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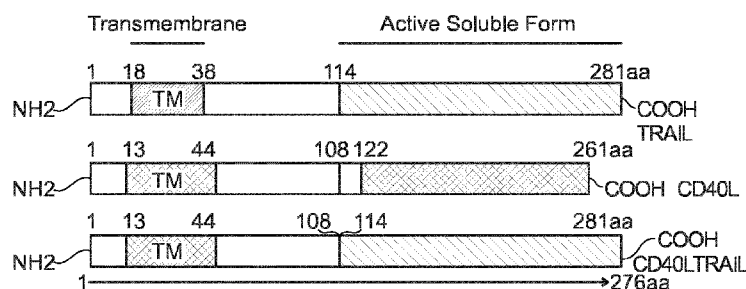


Fig. 10

- (57) **Abstract:** Provided are fusion proteins, polynucleotides and expression vectors with therapeutic utility in the prevention and/or treatment of cancer, as well as medical uses of these agents, and methods of treatment in which they are used. A fusion protein of the invention comprises an amino acid sequence that provides the apoptosis-inducing activity of TRAIL, and a membrane-anchoring amino acid sequence. The amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises the extracellular domain of TRAIL, or a fragment or variant thereof. A fusion protein comprising an amino acid sequence from TRAIL that is able to induce apoptosis, and a second sequence that anchors the first sequence to the cell membrane, is markedly more effective at reducing viability of TRAIL receptor positive cells than the naturally occurring ("wild type") TRAIL protein itself.



FUSION PROTEINS, POLYNUCLEOTIDES, EXPRESSION VECTORS, AND THEIR USES

The present invention relates to fusion proteins, polynucleotides encoding such fusion proteins, and expression vectors comprising such polynucleotides. The fusion proteins, polynucleotides and expression vectors may have therapeutic utility, particularly in the prevention and/or treatment of cancer, and the invention also relates to medical uses of these agents, and methods of treatment in which they are used.

Introduction

Cancer is one of the leading causes of mortality worldwide, and was responsible for approximately 13% of all human deaths in 2007. As the global population ages, mortality caused by cancer is expected to increase.

There is a clinical wish to develop targeted therapies that allow the selective killing of cancer cells, unlike chemotherapy or radiotherapy, both of which non-specifically kill cells of many types. Targeted therapies will allow the amount of non-specific cell death associated with treatment to be reduced, thus enhancing efficacy and giving rise to improved drug safety profiles.

TNF-Related Apoptosis-Inducing Ligand (TRAIL) is a death receptor ligand, a family of type II transmembrane proteins that signal to target cells on cell-cell contact or after protease-mediated release into the extracellular space. Soluble forms of TRAIL have recently been investigated as potential anti-cancer agents. The amino acid sequence of TRAIL is set out in SEQ ID NO: 1.

CD40 ligand (CD40L), also known as CD154, is a member of the tumour necrosis factor (TNF) superfamily of molecules. As its name implies, it binds to CD40 on antigen presenting cells. The amino acid sequence of CD40L is set out in SEQ ID NO: 2.

Summary of the invention

In a first aspect, the invention provides a fusion protein comprising an amino acid sequence that provides the apoptosis-inducing activity of TRAIL, and a membrane-anchoring amino acid sequence.

In a second aspect the invention provides a polynucleotide encoding a fusion protein according to the first aspect of the invention.

In a third aspect the invention provides an expression vector comprising a polynucleotide in accordance with the second aspect of the invention.

The invention also provides medical uses of the fusion proteins, polynucleotides, and expression vectors of the first, second, and third aspects of the invention. These agents are particularly useful as medicaments in the prevention and/or treatment of cancer.

In a fourth aspect the invention provides a pharmaceutical composition comprising an expression vector in accordance with the third aspect of the invention.

In a fifth aspect the invention provides a method of preventing and/or treating cancer, the method comprising providing to a subject in need of such treatment a therapeutically effective amount of a fusion protein in accordance with the first aspect of the invention.

In a sixth aspect the invention provides a method of preventing and/or treating cancer, the method comprising providing to a subject in need of such treatment an expression vector in accordance with the third aspect of the invention.

The present invention is based upon the inventors' surprising finding that a fusion protein comprising an amino acid sequence from TRAIL that is able to induce apoptosis, and a second sequence that anchors the first sequence to the cell membrane, is markedly more effective at reducing viability of TRAIL receptor positive cells than the naturally occurring ("wild type") TRAIL protein itself.

Particularly effective in this regard is an exemplary fusion protein (set out in SEQ ID NO: 3) in which the extracellular domain of TRAIL is combined with the transmembrane domain of CD40L. There have been no previous disclosures to suggest the particular effectiveness of this novel fusion protein.

The approach that has been adopted by the inventors, exemplified by their development of fusion proteins of the invention comprising amino acid sequences from TRAIL and CD40L, contrasts with previous, unsupported suggestions, that efficacy of proteins such as TRAIL and CD40L may be improved by inhibiting cleavage of the membrane bound ligand through the disruption of internal protease cleavage sites. In fact, as described in more detail in the

Examples section below, strategies directed towards the development of TRAIL derivatives in which endogenous cleavage sites are removed by mutation have proved to be unsuccessful. In contrast, the inventors' approach using fusion proteins is significantly more effective than the use of wild type TRAIL, wild type CD40L, or soluble TRAIL or CD40L.

The "Useful definitions" and "Detailed description of the invention" sections that follow provide, more details of further aspects of the invention, and preferred embodiments of the various aspects. Further details of the studies illustrating the benefits of the fusion proteins of the invention are described in the "Examples" section below.

Useful definitions

Certain terms used herein will now be further defined in order to further facilitate the understanding of the present invention.

"Fusion protein"

In accordance with its normal use, a fusion protein is taken to be a protein that comprises all or part of at least two naturally occurring proteins. It will be appreciated that naturally occurring proteins themselves do not meet this definition, and so a naturally occurring protein cannot be considered to constitute a "fusion protein" in accordance with the present invention. Thus, it may be preferred that if the amino acid sequence that provides the apoptosis-inducing activity of TRAIL consists of a sequence found in TRAIL, then membrane-anchoring amino acid sequence should not consist of the transmembrane region of TRAIL.

For the avoidance of doubt, a protein that essentially consists of sequence from a single naturally occurring protein, with the incorporation of some amino acid substitutions or deletions, but without the introduction of amino acid sequences from a second naturally occurring protein, does not constitute a fusion protein within the meaning of the present invention.

"Amino acid sequence that provides the apoptosis-inducing activity of TRAIL"

An "amino acid sequence that provides the apoptosis-inducing activity of TRAIL" is, for the purposes of the present invention any amino acid sequence that is capable of exerting the ability to induce apoptosis observed in respect of TRAIL.

The skilled person will readily be able to determine whether or not a sequence provides this activity, and in connection with this may consider whether or not the sequence is able to induce apoptosis, whether such apoptosis is induced only in cells that express TRAIL-R1 and/or TRAIL-R2, and whether the ability to induce apoptosis in this manner is inhibited by the presence of either neutralizing antibodies to TRAIL, or soluble TRAIL. Suitable assays by which the ability of an amino acid sequence to provide the apoptosis-inducing activity of TRAIL may be investigated are discussed further in the Examples section.

In a suitable embodiment, the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises the extracellular domain of TRAIL, or a fragment or variant thereof.

In its native form, the extracellular domain of wild type TRAIL comprises residues 39 to 281 of the amino acid sequence set out in SEQ ID NO: 1. However, one or more proteolytic cleavage sites are located within the sequence between residues 39 and 114, and these cleavage sites should be excluded from the sequence providing the apoptosis-inducing activity of TRAIL.

Thus a suitable fusion protein in accordance with the invention may comprise an amino acid sequence that provides the apoptosis-inducing activity of TRAIL from which proteolytic cleavage sites have been excluded by either deletion or replacement of amino acid residues required for cleavage. Alternatively, in a suitable embodiment residues 39 to 113 may be excluded from a fragment or variant of the wild type TRAIL sequence, and the sequence in the fusion protein used as an amino acid sequence that provides the apoptosis-inducing activity of TRAIL may be based only on the amino acid sequence found at positions 114 to 281 of in SEQ ID NO: 1. In a suitable embodiment, all of these residues may be used, as found in the fusion protein the sequence of which is set out in SEQ ID NO: 3.

In the case that the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises a variant of the extracellular domain of TRAIL, such a variant may, for example, share 90%, 95%, 96%, 97%, 98%, or 99% identity with the corresponding portion of the native TRAIL sequence (which may, for example, be the sequence at positions 114 to 281 of SEQ ID NO: 1). By the same token, a suitable variant may differ from the corresponding portion of the native TRAIL sequence (such as the sequence at positions 114 to 281 of SEQ ID NO: 1) by up to 50 amino acid residues, by up to 40 amino acid residues, by up to 30 amino acid residues, by up to 20 amino acid residues, or by up to 10 amino acid residues. A suitable derivative may differ from the corresponding portion of the native TRAIL

sequence (such as the sequence at positions 114 to 281 of SEQ ID NO: 1) by 9, 8, 7, 6, 5, 4, 3, 2 or just 1 amino acid residues.

In the case that the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises a fragment of the extracellular domain of TRAIL. It will be appreciated that fragments of this sort share 100% sequence identity with the corresponding portion of the native TRAIL sequence, but that they are truncated as compared to the native TRAIL sequence (as set out in SEQ ID NO: 1). Suitably such a fragment may, for example, consist of approximately 175, 170, or 168 contiguous amino acid residues of the native TRAIL sequence. Such a fragment may, consist of approximately 165, 160, 155, or 150 contiguous amino acid residues of the native TRAIL sequence.

“Membrane-anchoring amino acid sequence”

A “membrane-anchoring” amino acid sequence is, for the purposes of the present invention, an amino acid sequence that serves to anchor a fusion protein of the invention in the cell membrane of the cell expressing the protein. The orientation will be such that the amino acid sequence providing the apoptosis-inducing activity of TRAIL is found on the extracellular side of the membrane. This arrangement allows the fusion protein to interact with cells other than the cell in which the fusion protein has been expressed.

Merely by way of example, in a suitable embodiment the membrane-anchoring amino acid sequence may be selected from the group consisting of:

- a transmembrane domain;
- a glycosylphosphatidylinositol (GPI) anchor;
- a sequence motif that undergoes lipid modification thereby allowing membrane anchorage of the fusion protein;
- an antibody, or antigen-binding fragment thereof, the binding of which anchors the fusion protein to the membrane.

Membrane-anchoring sequences attach to the cell membrane (for example by embedding of amino acids, in the case of such sequences based on transmembrane domains, or through associated fatty acids, in the case of GPI anchors) in a manner that secures the fusion protein to the membrane.

Suitable embodiments of the membrane-anchoring domains set out above are discussed further below, however examples of membrane-anchoring sequences that go beyond those

referred to above will also be known to those skilled in the art, and these too may be used in the fusion proteins of the invention.

In the case of transmembrane domains for use in the fusion proteins of the invention, a suitable domain may, for example, be from a one-pass transmembrane type1 proteins for example CD4. Alternatively, a suitable domain may be from a one-pass transmembrane type2 protein, such as CD72. A suitable domain may be from a four -pass transmembrane protein (for example CD37), and may comprise one of more of the four individual transmembrane domains found in such proteins. Alternatively, a suitable domain may be from a multiple transmembrane attachment (type III) protein, such as C5aR, and may comprise one or one of more of the individual transmembrane domains found in proteins of this sort.

In order to serve their function, membrane-anchoring sequences comprising or based upon transmembrane domains should have sufficient length to allow the sequence to become embedded in the cell membrane.

In a suitable embodiment the membrane-anchoring amino acid sequence is derived from the transmembrane domain of CD40L. For present purposes, the transmembrane domain of CD40L should be taken as consisting of the amino acid residues 13 to 44 of SEQ ID NO: 2.

In a suitable embodiment, the membrane-anchoring amino acid sequence may comprise the transmembrane domain of CD40L, or a fragment or variant thereof.

A suitable variant may be one that shares 80%, 85% 90%, 95%, 96%, 97%, 98%, or 99% identity with the corresponding portion of the native CD40L sequence (which may comprise amino acid residues 13 to 44 of SEQ ID NO: 2). By an alternative definition, a suitable variant may differ from the corresponding portion of the native CD40L sequence (which may comprise amino acid residues 13 to 44 of SEQ ID NO: 2) by up to 30 residues, by up to 20 residues, or by up to 10 residues. For instance, a suitable variant of the transmembrane domain of CD40L (comprising amino acid residues 13 to 44 of SEQ ID NO: 2) may differ from the corresponding portion of the native CD40L sequence by 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residues.

Fragments of the CD40L transmembrane region may be selected with reference to the considerations above regarding the lengths of sequence necessary to achieve their function.

In embodiments of the invention where the fusion proteins make use of the CD40L transmembrane region, or a fragment or derivative thereof, the region, fragment or derivative may be provided as part of a longer sequence from CD40L.

Suitable membrane-anchoring sequences that allow lipid modification of the fusion proteins of the invention may facilitate conjugation of a lipid to the fusion protein *ex vivo*, or may induce lipid modification *in vivo*. Suitable membrane-anchoring sequences for that allow lipid modification of the fusion proteins of the invention may allow expression of the fusion protein at the cell surface.

By way of example a suitable sequence allowing lipid modification may be one that promotes N-myristoylation of the fusion protein. N-myristoylation is catalyzed by N-myristoyl transferase which generally recognises the sequence Met-Gly-X-X-X-Ser/Thr at the amino terminus. Other suitable sequences allowing lipid modification may include those that promote attachment of lipids selected from the group consisting of: fatty acids, isoprenoids, sterols, phospholipids, and glycosylphosphatidyl inositol (GPI). Examples of such sequences are well known to those skilled in the art.

It will also be appreciated that fusion proteins of the invention may comprise sequences that allow modification by more than one type of lipid. Merely by way of example, fusion proteins of the invention may comprise a sequence that promotes modification by myristate and a sequence that promotes modification by palmitate; a sequence that promotes modification by palmitate and a sequence that promotes modification by cholesterol; or a sequence that promotes modification by farnesyl and a sequence that promotes modification by palmitate.

Antibodies, or antigen-binding fragments thereof, suitable for use as membrane-anchoring amino acid sequences in the fusion proteins of the invention may bind to an antigen that is a cell surface moiety (for example, a moiety selected from the group consisting of: proteins; peptides; lipids; and polysaccharides associated with the membrane). For example, a suitable fusion protein may comprise an anti-CD37 antibody, or an antigen-binding fragment thereof.

Further examples of membrane-anchoring amino acid sequences that may be used in fusion proteins of the invention include suitable integral (intrinsic) or peripheral (extrinsic) proteins that leads to expression of the fusion protein at the cell surface.

“Therapeutic agents of the invention”

The ability of the fusion proteins of the invention to induce apoptosis in cancer cells means that these proteins (and indirectly the polynucleotides encoding the proteins, and expression vectors comprising such polynucleotides) have therapeutic utility in the prevention and/or treatment of cancer. The inventors believe that this utility is applicable in respect of a very wide range of cancers. Further examples of such cancers that may be prevented and/or treated using the fusion proteins of the invention are described elsewhere in the specification, and include, but are not limited to: cervical cancer; pancreatic cancer; liver cancer; head or neck cancer; and breast cancer.

For the sake of brevity, fusion proteins, polynucleotides or expression vectors in accordance with any of the aspects or embodiments of the invention described herein may all be referred to as “therapeutic agents of the invention”. Except for where the context requires otherwise, references to “therapeutic agents of the invention” in this manner may be considered equally applicable to any of the specific aspects or embodiments disclosed herein.

In keeping with their biological activity referred to above, and as set out elsewhere in the present specification, the invention provides medical uses of the fusion proteins, polynucleotides, and expression vectors. These medical uses may be for the prevention and/or treatment of cancer. Similarly, the invention provides methods of treatment, practiced to prevent and/or treat cancer, employing the fusion proteins, polynucleotides, or expression vectors described herein.

Specific forms of cancer in which the medical uses or methods of treatment described above may be of particular value are described further below.

In addition to the use of therapeutic agents in the treatment of cancer, it will also be appreciated that these therapeutic agents are suitable for use prophylactically in the prevention of cancer. In particular, therapeutic agents of the invention may be of prophylactic use in individuals identified as at high risk of developing cancers that are TRAIL Receptor (DR4 or DR5 or both) positive.

“A therapeutically effective amount”

A “therapeutically amount” of a therapeutic agent of the invention is an amount of such an agent that is, whether provided in a single dose or cumulatively over time, sufficient to induce a degree of cancer cell apoptosis that provides a therapeutic benefit. By way of example, a therapeutically effective amount of a therapeutic agent of the invention may be

one able to meet one or more of the following criteria: capable of effecting a reduction in tumour size; capable of slowing tumour growth.

While it will be appreciated that it is the fusion proteins of the invention that exert the ability to induce cancer cell apoptosis, the concept of a therapeutically effective amount in the context of the present disclosure applies not only to such proteins, but also to polynucleotides or expression vectors of the invention. While a therapeutically effective amount of a fusion protein of the invention is capable of “directly” inducing the requisite cancer cell apoptosis, the polynucleotides of the invention induce such apoptosis “indirectly” – by giving rise to expression of the biologically active fusion protein. Thus, in the contexts of polynucleotides or expression vectors of the invention, therapeutically effective amounts are amounts that are sufficient to bring about the expression of a therapeutically effective amount of the fusion protein of the invention.

Detailed description of the invention

As described elsewhere in the present specification, the fusion proteins of the invention, in which sequences providing the apoptosis-inducing activity of TRAIL are secured to the cell membrane by an anchoring sequence, have improved cancer cell-killing activity as compared to naturally occurring TRAIL. In a suitable embodiment the amino acid sequence that provides the apoptosis-inducing activity of TRAIL may be selected so that it does not contain a protease cleavage site. Suitably such a sequence may, for example, be a fragment chosen for the absence of a cleavage site, or a derivative in which a cleavage site has been disrupted.

Additionally or alternatively, a fusion protein of the invention may comprise a membrane-anchoring amino acid sequence selected so that it does not contain a protease cleavage site. As with the preceding paragraph such a sequence may, for example, be a fragment chosen for the absence of a cleavage site, or a derivative in which a cleavage site has been disrupted. The fragment of the CD40L transmembrane domain utilised in the specific fusion protein of SEQ ID NO: 3 represents an example of a membrane-anchoring sequence that has been chosen in this manner, since it ends before the cleavage site found at amino acid residues 110-116 of wild-type CD40L. It will be appreciated that omission of the cleavage site in this manner facilitates retention of the fusion protein on the cell surface. This chosen fragment of the CD40L transmembrane domain, or derivatives of this fragment, represents a preferred membrane-anchoring sequence for use in the fusion proteins of the present invention. Similarly, other fragments of the CD40L transmembrane region from which the

naturally occurring cleavage site at residues 110 to 116 has been omitted, or derivatives of such fragments, constitute further preferred examples of membrane-anchoring amino acid sequences suitable for use in the fusion proteins of the invention.

As has been referred to above, the therapeutic agents of the invention are suitable for use as medicaments for the prevention and/or treatment of cancer. In particular, the therapeutic agents of the invention are suitable for use as medicaments in the prevention and/or treatment of cancer that comprises cells expressing TRAIL-R1 (also referred to as DR4) and/or TRAIL-R2 (also referred to as DR5).

The determination of whether or not a cancer comprises cells that express TRAIL-R1 or TRAIL-R2 is a simple manner, and the skilled person will be able to identify many suitable assays by which the presence or absence of TRAIL-R1 and/or TRAIL-R2. Merely by way of example, immunolabelling of biopsy samples (such as histological samples) may be undertaken, for example using suitable commercially available antibodies. Examples of such antibodies include an antibody to TRAIL-R1 with the catalogue number "ab16955" and an antibody to TRAILR-2 with the catalogue number "ab16942", both of which are available from Millipore). Alternatively, a suitable approach may involve investigation of gene expression in a population of cells including the cancer cells of interest, for example by RT-PCR or other techniques known to those skilled in the art.

While assaying cancers for the presence of cells expressing TRAIL-R1 and/or TRAIL-R2 is not currently standard clinical procedure, this may be readily adopted once the benefits provided by the present invention have been appreciated.

In view of the above, it will be appreciated that subjects with cancer comprising cells positive for TRAIL-R1 and/or TRAILR-2 represent a preferred subgroup of patients that may derive benefit from the prevention and/or treatment of cancer through the medical use of therapeutic agents of the invention.

The invention also provides a method of determining suitability of a subject for treatment using the fusion proteins of the invention (for example in the methods of the invention), the method comprising assaying a sample comprising cells from the subject for the expression of TRAIL-R1 and/or TRAIL-R2, wherein the presence of TRAIL-R1 and/or TRAIL-R2 expression indicates that the subject may benefit from treatment using the fusion proteins of the invention methods of the invention, and wherein an absence of TRAIL-R1 and/or TRAIL-R2 expression indicates that the subject will not benefit from treatment using the fusion

proteins of the invention. Treatment using the fusion proteins of the invention may be achieved by provision of a nucleic acid encoding the fusion protein. Suitably, the method of determining suitability referred to above may further comprise a step of providing treatment using the fusion proteins of the invention (for example in the methods of treatment of the invention) when it is indicated that a subject will benefit from such treatment.

Polynucleotides encoding the fusion proteins of the invention represent a second aspect of the invention, and an example of such a polynucleotide is provided by SEQ ID NO:4 below.

As referred to above, expression vectors that comprising a polynucleotide encoding a fusion protein of the invention are themselves a third aspect of the present invention. Suitably, the vector may comprise a conditionally replicating virus.

DNA delivery systems, such as, but not limited to, the vectors of the invention, are suitable for use as therapeutic agents of the invention. The skilled person will be aware of a wide range of DNA delivery systems that may be employed for such use, and these DNA delivery systems, whether vectors or otherwise, may be used in the medical uses of the invention, incorporated in pharmaceutical compositions of the invention, and/or used in methods of treatment of the invention

As demonstrated further in the Examples below, the fusion proteins of the invention have been shown to be effective in inducing death of cancer cells derived from a range of different carcinomas types. Accordingly, the inventors believe that the therapeutic agents of the invention may be of use in the prevention and/or treatment of cancers including, but not limited to, the group consisting of: cervical cancer; pancreatic cancer; liver cancer; head or neck cancer; and breast cancer.

The skilled person will appreciate that there are many suitable routes of administration by which pharmaceutical compositions of the invention or expression vectors of the invention may be provided to a subject.

The invention will now be further defined with reference to the following Examples, and the accompanying Figures, in which:

Figure 1 illustrates PCR amplification of the CD40L N-terminal (1-324bp) and TRAIL C-terminal domain (340-846bp). 5 µl of the purified PCR products were run in 1% agarose gel containing 0.2 µg/ml ethidium bromide. The amplified fragment runs at an expected size.

Lane M is the DNA ladder, lane 1 is the amplified CD40L fragment, and lane 2 is the amplified TRAIL fragment;

Figure 2 shows agarose gel electrophoresis of the PCR amplified CD40LTRAIL fusion protein of the invention. 5 µl of the purified PCR product was run in 1% agarose gel containing 0.2 µg/ml ethidium bromide. The amplified fragment runs at the expected size of 831 bp;

Figure 3 illustrates analysis of the pShuttle-CMVCD40LTRAIL miniprep DNA. Empty pShuttle-CMV or pShuttle-CMVCD40LTRAIL were digested with *Sall* and *HindIII* for one hour and analysed using 1% agarose gel electrophoresis. Lane M is a DNA ladder, lane 1 is the empty vector, lane 2 is pShuttle-CMVncCD40LTRAIL;

Figure 4 sets out analysis of PAdshuttle-CMV-CD40LTRAIL recombinants. Kanamycin-positive selected clones were digested with *PacI* restriction endonuclease and the restriction products were analysed using 1% agarose gel electrophoresis. Lane M is DNA ladder; lane 1 is empty pAeasy-1 vector; lanes 2, 7 and 8 show the correct orientation of recombinant PAdshuttle-CMV-CD40L-TRAIL clones; lanes 3, 4 and 5 refer to pShuttle-CMV CD40LTRAIL; lane 6 refers to the recombinant PAdshuttle-CMV-CD40L-TRAIL but with the wrong orientation;

Figure 5 illustrates Confirmation of the retention of CD40L-TRAIL protein fusion expressed by RAdCD40LTRAIL at the cell surface. Panel A: EJ cells were infected with 50 MOI of either RAdTRAILwt (AdL) or RAdCD40LTRAIL (AdnL) or RAdMock (AdM) or left uninfected as a negative control. Samples were collected from the culture media 24 and 48 hours post-infection for sTRAIL measurement utilising a sTRAIL ELISA assay kit (R&D Biosystems MAB3751). Panel B: EJ cells infected with 50 MOI of RAdTRAILwt (AdL) or RAdCD40L-TRAIL (AdncL) RAdMock (AdM) or left uninfected as a negative control were grown for 48h, cells were lysed *in situ* and 60µg of total protein lysates were examined for TRAIL expression using specific antibody raised against TRAIL C-terminus domain. β-actin was also examined as a loading control;

Figure 6 illustrates the effect of RAdCD40LTRAIL compared to RAdTRAILwt on carcinoma cell viability. Panel A: The above indicated carcinoma cell lines (EJ, Hela, Panc1, HepG2) were either infected with of RAdMock (AdM), RAdTRAILwt (AdwL) or RAdCD40LTRAIL (AdncL) at the indicated MOI or left untreated as a negative control and seeded at a density of 6000 cells/100µl/well in 96 well microplate for 48 hours. Cell viability was then assessed

using the WST-1 assay reagent. Results are an average of triplet samples $\pm$ SD. Panel B: Pancreatic cell line Panc1 was either infected at 100 MOI with RAdMock, RAdTRAILwt or RAdCD40LTRAIL or left uninfected or treated with recombinant soluble TRAIL at 100ng/ml (sTRAIL) were treated with or without the pan-caspase inhibitor zVAD at a concentration of 30 μ M and seeded at a density of 6000 cells/100 μ l/well in 96 well microplate for 36 hours. Caspase 3/7 activity was then assessed utilizing the Caspase-Glo 3/7 Assay reagent according to the manufacturer's instructions (Promega, cat G8091). Results represents mean of triplet samples $\pm$ SD;

Figure 7 shows the results of FACS analysis of TRAILR1 and TRAILR2 expression in head and neck carcinoma cell lines BHY (a human oral SCC cell line); PE/CA PJ15 (a human oral SCC cell line of the tongue); and Hn (a human SCC cell line of the soft palate). FACS analysis was undertaken using: Anti-Human CD261 (also known as: TRAIL Receptor 1, TRAIL-R1, TRAILR1, DR4) PE, Clone: DJR1, Cat No: 12-6644-41; Anti-Human CD262 (also known as: Trail-R2, DR5) PE, Clone: DJR2-4 (a.k.a. 7-8), Cat. No: 12-9908-41; and Isotype: Mouse IgG1 K Isotype Control PE, Clone: P3.6.2.8.1, Cat No: 12-4714-42. All antibodies were purchased from ebioscience;

Figure 8 shows the effect of the membrane bound CD40L TRAIL fusion protein (AdncL) versus the wild-type TRAIL and recombinant soluble TRAIL (rsTRAIL) on cell viability. The human squamous cell carcinoma (SCC) cell lines BHY, HN and PE-CA- PJ15 were infected with 100 MOI of either RAdMock (AdM), RAdwtTRAIL (Adwt) or AdncCD40LTRAIL (AdncL) or treated with rsTRAIL at a concentration of 100ng/ml or left untreated as a negative control for 48 hours. Cell viability was then assessed with WST-1 assay (Roche cat no 05015944001). Results represent a mean of triplet samples $\pm$ SD;

Figure 9 illustrates inhibition of TRAIL binding to TRAIL receptors in RAdCD40LTRAIL-infected cells by neutralizing the expressed CD40LTRAIL via TRAIL monoclonal antibody or competing the binding by recombinant soluble TRAIL (rsTRAIL) restores cell viability. The pancreatic cell line Panc1 cells were infected with either RAdMock (AdM) or RAdTRAILwt (AdL) or RAdCD40LTRAIL (AdncL) at 100 MOI or left untreated as a negative control or treated with rsTRAIL at a concentration of 100 ng/ml, RAdCD40LTRAIL- infected cells were treated with an increasing concentrations (0.5, 1, 2 and 4 μ g/ml) of the neutralising mouse monoclonal TRAIL antibody (ab2219, 2E5), or at a fixed concentration (2 μ g/ml) of the monoclonal isotype mouse IgG1 control as a negative control, or treated with an increasing concentrations (50, 100 and 200ng/ml) of rsTRAIL or left untreated as a negative control. Cells were seeded at a density of 6000 cells/100 μ l/ well in 96 well microplate for 36 h. (A)

cell viability were examined by WST-1 assay reagent. Results are a mean of three triplicate samples $\pm$ SD, (B) microscopic pictures were taken for each treatment highlighting the visual effect of each treatment on the cells;

Figure 10 is a schematic representation of TRAIL, CD40L, and the CD40L/TRAIL fusion protein of SEQ ID NO:3;

Figure 11 shows sequence alignment between Fas ligand, CD40L, and TRAIL;

Figure 12 shows the results of ELISA for TRAIL in media conditioned by a range of cells: HEK 293 cells transfected with 1 μ g DNA of pShuttle wt TRAIL, pShuttle TRAILmt1, pShuttle TRAILmt2, pShuttle CMV, or left untransfected as a negative control. Samples were collected from the culture media 48 hours after transfection and assessed for the presence of sTRAIL; and

Figure 13 illustrates the sensitivity of HeLa and HepG2 cells to fusion proteins of the invention (as exemplified by SEQ ID NO:3).

Examples

1 Generation of polynucleotides, expression vectors, and fusion proteins in accordance with the invention

1.1 Generation of CD40L N-terminal fragment (1-324bp)

A pCLneoCD40L construct together with a couple of forward **CD40L₁Sal1F** (AGTC GAC ATG ATC GAA ACA TAC AAC CA) and reverse primers **CD40L_{324R}** (TTC TTT CTT CGT CTC CTC TTT GTT), were used under the following the PCR condition (93°C 5 mins (93°C 30 Sec, 58.1°C, 45 Sec, 72°C 2 mins) x 25, 72°C, 5 mins).

1.2 Generation of TRAIL C-terminus region (340-846bp)

A pcDNATRAIL construct together with a couple of forward **CD40L₃₂₄TRAIL₃₄₀F** (Tataatgttaaacaagaggagacgaagaaagaa GTG AGA GAA AGA GGT CCT CAG AGA GTA and reverse primer **TRAIL₈₄₆ HindIIIIR** (A AA GCT TTT AGC CAA CTA AAA AGG CC) were used to generate a TRAIL region (340-846bp) fused to a CD40L oligonucleotide spanning 291-324bp (red-colored sequence) under the following PCR reaction conditions 93°C 5 mins (93°C 30 Sec, 58.4°C, 45 Sec, 72°C 2 mins) x 25, 72°C, 5 mins.

1.3 Generation of the final CD40L-TRAIL fusion

To generate the CD40L (1-324bp) and TRAIL fragment (340-846bp), 100pg of CD40L PCR DNA fragment was equally mixed with TRAIL PCR DNA fragment. The CD40LTRAIL fusion fragment was then amplified by PCR using the forward primer **CD40L₁Sal1F** (AGTC GAC ATG ATC GAA ACA TAC AAC CA) and the reverse primer **TRAIL₈₄₆ HindIIIIR** (A AA GCT TTT AGC CAA CTA AAA AGG CC), the amplified fragment was then gel purified and analyzed by 1% agarose gel electrophoresis technique as shown in figure 2.

1.4 Cloning the CD40L-TRAIL DNA fusion into the pShuttle CMV vector.

The CD40L-TRAIL DNA fusion amplified fragment was gel purified and cloned into Topo2.1 plasmid, miniprep DNA was then digested with *Sall* and *HindIII* enzymes to release the CD40LTRAIL fusion, the purified CD40LTRAIL DNA fragment was then ligated into *Sall/HindIII* predigested and purified pShuttle-CMV vector, the sequence of CD40L-TRAIL fusion was then confirmed by sequence analysis and restriction digestions.

1.5 Generation of recombinant adenoviral plasmids by homologous recombination in *E. coli*

Once we confirmed the cloning of CD40L-TRAIL DNA fusion in frame with the CMV promoter in pShuttle-CMV vector, we used the AdEasy system (Quantum Biotechnology) using adenovirus vector and method as published by Chuan He and co-workers (He, Zhou et al. 1998).

The pAdEasy-1 adenoviral plasmid contains all Ad5 sequences except nucleotides 1–3,533 (encompassing the E1 genes) and nucleotides 28,130–30,820 (encompassing E3). The left arm of the pShuttle CMV vector contains the Ad5 nucleotides 34,931–35,935. The right arm contains Ad5 nucleotides 3,534–5,790 which mediate homologous recombination with the PAdEasy-1 vector in *E. coli*, plus inverted terminal repeat (ITR) and packaging signal sequences (nucleotides 1–480 of Ad5) required for viral production in mammalian cells. To insert the CD40L-TRAIL DNA fusion sequence into the Adenoviral backbone, the pShuttle CD40LTRAIL construct was homologously recombined with PAdEasy-1 vector.

Typically, 1.0 µg of pShuttle-CMVCD40LTRAIL vector was linearized with *PmeI*, purified by phenol/chloroform extraction, and mixed with 0.1 µg of pAdEasy-1 in a total volume of 6 µl. The DNA mixture was used to transform 20 µl of electrocompetent *E. coli* BJ5183 cells by electroporation method. The cell suspension was then plated onto LB-agar medium containing 50 µg/ml kanamycin and incubated at 37°C until colonies showed up. The smaller colonies (which usually represented the recombinants) were picked up and grown in 2 ml of L-Broth containing 50 µg/ml of kanamycin. Clones were first screened by analyzing their supercoiled sizes on agarose gels, comparing them to pAdEasy-1 control. Those clones that had inserts were further examined by *PacI* restriction endonuclease digestion. Once confirmed, supercoiled plasmid DNA was transformed into Topo 10F chemically competent cells for large-scale DNA amplification.

2 Cellular expression of fusion proteins

Production of adenoviruses in mammalian cells

Approximately 1.5×10^6 of 911 or HEK 293 cells were plated in 25 cm² flasks 24 hr before transfection, by which time they reached 50–70% confluency. Cells were washed once with 3 ml of OptiMEM, then 2.5 ml of OptiMEM was added to each flask and the flasks returned to the CO₂ incubator for 30 mins before transfection. 4 µg of recombinant adenoviral vector DNA, digested with *PacI* and phenol/chloroform purified and 20 µl of Lipofectamine were

added to 500µl of OptiMEM according to the manufacturer's instructions. After incubation at room temperature for 30 min, the transfection mix was added to the cells in the 25 cm² flasks. After 6 hours at 37°C, the medium containing the transfection mix was removed, and 6ml of growth medium with (30µM of the general caspase inhibitor zVAD) was added to inhibit possible cell death by CD40LTRAIL protein expression as HEK293 cells are TRAILR1 and 2 positive. Transfected cells were monitored and collected 7–10 days after transfection by scraping cells off flask and pelleting them along with any floating cells in the culture. The supernatant was discarded and the cell pellet were re-suspended in 2ml sterile PBS and viral particles were extracted by vigorous mixing with an equal volume of Arklone P and spinning for 10 min at 2000 rpm. The viral containing solution was collected and stored at -70°C or used to infect 3–5 x 10⁶ 293 cells in a 25 cm² flask. Four days later, viruses were harvested.

For large-scale recombinant adenoviral production, HEK 293 cells were plated out into twelve 150cm² flasks (IWAKI). At 80% confluency, the complete growth medium was removed and cells were supplemented with 5ml 2% FCS D-MEM with 1:100 dilution of the adenoviral extract solution. After 6 hours cells were supplemented with an additional 15ml of 2% FCS D-MEM medium (30µM of the general caspase inhibitor zVAD) and allowed to incubate at 37°C. 5-6 days post-infection, cells and medium were transferred to a 50ml tube and centrifuged at 2000rpm for 5 minutes at room temperature. The supernatant was discarded and the cell pellets were re-suspended in 10ml sterile PBS and viral particles were extracted by Arklone P.

3 *Expression of fusion proteins at the cell surface*

To test whether CD40LTRAIL protein delivered by RAdCD40LTRAIL vector resists the cleavage from cell surface, bladder carcinoma cells were either infected with 100 MOI of RAdMock, RAdTRAILwt (expressing wild-type TRAIL: 1-846bp) or RAd CD40LTRAIL or left uninfected as a negative control for 48h. Cell-free media were collected from each setting at 24 and 48h for sTRAIL assessment utilizing the sTRAIL ELISA assay kit (R&D Biosystems MAB3751). Cells were also lysed *in situ* for western blot analysis.

As shown in figure 5A, sTRAIL was detected in RAdTRAILwt-infected cells in a time dependent manner (at 48hs≥380 pg/ml), whereas less than 30pg/ml of sTRAIL were detected in cell-free medium obtained from RAdCD40LTRAIL-infected cells which could be attributed to TRAIL leaked from dead cells. Taken together these results indicate that the generated CD40L-TRAIL fusion protein is resistant to cleavage from membrane.

Wild-type TRAIL and CD40L-TRAIL were expressed from RAdTRAILwt and RAdCD40LTRAIL-infected cells respectively as determined by western blot analysis of total cell lysates (Figure 5B), using TRAIL specific antibody (Santa Cruz biotechnology Sc-1889) raised against the TRAIL c-terminus. CD40LTRAIL fusion runs slightly at a lower molecular weight compared to TRAIL wild-type protein as expected.

4 Fusion proteins of the invention reduce viability and induce apoptosis in cancer cell lines expressing TRAIL receptors

To examine the direct effect of the generated CD40L-TRAIL adenovirus compared to RAdTRAILwt, several carcinoma cell lines including the bladder carcinoma EJ, the cervical carcinoma Hela, the pancreatic carcinoma Panc1 and the liver HepG2 were either infected with RAdMock, RAdTRAILwt or RAdCD40LTRAIL at the indicated MOI as shown in figure 3a, or left untreated as a negative control and seeded at a density of 6000 cells/100µl/well in 96 well microplate for 48 hours. Cell viability was then assessed by WST-1 assay reagent. As shown in Figure 6A, Hela, Panc1 and HepG2 cells exhibit varying degrees of sensitivity toward the CD40L-TRAIL fusion protein and to a lesser extent to AdTRAILwt, however no cell viability reduction was observed in EJ where expression of TRAIL Receptor 1 and 2 is very low (data not shown). To examine whether this cell viability reduction is attributed to caspase activation and subsequent apoptosis induction, caspases 3,7 activity was assessed in Panc1 cells. As shown in figure 6B, higher caspase 3,7 activity was observed in RAdCD40LTRAIL-infected cells compared to RAdTRAIL wt control cells.

This was further illustrated in a study using head and neck cancer cell lines, the results of which are set out in figures 7 and 8. These results show that human squamous cell carcinoma (SCC) cell lines expressed the TRAIL receptors TRAILR1 and TRAILR2, and that transfection of these cell lines with expression vectors encoding the fusion protein of the invention set out in SEQ ID NO:3 significantly reduced viability of these cells.

5 Inhibition of binding of fusion proteins of the invention to TRAIL receptors restores cancer cell viability

It was important to confirm that the observed cell viability reduction is specifically due to CD40L-TRAIL expression. Therefore, we examined the effect of the neutralizing monoclonal antibody (ab2219, 2E5) on RAdCD40LTRAIL-induced cell viability reduction. Pancreatic carcinoma cells, Panc1, were infected with either RAdMock or RAdTRAILwt or

RA_{CD40L}TRAIL at 100 MOI or left untreated as a negative control or treated with rsTRAIL at a concentration of 100 ng/ml. Cells were then treated with increasing concentrations (0.5, 1, 2 and 4 µg/ml) of the neutralising mouse monoclonal TRAIL antibody (ab2219, 2E5), or at a fixed concentration (2 µg/ml) of the monoclonal isotype mouse IgG1 as a negative control, or treated with increasing concentrations (50, 100 and 200 ng/ml) of rsTRAIL or left untreated as a negative control. Cells were then seeded at a density of 6000 cells/100 µl/ well in 96 well microplate for 36h. Cell viability was then assessed by WST-1 assay reagent. As shown in figure 9, RA_{CD40L}TRAIL-infected Panc1 cells exhibited reduced cell viability. However, neutralizing the expressed CD40LTRAIL by the 2E5 neutralizing antibody significantly restored cell viability in a concentration-dependent way. This was caused by blocking CD40L-TRAIL binding to TRAIL receptors via antibody binding to the CD40LTRAIL, since the isotype control antibody was unable to restore cell viability. Furthermore, addition of soluble TRAIL into RA_{CD40L}TRAIL-infected cells resulted in partial restoration of cell viability, which could be attributed to the competition between soluble and membrane bound TRAIL on binding to the active TRAIL receptors. This result clearly shows the inhibitory effect of the CD40LTRAIL on TRAIL receptor-positive carcinoma cells.

6 Data to illustrate that the strategy to mutate the cleavage point of TRAIL did not work

The structures of TRAIL, CD40L and CD40LTRAIL fusion referred to below are shown schematically in Figure 10.

6.1 Generation of TRAIL mutant resistant to MMP cleavage

Previous work from our laboratory has shown that FasL lacking the ¹²⁸EKQI¹³¹ cleavage site is retained at the cell membrane and is more potent in inducing apoptosis than the wild-type protein. Furthermore, we also identified a CD40L cleavage site ¹¹⁰SFEMQKG¹¹⁶ by sequence alignment between FasL and CD40L (Elmetwali et al., 2010, Knox et al., 2003, Milner et al., 2002). Therefore, we sought to identify a possible TRAIL cleavage site and attempted to acquire information through sequence alignment between the published FasL and CD40L cleavage sites and TRAIL. This sequence alignment led us to an ¹⁰⁵EKQQ¹⁰⁸ site in TRAIL (Figure 11), which was similar to the FasL cleavage site ¹²⁸EKQI¹³¹, and, on this basis, we generated TRAIL mutants lacking the amino acid sequence from 105-108 and from 105 to 115. These TRAIL mutants were then cloned into pshuttle CMV vector.

However, neither the ¹⁰⁵EKQQ¹⁰⁸ nor the ¹⁰⁵EKQQHISPLV¹¹⁵ deletion mutation in TRAIL (mutants 1 and 2 respectively) were capable of blocking the cleavage of the TRAIL from the cell membrane (Figure 12). More extensive deletion mutations were not possible since these

would interfere with either the functional extracellular domain or the anchoring domain of the protein. For these reasons our alternative strategy of engineering the TRAIL-CD40L fusion protein was explored.

7 Data showing the performance benefits of the TRAIL fusion protein compared to anti-TRAIL-Receptor antibodies

Monoclonal antibodies targeting either TRAIL receptor 1 (Mapatumumab) or TRAIL receptor 2 (Lexatumumab) are currently being investigated in clinical trials, emphasising the importance of TRAIL as novel anti-cancer target. Because of the commercially sensitive nature of the development of these agents, it has not been possible to perform experiments to directly compare these antibodies with our TRAIL fusion protein. However, using published data, comparison of the ability of the antibodies and our fusion protein to induce apoptosis in comparable cell lines is possible. The antibodies can induce apoptosis in some cell lines. However, others are resistant to antibody alone and require combination with other agents such as cytotoxic chemotherapy to overcome this resistance. For example, Hela cells are resistant to lexatumumab unless used in combination with cisplatin (Clin Cancer Res 2009; 15: 2039-2047). In our experiments, Hela are relatively resistant to wild-type TRAIL unless administered with chemotherapy, consistent with the lexatumumab data. However, Hela are sensitive to our TRAIL fusion even when administered as a single agent, suggesting greater potency than both wild-type TRAIL and anti-TRAILR2 antibody (Figure 13). Similarly, the liver cancer cell line, HepG2, is resistant to mapatumumab (AACR, Cancer Res 2011; 71: suppl 8: abstract nr 4097) but is highly sensitive to the TRAIL fusion (Figure 13).

Furthermore, whilst these monoclonal antibodies target either TRAIL receptors 1 or 2, our fusion protein engages both receptors, which may facilitate a wider therapeutic spectrum.

Sequence information**SEQ ID NO:1**

Wild type TRAIL (underlined letters represent TRAIL sequence replaced by CD40L sequence in the fusion protein of SEQ ID NO:3)

MAMMEVQGGP SLGQTCVLIV IFTVLLQSLC VAVTYVYFTN ELKQMQDKYS KSGIACFLKE
DDSYWDPNDE ESMNSPCWQV KWQLRQLVRK MILRTSEETI STVQEKQQNI SPLVRERGPQ
RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR NGELVIHEKG
FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI LLMKSARNSC WSKDAEYGLY
SIYQGIGIFEL KENDRIFVSV TNEHLIDMDH EASFFGAFLV G

SEQ ID NO:2

Wild type CD40L (underlined letters represent CD40L sequence replaced by TRAIL sequence in the fusion protein of SEQ ID NO:3)

MIETYNQTSP RSAATGLPIS MKIFMYLLTV FLITQMIGSA LFAVYLHRR L
EDFVFMKTIQ RCNTGERSLS LLNCEEIKSQ FEGFVKDIML NKEETKKENS FEMQKGDQNP
QIAAHVISEA SSKTTSVLQW AEKGYTMSN NLVTLENGKQ LTVKRQGLYY IYAQVTFC
REASSQAPFI ASLCLKSPGR FERILLRAAN THSSAKPCGQ QSIHLGGVFE LQPGASVFN
VTDPSQVSHG TGFTSFGLLK L

SEQ ID NO: 3

A fusion protein of the invention (letters without underline are sequence based upon CD40L, while underlined letters represent the TRAIL C-terminal region)

MIETYNQTSP RSAATGLPIS MKIFMYLLTV FLITQMIGSA LFAVYLHRR L
EDFVFMKTIQ RCNTGERSLS LLNCEEIKSQ FEGFVKDIML NKEETKKEVR ERGPQRVA
ITGTRGRSNT LSSPNSKNEK ALGRKINSWE SSRSGHSFSL NLHLRNGELV IHEKGFYIY
SQTYFRFQEE IKENTKNDKQ MVQYIYKYTS YPDPILLMKS ARNSCWSKDA EYGLYSIYQG
GIFELKENDR IFVSVTNEHL IDMDHEASFF GAFLVG

SEQ ID NO: 4

The nucleotide sequence that encodes the fusion protein of SEQ ID NO: 3 (bases 1-324, encoding the CD40L N-terminal, are shown without underline, while bases 325-831bp encoding the TRAIL C-terminal region, and corresponding to 340-846bp from the wild type TRAIL sequence, are underlined). Note that the final codon, ACT, is a stop codon which is not translated.

ATGATCGAAACATACAACCAAACCTTCTCCCCGATCTGCGGCCACTGGACTGCCCATCAG
 CATGAAAATTTTTATGTATTTACTTACTGTTTTTCTTATCACCCAGATGATTGGGTCAGCA
 CTTTTTGCTGTGTATCTTCATAGAAGGTTGGACAAGATAGAAGATGAAAGGAATCTTCAT
 GAAGATTTTGTATTCATGAAAACGATACAGAGATGCAACACAGGAGAAAGATCCTTATC
 CTTACTGAACTGTGAGGAGATTAAAAGCCAGTTTGAAGGCTTTGTGAAGGATATAATGT
 TAAACAAAGAGGAGACGAAGAAAGAAGTGAGAGAAAGAGGTCCTCAGAGAGTAGCAGC
TCACATAACTGGGACCAGAGGAAGAAGCAACACATTGTCTTCTCCAACTCCAAGAATG
AAAAGGCTCTGGGCCGCAAATAAACTCCTGGGAATCATCAAGGAGTGGGCATTTCATT
CCTGAGCAACTTGCACTTGAGGAATGGTGAAGTGGTCATCCATGAAAAAGGGTTTTACT
ACATCTATTCCCAAACATACTTTTCGATTTTCAGGAGGAAATAAAAGAAAACACAAAGAACG
ACAAACAAATGGTCCAATATATTTACAAATACACAAGTTATCCTGACCCTATATTGTTGAT
GAAAAGTGCTAGAAATAGTTGTTGGTCTAAAGATGCAGAATATGGACTCTATTCCATCTA
TCAAGGGGGAATATTTGAGCTTAAGGAAAATGACAGAATTTTTGTTTCTGTAACAAATGA
GCACTTGATAGACATGGACCATGAAGCCAGTTTTTTTCGGGGCCTTTTTAGTTGGCTAA

Sequence alignment between the wild-type TRAIL sequence (query – SEQ ID NO: 5) and the TRAIL sequence used in the fusion protein (subject – SEQ ID NO: 6) is 99%. However the mismatched (TTT changed to TTC) nucleotide did not change the amino acid as the two triplet codons encode the same amino acid (Phe).

Query	339	AGTGAGAGAAAGAGGTCCTCAGAGAGTAGCAGCTCACATAACTGGGACCAGAGGAAGAAG	398
Sbjct	324	AGTGAGAGAAAGAGGTCCTCAGAGAGTAGCAGCTCACATAACTGGGACCAGAGGAAGAAG	383
Query	399	CAACACATTGTCTTCTCCAAACTCCAAGAATGAAAAGGCTCTGGGCCGCAAATAAACTC	458
Sbjct	384	CAACACATTGTCTTCTCCAAACTCCAAGAATGAAAAGGCTCTGGGCCGCAAATAAACTC	443
Query	459	CTGGGAATCATCAAGGAGTGGGCATTTCCTGAGCAACTTGCACTTGAGGAATGGTGA	518
Sbjct	444	CTGGGAATCATCAAGGAGTGGGCATTTCCTGAGCAACTTGCACTTGAGGAATGGTGA	503
Query	519	ACTGGTCATCCATGAAAAAGGGTTTTACTACATCTATTCCCAAACATACTTTTCGATTTC	578
Sbjct	504	ACTGGTCATCCATGAAAAAGGGTTTTACTACATCTATTCCCAAACATACTTTTCGATTTC	563

Query	579	GGAGGAAATAAAAGAAAACACAAAGAACGACAAACAAATGGTCCAATATATTTACAAATA	638
Sbjct	564	GGAGGAAATAAAAGAAAACACAAAGAACGACAAACAAATGGTCCAATATATTTACAAATA	623
Query	639	CACAAGTTATCCTGACCCTATATTGTTGATGAAAAGTGCTAGAAATAGTTGTTGGTCTAA	698
Sbjct	624	CACAAGTTATCCTGACCCTATATTGTTGATGAAAAGTGCTAGAAATAGTTGTTGGTCTAA	683
Query	699	AGATGCAGAATATGGACTCTATTCCATCTATCAAGGGGGAATATTTGAGCTTAAGGAAAA	758
Sbjct	684	AGATGCAGAATATGGACTCTATTCCATCTATCAAGGGGGAATATTTGAGCTTAAGGAAAA	743
Query	759	TGACAGAATTTTTGTTTCTGTAACAAATGAGCACTTGATAGACATGGACCATGAAGCCAG	818
Sbjct	744	TGACAGAATTTTTGTTTCTGTAACAAATGAGCACTTGATAGACATGGACCATGAAGCCAG	803
Query	819	TTTTTTGGGGCCTTTTAGTTGGCTAA	846
Sbjct	804	TTTTTTGGGGCCTTTTAGTTGGCTAA	831

% of identity between wild type TRAIL amino acid sequence (query – SEQ ID NO: 7) and TRAIL mutant sequence (subject – SEQ ID NO: 7) 100%

Query	1	VRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGE	60
		VRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGE	
Sbjct	1	VRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGE	60
Query	61	LVIHEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSK	120
		LVIHEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSK	
Sbjct	61	LVIHEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSK	120
Query	121	DAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG	168
		DAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG	
Sbjct	121	DAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG	168

Claims

1. A fusion protein comprising an amino acid sequence that provides the apoptosis-inducing activity of TRAIL, and a membrane-anchoring amino acid sequence.
2. A fusion protein according to claim 1, wherein the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises the extracellular domain of TRAIL, or a fragment or variant thereof.
3. A fusion protein according to claim 2, wherein the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises or consists of the extracellular domain of TRAIL.
4. A fusion protein according to claim 2, wherein the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises a variant of the extracellular domain of TRAIL, sharing 90%, 95%, 96%, 97%, 98%, or 99% identity with the corresponding portion of the native TRAIL sequence.
5. A fusion protein according to claim 2, wherein the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises a variant of the extracellular domain of TRAIL that differs from the corresponding portion of the native TRAIL sequence by up to 50 amino acid residues, by up to 40 amino acid residues, by up to 30 amino acid residues, by up to 20 amino acid residues, or by up to 10 amino acid residues, or by 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues.
6. A fusion protein according to claim 2, wherein the amino acid sequence that provides the apoptosis-inducing activity of TRAIL comprises a fragment of the extracellular domain of TRAIL.
7. A fusion protein according to claim 6, wherein the fragment consists of approximately 165, 160, 155, or 150 contiguous amino acid residues of the native TRAIL sequence.
8. A fusion protein according to claim 2 comprising amino acid residues 109 to 276 of SEQ ID NO: 3.
9. A fusion protein according to any preceding claim, wherein the membrane-anchoring amino acid sequence is selected from the group consisting of: a transmembrane domain; a

glycosylphosphatidylinositol (GPI) anchor; a sequence motif that undergoes lipid modification thereby allowing membrane anchorage of the fusion protein; and an epitope recognised by an antibody the binding of which anchors the fusion protein to the membrane.

10. A fusion protein according to claim 9, wherein the membrane-anchoring region comprises the transmembrane domain of CD40L, or a fragment or variant thereof.

11. A fusion protein according to claim 10, wherein the variant is one that shares 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity with the corresponding portion of the native CD40L sequence.

12. A fusion protein according to claim 10, wherein the variant is one that differs from the corresponding portion of the native CD40L sequence by up to 30 residues, by up to 20 residues, or by up to 10 residues, or by 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residues.

13. A fusion protein according to claim 10 comprising amino acid residues 1 to 108 of SEQ ID NO: 3.

14. A fusion protein comprising or consisting of the amino acid sequence set out in SEQ ID NO: 3.

15. A fusion protein according to any of claims 1 to 14 for use as a medicament.

16. A fusion protein according to claim 15, for use in the prevention and/or treatment of cancer.

17. A fusion protein for use according to claim 16, wherein the cancer comprises cells expressing TRAIL-R1 and/or TRAIL-R2.

18. A fusion protein for use according to claim 16 or claim 17, wherein the cancer is selected from the group consisting of: cervical cancer; pancreatic cancer; liver cancer; head or neck cancer; and breast cancer.

19. A polynucleotide encoding a fusion protein according to any of claims 1 to 18.

20. A polynucleotide according to claim 19, comprising DNA.

21. A polynucleotide according to claim 20, wherein the DNA comprises the sequence set out in SEQ ID NO. 4.
22. A polynucleotide according to claim 21, wherein the DNA consists of the sequence set out in SEQ ID NO. 4.
23. A polynucleotide according to claim 19, comprising RNA.
24. A polynucleotide according to any of claims 19 to 23 for use as a medicament.
25. A polynucleotide according to claim 24 for use in the prevention and/or treatment of cancer.
26. A polynucleotide for use according to claim 25, wherein the cancer comprises cells expressing TRAIL-R1 and/or TRAIL-R2.
27. A polynucleotide for use according to claim 25 or claim 26, wherein the cancer is selected from the group consisting of: cervical cancer; pancreatic cancer; liver cancer; head or neck cancer; and breast cancer.
28. An expression vector comprising a polynucleotide according to any of claims 19 to 27.
29. An expression vector according to any of claim 28 for use as a medicament.
30. An expression vector according to claim 29 for use in the prevention and/or treatment of cancer.
31. An expression vector for use according to claim 30, wherein the cancer comprises cells expressing TRAIL-R1 and/or TRAIL-R2.
32. An expression vector for use according to claim 30 or claim 31, wherein the cancer is selected from the group consisting of: cervical cancer; pancreatic cancer; liver cancer; head or neck cancer; and breast cancer.
33. A pharmaceutical composition comprising an expression vector according to any of claims 27 to 32.

34. A method of preventing and/or treating cancer, the method comprising providing to a subject in need of such treatment a therapeutically effective amount of a fusion protein according to any of claims 1 to 18.

35. A method of preventing and/or treating cancer according to claim 34, wherein the therapeutically effective amount of the fusion protein is provided by administration to the subject of a polynucleotide according to any of claims 19 to 27.

36. A method of preventing and/or treating cancer according to claim 35, wherein the therapeutically effective amount of the fusion protein is provided by administration to the subject of an expression vector according to any of claims 28 to 32.

37. A method of preventing and/or treating cancer according to any of claims 34 to 36, wherein the cancer comprises cells expressing TRAIL-R1 and/or TRAIL-R2.

38. A method of preventing and/or treating cancer according to any of claims 34 to 37, wherein the cancer is selected from the group consisting of: cervical cancer; pancreatic cancer; liver cancer; head or neck cancer; and breast cancer.

39. A method of preventing and/or treating cancer according to any of claims 34 to 38, further comprising a step of determining suitability of a subject for treatment using the methods of the invention, the method comprising assaying a sample comprising cells from the subject for the expression of TRAIL-R1 and/or TRAIL-R2, wherein the presence of TRAIL-R1 and/or TRAIL-R2 expression indicates that the subject may benefit from treatment with the methods of the invention, and wherein an absence of TRAIL-R1 and/or TRAIL-R2 expression indicates that the subject will not benefit from treatment with the methods of the invention.

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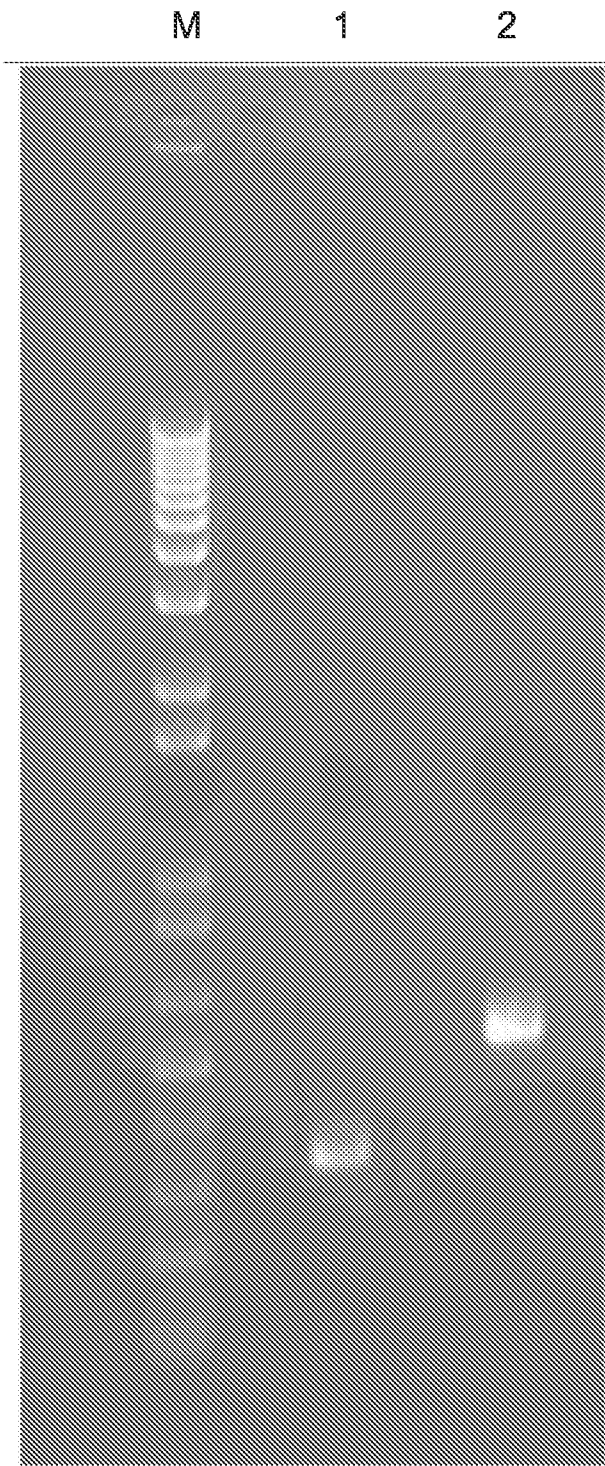


Fig. 1

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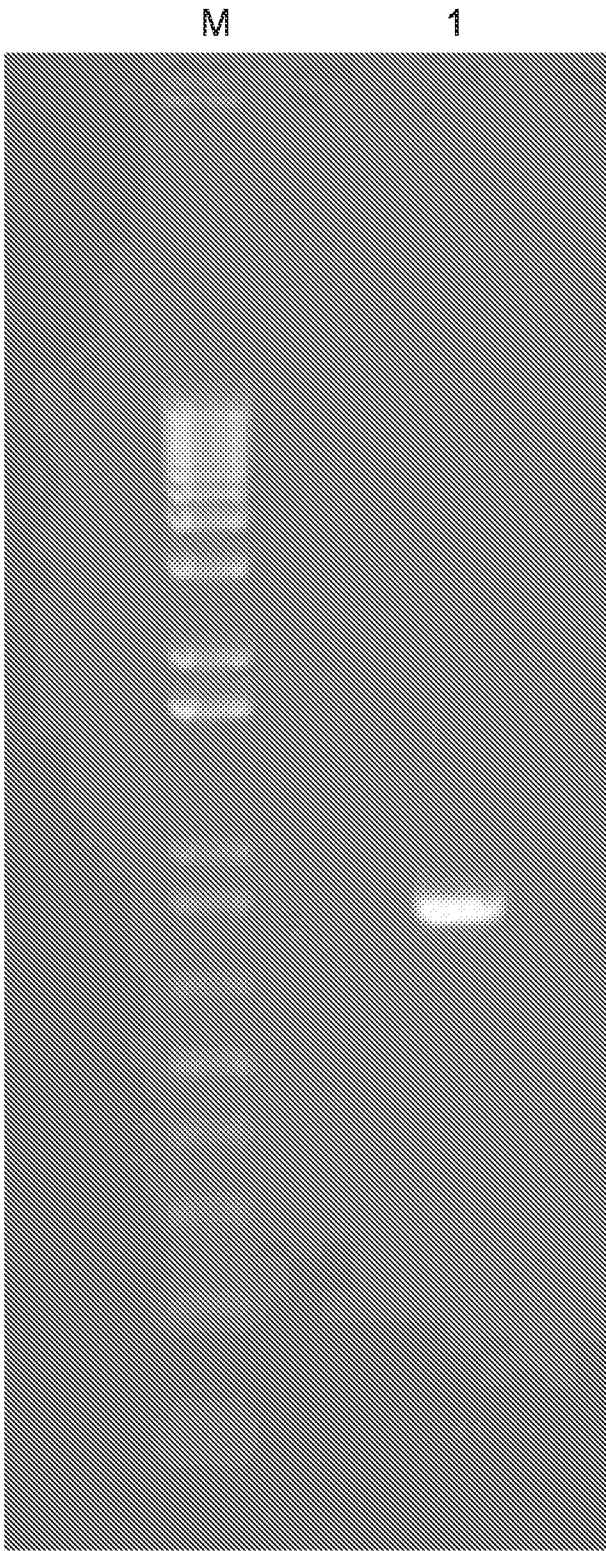


Fig. 2

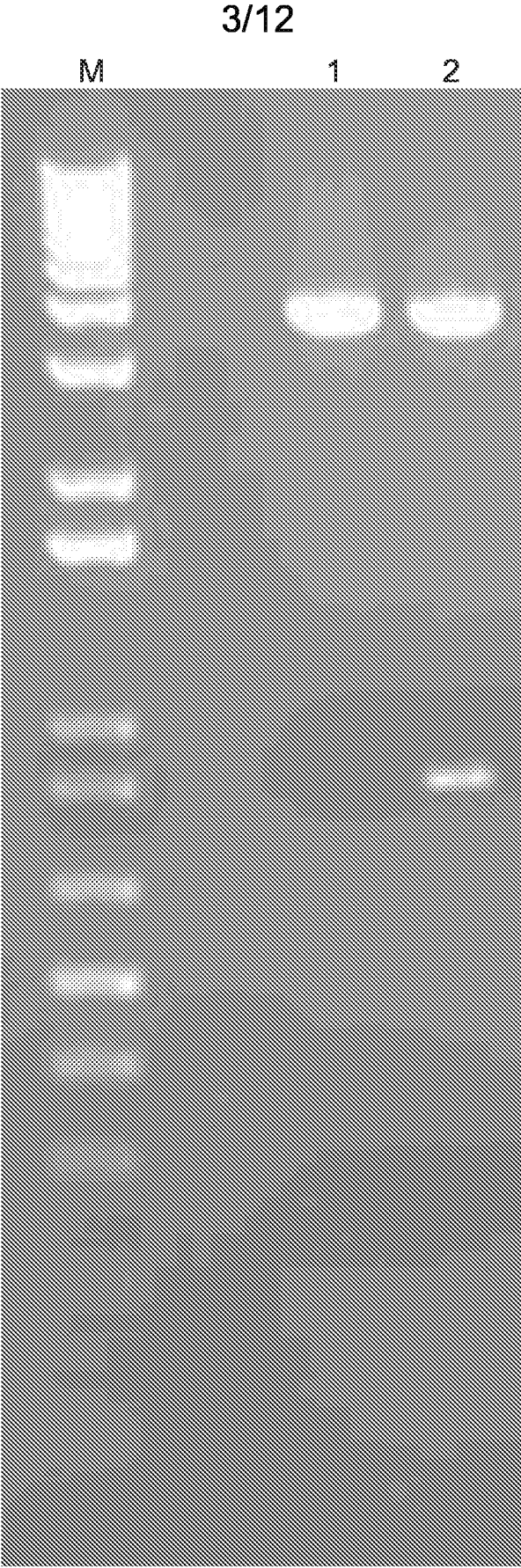


Fig. 3

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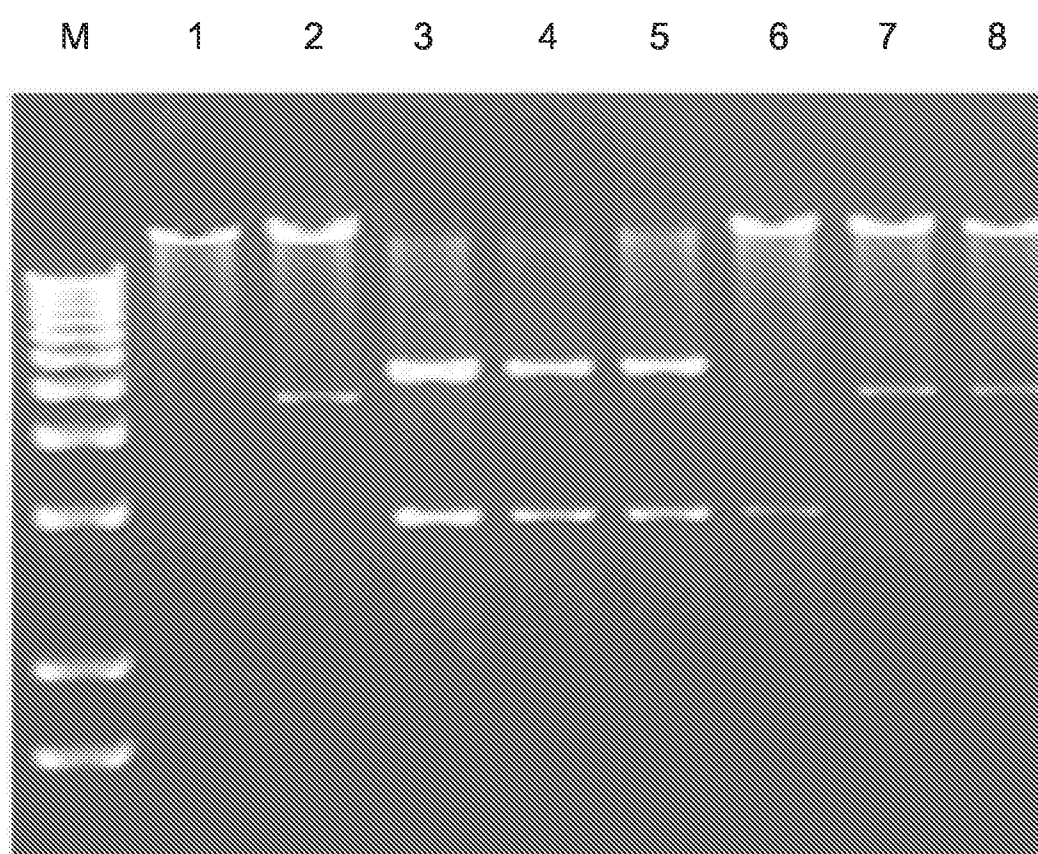


Fig. 4

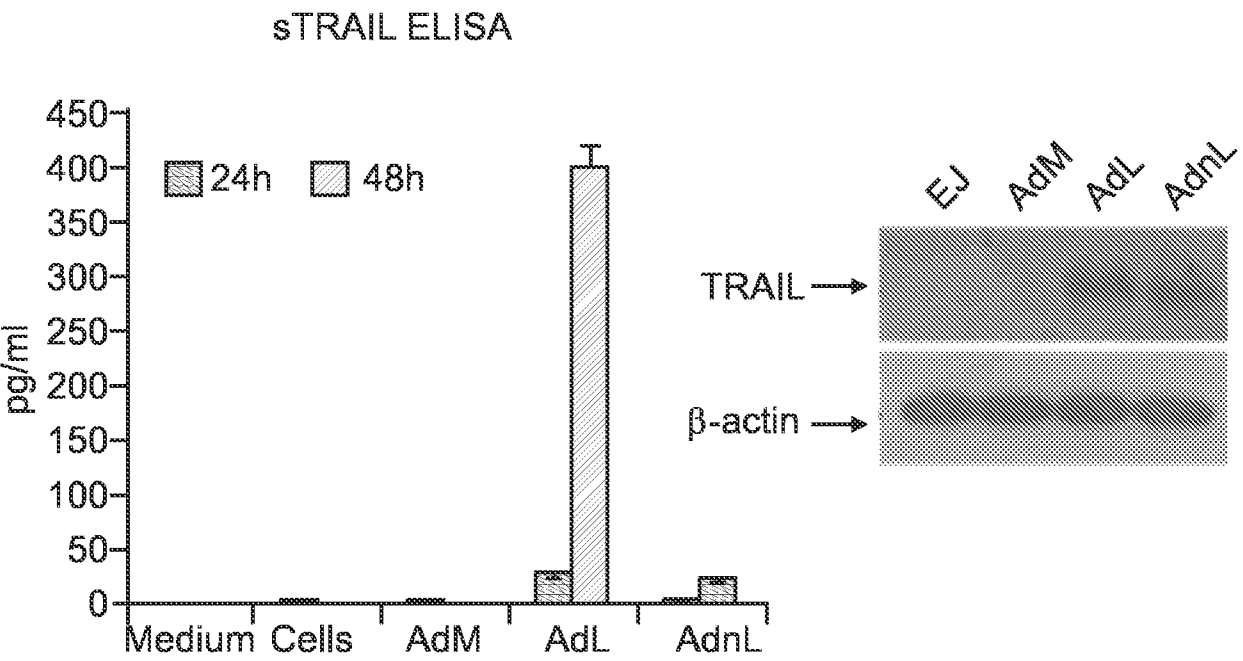


Fig. 5

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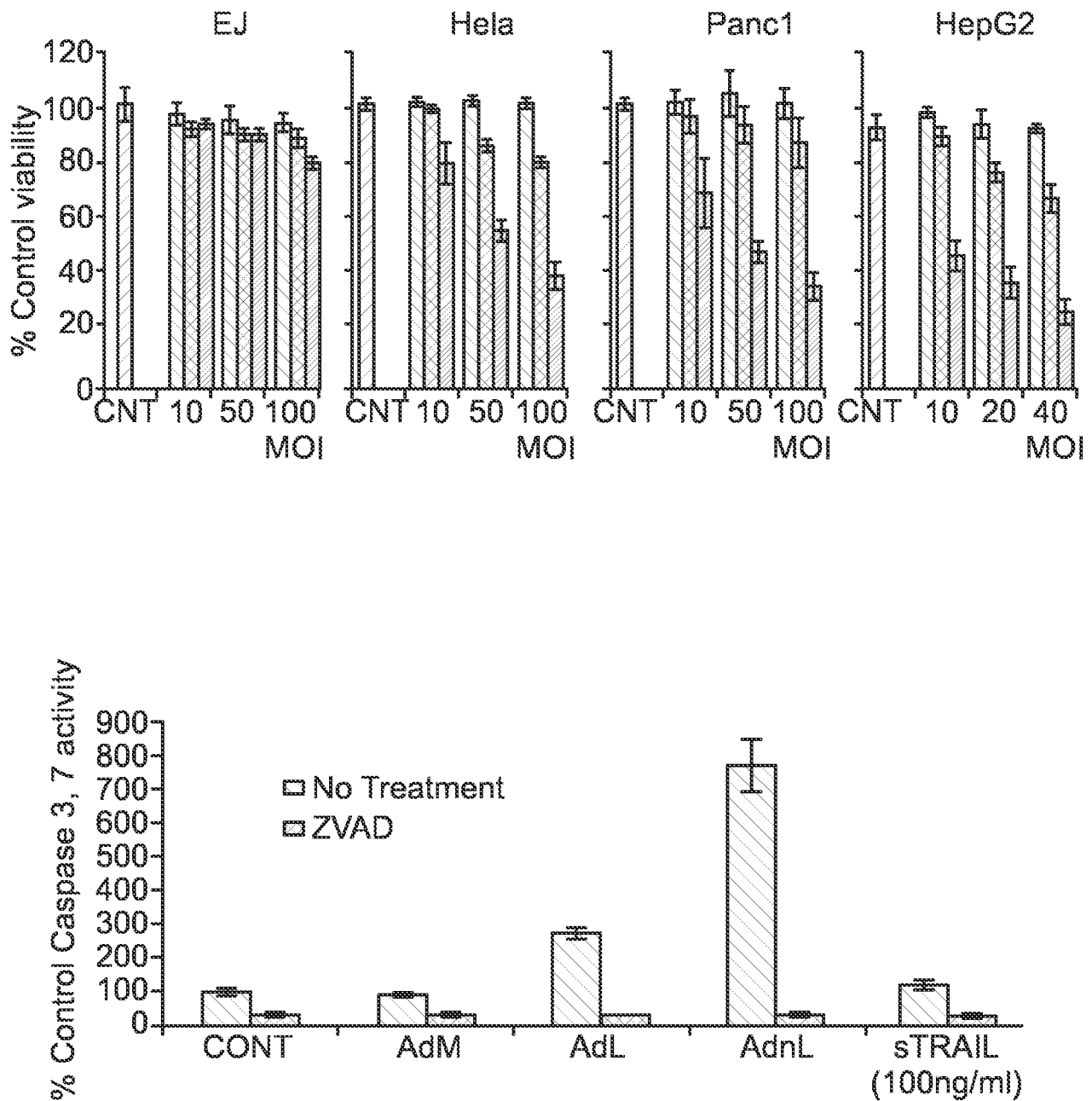
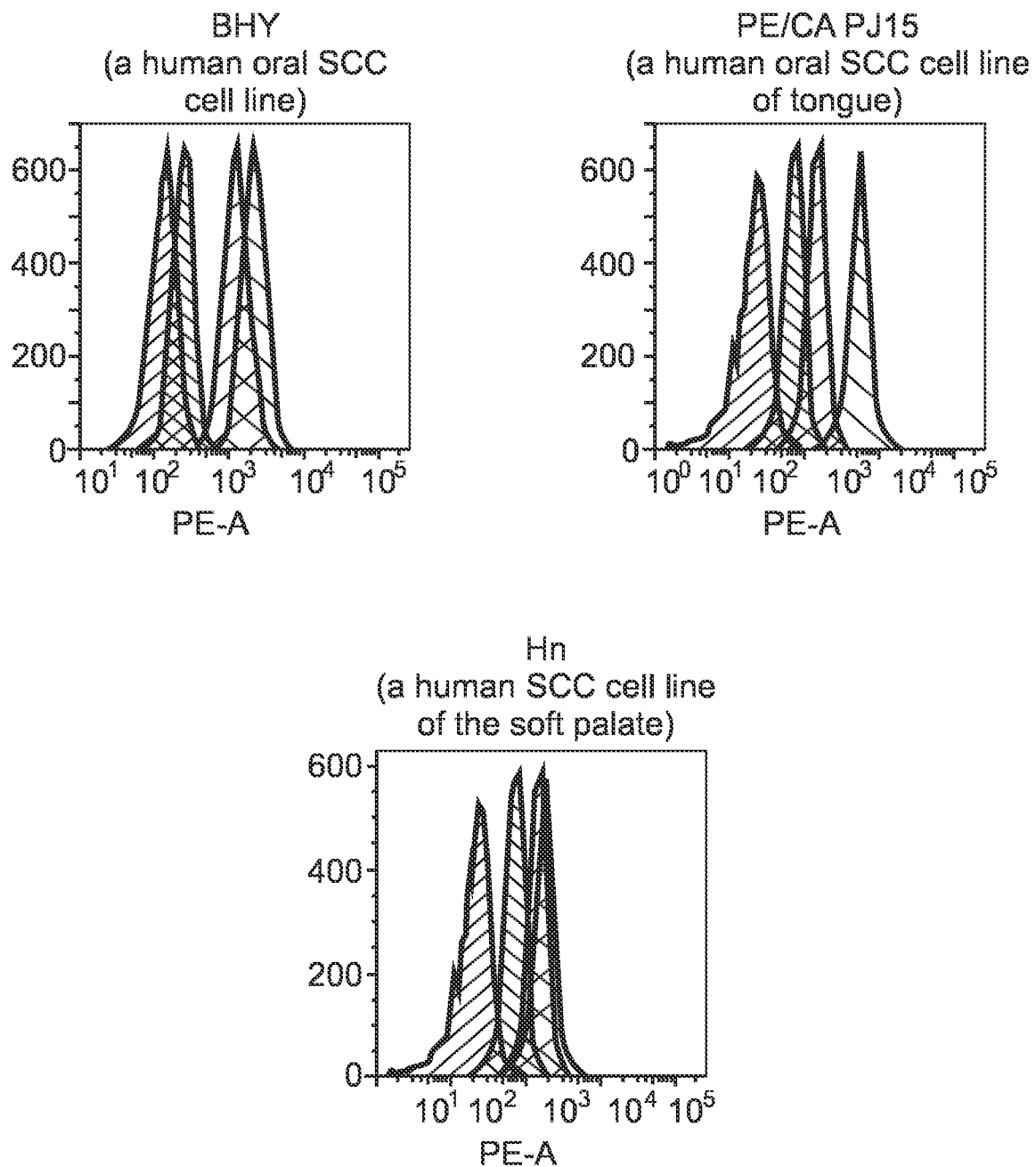


Fig. 6

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Peaks (left to right, save for Hn in which D5 peak is before DR4)

- Control unstained cells
- ▨ Isotype
- ▩ DR4
- ▤ D5

Fig. 7

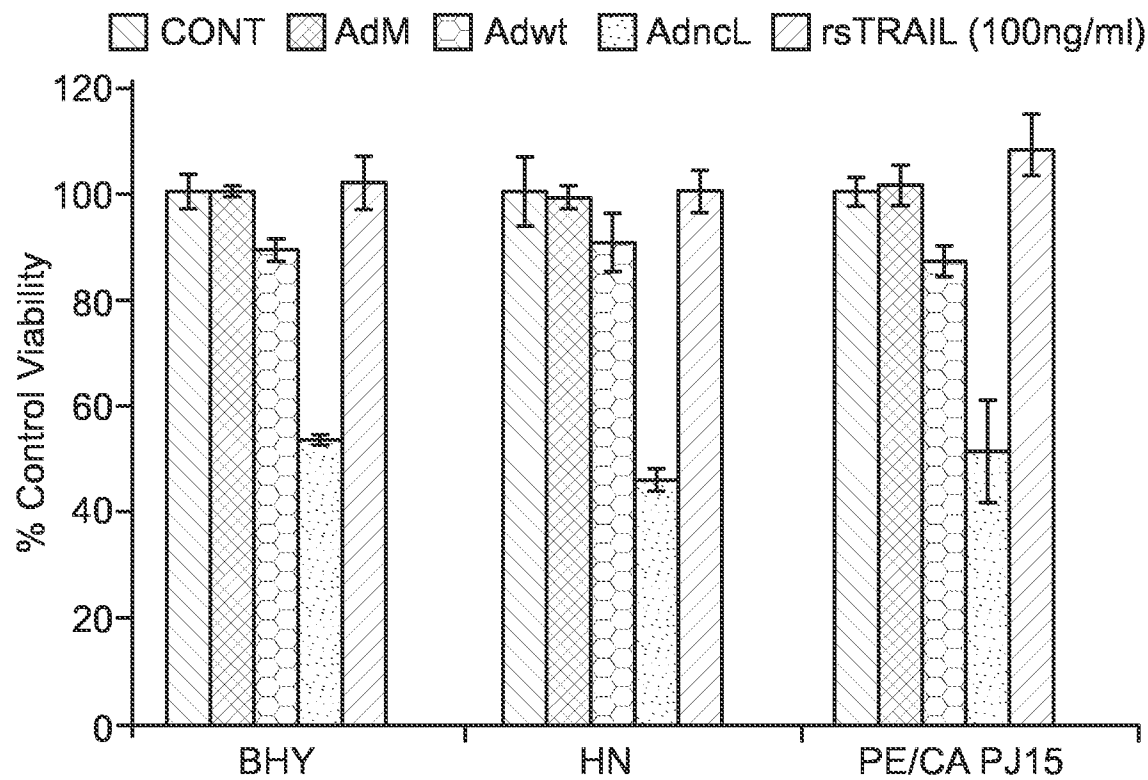


Fig. 8

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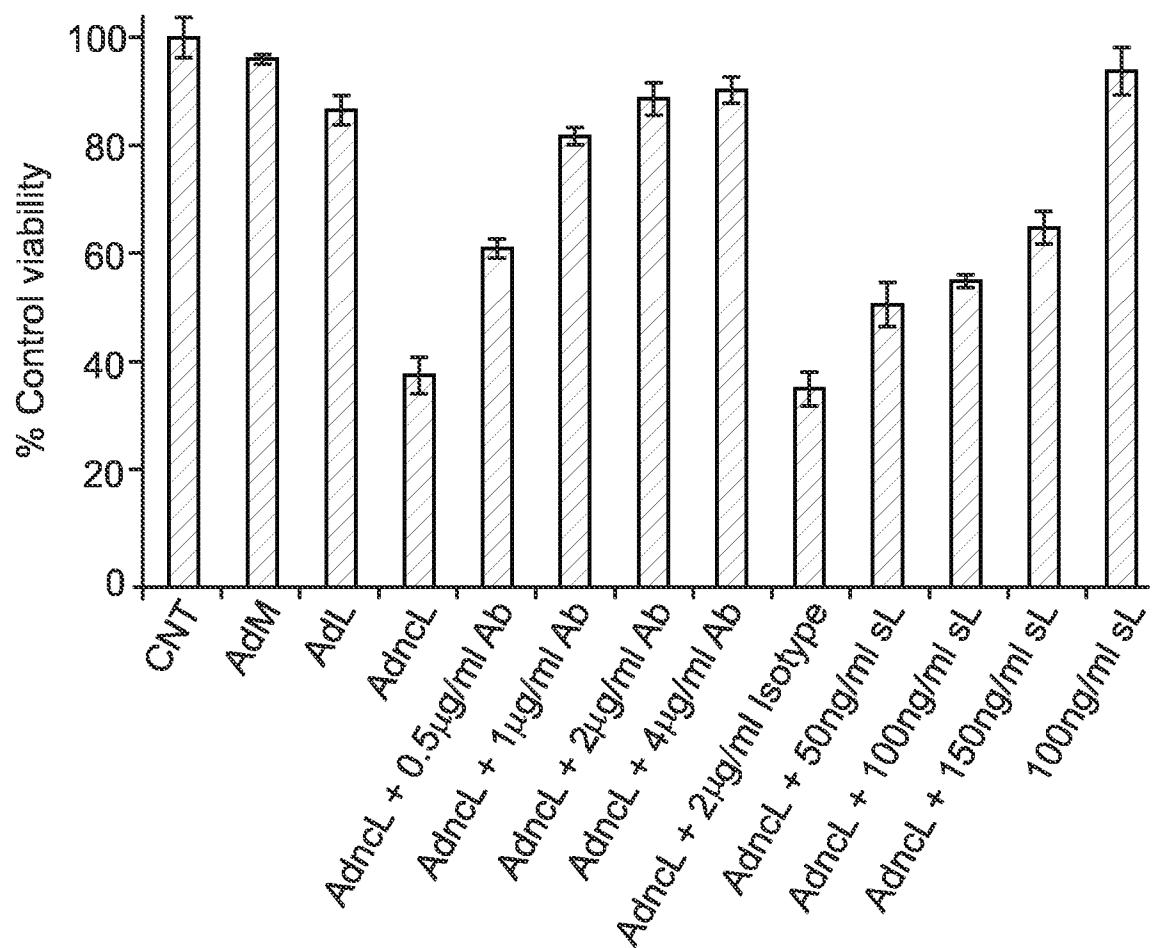


Fig. 9

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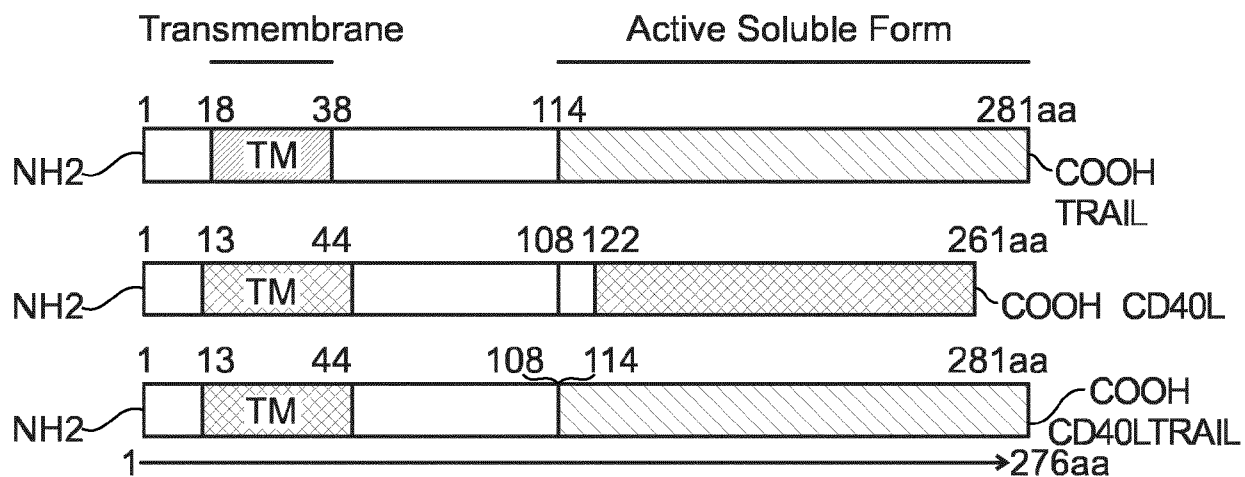


Fig. 10

	128	131
FasL:	SSL EKQI GHPSP	
	110	116
CD40L:	EN SF EMQK G DQN	
	105	107
TRAIL:	QEKQ Q HISPL	

Fig. 11

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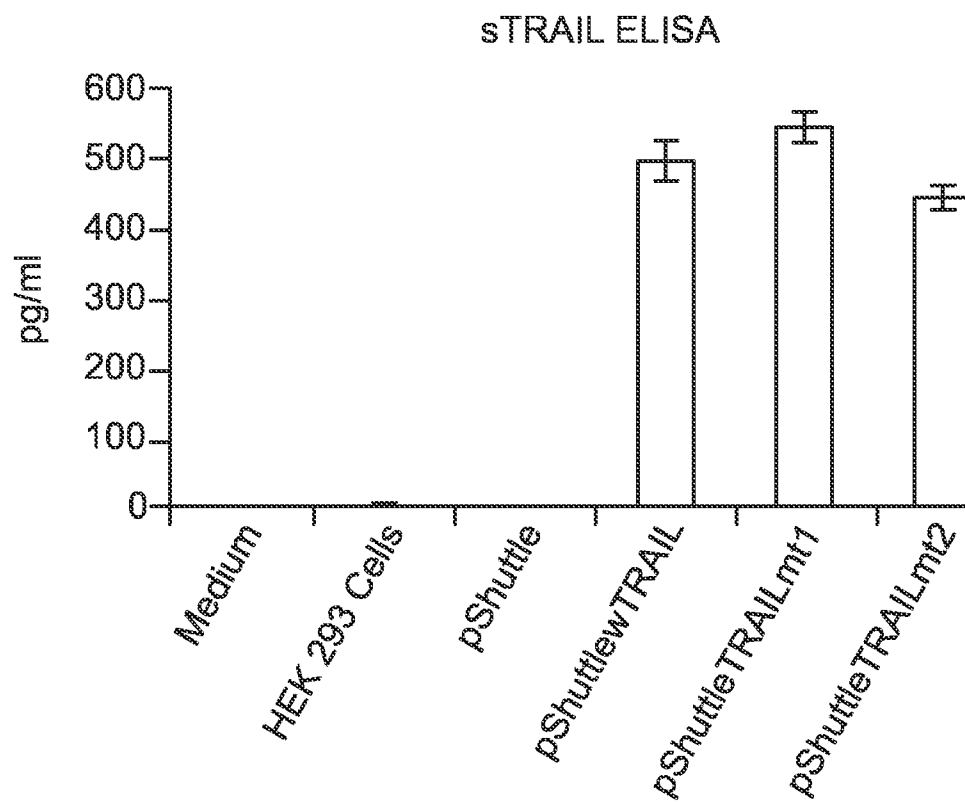


Fig. 12

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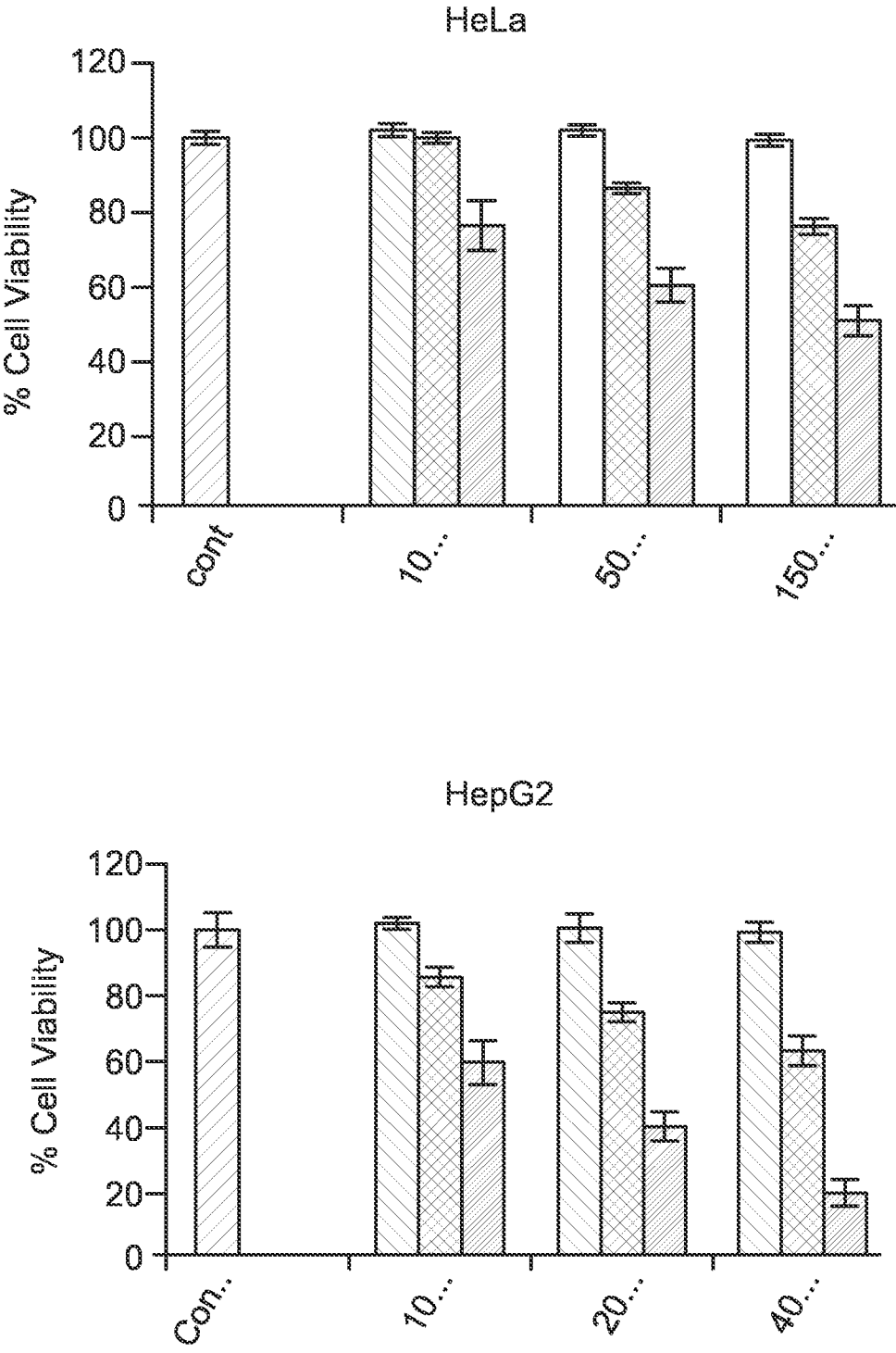


Fig. 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/051145

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/47
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

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

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

EPO-Internal

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Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

4 September 2015

Date of mailing of the international search report

10/09/2015

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Authorized officer

Keller, Yves

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2015/051145

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International application No

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International application No
PCT/GB2015/051145

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Information on patent family members

International application No

PCT/GB2015/051145

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