[0014] Despite the fact that much work has already been done in this area, no amatoxins have yet been coupled in a defined manner by controlling the DAR ratio. e.g. by using specific cysteines for such defined coupling. Furthermore, there doesn't seem to be a complete understanding about which amino acid positions to use for such cysteine-based conjugations. In particular, no information has yet been available about conjugates of amatoxins and antibodies via engineered cysteine residues. However, in light of the high toxicity of amatoxins, it is enormously important to identify constructs that display a high toxicity against the target cell of interest, while simultaneously showing excellent stability and tolerability. So far, this goal has not yet been achieved.

OBJECT OF THE INVENTION

[0015] Thus, there was still a great need for a cost-efficient and robust way of synthesizing conjugates comprising amatoxins and antibodies, in particular amatoxins linked to antibodies comprising specific cysteine residues, which conjugates are highly toxic towards target cells and simultaneously stable and highly tolerable. It was furthermore an object of the present invention to identify positions in antibody chains that can be mutated from a parental amino acid residue to a cysteine residue, which result in the formation of such highly toxic, stable and highly tolerable conjugates.

SUMMARY

[0016] The present disclosure is based on the unexpected observation that a limited number of specific wild-type amino acid residues can be identified, which can be mutated to single, unpaired cysteine residues, which result in the formation of highly toxic, stable and highly tolerable conjugates with amatoxins.

[0017] Thus, in one aspect the present disclosure refers to a conjugate of generic formula $(Ama – L – X – S)_n – Ab$, wherein Ama is an amatoxin, L is a linker, X is a moiety resulting from coupling of a thiol group to a thiol-reactive group, S is the sulphur atom of a cysteine amino acid residue, and Ab is an antibody sequence, or an -antigen-binding fragment, comprising said cysteine residue, wherein said cysteine residue is heavy chain 265Cys according to the EU numbering system, and wherein n indicates that one or more amatoxin molecules are coupled to one antibody or antigen-binding fragment. In one example, there is described a conjugate of generic formula: Ama – L – X – S – Ab,

wherein Ama is an amatoxin, L is a linker, X is a moiety resulting from coupling of a thiol group to a thiol-reactive group, S is the sulphur atom of a cysteine amino acid residue, and Ab is an antibody sequence, or a functional antibody fragment, comprising said cysteine residue, wherein said cysteine residue (i) is located in an antibody domain selected from CL, CH1, CH2, and CH3; (ii) is located at a position, where the germline sequence exhibiting the closest homology to the sequence of said antibody domain contains an amino acid residue different from cysteine; and (iii) is located a position that is solvent-exposed.

[0018] In another example, the present disclosure describes a conjugate of generic formula: Ama – L – X – S – Ab, wherein Ama is an amatoxin, L is a linker, X is a moiety resulting from coupling of a thiol group to a thiol-reactive group, S is the sulphur atom of a cysteine amino acid residue, and Ab is an antibody sequence, or a functional antibody fragment, comprising said cysteine residue, wherein said cysteine residue is selected from the list of heavy chain 118Cys, heavy chain 239Cys, and heavy chain 265Cys, in particular: heavy chain 118Cys, and heavy chain 265Cys.

[0019] In another aspect, the present disclosure refers to a method for synthesizing a conjugate of generic formula: $(Ama – L – X – S)_n – Ab$ by reacting a compound Ama – L – X' with an antibody, or an antigen-binding fragment, Ab – SH, wherein Ama is an amatoxin, L is a linker, X is a moiety resulting from coupling of a thiol group to a thiol-reactive group X', the group – SH is a thiol of a cysteine amino acid residue, and Ab is an antibody sequence, or sequence of an antigen-binding fragment, comprising said cysteine residue, wherein said cysteine residue is heavy chain 265Cys according to the EU numbering system, and wherein n indicates that one or more amatoxin molecules are coupled to one antibody or antigen-binding fragment. In a further example, there is disclosed a method for synthesizing a conjugate of generic formula: Ama – L – X – S – Ab by reacting a compound Ama – L – X', wherein X' is a thiol-reactive group, with an antibody Ab – SH, wherein the group – SH is a thiol of a cysteine amino acid residue, and Ab is an antibody sequence comprising said cysteine residue, wherein said cysteine residue is selected from the list of: heavy chain 118Cys, heavy chain 239Cys, and heavy chain 265Cys, in particular: heavy chain 118Cys, and heavy chain 265Cys.

[0020] In a further aspect, the present disclosure refers to a kit comprising (i) a compound Ama – L – X', wherein Ama is an amatoxin, L is a linker, X' is a thiol-reactive group, and (ii) an antibody, or an antigen-binding fragment, Ab – SH, wherein the group – SH is a thiol of a cysteine amino acid residue, and Ab is an antibody sequence, or sequence of an antigen-binding fragment, comprising said cysteine residue, wherein said cysteine residue is heavy chain 265Cys according to the EU numbering system. In another example, there is disclosed a kit comprising (i) a compound Ama – L – X', wherein X' is a thiol-reactive group, and (ii) an antibody Ab – SH, wherein the group – SH is a thiol of a cysteine amino acid residue, and Ab is an antibody sequence comprising said cysteine residue, wherein said cysteine residue is selected from the list of: heavy chain 118Cys, heavy chain 239Cys, and heavy chain 265Cys, in particular: heavy chain 118Cys, and heavy chain 265Cys.

[0021] In another example, there is disclosed a method for synthesizing the compound Ama – L – X', wherein X' is a maleimide group comprising the step of (a) reacting an amatoxin comprising a nucleophilic group with a compound Y-L-X'' wherein Y is a leaving group, and X'' is a protected maleimide group.

[0022] In a further aspect, the present disclosure refers to a pharmaceutical composition comprising the conjugate as disclosed herein.

[0023] In another aspect, the present disclosure refers to a conjugate as disclosed herein for use in the treatment of a disease associated with cells presenting a target, wherein said antibody, or functional antibody fragment, is specific for said target. In one example, there is disclosed a method of treating a disease associated with cells presenting a target, comprising the step of: contacting said cells with a conjugate according to the present disclosure, wherein said antibody is specific for said target.

BRIEF DESCRIPTION OF THE DRAWING

[0024] **Fig. 1** shows the structural formulae of different amatoxins. The numbers in bold type (1 to 8) designate the standard numbering of the eight amino acids forming the amatoxin. The standard designations of the atoms in amino acids 1, 3 and 4 are also shown (Greek letters α to γ, Greek letters α to δ, and numbers from 1' to 7', respectively).

[0025] **Fig. 2** shows a schematic view of an IgG1 molecule and the positions of the amino acid residues that have been mutated to cysteine residues and for toxin coupling.HC-A118C; HC-S131C; HC-S239C; HC-D265C; HC-E269C; HC-N297C; HC-A327C; HC-I332C; residues HC-D265 and HC-N297 are involved in Fc γ receptor binding; substitution will interfere with receptor binding and subsequent uptake in receptor-positive cells.

[0026] **Fig. 3** shows a schematic view of the expression vectors for production of Trastuzumab heavy and light chains according to Example 1.

CLAIMS

1. A conjugate of generic formula:

$$(Ama – L – X – S)_n – Ab,$$

wherein Ama is an amatoxin, L is a linker, X is a moiety resulting from coupling of a thiol group to a thiol-reactive group, S is the sulphur atom of a cysteine amino acid residue, and Ab is an antibody sequence, or an -antigen-binding fragment, comprising said cysteine residue, wherein said cysteine residue is heavy chain 265Cys according to the EU numbering system, and wherein n indicates that one or more amatoxin molecules are coupled to one antibody or antigen-binding fragment.

2. The conjugate of claim 1, wherein the linker is selected from a stable linker and a cleavable linker.

3. The conjugate of claim 1 or 2, wherein the thiol-reactive group is selected from bromo.acetamide, iodo acetamide, 4,6-dichloro-1,3,5-triazin-2-ylamino, 4-[5-(methylsulfonyl)-1H-tetrazole-1-yl]phenyl, 2-(methylsulfonyl)-benzo[d]thiazole-5-yl, 4-[5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl]-phenyl, 2-pyridyldithio-, 2-(5-nitro-pyridiyl)dithio-, methylthiosulfonyl and maleimide, particularly maleimide.

4. The conjugate of any one of claims 1 to 3, wherein n is selected from 1 and a value between 1 and 4.

5. The conjugate of claim 4, wherein n is 2.

6. A method for synthesizing a conjugate of generic formula:

$$(Ama – L – X – S)_n – Ab$$

by reacting a compound Ama – L – X’ with an antibody, or an antigen-binding fragment, Ab – SH, wherein Ama is an amatoxin, L is a linker, X is a moiety resulting from coupling of a thiol group to a thiol-reactive group X’, the group – SH is a thiol of a cysteine amino acid residue, and Ab is an antibody sequence, or sequence of an antigen-binding fragment, comprising said cysteine residue, wherein said cysteine residue is heavy chain 265Cys

according to the EU numbering system, and wherein n indicates that one or more amatoxin molecules are coupled to one antibody or antigen-binding fragment.

7. The method of claim 6, wherein n is selected from 1 and a value between 1 and 4.

8. The method of claim 7, wherein n is 2.

9. A kit comprising (i) a compound Ama – L – X', wherein Ama is an amatoxin, L is a linker, X' is a thiol-reactive group, and (ii) an antibody, or an antigen-binding fragment, Ab – SH, wherein the group – SH is a thiol of a cysteine amino acid residue, and Ab is an antibody sequence, or sequence of an antigen-binding fragment, comprising said cysteine residue, wherein said cysteine residue is heavy chain 265Cys according to the EU numbering system.

10. A pharmaceutical composition comprising the conjugate according any one of claims 1 to 5.

11. A conjugate according to any one of claims 1 to 5 for use in the treatment of a disease associated with cells presenting a target, wherein said antibody, or functional antibody fragment, is specific for said target.