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(54) **TUMOR TARGETED TNF-RELATED  
APOPTOSIS INDUCING LIGAND FUSION  
POLYPEPTIDE, METHODS AND USES  
THEREFOR**

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10, 2012.

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CPC ..... **C07K 14/705** (2013.01); **C07K 14/4747**  
(2013.01); **C12N 15/62** (2013.01); **C07K**  
**2319/00** (2013.01); **C07K 2319/21** (2013.01);  
**A61K 38/00** (2013.01)

(57) **ABSTRACT**

Fusion polypeptides comprising a TRAIL trimer and a targeting domain are disclosed. The targeting domain can be, in some embodiments, a sequence that binds MUC16, which is prevalent on some tumor cells such as pancreatic and ovarian tumor cells. A sequence that binds MUC 16 can be mesothelin or a MUC16-binding fragment thereof, such as amino acids 1-64 of mesothelin. A fusion polypeptide of the present teachings can induce apoptosis in a target cell such as a MUC16-expressing cancer cell. Also disclosed are nucleic acids encoding the fusion polypeptides, and methods of use of the fusion polypeptides and nucleic acids.

FIG.1

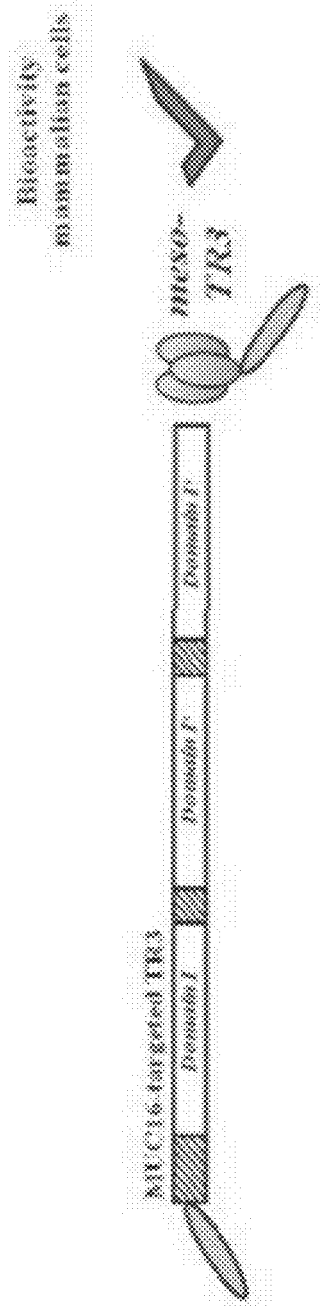


FIG. 2A

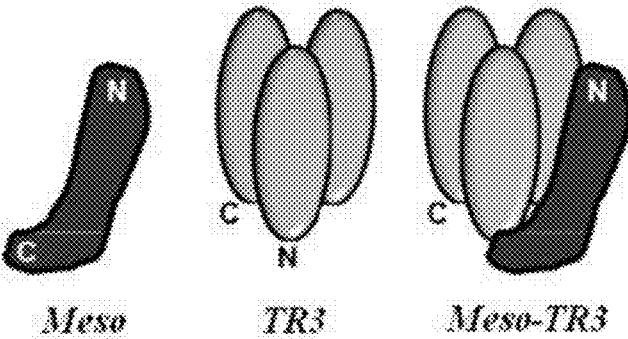
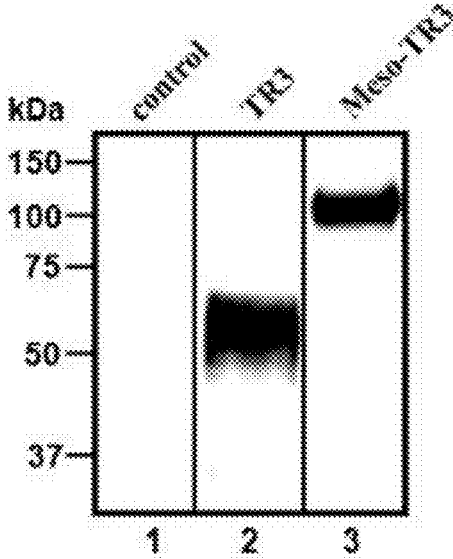


FIG. 2B



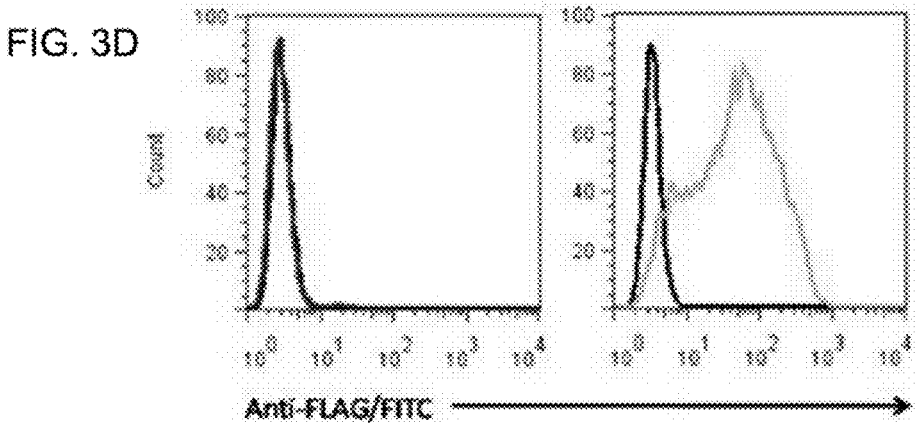
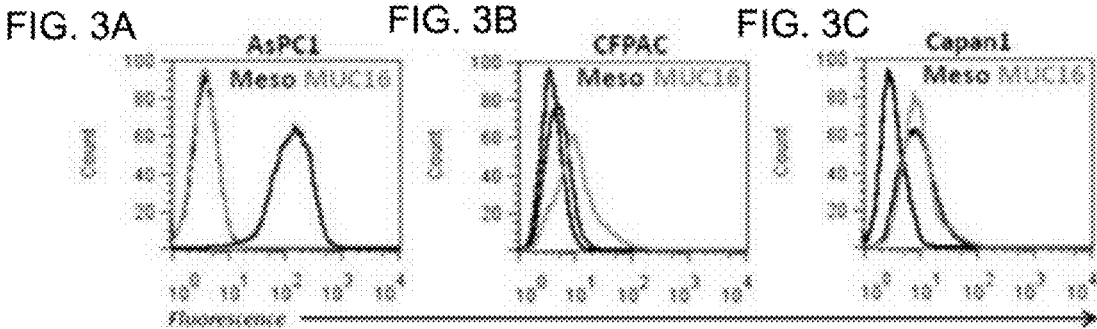


FIG. 4A

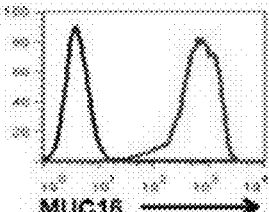


FIG. 4B

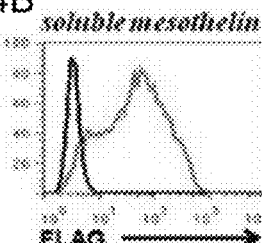


FIG. 4C

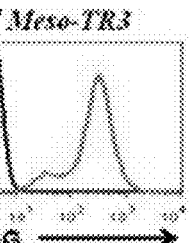


FIG. 4D

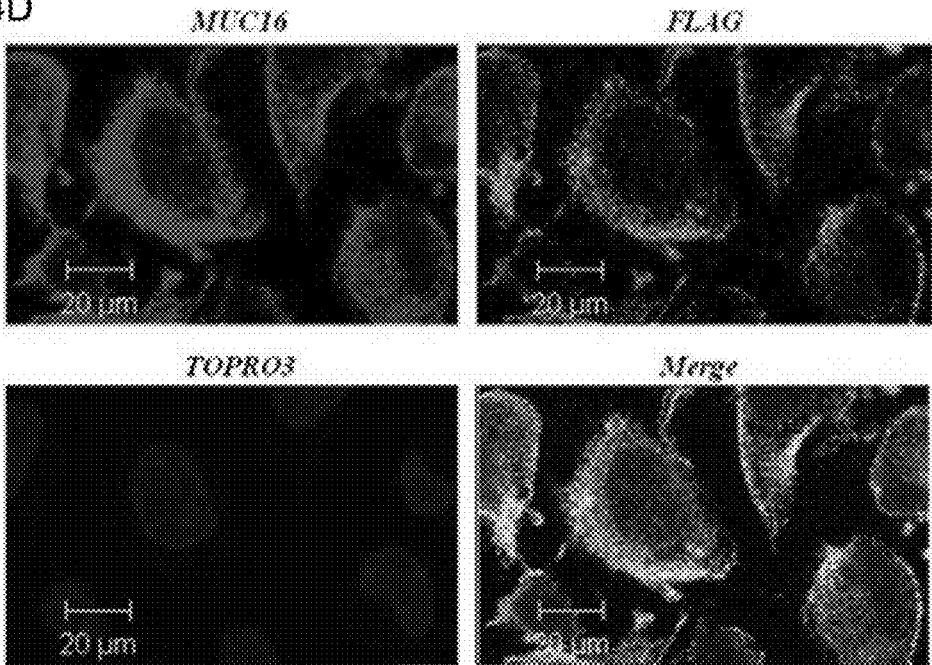


FIG. 5A

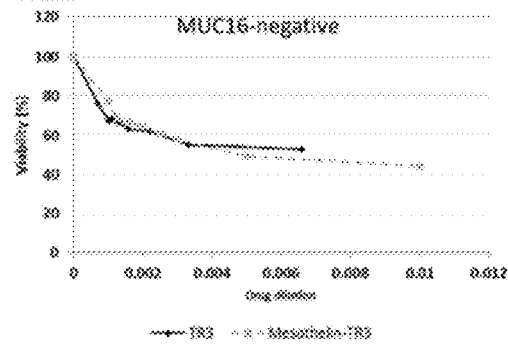


FIG. 5B

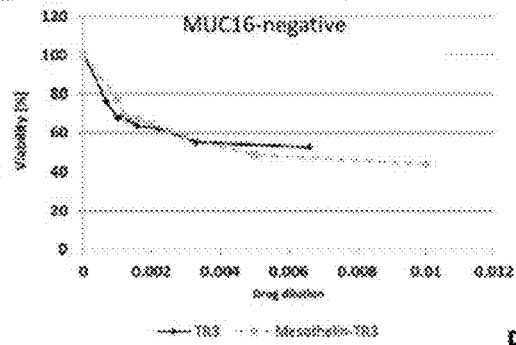


FIG. 5C

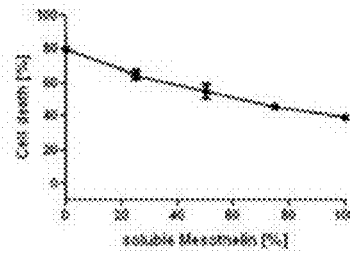


FIG. 5D

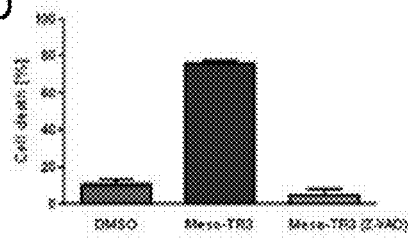


FIG. 5E

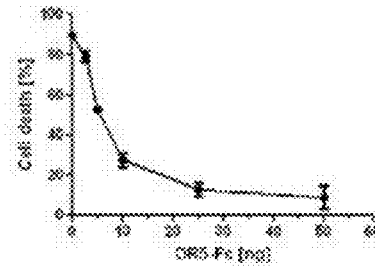


FIG. 6A *Jurkat* FIG. 6B *OVCAR3*

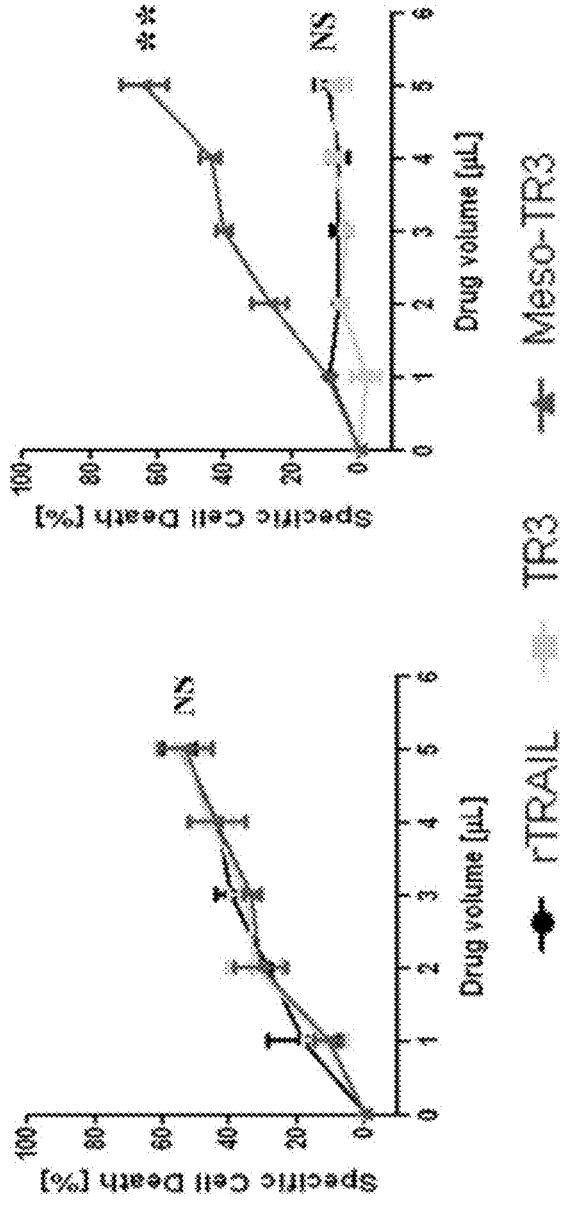


FIG. 7A

*OVCAR3*

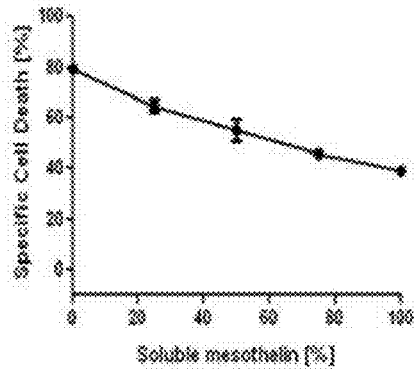


FIG. 7B

*OVCAR3*

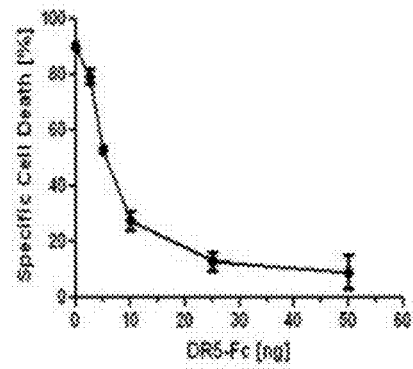


FIG. 7C

*OVCAR3*

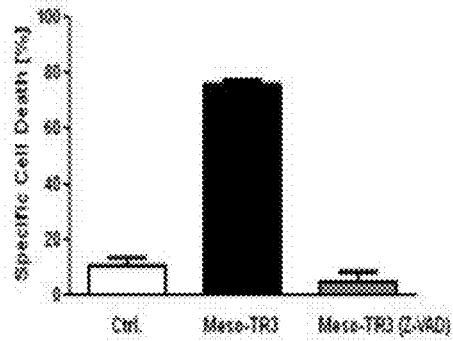


FIG. 7D

*Jurkat*

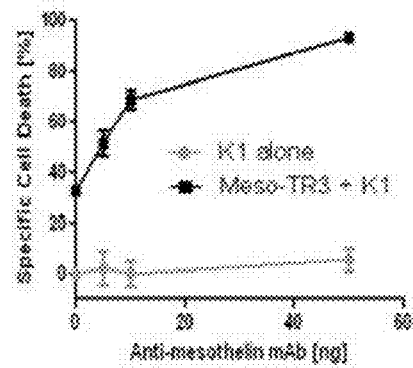




FIG. 8A

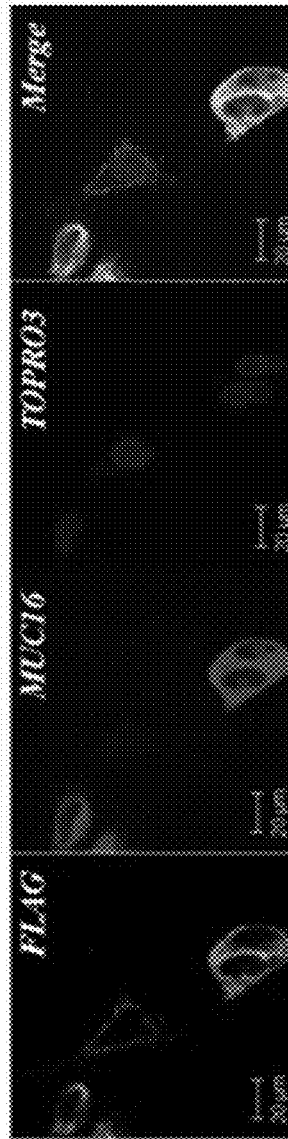


FIG. 8B

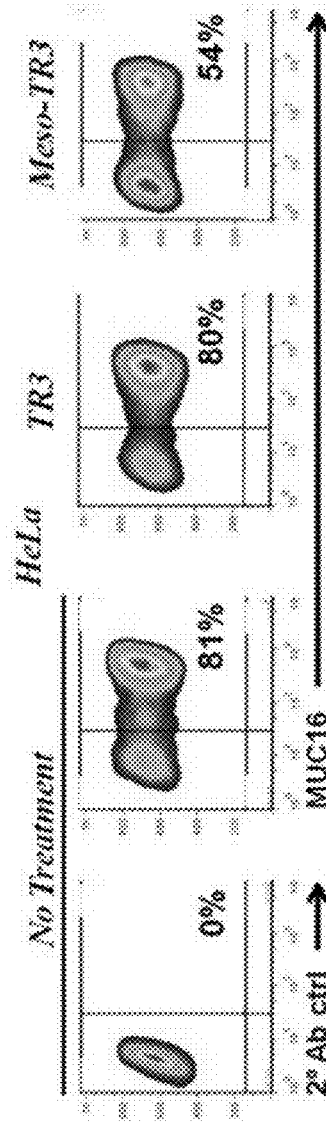


FIG. 9A

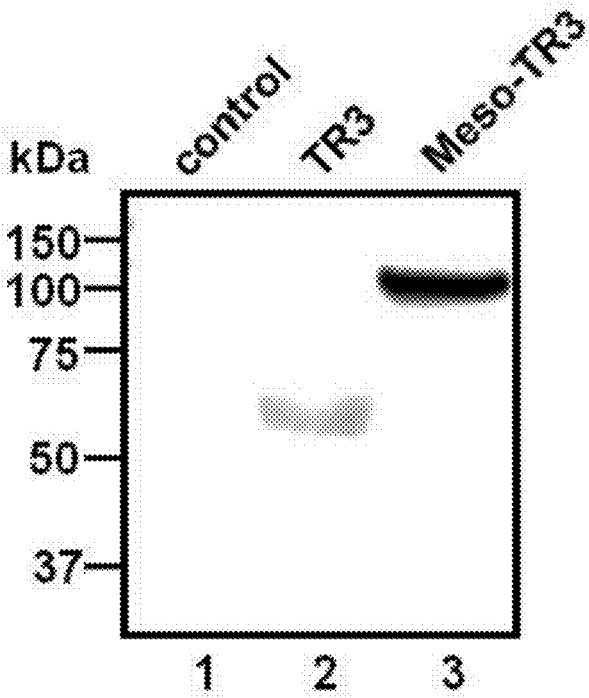
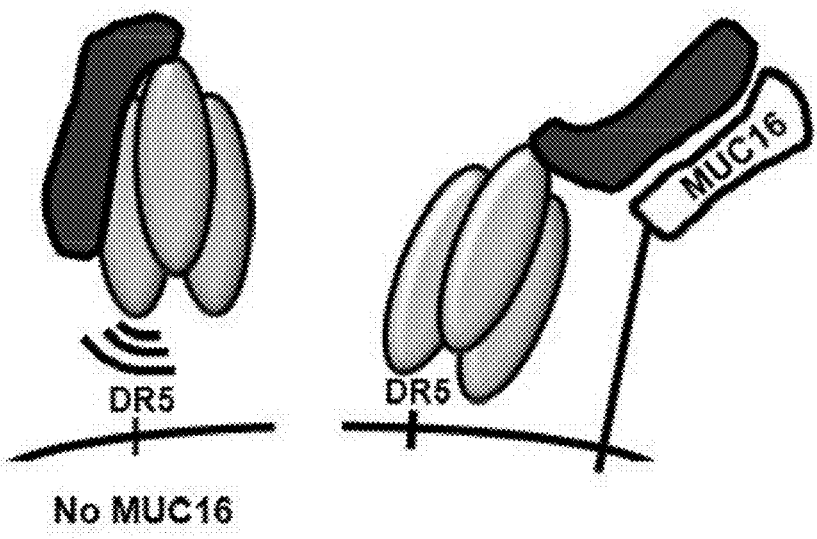


FIG. 9B



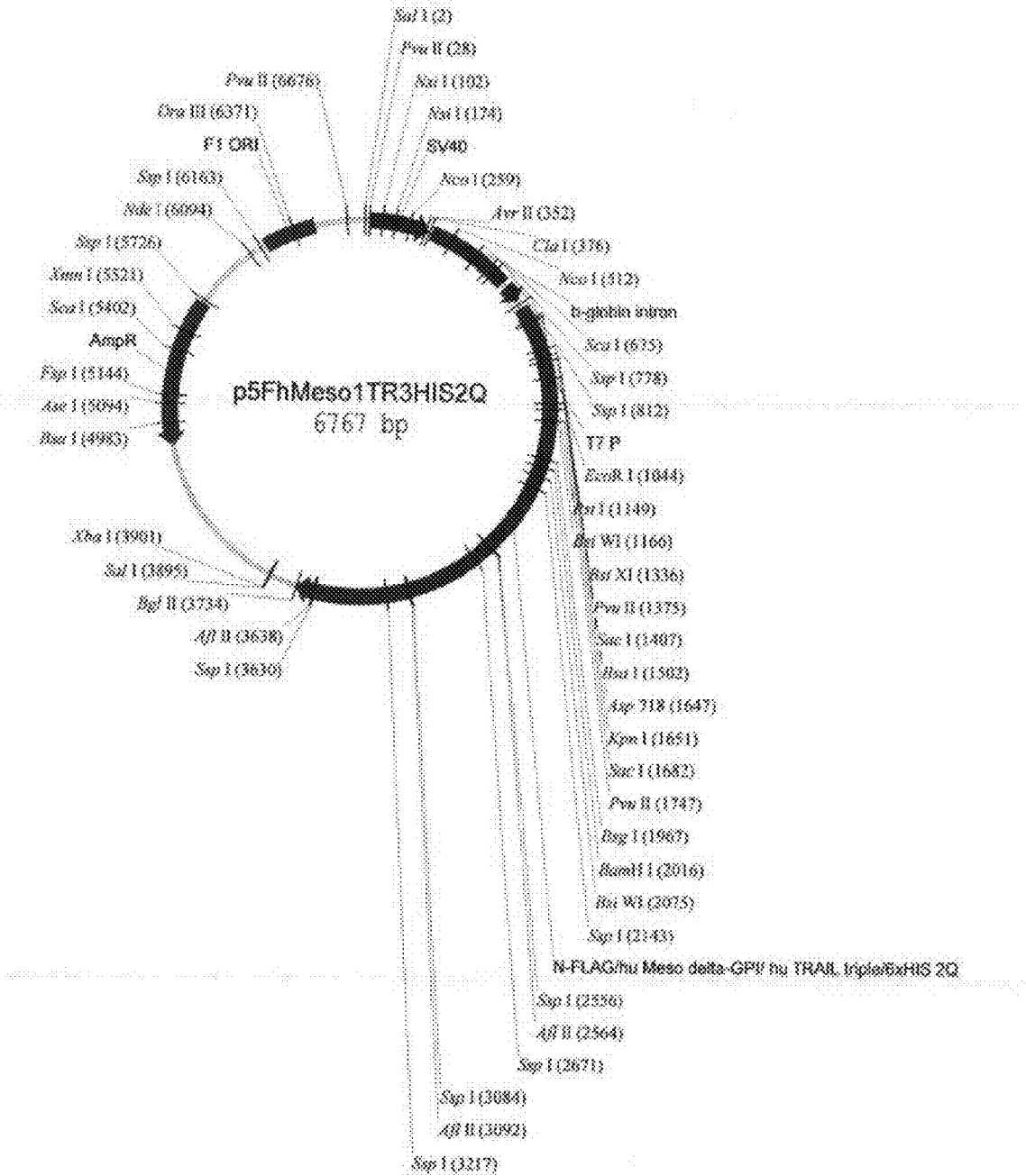


FIG. 10

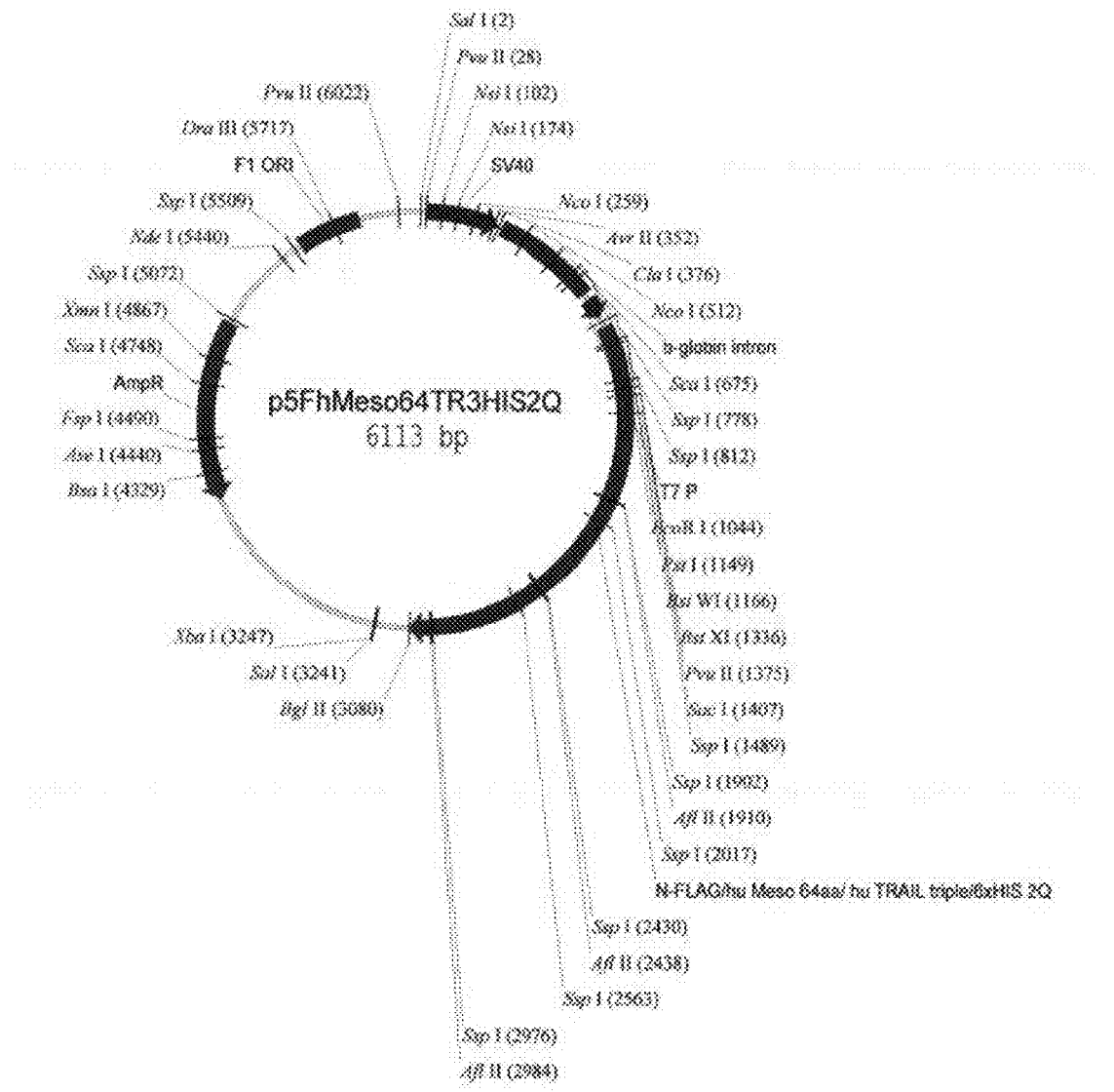


FIG. 11

FIG. 12A

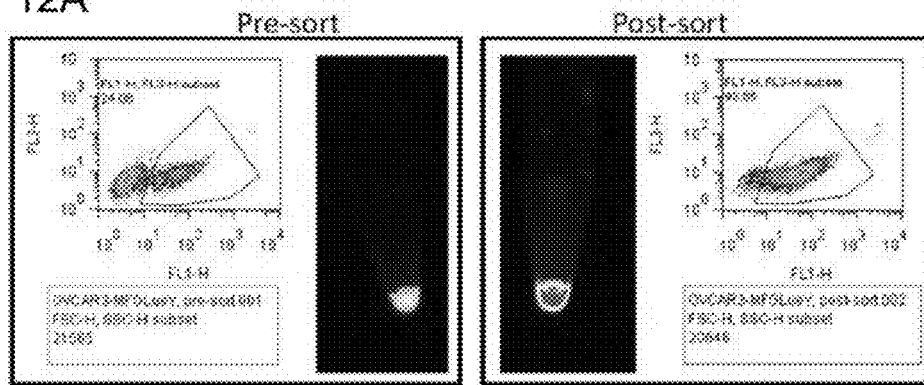


FIG. 12B

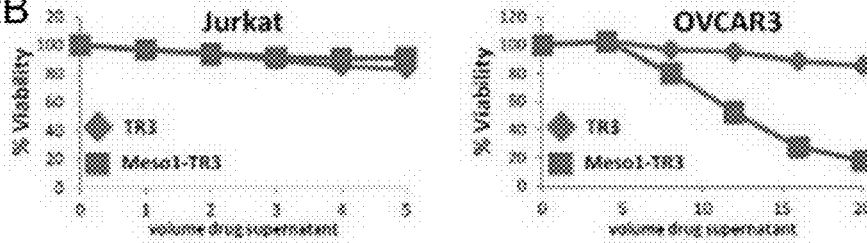


FIG. 12C

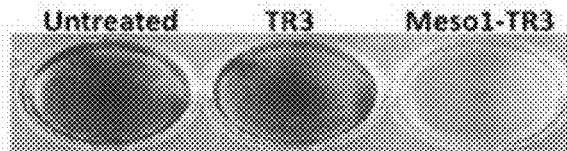


FIG. 12D

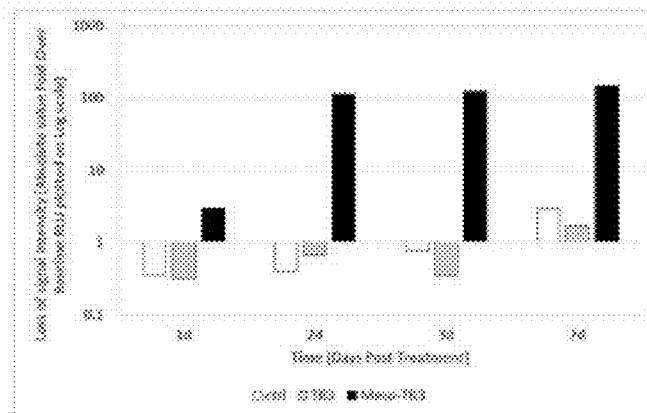


FIG. 13A

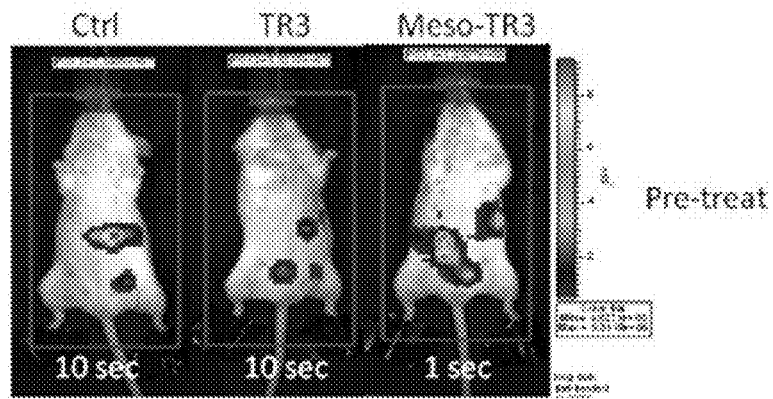


FIG. 13B

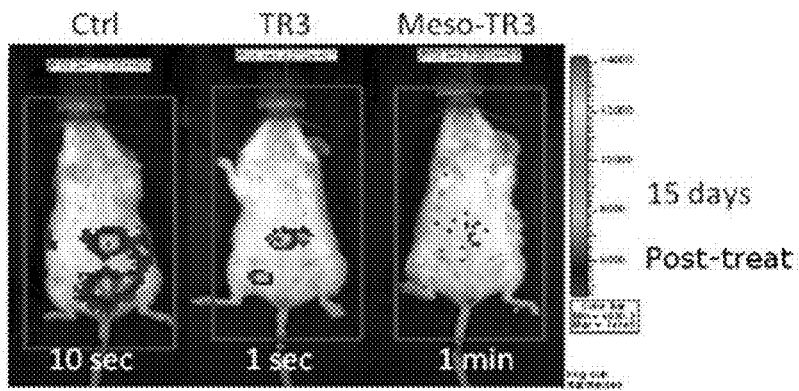
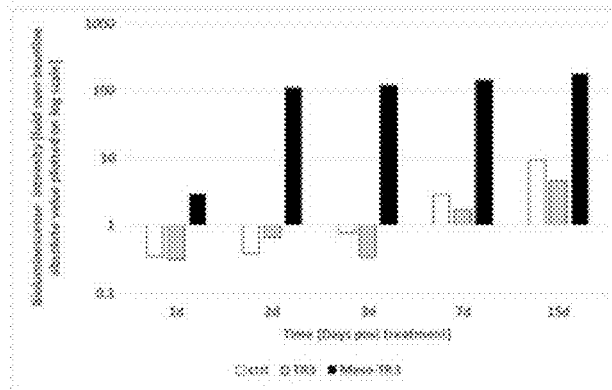


FIG. 13C



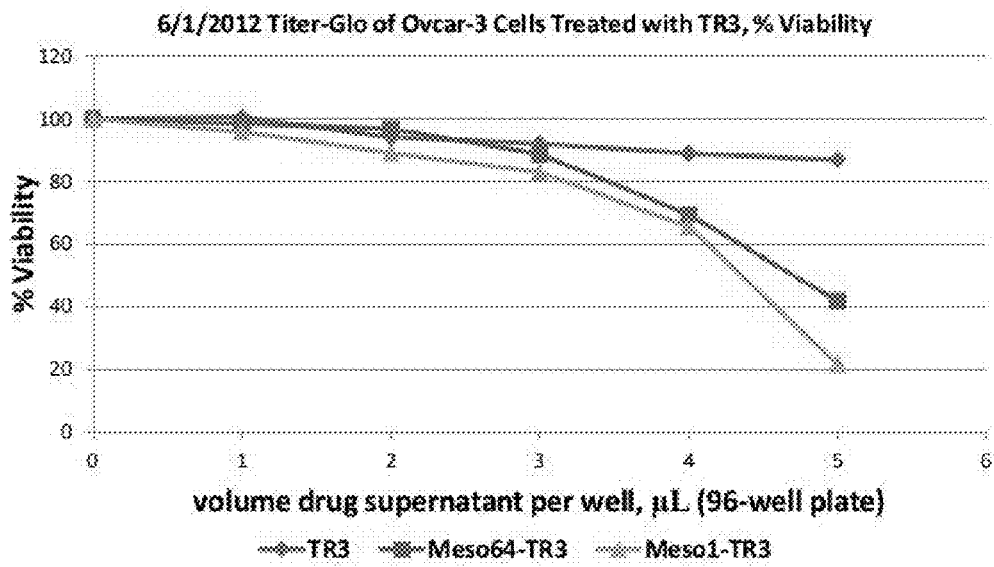
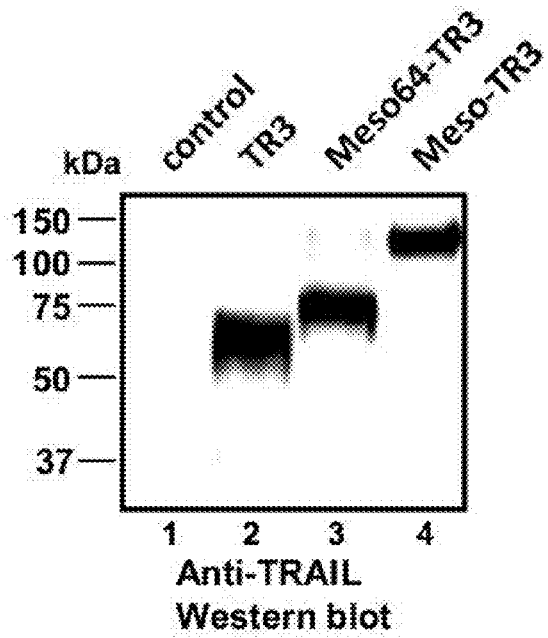


FIG. 14

**TUMOR TARGETED TNF-RELATED  
APOPTOSIS INDUCING LIGAND FUSION  
POLYPEPTIDE, METHODS AND USES  
THEREFOR**

CROSS-REFERENCE TO RELATED  
APPLICATION

**[0001]** This application is a Continuation of, and claims benefit of priority to U.S. Non-Provisional application Ser. No. 13/892,238, filed May 10, 2013. Application Ser. No. 13/892,238 claims the benefit of U.S. Provisional Patent Application 61/645,058 filed May 10, 2012. These applications are incorporated by reference, each in its entirety.

STATEMENT REGARDING FEDERALLY  
SPONSORED RESEARCH OR DEVELOPMENT

**[0002]** This invention was made with government support under Grants 5P30CA9184208 and 1R2 CA150945 awarded by the National Institutes of Health. The Government has certain rights in the invention.

INCORPORATION BY REFERENCE OF  
SEQUENCE LISTING

**[0003]** The Sequence Listing, which is a part of the present disclosure, includes a computer readable form and a written sequence listing comprising nucleotide and/or amino acid sequences. The sequence listing information recorded in computer readable form is identical to the written sequence listing. The subject matter of the Sequence Listing is incorporated herein by reference in its entirety.

INTRODUCTION

**[0004]** Pancreatic cancer is among those malignancies with the worst prognoses in the United States in 2010 (Jemal, A., et al. *CA Cancer J. Clin.* 60:277-300, 2010). There has been little progress in the management of the disease and the annual mortality rate remains nearly identical to the annual incidence rate. The five-year survival for pancreatic cancer patients is ~4%.

**[0005]** Transformed cancer cells can often be distinguished from normal tissues by changes in expression patterns of certain cellular markers. Two cell surface antigens with expression levels that can exceed normal levels in cancer cells are mesothelin and MUC16 (also known as CA-125).

**[0006]** Mesothelin is a GPI-linked cell surface glycoprotein that is believed to participate in tumor adhesion and dissemination including formation of metastases (Hassan, R., et al. *Clin. Cancer Res.* 10:3937-42, 2004). Mesothelin is expressed in mesothelial cells with limited expression in other normal cell types. Expression of mesothelin can be substantially up-regulated in human pancreas and ovarian cancers. For example, analyses of human pancreas cancers have shown greater than 3 fold up-regulation of mesothelin gene expression (Iacobuzio-Donahue, C. A., et al. *Cancer Res.* 63:8614-22, 2003). In one study, mesothelin expression was identified in pancreas adenocarcinomas (the far majority of pancreas cancers are ductal adenocarcinomas, PDACs) in all 60 patients examined by immunohistochemistry (Argani, P., et al. *Clin. Cancer Res.* 7:3862-8, 2001). In addition, mesothelin overexpression is commonly found in ovarian malignancies, lung cancer, and mesotheliomas (Ho, M., et al. *Clin. Cancer Res.* 13:1571-5, 2007; Mumminova, Z. E., et

al. *BMC Cancer.* 4:19, 2004; Ho, M., et al. *Clin. Cancer Res.* 11:3814-20, 2005). In addition, there is evidence that over-expression of mesothelin may be essential for progression of pancreas cancer, (Li, M., et al. *Mol. Cancer Ther.* 7:286-96, 2008). It has been shown that the N-terminal 64 amino acid sequence of mesothelin includes the minimal binding sequence required for MUC16 binding (Xiang, X., et al., *J. Cancer* 2: 280-291, 2011).

**[0007]** MUC16 (CA125) belongs to a group of mucins expressed on epithelial cells (Kufe, D. W. *Nat. Rev. Cancer.* 9:874-85, 2009). MUC16 is transmembrane anchored. In addition, patients with pancreatic cancer can have serum MUC16 levels that can be nearly 40-fold increased compared to healthy controls or patients with benign pancreatic lesions (Brand, R. E., et al. *Clin. Cancer Res.* 17:805-16, 2011). Membrane-bound MUC16 binds to native mesothelin with high affinity, whereas soluble MUC16 has only a weak affinity for mesothelin (Rump, A., et al. *J. Biol. Chem.* 279:9190-8, 2004; Bast, R. C., et al. *Int. J. Gynecol. Cancer.* 15:274-81, 2005; Gubbels, J. A., et al. *Mol. Cancer.* 5:50, 2006).

**[0008]** TNF-related apoptosis-inducing ligand (TRAIL) has been shown to exhibit potent apoptotic activity against tumor cells with lower toxicity to non-transformed cells following engagement with cellular receptors expressed abundantly on tumor cells (Falschlehner, C., et al. *J. Biochem. Cell Bio.* 39:1462-1475, 2007). TRAIL stimulates the extrinsic death pathway. Native, soluble TRAIL exists as a homotrimer in vivo (Kohlhaas, S. L., et al. *J. Biol. Chem.* 282: 12831-12841, 2007). The sequence of human TRAIL amino acids 91-281 is:

(SEQ ID NO: 1)

```
MILRTSEETISTVQEKQQNISPLVREVRGPQVAAHITGTRGRSNLSSP
NSKENKALGRKINSWESSRSRSGHSFLSNLHLRNGELVIEHKGFFYYIYSQT
YFRFQEEIKENTKNKQDMVQYIYKYTSYPDPILLMKSARNSCWSKDAEY
GLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFVVG.
```

**[0009]** Recombinant TRAIL has been produced in bacteria exclusively from monomeric cDNAs. However, the activity of recombinant TRAIL depends on trimerization (Spitzer, D., et al., *Mol. Cancer Ther.* 9: 2142-2151, 2010). Numerous design modifications have been used to generate molecules comprising trimerized TRAIL sequences, such as: tagging with FLAG sequence or His-tagging, with tag-mediated crosslinking; addition of leucine zipper [LZ] and/or isoleucine zipper [ILZ] sequences, with stabilization of TR3 trimers with cations [i.e., zinc] (Merino, D., et al. *Expert Opin. Ther. Targets.* 11: 1299-1314, 2007). However, such attempts to produce bioactive TRAIL from monomeric cDNAs in mammalian cells have failed. Such failures have been attributed to intermolecular disulfide bridge formation via TRAIL's unique cysteine at amino acid 230, resulting in a non-functional death receptor ligand (Bodmer, J. L., et al., *J. Biol. Chem.* 275: 20632-20637, 2000).

**[0010]** Previously, the present inventors developed bioactive TRAIL trimers ("TR3") (U.S. patent application Ser. No. 13/155,577, published as US Patent Application Publication 2011/0300629 A1; Spitzer, D., et al., *Mol. Cancer Ther.* 9: 2142-2151, 2010). Furthermore, the present inventors also developed numerous modifications to further enhance TR3's pharmacologic properties over conventional



TRAIL, including enhanced temperature stability and prolonged in vivo half-life (Spitzer, D., et al. *Mol. Cancer Ther.* 9:2142-51, 2010).

**[0011]** However, there is an unmet need for therapeutically active compositions that can induce cell death in tumor cell targets.

#### SUMMARY

**[0012]** In view of the unmet need for therapeutically effective reagents that target and cause death of tumor cells while minimizing toxicity to non-cancerous cells, the present inventors disclose fusion polypeptides comprising TRAIL trimers and targeting domains, and nucleic acids comprising sequences encoding such fusion polypeptides. In various embodiments, a fusion polypeptide of the present teachings can comprise, consist essentially of, or consist of a sequence of a TRAIL trimer plus a polypeptide sequence that can target a tumor cell such as, for example, a tumor cell that expresses abnormally high levels of a cell surface receptor such as MUC16. In various embodiments, a fusion polypeptide of the present teachings can comprise, consist essentially of, or consist of a sequence of a TRAIL trimer and a polypeptide sequence that can target a TRAIL trimer to a tumor cell such as, for example and without limitation, a pancreatic tumor cell or an ovarian cancer cell. In various embodiments, a fusion polypeptide of the present teachings can comprise, consist essentially of, or consist of a sequence of a TRAIL trimer plus a targeting sequence such as a mesothelin polypeptide. In various embodiments, the sequence of a mesothelin polypeptide can be that of a full length mesothelin, or a mesothelin of less than full length but retains MUC16 binding activity. In various embodiments, a fusion polypeptide of the present teachings can comprise, consist essentially of, or consist of a TRAIL trimer sequence plus a mesothelin sequence absent the GPI anchor. In various embodiments, a fusion polypeptide of the present teachings can comprise, consist essentially of, or consist of a TRAIL trimer sequence plus an N-terminal peptide sequence of mesothelin, such as, without limitation, the 64 amino acid sequence of the N-terminal of human mesothelin. In various embodiments, a fusion polypeptide of the present teachings can further comprise one or more linker sequences such as described in U.S. patent application Ser. No. 13/155,577 filed Jun. 8, 2011, published as US Patent Application Publication 2011/0300629 A1, and Spitzer, D., et al., *Mol. Cancer Ther.* 9: 2142-2151, 2010 which are hereby incorporated by reference, each in its entirety. In some configurations, a spacer can comprise, consist essentially of, or consist of one or more short consensus repeats (SCRs). In various configurations, a spacer can comprise, consist essentially of, or consist of one SCR, two SCRs, three SCRs or four SCRs. In some configurations, a fusion polypeptide can further comprise a tag sequence, such as, without limitation, a 6-His tag sequence and/or a FLAG sequence.

**[0013]** In various embodiments, a fusion polypeptide of the present teachings can be selected from the group consisting of complete mesothelin-TR3 (i.e., a fusion polypeptide comprising full-length mesothelin, plus TR3); mesothelinΔGPI-TR3 (i.e., a fusion polypeptide comprising mesothelin consisting of GPI-anchor-deleted mesothelin, plus TR3) and meso64-TR3 (i.e., a fusion polypeptide comprising a mesothelin consisting of the N-terminal 64 amino acids of mesothelin, plus TR3).

**[0014]** In various embodiments, the present teachings further include nucleic acids that encode any of the fusion polypeptides disclosed herein, as well as vectors such as viruses and plasmids comprising a nucleic acid that encodes any of the fusion polypeptides disclosed herein.

**[0015]** In some embodiments, a fusion polypeptide of the present teachings does not activate cell death pathways when contacted with a MUC16-negative cell at a concentration at which a TRAIL trimer alone (i.e., without mesothelin) activates cell death pathways in a MUC16-negative cell.

**[0016]** In some embodiments, a fusion polypeptide of the present teachings can bind to the surface of cells expressing MUC16, such as, for example, pancreatic or ovarian tumor cells.

**[0017]** In some embodiments, a fusion polypeptide of the present teachings can induce apoptosis in cells that express MUC16 such as tumor cells that express MUC16.

**[0018]** In some embodiments, a fusion polypeptide of the present teachings can block native binding sites of MUC16 in cells expressing MUC16, such as, for example, pancreatic or ovarian tumor cells.

**[0019]** In some embodiments, a fusion polypeptide of the present teachings can reduce metastatic potential of tumor cells that express MUC16.

**[0020]** Various embodiments of the present teachings include methods of treating cancer. In various configurations, these methods comprise administering to a subject in need thereof a therapeutically effective amount of a fusion polypeptide of the present teachings. In various configurations, the methods comprise administering to a subject in need thereof a therapeutically effective amount of a vector such as a plasmid or virus comprising a nucleic acid encoding a fusion polypeptide of the present teachings.

**[0021]** In various embodiments, methods of the present teachings include methods of inducing apoptosis in a cell that expresses MUC16 such as a tumor cell that expresses MUC16. In various configurations, these methods include contacting a cell that expresses MUC16 with a polypeptide of the present teachings, or a nucleic acid or vector of the present teachings. In various configurations, a fusion polypeptide or nucleic acid can be administered in an amount sufficient to cause apoptosis in a cell that expresses MUC16 without inducing apoptosis in other cells.

**[0022]** In various embodiments, methods of the present teachings include methods of blocking native binding sites of MUC16. In these methods, a fusion polypeptide of the present teachings or a nucleic acid encoding a fusion polypeptide of the present teachings is administered or applied to a cell expressing MUC16.

**[0023]** In various embodiments, methods of the present teachings include methods of reducing metastatic potential. In these methods, a fusion polypeptide of the present teachings or a nucleic acid encoding a fusion polypeptide of the present teachings is administered or applied to a cell expressing MUC16.

**[0024]** In various embodiments, methods of the present teachings include methods of killing MUC16-positive cells in a population of cells. In various configurations, these methods comprise contacting the cells of a population of cells with an effective amount of a fusion polypeptide or a nucleic acid of the present teachings, whereby >70% of MUC16-positive cells are killed, i.e., at a percentage greater than a "chemotherapeutic plateau."

## BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0026] FIG. 1 illustrates a fusion polypeptide of the present teachings.

[0027] FIG. 2A-B illustrate design and biochemical characterization of MUC16-targeted TRAIL.

[0028] FIG. 3A-D illustrate expression levels of mesothelin and MUC16 in pancreatic cancer cell lines (A, B, C) and mesothelin binding to MUC16-expressing target cells (D).

[0029] FIG. 4A-D illustrate Meso-TR3 binding to MUC16-expressing cancer targets.

[0030] FIG. 5A-E illustrate cell killing of MUC16-positive cells by a mesothelin-TR3 fusion polypeptide.

[0031] FIG. 6A-B illustrate that Meso-TR3 is a targeted therapeutic on MUC16-expressing tumor cells.

[0032] FIG. 7A-D illustrate phenotypic characterization of MUC16-targeted Meso-TR3.

[0033] FIG. 8A-B illustrate selective killing of MUC6-expressing tumor cells by a mesothelin-TR3 fusion polypeptide.

[0034] FIG. 9A-B illustrate that Meso-TR3 is fully activated on tumor cells expressing the biomarker MUC16.

[0035] FIG. 10 illustrates a restriction map of plasmid p5FhMeso64TR3HIS2Q.

[0036] FIG. 11 illustrates a restriction map of plasmid p5FhMeso1TR3HIS2Q.

[0037] FIG. 12A-D illustrate reduction of tumor burden by Meso-TR3 in an in vivo model of ovarian cancer.

[0038] FIG. 13A-C illustrate examples of reduction of tumor burden by Meso-TR3 in an in vivo model of ovarian cancer.

[0039] FIG. 14 illustrates production and killing potential of TR3, Meso64-TR3, and Meso-TR3.

## DETAILED DESCRIPTION

[0040] A desired feature of a therapeutic is that after systemic application, it seeks its target automatically, ignores all non-targets and, when arrived at its destination fully unleashes its intended pharmacologic activity, in analogy of a "magic bullet". Such a selective activity profile can be beneficial for the treatment of human malignancies, for example when treatment with conventional chemotherapy is known to be associated with debilitating side effects directly linked to an adverse impact on the quality of life of the patients.

Nearly 20 years ago, the TNF superfamily member TRAIL was identified as a potential cancer therapeutic because of its strong apoptosis induction on transformed cancer cells and lack of harmful side effects for the host. Since then, TRAIL has been evaluated in a number of clinical trials and found to be effective against several types of cancers (Herbst, R. S., et al., *J. Clin. Oncol.* 28:2839, 2010). Investigators have looked for ways to stabilize the bioactive trimer by a number of attempts, such as adding Zn<sup>2+</sup> to the production process which is believed to aid the coordination of the free cysteines (Mahalingam, D., et al., *Cancer Treat. Rev.* 35:280, 2009). Incorporation of targeting moieties directed against cancer-specific surface markers was also investigated. In these studies, cancer targeting was primarily achieved using

antibody fragments (scFv) on the basis of the conventional monomeric TRAIL platform (Bremer, E., et al., *Int. J. Cancer* 109:281, 2004; ten Cate, B., et al., *Leukemia* 23:1389, 2009). This technology turned out to be quite effective, despite a 1:1 stoichiometry of the targeting and effector domain of the fusion proteins which could potentially interfere with the formation of bioactive TRAIL trimers, resulting in unpredictable drug properties. In fact, we have produced scFv-TRAIL fusion proteins employing two different antibody fragments with one drug being constitutively active while the other drug was completely inactive in the absence of the target antigen.

[0041] The present inventors have recently designed a new method to produce bioactive soluble TRAIL from mammalian cells, designated TR3. Despite its much enhanced stability, this genetically fused TRAIL trimer has the capacity to serve as a drug platform for the design of targeted TRAIL therapy under stoichiometric control. This has been shown by fusing a scFv to the N-terminus of TR3 which resulted in a RBC-targeted scFv-TR3 fusion protein with a favorable 1:3 stoichiometry that was capable of tethering human TR3 to mouse RBCs which were converted into potent effector surfaces in analogy to nanoparticles, only capable of facilitating bystander killing (Spitzer, D., et al., *Mol. Cancer Ther.* 9:2142, 2010). In the instant application, we have described the in vitro characterization of a tumor-targeted variant of TR3 by harnessing the strong binding affinity of the two well described biomarkers mesothelin and MUC16. Instead of targeting TR3 via an antibody fragment to the desired cancer cells, the present inventors generated Meso-TR3, in which the mature form of secreted human mesothelin was placed at the N-terminus of human TR3. Meso-TR3 bound abundantly to endogenous MUC16, identical to soluble mesothelin itself and triggered a much enhanced death pathway than the parental drug TR3. These results had important implications because they confirmed that the mesothelin targeting domain was not masked by TR3 as it was still accessible to interact with membrane-associated MUC16. This interaction is important because it not only imparts target selectivity to Meso-TR3, but also serves to anchor soluble TRAIL to the surface of MUC16-positive cancer cells, thus converting it into a membrane bound TRAIL.

This conversion has been proposed to lead a more efficient receptor crosslinking (particularly important for DR5-mediated apoptosis), which in turn provides a more potent death signal resulting in an enhanced apoptosis compared to its soluble counterpart (Muhlenbeck, F., et al., *J. Biol. Chem.* 275:32208, 2000).

[0042] The importance of TRAIL receptor crosslinking in cell death is further exemplified by an enhanced induction of apoptosis noted in our experimental system upon adding mesothelin antibody to dimerize Meso-TR3, ultimately resulting in a more efficient TRAIL receptor crosslinking (FIG. 71)). Another potentially important aspect of the binding of mesothelin to MUC16 is that it may contribute to both homotypic (tumor cell-tumor cell) and heterotypic (tumor cell-mesothelial cell) cell interactions (Singh, A. P., et al., *Cancer Res.* 64:622, 2004). The latter type of cell interaction is believed to promote adherence of tumor cells to the peritoneum, resulting in metastatic spread of the primary lesion into the abdomen (Gubbels, J. A., et al., *Mol. Cancer* 5:50, 2006; Rump, A., *J. Biol. Chem.* 279:9190, 2004; Scholler, N., et al., *Cancer Lett.* 247:130, 2007). These

considerations suggest that by virtue of binding to MUC16, Meso-TR3 may also block the mesothelin/MUC16-dependent cell adhesion thus limiting the peritoneal dissemination of tumor cells in addition to facilitating enhanced TRAIL-mediated target cell death (Bergan, L., *Cancer Lett.* 255:263, 2007).

**[0043]** While the TR3 effector domain of Meso-TR3 did not seem to sterically interfere with binding the drug to MUC16, we noticed potential limitations with regard to TR3 binding to the DR5 receptor on MUC16-deficient targets. Based on semi-quantitative Western blot analysis, an 8-fold higher concentration of Meso-TR3 was required to achieve the same biological effect as untargeted TR3 on MUC16-deficient Jurkat cells. This finding was somewhat inconsistent with our earlier report in which we did not observe detrimental effects on the killing activity of a variety of domain additions engineered onto the TR3 drug platform (Spitzer, D., et al., *Mol. Cancer Ther.* 9:2142, 2010). A possible explanation for this finding is that, in its native state, the steric relationship between mesothelin and TR3 in the context of the Meso-TR3 fusion protein might be such that it partially masks the TR3 molecule and makes it less accessible to the death receptors in target antigen negative cells (FIG. 9B, left panel). However, when the mesothelin targeting moiety is bound to MUC16, exposure of the TR3 trimer is enabled and results in an unrestricted accessibility with the surface-associated death receptor(s). We therefore propose that these structural changes, in combination with a now membrane tethered TR3 are responsible for Meso-TR3 to acquire its full cytotoxic potential at the target cell membrane (FIG. 9B, right panel).

**[0044]** In summary, the present inventors have described the in vitro characterization of a downstream modification of the novel TRAIL-based drug platform TR3. Soluble Meso-TR3 targets the cancer biomarker MUC16 and exhibits all features of a traditional TRAIL-based cancer drug, combined with enhanced stability, killing capacity and favorable 1:3 stoichiometry of targeting (mesothelin) and effector domain (TR3). Methods

**[0045]** The methods and compositions described herein utilize laboratory techniques well known to skilled artisans, and can be found in references such as Sambrook and Russel

(2006), *Condensed Protocols from Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, ISBN 0879697717; Sambrook and Russel (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, ISBN 0879695773; Ausubel et al. (2002) *Short Protocols in Molecular Biology*. *Current Protocols*, ISBN 0471250929; Spector et al. (1998) *Cells: A Laboratory Manual*, Cold Spring Harbor Laboratory Press. ISBN 0879695226. As used herein, "TRAIL" can refer to full-length TRAIL polypeptide, or a domain thereof, such as TRAIL I domain (amino acids 91-113 human TRAIL) or TRAIL I' domain (amino acids 108-113 human TRAIL).

**[0046]** Non-limiting examples of fusion polypeptides of the present teachings include, in amino-terminal-to carboxy terminal order:

1. Mesothelin-TRAIL domain I-TRAIL domain I'-TRAIL domain I', wherein "mesothelin" is full-length human mesothelin; TRAIL domain I is human TRAIL fragment aa 91-113, TRAIL domain I' is human TRAIL fragment aa 108-113.

2. Mesothelin-TRAIL domain I-TRAIL domain I'-TRAIL domain I' wherein "mesothelin" is human mesothelin from which carboxy terminal sequence comprising the GPI anchor domain had been deleted; TRAIL domain I is human TRAIL fragment aa 91-113, TRAIL domain I' is human TRAIL fragment aa 108-113.

3. Mesothelin-TRAIL domain I-TRAIL domain I'-TRAIL domain I' wherein "mesothelin" consists of amino acids 1-64 of human mesothelin; TRAIL domain I is human TRAIL fragment aa 91-113, TRAIL domain I' is human TRAIL fragment aa 108-113.

4. Mesothelin-TRAIL domain I-TRAIL domain I'-TRAIL domain I' wherein "mesothelin" is a human mesothelin fragment that binds MUC16, such as without limitation amino acids 1-64; TRAIL domain I is human TRAIL fragment aa 91-113, TRAIL domain I' is human TRAIL fragment aa 108-113.

#### Vectors

**[0047]** Examples of vectors of the present teachings include, without limitation, plasmids of the following sequences.

```
p5FhMeso64TR3HIS2Q (6113 BP) (FIG. 10):
                                                                    (SEQ ID NO: 2)
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tccccaggct ccccagcagg cagaagtatg caaagcatgc atctcaatta gtcagcaacc 120
aggtgtggaa agtccccagg ctccccagca ggcagaagta tgcaaacgat gcatctcaat 180
tagtcagcaa ccatagtccc gccccaact cgcccaatcc cgcccaatcc tccgcccagt 240
tccgcccatt ctccgcccga tggctgacta atttttttta tttatgcaga ggccgaggcc 300
gcctcgcccc ctgagctatt ccagaagtag tgaggaggct tttttggagg cctaggcttt 360
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tttctttttc gctattgtaa aattcatgtt atatggaggg ggcaaatgtt tcagggtgtt 480
gtttagaatg ggaagatgct ccttgatca ccatggacc ccatgataat tttgtttcct 540
tcactctcta ctctgttgac aaccattgct tcctcttatt ctcttttcat tttctgtaac 600
tttttcgta aacttttagct tgcatttgta acgaattttt aaattcactt ttgtttatcc 660
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 ggtcatcatc ctgcctttct ctttatgggtt acaatgatat acaccgtttg agatgaggat 900  
 aaaatactct gagtccaaac cgggccctc tgctaaccat gttcatgctt tcttctttt 960  
 cctacagctc ctgggcaacg tgctggttat tgtgcgcct catcattttg gcaaagaatt 1020  
 gtaatacgcac tcaactatagg gcgaattcag gttctgtgga caatcacaat gggaaatcaa 1080  
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 ctggtcatcc atgaaaaagg gttttactac atctattccc aaacatactt tcgatttcag 1740  
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p5FhMeso1TR3HIS2Q (6767 BP) (FIG.11):

(SEQ ID NO: 3)

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(SEQ ID NO: 4)

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**[0048]** Polypeptides with anti-tumor activity of the present teachings include, without limitation, polypeptides of the following sequences. His tags, when present, are indicated with bold typeface.

TR3

(SEQ ID NO: 5)

MGIQGGSVLFGLLLVLAVFCHSGHSLQSYNPPRTPPMILRTSEETISTVQEKQNIISPLVR

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TR3 - HIS

(SEQ ID NO: 6)

MGIQGGSVLFGLLLVLAVFCHSGHSLQSYNPPRTPPMILRTSEETISTVQEKQONISPLVR  
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 AHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGMSFLSNLHLRNGELVIHEKGFYYI  
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TR3 - HIS2Q

(SEQ ID NO: 7)

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TR3 - HIS2V

(SEQ ID NO: 8)

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Meso-TR3

(SEQ ID NO: 9)

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Meso-TR3HIS2Q

(SEQ ID NO: 10)

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Meso64-TR3

(SEQ ID NO: 11)

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- continued

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## EXAMPLES

**[0049]** The present teachings including descriptions provided in the Examples that are not intended to limit the scope of any claim or aspect. Unless specifically presented in the past tense, an example can be a prophetic or an actual example. The following non-limiting examples are provided to further illustrate the present teachings. Those of skill in the art, in light of the present disclosure, will appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the present teachings.

### Example 1

**[0050]** This example illustrates design and biochemical characterization of the MUC16-targeted TRAIL trimer TR3 (FIG. 2). FIG. 2A is a schematic representation of proteins developed by the inventors. In these experiments, soluble mesothelin (Meso) containing an N-terminal FLAG tag (not shown), the parental TRAIL drug platform TR3 (center) and the MUC16-targeted mesothelin-TR3 fusion protein (Meso-TR3) were produced by transient transfection of HEK293T cells. FIG. 2B, depicts a Western blot analysis (reducing conditions) documents the molecular weights of TR3 ( $\approx 61$  kDa, lane 2) and Meso-TR3 ( $\approx 100$  kDa, lane 3) using anti-TRAIL pAb. Supernatant from mock-transfected HEK293T cells served as a negative control (lane 1).

**[0051]** Soluble mesothelin has been shown to bind to MUC16 rapidly and with high affinity (Gubbels, J. A., et al., *Mol. Cancer* 5:50, 2006). Since endogenous mesothelin is attached to the cell surface via a GPI anchor (Hassan, R., et al., *Clin. Cancer Res.* 10:3937, 2004; Chang, K., et al., *Proc. Natl. Acad. Sci. U.S.A.* 93:136, 1996), we designed a secreted form of the glycoprotein by deleting its GPI signal sequence (FIG. 2A, Meso). For immunologic detection purposes, we included a FLAG epitope tag, located at the amino-terminus of the secreted protein (not shown). The recombinant protein was produced in HEK293T cells and Western blot analysis confirmed its identity with a molecular weight of  $\approx 40$  kDa (not shown). To convert TR3 (FIG. 2A, center) into a MUC16-targeted cancer drug, we inserted the entire cDNA of soluble mesothelin (including the N-terminal FLAG tag) to the 5'-terminus of a TR3 expression plasmid (FIG. 2A, Meso-TR3). The resulting genetic constructs were expressed in mammalian 293T cells and characterized by Western blot analysis. Meso-TR3 was identified as a fusion protein with an apparent molecular weight of  $\approx 100$  kDa with the parental molecule TR3 being  $\approx 40$  kDa smaller (FIG. 21), consistent with the molecular weight of the mature and soluble form of human mesothelin.

### Example 2

**[0052]** This example illustrates that mesothelin binds to MUC16 in MUC16-expressing cells. In these experiments, various cancer cell lines were screened for expression of

mesothelin and MUC16. Briefly, cancer cell lines were incubated with antibodies against human mesothelin (K1, Santa Cruz) and human MUC16 (X75, AbCam). Primary antibody was detected with fluorescently labeled secondary antibody. The cells were then analyzed by flow cytometry. Mesothelin was expressed in all pancreatic cancer cell lines screened (AsPC1, CFPAC, Capan1) as well as ovarian cell line OVCAR3 (FIG. 3A-C, FIG. 4 A-C). MUC16 was only absent in AsPC3 (FIG. 3A). The presence of surface bound MUC16 is a prerequisite for the targeted delivery of TR3 to the cancer cells.

**[0053]** In order to confirm the MUC16 expression profile on OVCAR3 cells, we performed flow cytometry and were able to detect a strong surface expression with a homogeneous staining pattern for 100% of the cells (FIG. 4A). Next, we tested the ability of soluble, FLAG-tagged mesothelin to bind to membrane-bound MUC16 employing an *in vitro* binding assay using the same OVCAR3 cell line. Indeed, flow cytometry confirmed that soluble mesothelin was capable of binding to OVCAR3 cells (FIG. 4B). The staining pattern correlated well with the MUC16 expression profile of this cell line as nearly 100% of the cells were positive for the FLAG epitope tag, *i.e.* bound recombinant mesothelin. This pilot experiment was crucial as it confirmed not only the binding of recombinant mesothelin to native MUC16 on the target cells but also demonstrated accessibility of the epitope tag in the context of the mesothelin/MUC16 interaction.

**[0054]** In a next step, we asked if mesothelin protein, as part of the Meso-TR3 fusion protein, was capable of interacting with MUC16 on the OVCAR3 cell surface to facilitate membrane tethering of TR3. It was predicted that the multi-domain Meso-TR3 fusion protein could bind to OVCAR3 cells via two discrete mechanisms: 1) via the mesothelin/MUC16 interaction and 2) via the TR3/death receptor interaction [both DR4 and DR5 are expressed in OVCAR3 cells, not shown and Reis, C. R., *et al.*, *Cell Death. Dis.* 1:e83, 2010]. Since these circumstances would have complicated the interpretation of binding studies mediated exclusively via mesothelin, we first saturated the death receptor binding sites of Meso-TR3 with soluble death receptor 5 (DR5-Fc). In a following step, the Meso-TR3/DR5-Fc complexes were added to OVCAR3 cells in suspension. After several washing steps, the cells were stained for the presence of the FLAG epitope tag as evidence for drug binding to the OVCAR3 reporter cells. Using flow cytometry, we detected a strong and homogeneous fluorescence signal for cell-bound Meso-TR3, which was again nearly identical to the MUC16 staining profile and similar to the binding pattern of soluble mesothelin alone (FIG. 4C).

**[0055]** Further proof that Meso-TR3 and MUC16 do in fact co-localize on the plasma membrane of the target cells was obtained by employing confocal microscopy. Using the same detection system (anti-FLAG antibody) and death receptor blocking strategy (DR5-Fc pretreatment) as described above, the cells were now treated in an adherent state prior to washing, fixation, and immunostaining. Strong fluorescence signals were obtained for both the MUC16 epitope (red) and the FLAG tag of Meso-TR3 (green) (FIG. 4D). Importantly, the two signals overlapped (FIG. 4D, “merge”), suggesting that Meso-TR3 co-localizes with the mesothelin receptor MUC16 on the cancer cell membrane.

**[0056]** To demonstrate the targeting of mesothelin to cell surface MUC16, soluble FLAG-tagged mesothelin was gen-

erated in HEK293T cells. OVCAR3 cells were incubated with supernatant from HEK293T cells transfected with a secreted, FLAG-tagged form of human mesothelin. Following extensive washing to prevent detection of non-specific binding, mesothelin binding to MUC16 was assessed by staining for the FLAG tag. The cells were then analyzed by flow cytometry. There was a strong signal increase on the MUC16-positive OVCAR3 cancer cells, verifying that soluble mesothelin has a strong binding affinity for native MUC16 (FIG. 31). In FIG. 4, A presents a FACS-analysis of OVCAR3 cells assessed for expression of MUC16 (mAb X75) and a PE-conjugated secondary Ab (red line). The secondary Ab alone served to establish the background fluorescence (black line). In experiments illustrated in B, OVCAR3 cells in suspension were incubated with HEK293T-derived culture supernatant containing soluble mesothelin. Mesothelin binding was detected via anti-FLAG antibody staining (mAb M2) and a FITC-conjugated secondary Ab (green line). Cells treated with culture medium alone served as negative control (black line). In experiments illustrated in C, OVCAR3 cells in suspension were incubated with HEK293T-derived culture supernatant containing Meso-TR3.

**[0057]** To prevent binding of Meso-TR3 via TR3/death receptor interaction, Meso-TR3 was complexed with soluble DR5-Fc. Meso-TR3 binding was detected via anti-FLAG antibody staining similar to (B) using mAb M2, followed by FITC-conjugated secondary Ab (green line). Cells treated with culture medium alone served as negative control (black line). D, OVCAR3 cells were grown on 4-chamber slides and incubated the following day with Meso-TR3 complexed with DR5-Fc, similar to what has been described for (C). After washing, the cells were stained with a mixture of MUC16 pAb (red) and FLAG mAb (green), respectively. The cells were counterstained with TOPRO3 (blue, nuclei) and analyzed by confocal microscopy. The individual channels were overlaid to document co-localization of tumor marker and the targeted cancer drug (Merge). Original magnification: 63 $\times$ .

### Example 3

**[0058]** This example illustrates functional consequences of attaching the MUC16 targeting domain (mesothelin) to TR3.

**[0059]** TR3 and the fusion polypeptide mesothelin-TR3 (FIG. 1) were produced in HEK293T cells using standard transfection procedures. When MUC16-deficient Jurkat cells were treated with equimolar concentrations of TR3 and mesothelin-TR3, the cells were killed to the same degree (FIG. 5A).

**[0060]** In contrast, as shown in FIG. 5, when MUC16-high expressing OVCAR3 cells were treated with equimolar concentrations of TR3 and mesothelin-TR3, the mesothelin-TR3 was substantially more powerful in killing the cells than TR3 alone (SB).

OVCAR3 cells treated with mesothelin-TR3 can be rescued from cell death by adding increasing amounts of soluble mesothelin (SC). To determine whether cell death is caused by apoptosis, OVCAR3 cells were treated with mesothelin-TR3 in the presence of Z-VAD, a cell-permanent pan caspase inhibitor that inhibits the induction of apoptosis. In the presence of mesothelin-TR3, OVCAR3 cells were killed. However, with the addition of Z-VAD OVCAR3, cell death was minimal (SD).

**[0061]** To determine if the targeting of TR3 to the cell surface via mesothelin involves the native TR3 death pathway, OVCAR3 cells were treated with mesothelin-TR3 in the presence of increasing amounts of anti death receptor 5 (anti-DR5) antibody. Increasing amounts of anti-DR5 antibody inhibited the cancer cell killing by mesothelin-TR3, suggesting that the targeting of TR3 through mesothelin causes cell death via the native TR3 death pathway (SE).

#### Example 4

**[0062]** This example illustrates that mesothelin-TR3 is a targeted therapeutic on MUC16-expressing tumor cells, and that the mesothelin/MUC16 interaction can convert Meso-TR3 into a potent cancer drug (FIG. 6).

**[0063]** In order to compare the relative ability of cell death induction between Meso-TR3 and non-targeted TR3, it was important to establish the killing capacity of each drug mediated exclusively by the TR3 effector domain. Thus, we chose the TRAIL-sensitive T leukemia cell line Jurkat which lacks expression of MUC16 (not shown). We established the killing curves for both TR3 drugs and included recombinant TRAIL (rTRAIL) as an internal reference. At the drug concentrations chosen, all TRAIL drugs induced cell death to the same degree in the absence of the tumor marker MUC16 (FIG. 6A). This killing profile changed significantly when the same drug concentrations were used to treat MUC16-positive OVCAR3 cells, known to be sensitive to recombinant TRAIL (Lane, D., et al., *Cynecol. Oncol.* 93:594, 2004; Lane, D., et al., *Mol. Cancer Ther.* 5:509, 2006; Reis, C. R., et al., *Cell Death. Dis.* 1:e83, 2010). Non-targeted TR3 turned out to be quite inefficient with only ≈10% cell killing capacity at the highest dose used (FIG. 61). Importantly, TR3's killing profile was identical to that of rTRAIL, which is consistent with our earlier findings in that both drugs activate the extrinsic death pathway equally well and suggests that each trimer assumes the same native conformation (Spitzer, D., et al., *Mol. Cancer Ther.* 9:2142, 2010). Treatment with Meso-TR3, however, resulted in a much enhanced killing profile approaching 65% cell death at the highest drug dose employed (FIG. 6B). Linear regression analysis suggested a 7 to 12-fold stronger activity profile of Meso-TR3 when compared to TR3 and rTRAIL in OVCAR3 cells.

**[0064]** FIG. 6 shows the following: A, The cell killing profiles of TR3, Meso-TR3 and rTRAIL [0.2 ng/μL] were established on the MUC16-deficient T cell leukemia cell line Jurkat. NS, not significant (ANOVA). B, The same killing assay as in (A) using identical drug concentrations but the MUC16-positive ovarian cancer cell line OVCAR3 instead. \*\*, P<0.006; NS, not significant (ANOVA).

#### Example 5

**[0065]** This example illustrates that Meso-TR3 is phenotypically identical to conventional TRAIL (FIG. 7).

**[0066]** Based on the much enhanced killing profile of Meso-TR3 on MUC16-positive OVCAR3 cells, we hypothesized that the mesothelin/MUC16 interaction, i.e. the surface tethering of Meso-TR3 was responsible for the observed effects. To investigate this assumption, we performed a killing assay in the presence of increasing concentrations of soluble mesothelin to block the MUC16/Meso-TR3 interaction. As predicted, we were able to achieve a dose-dependent reduction in cell killing from 80% (no competitor) to 40% (highest competitor dose) (FIG. 7A).

We did not expect 100% rescue of the cells from apoptosis, because TR3 alone as well as recombinant rTRAIL exhibit baseline apoptosis-inducing activities in OVCAR3 cells, consistent with our observations.

**[0067]** In order to rule out phenotypic changes that might have been created following addition of the MUC16 targeting moiety mesothelin to the TR3 drug platform, we asked if the induction of cell death was purely mediated via the extrinsic death receptor pathway. Two lines of evidence suggest that this mechanism is well preserved following Meso-TR3 treatment. First, when soluble DR5-Fc was added to a standard killing assay using MUC16-positive OVCAR3 cells, Meso-TR3's killing capacity was nearly completely blunted, evidenced by a gradual decrease in cell death in a dose-dependent fashion from 90% in the absence of the soluble receptor to below 10% at the highest DR5-Fc concentration (FIG. 7B). As additional evidence for the involvement of the death receptor signaling cascade induced by Meso-TR3, the pan-caspase inhibitor Z-VAD-FMK blocked intracellular caspase activities and protected the cells completely from apoptosis (FIG. 7C).

**[0068]** Higher order TRAIL aggregates have been associated with increased activity due to more efficient death receptor clustering, especially regarding DR5 (Schneider, P., et al., *J. Exp. Med.* 187:1205, 1998.). In an attempt to recapitulate these observations, we treated Jurkat cells with Meso-TR3 in the presence of a mAb directed against the mesothelin moiety of the MUC16-targeted fusion protein. Using a sublethal dose of Meso-TR3 (33% cell death), we were able to demonstrate a dose-dependent augmentation of cell death to nearly 100% at the highest concentration of cross-linking antibody (FIG. 7D). These results strongly suggest that Meso-TR3 assumes a monomeric configuration in solution that can be further functionally enhanced by forming higher order aggregates (dimers), a concept just recently being utilized to treat highly vascularized cancers (Wilson, N. S., et al., *Cancer Cell* 22:80, 2012).

**[0069]** In FIG. 7, A, OVCAR3 cells were challenged with a constant amount of Meso-TR3 (80% specific cell death) and increasing concentrations of soluble mesothelin to study the impact of the mesothelin/MUC16 interaction of Meso-TR3. B, OVCAR3 cells were challenged with a constant amount of Meso-TR3 (90% specific cell death) and increasing concentrations of DR5-Fc to verify involvement of the extrinsic death pathway as a mechanism of Meso-TR3 killing. C, OVCAR3 cells were treated with a constant amount of Meso-TR3 (75% specific cell death) in the presence of Z-VAD-FMK, a pan-caspase inhibitor to block the extrinsic death pathway. Cells treated with DMSO were used as a control. D, MUC16-deficient Jurkat cells were treated with low dose Meso-TR3 (33% specific cell death) in the presence of anti-mesothelin mAb. Cross-linking of Meso-TR3 enhances target cell death to nearly 100%. Cells treated with anti-mesothelin Ab alone served as a control. Cells treated with medium alone were used as control. Error bars, ±SD. Results are representatives of at least 2 independent experiments done in triplicates.

#### Example 6

**[0070]** This example illustrates that mesothelin-TR3 selectively kills MUC16-expressing cells. In order to study drug selectivity aspects of Meso-TR3 toward MUC16-expressing targets, we took advantage of the fact that HeLa cells are composed of a native mix of MUC16-positive and



negative cells (80% and 20%, respectively). We therefore performed confocal microscopy on HeLa targets for tethering Meso-TR3. And indeed, those cells positive for the MUC16 tumor marker were heavily coated with Meso-TR3 (FIG. 8A). However, cells with a low or absent antigen expression were incapable of capturing Meso-TR3 and stained only weakly for the targeted drug (FIG. 8A, arrow). Based on these findings, we anticipated that Meso-TR3 would have a higher affinity for the MUC16-positive population within the mix and selectively eliminate these from the cell pool. And indeed, Meso-TR3 treatment resulted in a more than 30% reduction of MUC16-positive cells from 80% to 54% (FIG. 8B). In contrast, non-targeted TR3 was incapable of shifting the MUC16 ratio in this cervical cancer cell line due to the fact that it cannot discriminate between the two cell populations.

**[0071]** In these experiments (FIG. 8), HeLa cells were grown on 4-chamber slides and incubated the following day with Meso-TR3 complexed with DR5-Fc (8A). After washing, the cells were stained with a mixture of MUC16 pAb (red) and FLAG mAb (green), respectively. The cells were counterstained with TOPRO3 (blue, nuclei) and analyzed by confocal microscopy. The individual channels were overlaid to document co-localization of tumor marker and the targeted cancer drug (Merge). Original magnification: 63 $\times$ . B, HeLa cells were treated with TR3 and Meso-TR3 for 24 h. Two days post-treatment, the cells were assessed for changes in the MUC16 ratio using flow cytometry. Representative density plots are shown from experiments done at least twice in duplicates. These data indicate that Mesothelin-TR3 is more potent against MUC16-positive cells compared to TR3 alone.

#### Example 7

**[0072]** This example illustrates that Meso-TR3 is a cancer drug with prodrug properties and is fully activated on tumor cells expressing the biomarker MUC16 (FIG. 9).

Since the activity profiles of our TR3 drugs were routinely determined via functional apoptosis assays on reporter cells that lack the tumor marker MUC16 (compare FIG. 6A), we wanted to confirm that the drug input was similar for the respective TR3 variant. In order to do this, we employed semi-quantitative Western blot analysis, a detection method that does not rely on a native protein conformation, such as a TRAIL ELISA. When drug concentrations were analyzed that achieved identical killing capacities on MUC16-negative Jurkat cells, we consistently found much stronger signal intensities for Meso-TR3 compared to TR3 with a ratio of  $\approx$ 8 in favor of Meso-TR3 (FIG. 9A). These results suggest that, compared to TR3 alone, a significantly higher concentration of Meso-TR3 is required to achieve equivalent biological effects on MUC16-deficient cells (FIG. 9B).

**[0073]** In these experiments (FIG. 9), TR3 and Meso-TR3 preparations exerting identical killing profiles on MUC16-deficient tumor cells (A, compare with FIG. 6A) were subjected to semi-quantitative Western blot analysis under reducing conditions using anti-TRAIL pAb. The immunoreactive bands were quantified using QuantityOne<sup>®</sup> software (Bio-Rad, Hercules, Calif.) on a BioRad imaging system, with Meso-TR3 approximately 8-fold more abundant than 1TR3. B, Hypothetical proposed mechanism of Meso-TR3 activity. Without being limited by theory, the inventor have developed a hypothetical model. In this model, the mesothelin moiety of Meso-TR3 can partially

interfere with an unrestricted interaction of the TR3 domain and its death receptors (left panel). In the presence of MUC16 on the cancer cell surface, the mesothelin targeting domain can be removed from the TR3 surface thus enabling unrestricted access to and full activation of the death receptor-mediated extrinsic death pathway (right panel).

#### Example 8

**[0074]** These experiments, depicted in FIG. 12, illustrate that Meso-TR3 reduces the tumor burden in an in vivo mouse model of ovarian cancer. As shown in FIG. 12: A, ovarian cancer cell line OVCAR3 was genetically engineered, via retroviral infection, to stably express the luciferase-YFP fusion protein with a transduction efficiency of 24% (left panel, "Pre-sort", along with the corresponding luciferase activity following addition of luciferin substrate). In order to enrich the luciferase expressing cells, FACS sort was performed, resulting in a stable cell pool with more than 93% YFP (luciferase)-positive cells (right panel. Post-sort", along with the corresponding luciferase activity following addition of luciferin substrate). B, Meso-TR3 and the parental TR3 protein preparations were tested in apoptosis assays and show similar killing activity on MUC16-negative Jurkat cells (left panel). The same protein preparations were then applied to MUC6-positive OVCAR3 cells (adherent) and document the much increased killing profile of Meso-TR3 compared to the non-targeted TR3 parental molecule (right panel). C, OVCAR3 cell were first non-enzymatically detached from the culture flasks using EDTA and treated in suspension with TR3 and Meso-TR3 at equipotent concentrations on Jurkat cells (compare B, left panel). The cells were allowed to settle and the surviving cells that adhered following drug treatment were stained 2 days later with crystal violet. Of note, Meso-TR3 almost completely eliminated the cancer cells, in agreement to what has been documented above when the cells were treated in an adherent state (13, right panel). FIG. 12 D and FIG. 13: for the functional assessment of MUC16-targeted Meso-TR3 in vivo, SCID mice were injected i.p. with  $1 \times 10^6$  YFP-sorted OVCAR3 cells (93%). The next day, luciferase expression was monitored via non-invasive whole animal imaging and the mice were treated for 7 days with equivalent doses of TR3 and Meso-TR3 via the i.p. route and imaged at the indicated intervals. Of note, only the mouse treated with Meso-TR3 showed a substantial decrease in signal intensity, which was nearly 150-fold less than the initial luciferase activity and suggests enhanced and selective elimination of the labeled cells from the peritoneal location. In contrast, in mice treated with medium alone (ctrl) and TR3, the signal intensity did not change and support the results obtained from in vitro killing experiment.

#### Example 9

**[0075]** These experiments, depicted in FIG. 13 illustrate that Meso-TR3 reduces the tumor burden in an in vivo mouse model of ovarian cancer.

**[0076]** In these experiments, animals bearing MUC16-positive tumors expressing the luciferase-YFP fusion protein (as in Example 8) were treated with TR3, Meso-TR3, or control.

**[0077]** FIG. 13 illustrates examples of model animals treated with TR3, Meso-TR3, or control. Control, TR3 and

Meso-TR3 treated animals bearing ovarian cancer cell line OVCAR3 were imaged at the indicated times. In FIG. 13, A illustrates luciferase intensities prior to treatment, whereas B illustrates luciferase intensities 15 days post-treatment. Times beneath animals in A and B indicate duration of camera exposures. C illustrates a dramatic drop in image intensity in the animal receiving Meso-TR3 at 15 days. Note low level of signal obtained 15 days post-treatment in an animal which received Meso-TR3 even after a 1 min. camera exposure (B), whereas an animal receiving TR3 or control had much greater signals 15 days post-treatment. Data is normalized for photons/second. These data demonstrate therapeutic effectiveness of meso-1TR3 against tumors including MUC16-positive tumors.

## Example 10

**[0078]** This example illustrates production and killing potential of TR3, Meso64-TR3, and Meso-TR3. In these experiments, a Titer-Glo<sup>®</sup> assay (Promega Corporation, Madison, Wis.) was used in accordance with the supplier's instructions.

**[0079]** As shown in FIG. 14, the present inventors have demonstrated production in vitro of TR3, meso64-TR3, and Meso-TR3 (Western blot in upper panel). The present inventors also show the potency of Meso64-TR3 for killing Ovar-3 ovarian cancer cells, and the even greater potency of Meso1-TR3 for killing Ovar-3 ovarian cancer cells (cell killing curve in lower panel).

**[0080]** All references cited are hereby incorporated by reference, each in its entirety.

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&lt;210&gt; SEQ ID NO 3

&lt;211&gt; LENGTH: 6767

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: plasmid encoding fusion polypeptide

&lt;400&gt; SEQUENCE: 3

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&lt;210&gt; SEQ ID NO 4

&lt;211&gt; LENGTH: 5858

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: plasmid encoding fusion polypeptide

&lt;400&gt; SEQUENCE: 4

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What is claimed is:

1. A fusion polypeptide comprising:
  - a mesothelin polypeptide selected from the group consisting of meso64 and mesothelinΔGPI; and
  - three consecutive extracellular domains of TNF-related apoptosis-inducing ligand (TRAIL) domains fused together in a head-to-tail configuration.
2. A fusion polypeptide in accordance with claim 1, wherein the mesothelin polypeptide is meso64.
3. A fusion polypeptide in accordance with claim 1, wherein the mesothelin polypeptide is mesothelinΔGPI.
4. A fusion polypeptide in accordance with claim 1, further comprising a His-tag.
5. An anticancer therapeutic comprising the fusion polypeptide of claim 1.
6. A nucleic acid comprising a sequence encoding the fusion polypeptide of claim 1.
7. A vector comprising the nucleic acid of claim 6.
8. A vector of claim 7, wherein said vector is a plasmid.
9. A method of inducing apoptosis in a tumor cell, comprising contacting the tumor cell with the fusion polypeptide of claim 1.
10. The method of claim 9, wherein the tumor cell expresses MUC16.
11. The method of claim 9, wherein the tumor cell is an ovarian cancer cell.
12. The method of claim 9, wherein the tumor cell is a pancreatic cancer cell.
13. The method of claim 9 wherein the tumor cell is a breast cancer cell.
14. A method of treating a cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the fusion polypeptide of claim 1.
15. The method of claim 14, wherein the cancer comprises MUC16-positive cells.
16. The method of claim 14, wherein the cancer comprises ovarian cancer cells.
17. The method of claim 14, wherein the cancer comprises pancreatic cancer cells.
18. The method of claim 14, wherein the cancer comprises breast cancer cells.

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