is particularly suitable to select as the target (tumor) cell surface molecule for the sdAb, a cell-surface receptor known for its suitability to serve as the target for *e.g.* ADCs and AOCs. The conjugate of the

invention is therewith suitable for co-delivery of the effector molecule that is part of the conjugate, together with the saponin comprised by the very same conjugate of the invention, which conjugate is an improved ADC or an improved AOC comprising an sdAb and comprising a saponin. Targeting a tumor cell specific receptor with the conjugate of the invention promotes endocytosis and delivery of the saponin as part of the conjugate into the target cell endosome and/or lysosome. When the tumor cell is contacted with the conjugate of the invention, the effector molecule comprised by the conjugate of the invention is co-delivered into the endosome or lysosome, and under influence of the co-localized saponin, the effector molecule is subsequently transferred into the cytosol of the target cell. As explained herein earlier, the application of targeted saponin as part of the conjugate of the invention results in an about 100-fold to 1000-fold improvement of the potentiating effect of the saponin, when biological activity of the effector molecule comprised by the conjugate of the invention is considered, compared to the application of free saponin lacking a cell-targeting binding molecule such as a receptor ligand, an antibody or an sdAb.

Application of the small sdAb such as a camelid  $V_H$  in the conjugate of the invention prevents or slows down clearance of the conjugate of the invention from the circulation and from the body of a human subject to whom the conjugate was administered, when compared to clearance rates commonly observed for antibody based ADCs. In addition, due to the relatively small size of the sdAb, the risk for limiting or hampering the saponin activity inside a target cell due to the presence of the linked protein domain is limited, compared to the larger size of *e.g.* an antibody when such an antibody would be bound to the saponin. In general, the smaller the size of the molecule linked to the saponin, the smaller the risk for interference with the saponin activity inside cells due to the presence of the bound molecule, *e.g.* an sdAb such as a  $V_{HH}$ . Moreover, the relative small size of the sdAbs results in their rapid distribution in tissue, such as tumor tissue, allowing for improved reaching of target cells by the conjugate of the invention, and therewith to improved (extent of) binding to the target cells, compared to the relatively large-sized IgGs commonly applied in *e.g.* ADCs, OACs. One of the many benefits of applying sdAbs in the conjugates of the invention, is the absence of an Fc tail common to regular antibodies of *e.g.* the IgG type. Absence of an Fc tail in the sdAb in the conjugate of the invention prevents occurrence of Fc $\gamma$ -Receptor mediated off-target effects such as undesired side effects relating to Fc $\gamma$ -Receptor activation, when the conjugate is administered to a patient in need thereof. Absence of an Fc tail eliminates the risk of side effects generated by the binding of an Fc to cells of a patient to whom *e.g.* an antibody-based ADC is administered. The sdAb comprising conjugates of the invention do not bear this risk for Fc-mediated undesired side effects.

An embodiment is the conjugate of the invention, wherein the at least one effector molecule is covalently bound to at least one sdAb, preferably one, of the at least one sdAb and/or to at least one, preferably one, of the at least one saponin, either via a linker or bound directly to the sdAb and/or to the saponin, and/or wherein the at least one saponin is covalently bound to at least one sdAb, preferably one, of the at least one sdAb and/or to at least one effector molecule, preferably one, of the at least one effector molecule, either via a linker or bound directly to the sdAb and/or to the effector molecule.

An embodiment is the conjugate of the invention, wherein the conjugate comprises a trifunctional linker with each of the at least one sdAb, the at least one saponin and the at least one

effector molecule covalently bound to the trifunctional linker, preferably separately, either directly, or via a linker, and preferably, the conjugate comprises a trifunctional linker with one sdAb, the at least one saponin and at least one, preferably one, effector molecule covalently bound to the trifunctional linker, separately, either directly, or via a linker.

- 5       Coupling of the saponin to the sdAb and/or to the effector molecule via a linker provides flexibility when the binding site for coupling of the saponin to the sdAb and/or to the effector molecule is considered. Furthermore, such a linker may act as a spacer between the sdAb and the saponin and the effector molecule, such that the sdAb maintains its capability to bind to a binding site on a cell surface molecule and the saponin maintains its capability to enhance endosomal escape of the effector molecule  
10       comprised by the conjugate, and the effector molecule maintains its biological activity towards its intracellular binding partner.

- An embodiment is the conjugate of the invention, wherein the at least one saponin is covalently bound via a thio-ether bond to a sulfhydryl group in one of the at least one sdAb and/or in one of the at least one effector molecule, the covalent bonding preferably via linker N- $\epsilon$ -maleimidocaproic acid  
15       hydrazide (EMCH) that is covalently bound to an aldehyde group in position C<sub>23</sub> of the aglycone core structure of the saponin and that is covalently bound to the sulfhydryl group in the sdAb and/or in the effector molecule, such as a sulfhydryl group of a cysteine.

- An embodiment is the conjugate of the invention, wherein the at least one saponin is a bi-desmosidic triterpene saponin or derivative thereof belonging to the type of a 12,13-dehydrooleanane  
20       with optionally an aldehyde function in position C<sub>23</sub> and comprising a glucuronic acid unit in a first saccharide chain bound at the C<sub>3</sub>beta-OH group of the aglycone core structure of the saponin, wherein the saponin is covalently bound to an amino-acid residue of the at least one sdAb and/or of the at least one effector molecule via the carboxyl group of the glucuronic acid unit in the first saccharide chain, preferably via a linker, wherein the amino-acid residue preferably is selected from cysteine and lysine.

- 25       An embodiment is the conjugate of the invention, wherein the at least one saponin comprises a glucuronic acid unit in the first saccharide chain at the C<sub>3</sub>beta-OH group of the aglycone core structure of the saponin, which glucuronic acid unit is covalently bound to a linker, which linker is preferably covalently bound via an amide bond to an amine group in the at least one sdAb and/or in the at least one effector molecule, such as an amine group of a lysine or an N-terminus of the sdAb and/or of the  
30       effector molecule, preferably said linker is 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU).

- An embodiment is the conjugate of the invention, comprising more than one covalently bound saponin moieties of the at least one saponin, preferably 2, 3, 4, 5, 6, 8, 10, 16, 32, 64, 128 or 1-100 of such moieties, or any number of such moieties therein between, such as 7, 9, 12 saponin moieties. An  
35       embodiment is the conjugate of the invention, wherein the conjugate comprises 1 – 100 saponin moieties of the at least one saponin, preferably 2 – 64 saponin moieties, more preferably 4 – 32 saponin moieties, most preferably 8 – 16 saponin moieties, or any number therein between. Typically, the conjugate of the invention comprises 1, 2, 4, 8 or 16 saponin moieties (copies of the saponin molecule). For example, 4 saponin molecules are covalently linked to a G2 dendron and at least one, such as one  
40       or two, of the dendron-(saponin)<sub>4</sub> conjugate (saponin conjugate) is covalently linked to an sdAb. For

example, 8 saponin molecules are covalently linked to a G3 dendron and at least one, such as one or two, of the dendron-(saponin)<sub>8</sub> conjugate (saponin conjugate) is covalently linked to an sdAb. An embodiment is the conjugate of the invention wherein the conjugate comprises more than one saponin moieties wherein the saponin moieties are the same or different. That is to say, if more than one saponins are covalently linked to the sdAb(s) in the conjugate of the invention, these saponins can all be copies of the same saponin, or the saponin are different saponins. Preferred is the conjugate comprising multiple saponin moieties, wherein the saponins that are bound to the sdAb(s) are the same. For example, 2, 4, 8, 16 saponin molecules covalently linked to the sdAb's in the conjugate, for example 2-16 SO1861 copies or QS-21 copies, preferably SO1861.

An embodiment is the conjugate of the invention, wherein the more than one covalently bound saponin moieties are covalently bound directly to an amino-acid residue of the at least one sdAb and/or of the at least one effector molecule, preferably to a cysteine and/or to a lysine, and/or are covalently bound via a linker and/or via a cleavable linker.

An embodiment is the endosomal and/or lysosomal escape enhancing conjugate according to the invention, essentially having the molecular format of molecular structure (II):

**(saponin – linker –)<sub>a</sub> immunoglobulin – effector moiety**

(STRUCTURE (II)),

wherein a = 1-4, preferably 1, 2, 4, and wherein the immunoglobulin is preferably an sdAb,

or essentially having the molecular format of molecular structure (III):

**(saponin – dendron(-saponin)<sub>x</sub>)<sub>b</sub> – immunoglobulin – effector moiety**

(STRUCTURE (III)),

wherein x = between 1 and 100, preferably 1-63, 1-31, 1-15, 1-7, or 3; b = 1-4, preferably 1, 2, 4, and wherein the immunoglobulin is preferably an sdAb,

or essentially having the molecular format of molecular structure (IV):

**(saponin – trifunctional linker(-effector moiety))<sub>c</sub> – immunoglobulin**

(STRUCTURE (IV)),

wherein c = 1-4, preferably 1, 2, 4, and wherein the immunoglobulin is preferably an sdAb,

or essentially having the molecular format of molecular structure (V):



5

(STRUCTURE (V)),

wherein  $y$  = between 1 and 100, preferably 1-63, 1-31, 1-15, 1-7, or 3;  $d$  = 1-4, preferably 1, 2, 4, and wherein the immunoglobulin ('Ig') is preferably an sdAb.

10 Preferably,  $x$  or  $y$  is 3, 7 or 15. Preferably,  $b$  or  $d$  is 1, 2 or 4, although in some embodiments,  $b$  or  $d$  is 3, and when the immunoglobulin is an sdAb such as a  $V_{HH}$ ,  $a$  is preferably 1,  $b$  is preferably 1,  $c$  is preferably 1 and  $d$  is preferably 1. The Dendron is for example a G2, G3, G4 dendron or a G5 dendron. Preferably, the saponin is bound to the linker via a cleavable bond, such as a hydrazone bond that is  
15 lysosome). Preferably the linker is EMCH. Preferably, the trifunctional linker is the linker with Structure A as displayed hereunder. For Structure II and III, preferably, the effector moiety is bound to the Ig via a linker such as a cleavable linker.

An embodiment is the conjugate of the invention, wherein the more than one covalently bound saponin moieties are part of a covalent saponin conjugate comprising at least one oligomeric molecule  
20 or polymeric molecule and the more than one saponin covalently bound thereto, wherein the covalent saponin conjugate is covalently bound to at least one of the at least one sdAb and/or to at least one of the at least one effector molecule. An embodiment is the conjugate of the invention, wherein the at least one saponin is part of a covalent saponin conjugate comprising an oligomeric molecule or a polymeric molecule to which the saponin is covalently bound, and wherein the sdAb is also covalently bound to  
25 the same oligomeric molecule or polymeric molecule as to which the saponin is bound. Thus, the oligomeric molecule or the polymeric molecule comprises the one or more covalently bound saponin moieties and is covalently bound to a sdAb. The saponin and the sdAb are covalently bound to each other via the polymeric molecule or the oligomeric molecule. The oligomeric molecule or the polymeric molecule links the sdAb('s) and the saponin(s) together, providing a saponin-comprising conjugate of  
30 the invention. In addition, the effector molecule is either bound to the oligomeric molecule or the polymeric molecule, or is bound to the sdAb('s).

Such a covalent saponin conjugate of saponins bound to the oligomeric or polymeric molecule serves as a carrier (support, scaffold) for multiple saponin moieties, which can be bound to the sdAb comprised by the conjugate, via a single bond, preferably via a (cleavable) linker. Since the covalent  
35 saponin conjugate can bear any selected number of covalently bound saponin moieties, such as 1-200 saponin moieties, relating to the type of selected oligomeric or polymeric structure comprising binding sites for covalent linking these saponins, application of such covalent saponin conjugate provides freedom when the number of saponin moieties in the conjugate of the invention is considered. For example, for cytosolic delivery of the effector molecule comprised by the conjugate of the invention, the  
40 number of saponins present in the conjugate of the invention can be adapted by providing the covalent

saponin conjugate with a number of saponin moieties sufficient and enough for stimulating the cytosolic delivery of the effector molecule, when the covalent saponin conjugate is part of the conjugate of the invention, and when the effector molecule co-localizes with the saponins as integral part of the very same conjugate in the endosome or lysosome of a target cell in which the effector molecule should exert its biological activity.

Preferably, 1-8 of the covalent saponin conjugates are bound to the sdAb and/or to the effector molecule, more preferably 2-4 of such of such covalent saponin conjugates, wherein the at least one covalent saponin conjugate is optionally based on a dendron (for example a G2 dendron, G3 dendron, G4 dendron or a G5 dendron), wherein optionally 1-32 saponin moieties, preferably 2, 3, 4, 5, 6, 8, 10, 16, 32 of such moieties, or any number of such moieties therein between, such as 7, 9, 12 saponin moieties, are covalently bound to the oligomeric molecule or to the polymeric molecule of the at least one covalent saponin conjugate, either directly or via a linker. For example, 4 saponin moieties are bound to a G2 dendron, 8 saponin moieties are bound to a G3 dendron, 16 saponin moieties are bound to a G4 dendron, wherein the saponin for example is SO1861 or QS21, preferably SO1861.

An embodiment is the conjugate of the invention, wherein the more than one covalently bound saponin moieties are part of a covalent saponin conjugate comprising an oligomeric molecule or a polymeric molecule to which the saponin is covalently bound, and wherein the sdAb is also covalently bound to the same oligomeric molecule or polymeric molecule as to which the saponin is bound and wherein the effector moiety is covalently bound to the sdAb or to the oligomeric molecule or the polymeric molecule, preferably 1-8 of such oligomeric molecules or polymeric molecules comprising the saponin(s) is/are covalently bound to the sdAb, or 2-4 of the oligomeric molecules or polymeric molecules comprising the saponin(s) are covalently bound to the sdAb, wherein the oligomeric molecule or the polymeric molecule of the covalent saponin conjugate is optionally a dendron such as a G2 dendron, G3 dendron, G4 dendron or G5 dendron with 4, 8, 16 and 32 binding sites for covalently binding 4, 8, 16 or 32 saponin moieties, respectively, wherein optionally 1-32 saponin moieties, preferably 2, 3, 4, 5, 6, 8, 10, 16, 32 of such moieties, or any number of such moieties therein between, such as 7, 9, 12 saponin moieties, are covalently bound to the oligomeric molecule or to the polymeric molecule of the at least one covalent saponin conjugate, either directly or via a linker.

Preferably, one or two of the covalent saponin conjugates is/are bound to a single sdAb in the conjugate of the invention. For many purposes, coupling of a single saponin or coupling of a single covalent saponin conjugate to a single sdAb comprised by the conjugate, suffices for efficient stimulation of the effector molecule delivery into a target cell and into the cytosol of said cell, wherein the effector molecule is comprised by the conjugate of the invention. Typically, 4, 8 or 16 saponins are comprised by the conjugate of the invention, such as 4 or 8 saponins comprised by a single covalent saponin conjugate coupled to the sdAb in the conjugate of the invention. For example, the 4, 8 or 16 saponin moieties are covalently coupled to a G2 dendron, G3 dendron or G4 dendron, respectively. Typically, such conjugates of the invention comprise a single sdAb, to which the saponin or saponins or the covalent saponin conjugate(s) is/are bound, preferably a single saponin or a single covalent saponin conjugate is part of the conjugate.

An embodiment is the conjugate of the invention, wherein the oligomeric molecule or the polymeric molecule of the covalent saponin conjugate is a dendron, such as a G2 dendron, G3 dendron, G4 dendron or G5 dendron, wherein 1-32 saponin moieties, preferably 2, 3, 4 (for example for a G2 dendron), 5, 6, 8 (for example for a G3 dendron), 10, 16 (for example for a G4 dendron), 32 (for example for a G5 dendron) saponin moieties, or any number of saponin moieties therein between, such as 7, 9, 12 saponin moieties, are covalently bound to the oligomeric molecule or to the polymeric molecule of the covalent saponin conjugate.

10 An embodiment is the saponin-comprising conjugate of the invention, wherein 1-8 of the oligomeric molecules or polymeric molecules comprising the saponin(s) is/are covalently bound to the sdAb.

An embodiment is the saponin-comprising conjugate of the invention, wherein 2-4 of the oligomeric molecules or polymeric molecules comprising the saponin(s) are covalently bound to the sdAb.

15 An embodiment is the saponin-comprising conjugate of the invention, wherein the oligomeric molecule or the polymeric molecule of the covalent saponin conjugate is a dendron. Preferred is an oligomeric molecule or a polymeric molecule such as a dendron or a polyethylene glycol that is biologically sufficiently inactive or inert, preferably biologically inactive or inert.

20 An embodiment is the saponin-comprising conjugate of the invention, wherein the oligomeric molecule or the polymeric molecule of the covalent saponin conjugate is a G2 dendron, a G3 dendron, a G4 dendron or a G5 dendron with 4, 8, 16 and 32 binding sites for covalently binding 4, 8, 16 or 32 saponin moieties, respectively.

25 An embodiment is the saponin-comprising conjugate of the invention, wherein 1-32 saponin moieties, preferably 2, 3, 4, 5, 6, 8, 10, 16, 32 saponin moieties, or any number of saponin moieties therein between, such as 7, 9, 12 saponin moieties, are covalently bound to the oligomeric molecule or to the polymeric molecule of the covalent saponin conjugate. It is to be understood that "saponin-comprising conjugate" in the context of the invention is referring to the conjugate of at least one saponin molecule and at least one sdAb and at least one effector molecule and is not limited to a conjugate of the invention comprising a single sdAb and a single saponin molecule. For example, the conjugate comprises or consists of one or two sdAb's such as V<sub>HH</sub>('s), and 1-32 saponin molecules, such as 2, 4, 8, 16 saponin molecules, and at least one effector moiety.

An embodiment is the conjugate of the invention, wherein the at least one saponin is covalently bound to at least one of the at least one sdAb and/or to at least one of the at least one effector molecule via a cleavable linker.

35 An embodiment is the conjugate of the invention, wherein the cleavable linker is subject to cleavage under acidic conditions, reductive conditions, enzymatic conditions and/or light-induced conditions, and preferably the cleavable linker comprises a cleavable bond selected from a hydrazone bond and a hydrazide bond subject to cleavage under acidic conditions, and/or a bond susceptible to proteolysis, for example proteolysis by Cathepsin B, and/or a bond susceptible for cleavage under reductive conditions such as a disulfide bond.

An embodiment is the conjugate of the invention, wherein the cleavable linker is subject to cleavage *in vivo* under acidic conditions as for example present in endosomes and/or lysosomes of mammalian cells, preferably human cells, preferably at pH 4.0 – 6.5, and more preferably at pH  $\leq$  5.5.

Such cleavable linkers that are cleavable under the conditions as apparent in endosomes and  
5 lysosomes facilitates the delivery of free saponin inside the endosome or lysosome, upon cleavage (splitting off) of the saponin from the remainder of the conjugate of the invention. This way, the conjugate of the invention combines the benefits of cell-targeted delivery of the saponin upon specific binding of the sdAb, to the cell-surface molecule on the target cell, and of the presence of the free saponin inside the cell, *i.e.* inside the endosome (or lysosome), which contributes to the ability of the free saponin to  
10 stimulate and/or facilitate the delivery of the effector molecule comprised by the conjugate of the invention, out of the endosome (or lysosome) and into the cytosol of the target cell.

An embodiment is the conjugate of the invention, wherein the oligomeric molecule or the polymeric molecule of the covalent saponin conjugate is covalently bound to at least one of the at least one sdAb and/or to at least one of the at least one effector molecule, preferably to an amino-acid residue  
15 of the sdAb and/or of the effector molecule.

An embodiment is the conjugate of the invention, wherein the at least one saponin is covalently bound to the oligomeric molecule or to the polymeric molecule of the covalent saponin conjugate via a cleavable linker according to the invention.

An embodiment is the conjugate of the invention, wherein the at least one saponin is covalently  
20 bound to the oligomeric molecule or to the polymeric molecule of the covalent saponin conjugate via any one or more of an imine bond, a hydrazone bond, a hydrazide bond, an oxime bond, a 1,3-dioxolane bond, a disulfide bond, a thio-ether bond, an amide bond, a peptide bond or an ester bond, preferably via a linker.

An embodiment is the conjugate of the invention, wherein the at least one saponin comprises  
25 an aglycone core structure comprising an aldehyde function in position C<sub>23</sub> and the at least one saponin comprises optionally a glucuronic acid function in a first saccharide chain at the C<sub>3</sub>β-OH group of the aglycone core structure of the saponin, which aldehyde function is involved in the covalent bonding to the oligomeric molecule or to polymeric molecule of the covalent saponin conjugate, and/or, if present, the glucuronic acid function is involved in the covalent bonding to the oligomeric molecule or to the  
30 polymeric molecule of the covalent saponin conjugate, the bonding of the saponin either via a direct covalent bond, or via a linker, wherein the linker is a cleavable linker or a stable linker. Here, stable refers to a bond between the saponin and the sdAb or the effector molecule, or to a bond between the saponin and the oligomeric or polymeric structure, which bond remains intact (is not cleaved) under the acidic conditions inside a cell, in particular the acidic conditions in the endosome or lysosome of such a  
35 cell. In addition, such a stable bond remains intact (*i.e.* is not cleaved) in *e.g.* the circulation and in the organs of a human subject to whom the conjugate of the invention comprising the covalent saponin conjugate, is administered. In contrast, a cleavable linker in the context of the binding of a saponin to the sdAb or to the effector molecule comprised by the conjugate, or to an oligomeric structure or a polymeric structure refers to a bond that is cleaved under the acidic conditions as apparent inside  
40 endosomes and lysosomes of mammalian cells such as a human cell, *e.g.* a tumor cell, whereas such

cleavable linker remains intact (is not cleaved) when a conjugate comprising such cleavable bonds is present in the circulation or in organs, *i.e.* outside cells, of *e.g.* a human subject to whom the conjugate of the invention is administered.

5 An embodiment is the conjugate of the invention, wherein the aldehyde function in position C<sub>23</sub> of the aglycone core structure of the at least one saponin is covalently bound to linker EMCH, which EMCH is covalently bound via a thio-ether bond to a sulfhydryl group in the oligomeric molecule or in the polymeric molecule of the covalent saponin conjugate, such as a sulfhydryl group of a cysteine. Binding of the EMCH linker to the aldehyde group of the aglycone of the saponin results in formation of a hydrazone bond. Such a hydrazone bond is a typical example of a cleavable bond under the acidic  
10 conditions inside endosomes and lysosomes. A saponin that is coupled to the sdAb comprised by the conjugate of the invention or to the effector molecule comprised by the conjugate of the invention, or to an oligomeric structure or polymeric structure of a covalent saponin conjugate, wherein such a covalent saponin conjugate is coupled to the sdAb or to the effector molecule of the conjugate, is releasable from the conjugate of the invention once delivered in the endosome or lysosome of a target cell that exposes  
15 the cell-surface molecule to which the sdAb of the conjugate can bind. This way, saponin coupled to the sdAb or to the effector molecule in the conjugate of the invention is transferred from outside the cell into the endosome (or lysosome), and in the endosome (or the lysosome), the saponin is released from the remainder of the conjugate upon pH driven cleavage of the hydrazone bond. In the endosome (or the lysosome) the free saponin can exert its stimulatory activity when the delivery of the effector molecule comprised by the conjugate of the invention, into the cytosol is considered. Surprisingly, the inventors  
20 established that for the saponin it is not a prerequisite for endosomal escape enhancing activity of the saponin, that the saponin is present in endosomes or lysosomes in free form. Also saponins comprised by *e.g.* certain conjugates, are potentiating the delivery of an effector molecule, out of the endosome / lysosome into the cytosol of targeted cells, once the effector molecule and the saponin as part of a  
25 certain conjugate are both contacted with the same target cell.

An embodiment is the conjugate of the invention, wherein the glucuronic acid function in the first saccharide chain at the C<sub>3</sub>β-OH group of the aglycone core structure of the saponin is covalently bound to linker HATU, which HATU is covalently bound via an amide bond to an amine group in the oligomeric molecule or in the polymeric molecule of the covalent saponin conjugate, such as an amine  
30 group of a lysine or an N-terminus of a protein. When the HATU linker is coupled to the saponin and to the sdAb or the effector molecule of the conjugate of the invention, the saponin is for example bound to the N-terminus of the sdAb or the effector molecule (if such effector molecule is a proteinaceous effector molecule such as a protein toxin) or to the amine group of a lysine present in the sdAb or present in the effector molecule.

35 An embodiment is the conjugate of the invention, wherein the polymeric molecule or the oligomeric molecule of the covalent saponin conjugate is bound to at least one, preferably one, of the at least one sdAb and/or to at least one, preferably one, of the at least one effector molecule, preferably to an amino-acid residue of the sdAb and/or to an amino-acid residue of the effector molecule, involving a click chemistry group on the polymeric molecule or the oligomeric molecule of the covalent saponin

conjugate, the click chemistry group preferably selected from a tetrazine, an azide, an alkene or an alkyne, or a cyclic derivative of these groups, more preferably the click chemistry group is an azide.

An embodiment is the conjugate of the invention, wherein the polymeric molecule or the oligomeric molecule of the covalent saponin conjugate comprises a polymeric structure and/or an oligomeric structure selected from: a linear polymer, a branched polymer and/or a cyclic polymer, an oligomer, a dendrimer, a dendron such as a G2 dendron or a G3 dendron or a G4 dendron or a G5 dendron, a dendronized polymer, a dendronized oligomer, a DNA, a polypeptide, a poly-lysine, a poly-ethylene glycol, an oligo-ethylene glycol (OEG), such as OEG<sub>3</sub>, OEG<sub>4</sub> and OEG<sub>5</sub>, or an assembly of these polymeric structures and/or oligomeric structures which assembly is preferably built up by covalent cross-linking, preferably the polymeric molecule or the oligomeric molecule of the covalent saponin conjugate is a dendron such as a poly-amidoamine (PAMAM) dendrimer. An embodiment is the conjugate of the invention, wherein the polymeric molecule or the oligomeric molecule of the covalent saponin/sdAb/effector moiety conjugate comprises a polymeric structure and/or an oligomeric structure selected from: a dendrimer, a dendron, a dendronized polymer, a dendronized oligomer, a DNA, for example 2-200 nucleic acids, a poly-ethylene glycol, an oligo-ethylene glycol (OEG), such as OEG<sub>3</sub>, OEG<sub>4</sub> and OEG<sub>5</sub>. The oligomeric molecule or the polymeric molecule is selected for the absence of intrinsic biological activity. Typically, the selected oligomeric molecule or the polymeric molecule is an inert molecule when biological activity is considered that would or could pose a health risk or that would or could result in adverse events in a human subject when the saponin-comprising conjugate of the invention which comprises such an oligomeric molecule or polymeric molecule is administered to said human subject. Driven by the number of selected saponins to be incorporated in the conjugate of the invention, the type and size or length of the oligomeric structure or polymeric structure is selected. That is to say, the number of saponins to be coupled to the sdAb or to the effector molecule comprised by the conjugate, for formation of the conjugate of the invention, can determine the selection of a suitable oligomeric or polymeric structure, bearing the sufficient amount of binding sites for coupling the desired number of saponins, therewith providing a covalent saponin conjugate bearing the selected number of saponin moieties to be coupled to the sdAb or to the effector molecule, for provision of the conjugate of the invention. For example, length of an OEG or size of a Dendron or poly-lysine molecule determines the maximum number of saponins which can be covalently linked to such oligomeric or polymeric structure.

A conjugate according to the invention thus comprises at least one saponin. With "at least one" in this context is meant that the conjugate comprises one saponin molecule but may also comprise a couple (*e.g.* two, three or four) of saponins or a multitude (*e.g.* 10, 20 or 100) of saponins. Depending on the application, the conjugate may comprise a covalently bound scaffold (covalent saponin conjugate) with covalently bound saponins, wherein the scaffold may be designed such that it comprises a defined number of saponins. Preferably, a conjugate according to the invention comprises a defined number or range of saponins, rather than a random number. This is especially advantageous for drug development in relation to marketing authorization. A defined number in this respect means that a conjugate preferably comprises a previously defined number of saponins. This is, *e.g.*, achieved by designing a scaffold comprising a polymeric structure with a certain number of possible moieties for the

saponin(s) to attach. Under ideal circumstances, all of these moieties are coupled to a saponin and the scaffold than comprises the prior defined number of saponins. It is envisaged to offer a standard set of scaffolds, comprising, *e.g.*, two, four, eight, sixteen, thirty-two, sixty-four, *etc.*, saponins so that the optimal number can be easily tested by the user according to his needs. An embodiment is the conjugate of the invention comprising the scaffold of the invention (covalent saponin conjugate of the invention), wherein the saponin is present in a defined range as, *e.g.*, under non-ideal circumstances, not all moieties present in a polymeric structure bind a saponin. Such ranges may for instance be 2 – 4 saponin molecules per scaffold, 3 – 6 saponin molecules per scaffold, 4 – 8 saponin molecules per scaffold, 6 – 8 saponin molecules per scaffold, 6 – 12 saponin molecules per scaffold and so on. In such case, a conjugate comprising a scaffold according to the invention thus comprises 2, 3 or 4 saponins if the range is defined as 2 – 4.

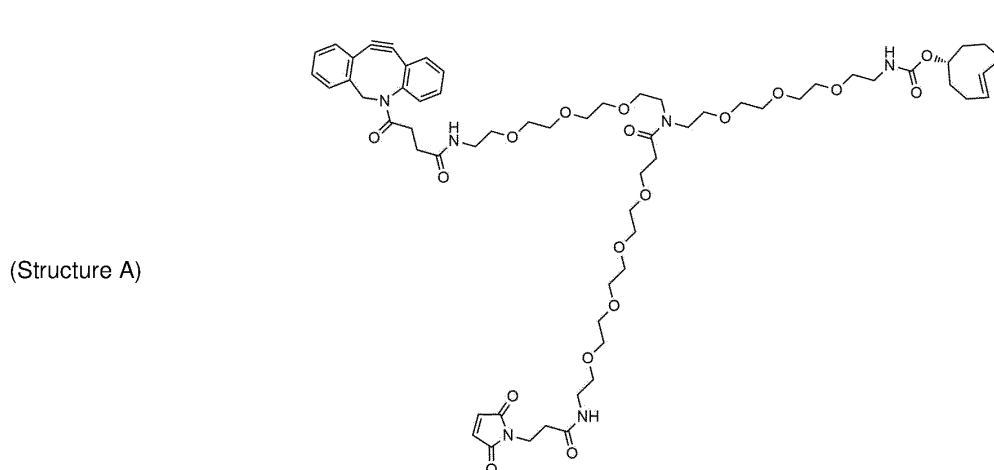
The scaffold is fundamentally independent of the type of saponin covalently bound to the scaffold, the scaffold subsequently (in sequential order) covalently coupled to the conjugate. Thus, the conjugate of the invention comprising the scaffold (covalent saponin conjugate of the invention) is the basis product for a platform technology. Since the at least one covalently bound saponin mediates intracellular delivery of the effector moiety bound to the cell-surface molecule targeting sdAb comprised by the conjugate of the invention, the scaffold technology according to the invention is a system that mediates controlled intracellular effector moiety delivery by saponins. The scaffold provides an optimized and functionally active unit that can be linked to the saponin(s) and to the cell-surface molecule targeting sdAb comprised by the conjugate, at a single and defined position in the sdAb.

An embodiment is the conjugate of the invention comprising a scaffold according to the invention (covalent saponin conjugate of the invention), wherein the number of monomers of the polymeric or oligomeric structure is an exactly defined number or range. Preferably, the polymeric or oligomeric structure comprises structures such as poly(amines), *e.g.*, polyethylenimine and poly(amidoamine), or structures such as polyethylene glycol, poly(esters), such as poly(lactides), poly(lactams), polylactide-co-glycolide copolymers, poly(dextrin), or a peptide or a protein, or structures such as natural and/or artificial polyamino acids, *e.g.* poly-lysine, DNA polymers, such as a DNA comprising 2-100 nucleotides, stabilized RNA polymers or PNA (peptide nucleic acid) polymers, for example comprising 2-200 nucleotides, either appearing as linear, branched or cyclic polymer, oligomer, dendrimer, dendron (for example any of a G2, G3, G4 or G5 dendron, for maximally covalently binding of 4, 8, 16 or 32 saponin moieties, respectively), dendronized polymer, dendronized oligomer or assemblies of these structures, either sheer or mixed. Preferably, the polymeric or oligomeric structures are biocompatible, wherein biocompatible means that the polymeric or oligomeric structure does not show substantial acute or chronic toxicity in organisms and can be either excreted as it is or fully degraded to excretable and/or physiological compounds by the body's metabolism. Assemblies can be built up by covalent cross-linking or non-covalent bonds and/or attraction. They can therefore also form nanogels, microgels, or hydrogels, or they can be attached to carriers such as inorganic nanoparticles, colloids, liposomes, micelles or particle-like structures comprising cholesterol and/or phospholipids. Said polymeric or oligomeric structures preferably bear an exactly defined number or range of coupling moieties (chemical groups) for the coupling of glycoside molecules (and/or effector molecules and/or carrier molecules such

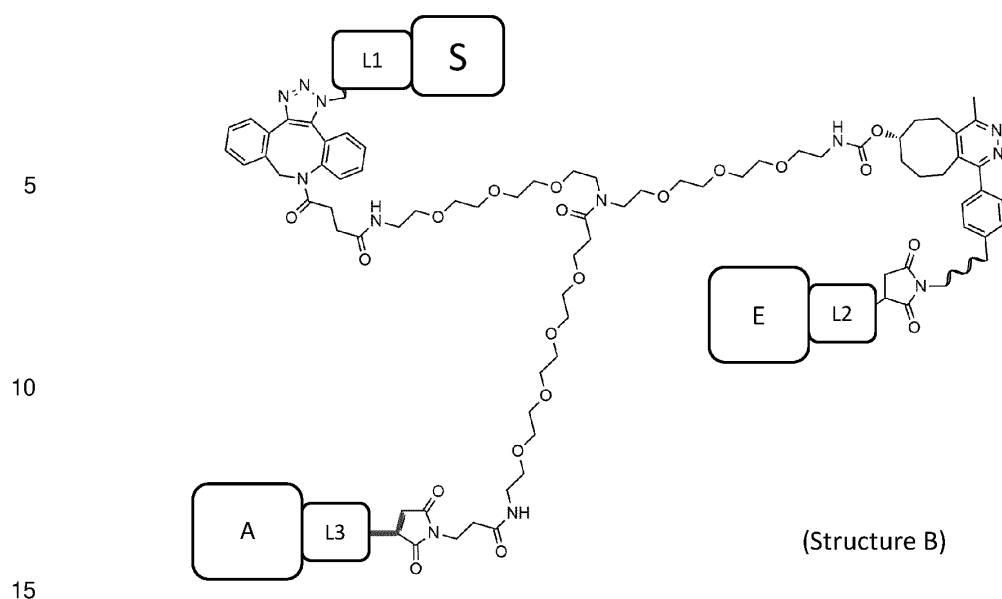
as a ligand, monoclonal antibody or a fragment thereof such as an sdAb). Preferably at least 50%, more preferably at least 75%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95%, more preferably at least 98%, more preferably at least 99%, most preferably (about) 100% of the exactly defined number or range of coupling moieties (chemical groups) in the polymeric or oligomeric structure is occupied by a glycoside molecule (saponin of the invention) in a scaffold according to the invention (covalent saponin conjugate of the invention).

Preferably, a dendron is a branched, clearly defined tree-like polymer with a single chemically addressable group at the origin of the tree, called the focal point. A dendrimer is a connection of two or more dendrons at their focal point. A dendronized polymer is a connection of the focal point of one or more dendrons to a polymer. In a preferred embodiment, a scaffold according to the invention is provided, wherein the polymeric or oligomeric structure comprises a linear, branched or cyclic polymer, oligomer, dendrimer, dendron, dendronized polymer, dendronized oligomer or assemblies of these structures, either sheer or mixed, wherein assemblies can be built up by covalent cross-linking or non-covalent attraction and can form nanogels, microgels, or hydrogels, and wherein, preferably, the polymer is a derivative of a poly(amine), *e.g.*, polyethylenimine and poly(amidoamine), and structures such as polyethylene glycol, poly(esters), such as poly(lactids), poly(lactams), polylactide-co-glycolide copolymers, and poly(dextrin), and structures such as natural and/or artificial polyamino acids such as poly-lysine, or a peptide or a protein or DNA polymers, such as a DNA comprising 2-100 nucleotides, stabilized RNA polymers or PNA (peptide nucleic acid) polymers, for example comprising 2-200 nucleotides. Preferably, the polymeric or oligomeric structures are biocompatible.

An embodiment is the conjugate of the invention, wherein the at least one saponin is covalently bound to at least one, preferably one, of the at least one sdAb and is covalently bound to at least one, preferably one, of the at least one effector molecule via a tri-functional linker, preferably the trifunctional linker represented by Structure A:



the conjugate preferably comprising the trifunctional linker of Structure A and having a molecular structure represented by Structure B:



wherein S is the at least one saponin or the covalent saponin conjugate according to the invention, E is the at least one, preferably one, effector molecule, A is the at least one sdAb such as a single sdAb, L1, L2 and L3 are each individually a bond between the trifunctional linker and the saponin or the covalent saponin conjugate, the effector molecule, and the sdAb, respectively, or L1, L2 and L3 are a linker, wherein L1, L2 and L3 are the same or different.

An example of a conjugate based on the trifunctional linker with Structure A and having a structure according to the structure generically depicted as Structure B, is depicted in Figure 44 as molecule 16; chemical synthesis of molecule 16 is depicted in Figures 40-44 (see also the Examples section, here below). The effector moiety E is BNA(ApoB), the saponin are four SO1861 molecules covalently linked to a dendron molecule, the sdAb is here a V<sub>HH</sub> produced by cell clone anti-HIVgp41 Q8C-tag and is thus an sdAb for binding to HIVgp41. In another example, the saponin is V<sub>HH</sub> 7D12 with the amino-acid sequence as depicted as SEQ ID NO: 75 and/or V<sub>HH</sub> 9G8 with the amino-acid sequence as depicted as SEQ ID NO: 76, or a linear multimer of one or more 7D12 domains and one or more 9G8 domains such as the tandem 7D12-9G8 (amino-acid sequence is according to SEQ ID NO: 73, for example).

Unless specifically indicated otherwise and in particular when relating to the endosomal escape mechanism of the saponin of the invention, whenever the word "endosome" or "endosomal escape" is used herein, it also includes the endolysosome and lysosome, and escape from the endolysosome and lysosome, respectively. After entering the cytosol, said substance might move to other cell units such as the nucleus.

In formal terms, a glycoside is any molecule in which a sugar group is bound through its anomeric carbon to another group via a glycosidic bond. Glycoside molecules, such as saponins, in the context of the invention are such molecules that are further able to enhance the effect of an effector moiety, without wishing to be bound by any theory, in particular by facilitating the endosomal escape of

the effector moiety. Without wishing to be bound by any theory, the glycoside molecules (saponins of the invention, such as those exemplified herein and in the claims) interact with the membranes of compartments and vesicles of the endocytic and recycling pathway and make them leaky for said effector moieties resulting in augmented endosomal escape. With the term “the scaffold is able to augment endosomal escape of the effector moiety” is meant that the at least one saponin (glycoside molecule), which is coupled via a linker or directly to the cell-surface molecule targeting antibody such as an sdAb or via the polymeric or oligomeric structure of the scaffold (covalent saponin conjugate of the invention), is able to enhance endosomal escape of an effector moiety when both molecules are within an endosome, *e.g.* a late endosome, optionally and preferably after the at least one saponin is released from the conjugate such as from a linker or polymeric or oligomeric structure comprised by said conjugate, *e.g.*, by cleavage of a cleavable bond between the at least one glycoside (saponin) and the conjugate (for example via a polymeric or oligomeric structure of a scaffold and/or via a linker). Even though a bond between the at least one saponin according to the invention and the cell-surface molecule targeting sdAb of the conjugate of the invention, optionally via a linker or a scaffold, may be a “stable bond”, that does not mean that such bond cannot be cleaved in the endosomes by, *e.g.*, enzymes. For instance, the saponin, optionally together with a linker or a part of the oligomeric or polymeric structure of a scaffold, may be cleaved off from the remaining linker fragment or oligomeric or polymeric structure. It could, for instance be that a protease cuts a (proteinaceous) linker or proteinaceous polymeric structure, *e.g.*, albumin, thereby releasing the at least one saponin. It is, however, preferred that the glycoside molecule (preferably saponin) is released in an active form, preferably in the original form that it had before it was (prepared to be) coupled to the cell-surface molecule targeting sdAb of the conjugate of the invention optionally via a linker and/or an oligomeric or polymeric scaffold (covalent saponin conjugate of the invention); thus the glycoside (saponin) has its natural structure after such cleavage or the glycoside (saponin) has (part of) a chemical group or linker bound thereto, after such cleavage, while glycoside biological activity (saponin biological activity), *e.g.* endosomal/lysosomal escape enhancing activity towards an effector moiety present in the same endosome or lysosome, is maintained or restored upon said cleavage of the bond between the glycoside (saponin) and the cell-surface molecule targeting antibody such as an sdAb, optionally comprising a linker and/or a scaffold of the invention. With regard to the present invention the term “stable” with respect to bonds between *e.g.* saponins and amino-acid residues of the cell-surface molecule targeting sdAb in the conjugate, a linker, a polymeric or oligomeric structures (of the scaffold, a.k.a. the covalent saponin conjugate of the invention), ligands, (monoclonal) immunoglobulins or binding domains or –fragments thereof, and/or effectors (effector moieties, effector molecules), is meant that the bond is not readily broken or at least not designed to be readily broken by, *e.g.*, pH differences, salt concentrations, or UV-light, reductive conditions. With regard to the present invention the term “cleavable” with respect to bonds between *e.g.* saponins and the cell-surface molecule targeting sdAb, linkers, amino-acid residues, polymeric or oligomeric structures of the covalent saponin conjugate, ligands, antibodies and/or effectors, is meant that the bond is designed to be readily broken by, *e.g.*, pH differences, salt concentrations, under reductive conditions, and the like. The skilled person is well aware of such cleavable bonds and how to prepare them.

Before the present invention one of the major hurdles of introducing ADCs and AOCs on the market was the small therapeutic window: a therapeutically effective dose of an ADC or an AOC is accompanied with (unacceptable) side effects, hampering development and implication in treatment of patients with the ADCs. By the application of the conjugate of the invention, such as ADC-saponin conjugate and AOC-saponin conjugate, it has now become possible to guide one or multiple glycoside molecules (saponin(s)) to a (target) cell, together with the ADC carrying a payload or together with a (monoclonal) antibody (sdAb) conjugated with an oligonucleotide such as a BNA according to the invention. In particular, it was previously not possible to specifically guide an effector moiety of an ADC or AOC or any other conjugate of a payload and a (proteinaceous) cell-surface molecule targeting molecule, and a (predefined, controllable) particular number or range of glycoside molecules (saponins) per effector moiety at the same time to the cytosol of cells, such as via the endocytic pathway of a cell.

A solution provided for by the invention comprises the covalent binding of at least one saponin to the cell-surface molecule targeting molecule of the conjugate of the invention, *i.e.* an sdAb. A further solution provided for by the invention comprises (first) polymerizing the glycoside molecules (saponins) using an oligomeric or polymeric scaffold, and providing the cell-surface molecule targeting molecule comprised by the conjugate of the invention with a cluster of covalently bound saponins, enabling re-monomerization of the one or more saponins at the intracellular site where the mode of action of the saponin is desired, *e.g.* after endocytosis. "Polymerizes" in this context means the reversible and/or irreversible multiple conjugation of saponin molecules to the sdAb, either via linker, or directly or via a polymeric or oligomeric structure to form a scaffold (covalent saponin conjugate of the invention) or the reversible and/or irreversible multiple conjugation of (modified) saponins thereby forming a polymeric or oligomeric structure to form a scaffold (covalent saponin conjugate of the invention). "Re-monomerization" in this context means the cleavage of the saponins from the conjugate, from the linker linking the saponin(s) to the cell-surface molecule targeting sdAb of the conjugate or from the scaffold, for example after endocytosis, and regaining the (native) chemical state of the unbound saponins, which unbound saponins may or may not comprise additional chemical groups such as a chemical group for linking the saponin to a linker, an amino-acid residue of the conjugate or to the scaffold, and/or a (chemical) linker bound to a chemical group of the saponin such as an aldehyde group or carboxylic acid group. Due to the complex chemistry of the saponins for example the 'polymerization' of saponins at a scaffold or other linking linker and their 're-monomerization' at a desired location such as intracellularly *e.g.* after endocytosis, was a challenging task. In particular, the chemical reactions used for providing the linkers and the scaffold comprising covalently linked glycosides for covalent binding to the conjugate, *e.g.* triterpenoid saponins (polymerization of the glycosides), normally occur in water-free organic solvents, but saponins and for example biocompatible polymers applied as a scaffold for bearing bound saponins, are water-soluble molecules. The chemical properties of the unmodified saponin further prohibited polymerization by itself and, one other possible solution, to bind multiple saponins (directly) to the effector molecule was estimated not to be very promising, as an effector molecule (drug, toxin, polypeptide or polynucleotide) does typically not provide sufficient binding sites and because the coupling product would become quite heterogeneous and/or coupling biologically active molecules such as a saponin and *e.g.* a peptide, a toxin, a nucleic acid together bears the risk for influencing and

hampering the activity of one or even both molecules bound together in such saponin-comprising conjugate. Further, there was a considerable risk that the effector moiety comprised by the conjugate of the invention loses its function when a saponin is coupled to the *e.g.* ADC or antibody-oligonucleotide conjugate (AOC). Embodiments of the present invention solves at least one of these drawbacks.

5       A second aspect of the invention relates to a pharmaceutical composition comprising the conjugate of the invention, and optionally a pharmaceutically acceptable excipient and/or pharmaceutically acceptable diluent.

Whether or not a conjugate of the invention comprising saponins, either or not further comprising one or more (cleavable) linkers and/or optionally a scaffold (covalent saponin conjugate of the invention),  
10   is able to disturb the acidic environment and inhibit the endosomal escape function of the at least one glycoside (saponin) can be easily determined with an assay as described in the examples section, and as known in the art. The inhibition is described as “fold amount increases of glycoside (saponin of the invention) necessary to induced 50% cell killing”. It is preferred that the scaffold does not lead to an increase that is at least the increase in glycoside molecules (saponins) necessary to obtain 50% cell  
15   killing observed when using Chloroquine as a positive control. Alternatively, and preferably, the conjugate comprising saponins, either or not further comprising one or more (cleavable) linkers and/or optionally a scaffold does not lead to an at least 4-fold increase of glycoside molecules to induce 50% cell killing, more preferably does not lead to an at least 2-fold increase. The fold increase is to be measured in assay, wherein Chloroquine, as a positive control, induces a 2-fold increase in glycoside  
20   amount, preferably saponin amount wherein the saponin is any one or more of the saponins of the invention (previous embodiments) to observe 50% cell killing.

As said before, the at least one saponin that is comprised by the conjugate according to the invention increases the efficacy of at least current and new effector moieties as defined in this invention. Potential side-effects will be decreased due to lowering of dosing of the effector moiety comprised by  
25   the conjugate, without lowering the efficacy. Therefore, the invention provides a conjugate according to the invention for use in medicine or for use as a medicament. A third aspect of the invention relates to a pharmaceutical composition of the invention, for use as a medicament.

A number of preferred features can be formulated for endosomal escape enhancers comprised by the conjugate of the invention, *i.e.* a saponin of the invention: (1) they are preferably not toxic and do  
30   not invoke an immune response, (2) they preferably do not mediate the cytosolic uptake of the effector moiety into off-target cells, (3) their presence at the site of action is preferably synchronized with the presence of the effector moiety, (4) they are preferably biodegradable or excretable, and (5) they preferably do not substantially interfere with biological processes of the organism unrelated to the biological activity of the effector molecule with which the endosomal escape enhancer is combined with,  
35   *e.g.* interact with hormones. Examples of saponins of the invention that fulfill the before mentioned criteria, at least to some extent, are bidesmosidic triterpenes, preferably bidesmosidic triterpene saponins, such as SO1861, SA1641, QS-21, GE1741, and the further saponins listed throughout the specification and more specifically in Table A1. Preferred is SO1861.

Also provided is the use of a conjugate according to the invention for manufacturing a  
40   medicament. Especially cancer medicines, and in particular the classical chemotherapy medicaments,

are notorious for their side effects. Because of targeting and synchronization in time and place of both the pharmaceutically active substance comprised by the conjugate and the saponin comprised by the very same conjugate molecule, a therapeutic conjugate according to the invention is especially valuable for use as a medicament, in particular for use in a method of treating cancer. The invention thus provides

5 a therapeutic conjugate according to the invention for use in a method of treating cancer. The invention also provides a therapeutic conjugate according to the invention for use in a method of treating acquired or hereditary disorders, in particular monogenic deficiency disorders. The therapeutic conjugate thus comprises the at least one saponin and the at least one effector moiety, and an sdAb for targeting the conjugate at an aberrant target cell such as a tumor cell or an auto-immune cell. Thus, an aspect of the

10 invention relates to a therapeutic conjugate according to the invention, wherein the conjugate comprises a covalently bound effector moiety and comprises a covalently bound saponin, and a cell-surface molecule binding antibody such as an sdAb, for use in a method for the treatment of a cancer or an auto-immune disease.

A further application of the conjugate of the invention in medicine is the substitution of

15 intracellular enzymes in target cells that produce these enzymes in insufficient amount or insufficient functionality. The resulting disease might be hereditary or acquired. In most cases, only symptomatic treatment is possible and for a number of rare diseases, insufficient treatment options lead to a shortened life span of concerned patients. An example for such a disease is phenylketonuria, which is an inborn error of metabolism that results in decreased metabolism of the amino acid phenylalanine.

20 The disease is characterized by mutations in the gene for the hepatic enzyme phenylalanine hydroxylase. Phenylketonuria is not curable to date. The incidence is approximately 1:10,000 with the highest known incidence in Turkey with 1:2,600. A cell-surface molecule targeting antibody comprised by the conjugate of the invention, preferably an sdAb such as a V<sub>HH</sub>, with bound phenylalanine hydroxylase or with a bound polynucleotide that encodes phenylalanine hydroxylase can be used to

25 target liver cells by use of a suitable specific antibody or sdAb, and to substitute the defect enzyme in hepatocytes. This is one example of use of the therapeutic conjugate of the invention comprising a saponin bound thereto and the enzyme or the oligonucleotide bound thereto according to the invention, for substitution or gene therapy. In a preferred embodiment, a therapeutic conjugate according to the invention for use in a method of gene therapy or substitution therapy is provided.

30 With the conjugate of the invention it has now become possible to design and manufacture a one-component, non-viral clinically applicable gene delivery technology. For example, the conjugate of the invention allows for development of non-viral based gene delivery technology, which enhances therapeutic efficacy with lower therapeutic dose thereby improving the health of patients. The conjugate of the invention, in particular when comprising a covalently bound cell-surface molecule targeting

35 antibody such as a monoclonal antibody or sdAb for binding to a (tumor, auto-immune) cell-surface specific molecule, and when bound to an effector moiety such as an oligonucleotide for example a BNA, allows for overcoming a longstanding and major bottleneck in the field of gene delivery, namely efficient, safe and cost-effective transfer of gene therapeutic products across the endosomal membrane into the cytosol/nucleosol. Indeed, gene therapy is one of the most promising treatment options for future

40 advanced therapies in a broad range of diseases. Successful gene delivery requires the recognition of

target cells as well as cytosolic and nucleosolic uptake of the gene. One of the major problems in the field of non-viral gene therapy is the inefficient and insufficiently safe delivery of genetic material for therapeutic use in patients.

Thus, when applying the conjugate of the invention, comprising a cell-targeting cell-surface molecule targeting molecule such as a ligand or preferably an antibody (fragment, domain thereof, preferably sdAb) and comprising an oligonucleotide such as an antisense BNA, the inventors now made it possible to overcome a longstanding and major bottleneck in the field of gene delivery: safe transfer of gene therapeutic products across the endosomal membrane into the cytosol/nucleosol. The conjugate of the invention represents technology designed for allowing targeting of any addressable cell type with all known genetic agents, thereby ensuring better patient therapy not limited to inherited disorders, but also for cancer therapy and therefore of importance for large patient groups. The technology based on the conjugate of the invention may comprise a polymeric or oligomeric scaffold (covalent saponin conjugate of the invention) that serves as a carrier for endosomal escape enhancers (EEEs), such as the saponins as exemplified herein, and the saponins of the embodiments according to the invention, for the cell-surface molecule targeting molecule such as a targeting ligand or (monoclonal) (tumor-cell specific) antibody, or a fragment thereof, or preferably an sdAb such as a V<sub>HH</sub>, and for the effector moiety, here an effector gene such as an LNA or BNA. Use of the conjugate of the invention, *e.g.* comprising a cell-targeting antibody (fragment) or sdAb and an oligonucleotide such as a BNA, has potential to bring any kind of biological macromolecules into the cytosol and the nucleus. Development of new targeting ligands, sdAbs and monoclonal (human, humanized) antibodies is under continuous investigation by numerous research groups and companies worldwide. The same for the oligonucleotides that are aimed for delivery in the cytosol of diseases cells such as cancer cells. The conjugate of the invention thus also presents as a molecular interface in which present and future targeting sdAbs and antibodies and present and future therapeutic oligonucleotides (as well as payloads such as protein toxins) are linked or can be linked to for example an oligomeric or polymeric scaffold module of the invention (covalent saponin conjugate of the invention) by click chemistry, allowing for customized drug applications and for future developments in the field of tissue and cell targeting techniques. The conjugate of the invention can comprise antibodies and ligands as the cell-surface molecule targeting molecule, but an sdAb is preferred. The worldwide market of gene therapeutics is rapidly growing and is covering potential treatments for a wide range of disease areas such as, cancer, cardiovascular diseases, Parkinson's, Alzheimer, HIV and many rare (monogenetic) diseases. The current viral vector-based gene therapeutic technologies have significant challenges, such as safety, manufacturing logistics, and associated high costs. The conjugate of the invention allows for use in a technology platform which represents an alternative for a current viral gene delivery technology. Therefore, the conjugate of the invention is suitable for implementing in approaches for developing non-viral gene treatments for diseases such as cancers, cardiovascular diseases, Parkinson's disease, Alzheimer's disease, HIV infection and many rare (monogenetic) diseases. The conjugate of the invention is suitable for developing novel treatments for transforming the field of antibody-drug conjugates (ADCs) and oligonucleotide-based therapeutics by making non-viral vector based gene therapeutics such as based on targeted antisense BNA. The application of the conjugate

of the invention, in particular in a covalent conjugate with an antibody such as an sdAb and an oligonucleotide such as a BNA and at least one saponin, is one of the many beneficial approaches made possible due to the present invention. For example, use of the conjugate of the invention now allows for exploitation of the endocytic pathway of mammalian cells. Endocytosis is exploited for the delivery of therapeutics, wherein the conjugate of the invention contributes to improved uptake and endosomal escape of *e.g.* siRNAs which are comprised by the conjugate. The conjugate of the invention is suitably used together with small molecules that act as delivery enhancers for *e.g.* payloads, oligonucleotides. Herewith, the conjugate of the invention bearing the covalently coupled oligonucleotide such as a BNA and bearing the covalently coupled cell targeting moiety such as a ligand and preferably an antibody (domain or fragment, preferably a V<sub>HH</sub>) and bearing the saponins of the invention, provides a solution for the current problem seen with current endosomal escape enhancers and gene therapeutic product, relating to their application as two components, thus complicating therapeutic approval and clinical applicability, since such a conjugate of the invention is a single-conjugate therapeutic molecule encompassing the saponin, gene product such as a BNA and the (tumor) cell targeting moiety such as a (monoclonal) antibody or sdAb. Thus the invention provides a non-viral gene delivery technology where endosomal escape enhancers (*e.g.* the glycosides of the embodiments of the invention and of the examples provided), gene therapeutic product (oligonucleotides according to the invention such as a BNA) and targeting ligand or antibody (according to *e.g.* the embodiments of the invention and the sdAbs exemplified here below in the Examples section) are all comprised by the conjugate of the invention. Such a conjugate of the invention thus provides therapeutic opportunities for current and future macromolecule drugs for a broad range of diseases and large patient groups. With the application of such a conjugate of the invention comprising at least one saponin, at least one oligonucleotide and at least one specific cell-targeting moiety such as an immunoglobulin or sdAb, the problem is addressed which is apparent for current methods of applying endosomal escape enhancers and gene therapeutic product separately, which current methods do not ensure that both compounds are at the same time at the site of interaction. This problem is now overcome by using the conjugate of the invention. That is to say, such a conjugate of the invention provides a non-viral gene delivery technology with increased synchronization (in time and place) of both compounds, *i.e.* the saponin and the gene product such as a BNA.

Gene therapies could help with hereditary, previously incurable diseases such as cystic fibrosis, chorea, Huntington's disease or hemophilia. However, currently some problems have not been overcome: for example, the therapeutic genes must precisely reach specific target cells in the body. On the other hand, the therapeutic genes should be absorbed by the targeted cells, but the therapeutic genes should not be destroyed. The current gene therapy approaches use viruses as a ferry for genes. However, these procedures involve considerable risks and cannot be transferred to the introduction of other biomolecules. An embodiment is the conjugate of the invention comprising (plant-derived) glycosides (*e.g.* any one of the saponins of the invention) for use a platform technology that allows not only delivery of genes when comprised by the conjugate as the carrier molecule, but also allows for the delivery of different therapeutic biomolecules to be introduced into target cells. Therefore, the conjugate of the invention is used for developing treatments based on nucleic acids for cystic fibrosis, chorea,

Huntington's disease or hemophilia. Herewith, with the conjugate of the invention, a new gene therapy strategy is available for improving the health of patients with genetic diseases, including those patients with cystic fibrosis, Huntington's disease, and hemophilia. As part of the invention, a non-viral gene delivery technology is developed that combines plant-derived endosomal escape enhancers (glycosides; *i.e.* the saponins of the invention), gene therapeutic products, and a targeting ligand (*i.e.* an sdAb) that are all comprised in a single conjugate. The resulting non-viral gene therapy based on the conjugate of the invention displays about 40 times increased delivery efficiency at a lower dosage over currently available strategies. Herewith, the conjugate of the invention is for use in clinical applications such as for the repair or replacement of defective genes, like in cystic fibrosis patients, and for the targeted delivery of specific genes, for instance, to destroy cancer cells. In fact, the conjugate of the invention is suitable for application in treatment regimens for any disease caused by a genetic defect – such as cystic fibrosis, Huntington's disease and hemophilia and which are currently incurable. Gene therapy which makes use of the conjugate of the invention helps in overcoming two current problems: Firstly, it is possible with the conjugate of the invention to deliver therapeutic genes to specific target cells in the body; secondly, the therapeutic genes enter the interior of these cells, but are not destroyed, due to the presence of saponin(s), the oligonucleotide product and a targeting moiety such as an antibody or an sdAb for binding a target cell, all covalently linked together in the conjugate of the invention, for example by using an oligomeric or polymeric scaffold of the invention (covalent saponin conjugate of the invention).

The present invention also provides a method of treating cancer, the method comprising administering a medicament comprising a therapeutic conjugate according to the invention to a patient in need thereof, preferably administering an effective dose of said medicament to a patient in need thereof, preferably a human cancer patient.

Considerations concerning forms suitable for administration are known in the art and include toxic effects, solubility, route of administration, and maintaining activity. For example, pharmacological compositions injected into the bloodstream should be soluble.

Suitable dosage forms, in part depend upon the use or the route of entry, for example transdermal or by injection. Such dosage forms should allow the compound to reach a target cell whether the target cell is present in a multicellular host. Other factors are known in the art, and include considerations such as toxicity and dosage form which retard the compound or composition from exerting its effect.

A fourth aspect of the invention relates to a pharmaceutical composition of the invention, for use in the treatment or the prophylaxis of any one or more of: a cancer, an auto-immune disease such as rheumatoid arthritis, an enzyme deficiency, a disease related to an enzyme deficiency, a gene defect, a disease relating to a gene defect, an infection such as a viral infection, hypercholesterolemia, primary hyperoxaluria, haemophilia A, haemophilia B, alpha-1 antitrypsin related liver disease, acute hepatic porphyria, an amyloidosis and transthyretin-mediated amyloidosis.

Surprisingly, the inventors found that a dose of an ADC, which does not result in any tumor-cell killing is sufficient and enough for efficient tumor-cell killing when such an ADC is contacted with the tumor cells in the presence of a saponin which is coupled to a tumor cell targeting antibody or V<sub>H</sub>H.

Examples are provided in Figures 2-7. For example, a dose of an ADC such as ADC anti-CD71 V<sub>HH</sub>-toxin, anti-HER2 V<sub>HH</sub>-toxin or anti-EGFR V<sub>HH</sub>-toxin does not exert a toxic effect on tumor cells when contacted with the tumor cells. Typical toxins are protein toxins, e.g. dianthin and saporin. However, when such an ADC is co-administered together with a conjugate comprising a saponin, efficient tumor cell killing is achieved. The conjugate comprising saponin is for example anti-HER2 V<sub>HH</sub> – SO1861, anti-CD71 V<sub>HH</sub> – SO1861, anti-EGFR V<sub>HH</sub> – SO1861, cetuximab-SO1861, trastuzumab-SO1861. The ADC is for example an ADC comprising a V<sub>HH</sub> capable of binding to HER2, CD71 or EGFR, and for example, the ADC comprises a protein toxin such as dianthin or saporin. By applying the conjugate comprising the saponin and a tumor cell targeting antibody or V<sub>HH</sub>, the saponin dose required to achieve an efficient biological activity of the effector molecule comprised by the ADC, *i.e.* an ADC or an AOC, inside a target cell, is about 100 to 1000 fold lower when the conjugate comprising the saponin is co-administered with the ADC, *i.e.* the ADC or the AOC to the target cells, compared to the dose of free saponin that is required when the ADC is co-administered to the target cell together with the free saponin. Herewith, the conjugate comprising the saponin potentiates the ADC, *i.e.* an sdAb comprising ADC or AOC, at a dose of the ADC or the AOC that would otherwise not be effective in tumor cells when it would be administered to a patient in need thereof in the absence of the targeted saponin, and in addition, herewith the conjugate comprising the saponin is already sufficiently effective when potentiation of the effector molecule of the ADC or AOC, is considered, already at a relatively low dose *i.e.* at a dose at which a free saponin that is not provided with a tumor-cell targeting binding molecule such as an sdAb, is not sufficiently effective in effector molecule potentiation. In combination, the inventors provided for an improved method for treating a human patient in need of treatment with a therapeutically effective amount of an ADC or an AOC comprising a tumor-cell targeting sdAb, since the ADC or the AOC is already effective at lower dose when co-administered to patients in the presence of a conjugate comprising a tumor cell targeting antibody or V<sub>HH</sub> and comprising saponin. One of the many benefits of this combination resides in the absence of an Fc tail in the sdAb part of the ADC and preferably also in the conjugate comprising the saponin. Absence of the Fc tail prevents unwanted binding of conjugates to off-target patient cells bearing Fc receptors, which binding could otherwise result in side-effects if such an Fc tail would be present, such as seen for many conventional ADCs, AOCs. Antibody-based ADCs and AOCs comprising an Fc tail suffer from a decreased efficacy due to the undesired binding of such IgG based ADCs, AOCs to Fc receptors. As a result of Fc receptor binding, the effective dose of such IgG-based ADCs and AOCs is lowered. Therewith, absence of the Fc tail provides for several benefits in this regard. Off-target and undesired binding of ADCs, AOCs and the improved ADCs and improved AOCs of the invention (*i.e.* the conjugate of the current invention) to Fc receptors cannot occur. Herewith, the ADCs, AOCs and the conjugate of the invention have a lower effective dose when target receptor mediated endocytosis and delivery of the ADC, AOC and the conjugate of the invention inside endosomes is considered, since no conjugate is 'lost' due to Fc receptor binding, a drawback seen with IgG-based conjugates. As a result, the therapeutic window of the ADC, AOC and the conjugates of the invention, all comprising cell-targeting sdAb instead of Fc-comprising antibody, is wider than the therapeutic window that would have been achieved when the sdAb is replaced by a conventional IgG comprising the Fc tail. Similarly, as a result, the therapeutic window of the conjugate comprising the

saponin is wider than the therapeutic window that would have been achieved when the sdAb in such a conjugate of a tumor-cell targeting sdAb and a saponin is replaced by a binding molecule for binding the cell-surface molecule on the target cell, such as a conventional IgG comprising the Fc tail.

5 An embodiment is the pharmaceutical composition for use of the invention, wherein the saponin is SO1861, a SO1861 derivative, QS-21, or a QS-21 derivative, preferably a SO1861 derivative or a QS-21 derivative, more preferably a SO1861 derivative according to the invention.

An embodiment is the pharmaceutical composition for use of the invention, wherein:

- said use is in the treatment or prevention of cancer in a human subject; and/or
- said use is in the treatment or prophylaxis of cancer in a patient in need thereof, wherein the  
10 at least one sdAb binds to a cell-surface molecule of the cell, preferably to a tumor-cell surface molecule of the cell, more preferably to a tumor cell-specific surface molecule of the cell; and/or
- the pharmaceutical composition, preferably a therapeutically effective amount of the pharmaceutical composition, is administered to a patient in need thereof, preferably a human patient.

A fifth aspect of the invention relates to an *in vitro* or *ex vivo* method for transferring the effector  
15 molecule of the invention from outside a cell to inside said cell, preferably to the cytosol of said cell, comprising the steps of:

- a) providing a cell which expresses on its cell surface the binding site for the at least one sdAb comprised by the conjugate of the invention, said binding site preferably being present on a cell-surface molecule of the cell, said cell preferably being selected from a liver cell, an aberrant cell  
20 such as a virally infected cell, an auto-immune cell, a cell comprising a gene defect, a cell comprising an enzyme deficiency and a tumor cell;
- b) providing the conjugate of the invention, said conjugate comprising the effector molecule to be transferred into the cell provided in step a); and
- c) contacting the cell of step a) *in vitro* or *ex vivo* with the conjugate of step b),  
25 therewith effecting the transfer of said conjugate comprising the effector molecule from outside the cell to inside said cell, and by effecting the transfer of said conjugate effecting the transfer of the effector molecule from outside the cell inside said cell, preferably into the cytosol of said cell.

A sixth aspect of the invention relates to an *in vitro* or *ex vivo* method for transferring the conjugate of the invention from outside a cell to inside said cell, comprising the steps of:

- a) providing a cell which expresses on its cell surface the binding site for the at least one sdAb comprised by the conjugate of the invention, said binding site preferably being present on a cell-surface molecule of the cell, said cell preferably being selected from a liver cell, an aberrant cell  
30 such as a virally infected cell, an auto-immune cell, a cell comprising a gene defect, a cell comprising an enzyme deficiency and a tumor cell;
- b) providing the conjugate of the invention; and
- c) contacting the cell of step a) *in vitro* or *ex vivo* with the conjugate of step b), therewith effecting  
35 the transfer of the conjugate from outside the cell to inside said cell.

TABLE A1. Saponins displaying (late) endosomal/lysosomal escape enhancing activity, and saponins comprising a structure reminiscent to such saponins displaying (late) endosomal/lysosomal escape enhancing activity

| Saponin Name                                    | Aglycone core                          | Carbohydrate substituent at the C-3beta-OH group              | Carbohydrate substituent at the C-28-OH group                                     |
|-------------------------------------------------|----------------------------------------|---------------------------------------------------------------|-----------------------------------------------------------------------------------|
| NP-005236                                       | 2alpha-Hydroxyoleanolic acid           | GlcA-                                                         | Glc/Gal-                                                                          |
| AMA-1                                           | 16alpha-Hydroxyoleanolic acid          | Glc-                                                          | Rha-(1→2)-[Xyl-(1→4)]-Rha-                                                        |
| AMR                                             | 16alpha-Hydroxyoleanolic acid          | Glc-                                                          | Rha-(1→2)-[Ara-(1→3)-Xyl-(1→4)]-Rha-                                              |
| alpha-Hederin                                   | Hederagenin (23-Hydroxyoleanolic acid) | Rha-(1→2)-Ara-                                                | -                                                                                 |
| NP-012672                                       | 16alpha,23-Dihydroxyoleanolic acid     | Ara/Xyl-(1→4)-Rha/Fuc-(1→2)-Glc/Gal-(1→2)-Rha/Fuc-(1→2)-GlcA- | Ara/Xyl-                                                                          |
| NP-017777                                       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→4)-Rha-(1→2)-[R-(→4)]-Fuc- (R = 4E-Methoxycinnamic acid)                   |
| NP-017778                                       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→4)-Rha-(1→2)-[R-(→4)]-Fuc- (R = 4Z-Methoxycinnamic acid)                   |
| NP-017774                                       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-4-OAc-Fuc-                                        |
| NP-018110 <sup>a</sup> , NP-017772 <sup>d</sup> | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-3,4-di-OAc-Fuc-                                   |
| NP-018109                                       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R-(→4)]-3-OAc-Fuc- (R = 4E-Methoxycinnamic acid) |
| NP-017888                                       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Glc-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-4-OAc-Fuc-                              |
| NP-017889                                       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-4-OAc-Fuc-                                          |
| NP-018108                                       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Ara/Xyl-(1→3)-Ara/Xyl-(1→4)-Rha/Fuc-(1→2)-[4-OAc-Rha/Fuc-(1→4)]-Rha/Fuc-          |
| SA1641 <sup>a</sup> , AE X55 <sup>b</sup>       | Gypsogenin                             | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Qui-(1→4)]-Fuc-                                    |
| NP-017674                                       | Quillaic acid                          | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-Fuc-                                    |
| NP-017810                                       | Quillaic acid                          | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-Fuc-                                              |
| AG1                                             | Quillaic acid                          | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-Fuc-                                              |
| NP-003881                                       | Quillaic acid                          | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                                   | Ara/Xyl-(1→4)-Rha/Fuc-(1→4)-[Glc/Gal-(1→2)]-Fuc-                                  |

|                                       |               |                             |                                                                                                                                                                       |
|---------------------------------------|---------------|-----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| NP-017676                             | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R-(→4)]-Fuc-<br>(R = 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)               |
| NP-017677                             | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R-(→4)]-Fuc-<br>(R = 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)                           |
| NP-017706                             | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-                                                                                                                  |
| NP-017705                             | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-                                                                                                      |
| NP-017773                             | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | 6-OAc-Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3-OAc-Rha-(1→3)]-Fuc-                                                                                                            |
| NP-017775                             | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3-OAc-Rha-(1→3)]-Fuc-                                                                                                                  |
| SA1657                                | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Qui-(1→4)]-Fuc-                                                                                                                        |
| AG2                                   | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-[Qui-(1→4)]-Fuc-                                                                                                                      |
| SO1861                                | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Xyl-(1→3)-4-OAc-Qui-(1→4)]-Fuc-                                                                                                        |
| GE1741                                | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3,4-di-OAc-Qui-(1→4)]-Fuc-                                                                                                             |
| SO1542                                | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-Fuc-                                                                                                                                  |
| SO1584                                | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | 6-OAc-Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-Fuc-                                                                                                                            |
| SO1658                                | Gypsogenin    | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Glc-(1→3)-[Xyl-(1→3)-Xyl-(1→4)]-Rha-(1→2)-Fuc-                                                                                                                        |
| SO1674                                | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Glc-(1→3)-[Xyl-(1→3)-Xyl-(1→4)]-Rha-(1→2)-Fuc-                                                                                                                        |
| SO1832                                | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Xyl-(1→3)-4-OAc-Qui-(1→4)]-Fuc-                                                                                                        |
| QS-7 (also referred to as QS1861)     | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4OAc-Fuc-                                                                                                   |
| QS-7 api (also referred to as QS1862) | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4OAc-Fuc-                                                                                                       |
| QS-17                                 | Quillaic acid | Gal-(1→2)-[Xyl-(1→3)]-GlcA- | Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R-(→4)]-Fuc-<br>(R = 5-O-[5-O-Rha-(1→2)-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid) |

|                                       |                                                                                  |                                                      |                                                                                                                                                             |
|---------------------------------------|----------------------------------------------------------------------------------|------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|
| QS-18                                 | Quillaic acid                                                                    | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                          | Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R-(→4)]-Fuc-<br>(R = 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid) |
| QS-21 A-apio                          | Quillaic acid                                                                    | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                          | Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R-(→4)]-Fuc-<br>(R = 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)                 |
| QS-21 A-xylo                          | Quillaic acid                                                                    | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                          | Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R-(→4)]-Fuc-<br>(R = 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)                 |
| QS-21 B-apio                          | Quillaic acid                                                                    | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                          | Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R-(→3)]-Fuc-<br>(R = 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)                 |
| QS-21 B-xylo                          | Quillaic acid                                                                    | Gal-(1→2)-[Xyl-(1→3)]-GlcA-                          | Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R-(→3)]-Fuc-<br>(R = 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)                 |
| beta-Aescin<br>(described: Aescin Ia) | Protoaescigenin-<br>21(2-methylbut-2-<br>enoate)-22-acetat                       | Glc-(1→2)-[Glc-(1→4)]-GlcA-                          | -                                                                                                                                                           |
| Teaseed saponin I                     | 23-Oxo-<br>barringtonenol C -<br>21,22-bis(2-<br>methylbut-2-enoate)             | Glc-(1→2)-Ara-(1→3)-[Gal-(1→2)]-GlcA-                | -                                                                                                                                                           |
| Teaseedsaponin J                      | 23-Oxo-<br>barringtonenol C -<br>21,22-bis(2-<br>methylbut-2-enoate)             | Xyl-(1→2)-Ara-(1→3)-[Gal-(1→2)]-GlcA-                | -                                                                                                                                                           |
| Assamsaponin F                        | 23-Oxo-<br>barringtonenol C -<br>21(2-methylbut-2-<br>enoate)-16,22-<br>diacetat | Glc-(1→2)-Ara-(1→3)-[Gal-(1→2)]-GlcA-                | -                                                                                                                                                           |
| Digitonin                             | Digitogenin                                                                      | Glc-(1→3)-Gal-(1→2)-[Xyl-(1→3)]-Glc-(1→4)-Gal-       | -                                                                                                                                                           |
| Primula acid 1                        | 3,16,28-<br>Trihydroxyoleanan-<br>12-en                                          | Rha-(1→2)-Gal-(1→3)-[Glc-(1→2)]-GlcA-                | -                                                                                                                                                           |
| AS64R                                 | Gypsogenic acid                                                                  | -                                                    | Glc-(1→3)-[Glc-(1→6)]-Gal-                                                                                                                                  |
|                                       |                                                                                  | <b>Carbohydrate substituent at the C-23-OH group</b> |                                                                                                                                                             |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                 |      |                            |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|------|----------------------------|
| AS6.2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Gypsogenic acid | Gal- | Glc-(1→3)-[Glc-(1→6)]-Gal- |
| Sapofectosid <sup>1</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Quillaic acid   |      |                            |
| Agrostemmoside E<br>(AG1856, AG2.8) <sup>2</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Quillaic acid   |      |                            |
| a, b: Different names refer to different isolates of the same structure<br>c, d: Different names refer to different isolates of the same structure<br>1: The structure of sapofectosid is provided in S. Sama <i>et al</i> , Sapofectosid – Ensuring non-toxic and effective DNA and RNA delivery, International Journal of Pharmaceutics, Volume 534, Issues 1–2, 20 December 2017, Pages 195-205.<br>2: The structure of Agrostemmoside E (also referred to as AG1856 or AG2.8) is given in Fig. 4 of J. Clochard <i>et al</i> , A new acetylated triterpene saponin from <i>Agrostemma githago</i> L. modulates gene delivery efficiently and shows a high cellular tolerance, International Journal of Pharmaceutics, Volume 589, 15 November 2020, 119822. |                 |      |                            |

An aspect of the invention relates to a kit comprising a container containing an endosomal escape enhancing conjugate according to the invention the kit further comprising instructions for using the conjugate.

- 5 An aspect of the invention relates to any of the following ADCs provided with at least one covalently linked saponin and AOCs provided with at least one covalently linked saponin, and their semi-finished conjugates, comprising the cell-surface molecule targeting molecule of the invention (*i.e.* an sdAb) and either comprising at least one effector moiety of the invention, providing an ADC or an AOC, or comprising at least one saponin of the invention, wherein the following antibodies are an sdAb or
- 10 multiple sdAb's such as two or three linearly covalently linked sdAb's (multivalent such as bivalent, multiparatopic such as biparatopic, multispecific, such as bispecific, or any combination thereof):  
 Anti-EGFR antibody – saponin, wherein for example the saponin is a saponin selected from Table A1;  
 Anti-EGFR antibody – triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising
- 15 a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin;  
 Anti-EGFR antibody – SO1861;  
 Anti-EGFR antibody – GE1741;  
 Anti-EGFR antibody – SA1641;  
 Anti-EGFR antibody – Quil-A;
- 20 Anti-EGFR antibody – QS-21;  
 Anti-EGFR antibody – saponins in water soluble saponin fraction of *Quillaja saponaria*;  
 sdAb derived from Cetuximab – saponin, wherein for example the saponin is a saponin selected from Table A1;  
 sdAb derived from Cetuximab – triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging
- 25 to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – SO1861;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – GE1741;
- 30 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – SA1641;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – Quil-A;

- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – QS-21;
- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – saponins in water soluble saponin fraction of Quillaja saponaria;
- Anti-HER2 antibody – saponin, wherein for example the saponin is a saponin selected from Table A1;
- 5 Anti-HER2 antibody – triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin;
- Anti-HER2 antibody – SO1861;
- Anti-HER2 antibody – GE1741;
- 10 Anti-HER2 antibody – SA1641;
- Anti-HER2 antibody – Quil-A;
- Anti-HER2 antibody – QS-21;
- Anti-HER2 antibody – saponins in water soluble saponin fraction of Quillaja saponaria;
- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – saponin, wherein for example the saponin is a saponin
- 15 selected from Table A1;
- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin;
- 20 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – SO1861;
- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – GE1741;
- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – SA1641;
- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – Quil-A;
- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – QS-21;
- 25 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – saponins in water soluble saponin fraction of Quillaja saponaria;
- Anti-CD71 antibody – saponin, wherein for example the saponin is a saponin selected from Table A1;
- Anti-CD71 antibody – triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising
- 30 a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin;
- Anti-CD71 antibody – SO1861;
- Anti-CD71 antibody – GE1741;
- Anti-CD71 antibody – SA1641;
- Anti-CD71 antibody – Quil-A;
- 35 Anti-CD71 antibody – QS-21;
- Anti-CD71 antibody – saponins in water soluble saponin fraction of Quillaja saponaria;
- Anti- HIVgp41 antibody – saponin, wherein for example the saponin is a saponin selected from Table A1;

Anti- HIVgp41 antibody – triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3 $\beta$ -OH group of the saponin;

Anti- HIVgp41 antibody – SO1861;

5 Anti- HIVgp41 antibody – GE1741;

Anti- HIVgp41 antibody – SA1641;

Anti- HIVgp41 antibody – Quil-A;

Anti- HIVgp41 antibody – QS-21;

Anti- HIVgp41 antibody – saponins in water soluble saponin fraction of *Quillaja saponaria*;

10 wherein the anti-HIVgp41 antibody for example is the VHH produced by clone Q8c;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – saponin, wherein for example the saponin is a saponin selected from Table A1;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and

15 optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3 $\beta$ -OH group of the saponin;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – SO1861;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – GE1741;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – SA1641;

20 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – Quil-A;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – QS-21;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – saponins in water soluble saponin fraction of *Quillaja saponaria*;

Anti-EGFR antibody – oligonucleotide;

Anti-EGFR antibody – antisense oligonucleotide;

25 Anti-EGFR antibody – siRNA;

Anti-EGFR antibody – antisense BNA;

Anti-EGFR antibody – antisense BNA(HSP27);

Anti-EGFR antibody – proteinaceous toxin;

Anti-EGFR antibody – ribosome inactivating protein;

30 Anti-EGFR antibody – dianthin;

Anti-EGFR antibody – saporin;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – oligonucleotide;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – antisense oligonucleotide;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – siRNA;

35 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – antisense BNA;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – antisense BNA(HSP27);

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – proteinaceous toxin;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – ribosome inactivating protein;

sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – dianthin;

- sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Cetuximab – saporin;  
 Anti-HER2 antibody – oligonucleotide;  
 Anti-HER2 antibody – antisense oligonucleotide;  
 Anti-HER2 antibody – siRNA;
- 5 Anti-HER2 antibody – antisense BNA;  
 Anti-HER2 antibody – antisense BNA(HSP27);  
 Anti-HER2 antibody – proteinaceous toxin;  
 Anti-HER2 antibody – ribosome inactivating protein;  
 Anti-HER2 antibody – dianthin;
- 10 Anti-HER2 antibody – saporin;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – oligonucleotide;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – antisense oligonucleotide;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – siRNA;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – antisense BNA;
- 15 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – antisense BNA(HSP27);  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – proteinaceous toxin;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – ribosome inactivating protein;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – dianthin;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of Trastuzumab – saporin;
- 20 Anti-CD71 antibody – oligonucleotide;  
 Anti-CD71 antibody – antisense oligonucleotide;  
 Anti-CD71 antibody – siRNA;  
 Anti-CD71 antibody – antisense BNA;  
 Anti-CD71 antibody – antisense BNA(HSP27);
- 25 Anti-CD71 antibody – proteinaceous toxin;  
 Anti-CD71 antibody – ribosome inactivating protein;  
 Anti-CD71 antibody – dianthin;  
 Anti-CD71 antibody – saporin;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – oligonucleotide;
- 30 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – antisense oligonucleotide;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – siRNA;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – antisense BNA;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – antisense BNA(HSP27);  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – proteinaceous toxin;
- 35 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – ribosome inactivating protein;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – dianthin;  
 sdAb derived from V<sub>H</sub> or V<sub>L</sub> of OKT-9 – saporin;  
 Anti-EGFR antibody (– oligonucleotide)(– saponin), wherein for example the saponin is a saponin  
 selected from Table A1, wherein the oligonucleotide is any one or more of antisense oligonucleotide,  
 40 siRNA, antisense BNA, and antisense BNA(HSP27), or wherein the saponin is any one or more of a

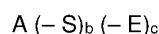
- triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin, SO1861, GE1741, SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, wherein
- 5 the anti-EGFR antibody preferably is Cetuximab;  
Anti-EGFR antibody (– proteinaceous toxin)(– saponin), wherein for example the saponin is a saponin selected from Table A1, wherein the proteinaceous toxin is any one or more of a ribosome inactivating protein, dianthin and saporin, or wherein the saponin is any one or more of a triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde
- 10 function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin, SO1861, GE1741, SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, wherein the anti-EGFR antibody preferably is Cetuximab;  
Anti-HER2 antibody (– oligonucleotide)(– saponin), wherein for example the saponin is a saponin selected from Table A1, wherein the oligonucleotide is any one or more of antisense oligonucleotide, siRNA, antisense BNA, and antisense BNA(HSP27), or wherein the saponin is any one or more of a triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin, SO1861, GE1741,
- 20 SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, wherein the anti-HER2 antibody preferably is trastuzumab;  
Anti-HER2 antibody (– proteinaceous toxin)(– saponin), wherein for example the saponin is a saponin selected from Table A1, wherein the proteinaceous toxin is any one or more of a ribosome inactivating protein, dianthin and saporin, or wherein the saponin is any one or more of a triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin, SO1861, GE1741, SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, wherein the anti-HER2 antibody preferably is trastuzumab;
- 25 Anti-CD71 antibody (– oligonucleotide)(– saponin), wherein for example the saponin is a saponin selected from Table A1, wherein the oligonucleotide is any one or more of antisense oligonucleotide, siRNA, antisense BNA, and antisense BNA(HSP27), or wherein the saponin is any one or more of a triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3beta-OH group of the saponin, SO1861, GE1741, SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, wherein the anti-CD71 antibody preferably is OKT-9; and  
Anti-CD71 antibody (– proteinaceous toxin)(– saponin), wherein for example the saponin is a saponin selected from Table A1, wherein the proteinaceous toxin is any one or more of a ribosome inactivating
- 40 protein, dianthin and saporin, or wherein the saponin is any one or more of a triterpenoid saponin and/or

a bidesmosidic triterpene saponin belonging to the type of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3 $\beta$ -OH group of the saponin, SO1861, GE1741, SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, wherein the anti-CD71 antibody preferably is OKT-9.

An embodiment is the semi-finished conjugate of the invention (sdAb-saponin or sdAb-effector moiety) or the conjugate of the invention, wherein the cell-surface molecule targeting molecule is selected from an sdAb derived from V<sub>H</sub> or V<sub>L</sub> of cetuximab, trastuzumab, OKT-9 (*i.e.* the sdAb is based on the V<sub>H</sub> or V<sub>L</sub> of such monoclonal antibodies and is capable of specifically binding to the target receptor on the cell-surface of a target cell), and/or wherein the effector moiety is selected from dianthin, saporin and antisense BNA(HSP27), antisense BNA(ApoB) and/or wherein the saponin is selected from Table A1, such as SO1861, GE1741, SA1641, Quil-A, QS-21 and saponins in water soluble saponin fraction of Quillaja saponaria, or a derivative thereof.

An embodiment is the conjugate according to the invention, wherein the cell-surface molecule targeting molecule is selected from an sdAb derived from V<sub>H</sub> or V<sub>L</sub> of cetuximab, trastuzumab, OKT-9 (*i.e.* the sdAb is based on the V<sub>H</sub> or V<sub>L</sub> of such monoclonal antibodies and is capable of specifically binding to the target receptor on the cell-surface of a target cell), and/or wherein the effector moiety is selected from dianthin, saporin and antisense BNA(HSP27) or antisense BNA(ApoB), and/or wherein the saponin is selected from Table A1, such as SO1861, GE1741, SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, or a derivative thereof.

An aspect of the invention relates to an ADC or an AOCs or a semi-finished ADC conjugate or a semi-finished AOC conjugate comprising the cell-surface molecule targeting sdAb of the invention and comprising at least one effector moiety of the invention and/or comprising at least one saponin of the invention, of Structure C:



(Structure C)

wherein A is the cell-surface molecule targeting sdAb;

S is the saponin;

E is the effector moiety;

b = 0 – 64, preferably 0, 1, 2, 3, 4, 8, 16, 32, 64 or any whole number or fraction therein between;

c = 0 – 8, preferably 0, 1, 2, 3, 4, 6, 8 or any whole number or fraction therein between,

wherein S is coupled to A and/or E, E is coupled to A and/or S, preferably S is coupled to A and E is coupled to A.

An embodiment is the Structure C of the invention, wherein A is an sdAb derived from an anti-EGFR antibody such as cetuximab, an anti-HER2 antibody such as trastuzumab, an anti-HIVgp41 antibody such as sdAb Q8c, an anti-CD71 antibody such as OKT-9, and/or wherein S is any one or more of a saponin, a triterpenoid saponin and/or a bidesmosidic triterpene saponin belonging to the type

of a 12,13-dehydrooleanane with an aldehyde function in position C-23 and optionally comprising a glucuronic acid function in a carbohydrate substituent at the C-3 $\beta$ -OH group of the saponin, SO1861, GE1741, SA1641, Quil-A, QS-21, and saponins in water soluble saponin fraction of Quillaja saponaria, or any of the saponins depicted in Table A1, and/or wherein E is any one or more of an oligonucleotide, an antisense oligonucleotide, an siRNA, an antisense BNA, and an antisense BNA(HSP27) or an antisense BNA(ApoB), and/or any one or more of a proteinaceous toxin, a ribosome inactivating protein, dianthin and saporin.

An embodiment is the Structure C of the invention, the conjugate of the invention or the semi-finished conjugate of the invention, wherein the saponin, if present, and/or the effector moiety, if present, is covalently coupled via at least one linker, such as a cleavable linker, and/or via a covalent saponin conjugate (*i.e.* at least one oligomeric or polymeric scaffold), such as a linker based on N- $\epsilon$ -maleimidocaproic acid hydrazide (EMCH), succinimidyl 3-(2-pyridyldithio)propionate or 3-(2-Pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), and such as a covalent saponin conjugate (a scaffold) based on a Dendron such as a G4-Dendron (See Figure 44, molecule 16 for an example) or a tri-functional linker such as the tri-functional linker of Structure A (see Figure 44, molecule 16 for an example), and/or wherein at least a lysine side chain and/or a cysteine side chain of the cell-surface molecule targeting antibody, preferably an sdAb such as a V<sub>HH</sub> according to the invention, is involved in the covalent bond with the saponin and/or the effector moiety and/or the linker and/or the cleavable linker and/or the covalent saponin conjugate, wherein preferably the saponin and/or the effector moiety is covalently linked to the cell-surface molecule targeting molecule, preferably an antibody such as an sdAb, wherein the covalent link comprises or consists of an amide bond, a hydrazone bond, a disulphide bond.

An aspect of the invention relates to the use of any of the aforementioned conjugates, ADCs comprising a covalently linked saponin, AOCs comprising a covalently linked saponin, semi-finished ADCs, semi-finished AOCs, as a medicament.

An aspect of the invention relates to the use of any of the aforementioned conjugates, ADCs comprising a covalently linked saponin, AOCs comprising a covalently linked saponin, semi-finished ADCs, semi-finished AOCs, for use in the treatment or prophylaxis of a cancer or an auto-immune disease.

## EXAMPLES AND EXEMPLARY EMBODIMENTS

### Example 1. V<sub>HH</sub>-SO1861 + mAb-saporin (1T2C and 2T2C)

The 1 target 2-components system (1T2C) is the combination treatment of V<sub>HH</sub>-SO1861 and mAb-protein toxin, where V<sub>HH</sub> and mAb recognize and bind the same cell surface receptor (Figure 1A). The 2 target 2-components system (2T2C) is the combination treatment of V<sub>HH</sub>-SO1861 and mAb-protein toxin, where V<sub>HH</sub> recognizes and binds a different cell surface receptor as the mAb (Figure 1B). SO1861-EMCH was conjugated (labile) via the terminal cysteine residue (Cys) to antiHER2V<sub>HH</sub>, with a DAR 1,

(HER2V<sub>HH</sub>-SO1861). HER2V<sub>HH</sub>-SO1861 was titrated on a fixed concentration of 10 pM CD71mab-saporin (CD71 monoclonal antibody conjugated to the protein toxin, saporin, with a DAR4) or 50 pM trastuzumab-saporin (trastuzumab conjugated to the protein toxin, saporin, with a DAR4). Targeted protein toxin mediated cell killing on SK-BR-3 (HER2<sup>++</sup>/CD71<sup>+</sup>) and MDA-MB-468 (HER2/CD71<sup>+</sup>) was determined. This revealed enhanced cell killing at low concentrations of HER2V<sub>HH</sub>-SO1861 in SK-BR-3 for both combinations with 10 pM CD71mab-saporin or 50 pM trastuzumab-saporin (IC<sub>50</sub> = 300 nM; Figure 2A). Equivalent concentrations of HER2V<sub>HH</sub>-SO1861 alone induced cell killing at high concentrations (IC<sub>50</sub> = 4.000 nM), whereas equivalent concentrations of HER2V<sub>HH</sub>, HER2V<sub>HH</sub> + CD71mab-saporin or HER2V<sub>HH</sub> + trastuzumab-saporin could not induce cell killing activity (IC<sub>50</sub> > 5000 nM; Figure 2A). In MDA-MB-468 (HER2/CD71<sup>+</sup>) the combination of HER2V<sub>HH</sub>-SO1861 + 10 pM CD71mab-saporin revealed cell killing activity at high concentrations (IC<sub>50</sub> = 2.000 nM; Figure 2B), whereas the combination of HER2V<sub>HH</sub>-SO1861 + 50 pM trastuzumab-saporin showed cell killing activity at much higher concentrations (IC<sub>50</sub> > 5.000 nM; Figure 2B). Equivalent concentrations of HER2V<sub>HH</sub>, HER2V<sub>HH</sub> + CD71mab-saporin or HER2V<sub>HH</sub> + trastuzumab-saporin could not induce cell killing activity in MDA-MB-468 cells (IC<sub>50</sub> > 5.000 nM; Figure 2B).

All this shows that conjugation of SO1861-EMCH to a HER2 targeting V<sub>HH</sub> enhances the endosomal escape and cytoplasmic delivery of a targeted protein toxin (targeting the same or different cell surface receptor) resulting in cell killing of HER2 expressing cells.

Next, trastuzumab-saporin or CD71mab-saporin was titrated on a fixed concentration of 900 nM HER2V<sub>HH</sub>-SO1861 and targeted protein toxin mediated cell killing on SK-BR-3 (HER2<sup>++</sup>/CD71<sup>+</sup>) and MDA-MB-468 (HER2/CD71<sup>+</sup>) was determined. This revealed that 900 nM HER2V<sub>HH</sub>-SO1861 in combination with low concentrations trastuzumab-saporin or CD71mab-saporin induced already efficient cell killing of SK-BR-3 (IC<sub>50</sub> = 0,0001 pM; Figure 3A), whereas CD71mab-saporin + 900 nM HER2V<sub>HH</sub> or trastuzumab-saporin + 900 nM HER2V<sub>HH</sub> could only induce cell killing at high concentrations (IC<sub>50</sub> = 50 pM; IC<sub>50</sub> = 400 pM resp; Figure 3). In MDA-MB-468 cells (HER2/CD71<sup>+</sup>) CD71mab-saporin + 900 nM HER2V<sub>HH</sub>-SO1861 showed cell killing at IC<sub>50</sub> = 0,01 pM, whereas trastuzumab-saporin + 900 nM HER2V<sub>HH</sub>-SO1861 showed activity at IC<sub>50</sub> = 2.000 pM. Trastuzumab-saporin + 900 nM HER2V<sub>HH</sub> or CD71mab-saporin + 900 nM HER2V<sub>HH</sub> showed cell killing only at (IC<sub>50</sub> > 10.000 pM and IC<sub>50</sub> = 20 pM resp. Figure 3B). All this shows that relatively low concentrations of trastuzumab-saporin or CD71mab-saporin can be effective and induce cell killing in combination with relatively low HER2V<sub>HH</sub>-SO1861 (DAR1) concentrations in HER2<sup>++</sup>/CD71<sup>+</sup> expressing cells.

#### **Example 2. V<sub>HH</sub>-SO1861 + V<sub>HH</sub>-dianthin (2T2C)**

The 2 target 2-components system (2T2C) is the combination treatment of V<sub>HH</sub>1-SO1861 and V<sub>HH</sub>2-protein toxin, where each V<sub>HH</sub> recognizes another cell surface receptor (Figure 1C). SO1861-EMCH was conjugated to the terminal cysteine residues of the V<sub>HH</sub> targeting HER2, producing HER2V<sub>HH</sub>-SO1861 (DAR1). HER2V<sub>HH</sub>-SO1861 was titrated on a fixed concentration of 50 pM CD71V<sub>HH</sub>-dianthin and targeted protein toxin mediated cell killing on SK-BR-3 (HER2<sup>++</sup>/CD71<sup>+</sup>) and MDA-MB-468 (HER2/CD71<sup>+</sup>) was determined. This revealed enhanced cell killing at relatively low concentrations of V<sub>HH</sub>HER2-L-SO1861 (SK-BR-3: IC<sub>50</sub> = 300 nM; Figure 4A). Equivalent concentrations of HER2V<sub>HH</sub>-

SO1861 alone induced cell killing at high concentrations ( $IC_{50} = 4.000 \text{ nM}$ ), whereas equivalent concentrations of  $HER2V_{HH}$ ,  $HER2V_{HH} + 50 \text{ pM } CD71V_{HH}$ -dianthin could not induce cell killing ( $IC_{50} > 5.000 \text{ nM}$ ; Figure 4A). In MDA-MB-468 ( $HER2/CD71^+$ ) the combination of  $HER2V_{HH}$ -SO1861 +  $50 \text{ pM } CD71V_{HH}$ -dianthin revealed cell killing activity at higher concentrations ( $IC_{50} = 600 \text{ nM}$ ; Figure 4B),  
 5 whereas equivalent concentrations of  $HER2V_{HH}$ ,  $HER2V_{HH}$ -SO1861 or  $HER2V_{HH} + 50 \text{ pM } CD71V_{HH}$ -dianthin could not induce cell killing activity ( $IC_{50} > 5.000 \text{ nM}$ ; Figure 4B).

Next,  $CD71V_{HH}$ -dianthin was titrated on a fixed concentration of  $900 \text{ nM } HER2V_{HH}$ -SO1861 and targeted protein toxin mediated cell killing on SK-BR-3 ( $HER2^{++}/CD71^+$ ) and MDA-MB-468 ( $HER2^-/CD71^+$ ) was determined. This revealed that  $900 \text{ nM } HER2V_{HH}$ -SO1861 in combination with low  
 10 concentrations  $CD71V_{HH}$ -dianthin induced efficient cell killing of SK-BR-3 cells ( $IC_{50} = 0,05 \text{ pM}$ ; Figure 5A), whereas  $CD71V_{HH}$ -dianthin or  $CD71V_{HH}$ -dianthin +  $900 \text{ nM } HER2V_{HH}$  could only induce cell killing at high concentrations ( $IC_{50} > 10.000 \text{ pM}$ ; Figure 5A). Besides,  $CD71V_{HH}$ -dianthin was also titrated on a fixed concentration of  $77 \text{ nM}$  trastuzumab-SO1861 (DAR4) and this revealed also a strong enhancement in cell killing activity in SK-BR-3 ( $HER2^{++}/CD71^+$ ) cells ( $IC_{50} < 0,0001 \text{ pM}$ ). In MDA-MB-  
 15 468 cells ( $HER2^-/CD71^+$ )  $CD71V_{HH}$ -dianthin +  $900 \text{ nM } HER2V_{HH}$ -SO1861 showed cell killing only at much higher concentrations ( $IC_{50} = 10 \text{ pM}$ , Figure 5B), whereas  $CD71V_{HH}$ -dianthin,  $CD71V_{HH}$ -dianthin +  $900 \text{ nM } HER2V_{HH}$  or  $CD71V_{HH}$ -dianthin + trastuzumab-SO1861 (DAR4) showed cell killing only at  $IC_{50} = 2.000 \text{ pM}$ ; Figure 5B).

All this shows that relatively low concentrations of  $V_{HH}CD71$ -dianthin can be effective and induce  
 20 cell killing in combination with low  $V_{HH}HER2$ -SO1861 conjugate concentrations in high  $HER2/CD71$  expressing cells.

The combination according to the invention in MDA-MB-468 cells ( $HER2^-/CD71^+$ ) did not reveal any cell killing activity. This shows that in the absence of sufficient receptor expression, effective intracellular delivered SO1861 concentrations are not reached (threshold) to induce endosomal escape  
 25 and cytoplasmic delivery of the protein toxin.

### Example 3. $V_{HH}$ -dianthin + mAb-SO1861 (1T2C and 2T2C)

The 1 target 2-components system (1T2C) is the combination treatment of mAb-SO1861 and  $V_{HH}$ -protein toxin, where mAb and  $V_{HH}$  recognize and bind the same cell surface receptor (Figure 1E). The  
 30 2 target 2-components system (2T2C) is also the combination treatment of mAb-SO1861 and  $V_{HH}$ -protein toxin, where the mAb and  $V_{HH}$  recognize another cell surface receptor (Figure 1D).

Dianthin-C (dianthin with a terminal cysteine) was conjugated to the terminal cysteine residues of the  $V_{HH}$  targeting HER2,  $V_{HH}$  targeting CD71 or  $V_{HH}$  targeting EGFR producing  $HER2V_{HH}$ -dianthin (DAR1),  $CD71V_{HH}$ -dianthin (DAR1) and  $EGFRV_{HH}$ -dianthin (DAR1).

$CD71V_{HH}$ -dianthin,  $HER2V_{HH}$ -dianthin or  $EGFRV_{HH}$ -dianthin was titrated on a fixed concentration of cetuximab-SO1861 (DAR4) and targeted protein toxin mediated cell killing on A431 ( $EGFR^{++}/HER2^{-}/CD71^+$ ) and A2058 ( $EGFR/HER2^{-}/CD71^+$ ) was determined. This revealed that very  
 35 low concentrations  $CD71V_{HH}$ -dianthin in combination with  $77 \text{ nM}$  cetuximab-SO1861 induced efficient cell killing of A431 cells ( $IC_{50} < 0,0001 \text{ pM}$ ; Figure 6A), whereas  $CD71V_{HH}$ -dianthin alone showed activity at  $IC_{50} = 2000 \text{ pM}$ . The other two combinations  $EGFRV_{HH}$ -dianthin +  $77 \text{ nM}$  cetuximab-SO1861  
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and HER2V<sub>HH</sub>-dianthin + 77 nM cetuximab-SO1861 showed efficient cell killing at respectively IC<sub>50</sub>= 20 pM and IC<sub>50</sub> = 50 pM, whereas EGFRV<sub>HH</sub>-dianthin or HER2V<sub>HH</sub>-dianthin alone could not induce efficient cell killing in A431 cells (IC<sub>50</sub>> 10.000 pM; Figure 6A). In A2058 cells (EGFR<sup>-</sup>/HER2<sup>+/+</sup>/CD71<sup>+</sup>), CD71V<sub>HH</sub>-dianthin and CD71V<sub>HH</sub>-dianthin + 77 nM cetuximab-SO1861 showed cell killing activity at  
 5 respectively, IC<sub>50</sub> = 3.000 pM and IC<sub>50</sub> = 1.000 pM, whereas all other treatments or combinations showed no cell killing up to IC<sub>50</sub> = 10.000 pM V<sub>HH</sub>-toxin in A2058 cells (Figure 6B).

This shows that cetuximab-SO1861 (DAR4) can efficiently induce endosomal escape of three different V<sub>HH</sub>-dianthin conjugates, thereby inducing enhanced cell killing in A431 cells.

Next, CD71V<sub>HH</sub>-dianthin, HER2V<sub>HH</sub>-dianthin or EGFRV<sub>HH</sub>-dianthin was titrated on a fixed  
 10 concentration of trastuzumab-SO1861 (DAR4) and targeted protein toxin mediated cell killing on SK-BR-3 (HER2<sup>++</sup>/EGFR<sup>-</sup>/CD71<sup>+</sup>) and MDA-MB-468 cells (HER2<sup>+</sup>/EGFR<sup>++</sup>/CD71<sup>+</sup>) was determined. This revealed that very low concentrations CD71V<sub>HH</sub>-dianthin in combination with 77 nM trastuzumab-SO1861 induced efficient cell killing of SK-BR-3 cells (IC<sub>50</sub>< 0,0001 pM; Figure 7A), whereas CD71V<sub>HH</sub>-dianthin alone showed activity at IC<sub>50</sub> = 10.000 pM. The other two combinations EGFRV<sub>HH</sub>-dianthin +  
 15 77 nM trastuzumab-SO1861 and HER2V<sub>HH</sub>-dianthin + 77 nM trastuzumab-SO1861 showed efficient cell killing at respectively IC<sub>50</sub> = 400 pM and IC<sub>50</sub> = 6 pM, whereas EGFRV<sub>HH</sub>-dianthin or HER2V<sub>HH</sub>-dianthin alone could not induce efficient cell killing in SK-BR-3 cells (IC<sub>50</sub> > 10.000 pM; Figure 7A). In MDA-MB-468 cells (HER2<sup>+</sup>/EGFR<sup>++</sup>/CD71<sup>+</sup>), CD71V<sub>HH</sub>-dianthin and CD71V<sub>HH</sub>-dianthin + 77 nM cetuximab-SO1861 showed cell killing activity at respectively, IC<sub>50</sub> = 3000 and IC<sub>50</sub> = 2000 pM., whereas all other  
 20 treatments or combinations showed no cell killing up to IC<sub>50</sub> = 10.000 pM V<sub>HH</sub>-dianthin in MDA-MB-468 cells (Figure 7B). This shows that trastuzumab-SO1861 (DAR4) can efficiently induce endosomal escape of three different V<sub>HH</sub>-dianthin conjugates, thereby inducing enhanced cell killing in SK-BR-3 cells.

## 25 **Materials and methods**

### *materials*

SO1861 was isolated and purified by Analyticon Discovery GmbH from raw plant extract obtained from *Saponaria officinalis*. V<sub>HH</sub> were purchased from QVQ, Utrecht, The Netherlands (HER2V<sub>HH</sub>: clone name: Q17c; CD71V<sub>HH</sub>: clone name: Q52c EGFRV<sub>HH</sub>: clone name: Q86c). Trastuzumab (Tras, Herceptin®,  
 30 Roche), Cetuximab (Cet, Erbitux®, Merck KGaA) were purchased from the pharmacy (Charite, Berlin). CD71 monoclonal antibody was purchased from BioCell (Okt9, #BE0023). Custom trastuzumab-saporin and antiCD71mab-saporin conjugate was produced and purchased from Advanced Targeting Systems (San Diego, CA). Dianthin-Cys (Dia-Cys, Dianthin mutant with a single C-terminal cysteine was  
 35 produced by Proteogenix, France.

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%, Sigma-Aldrich), 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent, 99%, Sigma-Aldrich), Zeba™ Spin Desalting Columns (2 mL, Thermo-Fisher), NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo-Fisher), NuPAGE™ MES SDS Running Buffer (Thermo-Fisher), Novex™ Sharp Pre-stained Protein Standard (Thermo-Fisher),  
 40 PageBlue™ Protein Staining Solution (Thermo-Fischer), Pierce™ BCA Protein Assay Kit (Thermo-

Fisher), N-Ethylmaleimide (NEM, 98%, Sigma-Aldrich), 1,4-Dithiothreitol (DTT, 98%, Sigma-Aldrich), Sephadex G25 (GE Healthcare), Sephadex G50 M (GE Healthcare), Superdex 200P (GE Healthcare), Isopropyl alcohol (IPA, 99.6%, VWR), Tris(hydroxymethyl)aminomethane (Tris, 99%, Sigma-Aldrich), Tris(hydroxymethyl)aminomethane hydrochloride (Tris.HCL, Sigma-Aldrich), L-Histidine (99%, Sigma-Aldrich), D-(+)-Trehalose dehydrate (99%, Sigma-Aldrich), Polyethylene glycol sorbitan monolaurate (TWEEN 20, Sigma-Aldrich), Dulbecco's Phosphate-Buffered Saline (DPBS, Thermo-Fisher), Guanidine hydrochloride (99%, Sigma-Aldrich), Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na<sub>2</sub>, 99%, Sigma-Aldrich), sterile filters 0.2 µm and 0.45 µm (Sartorius), Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Thermo-Fisher), Vivaspin T4 and T15 concentrator (Sartorius), Superdex 200PG (GE Healthcare), Tetra(ethylene glycol) succinimidyl 3-(2-pyridylidithio)propionate (PEG<sub>4</sub>-SPDP, Thermo-Fisher), HSP27 BNA disulfide oligonucleotide (Biosynthesis), [O-(7-Azabenzotriazol-1-yl)-N,N,N,N-tetramethyluronium-hexafluorophosphat] (HATU, 97%, Sigma-Aldrich), Dimethyl sulfoxide (DMSO, 99%, Sigma-Aldrich), N-(2-Aminoethyl)maleimide trifluoroacetate salt (AEM, 98%, Sigma-Aldrich), L-Cysteine (98.5%, Sigma-Aldrich), deionized water (DI) was freshly taken from Ultrapure Lab Water Systems (MilliQ, Merck), Nickel-nitrilotriacetic acid agarose (Ni-NTA agarose, Protino), Glycine (99.5%, VWR), 5,5-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB, 98%, Sigma-Aldrich), S-Acetylmercaptosuccinic anhydride Fluorescein (SAMSA reagent, Invitrogen) Sodium bicarbonate (99.7%, Sigma-Aldrich), Sodium carbonate (99.9%, Sigma-Aldrich), PD MiniTrap desalting columns with Sephadex G-25 resin (GE Healthcare), PD10 G25 desalting column (GE Healthcare), Zeba Spin Desalting Columns in 0.5, 2, 5, and 10 mL (Thermo-Fisher), Vivaspin Centrifugal Filters T4 10 kDa MWCO, T4 100 kDa MWCO, and T15 (Sartorius), Biosep s3000 aSEC column (Phenomenex), Vivacell Ultrafiltration Units 10 and 30 kDa MWCO (Sartorius), Nalgene Rapid-Flow filter (Thermo-Fisher),

## 25 *methods*

### **SO1861-EMCH synthesis**

To SO1861 (121 mg, 0.065 mmol) and EMCH.TFA (110 mg, 0.325 mmol) was added methanol (extra dry, 3.00 mL) and TFA (0.020 mL, 0.260 mmol). The reaction mixture stirred at room temperature. After 1.5 hours the reaction mixture was subjected to preparative MP-LC.<sup>1</sup> Fractions corresponding to the product were immediately pooled together, frozen and lyophilized overnight to give the title compound (120 mg, 90%) as a white fluffy solid. Purity based on LC-MS 96%.

LRMS (m/z): 2069 [M-1]<sup>-</sup>

LC-MS r.t. (min): 1.08<sup>4</sup>

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### **Cell viability assay**

After treatment the cells were incubated for 72 hr at 37°C before the cell viability was determined by a MTS-assay, performed according to the manufacturer's instruction (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). Briefly, the MTS solution was diluted 20× in DMEM without phenol red (PAN-Biotech GmbH) supplemented with 10% FBS. The cells were washed once with 200

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μL/PBS well, after which 100 μL diluted MTS solution was added/well. The plate was incubated for approximately 20-30 minutes at 37°C. Subsequently, the OD at 492 nm was measured on a Thermo Scientific Multiskan FC plate reader (Thermo Scientific). For quantification the background signal of 'medium only' wells was subtracted from all other wells, before the cell viability percentage of treated/untreated cells was calculated, by dividing the background corrected signal of treated wells over the background corrected signal of the untreated wells (x 100).

### FACS analysis

Cells were seeded in DMEM (PAN-Biotech GmbH) supplemented with 10% fetal calf serum (PAN-Biotech GmbH) and 1% penicillin/streptomycin (PAN-Biotech GmbH), at 500,000 c/plate in 10 cm dishes and incubated for 48 hrs (5% CO<sub>2</sub>, 37°C), until a confluency of 90% was reached. Next, the cells were trypsinized (TrypLE Express, Gibco Thermo Scientific) to single cells. 0.75 x 10<sup>6</sup> Cells were transferred to a 15 mL falcon tube and centrifuged (1,400 rpm, 3 min). The supernatant was discarded while leaving the cell pellet submerged. The pellet was dissociated by gentle tapping the falcon tube on a vortex shaker and the cells were washed with 4 mL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS). After washing the cells were resuspended in 3 mL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS) and divided equally over 3 round bottom FACS tubes (1 mL/tube). The cells were centrifuged again and resuspended in 200 μL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS) or 200 μL antibody solution; containing 5 μL antibody in 195 μL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS). APC Mouse IgG1, κ APC anti-human EGFR (#352906, Biolegend) was used to stain the EGFR receptor. PE anti-human HER2 APC anti-human CD340 (erbB2/HER-2) (#324408 Biolegend) was used to stain the HER2 receptor, PE Mouse IgG2a, κ Isotype Ctrl FC (#400212, Biolegend) was used as its matched isotype control. PE anti-human CD71 (#334106, Biolegend) was used to stain the CD71 receptor, PE Mouse IgG2a, κ Isotype Ctrl FC (#400212, Biolegend) was used as its matched isotype control. Samples were incubated for 30 min at 4°C on a tube roller mixer. Afterwards, the cells were washed 3x with cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS) and fixated for 20 min at room temperature using a 2% PFA solution in PBS. Cells were washed 2x with cold PBS, and resuspended in 250-350 μL cold PBS for FACS analysis. Samples were analyzed with a BD FACSCanto II flow cytometry system (BD Biosciences) and FlowJo software. Results of the analyses of the cell-surface expression of EGFR, HER2 and CD71 on the various cells is summarized in Table A2.

Table A2. Cell surface expression levels of EGFR, HER2 and CD71 of various cells

| Cell line  | EGFR<br>expression<br>level (MFI) | HER2<br>expression<br>level (MFI) | CD71<br>expression<br>level (MFI) |
|------------|-----------------------------------|-----------------------------------|-----------------------------------|
| MDA-MB-468 | 1656                              | 1                                 | 186                               |
| A431       | 1593                              | 10                                | 322                               |
| SK-BR-3    | 28                                | 1162                              | 331                               |
| A2058      | 1                                 | 5                                 | 59                                |

*Procedure for the conjugation of V<sub>HH</sub>-SO1861*

To an aliquot of V<sub>HH</sub> was added an aliquot of freshly prepared TCEP solution (10.0 mg/ml), the mixture vortexed briefly then incubated for 30 minutes at 20°C with roller-mixing. After incubation, the resulting V<sub>HH</sub>-SH was purified by gel filtration using zeba spin desalting column into TBS pH 7.5. To the resulting V<sub>HH</sub>-SH was added freshly prepared SPT-EMCH solution the mixture vortexed briefly then incubated overnight at 20°C.

After incubation, an aliquot of V<sub>HH</sub>-SO1861 mixture was removed and characterised by Ellman's assay to ascertain SO1861 incorporation. The conjugate was purified by 1.6 × 35 cm Superdex 200PG column eluting with DPBS pH 7.5 to give purified V<sub>HH</sub>-SO1861. The aliquot was filtered to 0.2 µm, concentrated and normalised to 1.0 mg/ml to afford V<sub>HH</sub>-SO1861.

*Procedure for the conjugation of V<sub>HH</sub>-Dianthin*

Dianthin-Cys was concentrated by ultrafiltration using a vivaspin T15 10KDa MWCO centrifugal filter and buffer exchanged into TBS pH 7.5. To the concentrated Dianthin-Cys was added an aliquot of freshly prepared TCEP solution (10.0 mg/ml), the mixture vortexed briefly then incubated for 60 minutes at 20 °C with roller-mixing. After incubation, the resulting Dianthin-SH was purified by gel filtration using a zeba spin desalting column then repeated centrifugal-wash cycles using a vivaspin T15 10KDa MWCO centrifugal filter into TBS pH 7.5. The resulting Dianthin-SH was reacted with freshly prepared DTME solution (10 mg/ml) in DMSO, the mixture vortexed briefly then incubated for 60 minutes at 20°C. After, the Dianthin-DTME was obtained following purification by gel filtration using a zeba spin desalting column into TBS pH 7.5. The Dianthin-DTME was stored at 20°C until conjugated. At the same time, an aliquot of V<sub>HH</sub> was concentrated by ultrafiltration using a vivaspin T15 10 KDa MWCO centrifugal filter and buffer exchanged into TBS pH 7.5. To the concentrated V<sub>HH</sub> was added an aliquot of freshly prepared TCEP solution (10.0 mg/ml), the mixture vortexed briefly then incubated for 60 minutes at 37°C with roller-mixing. After incubation, the resulting V<sub>HH</sub> was purified by gel filtration using a zeba spin desalting column then repeated centrifugal-wash cycles using a vivaspin T4 5 KDa MWCO centrifugal filter into TBS pH 7.5. An aliquot of the resulting V<sub>HH</sub>-SH was reacted with Dianthin-DTME, the mixture vortexed briefly then incubated overnight at 20°C. After, the reaction mixture was concentrated using a vivaspin T4 10 KDa MWCO centrifuge tube and purified by gel filtration using a 1.6 × 35 cm Superdex 200PG column eluting into DPBS pH 7.5.

**Antibody-(L-SO1861)<sup>4</sup>**

Trastuzumab, Cetuximab, are referred hereafter as "Ab". Ab was conjugated to the saponin SO1861-EMCH via Michael-type thiol-ene conjugation reaction at DARs of 1, 2, 3, 4, 5, and 6. The SO1861-EMCH molecule obtains a labile (L) hydrazone bond between its structure and its maleimide function generating a labile bond between the saponin and Ab. The procedure is exemplary described for Trastuzumab-(L-SO1861)<sub>4</sub>:

To a solution of Cetuximab (40 mg, 8.0 ml) was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Cetuximab divided into four portions (each of 9.73 mg, 4.864 mg/ml, 65 nmol) was added an aliquot of freshly prepared TCEP solution (0.5 – 2.0 mg/ml, 1.15 – 7.02 mole equivalents, 75 – 455 nmol), the mixtures vortexed briefly then incubated for 300 minutes at 20°C with roller-mixing. After incubation (prior to addition of SO1861-EMCH), a *ca.* 1 mg (0.210 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to Ab ratio = 2.0, 4.2, 5.9 and 6.8 respectively). To each of the bulk Ab-SH was added an aliquot of freshly prepared SO1861-EMCH solution (2 mg/ml, 1.3 mole equivalents per 'thiol', 0.15 – 0.61 µmol, 0.16 – 0.63 ml), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides each conjugation reaction, two aliquots of desalted Ab-SH (0.25 mg, 1.67 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 4.3 – 17.4 nmol, 2.2 – 8.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (2.2 – 8.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.200 ml aliquot of Ab – SO1861-EMCH mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls were characterized by Ellman's assay to obtain SO1861-EMCH incorporations. To the bulk Ab – SO1861-EMCH mixture was added an aliquot of freshly prepared NEM solution (2.5 mg/ml, 2.5 – 10 mole equivalents, 0.15 – 0.58 µmol) and the mixtures purified by zeba spin desalting columns eluting with DPBS pH 7.5 to give purified Cetuximab – (L-SO1861) conjugates. The products were normalized to 2.5 mg/ml and filtered to 0.2 µm prior to dispensing for biological evaluation. The reaction conditions and results for Trastuzumab-L-SO1861 conjugates and the reaction conditions and results for Cetuximab-L-SO1861 conjugates are summarized in Table A3 and Table A4.

**Table A3.** Summarized reaction conditions and results for Trastuzumab-L-SO1861 conjugates

| Batch                        | Ab feed            | TCEP feed mole equivalents | SO1861-EMCH feed | Obtained DAR | Purity by analytical SEC (%) | Yield (%) |
|------------------------------|--------------------|----------------------------|------------------|--------------|------------------------------|-----------|
| Tras-(L-SO1861) <sub>4</sub> | 9.91 mg<br>66 nmol | 3.83                       | 0.46 µmol        | 4.0          | 98.4                         | 81        |

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**Table A4.** Summarized reaction conditions and results for Cetuximab-L-SO1861 conjugates

| Batch                       | Ab feed            | TCEP feed mole equivalents | SO1861-EMCH feed | Obtained DAR | Purity by analytical SEC (%) | Yield (%) |
|-----------------------------|--------------------|----------------------------|------------------|--------------|------------------------------|-----------|
| Cet-(L-SO1861) <sub>4</sub> | 9.73 mg<br>65 nmol | 4.19                       | 0.46 µmol        | 4.1          | 99.0                         | 77        |

## Materials

Throughout the description, claims and drawings, 'VHH', 'Vhh', 'V<sub>hh</sub>' and 'V<sub>HH</sub>' should be understood as referring to the same type of single domain antibody. Similar for single domain antibodies of the type referred to as any of 'VH', 'Vh', 'V<sub>h</sub>' and 'V<sub>H</sub>'.

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HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, CD71-V<sub>HH</sub> (purchased), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%, Sigma-Aldrich), 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent, 99%, Sigma-Aldrich), Zeba™ Spin Desalting Columns (2 mL, Thermo-Fisher), NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo-Fisher), NuPAGE™ MES SDS Running Buffer (Thermo-Fisher), Novex™ Sharp Pre-stained Protein Standard (Thermo-Fisher), PageBlue™ Protein Staining Solution (Thermo-Fisher), Pierce™ BCA Protein Assay Kit (Thermo-Fisher), N-Ethylmaleimide (NEM, 98%, Sigma-Aldrich), 1,4-Dithiothreitol (DTT, 98%, Sigma-Aldrich), Sephadex G25 (GE Healthcare), Sephadex G50 M (GE Healthcare), Superdex 200P (GE Healthcare), Isopropyl alcohol (IPA, 99.6%, VWR), Tris(hydroxymethyl)aminomethane (Tris, 99%, Sigma-Aldrich), Tris(hydroxymethyl)aminomethane hydrochloride (Tris.HCL, Sigma-Aldrich), L-Histidine (99%, Sigma-Aldrich), D-(+)-Trehalose dehydrate (99%, Sigma-Aldrich), Polyethylene glycol sorbitan monolaurate (TWEEN 20, Sigma-Aldrich), Dulbecco's Phosphate-Buffered Saline (DPBS, Thermo-Fisher), Guanidine hydrochloride (99%, Sigma-Aldrich), Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na<sub>2</sub>, 99%, Sigma-Aldrich), sterile filters 0.2 µm and 0.45 µm (Sartorius), Vivaspin T4 and T15 concentrator (Sartorius), Superdex 200PG (GE Healthcare), HSP27 BNA disulfide oligonucleotide (Biosynthesis), [O-(7-Azabenzotriazol-1-yl)-N,N,N,N-tetramethyluronium-hexafluorophosphat] (HATU, 97%, Sigma-Aldrich), Dimethyl sulfoxide (DMSO, 99%, Sigma-Aldrich), N-(2-Aminoethyl)maleimide trifluoroacetate salt (AEM, 98%, Sigma-Aldrich), L-Cysteine (98.5%, Sigma-Aldrich), deionized water (DI) was freshly taken from Ultrapure Lab Water Systems (MilliQ, Merck), Nickel-nitrilotriacetic acid agarose (Ni-NTA agarose, Protino), Glycine (99.5%, VWR), 5,5-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB, 98%, Sigma-Aldrich), Sodium bicarbonate (99.7%, Sigma-Aldrich), Sodium carbonate (99.9%, Sigma-Aldrich), PD MiniTrap desalting columns with Sephadex G-25 resin (GE Healthcare), PD10 G25 desalting column (GE Healthcare), Zeba Spin Desalting Columns in 0.5, 2, 5, and 10 mL (Thermo-Fisher), Vivaspin Centrifugal Filters T4 10 kDa MWCO, T4 100 kDa MWCO, and T15 (Sartorius), Biosep s3000 aSEC column (Phenomenex), Vivacell Ultrafiltration Units 10 and 30 kDa MWCO (Sartorius), Nalgene Rapid-Flow filter (Thermo-Fisher), Acrylamide (99.9%, Sigma-Aldrich), Sodium dodecyl sulfate (98%, Sigma-Aldrich), Ammonium persulfate (APS, 98%, Sigma-Aldrich), Glycerol (99%, Sigma-Aldrich), Bromophenol Blue (Sigma-Aldrich), Polyethylene glycol dodecyl ether (Brij-35, Sigma-Aldrich). All SO1861 derivatives (SO1861-EMCH, SO1861-AEM, Dendron-[L-SO1861]<sub>n</sub>), all QS21 derivatives (QS21-EMCH, QS21-AEM, Dendron-[L-QS21]<sub>n</sub>), and trifunctional linker derivatives were produced in house.

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## Syntheses

### 1. V<sub>HH</sub>-[S-Trifunctional linker-(L-SO1861)-(L-HSP27 BNA)]<sub>4</sub>

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*HER2-V<sub>HH</sub>-[S-Tri-(L-SO1861)-(L-HSP27)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
*EGFR-V<sub>HH</sub>-[S-Tri-(L-SO1861)-(L-HSP27)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
*CD71-V<sub>HH</sub>-[S-Tri-(L-SO1861)-(L-HSP27)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,***

5 HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing HSP27 BNA derivatives which are referred hereafter as "HSP27-Mal". These HSP27-Mal derivatives were namely: 1) Mal-Trifunctional linker-(L-SO1861)-(L-HSP27 BNA), 2) Mal-Trifunctional linker-(blocked)-(L-HSP27 BNA). The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(L-SO1861)-(L-HSP27 BNA)]<sub>4</sub>:

10 Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution  
 15 (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12  
 20 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the HSP27 BNA-Mal derivatives **1** – **2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – HSP27 BNA derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer  
 25 (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain HSP27 BNA derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly  
 30 prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1** – **2** conjugates. The products were filtered to 0.2µm prior to dispensing for biological evaluation.

35 **2. V<sub>HH</sub>-[S-Trifunctional linker-(S-SO1861)-(L-HSP27 BNA)]<sub>4</sub>**

*HER2-V<sub>HH</sub>-[S-Tri-(S-SO1861)-(L-HSP27)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
*EGFR-V<sub>HH</sub>-[S-Tri-(S-SO1861)-(L-HSP27)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
*CD71-V<sub>HH</sub>-[S-Tri-(S-SO1861)-(L-HSP27)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,***

HER2- $V_{HH}$ , EGFR- $V_{HH}$ , and CD71- $V_{HH}$  are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing HSP27 BNA derivatives which are referred hereafter as "HSP27-Mal". These HSP27-Mal derivatives were namely: 1) Mal-Trifunctional linker-(S-SO1861)-(L-HSP27 BNA), 2) Mal-Trifunctional linker-(blocked)-(L-HSP27 BNA). The procedure is exemplary described for HER2- $V_{HH}$ -[S-Trifunctional linker-(S-SO1861)-(L-HSP27 BNA)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10  $\mu$ l/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA- $Na_2$  concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14  $\mu$ mol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32  $\mu$ mol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the HSP27 BNA-Mal derivatives **1** – **2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – HSP27 BNA derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50  $\mu$ g, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7  $\mu$ l of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7  $\mu$ l) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain HSP27 BNA derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6  $\times$  30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1** – **2** conjugates. The products were filtered to 0.2  $\mu$ m prior to dispensing for biological evaluation.

### 3. $V_{HH}$ -[S-Trifunctional linker-(S-dendron-(L-SO1861)<sub>n</sub>)-(L-HSP27 BNA)]<sub>4</sub>

*HER2- $V_{HH}$ -[S-Tri-(S-dendron-(L-SO1861)<sub>n</sub>)-(L-HSP27)]<sub>4</sub>, HER2- $V_{HH}$  -[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
EGFR- $V_{HH}$  -[S-Tri-(S-dendron-(L-SO1861)<sub>n</sub>)-(L-HSP27)]<sub>4</sub>, EGFR- $V_{HH}$  -[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
CD71- $V_{HH}$ -[S-Tri-(S-dendron-(L-SO1861)<sub>n</sub>)-(L-HSP27)]<sub>4</sub>, CD71- $V_{HH}$  -[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,*

HER2- $V_{HH}$ , EGFR- $V_{HH}$ , and CD71- $V_{HH}$  are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing HSP27 BNA derivatives which are referred hereafter as "HSP27-Mal". These HSP27-Mal derivatives were namely: 1) Mal-Trifunctional linker-(S-dendron-(L-SO1861)<sub>n</sub>)-(L-HSP27 BNA), 2) Mal-Trifunctional linker-(blocked)-(L-HSP27 BNA).

"n" refers to the number of SO1861 molecules that is 4, 8, or higher than 8. The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(S-dendron-(L-SO1861)<sub>4</sub>)-(L-HSP27 BNA)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the HSP27 BNA-Mal derivatives **1** – **2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – HSP27 BNA derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain HSP27 BNA derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1** – **2** conjugates. The products were filtered to 0.2 µm prior to dispensing for biological evaluation.

#### 4. V<sub>HH</sub>-[S-Trifunctional linker-(L-QS21)-(L-HSP27 BNA)]<sub>4</sub>

30 *HER2-V<sub>HH</sub>-[S-Tri-(L-QS21)-(L-HSP27)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
EGFR-V<sub>HH</sub>-[S-Tri-(L-QS21)-(L-HSP27)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
CD71-V<sub>HH</sub>-[S-Tri-(L-QS21)-(L-HSP27)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,*

HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing HSP27 BNA derivatives which are referred hereafter as "HSP27-Mal". These HSP27-Mal derivatives were namely: 1) Mal-Trifunctional linker-(L-QS21)-(L-HSP27 BNA), 2) Mal-Trifunctional linker-(blocked)-(L-HSP27 BNA). The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(L-QS21)-(L-HSP27 BNA)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127

mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14  $\mu$ mol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32  $\mu$ mol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the HSP27 BNA-Mal derivatives **1** – **2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – HSP27 BNA derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50  $\mu$ g, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7  $\mu$ l of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7  $\mu$ l) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain HSP27 BNA derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6  $\times$  30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1** – **2** conjugates. The products were filtered to 0.2  $\mu$ m prior to dispensing for biological evaluation.

#### 5. V<sub>HH</sub>-[S-Trifunctional linker-(S-QS21)-(L-HSP27 BNA)]<sub>4</sub>

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*HER2-V<sub>HH</sub>-[S-Tri-(S-QS21)-(L-HSP27)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
EGFR-V<sub>HH</sub>-[S-Tri-(S-QS21)-(L-HSP27)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
CD71-V<sub>HH</sub>-[S-Tri-(S-QS21)-(L-HSP27)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,*

30 HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing HSP27 BNA derivatives which are referred hereafter as "HSP27-Mal". These HSP27-Mal derivatives were namely: 1) Mal-Trifunctional linker-(S-QS21)-(L-HSP27 BNA), 2) Mal-Trifunctional linker-(blocked)-(L-HSP27 BNA). The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(S-QS21)-(L-HSP27 BNA)]<sub>4</sub>:

35 Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10  $\mu$ l/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

40 To Ab (2.1 mg, 0.5 mg/ml, 0.14  $\mu$ mol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32  $\mu$ mol), the mixture vortexed briefly then incubated for 90

minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the HSP27 BNA-Mal derivatives **1** – **2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – HSP27 BNA derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain HSP27 BNA derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1** – **2** conjugates. The products were filtered to 0.2 µm prior to dispensing for biological evaluation.

## 20 6. $V_{HH}$ -[S-Trifunctional linker-(S-dendron-(L-QS21)<sub>n</sub>)-(L-HSP27 BNA)]<sub>4</sub>

*HER2- $V_{HH}$ -[S-Tri-(S-dendron-(L-QS21)<sub>n</sub>)-(L-HSP27)]<sub>4</sub>, HER2- $V_{HH}$ -[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
EGFR- $V_{HH}$ -[S-Tri-(S-dendron-(L-QS21)<sub>n</sub>)-(L-HSP27)]<sub>4</sub>, EGFR- $V_{HH}$ -[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,  
CD71- $V_{HH}$ -[S-Tri-(S-dendron-(L-QS21)<sub>n</sub>)-(L-HSP27)]<sub>4</sub>, CD71- $V_{HH}$ -[S-Tri-(blocked)-(L-HSP27)]<sub>4</sub>,*

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HER2- $V_{HH}$ , EGFR- $V_{HH}$ , and CD71- $V_{HH}$  are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing HSP27 BNA derivatives which are referred hereafter as "HSP27-Mal". These HSP27-Mal derivatives were namely: 1) Mal-Trifunctional linker-(S-dendron-(L-QS21)<sub>n</sub>)-(L-HSP27 BNA), 2) Mal-Trifunctional linker-(blocked)-(L-HSP27 BNA). "n" refers to the number of QS21 molecules that is 4, 8, or higher than 8. The procedure is exemplary described for HER2- $V_{HH}$ -[S-Trifunctional linker-(S-dendron-(L-QS21)<sub>4</sub>)-(L-HSP27 BNA)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's

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assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the HSP27 BNA-Mal derivatives **1 – 2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – HSP27 BNA derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain HSP27 BNA derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1 – 2** conjugates. The products were filtered to 0.2 µm prior to dispensing for biological evaluation.

#### 7. $V_{HH}$ -[S-Trifunctional linker-(L-SO1861)-(L-dianthin)]<sub>4</sub>

*HER2- $V_{HH}$ -[S-Tri-(L-SO1861)-(L-dianthin)]<sub>4</sub>, HER2- $V_{HH}$ -[S-Tri-(blocked)-(L-dianthin)]<sub>4</sub>,  
EGFR- $V_{HH}$ -[S-Tri-(L-SO1861)-(L-dianthin)]<sub>4</sub>, EGFR- $V_{HH}$ -[S-Tri-(blocked)-(L-dianthin)]<sub>4</sub>,  
CD71- $V_{HH}$ -[S-Tri-(L-SO1861)-(L-dianthin)]<sub>4</sub>, CD71- $V_{HH}$ -[S-Tri-(blocked)-(L-dianthin)]<sub>4</sub>,*

HER2- $V_{HH}$ , EGFR- $V_{HH}$ , and CD71- $V_{HH}$  are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing dianthin derivatives which are referred hereafter as "dianthin -Mal". These dianthin -Mal derivatives were namely: 1) Mal-Trifunctional linker-(L-SO1861)-(L-dianthin), 2) Mal-Trifunctional linker-(blocked)-(L-dianthin). The procedure is exemplary described for HER2- $V_{HH}$ -[S-Trifunctional linker-(L-SO1861)-(L-dianthin)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA- $Na_2$  concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the dianthin -Mal derivatives **1 – 2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – dianthin derivatives

2 conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain dianthin derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1 – 2** conjugates. The products were filtered to 0.2µm prior to dispensing for biological evaluation.

#### 8. V<sub>HH</sub>-[S-Trifunctional linker-(S-SO1861)-(L- dianthin)]<sub>4</sub>

15 *HER2-V<sub>HH</sub>-[S-Tri-(S-SO1861)-(L- dianthin)]<sub>4</sub>, HER2-V<sub>HH</sub> -[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>, EGFR-V<sub>HH</sub> -[S-Tri-(S-SO1861)-(L- dianthin)]<sub>4</sub>, EGFR-V<sub>HH</sub> -[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(S-SO1861)-(L- dianthin)]<sub>4</sub>, CD71-V<sub>HH</sub> -[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>,*

HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing dianthin derivatives which are referred hereafter as "dianthin -Mal". These dianthin -Mal derivatives were namely: 1) Mal-Trifunctional linker-(S-SO1861)-(L- dianthin), 2) Mal-Trifunctional linker-(blocked)-(L- dianthin). The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(S-SO1861)-(L- dianthin)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the dianthin -Mal derivatives **1 – 2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – dianthin derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using

zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain dianthin derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1 – 2** conjugates. The products were filtered to 0.2 µm prior to dispensing for biological evaluation.

#### 9. $V_{HH}$ -[S-Trifunctional linker-(S-dendron-(L-SO1861)<sub>n</sub>)-(L- dianthin)]<sub>4</sub>

*HER2-V<sub>HH</sub>-[S-Tri-(S-dendron-(L-SO1861)<sub>n</sub>)-(L- dianthin)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(S-dendron-(L-SO1861)<sub>n</sub>)-(L- dianthin)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(S-dendron-(L-SO1861)<sub>n</sub>)-(L- dianthin)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>, HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing dianthin derivatives which are referred hereafter as "dianthin -Mal". These dianthin -Mal derivatives were namely: 1) Mal-Trifunctional linker-(S-dendron-(L-SO1861)<sub>n</sub>)-(L- dianthin), 2) Mal-Trifunctional linker-(blocked)-(L- dianthin). "n" refers to the number of SO1861 molecules that is 4, 8, or higher than 8. The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(S-dendron-(L-SO1861)<sub>4</sub>)-(L- dianthin)]<sub>4</sub>:*

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the dianthin -Mal derivatives **1 – 2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – dianthin derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain dianthin derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration

using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1 – 2** conjugates. The products were filtered to 0.2 µm prior to dispensing for biological evaluation.

#### 5 **10. V<sub>HH</sub>-[S-Trifunctional linker-(L-QS21)-(L-dianthin)]<sub>4</sub>**

*HER2-V<sub>HH</sub>-[S-Tri-(L-QS21)-(L-dianthin)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L-dianthin)]<sub>4</sub>,  
EGFR-V<sub>HH</sub>-[S-Tri-(L-QS21)-(L-dianthin)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L-dianthin)]<sub>4</sub>,  
CD71-V<sub>HH</sub>-[S-Tri-(L-QS21)-(L-dianthin)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L-dianthin)]<sub>4</sub>,*

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HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as “Ab”. Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing dianthin derivatives which are referred hereafter as “dianthin Mal”. These dianthin -Mal derivatives were namely: 1) Mal-Trifunctional linker-(L-QS21)-(L-dianthin), 2) Mal-Trifunctional linker-(blocked)-(L-dianthin). The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(L-QS21)-(L-dianthin)]<sub>4</sub>:

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Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

20

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 µmol) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the dianthin -Mal derivatives **1 – 2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – dianthin derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain dianthin derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1 – 2** conjugates. The products were filtered to 0.2 µm prior to dispensing for biological evaluation.

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**11. V<sub>HH</sub>-[S-Trifunctional linker-(S-QS21)-(L- dianthin)]<sub>4</sub>**

*HER2-V<sub>HH</sub>-[S-Tri-(S-QS21)-(L-dianthin)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>,  
*EGFR-V<sub>HH</sub>-[S-Tri-(S-QS21)-(L- dianthin)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>,  
 5 *CD71-V<sub>HH</sub>-[S-Tri-(S-QS21)-(L- dianthin)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>,***

HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing dianthin derivatives which are referred hereafter as "dianthin -Mal". These dianthin -Mal derivatives were namely: 1) Mal-Trifunctional linker-  
 10 (S-QS21)-(L- dianthin), 2) Mal-Trifunctional linker-(blocked)-(L- dianthin). The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(S-QS21)-(L- dianthin)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26  
 15 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the dianthin -Mal derivatives **1 – 2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – dianthin derivatives  
 25 **2** conjugation reaction two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside  
 30 positive and negative controls was characterized by Ellman's assay to obtain dianthin derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1 – 2** conjugates. The  
 35 products were filtered to 0.2 µm prior to dispensing for biological evaluation.

**12. V<sub>HH</sub>-[S-Trifunctional linker-(S-dendron-(L-QS21)<sub>n</sub>)-(L- dianthin)]<sub>4</sub>**

*HER2-V<sub>HH</sub>-[S-Tri-(S-dendron-(L-QS21)<sub>n</sub>)-(L-dianthin)]<sub>4</sub>, HER2-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>,  
 40 *EGFR-V<sub>HH</sub>-[S-Tri-(S-dendron-(L-QS21)<sub>n</sub>)-(L- dianthin)]<sub>4</sub>, EGFR-V<sub>HH</sub>-[S-Tri-(blocked)-(L- dianthin)]<sub>4</sub>,**

*CD71-V<sub>HH</sub>-[S-Tri-(S-dendron-(L-QS21)<sub>n</sub>)-(L-dianthin)]<sub>4</sub>, CD71-V<sub>HH</sub>-[S-Tri-(blocked)-(L-dianthin)]<sub>4</sub>,*

HER2-V<sub>HH</sub>, EGFR-V<sub>HH</sub>, and CD71-V<sub>HH</sub> are referred hereafter as "Ab". Ab was conjugated via Michael-type thiol-ene reaction to two different maleimide (Mal) bearing dianthin derivatives which are referred hereafter as "dianthin-Mal". These dianthin-Mal derivatives were namely: 1) Mal-Trifunctional linker-(S-dendron-(L-QS21)<sub>n</sub>)-(L-dianthin-Mal), 2) Mal-Trifunctional linker-(blocked)-(L-dianthin-Mal). "n" refers to the number of QS21 molecules that is 4, 8, or higher than 8. The procedure is exemplary described for HER2-V<sub>HH</sub>-[S-Trifunctional linker-(S-dendron-(L-QS21)<sub>4</sub>)-(L-dianthin-Mal)]<sub>4</sub>:

Ab was reconstituted to 21 mg/ml with deionized water (DI), then diluted to 5 mg/ml using histidine buffer pH 6. To a 20 mg (4.0 ml) aliquot was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5mM EDTA buffer pH 7.5.

To Ab (2.1 mg, 0.5 mg/ml, 0.14 µmol) was added an aliquot of freshly prepared TCEP solution (1.00 mg/ml, 2.35 mole equivalents, 0.32 µmol), the mixture vortexed briefly then incubated for 90 minutes at 20°C with roller-mixing. After incubation (prior to addition of construct), a ca. 0.2 mg (0.044 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to ab ratio = 4.0). The bulk Ab-SH was split into two aliquots (0.11 mg, 7.6 nmol and 0.12 mg, 8.3 nmol), and to each aliquot was added an aliquot of each of the dianthin-Mal derivatives **1** – **2** (freshly prepared in TBS pH 7.5, 2 mg/ml, 1.3 mole equivalents per 'thiol', 40 nmol and 43 nmol), the mixtures vortexed briefly then incubated for 120 minutes at 20°C. Besides the Ab – dianthin derivatives **2** conjugation reaction, two aliquots of desalted Ab-SH (50 µg, 3.3 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 17.3 nmol, 6.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (6.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.100 ml aliquot of Ab – construct **2** mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls was characterized by Ellman's assay to obtain dianthin derivatives **2** incorporation. To each bulk Ab – construct mixture was added an aliquot of freshly prepared NEM solution (0.25 mg/ml, 2.5 mole equivalents, 19 and 21 nmol) and the mixtures purified by gel filtration using a 1.6 × 30 cm Sephadex G50M eluting with DPBS pH 7.5 followed by repeated centrifugal filtration and washing using a 100 KDa MWCO concentrator to give purified Ab – construct **1** – **2** conjugates. The products were filtered to 0.2 µm prior to dispensing for biological evaluation.

#### Example 4

Conjugates of the invention. Figure 1F-I display four typical molecular assemblies or conjugates (covalent complexes) of the invention. These conjugates are manufactured and purified, for testing in cell-based bioassays, *in vivo* animal models, *etc.*

Fig. 1F is a cartoon representing an endosomal / lysosomal escape enhancing conjugate according to the invention, comprising at least one saponin moiety 'S' complexed with (covalently bound to) a targeting ligand such as an IgG (or an sdAb in some embodiments), wherein the saponin is linked

directly to the antibody, or is bound to the antibody via a (cleavable) linker, the antibody further complexed with (covalently bound to) at least one effector moiety 'E' via (cleavable) bond(s). The saponins are typically linked to the –SH groups of the cysteines in the ligand, here an antibody. The effector moiety/moieties is/are typically linked to the –SH groups of the cysteines in the ligand, here an antibody. Typically, the at least one saponin is selected from SA1641, SO1861, GE1741, QS-21, QS-7, or derivatives thereof, and combinations thereof, and the saponin SO1861 (derivative) is preferred. Typical cell-surface molecule targeting ligands selected for incorporation in the conjugate of the invention are immunoglobulins specific for (tumor) cell-surface receptors such as trastuzumab, cetuximab, anti-CD71 monoclonal antibody, or EGF for binding to EGFR. In Fig. 1F the cell-targeting ligand is an antibody specific for a cell-surface receptor. Typical targeted cell-surface molecules are HER2, EGFR, CD20, CD22, Folate receptor 1, CD146, CD56, CD19, CD138, CD27L, PSMA, CanAg, integrin-alphaV, CA6, CD33, mesothelin, Cripto, CD3, CD30, CD33, CD239, CD70, CD123, CD352, DLL3, CD25, ephrinA4, MUC1, Trop2, CD38, FGFR3, CD123, DLL3, CEACAM5, HER3, CD74, PTK7, Notch3, FGF2, C4.4A, FLT3, CD71. Also the known tumor-targeting antibodies are preferred for manufacturing a conjugate of the invention according to Figure 1F. Typically the effector moiety/moieties is/are selected from a (protein) toxin such as dianthin, saporin, ribosomal inactivating protein, or is/are an oligonucleotide such as an RNA, an siRNA, mRNA, BNA, or an enzyme. The saponin and the payload (effector moiety) are covalently coupled directly to the antibody or are linked to the antibody via a linker such as a cleavable linker, cleavable under acidic conditions, such as at a pH of 4.5 – 5.5.

Examples of endosomal / lysosomal escape enhancing conjugates of Fig. 1F that are manufactured and tested for activity by the current inventors are at least cetuximab, anti-CD71 monoclonal antibody, and trastuzumab coupled to (terminal) SO1861 and coupled to a payload such as HSP27 silencing ASO (BNA), dianthin, the enzyme Cre-recombinase. The term "terminal" in the context of the invention is to be understood as a molecule which is covalently linked to a single further molecule in the conjugates of the inventions. For example in the conjugate saponin – sdAb – effector moiety, it is to be understood that both the saponin and the effector moiety are terminal moieties in the conjugate, whereas the sdAb is the central moiety bearing the two terminal moieties.

Fig. 1G is a cartoon representing the endosomal / lysosomal escape enhancing conjugate according to the invention, comprising at least one saponin moiety 'S' complexed with a targeting ligand such as an IgG via a scaffold moiety such as a Dendron or PAMAM, wherein the saponin is linked directly to the dendron, or via a (cleavable) linker. The dendron moiety/moieties is/are typically linked to the –SH groups of the cysteines in the ligand (the antibody). Typically, the saponins are selected from SA1641, SO1861, GE1741, QS-21, QS-7 and combinations thereof and derivatives thereof, and the saponin SO1861 (derivative) is preferred. Typical cell-surface molecule targeting ligands selected for incorporation in the conjugate of the invention are immunoglobulins specific for (tumor) cell-surface receptors such as trastuzumab, anti-CD71 monoclonal antibody, cetuximab. Also the anti-tumor monoclonal antibodies known in the art are preferred for manufacturing a conjugate of the invention according to Figure 1G. The conjugates comprise the antibody which is further complexed with at least one effector moiety 'E' wherein the effector moiety/moieties is/are linked to the same scaffold such as a dendron to which the at least saponin moiety is coupled, the effector moiety coupled to the dendron via

(cleavable) bond(s) such as via a linker. Typically the antibody binds to any of cell-surface molecules HER2, EGFR, CD20, CD22, Folate receptor 1, CD146, CD56, CD19, CD138, CD27L, PSMA, CanAg, integrin- $\alpha$ V, CA6, CD33, mesothelin, Cripto, CD3, CD30, CD33, CD239, CD70, CD123, CD352, DLL3, CD25, ephrinA4, MUC1, Trop2, CD38, FGFR3, CD123, DLL3, CEACAM5, HER3, CD74, PTK7, Notch3, FGF2, C4.4A, FLT3, CD71.

Examples of endosomal / lysosomal escape enhancing conjugates of Fig. 1G that are manufactured and tested for activity by the current inventors are at least trastuzumab provided with at least a dendron, the at least one dendron bound to (terminal) saponin moiety/moieties and (terminal) payload moiety/moieties (effector moiety/moieties). The saponin is typically SO1861, the payload is typically BNA capable of silencing HSP27 (ASO (BNA)) or ApoB, or a (protein) toxin or an siRNA. The SO1861 (derivative) is coupled to the dendron via a cleavable hydrazone linkage (covalent bond).

### Example 5

SO1861 was conjugated (labile) via cysteine residues (Cys) and dianthin (protein toxin) was conjugated (stable) via lysine residues (Lys) to cetuximab (monoclonal antibody recognizing and binding human EGFR), resulting in the production of: Cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2</sup>. The conjugate was tested in a A431 (EGFR<sup>++</sup>) xenograph mouse tumor model for EGFR tumor targeted cell killing as illustrated in Figure 1J. Dosings started at day 12 when tumors reached ~150 mm<sup>3</sup> in size and tumor volume was determined after every dosing. Mice (n = 3) were treated (intraperitoneal; i.p.; dose escalation) at day 12: 0.5 mg/kg; day15: 1 mg/kg and day24: 1.5 mg/kg with cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2</sup> or cetuximab-(Lys-S-dianthin)<sup>1,6</sup>. At day 26, compared to the control group, tumor volume reduction was observed in the tumor bearing mice treated with cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2</sup> (Figure 8A). This shows that labile conjugation of SO1861 to an antibody-protein toxin (stable) conjugate enhances the targeted therapeutic efficacy of the tumor targeted antibody-protein toxin, thereby inducing a more effective tumor targeted therapy.

Next, SO1861 was conjugated (labile) via cysteine residues (Cys) and dianthin (protein toxin) was conjugated (labile) via lysine residues (Lys) to cetuximab (monoclonal antibody recognizing and binding human EGFR), resulting in the production of: Cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2</sup>. The conjugate was tested in a A431 (EGFR<sup>++</sup>) xenograph mouse tumor model for EGFR tumor targeted cell killing as illustrated in Figure 1J. Dosings started at day 12 when tumors reached ~150 mm<sup>3</sup> in size and tumor volume was determined after every dosing. Mice (n = 3) were treated (intraperitoneal; i.p.; dose escalation) at day 12: 0.5 mg/kg; day15: 1 mg/kg, day 24: 1.5 mg/kg with cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2</sup> or cetuximab-(Lys-L-dianthin)<sup>1,6</sup>. This revealed that after 35 days compared to the control, tumor bearing mice treated with cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2</sup> showed tumor growth inhibition (Figure 8B). When mice (n = 3; were treated (intravenous, i.v.; dose escalation) day 12: 0.5 mg/kg; day15: 1 mg/kg, day18: 2 mg/kg, day24: 2.5 mg/kg with the cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2</sup> according to the invention also tumor growth inhibition was observed compared to the control (data represents 1 mice, since 2 mice died during the treatments). This shows that labile conjugation of SO1861 to an antibody-protein toxin (labile) conjugate enhances the targeted

therapeutic efficacy of the tumor targeted antibody-protein toxin, thereby inducing a more effective tumor targeted therapy compared to the antibody-oligonucleotide conjugate that does not comprise saponin.

Next, SO1861-EMCH was conjugated via cysteine residues (Cys) to cetuximab (monoclonal antibody recognizing and binding human EGFR), with a DAR 3,9 and the antisense HSP27BNA oligo nucleotide (targeting and inducing degradation of the onco-target *hsp27* mRNA (gene silencing) in cancer cells) via a labile (L) linker to the lysine residues (Lys) of the antibody, with a DAR 1,8 resulting in the production of cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-HSP27BNA)<sup>1,8</sup>. Cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-HSP27BNA)<sup>1,8</sup> was tested in a A431 xenograph 'nude' mouse tumor model for EGFR-mediated tumor targeted HSP27 gene silencing, according to the invention as illustrated in Figure 1K. Dosing started at day 12 when tumors reached ~150 mm<sup>3</sup> in size and HSP27 mRNA expression was determined. For this, tumor samples were collected at 72 h after the first dosing and analysed for HSP27 gene expression levels compared to cellular control mRNA expression levels (reference genes). Tumor bearing mice (n = 3) treated (intraperitoneal; i.p.) with 30 mg/kg cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-HSP27BNA)<sup>1,8</sup> showed after 1 dosing 40% reduction in HSP27 mRNA expression in the tumors compared to single dosing of cetuximab-(Cys-L-SO1861)<sup>3,8</sup> or cetuximab-(Lys-L-HSP27BNA)<sup>1,5</sup> (Figure 9). Compared to the tumor of the vehicle control a reduction of 25% HSP27 gene expression was observed. This shows and enables that conjugation of SO1861 and HSP27BNA to the same targeting antibody, according to the invention, efficiently induces SO1861-mediated enhanced cytoplasmic delivery of a therapeutic antisense oligonucleotide in solid tumors of tumor bearing mice, inducing tumor targeted gene silencing.

In another example, a trifunctional linker scaffold was designed and produced with three specific chemical end groups for conjugation with SO1861 on one arm and the HSP27BNA on the other arm to produce SO1861-L-trifunctional linker-L-HSP27BNA. Next, SO1861-L-trifunctional linker-L-HSP27BNA was conjugated with its third arm to cysteine residues (Cys) of the anti-EGFR antibody, cetuximab (cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup>) and tested in a A431 xenograph 'nude' mouse tumor model for EGFR-mediated tumor targeted gene silencing activity, according to the invention as illustrated in Figure 1L. Dosings started at day 12 when tumors reached ~150 mm<sup>3</sup> in size and HSP27 mRNA expression was determined. For this, tumor samples were collected at 72 h after the first dosing and analysed for HSP27 gene expression levels compared to cellular control mRNA expression levels (reference genes). This revealed that 1 dosing of 30 mg/kg cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> resulted in a 40% reduction in HSP27 gene expression in the tumors compared to single dosing of 25 mg/kg cetuximab-(Cys-L-SO1861)<sup>3,8</sup> or 25 mg/kg cetuximab-(Lys-L-HSP27BNA)<sup>4</sup> mono therapies (Figure 10). Compared to the vehicle control tumors, a reduction of 25% HSP27 gene expression was observed in tumor bearing mice treated with 1 dosing of cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup>. This shows and enables that cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> efficiently induces SO1861-mediated enhanced cytoplasmic delivery of a therapeutic antisense oligonucleotide in a solid tumor of tumor bearing mice, inducing targeted gene silencing, *in vivo*.

#### Example 6

In another example according to the invention, SO1861 (labile) and the protein toxin, dianthin (labile or stable) were conjugated to the HER2 targeting antibody, trastuzumab. Trastuzumab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-dianthin)<sup>1,7</sup> or trastuzumab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2,0</sup>, were produced and tested for enhanced cell killing in SK-BR-3 (HER2<sup>++</sup>) and MDA-MB-468 (HER2<sup>-</sup>) cells as illustrated in Figure 1J. Both, trastuzumab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-dianthin)<sup>1,7</sup> (IC<sub>50</sub> = 0,8 nM) and trastuzumab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2,0</sup> (IC<sub>50</sub> = 0,8 nM) efficiently induces cell killing of SK-BR-3 cells (HER2<sup>++</sup>) (Figure 11A). This was not observed in SK-BR-3 cells treated with trastuzumab, trastuzumab-(Lys-L-dianthin)<sup>1,7</sup>, trastuzumab-(Lys-S-dianthin)<sup>1,7</sup> or trastuzumab-(L-SO1861)<sup>3,8</sup> alone (Figure 11A). In MDA-MB-468 cells (HER2<sup>-</sup>) no cell killing activity can be observed for any of the conjugates, according to the invention (Figure 11B). This shows that conjugation of SO1861 to an HER targeting antibody-protein toxin conjugate, efficiently induces SO1861-mediated enhanced cytoplasmic delivery of the protein toxin in the target cell resulting in target cell death.

In another example according to the invention, SO1861 (labile) and the protein toxin, dianthin (labile or stable) were conjugated to the EGFR targeting antibody, cetuximab. Cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2</sup> or cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-S-dianthin)<sup>2</sup>, was tested for enhanced cell killing in A431 cells (EGFR<sup>++</sup>) and A2058 cells (EGFR<sup>-</sup>) as illustrated in Figure 1J. Both, cetuximab-(Cys-L-SO1861)<sup>3,9</sup>(Lys-L-dianthin)<sup>2</sup> (IC<sub>50</sub> = 0,3 nM) and cetuximab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-S-dianthin)<sup>1,7</sup> (IC<sub>50</sub> = 0,3 nM) showed enhanced cell killing in A431 cells (EGFR<sup>++</sup>) compared to cetuximab-(Lys-L-dianthin)<sup>1,6</sup> (IC<sub>50</sub> = 2 pM), cetuximab-(Lys-S-dianthin)<sup>1,6</sup> (IC<sub>50</sub> = 2 pM) alone (Figure 11C). In A2058 cells (EGFR<sup>-</sup>) the combination according to the invention did not show any cell killing activity (IC<sub>50</sub> > 200 nM; Figure 11D). This shows that conjugation of SO1861 to an EGFR targeting antibody-protein toxin conjugate, efficiently enhances SO1861-mediated cytoplasmic delivery of the protein toxin in the target cell resulting in enhanced target cell death.

#### Example 7

In another example according to the invention, SO1861 (labile) and the HSP27BNA oligonucleotide (labile, L) were conjugated to the EGFR targeting antibody, cetuximab. Cetuximab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-HSP27BNA)<sup>3,8</sup> was tested for enhanced HSP27 gene silencing in A431 cells (EGFR<sup>++</sup>) and A2058 (EGFR<sup>-</sup>) cells, according to the invention as illustrated in Figure 1K. Cetuximab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-HSP27BNA)<sup>3,8</sup> efficiently induces HSP27 gene silencing in A431 cells (IC<sub>50</sub> = 3 nM) compared to cetuximab, cetuximab-(Lys-L-HSP27BNA)<sup>3,9</sup> or cetuximab-(Cys-L-SO1861)<sup>3,8</sup> alone (Figure 12A). In A2058 cells (EGFR<sup>-</sup>) no gene silencing activity can be observed with cetuximab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-HSP27BNA)<sup>3,8</sup> (IC<sub>50</sub> > 100 nM; Figure 12B). This shows and enables that conjugation of SO1861 and HSP27BNA to the same targeting antibody, according to the invention, efficiently induces SO1861-mediated enhanced cytoplasmic delivery of a therapeutic antisense oligo nucleotide in the target cells, inducing targeted gene silencing.

In another example according to the invention, SO1861 (labile) and the HSP27BNA oligo (labile) were conjugated to the HER2 targeting antibody, trastuzumab. Trastuzumab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-HSP27BNA)<sup>3,5</sup> was tested for enhanced HSP27 gene silencing in SK-BR-3 cells (HER2<sup>++</sup>) cells, according to the invention as illustrated in Figure 1K. Trastuzumab-(Cys-L-SO1861)<sup>3,8</sup>(Lys-L-

HSP27BNA)<sup>3,5</sup> efficiently induces HSP27 gene silencing in SK-BR-3 cells (IC<sub>50</sub> = 9 nM) compared to trastuzumab-(Lys-L-HSP27BNA)<sup>4,4</sup> alone (Figure 13). This shows and enables that conjugation of SO1861 and HSP27BNA to an HER2 targeting antibody, according to the invention, efficiently induces SO1861-mediated enhanced cytoplasmic delivery of a therapeutic antisense oligo nucleotide in the target cells, inducing targeted gene silencing.

In another example, cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> was tested for enhanced HSP27 gene silencing in A431 (EGFR<sup>++</sup>) and A2058 (EGFR<sup>-</sup>) cells according to the invention as illustrated in Figure 1L. Cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> efficiently induces HSP27 gene silencing in A431 cells (IC<sub>50</sub> = 2 nM) compared to Cetuximab-(Lys-L-HSP27BNA)<sup>4</sup> or Cetuximab-(Cys-L-SO1861)<sup>3,7</sup> alone (Figure 14A). In A2058 cells (EGFR<sup>-</sup>) gene silencing activity was only observed at high (> 80 nM) concentrations of Cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> (IC<sub>50</sub> = 100 nM; Figure 14B). This shows and enables that in high EGFR expressing cells cetuximab-Cys-(SO1861-L-trifunctional linker-L-HSP27BNA)<sup>3,7</sup> efficiently induces SO1861-mediated enhanced cytoplasmic delivery of a therapeutic antisense oligo nucleotide in the target cells, inducing targeted gene silencing.

**Summary of a number of embodiments relating to antibodies and receptor ligands covalently bound to a protein toxin or to a saponin**

**mAb:** trastuzumab (HER2) or cetuximab (EGFR)

**Ligand:** EGF

**Protein toxin:** Ribosome inactivating protein, saporin or dianthin

**endosomal escape enhancing conjugates of saponin with a ligand:**

**mAb-SO1861 endosomal escape enhancing conjugates**

- contain a cleavable hydrazone linker
- Trastuzumab-SO1861                      DAR 4.0
- Cetuximab -SO1861                      DAR 3.7;

**the endosomal escape enhancing conjugates of saponin with a ligand combined in *in vitro* or *in vivo* test models, with:**

**mAb/ligand-protein toxin conjugates**

- contain a non-cleavable chemical linker or are recombinant fusion proteins
- Trastuzumab-Saporin                      DAR 3.0
- Cetuximab-Saporin                      DAR 2.6
- Trastuzumab-Dianthin                      DAR 1.0
- EGF-Dianthin (fusion protein)              DAR 1.0
- IgG-Saporin                      DAR 2.2

For examples 8-13:

*Materials:*

Trastuzumab and cetuximab were purchased from the pharmacy (Charite, Berlin). SO1861 was isolated and purified by Analyticon Discovery GmbH from raw plant extract obtained from *Saponaria officinalis* L.

*Methods***SO1861-EMCH synthesis**

SO1861 was from Saponaria officinalis L (Analyticon Discovery GmbH), and was coupled to EMCH according to conventional steps known in the art.

**Conjugation of SO1861 to antibodies**

Custom production of trastuzumab-SO1861 and cetuximab-SO1861 was performed by FleetBioprocessing (UK). SO1861-EMCH was conjugated to cysteines of the antibody.

**Conjugation of Saporin to Trastuzumab and cetuximab**

Custom trastuzumab-saporin and cetuximab-saporin conjugates were produced and purchased from Advanced Targeting Systems (San Diego, CA). IgG-saporin and saporin was purchased from Advanced Targeting Systems

**FACS analyses**

FACS analysis was performed on a BD FACSCanto II, data analysis with FlowJo V10 software, FACS antibodies were: 1) Isotype: APC Mouse IgG1,  $\kappa$  Isotype Ctrl (FC) (400122, Biolegend). EGFR: APC anti-human EGFR (352906, Biolegend) HER2: APC anti-human CD340 (erbB2/HER-2) (324408, Biolegend).

**Dianthin production**

Dianthin was expressed in a bacterium culture and the protein was purified following conventional cell culturing and protein purification steps known in the art.

**Conjugation of antibody to dianthin**

Conjugation of antibody and dianthin was according to common procedures known in the art.

**Cell culture**

Cells were cultured in DMEM (PAN-Biotech GmbH) supplemented with 10% fetal bovine serum (FBS) (PAN-Biotech GmbH) at 37°C and 5% CO<sub>2</sub>.

**Cell viability assay**

Cells were seeded in a 96 well plate at 5.000-10.000 c/w in 100  $\mu$ L/well and incubated overnight at 37°C. The next day 10x concentrated treatment-mix samples were prepared in PBS, which contain antibody-conjugated SO1861 (*i.e.* a 'binding molecule' or an 'endosomal escape enhancing conjugate' of the invention) and targeted-toxin (*i.e.* a 'binding molecule') both at 10x final concentration. The media was removed from the cell culture plate and replaced by 180  $\mu$ L culture media, followed by the addition of 20  $\mu$ L treatment-mix/well. For control, 10x treatment-mix samples were prepared that contained the corresponding concentrations of only antibody-conjugated SO1861, only antibody, only SO1861, only targeted-toxin, or PBS without compound as vehicle control. In the case that endosomal acidification inhibitors (chloroquine (Sigma Aldrich) or bafilomycin A1 (Enzo Life Sciences)) were used, the cell culture media in step 1 of treatment was replaced by 180  $\mu$ L media containing 1  $\mu$ M chloroquine or

0.2  $\mu$ M bafilomycin A1. The plate was incubated for 1 hour at 37°C, before the 10x treatment-mix samples were added. The remaining incubation and treatment steps were performed according to standard procedures known in the field.

After treatment the cells were incubated for 72 hr at 37°C before the cell viability was determined by a MTS-assay, performed according to the manufacturer's instruction (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). Briefly, the MTS solution was diluted 20x in DMEM without phenol red (PAN-Biotech GmbH) supplemented with 10% FBS (PAN-Biotech GmbH). The cells were washed once with 200  $\mu$ L PBS per well, after which 100  $\mu$ L diluted MTS solution was added per well. The plate was incubated for approximately 20-30 minutes at 37°C. Subsequently, the optical density at 492 nm was measured on a Thermo Scientific Multiskan FC plate reader (Thermo Scientific). For quantification the background signal of 'medium only' wells was subtracted from all other wells, before the ratio of untreated/treated cells was calculated, by dividing the background corrected signal of untreated wells over the background corrected signal of the treated wells.

## Results

### Example 8. 1 target 2-component system

1 target 2-components system is the combination treatment of mAb1-protein toxin and mAb1-SO1861 (see Figure 1A, E), whereas the 2 target 2-component system is the combination of mAb1-protein toxin and mAb2-SO1861 or mAb2-protein toxin + mAb1-SO1861 (Figure 1B-D). The 1-target 1 component system of the invention (the same ligand such as an antibody such as an IgG or an sdAb such as a V<sub>HH</sub> is applied for covalently conjugating saponin to a first portion of said ligand and for covalently conjugating an effector molecule to a second separate portion of said ligand) comprises for example mAb1-SO1861 and mAb1-effector moiety (See Figure 1F-L).

Cetuximab-SO1861 (monoclonal antibody recognizing and binding EGFR, conjugated to the saponin molecule, SO1861; an endosomal escape enhancing conjugate) was titrated (calculated on concentration SO1861) on a fixed concentration of 10 pM cetuximab-saporin (monoclonal antibody recognizing and binding EGFR, conjugated to the protein toxin, saporin) and cell killing on high EGFR expressing cells was determined. High EGFR expressing cells (A431 or CaSKi) showed efficient cell killing when 10 pM cetuximab-saporin was combined with high concentrations of non-targeted unconjugated SO1861 (A431: [SO1861] IC<sub>50</sub> = 600 nM and Caski: [SO1861] IC<sub>50</sub> = 700 nM; Figure 15A, 15B; Table A6). However, when cetuximab-saporin was combined with cetuximab-SO1861 strong cell killing was induced already at low concentrations of SO1861 (A431: [SO1861] IC<sub>50</sub> = 5 nM and Caski [SO1861] IC<sub>50</sub> = 8 nM; Figure 15A, 15B ; Table A6). This shows that targeted conjugated SO1861 is more effective in inducing endosomal escape compared to non-targeted unconjugated SO1861. Next, cetuximab-saporin was titrated on a fixed concentration of 300 nM cetuximab-SO1861 and targeted protein toxin mediated cell killing on EGFR expressing cells was determined. High EGFR expressing cells (A431 or CaSKi) show cell killing only at high cetuximab-saporin concentrations in combination with non-targeted unconjugated 300nM SO1861 (A431: [toxin] IC<sub>50</sub> = 40 pM; CaSKi: [toxin] IC<sub>50</sub> = 40 pM; Figure 15C, 15D; Table A7) whereas 300nM cetuximab-SO1861 in combination with low cetuximab-

saporin concentrations already induced efficient cell killing (A431: [toxin] IC<sub>50</sub> = 0.4 pM; CaSki: [toxin] IC<sub>50</sub> = 2 pM; Figure 15C and 15D; Table A7). Highest cell killing efficiency is achieved when high concentrations of non-targeted unconjugated SO1861 (1500 nM) is combined with low concentrations of cetuximab-saporin (A431: [toxin] IC<sub>50</sub> = 0,03 pM; CaSki: [toxin] IC<sub>50</sub> = 0,02 pM; Figure 15C, 15D; Table A7). All this shows that when conjugated to cetuximab, relatively low concentrations of SO1861 efficiently can kill high EGFR expressing cells in combination with relatively low concentrations of cetuximab-saporin. High concentrations (1500 nM) of non-targeted unconjugated SO1861 in combination with low concentrations of cetuximab-saporin is still most effective since receptor competition does not play a role for SO1861 to enter the cell, since in the 1-target 2-component system both conjugates compete for the same EGFR receptor. The receptor competition principle is also clearly shown in the cetuximab-toxin titration treatments without (A431: [toxin] IC<sub>50</sub> = 40 pM; Caski: [toxin] IC<sub>50</sub> = 40 pM) or with 75nM cetuximab (A431: [toxin] IC<sub>50</sub> = 1000 pM; Caski: from IC<sub>50</sub> = 1000 pM; Figure 15C, 15D).

Next, cetuximab-SO1861 was titrated (calculated on concentration SO1861) on a fixed concentration of 10 pM cetuximab-saporin and cell killing on low/no EGFR expressing cells was determined. Low EGFR expressing cells (HeLa) showed only cell killing when 10 pM cetuximab-saporin was combined with high concentrations of non-targeted unconjugated SO1861, whereas A2058 cells that do not express EGFR (A2058) were not sensitive at all (HeLa: [SO1861] IC<sub>50</sub> = 1000 nM; A2058: [SO1861] IC<sub>50</sub> > 1000 nM; Figure 16A, 16B; Table A6). The combination of 10 pM cetuximab-saporin with increasing concentrations of cetuximab-SO1861 did not induce any significant cell killing in both cell lines (HeLa: [SO1861] IC<sub>50</sub> = 1000 nM; A2058: [SO1861] IC<sub>50</sub> > 1000 nM; Figure 16A, 16B; Table A6). This shows that in the absence of sufficient receptor expression, effective intracellular SO1861 concentrations are not reached (threshold) to induce endosomal protein toxin escape and toxin-mediated cell killing. Next, cetuximab-saporin was titrated on a fixed concentration of 300nM cetuximab-SO1861 and targeted protein toxin mediated cell killing on low/no EGFR expressing cells was determined. Low EGFR expressing cells (HeLa) show cell killing only at very high cetuximab-saporin concentrations in combination with 278 nM cetuximab-SO1861 or 300 nM unconjugated SO1861, whereas A2058 cells (EGFR) are not sensitive at any of the tested concentrations (HeLa: [toxin] IC<sub>50</sub> = 60 pM; A2058: [toxin] IC<sub>50</sub> > 10.000 pM; Figure 16C, 16D; Table A7). High concentrations of unconjugated SO1861 (1500 nM) in combination with low concentrations of cetuximab-saporin in low EGFR expressing cells (HeLa) show efficient cell killing, whereas in A2058 cells only at very high cetuximab-saporin concentrations in combination with 1500nM SO1861 non-targeted, a-specific cell killing is induced (Hela: [toxin] IC<sub>50</sub> = 0,03 pM; A2058: [toxin] IC<sub>50</sub> = 20 pM; Figure 16C, 16D; Table A5). All this shows that cells with low or no EGFR receptor expression are not susceptible for the combination of cetuximab-SO1861 + cetuximab-saporin, due to a lack of sufficient EGFR receptor that facilitates the entry of sufficient SO1861 and toxin within the cell.

Trastuzumab-SO1861 (monoclonal antibody recognizing and binding HER2, conjugated to the saporin molecule, SO1861; an endosomal escape enhancing conjugate according to the invention) was titrated (calculated on concentration SO1861) on a fixed concentration of 50 pM trastuzumab-saporin (monoclonal antibody recognizing and binding HER2, conjugated to the protein toxin, saporin) and cell

killing on high HER2 expressing cells was determined. High HER2 expressing cells (SKBR3) showed efficient cell killing when 50 pM trastuzumab-saporin was combined with high concentrations of non-targeted unconjugated SO1861 (SKBR3; Figure 17A, 17B; Table A6). However, when trastuzumab-saporin was combined with trastuzumab-SO1861 strong cell killing was induced already at low concentrations of SO1861 (SKBR3; Figure 17A, 17B; Table A6). This shows that targeted conjugated SO1861 is more effective in inducing endosomal escape compared to non-targeted unconjugated SO1861. Next, trastuzumab-saporin was titrated on a fixed concentration of 50 nM trastuzumab-SO1861 and targeted protein toxin mediated cell killing on HER2 expressing cells was determined. High HER2 expressing cells (SKBR3 or BT474) show cell killing only at high trastuzumab-saporin concentrations in combination with non-targeted unconjugated 10 nM SO1861 (Table A7) whereas 10 nM trastuzumab-SO1861 in combination with low trastuzumab-saporin concentrations already induced efficient cell killing (Table A7). Highest cell killing efficiency is achieved when high concentrations of non-targeted unconjugated SO1861 (1500 nM) is combined with low concentrations of trastuzumab-saporin (Table A7). All this shows that when conjugated to trastuzumab, low concentrations of SO1861 efficiently can kill high HER2 expressing cells in combination with relatively low concentrations of trastuzumab-saporin. High concentrations (1500 nM) of non-targeted unconjugated SO1861 in combination with low concentrations of trastuzumab-saporin is still most effective, since receptor competition does not play a role for SO1861 to enter the cell, since in the 1-target 2-component system both conjugates compete for the same EGFR receptor. The receptor competition principle is also clearly shown in the trastuzumab-toxin titration treatments without or with 2,5 nM trastuzumab (SKBR3: [toxin] IC<sub>50</sub> = 1000 nM).

Next, trastuzumab-SO1861 was titrated (calculated on concentration SO1861) on a fixed concentration of 50 pM trastuzumab-saporin and cell killing on low/no EGFR expressing cells was determined. Low EGFR expressing cells (JIMT1; A431) showed only cell killing when 50 pM trastuzumab-saporin was combined with high concentrations of non-targeted unconjugated SO1861 (JIMT1: [SO1861] IC<sub>50</sub> >1000 nM; A431: [SO1861] IC<sub>50</sub> >1000 nM; Figure 18A, 18B; Table A6). The combination of 50 pM trastuzumab-saporin with increasing concentrations of trastuzumab-SO1861 did not induce any significant cell killing in both cell lines (JIMT1: [SO1861] IC<sub>50</sub> >1000 nM; A431: [SO1861] IC<sub>50</sub> >1000 nM; Figure 18A, 18B; Table A6). This shows that in the absence of sufficient receptor expression, effective intracellular SO1861 concentrations are not reached (threshold) to induce endosomal protein toxin escape and toxin-mediated cell killing. Next, trastuzumab-saporin was titrated on a fixed concentration of 10 nM trastuzumab-SO1861 and targeted protein toxin mediated cell killing on low/no HER2 expressing cells was determined. Low HER2 expressing cells (JIMT1; A431) show no significant cell killing at high trastuzumab-saporin concentrations in combination with 10 nM trastuzumab-SO1861 (JIMT-1: [toxin] IC<sub>50</sub> > 10.000 pM; A431: [toxin] IC<sub>50</sub> > 10.000 pM; Figure 18C, 18D; Table A7). High concentrations of unconjugated SO1861 (1500 nM) in combination with low concentrations of trastuzumab-saporin in low HER2 expressing cells (show efficient cell killing (JIMT1: [toxin] IC<sub>50</sub> = 0,1 pM; A431: [toxin] IC<sub>50</sub> = 0,8 pM; Figure 18C, 18D; Table A5). All this shows that cells with low HER2 receptor expression are not susceptible for the combination of trastuzumab-SO1861 +

trastuzumab-saporin, due to a lack of sufficient HER2 receptor that facilitates the entry of sufficient SO1861 and toxin within the cell.

**Example 9. 2 target 2-component system**

5 Cetuximab-SO1861 was titrated (calculated on concentration SO1861) on a fixed concentration of 50 pM trastuzumab-saporin and cell killing on high EGFR/low HER2 expressing cells was determined. A431 and CaSki cells showed efficient cell killing when 50 pM trastuzumab-saporin was combined with high concentrations of non-targeted unconjugated SO1861 (A431 and Caski: [SO1861] IC<sub>50</sub> = 1000 nM; Figure 19A, 19B; Table A6). However, when trastuzumab-saporin was combined with cetuximab-SO1861 strong cell killing was induced already at low concentrations of SO1861 (A431: [SO1861] IC<sub>50</sub> = 12 nM and Caski [SO1861] IC<sub>50</sub> = 40 nM; Figure 19A, 19B; Table A6). This shows that targeted conjugated SO1861 is more effective in inducing endosomal escape compared to non-targeted unconjugated SO1861. Next, trastuzumab-saporin was titrated on a fixed concentration of 300 nM cetuximab-SO1861 and targeted protein toxin mediated cell killing was determined on high EGFR/low HER2 expressing cells (A431 and CaSki) No effective cell killing was observed with high trastuzumab-saporin concentrations in combination with non-targeted unconjugated 300 nM SO1861 (A431 and Caski: [toxin] IC<sub>50</sub> > 10.000 pM; Figure 19C, 19D; Table A7) whereas 300 nM cetuximab-SO1861 in combination with low trastuzumab-saporin concentrations already induced efficient cell killing (A431: [toxin] IC<sub>50</sub> = 3 pM; Caski: [toxin] IC<sub>50</sub> = 1 pM; Figure 19C and 19D; Table A7). In A431 cells, comparable cell killing efficiency is achieved when high concentrations (1500 nM) of non-targeted unconjugated SO1861 is combined with low concentrations of trastuzumab-saporin (A431: [toxin] IC<sub>50</sub> = 1 pM; see Figure 19C; Table A7). In Caski cells the response was slightly stronger compared to the combination of cetuximab-SO1861 and Trastuzumab-saporin, due to the fact that the EGFR expression in these cells is significantly lower compared to A431 and thus targeted delivery of SO1861 to Caski cells is less sufficient (Caski: [toxin] IC<sub>50</sub> = 0,2 pM see Figure 19D; Table A7). All this shows that when conjugated to cetuximab, low concentrations of SO1861 efficiently can kill high EGFR expressing cells in combination with relatively low concentrations of trastuzumab-saporin. High concentrations (1500 nM) of non-targeted unconjugated SO1861 in combination with low concentrations of trastuzumab-saporin has comparable activity, since receptor competition does not play a role for SO1861 to enter the cell, since in the 2-target 2-component system both conjugates are delivered via different receptors, SO1861 via EGFR and toxin via HER2 receptor.

Next, cetuximab-SO1861 was titrated (calculated on concentration SO1861) on a fixed concentration of 50 pM trastuzumab-saporin and cell killing on low/no EGFR/HER2 expressing cells was determined. Low EGFR/HER2 expressing cells showed only cell killing when 50 pM trastuzumab-saporin was combined with high concentrations of non-targeted unconjugated SO1861 (HeLa: [SO1861] IC<sub>50</sub> > 1000 nM; A2058: [SO1861] IC<sub>50</sub> > 1000 nM; Figure 20A, 20B; Table A6). The combination of 50 pM trastuzumab-saporin with increasing concentrations of cetuximab-SO1861 only showed significant cell killing at high concentrations of cetuximab-SO1861 in both cell lines (HeLa: [SO1861] IC<sub>50</sub> > 1000 nM; A2058: [SO1861] IC<sub>50</sub> > 1000 nM; Figure 20A, 20B; Table A6). This shows that in the absence of sufficient receptor expression, effective intracellular SO1861 concentrations are not reached

(threshold) to induce endosomal protein toxin escape and toxin-mediated cell killing. Next, trastuzumab-saporin was titrated on a fixed concentration of cetuximab-SO1861 and targeted protein toxin mediated cell killing on low/no EGFR/HER2 expressing cells was determined. Low/no EGFR/HER2 expressing cells (HeLa and A2058) show no significant cell killing at high trastuzumab-saporin concentrations in combination with 278 nM cetuximab-SO1861 (HeLa: [toxin] IC<sub>50</sub> > 10.000 pM; A2058: [toxin] IC<sub>50</sub> > 10.000 pM; Figure 20C, 20D; Table A7). High concentrations of unconjugated SO1861 (1500 nM) in combination with low concentrations of trastuzumab-saporin show efficient cell killing (HeLa: [toxin] IC<sub>50</sub> = 0,4 pM; A2058: [toxin] IC<sub>50</sub> = 0,5 pM; Figure 20C, 20D; Table A5). All this shows that cells with low/no EGFR/low HER2 expression are not susceptible for the combination of cetuximab-SO1861 + trastuzumab-saporin, due to a lack of sufficient EGFR receptor that facilitates the entry of sufficient SO1861 to ensure efficient cytoplasmic delivery of the toxin within the cell.

Next, Trastuzumab-SO1861 was titrated (calculated on concentration SO1861) on a fixed concentration of 1.5 pM EGF-dianthin and cell killing on high HER2/low EGFR expressing cells was determined. SKBR3 showed efficient cell killing when 1.5 pM EGF-dianthin was combined with high concentrations of non-targeted unconjugated SO1861 (SKBR3: [SO1861] IC<sub>50</sub> = 800 nM; Figure 21A; Table A6). However, when EGF-dianthin was combined with trastuzumab-SO1861 strong cell killing was induced already at low concentrations of conjugated SO1861 (SKBR3: [SO1861] IC<sub>50</sub> = 2 nM; Figure 21A; Table A6). This shows that targeted conjugated SO1861 is more effective in inducing endosomal escape compared to non-targeted unconjugated SO1861. Next, EGF-dianthin was titrated on a fixed concentration of trastuzumab-SO1861 and targeted protein toxin mediated cell killing was determined on SKBR3. No effective cell killing was observed with high EGF-dianthin concentrations in combination with non-targeted unconjugated 10 nM SO1861 (SKBR3 (shown) and BT474 (not shown): [toxin] IC<sub>50</sub> > 10.000 pM; Figure 21B; Table A7) whereas 9.4 nM trastuzumab-SO1861 in combination with low EGF-dianthin concentrations already induced efficient cell killing (SKBR3: [toxin] IC<sub>50</sub> = 3 pM (shown); BT474: [toxin] IC<sub>50</sub> = 1 pM (not shown); Figure 21B; Table A7). Comparable cell killing efficiency is achieved when high concentrations (1075 nM) of non-targeted unconjugated SO1861 is combined with low concentrations of EGF-dianthin; Figure 21B; Table A7). All this shows that when conjugated to trastuzumab, low concentrations of SO1861 efficiently can kill high HER2 expressing cells in combination with relatively low concentrations of EGF-dianthin. High concentrations (1500 nM) of non-targeted unconjugated SO1861 in combination with low concentrations of EGF-dianthin has comparable activity, since receptor competition does not play a role for SO1861 to enter the cell, since in the 2-target 2-component system both conjugates are delivered via different receptors, SO1861 via HER2 and toxin via EGFR receptor.

Next, Trastuzumab-SO1861 was titrated (calculated on concentration SO1861) on a fixed concentration of 5 pM cetuximab-saporin and cell killing on low HER2, low/high EGFR expressing cells was determined showed cell killing when 5 pM cetuximab-saporin was combined with high concentrations of non-targeted unconjugated SO1861 (JIMT-1: [SO1861] IC<sub>50</sub> > 1000 nM; A431: [SO1861] IC<sub>50</sub> > 1000 nM; Figure 22A, 22B; Table A6). The combination of 5 pM cetuximab-saporin with increasing concentrations of trastuzumab-SO1861 showed cell killing only at high concentrations of cetuximab-SO1861 in both cell lines (JIMT-1: [SO1861] IC<sub>50</sub> > 1000 nM; A431: [SO1861] IC<sub>50</sub> >

1000 nM; Figure 22A, 22B; Table A6). This shows that in the absence of sufficient receptor expression, effective intracellular SO1861 concentrations are not reached (threshold) to induce endosomal protein toxin escape and toxin-mediated cell killing. Next, cetuximab-saporin was titrated on a fixed concentration of trastuzumab-SO1861 and targeted protein toxin mediated cell killing on JIMT-1 and A431 was determined. Cell killing was observed only at high cetuximab-saporin concentrations in combination with 10 nM trastuzumab-SO1861 (JIMT-1: [toxin] IC<sub>50</sub> > 90 pM; A431: [toxin] IC<sub>50</sub> > 20 pM; Figure 22C, 22D; Table A7). High concentrations of unconjugated SO1861 (1500 nM) in combination with low concentrations of cetuximab-saporin show efficient cell killing (JIMT-1: [toxin] IC<sub>50</sub> = 0,02 pM; A431: [toxin] IC<sub>50</sub> = 0,03 pM; Figure 22C, 22D; Table A5). All this shows that cells with low HER2, low/high EGFR expression are not susceptible for the combination of trastuzumab-SO1861 + cetuximab-saporin, due to a lack of sufficient HER2 receptor that facilitates the entry of sufficient SO1861 to ensure efficient cytoplasmic delivery of the toxin within the cell. Even a very high EGFR expression in A431 cells did not result in efficient cell killing by cetuximab-saporin, since the threshold of SO1861 was not reached due to a lack of HER2 receptors that could facilitate the uptake of SO1861 via trastuzumab-SO1861.

#### Example 10.

The 2 targeted 2 component system results in cell killing of very low target expressing cells. In A431 cells T-DM1 kills cells at nanomolar concentrations, whereas the targeted 2 component system can efficiently kill cells at picomolar concentrations (~7000 fold decrease in toxin concentration) (Figure 23)

#### Example 11. Mechanism of action

When endosomal acidification is blocked the targeted 2 component system is not active, due to the fact that SPT001 (a plant-derived saponin, SO1861) is only active at low endosomal pH.

#### Example 12

Endosomal acidification inhibitors block the targeted 2-component system activity showing that SO1861 function is reduced when acidification of endosomes is blocked.

#### Example 13

Figure 24A-E displays the relative cell viability when trastuzumab (Fig. 24A), cetuximab (Fig. 24B) or T-DM1 (Fig. 24C), free toxins saporin (Fig. 24D) and dianthin (Fig. 24D), saporin coupled to a non-cell binding IgG (Fig. 24D), and saporin coupled to a non-cell binding IgG combined with free saponin SO1861 (Fig. 24E) are contacted with the indicated cell lines SK-BR-3, JIMT-1, MDA-MB-468, A431, CaSki, HeLa, A2058, BT-474.

Trastuzumab and cetuximab do not or hardly influence cell viability when exposed to most of the cell lines, with some effect on cell viability when trastuzumab is exposed to SK-BR-3 cells at relatively high dose, and with some effect on cell viability when cetuximab is exposed to MDA-MB-468 cells at relatively high dose.

TDM-1, or ado-trastuzumab emtansine, is a targeted therapy approved by the U.S. Food and Drug Administration to treat: HER2-positive metastatic breast cancer that has previously been treated with Herceptin (chemical name: trastuzumab) and taxane chemotherapy; early-stage HER2-positive breast cancer after surgery if residual disease was found after neoadjuvant (before surgery) treatment with Herceptin and taxane chemotherapy. The TDM-1 is a combination of Herceptin and the chemotherapy medicine emtansine. Fig. 24C shows that the TDM-1 results in decreased cell viability for all cell lines tested.

The free toxins saporin and dianthin and the toxin saporin coupled to a control IgG with no affinity for any of the cell surface molecules on the cell lines tested, do not or hardly have any influence on cell viability over a wide range of concentrations toxin tested, up to 10000 pM.

When the toxin saporin is coupled to a non-cell binding IgG, combining the conjugate with the free saponin SO1861 results in an IgG-saporin dose dependent decrease of the relative cell viability (Fig. 24E).

**Table A5.** Summary of IC50 values for mAb, toxin, ligand toxin or mAb-toxin monotherapy +/- SO1861.

| Cell line  | EGFR expressi on level (MFI) | HER2 expressi on level (MFI) | Trastuzu mab (nM) | Cetuxim ab (nM) | T-DM1 (nM) | Saporin (pM) |                |           |                | Trastuzumab-Saporin (pM) |                | Trastuzumab-Dianthin (pM) |                | Cetuximab-Saporin (pM) |                | EGF-Dianthin (pM) |                |
|------------|------------------------------|------------------------------|-------------------|-----------------|------------|--------------|----------------|-----------|----------------|--------------------------|----------------|---------------------------|----------------|------------------------|----------------|-------------------|----------------|
|            |                              |                              |                   |                 |            | No SO1861    | 1500 nM SO1861 | No SO1861 | 1500 nM SO1861 | No SO1861                | 1500 nM SO1861 | No SO1861                 | 1500 nM SO1861 | No SO1861              | 1500 nM SO1861 | No SO1861         | 1500 nM SO1861 |
| MDA-MB-468 | 1656                         | 1                            | > 3.000           | > 3.000 *       | 60         | > 10.000     | 20             | > 10.000  | 30             | > 10.000                 | 25             | > 10.000                  | 200            | 180                    | 0,06           | 240               | 0,09           |
| A431       | 1593                         | 10                           | > 3.000           | > 3.000         | 80         | > 10.000     | n.d.           | > 10.000  | 90             | > 10.000                 | 0,8            | > 10.000                  | 60             | 40                     | 0,03           | 700               | 0,03           |
| CaSki      | 481                          | 12                           | > 3.000           | > 3.000         | 40         | > 10.000     | 15             | > 10.000  | 60             | > 10.000                 | 0,2            | > 10.000                  | 5              | 40                     | 0,02           | 2000              | 0,03           |
| SK-BR-3    | 28                           | 1162                         | > 3.000 **        | > 3.000         | 1          | > 10.000     | n.d.           | > 10.000  | 100            | 4500                     | 0,05           | > 10.000                  | 1              | n.d.                   | n.d.           | > 10.000          | 0,5            |
| JIMT-1     | 58                           | 74                           | > 3.000           | > 3.000         | 10         | > 10.000     | 30             | > 10.000  | 90             | > 10.000                 | 0,1            | > 10.000                  | 40             | 300                    | 0,02           | > 10.000          | 0,07           |
| HeLa       | 91                           | 7                            | > 3.000           | > 3.000         | 40         | > 10.000     | 30             | > 10.000  | 200            | > 10.000                 | 0,4            | > 10.000                  | 18             | 60                     | 0,03           | 3800              | 0,04           |
| A2058      | 1                            | 5                            | > 3.000           | > 3.000         | 30         | > 10.000     | 25             | > 10.000  | 200            | > 10.000                 | 0,5            | > 10.000                  | 5              | > 10.000               | 20             | > 10.000          | 30             |



**Table A7.** Data summary of IC50 values for the targeted 2-component system, mAb-toxin titration with fixed [mAb-SO1861]. IC50 for each value is calculated as a percentage relative to the component(s) with constant concentration for that treatment (100%)

| 1-target 2-component |                             |                             |                                                        |                                                   | 2-target 2-component                                       |                                                             |                                                          |                                                            |                                                          |                                                     |
|----------------------|-----------------------------|-----------------------------|--------------------------------------------------------|---------------------------------------------------|------------------------------------------------------------|-------------------------------------------------------------|----------------------------------------------------------|------------------------------------------------------------|----------------------------------------------------------|-----------------------------------------------------|
| Cell line            | EGFR expression level (MFI) | HER2 expression level (MFI) | Both SO1861 and toxin to EGFR                          |                                                   | Both SO1861 and toxin to HER2                              |                                                             | SO1861 to EGFR; toxin to HER2                            |                                                            | SO1861 to HER2; toxin to EGFR                            |                                                     |
|                      |                             |                             | 278 nM Cetuximab-SO1861 + Cetuximab-Saporin (IC50, pM) | 278 nM Cetuximab-SO1861 + EGF-Dianthin (IC50, pM) | 300 nM Trastuzumab-SO1861 + Trastuzumab-Saporin (IC50, pM) | 300 nM Trastuzumab-SO1861 + Trastuzumab-Dianthin (IC50, pM) | 278 nM Cetuximab-SO1861 + Trastuzumab-Saporin (IC50, pM) | 1388 nM Cetuximab-SO1861 + Trastuzumab-Dianthin (IC50, pM) | 300 nM Trastuzumab-SO1861 + Cetuximab-Saporin (IC50, pM) | 300 nM Trastuzumab-SO1861 + EGF-Dianthin (IC50, pM) |
| MDA-MB-468           | 1656                        | 1                           | 0.5                                                    | 1                                                 | > 10.000                                                   | > 10.000                                                    | 55                                                       | 500                                                        | 100                                                      | 750                                                 |
| A431                 | 1593                        | 10                          | 0.4                                                    | 0.4                                               | > 10.000                                                   | > 10.000                                                    | 3                                                        | 30                                                         | 20                                                       | 2.300                                               |
| CaSki                | 481                         | 12                          | 2                                                      | 1                                                 | > 10.000                                                   | > 10.000                                                    | 1                                                        | 25                                                         | 14                                                       | 1.600                                               |
| SK-BR-3              | 28                          | 1162                        | n.d.                                                   | n.d.                                              | 20                                                         | n.d.                                                        | n.d.                                                     | n.d.                                                       | n.d.                                                     | 2                                                   |
| JIMT-1               | 58                          | 74                          | > 10.000                                               | > 10.000                                          | > 10.000                                                   | > 10.000                                                    | 3.000                                                    | > 10.000                                                   | 90                                                       | 4.000                                               |
| HeLa                 | 91                          | 7                           | 6.000                                                  | > 10.000                                          | > 10.000                                                   | > 10.000                                                    | > 10.000                                                 | 10.000                                                     | 50                                                       | 4.000                                               |
| A2058                | 1                           | 5                           | > 10.000                                               | > 10.000                                          | > 1.0000                                                   | > 10.000                                                    | > 10.000                                                 | > 10.000                                                   | > 10000                                                  | > 10.000                                            |

5

#### EXAMPLE 14 – TREATING A MAMMALIAN TUMOR-BEARING ANIMAL WITH A CONJUGATE OF THE INVENTION IN COMBINATION WITH AN ADC RESULTS IN SURVIVAL AND TUMOR REGRESSION

Female Balb/c nude mice were injected subcutaneously with a suspension of human A431 tumor cells.

- 10 Under the skin of the mice, a human epidermal carcinoma developed in the xenograft animal tumor model. After injection of the tumor cells, the xenograft tumor was allowed to develop to a size of approximately 170-180 mm<sup>3</sup>. The A431 tumor cells have the following characteristics: high EGFR expressors, medium CD71 expressors, low HER2 expressors.

In Table 8A, the results of the treatment of control mice and tumor-bearing mice are presented. Tumor-bearing mice were treated with the indicated antibodies directed to either human Her2/neu, human EGFR, or human CD71, which are cell-surface receptors on the xenograft tumor. Cetuximab was covalently conjugated with saponin SO1861. The SO1861 was first provided with the linker EMCH (N-ε-maleimidocaproic acid hydrazide), which EMCH is a maleimide-and-hydrazide crosslinker for covalently conjugating sulfhydryls (reduced cysteines of the antibody) to carbonyls (aldehyde or ketones; here the carbonyl of the aldehyde at position C-23 of the saponin). The saponin-EMCH was covalently coupled to reduced cysteines of the Cetuximab, forming a covalent thio-ether bond between the EMCH and the cysteine side chain. The ADCs trastuzumab-saporin (covalent conjugate) and anti-CD71 mAb (OKT-9, IgG) – saporin (covalent conjugate) were tested for their tumor-attacking efficacy in the mice, measured as tumor volume in time after start of the treatment with the ADCs. The dose of the ADCs was sub-optimal in the tumor model. That is to say, from previous experiments, it was established at which sub-optimal dose of the ADCs no tumor-regression or arrest of tumor growth would be observable.

15

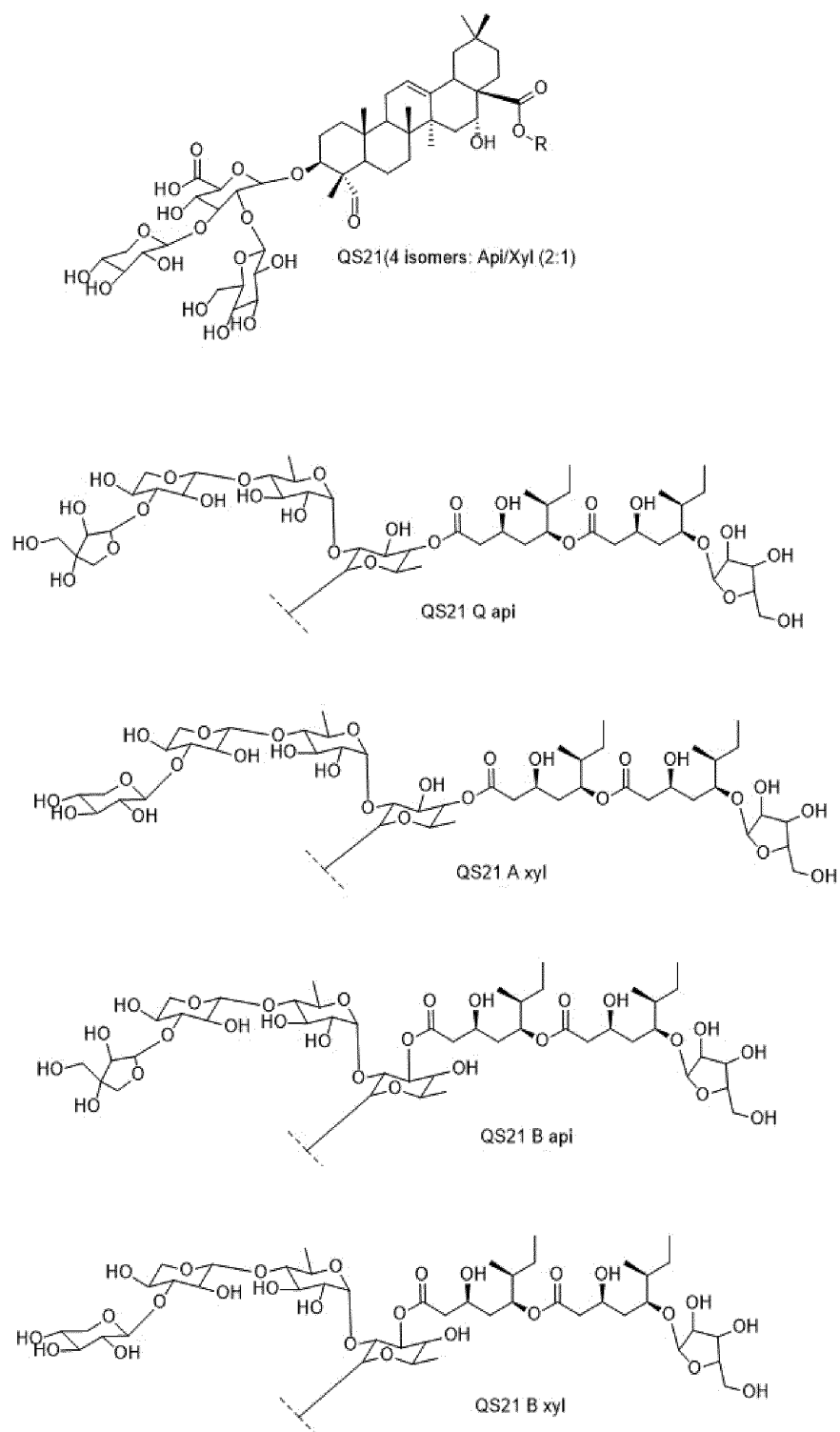
| <b>TABLE 8A: RESULTS OF TREATING A MAMMALIAN TUMOR-BEARING ANIMAL WITH A CONJUGATE OF THE INVENTION IN COMBINATION WITH AN ADC RESULTS IN SURVIVAL AND TUMOR REGRESSION</b> |                                 |                                                                                            |                                                                                                                            |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|--------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|
| <b>Treatment group</b>                                                                                                                                                      | <b>Patient / healthy animal</b> | <b>treatment</b>                                                                           | <b>tumor size (volume in mm<sup>3</sup> or '+' for growth, '-' for regression, and 'stable' for growth nor regression)</b> |
| 1                                                                                                                                                                           | xenograft                       | vehicle                                                                                    | 2000 mm <sup>3</sup> (death/euthanasia)                                                                                    |
| 2                                                                                                                                                                           | xenograft                       | Trastuzumab-saporin                                                                        | 2000 mm <sup>3</sup> (death/euthanasia)                                                                                    |
| 3                                                                                                                                                                           | xenograft                       | Anti-CD71 mAb OKT-9 – saporin (covalent conjugate)                                         | 2000 mm <sup>3</sup> (death/euthanasia)                                                                                    |
| 4                                                                                                                                                                           | xenograft                       | Cetuximab-SO1861 (covalent conjugate)                                                      | 2000 mm <sup>3</sup> (death/euthanasia)                                                                                    |
| 5                                                                                                                                                                           | xenograft                       | Cetuximab                                                                                  | > 170 mm <sup>3</sup> , but < 2000 mm <sup>3</sup> (death/euthanasia)                                                      |
| 6                                                                                                                                                                           | xenograft                       | Trastuzumab-saporin (covalent conjugate) + Cetuximab-SO1861 (covalent conjugate)           | Tumor regression from 180 mm <sup>3</sup> at the start of treatment back to 80 mm <sup>3</sup> (survival)                  |
| 7                                                                                                                                                                           | xenograft                       | Anti-CD71 mAb OKT-9 - saporin (covalent conjugate) + Cetuximab-SO1861 (covalent conjugate) | Tumor regression from 180 mm <sup>3</sup> at the start of treatment back to 40 mm <sup>3</sup> (survival)                  |

These results demonstrate that the combination therapy of an ADC at a dose which is ineffective when treatment of tumor-bearing mice with the ADC alone is considered (tumor growths, death of the mice is not prevented (euthanasia)), with a conjugate of the invention consisting of a tumor-cell specific receptor targeting antibody covalently bound to a saponin, *i.e.* SO1861, the covalent conjugate administered to the mice suffering from cancer, at a non-effective dose when administered alone (tumor growths, death of the mice is not prevented (euthanasia)), provides an efficient and efficacious treatment regimen, expressed as tumors in regression and prolonged survival of the treated animals (beyond the duration of the experiment). The sub-optimal dose of ADC combined with a covalently bound saponin-comprising conjugate of the invention which has no anti-tumor activity when administered alone, thus provide for an effective treatment option for cancer patients, wherein a relative low dose of the ADC is efficacious. A lower dose of ADC bears the promise of less risk for adverse events, or even no side effects at all. In addition, the stimulatory effect of the saponin-bearing conjugate when the efficacy of the ADC is considered, shows that ADCs which previously have proven to lack efficacy when tumor patient treatment is concerned, may gain renewed attention and value, since ADC efficacy is improved in combination therapy setting, as the current example demonstrated. Reference is made to ADCs known in the art which were previously investigated in the human clinical setting, but then were for some ADCs retracted from further clinical investigation. Especially the ADCs for which clinical development was terminated due to observed lack of efficacy and/or due to occurrence of unacceptable adverse event are ADCs which may gain renewed value for cancer patients when combined with a covalently bound saponin-comprising conjugate, such as the cetuximab-saponin tested.

**EXAMPLE 15 – saponins mixture of Quillaja saponaria comprising QS-21, with endosomal/lysosomal escape enhancing activity**

Scheme Q displays the common molecular structure of a series of QS-21 saponins (in part adapted from: Conrado Pedebos, Laércio Pol-Fachin, Ramon Pons, Cilaine V. Teixeira Hugo Verli, Atomic Model and Micelle Dynamics of QS-21 Saponin, Molecules 2014, 19, 3744-3760; four isoforms, wherein each of the depicted glycans can be bound as the R group). A mixture of water-soluble saponins obtained from Quillaja saponaria (Sigma-Aldrich, product No. S4521; Roth, Item No. 6857; InvivoGen, product 'Quil-A') may be applied in an endosomal/lysosomal escape enhancing conjugate, composition and combination of the invention, based on endosomal/lysosomal escape enhancing properties of at least one individual saponin present in the mixture, *e.g.* QS-21, or based on a combination of two or more of the saponins comprised by the mixture, such as QS-21 and QS-7.

The inventors demonstrated that the mixture of saponins from Quillaja saponaria at 2,5 microgram/ml dose was capable of enhancing endosomal escape of dianthin, as tested with mammalian tumor cells in a cell-based bioassay. The effector molecule exposed to the cells was dianthin covalently coupled to the ligand EGF: EGF-dianthin. Cells tested were tumor cell lines HeLa for free saponins, and A431, MDA-MB-468, CaSki and A2058 for testing the saponins when covalently coupled to cetuximab.



(Scheme Q)

**Example 16**

The 1 target 2-components system (1T2C) is the combination treatment of mAb1-protein toxin and mAb1-SO1861, as illustrated in Figure 1A, E. SO1861-EMCH was conjugated via cysteine residues (Cys) and HSP27BNA oligo was conjugated via lysine residues to cetuximab (monoclonal antibody recognizing and binding human EGFR), both with a DAR 4 resulting in the production of 2 conjugates: cetuximab-(Cys-L-SO1861)<sup>4</sup> and cetuximab-(Lys-L-HSP27BNA)<sup>4</sup>. The combination of cetuximab-(Cys-L-SO1861)<sup>4</sup> (intraperitoneal administration, (i.p.)) and cetuximab-(Lys-L-HSP27BNA)<sup>4</sup> (intravenous administration, (i.v.)) was tested in a A431 xenograph mouse tumor model for EGFR tumor targeted gene silencing activity. Dosings started at day 12 when tumors reached ~150 mm<sup>3</sup> in size and tumor samples were collected at 72 h after the first dosing and analysed for HSP27 gene expression compared to control gene mRNA expression levels (reference genes). This revealed that 1 dosing of 50 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup> + 25 mg/kg cetuximab-(Lys-L-HSP27BNA)<sup>4</sup> resulted in a 50% reduction in HSP27 gene expression in the A431 tumors compared to single dosing of cetuximab-(Cys-L-SO1861)<sup>4</sup> or cetuximab-(Lys-L-HSP27BNA)<sup>4</sup> mono therapies (Figure 25). Compared to the vehicle control tumors, a reduction of 40% HSP27 gene silencing was observed. This shows and enables that the combination of cetuximab-conjugated SO1861 + cetuximab-conjugated HSP27BNA oligo, according to the 1T2C invention, induces efficient targeted delivery of a therapeutic antisense oligo nucleotide in the cytoplasm of solid tumor cells, thereby inducing tumor targeted gene silencing, *in vivo*.

Next, SO1861-EMCH was conjugated via cysteine residues (Cys) to trastuzumab (monoclonal antibody recognizing and binding human HER2), with a DAR 4 resulting in the production of trastuzumab-(Cys-L-SO1861)<sup>4</sup>. The combination of trastuzumab-(Cys-L-SO1861)<sup>4</sup> and trastuzumab-saporin (trastuzumab protein toxin conjugate) was tested in a mouse tumor model (patient derived xenograph tumor model, PDX) with high HER2 expression levels and resistant for trastuzumab mono therapy. The combination, according to the 1T2C invention of 40 mg/kg trastuzumab-(Cys-L-SO1861)<sup>4</sup> (intraperitoneal administration, (i.p.)) + 0.03 (Day1, 8)/ 0.02 (Day 15, 22, 30, 36, 43) mg/kg trastuzumab-saporin (intravenous administration, (i.v.)) revealed strong tumor growth inhibition compared to the vehicle control and the 40 mg/kg trastuzumab-(Cys-L-SO1861)<sup>4</sup> or 0.03/0.02 mg/kg trastuzumab-saporin mono therapies (Figure 26). Besides, in tumor bearing mice that were treated with a lower dosing combination (40 mg/kg trastuzumab-(Cys-L-SO1861)<sup>4</sup> + 0.01 mg/kg trastuzumab-saporin) no tumor growth inhibiting activity was observed (Figure 26). This shows and enables that the 1T2C combination of trastuzumab conjugated SO1861 + trastuzumab conjugated protein toxin induces efficient targeted delivery of a therapeutic protein toxin in the cytoplasm of solid tumor cells, thereby inducing tumor cell death and tumor growth inhibition, *in vivo*.

**Example 17. 2 target 2-component system (in vivo)**

The 2 target 2-components system (2T2C) is the combination treatment of mAb1-SO1861 and mAb2-protein toxin, (Figure 1B-D). SO1861-EMCH was conjugated via cysteine residues (Cys) to cetuximab (monoclonal antibody recognizing and binding human EGFR), with a DAR 4 resulting in the production of: cetuximab-(Cys-L-SO1861)<sup>4</sup>. The combination of cetuximab-(Cys-L-SO1861)<sup>4</sup> and trastuzumab-

saporin or CD71mab-saporin was tested in a A431 (EGFR<sup>++</sup>/HER2<sup>+/-</sup>/CD71<sup>+</sup>) xenograph 'nude' mouse tumor model for EGFR tumor targeted cell killing as illustrated in Figure 1B-D. Dose escalation was performed to determine the therapeutic efficacy (Day 9: 0.3 mg/kg trastuzumab-saporin or 0.1 mg/kg CD71mab-saporin + 5 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup>; Day 14, 18: 0.1 mg/kg trastuzumab-saporin or 0.05 mg/kg CD71mab-saporin + 5 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup>; Day 21: 0.05 mg/kg trastuzumab-saporin or 0.05 mg/kg CD71mab-saporin + 15 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup>; Day 28: 0.02 mg/kg trastuzumab-saporin or 0.02 mg/kg CD71mab-saporin + 15 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup> trastuzumab-saporin/cetuximab-SO1861. Controls were on the same dosing scheme respectively, only cetuximab (i.v.) was given 25 mg/kg every treatment day). At day 32 (dashed vertical line), day 35 and day 39 the inventors started the combination, according to the 2T2C invention of 25 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup> (intraperitoneal injection (i.p.) + 0.02 mg/kg trastuzumab-saporin or 0.02 CD71mab-saporin (intravenous administration, (i.v.)) and this revealed strong tumor regression for both 2T2C combination groups compared to the vehicle control, 25 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup> or 0.02 mg/kg trastuzumab-saporin/CD71mab-saporin mono therapies (Figure 27). The 2T2C system even outcompetes cetuximab, the clinically used monoclonal antibody against EGFR. Next the inventors performed the same experiment but then the test was started with 25 mg/kg cetuximab-(Cys-L-SO1861)<sup>4</sup> (intraperitoneal injection (i.p.) + 0.03 mg/kg trastuzumab-saporin or 0.03 CD71mab-saporin (intravenous administration, (i.v.)) treatment with a dosing at day 9 and 14 and thereafter 1 dosing per week. The 2T2C system according to the invention showed tumor regression in all mice and even in 1 mice in both 2T2C groups, complete tumor eradication (tumor volume= 0 mm<sup>3</sup>) (Figure 28). Also here the controls showed a strong increased in tumor volume whereas the positive control for this A431 mice model, cetuximab showed only tumor growth inhibition, but no regression (Figure 28). This shows and enables the 2T2C system approach, of cetuximab conjugated SO1861 + trastuzumab conjugated protein toxin or CD71mab conjugated protein toxin inducing highly efficient targeted delivery of a therapeutic protein toxin in the cytoplasm of solid tumors of tumor bearing mice, *in vivo*, thereby inducing even full tumor eradication in some mice and strong tumor regression in others even in large size tumors (2000 mm<sup>3</sup>).

#### **Example 18. 2 target 2-component system (in vitro)**

##### **Results**

The 2 target 2-components system (2T2C) is the combination treatment of mAb1-SO1861 and mAb2-protein toxin, (Figure 1B-D). SO1861-EMCH was conjugated via cysteine residues (Cys) to cetuximab (monoclonal antibody recognizing and binding human EGFR), with a DAR 3,7 (cetuximab-(Cys-L-SO1861)<sup>3,7</sup>). Cetuximab-(Cys-L-SO1861)<sup>3,7</sup> was titrated on a fixed concentration of 50 pM trastuzumab-saporin (trastuzumab, conjugated to the protein toxin, saporin) and targeted protein toxin mediated cell killing on EGFR/HER2 expressing cells (A431, EGFR<sup>++</sup>/HER2<sup>+/-</sup>; CaSKi, EGFR<sup>+</sup>/HER2<sup>+/-</sup>) was determined as illustrated in Figure 1B-D. This revealed strong cell killing at low concentrations of cetuximab-(Cys-L-SO1861)<sup>3,7</sup> (A431: IC<sub>50</sub> = 3 nM and CaSKi IC<sub>50</sub> = 10 nM; Figure 29A, 29B) whereas equivalent concentrations cetuximab, cetuximab-(Cys-L-SO1861)<sup>3,7</sup> or cetuximab + 50 pM trastuzumab-saporin could not induce any cell killing activity in EGFR/HER2 expressing cells. This shows that

relatively low concentrations of cetuximab-SO1861 conjugate efficiently enhances endosomal escape of the trastuzumab conjugated protein toxin (at non-effective concentrations), thereby inducing efficient cell killing of high EGFR/low HER2 expressing cells.

Next, trastuzumab-saporin was titrated on a fixed concentration of 75 nM cetuximab-(Cys-L-SO1861)<sup>3,7</sup> and targeted protein toxin mediated cell killing on EGFR/HER2 expressing cells was determined. This revealed that 75 nM cetuximab-(Cys-L-SO1861)<sup>3,7</sup> in combination with low concentrations trastuzumab-saporin induced already efficient cell killing in EGFR/HER2 expressing cells (A431: IC<sub>50</sub> = 5 pM; and CaSKI: IC<sub>50</sub> = 1 pM; Figure 29C and 29D), whereas trastuzumab-saporin alone or trastuzumab-saporin + 75 nM cetuximab did not show significant cell killing activity (IC<sub>50</sub> > 10.000 pM) in both cell lines (Figure 29C, 29D). All this shows that relatively low concentrations of trastuzumab-saporin can be effective and induce cell killing in combination with low cetuximab-SO1861 conjugate concentrations in high EGFR/low HER2 expressing cells.

Next, cetuximab-(Cys-L-SO1861)<sup>3,7</sup> was titrated on a fixed concentration of 50 pM trastuzumab-saporin and targeted protein toxin-mediated cell killing on HeLa (EGFR<sup>+/+</sup>/HER2<sup>+/+</sup>) or A2058 (EGFR<sup>-</sup>/HER2<sup>+/+</sup>) was determined as illustrated in Figure 1B-D. Both HeLa (EGFR<sup>+/+</sup>/HER2<sup>+/+</sup>) and A2058 (EGFR<sup>-</sup>/HER2<sup>+/+</sup>) cells do not show cell killing at low concentrations of cetuximab-(Cys-L-SO1861)<sup>3,7</sup> + 50 pM trastuzumab-saporin (HeLa: IC<sub>50</sub> = 400 nM; A2058: IC<sub>50</sub> > 400 nM; Figure 30A, 30B). This shows that in the absence of sufficient receptor expression, effective intracellular delivered SO1861 concentrations are not reached (threshold) to induce endosomal escape and cytoplasmic delivery of the protein toxin.

Next, trastuzumab-saporin was titrated on a fixed concentration of 75 nM cetuximab-(Cys-L-SO1861)<sup>3,7</sup> and targeted protein toxin mediated cell killing on HeLa (EGFR<sup>+/+</sup>/HER2<sup>+/+</sup>) or A2058 (EGFR<sup>-</sup>/HER2<sup>+/+</sup>) was determined. Both HeLa (EGFR<sup>+/+</sup>/HER2<sup>+/+</sup>) and A2058 (EGFR<sup>-</sup>/HER2<sup>+/+</sup>) cells showed no cell killing activity (HeLa: IC<sub>50</sub> > 10.000 pM; A2058: IC<sub>50</sub> > 10.000 pM; Figure 30C, 30D). All this shows that cells with low or no EGFR receptor expression are not susceptible for the combination of cetuximab-(Cys-L-SO1861)<sup>3,7</sup> + trastuzumab-saporin, due to a lack of sufficient EGFR receptor that facilitates the antibody-mediated delivery of sufficient SO1861 (threshold) to ensure endosomal escape of the toxin within the cytoplasm of the cell.

Next, SO1861-EMCH was conjugated via cysteine residues (Cys) to trastuzumab (monoclonal antibody recognizing and binding human HER2), with a DAR 4 (trastuzumab-(Cys-L-SO1861)<sup>4</sup>). Trastuzumab-(Cys-L-SO1861)<sup>4</sup> was titrated on a fixed concentration of 1.5 pM EGFdianthin (EGFR targeted ligand toxin fusion protein) and targeted protein toxin mediated cell killing on HER2/EGFR expressing cells (SK-BR-3: HER2<sup>++</sup>/EGFR<sup>+/+</sup>) was determined. This revealed strong cell killing at low concentrations of trastuzumab-(Cys-L-SO1861)<sup>4</sup> + 1.5 pM EGFdianthin (SK-BR-3: IC<sub>50</sub> = 1 nM; Figure 31A) whereas equivalent concentrations trastuzumab, trastuzumab-(Cys-L-SO1861)<sup>4</sup> or trastuzumab + 1.5 pM EGFdianthin could not induce any cell killing activity in HER2<sup>++</sup>/EGFR<sup>+/+</sup> expressing cells. This shows that trastuzumab conjugated SO1861 efficiently enhances endosomal escape of the EGF fusion protein toxin (at non-effective concentrations), thereby inducing cell killing of high HER2/low EGFR expressing cells.

Next, EGFdianthin was titrated on a fixed concentration of 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and targeted protein toxin mediated cell killing on SK-BR-3 (HER2<sup>++</sup>/EGFR<sup>+/+</sup>) expressing cells

was determined. This revealed that 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> in combination with low concentrations EGFdianthin induced already efficient cell killing in HER2/EGFR expressing cells (SK-BR-3: IC<sub>50</sub> = 1 pM) (Figure 31B), whereas EGFdianthin alone or EGFdianthin + 2.5 nM trastuzumab showed no cell killing activity (IC<sub>50</sub> > 10.000 pM) (Figure 31B). All this shows that relatively low concentrations of EGFdianthin can be effective and induce cell killing only in combination with low

Next, trastuzumab-(Cys-L-SO1861)<sup>4</sup> was titrated on a fixed concentration of 1.5 pM EGFdianthin and targeted protein toxin mediated cell killing on JIMT-1 (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) or MDA-MB-468: HER2/EGFR<sup>++</sup>) was determined. Both cell lines were not sensitive for any combination of trastuzumab-(Cys-L-SO1861)<sup>4</sup> + 1.5 pM EGFdianthin (JIMT-1: IC<sub>50</sub> > 1000 nM; MDA-MB-468: IC<sub>50</sub> > 1000 nM; Figure 32A, 32B). This shows that in the absence of sufficient HER2 receptor expression, effective intracellular delivered SO1861 concentrations are not reached (threshold) to induce endosomal escape and cytoplasmic delivery of the protein toxin.

Next, EGFdianthin was titrated on a fixed concentration of 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and targeted protein toxin mediated cell killing on JIMT-1 (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) or MDA-MB-468 (HER2/EGFR<sup>++</sup>) was determined. Both cell lines showed cell killing at high EGFdianthin concentrations with or without 2,5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> (JIMT-1: IC<sub>50</sub> = 10.000 pM; MDA-MB-468: IC<sub>50</sub> = 200 pM Figure 32C, 32D).

All this shows that cells with low or no HER2 receptor expression are not susceptible for the combination of trastuzumab-(Cys-L-SO1861)<sup>3,7</sup> + 1.5 pM EGFdianthin, due to a lack of sufficient HER2 receptor that facilitates the antibody-mediated delivery of sufficient SO1861 (threshold) to ensure endosomal escape of the toxin within the cytoplasm of the cell.

Next, SO1861-EMCH was conjugated via cysteine residues (Cys) to trastuzumab (monoclonal antibody recognizing and binding human HER2), with a DAR 4, (trastuzumab-(Cys-L-SO1861)<sup>4</sup>). Trastuzumab-(Cys-L-SO1861)<sup>4</sup> was titrated on a fixed concentration of 5 pM cetuximab-saporin (EGFR targeting antibody-protein toxin conjugate) and targeted protein toxin mediated cell killing on HER2/EGFR expressing cells (SK-BR-3: HER2<sup>++</sup>/EGFR<sup>+/+</sup>) was determined as illustrated in Figure 1B-D. This revealed strong cell killing at low concentrations of trastuzumab-(Cys-L-SO1861)<sup>4</sup> + 5 pM cetuximab-saporin (SK-BR-3: IC<sub>50</sub> = 1 nM; Figure 33A) whereas equivalent concentrations trastuzumab, trastuzumab-(Cys-L-SO1861)<sup>4</sup> or trastuzumab + 5 pM cetuximab-saporin could not induce any cell killing activity in HER2<sup>++</sup>/EGFR<sup>+/+</sup> expressing cells. This shows that trastuzumab conjugated SO1861 efficiently enhances endosomal escape of the cetuximab conjugated protein toxin (at non-effective concentrations), thereby inducing cell killing of HER2<sup>++</sup>/EGFR<sup>+/+</sup> expressing cells.

Next, cetuximab-saporin was titrated on a fixed concentration of 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and 75 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and targeted protein toxin mediated cell killing on HER2/EGFR expressing cells (SK-BR-3: HER2<sup>++</sup>/EGFR<sup>+/+</sup>) was determined. This revealed that 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> in combination with low concentrations cetuximab-saporin induced already efficient cell killing in SK-BR-3 cells (SK-BR-3: IC<sub>50</sub> = 1 pM; Figure 33B), whereas cetuximab-saporin alone or cetuximab-saporin + 2.5 nM trastuzumab showed cell killing only at high concentrations trastuzumab-saporin (SK-BR-3: IC<sub>50</sub> > 4000 pM; Figure 33B). All this shows that relatively low

concentrations of cetuximab-saporin can be effective and induce cell killing only in combination with low trastuzumab-(Cys-L-SO1861)<sup>4</sup> concentrations in HER2<sup>+/+</sup>/EGFR<sup>+/+</sup> expressing cells.

Next, trastuzumab-(Cys-L-SO1861)<sup>4</sup> was titrated on a fixed concentration of 5 pM cetuximab-saporin and targeted protein toxin mediated cell killing on JIMT-1 (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) and MDA-MB-468 (HER2<sup>-</sup>/EGFR<sup>+/+</sup>) cells was determined. Both cell lines were not sensitive for the combination of trastuzumab-(Cys-L-SO1861)<sup>4</sup> + 5 pM cetuximab-saporin (JIMT-1: IC<sub>50</sub> > 1000 nM; MDA-MB-468: IC<sub>50</sub> > 1000 nM; Figure 34A, 34B). This shows that in the absence of sufficient HER2 receptor expression, effective intracellular delivered SO1861 concentrations are not reached (threshold) to induce endosomal escape and cytoplasmic delivery of the protein toxin.

Next, cetuximab-saporin was titrated on a fixed concentration of 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> and targeted protein toxin mediated cell killing on JIMT-1 (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>) and MDA-MB-468 (HER2<sup>-</sup>/EGFR<sup>+/+</sup>) cells was determined. Both cell lines showed cell killing at similar cetuximab-saporin concentrations with or without 2.5 nM trastuzumab-(Cys-L-SO1861)<sup>4</sup> (JIMT-1: IC<sub>50</sub> = 80 pM; MDA-MB-468: IC<sub>50</sub> = 100 pM; Figure 34C, 34D).

All this shows that cells with low or no HER2 receptor expression are not susceptible for the combination of trastuzumab-(Cys-L-SO1861)<sup>4</sup> + cetuximab-saporin, due to a lack of sufficient HER2 receptor that facilitates the antibody-mediated delivery of sufficient SO1861 (threshold) to ensure endosomal escape of the toxin within the cytoplasm of the cell.

#### **Example 19. SO1861 + EGFR/HER2/CD71 targeted V<sub>HH</sub>-protein toxin**

SO1861 was titrated on a fixed concentration of 50 pM V<sub>HH</sub>-CD71-dianthin, 50 pM V<sub>HH</sub>-HER2-dianthin or 50 pM V<sub>HH</sub>-EGFR-dianthin. Targeted protein toxin-mediated cell killing on A431 (EGFR<sup>+/+</sup>/HER2<sup>+/+</sup>/CD71<sup>+</sup>), A2058 (EGFR<sup>-</sup>/HER2<sup>+/+</sup>/CD71<sup>+</sup>), SK-BR-3 (HER2<sup>+/+</sup>/EGFR<sup>+/+</sup>/CD71<sup>+</sup>) and MDA-MB-468 (HER2<sup>-</sup>/EGFR<sup>+/+</sup>/CD71<sup>+</sup>) was determined. This revealed that SO1861 + 50 pM V<sub>HH</sub>-CD71-dianthin showed activity at IC<sub>50</sub> = 200 nM in A431 (CD71<sup>+</sup>) and A2058 (CD71<sup>+</sup>) cells, whereas SO1861 + 50 pM V<sub>HH</sub>-EGFR-dianthin showed activity at IC<sub>50</sub> = 400 nM in A431 (EGFR<sup>+/+</sup>), but no activity in A2058 (EGFR<sup>-</sup>) (Figure 35). SO1861 + 50 pM V<sub>HH</sub>-HER2-dianthin showed no activity with any of the tolerated SO1861 concentrations (SO1861 tolerated concentration IC<sub>50</sub> = 1000 nM) in A431 (HER2<sup>+/+</sup>), whereas in A2058 (HER2<sup>+/+</sup>), only slight activity could be detected at IC<sub>50</sub> = 700 nM SO1861 (Figure 35). Furthermore, SO1861 + 50 pM V<sub>HH</sub>-CD71-dianthin or SO1861 + 50 pM V<sub>HH</sub>-HER2-dianthin showed activity at IC<sub>50</sub> = 200 nM in SK-BR-3 (HER2<sup>+/+</sup>/CD71<sup>+</sup>), whereas in MDA-MB-468 (HER2<sup>-</sup>/CD71<sup>+</sup>) cells, SO1861 + 50 pM V<sub>HH</sub>-CD71-dianthin showed activity at IC<sub>50</sub> = 200 nM and SO1861 + 50 pM V<sub>HH</sub>-HER2-dianthin showed no enhanced activity compared to SO1861 alone (Figure 36). SO1861 + 50 pM V<sub>HH</sub>-EGFR-dianthin showed activity at IC<sub>50</sub> = 700 nM in SK-BR-3 (EGFR<sup>+/+</sup>) and strong activity (SO1861 IC<sub>50</sub> = 300 nM) in MDA-MB-468 (EGFR<sup>+/+</sup>) (Figure 36).

#### **Example 20 V<sub>HH</sub>-EGFR-dianthin + SO1861-EMCH or mAb-SO1861**

The 1 target 2-components system (1T2C) is the combination treatment of mAb-SO1861 and V<sub>HH</sub>-protein toxin, where mAb and V<sub>HH</sub> recognize and bind the same cell surface receptor (Figure 1E).

$V_{HH}$ -EGFR-dianthin (conjugate) was titrated alone or on a fixed concentration of 4000 nM SO1861-EMCH or 76.9 nM cetuximab-SO1861 (DAR4) and targeted protein toxin mediated cell killing on A431 (EGFR<sup>++</sup>) and A2058 (EGFR<sup>-</sup>) was determined. In A431 cells (EGFR<sup>++</sup>) the combination of  $V_{HH}$ -EGFR-dianthin + 4000 nM SO1861-EMCH revealed an IC<sub>50</sub> = 4 pM compared to  $V_{HH}$ -EGFR-dianthin alone (IC<sub>50</sub> > 10.000 pM), whereas t $V_{HH}$ -EGFR-dianthin + 76.9 nM cetuximab-SO1861 (DAR4) revealed an IC<sub>50</sub> = 400 pM (Figure 45A). In A2058 cells (EGFR<sup>-</sup>), only the combination of  $V_{HH}$ -EGFR-dianthin + 4000 nM SO1861-EMCH showed activity at high concentrations (IC<sub>50</sub> = 1000 pM), whereas  $V_{HH}$ -EGFR-dianthin alone or  $V_{HH}$ -EGFR-dianthin + 76.9 nM cetuximab-SO1861 (DAR4) showed no activity up to 10.000 pM (Figure 45B).

**Example 21.** *Bivalent- $V_{HH}$ -EGFR-dianthin or mAb-toxin + SO1861-EMCH or mAb-SO1861*

The 1 target 2-components system (1T2C) is the combination treatment of mAb-SO1861 and  $V_{HH}$ -protein toxin, where mAb and  $V_{HH}$  recognize and bind the same cell surface receptor (Figure 1E).

Cetuximab-saporin (conjugate) or bivalent $V_{HH}$ -EGFR-dianthin (recombinant fusion protein) was titrated alone or on a fixed concentration of 4000 nM SO1861-EMCH or 76.9 nM cetuximab-SO1861 (DAR4) and targeted protein toxin mediated cell killing on MDA-MB-468 (EGFR<sup>++</sup>), A431 (EGFR<sup>++</sup>) and A2058 (EGFR<sup>-</sup>) was determined. In MDA-MB-468 cells (EGFR<sup>++</sup>) the combination of bivalent $V_{HH}$ -EGFR-dianthin + 4000 nM SO1861-EMCH, cetuximab-saporin + 4000 nM SO1861-EMCH, bivalent $V_{HH}$ -EGFR-dianthin + 76.9 nM cetuximab-SO1861 (DAR4) or cetuximab-saporin + 76.9 nM cetuximab-SO1861 (DAR4) revealed a strong increase in EGFR targeted toxin-mediated the cell killing activity (IC<sub>50</sub> = 0,5 pM) compared to bivalent $V_{HH}$ -EGFR-dianthin or cetuximab-saporin alone (IC<sub>50</sub> = 4000 pM or IC<sub>50</sub> = 300 pM, respectively) (Figure 37A). In A431 cells (EGFR<sup>++</sup>) the combination of bivalent $V_{HH}$ -EGFR-dianthin + 4000 nM SO1861-EMCH revealed an IC<sub>50</sub> = 0,05 pM compared to bivalent $V_{HH}$ -EGFR-dianthin alone (IC<sub>50</sub> = 300 pM), cetuximab-saporin + 4000 nM SO1861-EMCH revealed IC<sub>50</sub> = 3 pM compared to cetuximab-saporin alone (IC<sub>50</sub> = 4000 pM), bivalent $V_{HH}$ -EGFR-dianthin + 76.9 nM cetuximab-SO1861 (DAR4) or cetuximab-saporin + 76.9 nM cetuximab-SO1861 (DAR4) revealed both an IC<sub>50</sub> = 10 pM (Figure 37B).

A2058 cells (EGFR<sup>-</sup>), only the combination of bivalent $V_{HH}$ -EGFR-dianthin + 4000 nM SO1861-EMCH or cetuximab-saporin + 4000nM SO1861-EMCH showed activity at high concentrations (IC<sub>50</sub> = 50 pM and IC<sub>50</sub> > 100 pM, respectively), whereas bivalent $V_{HH}$ -EGFR-dianthin, cetuximab-saporin alone or bivalent $V_{HH}$ -EGFR-dianthin + 76.9 nM cetuximab-SO1861 (DAR4) or cetuximab-saporin + 76.9 nM cetuximab-SO1861 (DAR4) showed no activity up to 10.000 pM (Figure 37C).

**Example 22.** *SO1861 + Bivalent- $V_{HH}$ -EGFR-dianthin*

SO1861 or Cetuximab-SO1861 (DAR4) was titrated on a fixed concentration of 1 pM or 5 pM bivalent $V_{HH}$ -EGFR-dianthin and targeted protein toxin mediated cell killing on MDA-MB-468 cells (EGFR<sup>++</sup>) and A2058 cells (EGFR<sup>-</sup>) was determined. In MDA-MB-468 cells (EGFR<sup>++</sup>) this revealed that very low concentrations cetuximab conjugated SO1861 in combination with 1 pM or 5 pM bivalent $V_{HH}$ -

EGFR-dianthin induced efficient cell killing ( $IC_{50} = 20$  nM), whereas unconjugated SO1861 + 1 pM or 5 pM bivalent $V_{HH}$ -EGFR-dianthin showed activity at  $IC_{50} = 1000$  nM (Figure 38A). In A2058 (EGFR<sup>-</sup>) activity was only observed at  $IC_{50} > 1000$  nM (Figure 38B).

5 **Example 23. mAb-SO1861 + bivalent $V_{HH}$ -EGFR-dianthin (1T2C and 2T2C)**

The 1 target 2-components system (1T2C) is the combination treatment of mAb-SO1861 and  $V_{HH}$ -protein toxin, where mAb and  $V_{HH}$  recognize and bind the same cell surface receptor (Figure 1E). The 2 target 2-components system (2T2C) is also the combination treatment of mAb-SO1861 and  $V_{HH}$ -protein toxin, where the mAb and  $V_{HH}$  recognize another cell surface receptor (Figure 1D).

10 Bivalent $V_{HH}$ -EGFR-dianthin (with the amino-acid sequence depicted as the sequence of SEQ ID NO: 73) was produced as recombinant fusion protein. Cetuximab-SO1861 (DAR4) was titrated on a fixed (non-effective) concentration of 50 pM bivalent $V_{HH}$ -EGFR-dianthin and targeted protein toxin mediated cell killing on A431 (EGFR<sup>++</sup>/HER2<sup>+/-</sup>), MDA-MB-468 (EGFR<sup>++</sup>/HER2<sup>+/-</sup>), SK-BR-3 (HER2<sup>++</sup>/EGFR<sup>+</sup>) and A2058 (EGFR<sup>-</sup>/HER2<sup>+/-</sup>) was determined. This revealed that very low  
15 concentrations cetuximab-SO1861 (DAR4) in combination with 50 pM bivalent $V_{HH}$ -EGFR-dianthin induced efficient cell killing of MDA-MB-468 (EGFR<sup>++</sup>) cells ( $IC_{50} = 0,5$  nM) and A431 (EGFR<sup>++</sup>) cells ( $IC_{50} = 4$  nM) whereas on SK-BR-3 (EGFR<sup>+</sup>) or A2058 (EGFR<sup>-</sup>) cells activity was detected only with very high concentrations of cetuximab-SO1861 ( $IC_{50} = 200$  nM and  $IC_{50} = 400$  nM respectively) (Figure 39).

20 This shows that low concentrations of cetuximab-SO1861 (DAR4) can efficiently induce endosomal escape of a bivalent $V_{HH}$ -EGFR-dianthin only in high EGFR expressing cells, thereby inducing enhanced cell killing.

Next, trastuzumab-SO1861 (DAR4) was titrated on a fixed (non-effective) concentration of 50 pM bivalent $V_{HH}$ -EGFR-dianthin and targeted protein toxin-mediated cell killing on A431  
25 (EGFR<sup>++</sup>/HER2<sup>+/-</sup>), A2058 (EGFR<sup>-</sup>/HER2<sup>+/-</sup>), SK-BR-3 (HER2<sup>++</sup>/EGFR<sup>+</sup>) and MDA-MB-468 cells (HER2<sup>-</sup>/EGFR<sup>++</sup>) was determined. This revealed that very low concentrations trastuzumab-SO1861 (DAR4) in combination with 50 pM bivalent $V_{HH}$ -EGFR-dianthin induced efficient cell killing of SK-BR-3 cells ( $IC_{50} = 0,3$  nM), whereas the combination showed only at very high concentrations trastuzumab-SO1861, cell killing activity in A431 (HER2<sup>+/-</sup>), A2058 (HER2<sup>+/-</sup>) and MDA-MB-468 (HER2<sup>-</sup>) cells ( $IC_{50} = 400$  nM)  
30 (Figure 39). This shows that low concentrations of trastuzumab-SO1861 (DAR4) can efficiently induce endosomal escape of a bivalent $V_{HH}$ -EGFR-dianthin only in high HER2 expressing cells, thereby inducing enhanced cell killing.

**Materials and methods**

35

*materials*

SO1861 was isolated and purified by Analyticon Discovery GmbH from raw plant extract obtained from *Saponaria officinalis*. Trastuzumab (Tras, Herceptin®, Roche), Cetuximab (Cet, Erbitux®, Merck KGaA) were purchased from the pharmacy (Charite, Berlin). Bivalent- $V_{HH}$ -EGFR-dianthin fusion (SEQ ID NO:  
40 73) was produced as recombinant protein in E.coli according to standard procedures at GenScript

(Leiden, The Netherlands). Monovalent V<sub>HH</sub> were purchased from QVQ, Utrecht, The Netherlands (V<sub>HH</sub>-HER2: clone name: Q17c; V<sub>HH</sub>-CD71: clone name: Q52c V<sub>HH</sub>-EGFR: clone name: Q86c). Cetuximab-saporin conjugates were produced and purchased from Advanced Targeting Systems (San Diego, CA). Dianthin-cys was produced and purchased from Proteogenix, France.

5 Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%, Sigma-Aldrich), 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent, 99%, Sigma-Aldrich), Zeba™ Spin Desalting Columns (2 mL, Thermo-Fisher), NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo-Fisher), NuPAGE™ MES SDS Running Buffer (Thermo-Fisher), Novex™ Sharp Pre-stained Protein Standard (Thermo-Fisher), PageBlue™ Protein Staining Solution (Thermo-Fischer), Pierce™ BCA Protein Assay Kit (Thermo-Fisher), N-Ethylmaleimide (NEM, 98%, Sigma-Aldrich), 1,4-Dithiothreitol (DTT, 98%, Sigma-Aldrich), Sephadex G25 (GE Healthcare), Sephadex G50 M (GE Healthcare), Superdex 200P (GE Healthcare), Isopropyl alcohol (IPA, 99.6%, VWR), Tris(hydroxymethyl)aminomethane (Tris, 99%, Sigma-Aldrich), Tris(hydroxymethyl)aminomethane hydrochloride (Tris.HCL, Sigma-Aldrich), L-Histidine (99%, Sigma-Aldrich), D-(+)-Trehalose dehydrate (99%, Sigma-Aldrich), Polyethylene glycol sorbitan monolaurate (TWEEN 20, Sigma-Aldrich), Dulbecco's Phosphate-Buffered Saline (DPBS, Thermo-Fisher), Guanidine hydrochloride (99%, Sigma-Aldrich), Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na<sub>2</sub>, 99%, Sigma-Aldrich), sterile filters 0.2 µm and 0.45 µm (Sartorius), Vivaspin T4 and T15 concentrator (Sartorius), Superdex 200PG (GE Healthcare), Tetra(ethylene glycol), Dimethyl sulfoxide (DMSO, 99%, Sigma-Aldrich), N-(2-Aminoethyl)maleimide trifluoroacetate salt (AEM, 98%, Sigma-Aldrich), L-Cysteine (98.5%, Sigma-Aldrich), deionized water (DI) was freshly taken from Ultrapure Lab Water Systems (MilliQ, Merck), Nickel-nitrilotriacetic acid agarose (Ni-NTA agarose, Protino), Glycine (99.5%, VWR), 5,5-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB, 98%, Sigma-Aldrich), S-Acetylmercaptosuccinic anhydride Fluorescein (SAMSA reagent, Invitrogen) Sodium bicarbonate (99.7%, Sigma-Aldrich), Sodium carbonate (99.9%, Sigma-Aldrich), PD MiniTrap desalting columns with Sephadex G-25 resin (GE Healthcare), PD10 G25 desalting column (GE Healthcare), Zeba Spin Desalting Columns in 0.5, 2, 5, and 10 mL (Thermo-Fisher), Vivaspin Centrifugal Filters T4 10 kDa MWCO, T4 100 kDa MWCO, and T15 (Sartorius), Biosep s3000 aSEC column (Phenomenex), Vivacell Ultrafiltration Units 10 and 30 kDa MWCO (Sartorius), Nalgene Rapid-Flow filter (Thermo-Fisher),

### 30 *Methods*

#### **SO1861-EMCH synthesis**

To SO1861 (121 mg, 0.065 mmol) and EMCH.TFA (110 mg, 0.325 mmol) was added methanol (extra dry, 3.00 mL) and TFA (0.020 mL, 0.260 mmol). The reaction mixture stirred at room temperature. After 1.5 hours the reaction mixture was subjected to preparative MP-LC.<sup>1</sup> Fractions corresponding to the product were immediately pooled together, frozen and lyophilized overnight to give the title compound (120 mg, 90%) as a white fluffy solid. Purity based on LC-MS 96%.

LRMS (m/z): 2069 [M-1]<sup>-</sup>

LC-MS r.t. (min): 1.08<sup>4</sup>

**Cell viability assay**

After treatment the cells were incubated for 72 hr at 37°C before the cell viability was determined by a MTS-assay, performed according to the manufacturer's instruction (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). Briefly, the MTS solution was diluted 20x in DMEM without phenol red (PAN-Biotech GmbH) supplemented with 10% FBS. The cells were washed once with 200 µL/PBS well, after which 100 µL diluted MTS solution was added/well. The plate was incubated for approximately 20-30 minutes at 37°C. Subsequently, the OD at 492 nm was measured on a Thermo Scientific Multiskan FC plate reader (Thermo Scientific). For quantification the background signal of 'medium only' wells was subtracted from all other wells, before the cell viability percentage of treated/untreated cells was calculated, by dividing the background corrected signal of treated wells over the background corrected signal of the untreated wells (x 100).

**FACS analysis**

Cells were seeded in DMEM (PAN-Biotech GmbH) supplemented with 10% fetal calf serum (PAN-Biotech GmbH) and 1% penicillin/streptomycin (PAN-Biotech GmbH), at 500,000 c/plate in 10 cm dishes and incubated for 48 hrs (5% CO<sub>2</sub>, 37°C), until a confluency of 90% was reached. Next, the cells were trypsinized (TrypLE Express, Gibco Thermo Scientific) to single cells. 0.75 x 10<sup>6</sup> Cells were transferred to a 15 mL falcon tube and centrifuged (1,400 rpm, 3 min). The supernatant was discarded while leaving the cell pellet submerged. The pellet was dissociated by gentle tapping the falcon tube on a vortex shaker and the cells were washed with 4 mL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS). After washing the cells were resuspended in 3 mL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS) and divided equally over 3 round bottom FACS tubes (1 mL/tube). The cells were centrifuged again and resuspended in 200 µL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS) or 200 µL antibody solution; containing 5 µL antibody in 195 µL cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS). APC Mouse IgG1, κ APC anti-human EGFR (#352906, Biolegend) was used to stain the EGFR receptor. PE anti-human HER2 APC anti-human CD340 (erbB2/HER-2) (#324408 Biolegend) was used to stain the HER2 receptor, PE Mouse IgG2a, κ Isotype Ctrl FC (#400212, Biolegend) was used as its matched isotype control. PE anti-human CD71 (#334106, Biolegend) was used to stain the CD71 receptor, PE Mouse IgG2a, κ Isotype Ctrl FC (#400212, Biolegend) was used as its matched isotype control. Samples were incubated for 30 min at 4 °C on a tube roller mixer. Afterwards, the cells were washed 3x with cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free, 2% FBS) and fixated for 20 min at room temperature using a 2% PFA solution in PBS. Cells were washed 2x with cold PBS, and resuspended in 250-350 µL cold PBS for FACS analysis. Samples were analyzed with a BD FACSCanto II flow cytometry system (BD Biosciences) and FlowJo software. FACS data per cell lines are in Table A2

**Procedure for the conjugation of V<sub>HH</sub>-Dianthin**

Dianthin-Cys was concentrated by ultrafiltration using a vivaspin T15 10KDa MWCO centrifugal filter and buffer exchanged into TBS pH 7.5. To the concentrated Dianthin-Cys was added an aliquot of freshly prepared TCEP solution (10.0 mg/ml), the mixture vortexed briefly then incubated for 60 minutes at 20°C with roller-mixing. After incubation, the resulting Dianthin-SH was purified by gel filtration using

a zeba spin desalting column then repeated centrifugal-wash cycles using a vivaspin T15 10KDa MWCO centrifugal filter into TBS pH 7.5. The resulting Dianthin-SH was reacted with freshly prepared DTME solution (10 mg/ml) in DMSO, the mixture vortexed briefly then incubated for 60 minutes at 20°C. After, the Dianthin-DTME was obtained following purification by gel filtration using a zeba spin desalting column into TBS pH 7.5. The Dianthin-DTME was stored at 20°C until conjugated. At the same time, an aliquot of V<sub>HH</sub> was concentrated by ultrafiltration using a vivaspin T15 10 KDa MWCO centrifugal filter and buffer exchanged into TBS pH 7.5. To the concentrated V<sub>HH</sub> was added an aliquot of freshly prepared TCEP solution (10.0 mg/ml), the mixture vortexed briefly then incubated for 60 minutes at 37°C with roller-mixing. After incubation, the resulting V<sub>HH</sub> was purified by gel filtration using a zeba spin desalting column then repeated centrifugal-wash cycles using a vivaspin T4 5KDa MWCO centrifugal filter into TBS pH 7.5. An aliquot of the resulting V<sub>HH</sub>-SH was reacted with Dianthin-DTME, the mixture vortexed briefly then incubated overnight at 20°C. After, the reaction mixture was concentrated using a vivaspin T4 10 KDa MWCO centrifuge tube and purified by gel filtration using a 1.6 × 35 cm Superdex 200PG column eluting into DPBS pH 7.5.

#### Antibody-(L-SO1861)<sup>4</sup>

Trastuzumab, Cetuximab, are referred hereafter as "Ab". Ab was conjugated to the saponin SO1861-EMCH via Michael-type thiol-ene conjugation reaction at DARs of 1, 2, 3, 4, 5, and 6. The SO1861-EMCH molecule obtains a labil (L) hydrazone bond between its structure and its maleimide function generating a labil bond between the saponin and Ab. The procedure is exemplary described for Trastuzumab-(L-SO1861)<sub>4</sub> (See Table A3):

To a solution of Cetuximab (40 mg, 8.0 ml) was added 10 µl/ml each of Tris concentrate (127 mg/ml, 1.05 M), Tris.HCl concentrate (623 mg/ml, 3.95 M) and EDTA-Na<sub>2</sub> concentrate (95 mg/ml, 0.26 M) to give a 50 mM TBS, 2.5 mM EDTA buffer pH 7.5.

To Cetuximab divided into four portions (each of 9.73 mg, 4.864 mg/ml, 65 nmol) was added an aliquot of freshly prepared TCEP solution (0.5 – 2.0 mg/ml, 1.15 – 7.02 mole equivalents, 75 – 455 nmol), the mixtures vortexed briefly then incubated for 300 minutes at 20 °C with roller-mixing. After incubation (prior to addition of SO1861-EMCH), a ca. 1 mg (0.210 ml) aliquot of Ab-SH was removed from each mixture and purified by gel filtration using a zeba spin desalting column into TBS pH 7.5. These aliquots were characterized by UV-vis analysis and Ellman's assay (thiol to Ab ratio = 2.0, 4.2, 5.9 and 6.8 respectively). To each of the bulk Ab-SH was added an aliquot of freshly prepared SO1861-EMCH solution (2 mg/ml, 1.3 mole equivalents per 'thiol', 0.15 – 0.61 µmol, 0.16 – 0.63 ml), the mixtures vortexed briefly then incubated for 120 minutes at 20 °C. Besides each conjugation reaction, two aliquots of desalted Ab-SH (0.25 mg, 1.67 nmol) were reacted with NEM (1.3 mole equivalents per 'thiol', 4.3 – 17.4 nmol, 2.2 – 8.7 µl of a 0.25 mg/ml solution) or TBS pH 7.5 buffer (2.2 – 8.7 µl) for 120 minutes at 20°C, as positive and negative controls, respectively. After incubation (prior to addition of NEM), a 0.200 ml aliquot of Ab – SO1861-EMCH mixture was removed and purified by gel filtration using zeba spin desalting column into TBS pH 7.5. This aliquot was characterized by UV-vis and alongside positive and negative controls were characterized by Ellman's assay to obtain SO1861-EMCH incorporations. To the bulk Ab – SO1861-EMCH mixture was added an aliquot of freshly prepared NEM solution (2.5 mg/ml,

2.5 – 10 mole equivalents, 0.15 – 0.58  $\mu\text{mol}$ ) and the mixtures purified by zeba spin desalting columns eluting with DPBS pH 7.5 to give purified Cetuximab – (L-SO1861) conjugates (See Table A4). The products were normalized to 2.5 mg/ml and filtered to 0.2  $\mu\text{m}$  prior to dispensing for biological evaluation.

## 5 Materials and methods

### Abbreviations

|    |          |                                                                                                                 |  |
|----|----------|-----------------------------------------------------------------------------------------------------------------|--|
|    | AEM      | <i>N</i> -(2-Aminoethyl)maleimide trifluoroacetate salt                                                         |  |
|    | AMPD     | 2-Amino-2-methyl-1,3-propanediol                                                                                |  |
| 10 | BOP      | (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate                                        |  |
|    | DIPEA    | <i>N,N</i> -diisopropylethylamine                                                                               |  |
|    | DMF      | <i>N,N</i> -dimethylformamide                                                                                   |  |
|    | DTME     | Dithiobismaleimidoethane                                                                                        |  |
|    | DTT      | Dithiothreitol                                                                                                  |  |
| 15 | EDCI.HCl | 3-((Ethylimino)methyleneamino)- <i>N,N</i> -dimethylpropan-1-aminium chloride                                   |  |
|    | EDTA     | Ethylenediaminetetraacetic acid                                                                                 |  |
|    | EMCH.TFA | <i>N</i> -( $\epsilon$ -maleimidocaproic acid) hydrazide, trifluoroacetic acid salt                             |  |
|    | HATU     | 1-[Bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i> ]pyridinium 3-oxid hexafluorophosphate |  |
| 20 | min      | minutes                                                                                                         |  |
|    | NMM      | 4-Methylmorpholine                                                                                              |  |
|    | r.t.     | retention time                                                                                                  |  |
|    | SEC      | Size exclusion chromatography                                                                                   |  |
|    | TBEU     | (Tris-(hydroxymethyl)-aminomethan)-Borat-EDTA-Urea                                                              |  |
| 25 | TCEP     | tris(2-carboxyethyl)phosphine hydrochloride                                                                     |  |
|    | Temp     | temperature                                                                                                     |  |
|    | TFA      | trifluoroacetic acid                                                                                            |  |

### Analytical methods

30

#### LC-MS method 1

Apparatus: Waters IClass; Bin. Pump: UPIBSM, SM: UPISMFTN with SO; UPCMA, PDA: UPPDATC, 210-320 nm, SQD: ACQ-SQD2 ESI, mass ranges depending on the molecular weight of the product:

neg or neg/pos within in a range of 1500-2400 or 2000-3000; ELSD: gas pressure 40 psi, drift tube temp:

35 50°C; column: Acquity C18, 50×2.1 mm, 1.7  $\mu\text{m}$  Temp: 60°C, Flow: 0.6 mL/min, lin. Gradient depending on the polarity of the product:

$A_{t_0} = 2\% \text{ A}$ ,  $t_{5.0\text{min}} = 50\% \text{ A}$ ,  $t_{6.0\text{min}} = 98\% \text{ A}$

$B_{t_0} = 2\% \text{ A}$ ,  $t_{5.0\text{min}} = 98\% \text{ A}$ ,  $t_{6.0\text{min}} = 98\% \text{ A}$

Posttime: 1.0 min, Eluent A: acetonitrile, Eluent B: 10 mM ammonium bicarbonate in water (pH=9.5).

## LC-MS method 2

- Apparatus: Waters IClass; Bin. Pump: UPIBSM, SM: UPISMFTN with SO; UPCMA, PDA: UPPDATC, 210-320 nm, SQD: ACQ-SQD2 ESI, mass ranges depending on the molecular weight of the product: pos/neg 100-800 or neg 2000-3000; ELSD: gas pressure 40 psi, drift tube temp: 50°C; column: Waters XSelect™ CSH C18, 50×2.1 mm, 2.5 µm, Temp: 25°C, Flow: 0.5 mL/min, Gradient:  $t_{0min} = 5\%$  A,  $t_{2.0min} = 98\%$  A,  $t_{2.7min} = 98\%$  A, Posttime: 0.3 min, Eluent A: acetonitrile, Eluent B: 10 mM ammonium bicarbonate in water (pH=9.5).

## LC-MS method 3

- Apparatus: Waters IClass; Bin. Pump: UPIBSM, SM: UPISMFTN with SO; UPCMA, PDA: UPPDATC, 210-320 nm, SQD: ACQ-SQD2 ESI, mass ranges depending on the molecular weight of the product: pos/neg 105-800, 500-1200 or 1500-2500; ELSD: gas pressure 40 psi, drift tube temp: 50°C; column: Waters XSelect™ CSH C18, 50×2.1 mm, 2.5 µm, Temp: 40°C, Flow: 0.5 mL/min, Gradient:  $t_{0min} = 5\%$  A,  $t_{2.0min} = 98\%$  A,  $t_{2.7min} = 98\%$  A, Posttime: 0.3 min, Eluent A: 0.1% formic acid in acetonitrile, Eluent B: 0.1% formic acid in water.

## LC-MS method 4

- Apparatus: Waters IClass; Bin. Pump: UPIBSM, SM: UPISMFTN with SO; UPCMA, PDA: UPPDATC, 210-320 nm, SQD: ACQ-SQD2 ESI, mass ranges depending on the molecular weight of the product: pos/neg 100-800 or neg 2000-3000; ELSD: gas pressure 40 psi, drift tube temp: 50°C column: Waters Acquity Shield RP18, 50×2.1 mm, 1.7 µm, Temp: 25°C, Flow: 0.5 mL/min, Gradient:  $t_{0min} = 5\%$  A,  $t_{2.0min} = 98\%$  A,  $t_{2.7min} = 98\%$  A, Posttime: 0.3 min, Eluent A: acetonitrile, Eluent B: 10 mM ammonium bicarbonate in water (pH = 9.5).

## 25 Preparative methods

## Preparative MP-LC method 1

- Instrument type: Reveleris™ prep MPLC; column: Waters XSelect™ CSH C18 (145×25 mm, 10µm); Flow: 40 mL/min; Column temp: room temperature; Eluent A: 10 mM ammoniumbicarbonate in water pH = 9.0; Eluent B: 99% acetonitrile + 1% 10 mM ammoniumbicarbonate in water; Gradient:
- $t_{0min} = 5\%$  B,  $t_{1min} = 5\%$  B,  $t_{2min} = 10\%$  B,  $t_{17min} = 50\%$  B,  $t_{18min} = 100\%$  B,  $t_{23min} = 100\%$  B
- $t_{0min} = 5\%$  B,  $t_{1min} = 5\%$  B,  $t_{2min} = 20\%$  B,  $t_{17min} = 60\%$  B,  $t_{18min} = 100\%$  B,  $t_{23min} = 100\%$  B
- ; Detection UV: 210, 235, 254 nm and ELSD.

## Preparative MP-LC method 2

- Instrument type: Reveleris™ prep MPLC; Column: Phenomenex LUNA C18(3) (150×25 mm, 10µm); Flow: 40 mL/min; Column temp: room temperature; Eluent A: 0.1% (v/v) Formic acid in water, Eluent B: 0.1% (v/v) Formic acid in acetonitrile; Gradient:

$A_{t_{0min}} = 5\% \text{ B}$ ,  $t_{1min} = 5\% \text{ B}$ ,  $t_{2min} = 20\% \text{ B}$ ,  $t_{17min} = 60\% \text{ B}$ ,  $t_{18min} = 100\% \text{ B}$ ,  $t_{23min} = 100\% \text{ B}$

$B_{t_{0min}} = 2\% \text{ B}$ ,  $t_{1min} = 2\% \text{ B}$ ,  $t_{2min} = 2\% \text{ B}$ ,  $t_{17min} = 30\% \text{ B}$ ,  $t_{18min} = 100\% \text{ B}$ ,  $t_{23min} = 100\% \text{ B}$

$C_{t_{0min}} = 5\% \text{ B}$ ,  $t_{1min} = 5\% \text{ B}$ ,  $t_{2min} = 10\% \text{ B}$ ,  $t_{17min} = 50\% \text{ B}$ ,  $t_{18min} = 100\% \text{ B}$ ,  $t_{23min} = 100\% \text{ B}$

$D_{t_{0min}} = 5\% \text{ B}$ ,  $t_{1min} = 5\% \text{ B}$ ,  $t_{2min} = 5\% \text{ B}$ ,  $t_{17min} = 40\% \text{ B}$ ,  $t_{18min} = 100\% \text{ B}$ ,  $t_{23min} = 100\% \text{ B}$

5 ; Detection UV : 210, 235, 254 nm and ELSD.

#### Preparative LC-MS method 3

MS instrument type: Agilent Technologies G6130B Quadrupole; HPLC instrument type: Agilent Technologies 1290 preparative LC; Column: Waters XSelect™ CSH (C18, 150×19mm, 10µm); Flow: 25  
10 ml/min; Column temp: room temperature; Eluent A: 100% acetonitrile; Eluent B: 10 mM ammonium bicarbonate in water pH = 9.0; Gradient:

$A_{t_0} = 20\% \text{ A}$ ,  $t_{2.5min} = 20\% \text{ A}$ ,  $t_{11min} = 60\% \text{ A}$ ,  $t_{13min} = 100\% \text{ A}$ ,  $t_{17min} = 100\% \text{ A}$

$B_{t_0} = 5\% \text{ A}$ ,  $t_{2.5min} = 5\% \text{ A}$ ,  $t_{11min} = 40\% \text{ A}$ ,  $t_{13min} = 100\% \text{ A}$ ,  $t_{17min} = 100\% \text{ A}$

; Detection: DAD (210 nm); Detection: MSD (ESI pos/neg) mass range: 100 – 800; Fraction collection  
15 based on DAD.

#### Preparative LC-MS method 4

MS instrument type: Agilent Technologies G6130B Quadrupole; HPLC instrument type: Agilent Technologies 1290 preparative LC; Column: Waters XBridge Protein (C4, 150×19mm, 10 µm); Flow: 25  
20 ml/min; Column temp: room temperature; Eluent A: 100% acetonitrile; Eluent B: 10 mM ammonium bicarbonate in water pH = 9.0; Gradient:

$A_{t_0} = 2\% \text{ A}$ ,  $t_{2.5min} = 2\% \text{ A}$ ,  $t_{11min} = 30\% \text{ A}$ ,  $t_{13min} = 100\% \text{ A}$ ,  $t_{17min} = 100\% \text{ A}$

$B_{t_0} = 10\% \text{ A}$ ,  $t_{2.5min} = 10\% \text{ A}$ ,  $t_{11min} = 50\% \text{ A}$ ,  $t_{13min} = 100\% \text{ A}$ ,  $t_{17min} = 100\% \text{ A}$

$C_{t_0} = 5\% \text{ A}$ ,  $t_{2.5min} = 5\% \text{ A}$ ,  $t_{11min} = 40\% \text{ A}$ ,  $t_{13min} = 100\% \text{ A}$ ,  $t_{17min} = 100\% \text{ A}$

25 ; Detection: DAD (210 nm); Detection: MSD (ESI pos/neg) mass range: 100 – 800; Fraction collection based on DAD

#### Flash chromatography

Grace Reveleris X2® C-815 Flash; Solvent delivery system: 3-piston pump with auto-priming, 4  
30 independent channels with up to 4 solvents in a single run, auto-switches lines when solvent depletes; maximum pump flow rate 250 mL/min; maximum pressure 50 bar (725 psi); Detection: UV 200-400 nm, combination of up to 4 UV signals and scan of entire UV range, ELSD; Column sizes: 4-330g on instrument, luer type, 750g up to 3000g with optional holder.

35 UV-vis spectrophotometry

Protein concentrations were determined using a Thermo Nanodrop 2000 spectrometer and the following mass  $\epsilon_{280}$  values ((mg/ml) $^{-1}$  cm $^{-1}$ ); Q8c (1.772), Q52c, (1.769), Q17c (1.802) and Q86(c) (2.246). Oligo concentrations were determined using a molar  $\epsilon_{260}$  value of 153,000 M $^{-1}$  cm $^{-1}$ .

5 Ellman's assay was carried out using a Perkin Elmer Lambda 25 Spectrophotometer and a literature molar  $\epsilon_{412}$  value of 14150 M $^{-1}$  cm $^{-1}$  for TNB. Experimentally determined molar  $\epsilon_{495}$  = 58,700 M $^{-1}$  cm $^{-1}$  and  $R_{z280:495}$  = 0.428 were used for SAMSA-fluorescein.

## SEC

10 The conjugates were analysed by SEC using an Akta purifier 10 system and Biosep SEC-s3000 column eluting with DPBS:IPA (85:15). Conjugate purity was determined by integration of the Conjugate peak with respect to impurities/aggregate forms.

## SDS-PAGE and Western Blotting

15 Native proteins and conjugates were analysed under heat denaturing non-reducing and reducing conditions by SDS-PAGE against a protein ladder using a 4-12% bis-tris gel and MES as running buffer (200V, ~40 minutes). Samples were prepared to 0.5 mg/ml, comprising LDS sample buffer and MOPS running buffer as diluent. For reducing samples, DTT was added to a final concentration of 50mM. Samples were heat treated for 2 minutes at 90-95°C and 5  $\mu$ g (10  $\mu$ l) added to each well. Protein ladder (10  $\mu$ l) was loaded without pre-treatment. Empty lines were filled with 1 $\times$  LDS sample buffer (10  $\mu$ l).  
20 After the gel was run, it was washed thrice with DI water (100 ml) with shaking (15 minutes, 200 rpm). Coomassie staining was performed by shaker-incubating the gel with PAGEBlue protein stain (30 ml) (60 minutes, 200 rpm). Excess staining solution was removed, rinsed twice with DI water (100 ml) and destained with DI water (100 ml) (60 minutes, 200 rpm). The resulting gel was imaged and processed using imageJ.

25

## TBEU-PAGE

Native protein, conjugates and BNA standard were analysed under heat denaturing non-reducing and reducing conditions by TBEU-PAGE against an oligo ladder using a 15% TBE-Urea gel and TBE as running buffer (180V, ~60 minutes). Samples were prepared to 0.5 mg/ml, and BNA standard was  
30 prepared to 20  $\mu$ g/ml, respectively, all comprising TBE Urea sample buffer and purified H<sub>2</sub>O as diluent. Samples and standards were heat treated for 3 minutes at 70°C and 10  $\mu$ l added to each well, equating to 5  $\mu$ g of protein and conjugate samples, and 0.2  $\mu$ g of BNA, per lane. Oligo ladder reconstituted to 0.1  $\mu$ g/band/ml in TE pH 7.5 (2  $\mu$ l) was loaded without pre-treatment. After the gel was run, it was stained with freshly prepared ethidium bromide solution (1  $\mu$ g/ml) with shaking (40 minutes, 200 rpm).  
35 The resulting gel was visualised by UV epi-illumination (254 nm), imaged and processed using imageJ.

## MALDI-TOF-MS

MALDI-TOF spectra were recorded on a MALDI-Mass Spectrometer (Bruker Ultraflex III). Typically, the sample dissolved in MilliQ water in nanomolar to micromolar range was spotted on the target (MTP 384 target plate polished steel T F, Bruker Daltons) using either super-DHB (99%, Fluka) or sinapinic acid (SA, 99%, Sigma-Aldrich) as the matrix dissolved in acetonitrile (MADLI-TOF-MS tested, Sigma) / 0.1% TFA (7:3 v/v) via the dried-droplet-method. PepMix (Peptide Calibration Standard, Bruker Daltons) or ProteMass (Protein Calibration Standard, Sigma-Aldrich) served as calibration standards.

#### **V<sub>HH</sub>-L-BNA synthesis (Figure 40)**

Intermediate 1:

#### **BNA-DTME (molecule 3)**

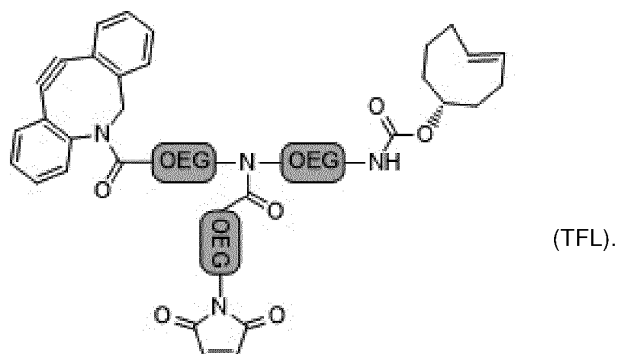
To an aliquot of ApoB BNA disulfide (9.4 mg, 1.6  $\mu$ mol, 1.00 ml) prepared by reconstitution of lyophilised BNA in TBS pH 7.5 to 10 mg/ml, was added an aliquot of freshly prepared DTT solution (50.0 mg/ml, 10 mole equivalents, 13.0  $\mu$ mol), the mixture vortexed briefly then incubated for 60 minutes at 37°C with roller-mixing. After incubation, the resulting BNA-SH was concentrated to <1 ml and purified by gel filtration using a PD10 G25 desalting column eluting with TBS pH 7.5. The resulting BNA-SH (6.4 mg, 1.1  $\mu$ mol, 2.00 ml) was reacted with freshly prepared DTME solution (20 mg/ml, 10 mole equivalents, 11.0  $\mu$ mol, 0.171 ml) in DMSO, the mixture vortexed briefly then incubated for 60 minutes at 20°C. After, the BNA-DTME (5.4 mg, 0.92  $\mu$ mol, 1.22 mg/ml) was obtained following purification by gel filtration using a PD10 G25 desalting column into TBS pH 7.5. The BNA-DTME was stored at 20°C until conjugated.

#### **V<sub>HH</sub>-L-BNA (molecule 5)**

An aliquot of V<sub>HH</sub> (3.0 mg, 0.2  $\mu$ mol) was concentrated by ultrafiltration using a vivaspin T4 5 kDa MWCO centrifugal filter and buffer exchanged into TBS pH 7.5. To the concentrated V<sub>HH</sub> (2.8 mg, 0.19  $\mu$ mol, 2.83 mg/ml) was added an aliquot of freshly prepared TCEP solution (5.0 mg/ml, 4 mole equivalents, 0.77  $\mu$ mol), the mixture vortexed briefly then incubated for 60 minutes at 37 °C with roller-mixing. After incubation, the resulting V<sub>HH</sub>-SH was purified by gel filtration using a zeba spin desalting column then repeated centrifugal-wash cycles using a vivaspin T4 5 kDa MWCO centrifugal filter into TBS pH 7.5. The resulting V<sub>HH</sub>-SH (2.0 mg, 0.13  $\mu$ mol, 0.90 mg/ml, VHH:SH = 1.0) was reacted with BNA-DTME (1.1 mole equivalents, 0.83 mg, 0.14  $\mu$ mol), the mixture vortexed briefly then incubated overnight at 20°C. After, the reaction mixture was concentrated using a vivaspin T4 10 kDa MWCO centrifuge tube and purified by gel filtration using a 1.6 × 35 cm Superdex 200PG column eluting into DPBS pH 7.5. The product fractions were collected, pooled and concentrated to <1ml using a vivaspin T4 10KDa MWCO centrifuge tubes. Yield: 0.81 mg, 0.40 mg/ml, 29%.

#### **Trifunctional linker-(L-SO1861)-(L-BNA)-(V<sub>HH</sub>) (Figure 41, 42)**

Referring to Figure 41-44, the conjugate comprising  $V_{HH}$ , saponin SO1861 and ApoB BNA was synthesized, using the trifunctional linker TFL with molecular structure:



The conjugate SO1861-L-ApoB BNA- $V_{HH}$  synthesis ( $V_{HH}$ -ApoB BNA-SO1861 conjugate) was formed.

10 **SO1861-L-azide** (molecule 7)

To SO1861 60 mg, 0.032 mmol) and 1-azido-3,6,9,12-tetraoxapentadecane-15-hydrazide (39.3 mg, 0.129 mmol) was added methanol (extra dry, 1.00 mL) and TFA (9.86  $\mu$ L, 0.129 mmol) and the reaction mixture was shaken for 1 min and left standing at room temperature. After 2 hours the reaction mixture was subjected to preparative MP-LC.<sup>1</sup> Fractions corresponding to the product were immediately pooled together, frozen and lyophilized overnight to give the title compound (58.4 mg, 84%) as a white fluffy solid. Purity based on LC-MS 100%.

LRMS (m/z): 2150 [M-1]<sup>1-</sup>

LC-MS r.t. (min): 1.10<sup>3B</sup>

Intermediate 2:

20 **Trifunctional linker-(L-SO1861)-(TCO)-(Maleimide)** (molecule 8)

A solution of TFL-(DBCO)-(TCO)-(Maleimide) (15 mg, 12.5  $\mu$ mol) in DMF (2.0 mL) was added to SO1861-L-azide (26.8 mg, 12.5  $\mu$ mol). The reaction mixture was shaken for 30 min and left standing at room temperature. After 30 min the reaction mixture was submitted to preparative MP-LC.<sup>1C</sup> Fractions corresponding to the product were immediately pooled together, frozen and lyophilized overnight to give the title compound (31.4 mg, 75%) as a white fluffy solid. Purity based on LC-MS 96%.

MS (m/z): 3354 [M-1]<sup>1-</sup>

Intermediate 3:

**Trifunctional linker-(L-SO1861)-(TCO)-(V<sub>HH</sub>) (molecule 9)**

An aliquot of V<sub>HH</sub> (3.0 mg, 0.2  $\mu$ mol) was concentrated by ultrafiltration using a vivaspin T4 5 kDa MWCO centrifugal filter and buffer exchanged into TBS pH 7.5. To the concentrated V<sub>HH</sub> (2.8 mg, 0.19  $\mu$ mol, 2.83 mg/ml) was added an aliquot of freshly prepared TCEP solution (5.0 mg/ml, 4 mole equivalents, 0.77  $\mu$ mol), the mixture vortexed briefly then incubated for 60 minutes at 37°C with roller-mixing. After incubation, the resulting V<sub>HH</sub> was purified by gel filtration using a zeba spin desalting column then repeated centrifugal-wash cycles using a vivaspin T4 5 kDa MWCO centrifugal filter into TBS pH 7.5. The resulting V<sub>HH</sub>-SH (2.0 mg, 0.13  $\mu$ mol, 0.90 mg/ml, VHH Q8c:SH = 1.0) was reacted with TFL-(L-SO1861)-(TCO)-(Maleimide) (1.1 mole equivalents, 0.5 mg, 0.15  $\mu$ mol), the mixture vortexed briefly then incubated overnight at 20°C. After, the reaction mixture was concentrated using a vivaspin T4 10 kDa MWCO centrifuge tube and purified by gel filtration using a 1.6  $\times$  35 cm Superdex 200PG column eluting into DPBS pH 7.5. The product fractions were collected, pooled and concentrated to <1 ml using a vivaspin T4 10 kDa MWCO centrifuge tubes. Yield: 1.3 mg, 0.40 mg/ml, 54%.

MS (m/z): 18062 [M + Na]<sup>1+</sup>

15 Intermediate 4:

**methyldetrazine-L-ApoB BNA (molecule 11)**

To ApoB BNA-disulfide (5.00 mg, 0.686  $\mu$ mol) was added a solution of 20 mM ammonium bicarbonate with 2.5 mM TCEP (1.00 mL, 2.5  $\mu$ mol). The reaction mixture was shaken for 1 min and left standing at room temperature. After 1 hour the reaction mixture was filtered by using a centrifugal filter with a molecular weight cut-off of 3000 Da (5000  $\times$  g for 30 min, 2  $\times$  0.50 mL). Next, the residue solution was washed twice with a solution of 20 mM ammonium bicarbonate with 2.5 mM TCEP (0.50 mL), each time filtered under the same conditions described above. As next, the residue solution was diluted with 20 mM ammonium bicarbonate (1.50 mL) and the resulting mixture was directly added to a solution of (E)-1-(4-((2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl)hydrazineylidene)methyl)benzamido)-N-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)-3,6,9,12-tetraoxapentadecan-15-amide (1.36 mg, 1.73  $\mu$ mol) in acetonitrile (0.5 mL). The reaction mixture was shaken for 1 min and left standing at room temperature. After 30 min the reaction mixture was frozen and lyophilized overnight to yield the crude title product as a pink fluffy solid. To the crude product was added a solution of 20 mM ammonium bicarbonate (1.50 mL) and the resulting suspension was filtered over a 0.45  $\mu$ m syringe filter. The filtrate was lyophilized overnight to yield the title product (5.44 mg, quant) as a pink fluffy solid. Purity based on LC-MS 90%.

LRMS (m/z): 2648 [M-3]<sup>3-</sup>

LC-MS r.t. (min): 0.62<sup>4</sup>

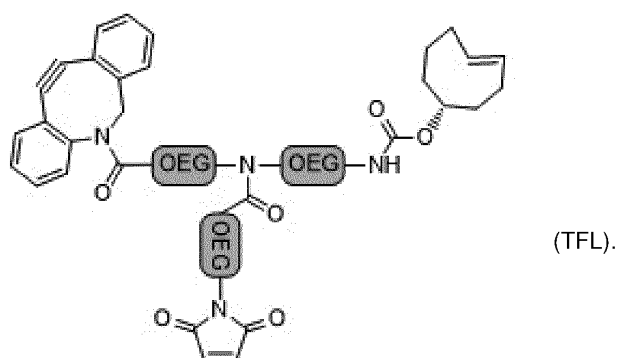
35

**Trifunctional linker-(L-SO1861)-(L-BNA)-(V<sub>HH</sub>) (molecule 12)**

To a solution of trifunctional linker-(L-SO1861)-(TCO)-(V<sub>HH</sub>) (3 mg, 0.16  $\mu$ mol, 0.40 mg/ml) dissolved in DPBS pH 7.5 Methyltetrazine-BNA oligo (0.63 mg, 96 nmol) was added. The reaction mixture was shaken for 1 min and was incubated overnight. After, the reaction mixture was concentrated using a vivaspin T4 10 kDa MWCO centrifuge tube and purified by gel filtration using a 1.6  $\times$  35 cm Superdex 200PG column eluting into DPBS pH 7.5. The product fractions were collected, pooled and concentrated to <1ml using a vivaspin T4 10KDa MWCO centrifuge tubes. Yield: 2.4 mg, 0.40 mg/ml, 61%.

#### Trifunctional linker-(dendron(-L-SO1861)<sup>4</sup>)-(L-BNA)-(V<sub>HH</sub>) (Figure 43 and 44)

Referring to Figure 41-44, the conjugate comprising V<sub>HH</sub>, dendron(-L-SO1861)<sup>4</sup> and ApoB BNA was synthesized, using the trifunctional linker (TFL) with molecular structure:



The conjugate dendron(-L-SO1861)<sup>4</sup>-L-BNA-V<sub>HH</sub> synthesis (V<sub>HH</sub>-BNA-dendron(-L-SO1861)<sup>4</sup> conjugate) was formed.

#### dendron(-L-SO1861)<sup>4</sup>-azide (molecule 13)

Dendron(SO1861)<sup>4</sup>-amine (6.81 mg, 0.748  $\mu$ mol) and 2,5-dioxopyrrolidin-1-yl 1-azido-3,6,9,12-tetraoxapentadecan-15-oate (2.90 mg, 7.48  $\mu$ mol) were dissolved in DMF(1.00 mL). Next, DIPEA (1.302  $\mu$ L, 7.48  $\mu$ mol) was added and the mixture was shaken for 1 min and left standing at room temperature. After 2 hours the reaction mixture was subjected to preparative LC-MS.<sup>30</sup> Fractions corresponding to the product were immediately pooled together, frozen and lyophilized overnight to give the title compound (5.86 mg, 84%) as a white fluffy solid. Purity based on LC-MS 90%.

LRMS (m/z): 2344 [M-4]<sup>+</sup>

LC-MS r.t. (min): 4.78<sup>5B</sup>

Intermediate 5:

#### Trifunctional linker-(dendron(-L-SO1861)<sup>4</sup>)-(TCO)-(Maleimide) (molecule 14)

A solution of TFL-(DBCO)-(TCO)-(Maleimide) (5 mg, 4.1  $\mu$ mol) in DMF (2.0 mL) was added to dendron(-L-SO1861)<sub>4</sub>-azide (39.4 mg, 4.2  $\mu$ mol). The reaction mixture was shaken for 90 min and left standing at room temperature. After 90 min the reaction mixture was submitted to preparative MP-LC.<sup>1C</sup> Fractions corresponding to the product were immediately pooled together, frozen and lyophilized overnight to give the title compound (31.2 mg, 72%) as a white fluffy solid. Purity based on LC-MS 96%.

MS (m/z): 10611 [M+Na]<sup>1+</sup>

Intermediate 6:

**Trifunctional linker-(dendron(-L-SO1861)<sup>4</sup>)-(TCO)-(V<sub>HH</sub>) (molecule 15)**

An aliquot of V<sub>HH</sub> (3.0 mg, 0.2  $\mu$ mol) was concentrated by ultrafiltration using a vivaspin T4 5 kDa MWCO centrifugal filter and buffer exchanged into TBS pH 7.5. To the concentrated V<sub>HH</sub> (2.8 mg, 0.19  $\mu$ mol, 2.83 mg/ml) was added an aliquot of freshly prepared TCEP solution (5.0 mg/ml, 4 mole equivalents, 0.77  $\mu$ mol), the mixture vortexed briefly then incubated for 60 minutes at 37°C with roller-mixing. After incubation, the resulting V<sub>HH</sub>-SH was purified by gel filtration using a zeba spin desalting column then repeated centrifugal-wash cycles using a vivaspin T4 5 kDa MWCO centrifugal filter into TBS pH 7.5. The resulting V<sub>HH</sub>-SH (2.0 mg, 0.13  $\mu$ mol, 0.90 mg/ml, V<sub>HH</sub>:SH = 1.0) was reacted with TFL-(dendron(-L-SO1861)<sup>4</sup>)-(TCO)-(Maleimide) (1.1 mole equivalents, 1.5 mg, 0.14  $\mu$ mol), the mixture vortexed briefly then incubated overnight at 20°C. After, the reaction mixture was concentrated using a vivaspin T4 10 kDa MWCO centrifuge tube and purified by gel filtration using a 1.6 × 35 cm Superdex 200PG column eluting into DPBS pH 7.5. The product fractions were collected, pooled and concentrated to <1ml using a vivaspin T4 10kDa MWCO centrifuge tubes. Yield: 0.95 mg, 0.40 mg/ml, 29%. The V<sub>HH</sub> is for example V<sub>HH</sub> Q8c:SH which is an sdAb for binding to HIVgp41 and produced by clone anti-HIVgp41 Q8C-tag, or the V<sub>HH</sub> is for example V<sub>HH</sub> 7D12 (SEQ ID NO: 75) or V<sub>HH</sub> 9G8 (SEQ ID NO: 76), or the tandem of two V<sub>HH</sub>'s 7D12-9G8 (SEQ ID NO: 74).

MS (m/z): 25295 [M + Na]<sup>1+</sup>

Intermediate 4:

**methyltetrazine-L-ApoB BNA (molecule 11)**

To ApoB BNA disulfide (5.00 mg, 0.686  $\mu$ mol) was added a solution of 20 mM ammonium bicarbonate with 2.5 mM TCEP (1.00 mL, 2.5  $\mu$ mol). The reaction mixture was shaken for 1 min and left standing at room temperature. After 1 hour the reaction mixture was filtered by using a centrifugal filter with a molecular weight cut-off of 3000 Da (5000 × g for 30 min, 2 × 0.50 mL). Next, the residue solution was washed twice with a solution of 20 mM ammonium bicarbonate with 2.5 mM TCEP (0.50 mL), each time filtered under the same conditions described above. As next, the residue solution was diluted with 20 mM ammonium bicarbonate (1.50 mL) and the resulting mixture was directly added to a solution of (E)-1-(4-(((2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl)hydrazineylidene)methyl)benzamido)-N-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)-3,6,9,12-tetraoxapentadecan-15-amide (1.36 mg, 1.73  $\mu$ mol)

in acetonitrile (0.5 mL). The reaction mixture was shaken for 1 min and left standing at room temperature. After 30 min the reaction mixture was frozen and lyophilized overnight to yield the crude title product as a pink fluffy solid. To the crude product was added a solution of 20 mM ammonium bicarbonate (1.50 mL) and the resulting suspension was filtered over a 0.45 µm syringe filter. The filtrate was lyophilized overnight to yield the title product (5.44 mg, quant) as a pink fluffy solid. Purity based on LC-MS 90%.

LRMS (m/z): 2648 [M-3]<sup>3-</sup>

LC-MS r.t. (min): 0.62<sup>4</sup>

#### Trifunctional linker-(dendron(-L-SO1861)<sup>4</sup>)-(L-BNA)-(V<sub>HH</sub>) (molecule 16)

To a solution of TFL-(dendron(-L-SO1861)<sup>4</sup>)-(TCO)-(V<sub>HH</sub>) (2.2 mg, 87 nmol, 0.40 mg/ml) dissolved in DPBS pH 7.5 Methyltetrazine-BNA (0.4 mg, 60 nmol) was added. The reaction mixture was shaken for 1 min and was incubated overnight. After, the reaction mixture was concentrated using a vivaspin T4 10 kDa MWCO centrifuge tube and purified by gel filtration using a 1.6 × 35 cm Superdex 200PG column eluting into DPBS pH 7.5. The product fractions were collected, pooled and concentrated to <1 ml using a vivaspin T4 10 kDa MWCO centrifuge tubes. Yield: 2.0 mg, 0.40 mg/ml, 73%.

#### LITERATURE REFERENCES

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- Marta Kijanka & Frank-Jan Warnders & Mohamed El Khattabi & Marjolijn Lub-de Hooge & Gooitzen M. van Dam & Vasilis Ntziachristos & Liesbeth de Vries & Sabrina Oliveira & Paul M. P. van Bergen en Henegouwen, "Rapid optical imaging of human breast tumour xenografts using anti-HER2 V<sub>HH</sub>s site-directly conjugated to IRDye 800CW for image-guided surgery", Eur J Nucl Med Mol Imaging (2013) 40:1718–1729 DOI 10.1007/s00259-013-2471-2
- Karen Mercier, Raimond Heukers and Chiraz Frydman, "Surface Plasmon Resonance imaging (SPRi) - Production of a single domain antibody Q17c directed against recombinant HER2 protein and its binding study by Surface Plasmon Resonance imaging technology", Horiba Application Note Pharmaceuticals SPRi 42, 2019

#### SEQ ID NOs

SEQ ID NO: 1: Amino-acid coding DNA sequence of Anti-HER2 sdAb 2Rb17c from camelid

gaagttcagctgcaggaatctgggtgggtctggtcagccgggtggtctctgcgtctgtcttcgcggcgctctggttcattcttctaacgacgcg  
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- 5 SEQ ID NO: 2: Amino-acid sequence of Anti-HER2 sdAb 2Rb17c from camelid  
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GRFTISRDNARKRTLYLQMNSLKDEDTALYYCVTGYGVTKTPTGQGTQVTVSSHHHHHHSPSTPPTPS  
PSTPPC
- 10 SEQ ID NO: 3: Amino-acid coding DNA sequence of Anti-HER2 sdAb NB2 from *Camelus dromedarius*  
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- 15 SEQ ID NO: 4: Amino-acid sequence of Anti-HER2 sdAb NB2 from *Camelus dromedarius*  
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- 20 SEQ ID NO: 5: Amino-acid coding DNA sequence of Anti-HER2 sdAb pcNB2, a synthetic construct  
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- 25 SEQ ID NO: 6: Amino-acid sequence of Anti-HER2 sdAb pcNB2, a synthetic construct  
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- 30 SEQ ID NO: 7: amino-acid coding DNA sequence of Anti-HER1 sdAb 7D12 from camelid  
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- 35 SEQ ID NO: 8: amino-acid sequence of Anti-HER1 sdAb 7D12 from camelid  
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- 40 SEQ ID NO: 9: amino-acid coding DNA sequence of Anti-HER1 sdAb 9G8 from camelid

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5

SEQ ID NO: 10: amino-acid sequence of Anti-HER1 sdAb 9G8 from camelid

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- 10 SEQ ID NO: 11: Amino-acid coding DNA sequence of Anti-VGFR2 sdAb NTV1, a synthetic construct  
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15

SEQ ID NO: 12: Amino-acid sequence of Anti-VGFR2 sdAb NTV1, a synthetic construct

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- 20 SEQ ID NO: 13: Amino-acid coding DNA sequence of Anti-VGFR2 sdAb NTV2, a synthetic construct  
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25

SEQ ID NO: 14: Amino-acid sequence of Anti-VGFR2 sdAb NTV2, a synthetic construct

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- 30 SEQ ID NO: 15: Amino-acid coding DNA sequence of Anti-VGFR2 sdAb NTV3, a synthetic construct  
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35

SEQ ID NO: 16: Amino-acid sequence of Anti-VGFR2 sdAb NTV3, a synthetic construct

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- 40 SEQ ID NO: 17: Amino-acid coding DNA sequence of Anti-VGFR2 sdAb NTV4, a synthetic construct

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5

SEQ ID NO: 18: Amino-acid sequence of Anti-VGFR2 sdAb NTV4, a synthetic construct  
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GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAAVHEKAADMNFWGQGLTVTVSS

10 SEQ ID NO: 19: amino-acid coding DNA sequence of Anti-human CD19 sdAb SRB-85 from Bactrian camel

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ctcttgaccgcgacgcggttaaagggtggttcgtcagccgccgggtaaaggctggaatgggttctctgcgtatctctcaggacaacgcga  
aaaacacccgttacctgcagatgaactctctgaaaccggaagacaccgcgatgtactactgcgcgtgtctaaatgctacacccgtgtttacga  
15 ctactggggtcaggttaccaggttaccgtttctctggt

SEQ ID NO: 20: amino-acid sequence of Anti-human CD19 sdAb SRB-85 from Bactrian camel  
EVQLLESGGGLVQPGGSLRSCEASGFNAMTSIDSWTDAVKGWVRQPPGKGLEWVSRFAISQDNAK  
NTVYLQMNSLKPEDTAMYYCALSKCYTRVYDYWGQGTQVTVSS

20

SEQ ID NO: 21: amino-acid coding DNA sequence of Anti-human CD19 sdAb SRB-37 from Bactrian camel

gaagttcagctgcaggaatctggtggtggtctggttcagccgggtggttctctgcgtctgtcttgcgcggcgctggtttcatctacatggttggtatca  
aaaccgaacgtgacggtgttaaagggtggttcgtcagggcgccgggtaaaggctggaatggctgtctcttaccatcccgcgtgacaacgc  
25 gaaaaacacccgtgtacctgcagatgaacaacctgaaatctgaagacaccgcgtgtactactgcgcgaccgaagaaaacgactgggtgta  
gggtaccaggttaccgtttctctggt

SEQ ID NO: 22: amino-acid sequence of Anti-human CD19 sdAb SRB-37 from Bactrian camel  
EVQLQESGGGLVQPGGSLRLSCAASGFIYMGVGIKTERDGVKGWVRQAPGKGLEWLSRFTIPRDNK  
30 NTLYLQMNNLKSEDTALYYCATEENDWGQGTQVTVSS

SEQ ID NO: 23: Amino-acid coding DNA sequence of Anti-CTLA-4 sdAb NB16 from *Camelus dromedarius*

caggttcagctgcaggaatctggtggtggttctggttcagggcggtggttctctgcgtctgtcttgaccgcgtctggtttcggtgttgacggtaccgac  
35 atgggttggtaccgtcagggcgccgggtaacgaatgcgaactggttctctatctcttctatcggtatcggttactactctgaatctgtaaaggctggtt  
caccatctctcgtgacaacgcgaaaaacacccgttacctgcagatgaactctctgcgtccggacgacaccgcggttactactgcggtcgtcgtt  
ggatcgggttaccgttcggttaactggggtcgtggtaccaggttaccgtttctct

SEQ ID NO: 24: Amino-acid sequence of Anti-CTLA-4 sdAb NB16 from *Camelus dromedarius*

QVQLQESGGGSVQAGGSLRLSCTASGFGVDGTDGMGWYRQAPGNECELVSSISSIGIGYYSESVKGR  
FTISRDNANKNTVYLMNSLRPDDTAVYYCGRRWIGYRCGNWGRGTQVTVSS

5 SEQ ID NO: 25: Amino-acid coding DNA sequence of Anti-CTLA-4 sdAb NB36 from *Camelus dromedarius*

caggttcagctgcaggaatctggtggtggttctgtcaggcgggtggttctctcgctctgtcttgaccggttctcgttacacctacacccatgggttg  
ttccgtcaggcgcgggtaagaacgtgaaggtgtgttgatcaccggttcggttctcgttctacgaggactctgttaaaggctgttcacat  
ctctcgtgacaacgcgaacaacacccatctctgcagatgaactctctgaaaccggaagactctgcgatgtactactgcgcggcgctggttctt  
ctggtacctctacaaatggaacgaatacgggttctacaactactggggtcagggtaccaggtaccggttcttct

10

SEQ ID NO: 26: Amino-acid sequence of Anti-CTLA-4 sdAb NB36 from *Camelus dromedarius*  
QVQLQESGGGSVQAGGSLRLSCTGSRITYTMGWFRQAPGKEREGVVAITAFGSPFYADSVKGRFTI  
SRDNANNTIFLQMNSLKPEDSAMYCAARGSSGTSYKWNEYGSYNYWGQGTQVTVSS

15 SEQ ID NO: 27: Amino-acid coding DNA sequence of Anti-CTLA-4 sdAb NB91 from *Camelus dromedarius*

caggttcagctgcaggaatctggtggtggttctgtcaggcgggtggttctctcgctctgtcttgccgcggtctaaatacacctcttgcattgggttg  
ttccgtcaggcgcgggtaagaacgtgaaggtgtgtgcacatcgactctggtccggtaccctgtacgaggactctgttaaaggctgttcacc  
atctctaaagacaacgcgaaaaacacccgtacctggaatgtctaccctgaaaccggacgacaccgcgatgtactactgcgcggcggggtc  
20 cgatgtactctggttcttgcactacaactactggggtcagggtaccaggtaccggttcttct

25 SEQ ID NO: 28: Amino-acid sequence of Anti-CTLA-4 sdAb NB91 from *Camelus dromedarius*  
QVQLQESGGGSVQAGGSLRLSCAASKYTSCMGWFRQAPGKEREVVAHIDSGPRTLYADSVKGRFTI  
SKDNAKNTLYLEMSTLKPDDTAMYYCAAGPMYSGSCNYYWGQGTQVTVSS

SEQ ID NO: 29: Amino-acid coding DNA sequence of Anti-human PD-L1 sdAb A1 from *Camelus dromedarius*

Caggttcagctgcaggaatctggtggtggttctgtcaggcgggtggttctctcgctctgtcttgccgcggtctggttaccctggactactacgc  
gatcgggtggttcctcaggcgcgggtaagaacgtgaaggtgttcttgcacatctctcttgacggttctacactactacgcggactctgttaaag  
30 gtcgtttaccatctctcgtgacaacgcgaaaaacacccgttacctgcagatgttctctgaaaccggaagacaccgcggttactactgcggta  
tctctggttcttgcctgctggaagactacggtatggactactggggtaaagggtaccaggtaccggttcttct

35 SEQ ID NO: 30: Amino-acid sequence of Anti-human PD-L1 sdAb A1 from *Camelus dromedarius*  
QVQLQESGGGLVQPGGSLRLSCAASGFTLDYYAIGWFRQAPGKEREGVSCISSDGSSTYYADSVKG  
RFTISRDNANKNTVYLMSSSLKPEDTAVYYCGISGSCLEDYGM DYWGKGTQVTVSS

SEQ ID NO: 31: Amino-acid coding DNA sequence of Anti-human PD-L1 sdAb B1 from *Camelus dromedarius*

40 caggttcagctgcaggaatctggtggtggttctgtcaccgggtggttctctcgctctgtcttgccgcggtctggttctcttgacaactacgcg  
atcgggtggttcctcaggcgcgggtaagaacgtgaaggtgttcttgcacatctcttgggttctgaaggctcgttactacgcggactctgttaa

ggtcgtttcaccatctctcgtgacaacgcgaaaaacaccgcgttctcgcagatgaactctctgaaaccggaagacaccgcggactactactgc  
gcgaccgttggtttctgctctctcagtacggtatggaattcgttggtgactactgggtcaggtaccaggtaccggtttcttct

SEQ ID NO: 32: Amino-acid sequence of Anti-human PD-L1 sdAb B1 from *Camelus dromedaries*

5 QVQLQESGGGLVHPGGSRLRLSCAASGFSLDNYAIGWFRQAPGKEREGVSCISSGSEGRRRYYADSVK  
GRFTISRDNKNTAFLQMNSLKPEDTADYYCATVGFCSQYGMFVGDYWGQGTQVTVSS

SEQ ID NO: 33: Amino-acid sequence of Anti-mouse serum albumin sdAb MSA21 (organism: artificial  
sequence)

10 QVQLQESGGGLVQPGGSRLRLSCEASGFTFSRFGMTWVRQAPGKGVEWVSGISLGDSTLYADSVK  
GRFTISRDNKNTLYLQMNSLKPEDTAVYYCTIGGSLNPGGQGTQVTVSS

SEQ ID NO: 34: Amino-acid sequence of Anti-human serum albumin sdAb Alb-1 (organism: artificial  
sequence)

15 AVQLVESGGGLVQPGNSRLRLSCAASGFTFRSFGMSWVRQAPGKEPEWVSSISGSGSDTLYADSVKG  
RFTISRDNKNTLYLQMNSLKPEDTAVYYCTIGGSLSRSSQGTQVTVSS

SEQ ID NO: 35: Amino-acid sequence of Anti-human serum albumin sdAb Alb23 (Humanized, optimized  
Alb1) (organism: artificial sequence)

20 EVQLLES GGGLVQPGGSRLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVK  
GRFTISRDNKNTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTQVTVSS

SEQ ID NO: 36: Amino-acid sequence of Anti-EGFR V<sub>HH</sub> 7A5 (organism: artificial; recombinant peptide)

EVQLVESGGGLVQAGGSRLRLSCAASDRTFSSNMGWFRQAPGKEREFVAAIGWGGLETHYSDSVK  
25 GRFTISRDNKNTVYLQMNSLKPEDTARYYCAVSSTRTVIYTLPRMYNYWGQGTQVTVSS

SEQ ID NO: 37: Amino-acid sequence of Anti-EGFR V<sub>HH</sub> 7D12 (organism: artificial; recombinant  
peptide)

QVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMGWFRQAPGKEREFVSGISWRGDSTGYADSVK  
30 GRFTISRDNKNTVDLQMNSLKPEDTAIYYCAAAGSAWYGTLYEYDYWGQGTQVTVSS

SEQ ID NO: 38: Amino-acid sequence of Anti-EGFR V<sub>HH</sub> 7C12 (organism: artificial; recombinant  
peptide)

AVQLVESGGGSVQAGGSRLRLTCAASGRTSRSYGMGWFRQAPGKEREFVSGISWRGDSTGYADSVK  
35 GRFTISRDNKNTVDLQMNSLKPEDTAIYYCAAAGSTWYGTLYEYDYWGQGTQVTVSS

SEQ ID NO: 39: Amino-acid sequence of Anti-insulin-like growth factor 1 receptor V<sub>HH</sub> 4B11 (organism:  
artificial; recombinant peptide)

EVQLVESGGGLVQPGGSRLRLSCAASGSIFTFNAMGWYRQAPGKQRELVAVIISGGSTHYVDSVKGRF  
40 TISRDNKKNMYYLQMNSLKPEDTAVYYCNVKKFGDYWGQGTQVTVSS

SEQ ID NO: 40: Amino-acid sequence of Anti-insulin-like growth factor 1 receptor V<sub>HH</sub> 3G7 (organism: artificial; recombinant peptide)

DVQLVESGGGLVQAGGSLRLSCAASESISTINVMAWYRQAPGKQRELVAEITRSGRTNYVDSVKGRF  
5 TISRDNANKNTMYLQMNSLNLEDTAVYYCRTIDGSWREYWGGGTQVTVSS

SEQ ID NO: 41: Amino-acid sequence of Anti-insulin-like growth factor 1 receptor V<sub>HH</sub> 2C7 (organism: artificial; recombinant peptide)

QVKLEESGGGLVQPGGSLRLSCVASGRTFSNYAIVIGWFRQAPGQEREFVAAINWNSRSTYYADSVK  
10 GRFTISRLNARNTVYLMNRLKPEDTAVYDCAASHSDYGGTNANLYDYWGQGTQVTVSS

SEQ ID NO: 42: Amino-acid sequence of Anti-insulin-like growth factor 1 receptor V<sub>HH</sub> 1C7 (organism: artificial; recombinant peptide)

QVKLEESGGGLVQAGGSLRLSCVASGRTFSRTANAWFRQAPGKEREFVATITWNSGTTRYADSVKG  
15 RFFISKDSAKNTIYLEMNSLEPEDTAVYYCAATAAAVITPTRGYNYWGQGTQVTVSS

SEQ ID NO: 43: Amino-acid sequence of Anti-CEACAM V<sub>HH</sub> NbCEA5 (organism: artificial; recombinant peptide)

EVQLVESGGGSVQAGGSLRLSCAASGDTYGSYWMGWFRQAPGKEREGVAAINRGGGYTVYADSV  
20 KGRFTISRDTAKNTVYLMNSLRPDDTADYYCAASGVLGGLHEDWFNYWGQGTQVTVSS

SEQ ID NO: 44: Amino-acid sequence of Anti-CEACAM V<sub>HH</sub> CEA5 (organism: artificial; recombinant peptide)

EVQLVESGGGSVQAGGSLRLSCAASGDTYGSYWMGWFRQAPGQEREVAAINRGGGYTVYADSV  
25 KGRFTISRDNANKNTLYLQMNSLRPDDTADYYCAASGVLGGLHEDWFNYWGQGTQVTVSS

SEQ ID NO: 45: Amino-acid sequence of Anti-CD123 V<sub>HH</sub> 57A07 (organism: artificial; recombinant peptide)

EVQLVESGGGLVQAGGSLRLSCAASGSIFSGNVMGWYRRQAPGKEREWVAAIASGGSIIYRDSVKG  
30 RFTISRDNANKNTVYLMNSLKPEDTAVYYCNSHPPTLPYWGLGTQVTVSS

SEQ ID NO: 46: Amino-acid sequence of Anti-CD123 V<sub>HH</sub> 57B04 (organism: artificial; recombinant peptide)

EVQLVESGGGLVQPGGSLRLSCAASGINFRFNSMGWRRRAPGKEREWVAITSGDITNYRDSVRG  
35 RFTISRDNVKNNTVYLMNTLKLEDTAVYYCNTFPPIADYWGLGTQVTVSS

SEQ ID NO: 47: Amino-acid sequence of Anti-CD123 V<sub>HH</sub> 51D09 (organism: artificial; recombinant peptide)

EVQLVESGGGLVQPGGSLRLSCAASGSIFSGNTMGWYRQAPGKQRELVAAISSGGSTDYADSVKGR  
40 FTISRDNKNTVYLMNSLRPEDTAVYYCNAAILLYRLYGYEEGDYWGLGTQVTVSS

SEQ ID NO: 48: Amino-acid sequence of Anti-CD123 V<sub>HH</sub> 55C05 (organism: artificial; recombinant peptide)

EVQLVESGGGLVPAGDSLRLSCVASGRSLNTYTMGWFRQAPGKECEEVAAINWNGVYRDYADSAK  
GRETASRDNAMNTVFLQMNSLKPEDTAVYFCATATQGWDRHTEPSDFGSWGLGTQVTVSS

5

SEQ ID NO: 49: Amino-acid sequence of Anti-CD123 V<sub>HH</sub> 50F07 (organism: artificial; recombinant peptide)

EVQLVESGGGLVQPGGSLRLSCTGSGSTFSINAMGWYRQAPGKQRELVAAITSGGRTNYADSVKGR  
FTISRDNSKNTVYLQMNSLRPEDTAVYYCNARISAGTAFWLWSDYEWGLGTLTVTVSS

10

SEQ ID NO: 50: Amino-acid sequence of Anti-CD123 V<sub>HH</sub> 55F03 (organism: artificial; recombinant peptide)

EVQLVESGGGLVQAGGPLRLSCAASGRTFSSYVMGWFRQAPGKEREFVAAIYWSNGKTQYTDSVK  
GRFTISGDNAKNTVYLQMNSLNPEDTAVYYCVADKDETFRTLPYADYWGLGTQVTVSS

15

SEQ ID NO: 51: Amino-acid sequence of Anti-CD123 V<sub>HH</sub> 55A01 (organism: artificial; recombinant peptide)

EVQLVESGGGSVQAGGSLRLSCTTSGRALNMYVMGWFRQAPGNEREFVAATSSSGGSTSYPDVK  
GRFTISRDNAKNTVYLQMNSLKPEDTAAYRCAASPYVSTPTMNILEEYRYWGLGTQVTVSS

20

SEQ ID NO: 52: Amino-acid sequence of Anti-c-MET V<sub>HH</sub> 04E09 (organism: artificial sequence)

EVQLVESGGGLVQPGGSLRLSCAASGFILDYFAIGWFRQAPGKEREGVLCIDASDDITYYADSVKGRF  
TISRDNKNTVYLQMNSLKPEDTGYYCATPIGLSSCLLEYDYDYWGQGLTVTVSS

25

SEQ ID NO: 53: Amino-acid sequence of Anti-c-MET V<sub>HH</sub> 06B08 (organism: artificial sequence)

EVQLVESGGGLVQAGGSLRLSCAASGRTISRYTMGWFRQAPGKEREFVAAISWSDNTNYADSVKGR  
RFTISRPNKNTMYLQMNSLKPEDTAVYYCAADYRSGSYQASEWTRPSGYDYWGQGLTVTVSS

SEQ ID NO: 54: Amino-acid sequence of Anti-c-MET V<sub>HH</sub> 06C12 (organism: artificial sequence)

30 EVQLVESGGGLVQPGGSLRLSCAASGFSLDYFAIGWFRQAPGKEREEISCISNSDGSTYYANSVKGR  
FTISIDNAKNTVYLMNTSLKPEDTAVYYCATPVGLGPFCKTTNDYDYSQGLTVTVSS

SEQ ID NO: 55: Amino-acid sequence of Anti-c-MET V<sub>HH</sub> 06F10 (organism: artificial sequence)

35 EVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAINWFRQAPGKEREGVSCISGGDGSTYYADSVKG  
RFTISRDNKNTVYLQMNSLKPEDTAVYYCATALGLSSSCHGDGYDYWGQGLTVTVSS

SEQ ID NO: 56: Amino-acid sequence of Anti-Her3 V<sub>HH</sub> 21F6 (organism: artificial sequence)

EVQLVESGGGLVQAGGSLRLSCAASGRTYYLNAMGWFRQGPGRDREFVAAIDWSDGNKDYADSVK  
GRFTISRDNKNTVYLQMNSLKPEDTAVYYCAADTPPWGPMIYIESYDSWGQGLTVTVSS

40

SEQ ID NO: 57: Amino-acid sequence of Anti-Her3 V<sub>HH</sub> 4C7 (organism: artificial sequence)  
EVQLVESGGGLVQAGGSLRLSCAASGFTFSSYPMSWVRQAPGKGPAAVSTVSPGGITTSYADSVKGR  
RFTISRDNANKNTLYLQMNSLKPEDTAVYYCLRDNLNRRGQGTQVTVSS

- 5 SEQ ID NO: 58: Amino-acid sequence of Anti-Her3 V<sub>HH</sub> 17B5 (organism: artificial sequence)  
EVQLVESGGGLVQPGGSLRLSCAASGSIGGLNMAWYRQAPGKERELVAGIFGVGSTRYADSVKGR  
FTISRDIKNTVFLQMNSLNSEDVAVYYCRMSSVTRGSSDYWGQGTQVTVSS

- SEQ ID NO: 59: Amino-acid sequence of Anti-Her3 V<sub>HH</sub> 18G11 (organism: artificial sequence)  
10 EVQLVESGGGLVQPGGSLRLSCAASGTLFKINAMGWYRQAPGKRRELVALITSSDITDYAESVEGRF  
TISRDNNTWNAVYLMNSLKPEDTAVYYCHSDHYSMGVPEKRVIMYGQGTQVTVSS

- SEQ ID NO: 60: Amino-acid sequence of Anti-Her3 V<sub>HH</sub> 34C7 (organism: artificial sequence)  
EVQLVESGGGLVQPGGSLGLSCVASGSIFRINAMAWYRQAPGKQRELVAEITAGGSTNYADSVKGRF  
15 TISVDNAWNTLYLQMNSLKVEDTAVYYCNLDHYTTWDRRSAYWGQGTQVTVSS

- SEQ ID NO: 61: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 47D5 (organism: llama)  
KVQLVESGGGLVQPGGSLRLSCAASGSIFGFNDMAWYRQAPGKQRELVALISRVTSSADSVKGR  
FTISRVNAKDTVYLMNSLKPEDTAVYYCYMDQRLDGSTLAYWGQGTQVTVSS

- 20 SEQ ID NO: 62: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 2D3 (organism: llama)  
EVQLVESGGGLVQPGGSLRLSCAASGFTFDDYAMSWVRQVPGKGLEWVSSINWSGTHTDYADSVK  
GRFTISRNNANNTLYLQMNSLKSSEDVAVYYCAKNWRDAGTTWFEKSGSAGQGTQVTVSS

- 25 SEQ ID NO: 63: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 5F7 (organism: llama)  
EVQLVESGGGLVQPGGSLRLSCAASGFTFSINTMGWYRQAPGKQRELVALISSIGDITYADSVKGRF  
TISRDNANKNTVYLMNSLKPEDTAVYYCKRFRATAAQGTDYWGQGTQVTVSS

- SEQ ID NO: 64: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 13D11 (organism: llama)  
30 EVQLVESGGGLVHPGGSLRLSCVSGFSLDDYGMTWVRRAPGKGLEWVSSINWSGTHTDYADSVK  
GRFTISRDNANKNTLYLQMNSLNPEDTAVYYCGQGKIVPTNPRGHGTQVTVSS

- SEQ ID NO: 65: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 2B4 (organism: llama)  
EVQLVESGGGLVQPGGSLRLSCVSGFSLDDYAMTWVRQAPGKGLEWVSSINWSGTHTDYADSVK  
35 GRFTISRDNANKNTLYLQMNSLSPEDTAVYYCNQGWKIRPTIPMGHGTQVTVSS

- SEQ ID NO: 66: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 2G2 (organism: llama)  
EVQLVESGGGLVQPGGSLRLSCVASGFSLDDYGMTWVRQAPGKGLEWVSSINWSGTHTDYDTPVK  
GRFTISRDNANKNTLYLQMNSLTPEDTAVYYCNRGWKIVPTDLGGHGTQVTVSS

SEQ ID NO: 67: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 13G11 (organism: llama)  
EVQLVESGGGLVQAGGSLRLSCAASGRTFISNYAMGWFRQAPGKEREFVATINWSGSHSDYADSVK  
GRFTISRDNKNTVYVYLMNNLKSSEDVAVYYCAPGWGTAPLSTSVYWGQGTQVTVSS

- 5 SEQ ID NO: 68: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 12E33 (organism: llama)  
EVQLVESGGGMVQAGGSLRLSCAASGLTISNYMGWFRQAPGKEREFVSSINWSGTHTYDADFVK  
GRFIISRDNKNTVYVYVQINSLKPEDTAVYYCAAGGWGTGRYNYWGQGTQVTVSS

- SEQ ID NO: 69: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 13F21 (organism: llama)  
10 EVQLVESGGGLVQSGGSLRLSCVASGTIVSINATSWYRQAPGNQRELVATIIGDGRTHYADSVKDRFT  
ISRDAANLVYVYLMNLSLKPEDTAIYSCNANGIESYGWGNRHFNYWTVGTQVTVSS

- SEQ ID NO: 70: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 11A101 (organism: llama)  
EVQLVESGGGLVQAGGSLRLSCAASGRTFNAMGWFRQAPGKEREFVAAISRSPGVITYYADSVKGRF  
15 TTSRDNKNTVYVYVQMNLSLKPEDTAVYYCAADFYLATLAHEYDYWGQGTQVTVSS

- SEQ ID NO: 71: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 11A22 (organism: llama)  
EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMAWFRQAPGTEREFIAGIRWSDGSTYYADSVKG  
RFTISRDNKNTVYVYVQMNLSLKPEDTAVYYCAADFVYSTLAHEYDYWGQGTQVTVSS  
20

SEQ ID NO: 72: Amino-acid sequence of Anti-Her2 V<sub>HH</sub> 12D44 (organism: llama)  
KVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMAWFRQAPGTEREFIAGIRWSDGSTYYADSVKG  
RFTISRANAKNTVYVYVQMNGLKPEDTAVYYCAADFVYSTLAHEYDYWGQGTQVTVSS

- 25 SEQ ID NO: 73: Amino acid sequence of bivalent VHHEGFR-dianthin-Cys  
QVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMWFRQAPGKEREFVSGISWRGDSTGYADSVK  
GRFTISRDNKNTVDLQMNLSLKPEDTAIYYCAAAGSAWYGTLYEYDYWGQGTQVTVSSGGGGSGG  
GGSEVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVAINWSSGSTYYAD  
SVKGRFTISRDNKNTMYLQMNLSLKPEDTAVYYCAAGYQINSGNYNFKDYEYDYWGQGTQVTVSSG  
30 GGGSGGGGSAATAYTLNLANPSASQYSSFLDQIRNNVRDTSLIYGGTDVAVIGAPSTTDKFLRLNFQ  
GPRGTVSLGLRRENLYVVAYLAMDNANVNRAYYFKNQITSAELTALFPEVVVANQKQLEYGEDYQAIE  
KNAKITTGDQSRKELGLGINLLITMIDGVNKKVRVVKDEARFLIAIQMTAEAAFRFYIQNLVTKNFPNKF  
DSENKVIQFQVSWSKISTAIFGDCKNGVFNKDYDFGFGKVRQAKDLQMGLLKYLGRPKGGGGSGGG  
GSHRWCCPGCCKTFGGGGSHHHHHHHHHHRWCCPGCCKTF

- 35 SEQ ID NO: 74: Amino acid sequence of bivalent VHHEGFR-Cys  
QVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMWFRQAPGKEREFVSGISWRGDSTGYADSVK  
GRFTISRDNKNTVDLQMNLSLKPEDTAIYYCAAAGSAWYGTLYEYDYWGQGTQVTVSSGGGGSGG  
GGSEVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVAINWSSGSTYYAD

SVKGRFTISRDNANTMYLQMNSLKPEDTAVYYCAAGYQINSGNYNFKDYEDYWGQGTQVTVSSH  
HHHHHHHHHHHRWCCPGCCKTF

SEQ ID NO: 75: Amino acid sequence of VHH 7D12

5 QVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMGWFRQAPGKEREFVSGISWRGDSTGYADSVK  
GRFTISRDNANTVLDQMNSLKPEDTAIYYCAAAGSAWYGTLYEYDYWGQGTQVTVSS

SEQ ID NO: 76: Amino acid sequence of VHH 9G8

10 EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVAINWSSGSTYYADSVK  
GRFTISRDNANTMYLQMNSLKPEDTAVYYCAAGYQINSGNYNFKDYEDYWGQGTQVTVSS

## CLAIMS

1. Conjugate for transferring an effector molecule from outside a cell into said cell, the conjugate comprising at least one effector molecule to be transferred into the cell, at least one single-domain antibody (sdAb) and at least one saponin, covalently bound to each other, directly or via at least one linker, wherein the at least one saponin is a mono-desmosidic triterpene glycoside or is a bi-desmosidic triterpene glycoside, and wherein the sdAb is capable of binding to a cell-surface molecule of said cell.
2. Conjugate of claim 1, comprising at least one sdAb which is any one or more of: a  $V_H$  domain derived from a heavy chain of an antibody, preferably of immunoglobulin G origin, preferably of human origin; a  $V_L$  domain derived from a light chain of an antibody, preferably of immunoglobulin G origin, preferably of human origin; a  $V_{HH}$  domain such as derived from a heavy-chain only antibody (HCAb) such as from *Camelidae* origin or *Ig-NAR* origin such as a variable heavy chain new antigen receptor ( $V_{NAR}$ ) domain, preferably the HCAb is from *Camelidae* origin; and preferably the at least one sdAb is a  $V_{HH}$  domain derived from an HCAb from *Camelidae* origin (camelid  $V_H$ ) such as derived from an HCAb from camel, lama, alpaca, dromedary, vicuna, guanaco and Bactrian camel.
3. Conjugate of claim 1 or 2, comprising at least two sdAbs with a single first sdAb covalently bound to one of the at least one effector molecule and/or to one of the at least one saponin, or with two or more sdAbs of which at least one sdAb is bound to the at least one effector molecule and/or of which at least one sdAb is bound to the at least one saponin, or with all of the at least two sdAbs each bound separately to either an effector molecule of the at least one effector molecule or to a saponin of the at least one saponin, or both.
4. Conjugate of any one of the claims 1-3, wherein the at least one sdAb comprises at least two sdAbs, which are the same sdAbs, preferably two to eight sdAbs, more preferably two – four sdAbs, or wherein the at least one sdAbs are at least two sdAbs which are biparatopic, preferably two sdAbs which are biparatopic.
5. Conjugate of any one of the claims 1-4, comprising one – eight sdAbs, capable of binding to a same binding site on a cell-surface molecule, wherein the at least one effector molecule and/or the at least one saponin is/are bound to a single first sdAb of the one – eight sdAbs or wherein the at least one effector molecule and/or the at least one saponin is/are bound to two or more of the sdAbs, if present, wherein the at least one effector molecule and the at least one saponin are bound to the same sdAb or are bound to different sdAbs, wherein preferably each of the at least one effector molecule is bound to a separate sdAb and/or each of the at least one saponin is bound to a separate sdAb, wherein an effector molecule and a saponin are bound to the same sdAb or are bound to separate sdAbs.
6. Conjugate of any one of the claims 1-5, wherein the at least one sdAb is a single sdAb or are at least two, preferably two sdAbs, wherein the sdAb(s) is/are capable of binding to a cell-surface molecule of

the cell such as HIVgp41 or wherein the sdAb(s) is/are capable of binding to a cell-surface receptor of the cell, such as a tumor-cell surface receptor of the cell, preferably a tumor-cell specific receptor, more preferably to a receptor selected from any one or more of: CD71, CA125, EpCAM(17-1A), CD52, CEA, CD44v6, FAP, EGF-IR, integrin, syndecan-1, vascular integrin alpha-V beta-3, HER2, EGFR, CD20, CD22, Folate receptor 1, CD146, CD56, CD19, CD138, CD27L receptor, prostate specific membrane antigen (PSMA), CanAg, integrin-alphaV, CA6, CD33, mesothelin, Cripto, CD3, CD30, CD239, CD70, CD123, CD352, DLL3, CD25, ephrinA4, MUC-1, Trop2, CEACAM5, CEACAM6, HER3, CD74, PTK7, Notch3, FGF2, C4.4A, FLT3, CD38, FGFR3, CD7, PD-L1, CTLA-4, CD52, PDGFRA, VEGFR1, VEGFR2, c-Met (HGFR), EGFR1, RANKL, ADAMTS5, CD16, CXCR7 (ACKR3), glucocorticoid-induced TNFR-related protein (GITR), most preferably selected from: HER2, c-Met, VEGFR2, CXCR7, CD71, EGFR and EGFR1.

7. Conjugate of any one of the claims 1-6, wherein the at least one sdAb is a single sdAb or are at least two, preferably two, wherein the sdAb(s) is/are selected from: an anti-CD71 sdAb, an anti-HER2 sdAb, an anti-CD20 sdAb, an anti-CA125 sdAb, an anti-EpCAM (17-1A) sdAb, an anti-EGFR sdAb, an anti-CD30 sdAb, an anti-CD33 sdAb, an anti-vascular integrin alpha-v beta-3 sdAb, an anti-CD52 sdAb, an anti-CD22 sdAb, an anti-CEA sdAb, an anti-CD44v6 sdAb, an anti-FAP sdAb, an anti-CD19 sdAb, an anti-CanAg sdAb, an anti-CD56 sdAb, an anti-CD38 sdAb, an anti-CA6 sdAb, an anti-IGF-1R sdAb, an anti-integrin sdAb, an anti-syndecan-1 sdAb, an anti-CD79b, an anti-c-Met sdAb, an anti-EGFR1 sdAb, an anti-VEGFR2 sdAb, an anti-CXCR7 sdAb, an anti-HIVgp41, wherein the sdAb(s) is/are preferably  $V_{HH}(s)$ , more preferably camelid  $V_H(s)$ .

8. Conjugate of any one of the claims 1-7, wherein the at least one sdAb comprises an sdAb that is capable of binding to HER2, CD71, HIVgp41 and/or EGFR, wherein said sdAb is preferably a  $V_{HH}$ , more preferably a camelid  $V_H$ .

9. Conjugate of any one of the claims 1-8, wherein the at least one sdAb comprises an sdAb for binding to HER2 selected from: sdAb produced by clone 11A4, clone 18C3, clone 22G12, clone Q17 or clone Q17-C-tag; or comprises an sdAb for binding to EGFR and produced by clone anti-EGFR Q86-C-tag; or comprises an sdAb for binding to CD71 and produced by clone anti-CD71 Q52-C-tag; or comprises an sdAb for binding to HIVgp41 and produced by clone anti-HIVgp41 Q8C-tag; or comprises an sdAb encoded by a cDNA of any one of the SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 and 31; or comprises any one of the sdAbs with an amino-acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36-72, or comprises a tandem of two sdAb's with an amino-acid sequence of SEQ ID NO: 74 or comprises  $V_{HH}$  7D12 with the amino-acid sequence as depicted as SEQ ID NO: 75 and/or  $V_{HH}$  9G8 with the amino-acid sequence as depicted as SEQ ID NO: 76, wherein optionally the conjugate further comprises a further sdAb, different from the at least one sdAb, the further sdAb for binding to albumin, such as any one or more of the further sdAbs with an amino-acid sequence of SEQ ID NO: 33, 34 and 35, preferably the further sdAb is a  $V_{HH}$ , more preferably a camelid  $V_H$ .

10. Conjugate of any one of the claims 1-9, wherein the effector molecule comprises or consists of at least one of a small molecule such as a drug molecule, a toxin such as a protein toxin, an oligonucleotide such as a BNA, a xeno nucleic acid or an siRNA, an enzyme, a peptide, a protein, or any combination thereof.

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11. Conjugate of any one of the claims 1-10, wherein the at least one effector molecule is selected from any one or more of a vector, a gene, a cell suicide inducing transgene, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), anti-sense oligonucleotide (ASO, AON), short interfering RNA (siRNA), anti-microRNA (anti-miRNA), DNA aptamer, RNA aptamer, mRNA, mini-circle DNA, peptide nucleic acid (PNA), phosphoramidate morpholino oligomer (PMO), locked nucleic acid (LNA), bridged nucleic acid (BNA), 2'-deoxy-2'-fluoroarabino nucleic acid (FANA), 2'-O-methoxyethyl-RNA (MOE), 3'-fluoro hexitol nucleic acid (FHNA), a plasmid, glycol nucleic acid (GNA) and threose nucleic acid (TNA), or a derivative thereof, more preferably a BNA, for example a BNA for silencing HSP27 protein expression or a BNA for silencing apolipoprotein B expression.

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12. Conjugate of any one of the previous claims, wherein the at least one effector molecule is an oligonucleotide selected from any one or more of a(n): short interfering RNA (siRNA), short hairpin RNA (shRNA), anti-hairpin-shaped microRNA (miRNA), single-stranded RNA, aptamer RNA, double-stranded RNA (dsRNA), anti-microRNA (anti-miRNA, anti-miR), antisense oligonucleotide (ASO), mRNA, DNA, antisense DNA, locked nucleic acid (LNA), bridged nucleic acid (BNA), 2'-O,4'-aminoethylene bridged nucleic Acid (BNA<sup>NC</sup>), BNA-based siRNA, and BNA-based antisense oligonucleotide (BNA-AON).

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13. Conjugate of any one of the previous claims, wherein the at least one effector molecule is an oligonucleotide selected from any one of an anti-miRNA, a BNA-AON or an siRNA, such as BNA-based siRNA, preferably selected from chemically modified siRNA, metabolically stable siRNA and chemically modified, metabolically stable siRNA.

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14. Conjugate of any one of the previous claims, wherein the at least one effector molecule is an oligonucleotide that is capable of silencing a gene, when present in a cell comprising such gene, wherein the gene is any one of genes: apolipoprotein B (apoB), transthyretin (TTR), proprotein convertase subtilisin/kexin type 9 (PCSK9), delta-aminolevulinate synthase 1 (ALAS1), antithrombin 3 (AT3), glycolate oxidase (GO), complement component C5 (CC5), X gene of hepatitis B virus (HBV), S gene of HBV, alpha-1 antitrypsin (AAT) and lactate dehydrogenase (LDH), and/or is capable of targeting an aberrant miRNA when present in a cell comprising such aberrant miRNA.

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15. Conjugate of any one of the previous claims, wherein the effector molecule is an oligonucleotide that is capable of targeting an mRNA, when present in a cell comprising such mRNA, wherein the mRNA is involved in expression of any one of proteins: apoB, TTR, PCSK9, ALAS1, AT3, GO, CC5, expression product of X gene of HBV, expression product of S gene of HBV, AAT and LDH, or is capable of

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antagonizing or restore an miRNA function such as inhibiting an oncogenic miRNA (onco-miR) or suppression of expression of an onco-miR, when present in a cell comprising such an miRNA.

16. Conjugate of any one of the claims 1-15, wherein the at least one effector molecule comprises or, when dependent on any one of the claims 1-9, consists of at least one proteinaceous molecule, preferably selected from any one or more of a peptide, a protein, an enzyme and a protein toxin.

17. Conjugate of any one of the claims 1-16, wherein the at least one effector molecule comprises or, when dependent on any one of the claims 1-9, consists of at least one of: urease and Cre-recombinase, a proteinaceous toxin, a ribosome-inactivating protein, a protein toxin, a bacterial toxin, a plant toxin, more preferably selected from any one or more of a viral toxin such as apoptin; a bacterial toxin such as Shiga toxin, Shiga-like toxin, *Pseudomonas aeruginosa* exotoxin (PE) or exotoxin A of PE, full-length or truncated diphtheria toxin (DT), cholera toxin; a fungal toxin such as alpha-sarcin; a plant toxin including ribosome-inactivating proteins and the A chain of type 2 ribosome-inactivating proteins such as dianthin e.g. dianthin-30 or dianthin-32, saporin e.g. saporin-S3 or saporin-S6, bouganin or de-immunized derivative debouganin of bouganin, shiga-like toxin A, pokeweed antiviral protein, ricin, ricin A chain, modeccin, modeccin A chain, abrin, abrin A chain, volkensin, volkensin A chain, viscumin, viscumin A chain; or an animal or human toxin such as frog RNase, or granzyme B or human angiogenin, or any toxic fragment or toxic derivative thereof; preferably the protein toxin is dianthin and/or saporin.

18. Conjugate of any one of the claims 1-17, wherein the at least one effector molecule comprises or, when dependent on any one of the claims 1-9, consists of at least one payload.

19. Conjugate of any one of the claims 1-18, wherein the at least one effector molecule comprises or, when dependent on any one of the claims 1-9, consists of at least one of: a toxin targeting ribosomes, a toxin targeting elongation factors, a toxin targeting tubulin, a toxin targeting DNA and a toxin targeting RNA, more preferably any one or more of emtansine, pasudotox, maytansinoid derivative DM1, maytansinoid derivative DM4, monomethyl auristatin E (MMAE, vedotin), monomethyl auristatin F (MMAF, mafodotin), a Calicheamicin, N-Acetyl-γ-calicheamicin, a pyrrolobenzodiazepine (PBD) dimer, a benzodiazepine, a CC-1065 analogue, a duocarmycin, Doxorubicin, paclitaxel, docetaxel, cisplatin, cyclophosphamide, etoposide, docetaxel, 5-fluorouracyl (5-FU), mitoxantrone, a tubulysin, an indolinobenzodiazepine, AZ13599185, a cryptophycin, rhizoxin, methotrexate, an anthracycline, a camptothecin analogue, SN-38, DX-8951f, exatecan mesylate, truncated form of *Pseudomonas aeruginosa* exotoxin (PE38), a Duocarmycin derivative, an amanitin, α-amanitin, a spliceostatin, a thailanstatin, ozogamicin, tesirine, Amberstatin269 and soravtansine, or a derivative thereof.

20. Conjugate of any one of the claims 1-19, wherein the conjugate comprises an antibody-drug conjugate (ADC), such as an ADC comprising at least one sdAb derived from: gemtuzumab ozogamicin, brentuximab vedotin, trastuzumab emtansine, inotuzumab ozogamicin, moxetumomab pasudotox and polatuzumab vedotin, and/or comprising at least one effector molecule which is a toxin present in any

one or more of: gemtuzumab ozogamicin, brentuximab vedotin, trastuzumab emtansine, inotuzumab ozogamicin, moxetumomab pasudotox and polatuzumab vedotin, and/or selected from dianthin and saporin.

- 5 21. Conjugate of any one of the previous claims, wherein the at least one saponin comprises an aglycone core structure selected from:

2alpha-hydroxy oleanolic acid;  
 16alpha-hydroxy oleanolic acid;  
 hederagenin (23-hydroxy oleanolic acid);  
 10 16alpha,23-dihydroxy oleanolic acid;  
 gypsogenin;  
 quillaic acid;  
 protoaescigenin-21(2-methylbut-2-enoate)-22-acetate;  
 23-oxo-barringtonenol C-21,22-bis(2-methylbut-2-enoate);  
 15 23-oxo-barringtonenol C-21(2-methylbut-2-enoate)-16,22-diacetate;  
 digitogenin;  
 3,16,28-trihydroxy oleanan-12-en;  
 gypsogenic acid; or  
 a derivative thereof,

- 20 preferably, the at least one saponin comprises an aglycone core structure selected from quillaic acid and gypsogenin, more preferably the at least one saponin comprises aglycone core structure quillaic acid.

22. Conjugate of any one of the previous claims, wherein the at least one saponin comprises one or  
 25 both of: a first saccharide chain bound to the C<sub>3</sub> atom or to the C<sub>28</sub> atom of the aglycone core structure of the at least one saponin, preferably bound to the C<sub>3</sub> atom, and a second saccharide chain bound to the C<sub>28</sub> atom of the aglycone core structure of the at least one saponin, and preferably the at least one saponin comprises the first and the second saccharide chain.

- 30 23. Conjugate of claim 22, wherein

- the at least one saponin comprises the first saccharide chain selected from:

- GlcA-,  
 Glc-,  
 Gal-,  
 35 Rha-(1→2)-Ara-,  
 Gal-(1→2)-[Xyl-(1→3)]-GlcA-,  
 Glc-(1→2)-[Glc-(1→4)]-GlcA-,  
 Glc-(1→2)-Ara-(1→3)-[Gal-(1→2)]-GlcA-,  
 Xyl-(1→2)-Ara-(1→3)-[Gal-(1→2)]-GlcA-,  
 40 Glc-(1→3)-Gal-(1→2)-[Xyl-(1→3)]-Glc-(1→4)-Gal-,

- Rha-(1→2)-Gal-(1→3)-[Glc-(1→2)]-GlcA-,  
 Ara-(1→4)-Rha-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 Ara-(1→4)-Fuc-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 Ara-(1→4)-Rha-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 5 Ara-(1→4)-Fuc-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 Ara-(1→4)-Rha-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 Ara-(1→4)-Fuc-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 Ara-(1→4)-Rha-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-,  
 Ara-(1→4)-Fuc-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-,  
 10 Xyl-(1→4)-Rha-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 Xyl-(1→4)-Fuc-(1→2)-Glc-(1→2)-Rha-(1→2)-GlcA-,  
 Xyl-(1→4)-Rha-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 Xyl-(1→4)-Fuc-(1→2)-Gal-(1→2)-Rha-(1→2)-GlcA-,  
 Xyl-(1→4)-Rha-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 15 Xyl-(1→4)-Fuc-(1→2)-Glc-(1→2)-Fuc-(1→2)-GlcA-,  
 Xyl-(1→4)-Rha-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-,  
 Xyl-(1→4)-Fuc-(1→2)-Gal-(1→2)-Fuc-(1→2)-GlcA-, and  
 any derivative thereof,  
 and/or
- 20 • the at least one saponin comprises the second saccharide chain selected from:  
 Glc-,  
 Gal-,  
 Rha-(1→2)-[Xyl-(1→4)]-Rha-,  
 Rha-(1→2)-[Ara-(1→3)-Xyl-(1→4)]-Rha-,  
 25 Ara-,  
 Xyl-,  
 Xyl-(1→4)-Rha-(1→2)-[R1-(→4)]-Fuc- wherein R1 is 4E-Methoxycinnamic acid,  
 Xyl-(1→4)-Rha-(1→2)-[R2-(→4)]-Fuc- wherein R2 is 4Z-Methoxycinnamic acid,  
 Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-4-OAc-Fuc-,  
 30 Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-3,4-di-OAc-Fuc-,  
 Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R3-(→4)]-3-OAc-Fuc- wherein R3 is 4E-Methoxycinnamic acid,  
 Glc-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-4-OAc-Fuc-,  
 Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-4-OAc-Fuc-,  
 (Ara- or Xyl-)(1→3)-(Ara- or Xyl-)(1→4)-(Rha- or Fuc-)(1→2)-[4-OAc-(Rha- or Fuc-)(1→4)]-(Rha- or  
 35 Fuc-),  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Qui-(1→4)]-Fuc-,  
 Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-Fuc-,  
 Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-Fuc-,  
 Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-Fuc-,  
 40 Ara/Xyl-(1→4)-Rha/Fuc-(1→4)-[Glc/Gal-(1→2)]-Fuc-,

- Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R4-(→4)]-Fuc- wherein R4 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R5-(→4)]-Fuc- wherein R5 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 5 Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,  
 Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,  
 6-OAc-Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3-OAc-Rha-(1→3)]-Fuc-,  
 Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3-OAc-Rha-(1→3)]-Fuc-,  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Qui-(1→4)]-Fuc-,  
 10 Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-[Qui-(1→4)]-Fuc-,  
 Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Xyl-(1→3)-4-OAc-Qui-(1→4)]-Fuc-,  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[3,4-di-OAc-Qui-(1→4)]-Fuc-,  
 Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-Fuc-,  
 6-OAc-Glc-(1→3)-[Xyl-(1→4)]-Rha-(1→2)-Fuc-,  
 15 Glc-(1→3)-[Xyl-(1→3)-Xyl-(1→4)]-Rha-(1→2)-Fuc-,  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Xyl-(1→3)-4-OAc-Qui-(1→4)]-Fuc-,  
 Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,  
 Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)]-4-OAc-Fuc-,  
 Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R6-(→4)]-Fuc- wherein R6 is 5-O-[5-O-Rha-(1→2)-  
 20 Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R7-(→4)]-Fuc- wherein R7 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Api/Xyl-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[R8-(→4)]-Fuc- wherein R8 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 25 Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R9-(→4)]-Fuc- wherein R9 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R10-(→4)]-Fuc- wherein R10 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R11-(→3)]-Fuc- wherein R11 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 30 methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid),  
 Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[R12-(→3)]-Fuc- wherein R12 is 5-O-[5-O-Ara/Api-3,5-dihydroxy-6-methyl-octanoyl]-3,5-dihydroxy-6-methyl-octanoic acid)  
 Glc-(1→3)-[Glc-(1→6)]-Gal-, and  
 any derivative thereof.

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24. Conjugate of any one of the claims 1-23, wherein the at least one saponin comprises the first saccharide chain and comprises the second saccharide chain of claim 22 or 23, wherein the first saccharide chain comprises more than one saccharide moiety and the second saccharide chain comprises more than one saccharide moiety, and wherein the aglycone core structure preferably is

quillaic acid or gypsogenin, more preferably quillaic acid, wherein one, two or three, preferably one or two, of:

- i. an aldehyde group in the aglycone core structure has been derivatised,
- ii. a carboxyl group of a glucuronic acid moiety in the first saccharide chain has been derivatised,
- 5 and
- iii. at least one acetoxyl group (Me(CO)O-) in the second saccharide chain has been derivatised.

25. Conjugate of any one of the claims 1-24, wherein the at least one saponin comprises:

- i. an aglycone core structure comprising an aldehyde group which has been derivatised by:
  - 10 - reduction to an alcohol;
  - transformation into a hydrazone bond through reaction with N-ε-maleimidocaproic acid hydrazide (EMCH) wherein the maleimide group of the EMCH is optionally derivatised by formation of a thioether bond with mercaptoethanol;
  - transformation into a hydrazone bond through reaction with N-[β-maleimidopropionic acid] hydrazide (BMPH) wherein the maleimide group of the BMPH is optionally derivatised by
 15 formation of a thioether bond with mercaptoethanol; or
  - transformation into a hydrazone bond through reaction with N-[κ-maleimidoundecanoic acid] hydrazide (KMUH) wherein the maleimide group of the KMUH is optionally derivatised by formation of a thioether bond with mercaptoethanol;
- 20 ii. a first saccharide chain comprising a carboxyl group, preferably a carboxyl group of a glucuronic acid moiety, which has been derivatised by transformation into an amide bond through reaction with 2-amino-2-methyl-1,3-propanediol (AMPD) or N-(2-aminoethyl)maleimide (AEM);
- iii. a second saccharide chain comprising an acetoxyl group (Me(CO)O-) which has been
 25 derivatised by transformation into a hydroxyl group (HO-) by deacetylation; or
- iv. any combination of two or three derivatisations i., ii. and/or iii., preferably any combination of two derivatisations i., ii. and/or iii.

26. Conjugate of any one of the claims 1-25, wherein the at least one saponin is any one or more of:
- 30 *Quillaja* bark saponin, dipsacoside B, saikosaponin A, saikosaponin D, macranthoidin A, esculentoside A, phytolaccagenin, aescinate, AS6.2, NP-005236, AMA-1, AMR, alpha-Hederin, NP-012672, NP-017777, NP-017778, NP-017774, NP-018110, NP-017772, NP-018109, NP-017888, NP-017889, NP-018108, SA1641, AE X55, NP-017674, NP-017810, AG1, NP-003881, NP-017676, NP-017677, NP-017706, NP-017705, NP-017773, NP-017775, SA1657, AG2, SO1861, GE1741, SO1542, SO1584,
  - 35 SO1658, SO1674, SO1832, SO1904, SO1862, QS-7, QS1861, QS-7 api, QS1862, QS-17, QS-18, QS-21 A-apio, QS-21 A-xylo, QS-21 B-apio, QS-21 B-xylo, beta-Aescin, Aescin Ia, Teaseed saponin I, Teaseedsaponin J, Assamsaponin F, Digitonin, Primula acid 1 and AS64R, or a derivative thereof, or a stereoisomer thereof, and/or any combinations thereof, preferably any one or more of QS-21 or a QS-21 derivative, SO1861 or a SO1861 derivative, SA1641 or a SA1641 derivative and GE1741 or a

GE1741 derivative, more preferably a QS-21 derivative or a SO1861 derivative, most preferably a SO1861 derivative, such as a saponin derivative of claim 24 or 25.

27. Conjugate of any one of the claims 1-26, wherein the at least one saponin is any one or more of:  
5 SO1861, SA1657, GE1741, SA1641, QS-21, QS-21A, QS-21 A-api, QS-21 A-xyl, QS-21B, QS-21 B-api, QS-21 B-xyl, QS-7-xyl, QS-7-api, QS-17-api, QS-17-xyl, QS1861, QS1862, Quillajasaponin, Saponinum album, QS-18, Quil-A, Gyp1, gypsoside A, AG1, AG2, SO1542, SO1584, SO1658, SO1674, SO1832, SO1862, SO1904, or a derivative thereof, or a stereoisomer thereof, and/or any combinations thereof, preferably the saponin derivative is a SO1861 derivative and/or a GE1741 derivative and/or a  
10 SA1641 derivative and/or a QS-21 derivative, more preferably the saponin derivative is a SO1861 derivative or a QS21 derivative, most preferably, the saponin derivative is a SO1861 derivative according to claim 24 or 25.

28. Conjugate of any one of the claims 1-27, wherein the at least one sdAb comprises an sdAb for  
15 binding to a cell-surface molecule of the cell wherein the cell is an aberrant cell such as a tumor cell, an auto-immune cell, an infected cell such as a virally infected cell, or a cell comprising a gene defect or an enzyme defect.

29. Conjugate of any one of the claims 1-28, wherein the at least one sdAb comprises an sdAb for  
20 binding to a cell-surface molecule of the cell, the sdAb derived from or based on any one or more of immunoglobulins: an anti-CD71 antibody such as IgG type OKT-9, an anti-HER2 antibody such as trastuzumab (Herceptin), pertuzumab, an anti-CD20 antibody such as rituximab, ofatumumab, tositumomab, obinutuzumab ibritumomab, an anti-CA125 antibody such as oregovomab, an anti-EpCAM (17-1A) antibody such as edrecolomab, an anti-EGFR antibody such as cetuximab,  
25 matuzumab, panitumumab, nimotuzumab, an anti-CD30 antibody such as brentuximab, an anti-CD33 antibody such as gemtuzumab, huMy9-6, an anti-vascular integrin alpha-v beta-3 antibody such as etaracizumab, an anti-CD52 antibody such as alemtuzumab, an anti-CD22 antibody such as epratuzumab, pinatuzumab, binding fragment (Fv) of anti-CD22 antibody moxetumomab, humanized monoclonal antibody inotuzumab, an anti-CEA antibody such as labetuzumab, an anti-CD44v6 antibody  
30 such as bivatuzumab, an anti-FAP antibody such as sibrotuzumab, an anti-CD19 antibody such as huB4, an anti-CanAg antibody such as huC242, an anti-CD56 antibody such as huN901, an anti-CD38 antibody such as daratumumab, OKT-10 anti-CD38 monoclonal antibody, an anti-CA6 antibody such as DS6, an anti-IGF-1R antibody such as cixutumumab, 3B7, an anti-integrin antibody such as CNTO 95, an anti-syndecan-1 antibody such as B-B4, an anti-CD79b such as polatuzumab, an anti-HIVgp41  
35 antibody, preferably any one of an anti-HIVgp41 antibody, an anti-CD71 antibody, an anti-HER2 antibody and an anti-EGFR antibody, more preferably any one of: trastuzumab, pertuzumab, cetuximab, matuzumab, an anti-CD71 antibody, OKT-9, most preferably trastuzumab, cetuximab, the anti-CD71 antibody OKT-9.

30. Conjugate of any one of the claims 1-29, wherein the at least one effector molecule is covalently bound to at least one sdAb, preferably one, of the at least one sdAb and/or to at least one, preferably one, of the at least one saponin, either via a linker or bound directly to the sdAb and/or to the saponin, and/or wherein the at least one saponin is covalently bound to at least one sdAb, preferably one, of the at least one sdAb and/or to at least one effector molecule, preferably one, of the at least one effector molecule, either via a linker or bound directly to the sdAb and/or to the effector molecule.
31. Conjugate of any one of the claims 1-30, wherein the conjugate comprises a trifunctional linker with each of the at least one sdAb, the at least one saponin and the at least one effector molecule covalently bound to the trifunctional linker, preferably separately, either directly, or via a linker, and preferably, the conjugate comprises a trifunctional linker with one sdAb, the at least one saponin and at least one, preferably one, effector molecule covalently bound to the trifunctional linker, separately, either directly, or via a linker.
32. Conjugate of any one of the previous claims, wherein the at least one saponin is covalently bound via a thio-ether bond to a sulfhydryl group in one of the at least one sdAb and/or in one of the at least one effector molecule, the covalent bonding preferably via linker N-ε-maleimidocaproic acid hydrazide (EMCH) that is covalently bound to an aldehyde group in position C<sub>23</sub> of the aglycone core structure of the saponin and that is covalently bound to the sulfhydryl group in the sdAb and/or in the effector molecule, such as a sulfhydryl group of a cysteine.
33. Conjugate of any one of the previous claims, wherein the at least one saponin is a bi-desmosidic triterpene saponin or derivative thereof belonging to the type of a 12,13-dehydrooleanane with optionally an aldehyde function in position C<sub>23</sub> and comprising a glucuronic acid unit in a first saccharide chain bound at the C<sub>3</sub>β-OH group of the aglycone core structure of the saponin, wherein the saponin is covalently bound to an amino-acid residue of the at least one sdAb and/or of the at least one effector molecule via the carboxyl group of the glucuronic acid unit in the first saccharide chain, preferably via a linker, wherein the amino-acid residue preferably is selected from cysteine and lysine.
34. Conjugate of claim 33, wherein the at least one saponin comprises a glucuronic acid unit in the first saccharide chain at the C<sub>3</sub>β-OH group of the aglycone core structure of the saponin, which glucuronic acid unit is covalently bound to a linker, which linker is preferably covalently bound via an amide bond to an amine group in the at least one sdAb and/or in the at least one effector molecule, such as an amine group of a lysine or an N-terminus of the sdAb and/or of the effector molecule, preferably said linker is 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU).
35. Conjugate of any one of the previous claims, comprising more than one covalently bound saponin moieties of the at least one saponin, preferably 2, 3, 4, 5, 6, 8, 10, 16, 32, 64, 128 or 1-100 of such moieties, or any number of such moieties therein between, such as 7, 9, 12 saponin moieties.

36. Conjugate of claim 35, wherein the more than one covalently bound saponin moieties are covalently bound directly to an amino-acid residue of the at least one sdAb and/or of the at least one effector molecule, preferably to a cysteine and/or to a lysine, and/or are covalently bound via a linker and/or via a cleavable linker.

37. Conjugate of claim 35, wherein the more than one covalently bound saponin moieties are part of a covalent saponin conjugate comprising an oligomeric molecule or a polymeric molecule to which the saponin is covalently bound, and wherein the sdAb is also covalently bound to the same oligomeric molecule or polymeric molecule as to which the saponin is bound and wherein the effector moiety is covalently bound to the sdAb or to the oligomeric molecule or the polymeric molecule, preferably 1-8 of such oligomeric molecules or polymeric molecules comprising the saponin(s) is/are covalently bound to the sdAb, or 2-4 of the oligomeric molecules or polymeric molecules comprising the saponin(s) are covalently bound to the sdAb, wherein the oligomeric molecule or the polymeric molecule of the covalent saponin conjugate is optionally a dendron such as a G2 dendron, G3 dendron, G4 dendron or G5 dendron with 4, 8, 16 and 32 binding sites for covalently binding 4, 8, 16 or 32 saponin moieties, respectively, wherein optionally 1-32 saponin moieties, preferably 2, 3, 4, 5, 6, 8, 10, 16, 32 of such moieties, or any number of such moieties therein between, such as 7, 9, 12 saponin moieties, are covalently bound to the oligomeric molecule or to the polymeric molecule of the at least one covalent saponin conjugate, either directly or via a linker.

38. Conjugate of any one of the previous claims, wherein the at least one saponin is covalently bound to at least one of the at least one sdAb and/or to at least one of the at least one effector molecule via a cleavable linker.

39. Conjugate of claim 38, wherein the cleavable linker is subject to cleavage under acidic conditions, reductive conditions, enzymatic conditions and/or light-induced conditions, and preferably the cleavable linker comprises a cleavable bond selected from a hydrazone bond and a hydrazide bond subject to cleavage under acidic conditions, and/or a bond susceptible to proteolysis, for example proteolysis by Cathepsin B, and/or a bond susceptible for cleavage under reductive conditions such as a disulfide bond.

40. Conjugate of claim 38 or 39, wherein the cleavable linker is subject to cleavage *in vivo* under acidic conditions as for example present in endosomes and/or lysosomes of mammalian cells, preferably human cells, preferably at pH 4.0 – 6.5, and more preferably at pH  $\leq$  5.5.

41. Conjugate of claim 37 or any one of claims 38-40 when dependent on claim 37, wherein the oligomeric molecule or the polymeric molecule of the covalent saponin conjugate is covalently bound to at least one of the at least one sdAb and/or to at least one of the at least one effector molecule, preferably to an amino-acid residue of the sdAb and/or of the effector molecule.

42. Conjugate of claim 41, wherein the at least one saponin is covalently bound to the oligomeric molecule or to the polymeric molecule of the covalent saponin conjugate via a cleavable linker according to any one of the claims 38-40.

5

43. Conjugate of claim 41 or 42, wherein the at least one saponin is covalently bound to the oligomeric molecule or to the polymeric molecule of the covalent saponin conjugate via any one or more of an imine bond, a hydrazone bond, a hydrazide bond, an oxime bond, a 1,3-dioxolane bond, a disulfide bond, a thio-ether bond, an amide bond, a peptide bond or an ester bond, preferably via a linker.

10

44. Conjugate of any one of the claims 41-43, wherein the at least one saponin comprises an aglycone core structure comprising an aldehyde function in position C<sub>23</sub> and the at least one saponin comprises optionally a glucuronic acid function in a first saccharide chain at the C<sub>3</sub>β-OH group of the aglycone core structure of the saponin, which aldehyde function is involved in the covalent bonding to the oligomeric molecule or to polymeric molecule of the covalent saponin conjugate, and/or, if present, the glucuronic acid function is involved in the covalent bonding to the oligomeric molecule or to the polymeric molecule of the covalent saponin conjugate, the bonding of the saponin either via a direct covalent bond, or via a linker.

20

45. Conjugate of claim 44, wherein the aldehyde function in position C<sub>23</sub> of the aglycone core structure of the at least one saponin is covalently bound to linker EMCH, which EMCH is covalently bound via a thio-ether bond to a sulfhydryl group in the oligomeric molecule or in the polymeric molecule of the covalent saponin conjugate, such as a sulfhydryl group of a cysteine.

25

46. Conjugate of claim 44 or 45, wherein the glucuronic acid function in the first saccharide chain at the C<sub>3</sub>β-OH group of the aglycone core structure of the saponin is covalently bound to linker HATU, which HATU is covalently bound via an amide bond to an amine group in the oligomeric molecule or in the polymeric molecule of the covalent saponin conjugate, such as an amine group of a lysine or an N-terminus of a protein.

30

47. Conjugate of any one of the claims 41-46, wherein the polymeric molecule or the oligomeric molecule of the covalent saponin conjugate is bound to at least one, preferably one, of the at least one sdAb and/or to at least one, preferably one, of the at least one effector molecule, preferably to an amino-acid residue of the sdAb and/or to an amino-acid residue of the effector molecule, involving a click chemistry group on the polymeric molecule or the oligomeric molecule of the covalent saponin conjugate, the click chemistry group preferably selected from a tetrazine, an azide, an alkene or an alkyne, or a cyclic derivative of these groups, more preferably the click chemistry group is an azide.

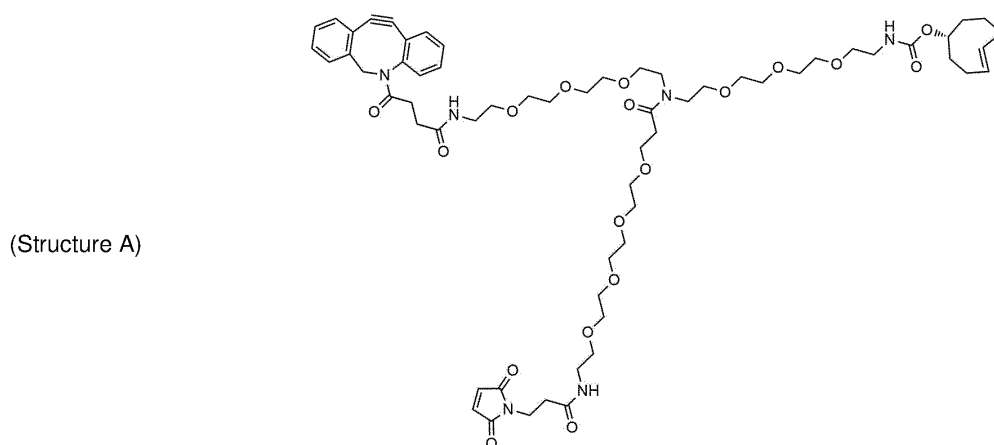
35

48. Conjugate of any one of the claims 41-47, wherein the polymeric molecule or the oligomeric molecule of the covalent saponin conjugate comprises a polymeric structure and/or an oligomeric structure

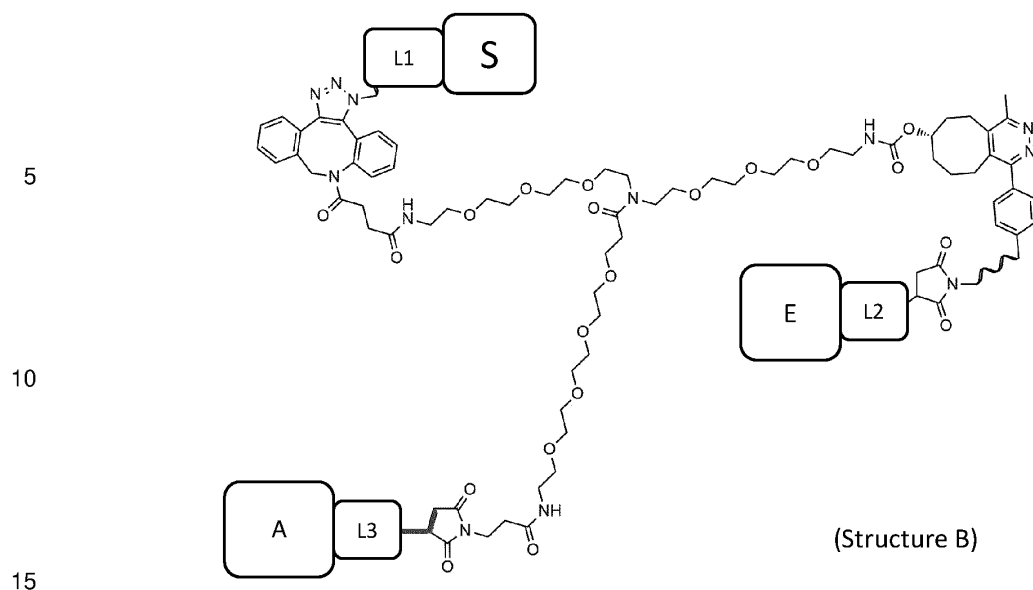
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selected from: a linear polymer, a branched polymer and/or a cyclic polymer, an oligomer, a dendrimer, a dendron such as a G2 dendron or a G3 dendron, a dendronized polymer, a dendronized oligomer, a DNA, a polypeptide, a poly-lysine, a poly-ethylene glycol, an oligo-ethylene glycol (OEG), such as OEG<sub>3</sub>, OEG<sub>4</sub> and OEG<sub>5</sub>, or an assembly of these polymeric structures and/or oligomeric structures which assembly is preferably built up by covalent cross-linking, preferably the polymeric molecule or the oligomeric molecule of the covalent saponin conjugate is a dendron such as a poly-amidoamine (PAMAM) dendrimer.

49. Conjugate according to any one of claim 1 – 48, wherein the at least one saponin is covalently bound to at least one, preferably one, of the at least one sdAb and is covalently bound to at least one, preferably one, of the at least one effector molecule via a tri-functional linker, preferably the trifunctional linker represented by Structure A:



the conjugate preferably comprising the trifunctional linker of Structure A and having a molecular structure represented by Structure B:



wherein S is the at least one saponin or the covalent saponin conjugate of any one of the claims 37 and 41-48, E is the at least one, preferably one, effector molecule, A is the at least one sdAb such as a single sdAb, L1, L2 and L3 are each individually a bond between the trifunctional linker and the saponin or the covalent saponin conjugate, the effector molecule, and the sdAb, respectively, or L1, L2 and L3 are a linker, wherein L1, L2 and L3 are the same or different.

50. Pharmaceutical composition comprising the conjugate of any one of the claims 1-49, and optionally a pharmaceutically acceptable excipient and/or pharmaceutically acceptable diluent.

51. Pharmaceutical composition of claim 50, for use as a medicament.

52. Pharmaceutical composition of claim 50, for use in the treatment or the prophylaxis of any one or more of: a cancer, an auto-immune disease such as rheumatoid arthritis, an enzyme deficiency, a disease related to an enzyme deficiency, a gene defect, a disease relating to a gene defect, an infection such as a viral infection, hypercholesterolemia, primary hyperoxaluria, haemophilia A, haemophilia B, alpha-1 antitrypsin related liver disease, acute hepatic porphyria, an amyloidosis and transthyretin-mediated amyloidosis.

53. Pharmaceutical composition of claim 51 or 52, wherein the saponin is SO1861, a SO1861 derivative, QS-21, or a QS-21 derivative, preferably a SO1861 derivative or a QS-21 derivative, more preferably a SO1861 derivative of any one of the claims 24-27.

54. Pharmaceutical composition for use of claim 52 or 53, wherein:

- said use is in the treatment or prevention of cancer in a human subject; and/or
  - said use is in the treatment or prophylaxis of cancer in a patient in need thereof, wherein the at least one sdAb binds to a cell-surface molecule of the cell, preferably to a tumor-cell surface molecule of the cell, more preferably to a tumor cell-specific surface molecule of the cell; and/or
- 5        - the pharmaceutical composition, preferably a therapeutically effective amount of the pharmaceutical composition, is administered to a patient in need thereof, preferably a human patient.

55. *In vitro* or *ex vivo* method for transferring the effector molecule of any one of the claims 1-49 from outside a cell to inside said cell, preferably to the cytosol of said cell, comprising the steps of:

- 10        a) providing a cell which expresses on its cell surface the binding site for the at least one sdAb comprised by the conjugate of any one of the claims 1-49, said binding site preferably present on a cell-surface molecule of the cell according to any one of the claims 4-9, 20, 28 or 29, said cell preferably being selected from a liver cell, an aberrant cell such as a virally infected cell, an auto-immune cell, a cell comprising a gene defect, a cell comprising an enzyme deficiency and
- 15        a tumor cell;
- b) providing the conjugate of any one of the claims 1-49, said conjugate comprising the effector molecule to be transferred into the cell provided in step a); and
- c) contacting the cell of step a) *in vitro* or *ex vivo* with the conjugate of step b), therewith effecting the transfer of said conjugate comprising the effector molecule from outside the cell
- 20        to inside said cell, and by effecting the transfer of said conjugate effecting the transfer of the effector molecule from outside the cell to inside said cell, preferably into the cytosol of said cell.

56. *In vitro* or *ex vivo* method for transferring the conjugate of any one of the claims 1-49 from outside a cell to inside said cell, comprising the steps of:

- 25        a) providing a cell which expresses on its cell surface the binding site for the at least one sdAb comprised by the conjugate of any one of the claims 1-49, said binding site preferably present on a cell-surface molecule of the cell according to any one of the claims 4-9, 20, 28 or 29, said cell preferably being selected from a liver cell, an aberrant cell such as a virally infected cell, an auto-immune cell, a cell comprising a gene defect, a cell comprising an enzyme deficiency and
- 30        a tumor cell;
- b) providing the conjugate of any one of the claims 1-49; and
- c) contacting the cell of step a) *in vitro* or *ex vivo* with the conjugate of step b), therewith effecting the transfer of the conjugate from outside the cell to inside said cell.

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