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(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101 Rue de Tolbiac, 75013 Paris (FR). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3 rue Michel Ange, 75016 Paris (FR). **INSTITUT DE RECHERCHE POUR LE DÉVELOPPEMENT (IRD)** [FR/FR]; 44, bd de Dunkerque, 13002 Marseille (FR). **UNIVERSITE DE LA REUNION SAINT DENIS** [FR/FR]; 15 avenue René Cassin, SAINT DENIS, 97490 (RE).

(72) Inventors: **DESPRES, Philippe**; PROCESSUS INFECTIEUX EN MILIEU INSULAIRE TROPICAL (PIMIT) - U1187, UNIVERSITE DE LA REUNION - PLATEFORME DU CYROI, 2 RUE MAXIME RIVIERE, CYCLOTRON REUNION OCEAN INDIEN, SAINTE CLOTILDE, 97490 (RE). **GADEA, Gilles**; PROCESSUS INFECTIEUX EN MILIEU INSULAIRE TROPICAL (PIMIT) - U1187, UNIVERSITE DE LA REUNION - PLATEFORME DU CYROI, 2 RUE MAXIME RIVIERE, CYCLOTRON REUNION OCEAN INDIEN, SAINTE CLOTILDE, 97490 (RE). **VANWALSCHAPPEL, Bénédicte**; PROCESSUS INFECTIEUX EN MILIEU INSULAIRE TROPICAL (PIMIT) - U1187, UNIVERSITE DE LA REUNION - PLATEFORME DU CYROI, 2 RUE MAXIME RIVIERE, CYCLOTRON REUNION OCEAN INDIEN, SAINTE CLOTILDE, 97491 (RE).

(74) Agent: **INSERM TRANSFERT**; 7 rue Watt, 75013 Paris (FR).

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(54) Title: USE OF THE LAST C-TERMINAL RESIDUES M31/41 OF ZIKV M ECTODOMAIN FOR TRIGGERING APOPTOTIC CELL DEATH

(57) Abstract: Apoptosis is the main mechanism by which Mosquito-borne Zika virus infection causes cell death. Here, the inventors aimed to determine whether the last C-terminal residues M31/41 of ZIKV M ectodomain can trigger apoptotic cell death. Apoptosis was detected in human hepatoma Huh7 cells expressing of a recombinant GFP which includes ZIKV M oligopeptide at its C-terminus. The triggering of apoptosis requires translocation of ZIKV M oligopeptide in the secretory pathway and involves caspase-3 activation. The inventors then assessed whether ZIKV M oligopeptide has ability to confer death-promoting activity to a secreted tumor-associate antigen such as megakaryocyte-potentiating factor (MPF) which is a cleaved product of human mesothelin. Expression of MPF with ZIKV M oligopeptide linked to its C-terminus resulted in induction of apoptosis in human pulmonary adenocarcinoma A549 cells as well as human hepatoma Huh 7 cells. Given that ZIKV has been proposed as an oncolytic virus for cancer therapy, the ability of ZIKV sequence RVENWIFRNPG (called ZAMP for *ZikaApoptosis M Peptide*) (SEQ ID NO:1) to confer pro-apoptotic activity to a tumor-associated antigen such as MPF opens up new attractive perspectives for ZAMP as innovative anti-cancer agent.



USE OF THE LAST C-TERMINAL RESIDUES M31/41 OF ZIKV M ECTODOMAIN FOR TRIGGERING APOPTOTIC CELL DEATH

5 FIELD OF THE INVENTION:

The present invention is in the field of medicine, in particular virology and oncology.

BACKGROUND OF THE INVENTION:

10 Zika virus is an emergent mosquito-borne enveloped RNA virus belonging to *flavivirus* genus of *Flaviviridae* family. ZIKV is a neurotropic pathogen that mainly targets the central nervous system (CNS) [1], leading to several neurological diseases such as congenital neurological disorders and Guillain-Barré syndrome in adults [2, 3]. ZIKV strains are clustered into two major lineages, the African and Asian genotypes [4], the latter being responsible for the current epidemics with million cases of infection reported in particular, in South America.

15 In addition to its conventional transmission by infected-mosquito bite, human-to-human sexual or maternal-to-fetal transmission has been confirmed during the recent epidemics.

Like other flaviviruses such as dengue virus (DENV), yellow fever virus (YFV), and West Nile virus (WNV), ZIKV contains a single genomic RNA encoding a large polyprotein that is co- and post-translationally processed into three structural proteins C, prM (the

20 intracellular precursor of the small membrane protein M), and E followed by seven non-structural proteins NS1 to NS5. The processing of prM in mature M protein (75 amino acids) by host furin/subtilisin protease family occurs in a post-Golgi compartment leading to release of mature and infectious virus particles. The M protein consists of an ectodomain (hereafter referred as ectoM) composed of amino acids M-1/40 followed by a transmembrane-anchoring

25 region including two transmembrane domains (TMDs) (**Fig. 1A**). It is of note that dengue M sequences are highly conserved among the four serotypes unlike other structural proteins.

It has recently been reported that expression of mature DENV M protein leads to inflammasome activation [5]. Historically, it had been demonstrated that expression of DENV ectoM conjugated to a reporter protein such as GFP can trigger apoptosis in human hepatoma

30 cells [6]. The death-promoting activity is associated with a localization of DENV ectoM protein in a post-Golgi compartment [6]. Mutational analysis allowed to restrain the pro-apoptotic viral sequence to the last C-terminal amino-acid residues M-32/40 of DENV ectoM which had been named ApoptoM [6]. Although the mechanism of ApoptoM-mediated cell death remains to be better understood, apoptosis triggered by ApoptoM was associated with a mitochondrial

dysfunction leading to activation of apoptosis executioner caspase-3 [7]. However, the role of the last C-terminal residues of ZIKV M ectodomain for triggering apoptotic cell death has not yet been investigated.

5 SUMMARY OF THE INVENTION:

The present invention is defined by the claims. In particular, the present invention relates to the use of the last C-terminal residues M31/41 of ZIKV M ectodomain for triggering apoptotic cell death.

10 DETAILED DESCRIPTION OF THE INVENTION:

Mosquito-borne Zika virus (ZIKV) is an emerging flavivirus of medical concern associated with severe neurological disorders. Apoptosis is the main mechanism by which ZIKV infection causes cell death. The inventors previously reported that expression of flavivirus M ectodomain (residues M-1/41) resulted in apoptotic cell death. Here, they aimed to determine whether the last C-terminal residues M31/41 of ZIKV M ectodomain can trigger apoptotic cell death. Apoptosis was detected in human hepatoma Huh7 cells expressing of a recombinant GFP which includes ZIKV M oligopeptide at its C-terminus. The triggering of apoptosis requires translocation of ZIKV M oligopeptide in the secretory pathway and involves caspase-3 activation. The inventors then assessed whether ZIKV M oligopeptide has ability to confer death-promoting activity to a secreted tumor-associate antigen such as megakaryocyte-potentiating factor (MPF) which is a cleaved product of human mesothelin. Expression of MPF with ZIKV M oligopeptide linked to its C-terminus resulted in induction of apoptosis in human pulmonary adenocarcinoma A549 cells as well as Huh 7 cells. Given that ZIKV has been proposed as an oncolytic virus for cancer therapy, the ability of ZIKV sequence RVENWIFRNPG (called ZAMP for *Zika Apoptosis M Peptide*) (SEQ ID NO:1) to confer pro-apoptotic activity to a tumor-associated antigen such as MPF opens up new attractive perspectives for ZAMP as innovative anti-cancer agent.

Main definitions:

30 As used herein, the terms “**polypeptide**”, “**peptide**”, and “**protein**” are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component. Polypeptides when discussed in the context of gene therapy refer to the respective intact polypeptide, or any

fragment or genetically engineered derivative thereof, which retains the desired biochemical function of the intact protein.

As used herein, the expression “**derived from**” refers to a process whereby a first component (e.g., a first polypeptide), or information from that first component, is used to isolate, derive or make a different second component (e.g., a second polypeptide that is different from the first one).

As used herein, the term “**having the amino sequence**” has to be understood as consisting or comprising the amino sequence.

As used herein, the term “**signal peptide**” refers to a leader sequence ensuring entry into the secretory pathway

As used herein, the term “**polynucleotide**” refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

As used herein, the term “**encoding**” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as, for example, a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (e.g., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene, cDNA, or RNA, encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred

to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase "nucleotide sequence that encodes a protein or a RNA" may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

As used herein, the terms "**vector**", "**cloning vector**" and "**expression vector**" mean the vehicle by which a DNA or RNA sequence (*e.g.*, a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.*, transcription and translation) of the introduced sequence.

As used herein, the term "**promoter/regulatory sequence**" refers to a nucleic acid sequence (such as, for example, a DNA sequence) recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence, thereby allowing the expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

As used herein, the term "**operably linked**" or "**transcriptional control**" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences can be contiguous with each other and, *e.g.*, where necessary to join two protein coding regions, are in the same reading frame.

As used herein, the term "**transformation**" means the introduction of a "foreign" (*i.e.*, extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme

coded by the introduced gene or sequence. A host cell that receives and expresses introduced DNA or RNA has been "transformed".

As used herein, the term "**expression system**" means a host cell and compatible vector under suitable conditions, *e.g.*, for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

As used herein, the "**percent identity**" between the two sequences is a function of the number of identical positions shared by the sequences (i. e., % identity = number of identical positions/total number of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described below. The percent identity between two amino acid sequences can be determined using the Needleman and Wunsch algorithm (NEEDLEMAN, and Wunsch). The percent identity between two nucleotide or amino acid sequences may also be determined using for example algorithms such as EMBOSS Needle (pair wise alignment; available at www.ebi.ac.uk). For example, EMBOSS Needle may be used with a BLOSUM62 matrix, a "gap open penalty" of 10, a "gap extend penalty" of 0.5, a false "end gap penalty", an "end gap open penalty" of 10 and an "end gap extend penalty" of 0.5. In general, the "percent identity" is a function of the number of matching positions divided by the number of positions compared and multiplied by 100. For instance, if 6 out of 10 sequence positions are identical between the two compared sequences after alignment, then the identity is 60%. The % identity is typically determined over the whole length of the query sequence on which the analysis is performed. Two molecules having the same primary amino acid sequence or nucleic acid sequence are identical irrespective of any chemical and/or biological modification. According to the invention a first amino acid sequence having at least 80% of identity with a second amino acid sequence means that the first sequence has 80; 81; 82; 83; 84; 85; 86; 87; 88; 89; 90; 91; 92; 93; 94; 95; 96; 97; 98; 99 or 100% of identity with the second amino acid sequence.

As used herein, the term "**conjugate**" or interchangeably "**conjugated polypeptide**" is intended to indicate a composite or chimeric molecule formed by the covalent attachment of one or more polypeptides. The term "**covalent attachment**" "or "**conjugation**" means that the polypeptide and the non-peptide moiety are either directly covalently joined to one another, or

else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. A particular conjugate is a fusion protein.

- 5 As used herein, the term “**fusion protein**” indicates a protein created through the attaching of two or more polypeptides which originated from separate proteins. In particular fusion proteins can be created by recombinant DNA technology and are typically used in biological research or therapeutics. Fusion proteins can also be created through chemical covalent conjugation with or without a linker between the polypeptides portion of the fusion proteins. In the fusion protein
10 the two or more polypeptide are fused directly or via a linker.

As used herein, the term “**heterologous polypeptide**” refers to a polypeptide which does not derive from the same protein to which said heterologous polypeptide is fused.

- 15 As used herein, the term “**directly**” means that the first amino acid at the N-terminal end of a first polypeptide is fused to the last amino acid at the C-terminal end of a second polypeptide. This direct fusion can occur naturally as described in (Vigneron et al., Science 2004, PMID 15001714), (Warren et al., Science 2006, PMID 16960008), (Berkers et al., J. Immunol. 2015a, PMID 26401000), (Berkers et al., J. Immunol. 2015b, PMID 26401003), (DeLong et al., Science
20 2016, PMID 26912858) (Liepe et al., Science 2016, PMID 27846572), (Babon et al., Nat. Med. 2016, PMID 27798614).

- As used herein, the term “**linker**” or “**spacer**” has its general meaning in the art and refers to an amino acid sequence of a length sufficient to ensure that the proteins form proper secondary
25 and tertiary structures. In some embodiments, the linker is a peptidic linker which comprises at least one, but less than 30 amino acids e.g., a peptidic linker of 2-30 amino acids, preferably of 10-30 amino acids, more preferably of 15-30 amino acids, still more preferably of 19-27 amino acids, most preferably of 20-26 amino acids. Typically, the linker has 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; 15; 16; 17; 18; 19; 20; 21; 22; 23; 24; 25; 26; 27; 28; 29; 30 amino acid residues.
30 Typically, linkers are those which allow the compound to adopt a proper conformation (i.e., a conformation allowing a proper signal transducing activity through the IL-15Rbeta/gamma signalling pathway). The most suitable linker sequences (1) will adopt a flexible extended conformation, (2) will not exhibit a propensity for developing ordered secondary structure which could interact with the functional domains of fusion proteins, and (3) will have minimal

hydrophobic or charged character which could promote interaction with the functional protein domains.

As used herein, the term “**tumor antigen**” includes both tumor specific antigen (TSA) and tumor associated antigen (TAA). A tumor specific antigen is known as an antigen that is expressed only by tumor cells while tumor associated antigen are expressed on tumor cells but may also be expressed on some normal cells.

As used herein, the term “**mesothelin**” has its general meaning in the art and refers to the polypeptide encoded by the MSLN gene. An exemplary amino acid sequence for mesothelin is as set forth in **SEQ ID NO:2**. The signal peptide of mesothelin consists of the of the amino acid sequence that ranges from the amino acid residue at position 1 to the amino acid at position 36 in **SEQ ID NO:2**. The Megakaryocyte-potentiating factor (MPF) typically consists of the amino acid sequence that ranges from the amino acid residue at position 37 to the amino acid at position 286 in **SEQ ID NO:2**.

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SEQ ID NO:2 >sp|Q13421|MSLN_HUMAN Mesothelin OS=Homo sapiens OX=9606
GN=MSLN PE=1 SV=2. The Megakaryocyte-potentiating factor is
underlined in the sequence.
MALPTARPLLGSCGTPALGSLFLFLFSLGWVQPSRTLAGETGQEAAPLDGVLANPPNISS
LSPRQLLGFPCEVSGSLSTERVRELAVALAQKNVKLSTEQRLRCLAHRLSEPPEDLDALPL
DLLLFLNPDAFSGPQACTRFFSRITKANVDLLPRGAPERQRLLPAAALACWGVRSLLSEA
DVRALGGLACDLPGRFVAESA EVLLPRLVSCPGPLDQDQQAARAALQGGGPPYGPPSTW
SVSTMDALRGLLPVLGQPIIRSIPQGIVA AWRQRSSRDPSWRQPERTILRPRFRREVEKT
ACPSGKKAREIDESLI FYKKWELEACVDAALLATQMDRVNAIPFTYEQLDVLKHKLDELY
PQGYPESVIQHLGYLFLKMSPEDIRKWNVTSL ETLKALLEVNKGHEMSQAPRRPLPQVA
TLIDRFVKGRGQLDKDTLDTLTA FYPGYLCSL SPEELSSVPPSSIWAVRPQDLDTCDPRQ
LDVLYPKARLAFQNMNGSEYFVKIQS FLGGA PTEDLKALSQQNVSMDLATFMKLRTDAVL
PLTVAEVQKLLGPHVEGLKAEERHRPVRDWILRQRQDDLDLTLGLGLQG GIPNGYLVLDLS
MQEALSGTPCLLGPGPVLTVLALLLASTLA

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As used herein, the term “**oncolytic virus**” refers to a virus capable of selectively replicating in dividing cells (e.g. a proliferative cell such as a cancer cell) with the aim of slowing the growth and/or lysing said dividing cell, either in vitro or in vivo, while showing no or minimal replication in non-dividing cells. Typically, an oncolytic virus contains a viral genome packaged into a viral particle (or virion) and is infectious (i.e. capable of infecting and entering into a host cell or subject).

As used herein, the term “**cancer**” has its general meaning in the art and includes, but is not limited to, solid tumors and blood borne tumors. The term cancer includes diseases of the skin,

tissues, organs, bone, cartilage, blood and vessels. The term "**cancer**" further encompasses both primary and metastatic cancers. Examples of cancers that may be treated by methods and compositions of the invention include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; bronchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometrioid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous; adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and rhabdomyosarcoma, malignant; Sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant;

dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

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As used herein, the term "**treatment**" or "**treat**" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a patient having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a patient beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen

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may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular interval, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., pain, disease manifestation, etc.]).

As used herein, the term "**pharmaceutical composition**" refers to a composition described herein, or pharmaceutically acceptable salts thereof, with other agents such as carriers and/or excipients. The pharmaceutical compositions as provided herewith typically include a pharmaceutically acceptable carrier.

As used herein, the term "**pharmaceutically acceptable carrier**" includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical-Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980) discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof.

As used herein, the expression "**therapeutically effective amount**" is meant a sufficient amount of the active ingredient of the present invention to induce an immune response at a reasonable benefit/risk ratio applicable to the medical treatment.

Polypeptides of the present invention:

The first object of the present invention relates to the peptide that derives from the ZIKV M oligopeptide and having the amino acid sequence as set forth in **SEQ ID NO:1** (RVENWIFRNPG).

In some embodiments, the present invention relates to a peptide that derives from the ZIKV M oligopeptide and comprising the amino acid sequence as set forth in **SEQ ID NO:1** (RVENWIFRNPG).

- 5 In some embodiments, the present invention relates to a peptide that derives from the ZIKV M oligopeptide consisting in **SEQ ID NO:1** (RVENWIFRNPG).

In some embodiments, the present invention relates to a conjugate wherein a heterologous polypeptide is conjugated or fused to the peptide of the present invention.

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In some embodiments, the heterologous polypeptide is conjugated to the peptide of the present invention of the present invention by using chemical coupling. Several methods are known in the art for the attachment or conjugation of an antibody to its conjugate moiety. Examples of linker types that have been used to conjugate a moiety to an antibody include, but are not limited to, hydrazones, thioethers, esters, disulfides and peptide-containing linkers, such as valine-citruline linker. A linker can be chosen that is, for example, susceptible to cleavage by low pH within the lysosomal compartment or susceptible to cleavage by proteases, such as proteases preferentially expressed in tumor tissue such as cathepsins (e.g., cathepsins B, C, D). Techniques for conjugating polypeptides and in particular, are well-known in the art (See, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy," in Monoclonal Antibodies And Cancer Therapy (Reisfeld et al. eds., Alan R. Liss, Inc., 1985); Hellstrom et al., "Antibodies For Drug Delivery," in Controlled Drug Delivery (Robinson et al. eds., Marcel Dekker, Inc., 2nd ed. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological And Clinical Applications (Pinchera et al. eds., 1985); "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody In Cancer Therapy," in Monoclonal Antibodies For Cancer Detection And Therapy (Baldwin et al. eds., Academic Press, 1985); and Thorpe et al., 1982, Immunol. Rev. 62:119-58; see also, e.g., PCT publication WO 89/12624.)

- 20
25
30 In some embodiments, the heterologous polypeptide is fused to the peptide of the present invention to form a fusion protein.

In some embodiments, the heterologous polypeptide can be fused to the N-terminus or C-terminus of the peptide of the present invention.

In some embodiments, the heterologous polypeptide is a tumor antigen. Tumor specific antigens and tumor associated antigens have been described in the art. Such tumor antigen can be, but is not limited to human epithelial cell mucin (Muc-1; a 20 amino acid core repeat for Muc-1 glycoprotein, present on breast cancer cells and pancreatic cancer cells), the Ha-ras oncogene product, p53, carcino-embryonic antigen (CEA), the raf oncogene product, GD2, GD3, GM2, TF, sTn, MAGE-1, MAGE-3, tyrosinase, gp75, Melan-A/Mart-1, gp100, HER2/neu, EBV-LMP 1 & 2, HPV-F4, 6, 7, prostatic serum antigen (PSA), alpha-fetoprotein (AFP), CO17-1A, GA733, gp72, p53, the ras oncogene product, proteinase 3, Wilm's tumor antigen-1, telomerase, HPV E7 and melanoma gangliosides, as well as any other tumor antigens now known or identified in the future. Carcinoembryonic antigen (CEA), and alpha-fetoprotein (AFP) are two examples of such tumor antigens. AFP levels rise in patients with hepatocellular carcinoma: 69% of patients with liver cancer express high levels of AFP in their serum. CEA is a serum glycoprotein of 200 kDa found in adenocarcinoma of colon, as well as cancers of the lung and genitourinary tract. Yet another examples include the MICA/B ligands of NKG2D. These molecules are expressed on many types of tumors, but not normally on healthy cells. Additional specific examples of tumor antigens include epithelial cell adhesion molecule (Ep-CAM/TACSTD1), mesothelin, tumor-associated glycoprotein 72 (TAG-72), gp100, Melan-A, MART-1, KDR, RCAS1, MDA7, cancer-associated viral vaccines (e.g., human papillomavirus antigens), prostate specific antigen (PSA, PSMA), RAGE (renal antigen), CAMEL (CTL-recognized antigen on melanoma), CT antigens (such as MAGE-B5, -B6, -C2, -C3, and D; Mage-12; CT10; NY-ESO-1, SSX-2, GAGE, BAGE, MAGE, and SAGE), mucin antigens (e.g., MUC1, mucin-CA125, etc.), cancer-associated ganglioside antigens, tyrosinase, gp75, C-myc, Mart1, MelanA, MUM-1, MUM-2, MUM-3, HLA-B7, Ep-CAM, tumor-derived heat shock proteins, and the like (see also, e.g., Acres et al., Curr Opin Mol Ther 2004 February, 6:40-7; Taylor-Papadimitriou et al., Biochim Biophys Acta. 1999 Oct. 8; 1455(2-3):301-13; Emens et al., Cancer Biol Ther. 2003 July-August; 2(4 Suppl 1):S161-8; and Ohshima et al., Int J Cancer. 2001 Jul. 1; 93(1):91-6). Other exemplary tumor antigen targets include CA 195 tumor-associated antigen-like antigen (see, e.g., U.S. Pat. No. 5,324,822) and female urine squamous cell carcinoma-like antigens (see, e.g., U.S. Pat. No. 5,306,811), and the breast cell tumor antigens described in U.S. Pat. No. 4,960,716.

In some embodiments, the heterologous polypeptide is mesothelin and more particularly the megakaryocyte-potentiating factor (MPF, 32-kDa) that derives from mesothelin. In some embodiments, the heterologous polypeptide consists of the amino acid sequence that has at least

80% of identity with the amino acid sequence that ranges from the amino acid residue at position 37 to the amino acid at position 286 in **SEQ ID NO:2**.

In some embodiments, the peptide of the present invention is fused either directly or via a linker to the heterologous polypeptide. As used herein, the term "directly" means that the (first or last) amino acid at the terminal end (N or C-terminal end) of the peptide of the present invention is fused to the (first or last) amino acid at the terminal end (N or C-terminal end) of heterologous polypeptide. This direct fusion can occur naturally as described in (Vigneron et al., Science 2004, PMID 15001714), (Warren et al., Science 2006, PMID 16960008), (Berkers et al., J. Immunol. 2015a, PMID 26401000), (Berkers et al., J. Immunol. 2015b, PMID 26401003), (DeLong et al., Science 2016, PMID 26912858) (Liepe et al., Science 2016, PMID 27846572), (Babon et al., Nat. Med. 2016, PMID 27798614).

In some embodiments, the linker is a peptidic linker which comprises at least one, but less than 30 amino acids e.g., a peptidic linker of 2-30 amino acids, preferably of 10-30 amino acids, more preferably of 15-30 amino acids, still more preferably of 19-27 amino acids, most preferably of 20-26 amino acids. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser (i.e., G, N or S). Virtually any permutation of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr, Ala, Leu, Gln (i.e., T, A, L, Q) may also be used in the linker sequence. The length of the linker sequence may vary without significantly affecting the biological activity of the fusion protein. If used for therapeutical purposes, the linker is preferably non-immunogenic. Exemplary linker sequences are described in U.S. Pat. Nos. 5,073,627 and 5,108,910. Further preferred examples of linker sequences are Gly/Ser linkers of different length including (gly4ser)₃, (gly4ser)₄, (gly4ser), (gly3ser), gly3, and (gly3ser)₂.

In some embodiments, the linker is a glycine-serine linker having the amino acid sequence as set forth in **SEQ ID NO:3** (GGGSGGG).

In some embodiments, the fusion protein of the present invention comprises the amino acid sequence as set forth in **SEQ ID NO:4**.

SEQ ID NO:4>MPF-linker-ZAMP
LAGETGQEAAPLDGVLANPPNIISSLSRQLLGFPCEVSGLSTERVRELAVALAQKNVKLSTEQLRCLA
HRLSEPPEDLDALPLDLLLFLNPDAFSGPQACTRFFSRITKANVDLLPRGAPERQRLPAALACWGVRG

SLLEADVRLGGLACDLPGRFVAESA EVLLPRLVSCPGPLDQDQQAARAALQGGGPPYGPPSTWSVS
TMDALRGLLPVLGQPIIRSI PQGIVA AWRQRSSRDPSWRQPER **GGGSGGGRVENWIFRNPG**

In some embodiments, the fusion protein of the present invention comprises a signal peptide, preferably at its N-terminal end. Signal peptides are well known in the art. In some embodiments, the signal peptide consists of the signal peptide of mesothelin.

In some embodiments, the fusion protein of the present invention consists of the amino acid sequence as set forth in **SEQ ID NO:5**

SEQ ID NO:4>Signal peptide-MPF-linker-ZAMP
MALPTARPLLGSCGTPALGSLFLFSLGWVQPSRTIAGETGQEAAPLDGVLNPPNIISSLSRQLLGF
PCAEVSGLSTERVRELAVALAQKNVKLSTEQLRCLAHRLSEPPEDLDALPLDLLFLNPDAFSGPQACT
RFFSRITKANVDLLPRGAPERQRLLPAAALACWGVRSLLSEADVRLGGLACDLPGRFVAESA EVLLPRL
LVSCPGPLDQDQQAARAALQGGGPPYGPPSTWSVSTMDALRGLLPVLGQPIIRSI PQGIVA AWRQRSS
RDPSWRQPER **GGGSGGGRVENWIFRNPG**

The peptide of the present invention as well as fusion proteins comprising thereof may be produced by any technique known per se in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination. Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce said polypeptides, by standard techniques for production of polypeptides. For instance, they can be synthesized using well-known solid phase method, preferably using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions. Alternatively, the polypeptides and fusions proteins of the invention can be synthesized by recombinant DNA techniques as is now well-known in the art. For example, these fragments can be obtained as DNA expression products after incorporation of DNA sequences encoding the desired (poly) peptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired polypeptide, from which they can be later isolated using well-known techniques.

Polynucleotides and vectors of the present invention:

A further object of the present invention relates to a polynucleotide that encodes for the peptide and the conjugates including the fusion proteins of the present invention.

Typically, said polynucleotide is a DNA or RNA molecule, which may be included in any suitable vector, such as a plasmid, cosmid, episome, artificial chromosome, phage or a viral vector.

A wide variety of methods exist to deliver polynucleotides to subjects, as defined herein. For example, the polynucleotide of the present invention can be formulated with cationic polymers including cationic liposomes. Other liposomes also represent effective means to formulate and deliver self-acid nucleic molecule. Alternatively, the DNA can be incorporated into a viral vector, viral particle, or bacterium for pharmacologic delivery. Viral vectors can be infection competent, attenuated (with mutations that reduce capacity to induce disease), or replication-deficient. Methods utilizing DNA to prevent the deposition, accumulation, or activity of pathogenic self-proteins may be enhanced by use of viral vectors or other delivery systems that increase humoral responses against the encoded autoantigen. In some embodiments, the DNA can be conjugated to solid supports including gold particles, polysaccharide-based supports, or other particles or beads that can be injected, inhaled, or delivered by particle bombardment (ballistic delivery). Methods for delivering nucleic acid preparations are known in the art. See, for example, U.S. Patent Nos. 5,399,346, 5,580,859, and 5,589,466. A number of viral based systems have been developed for transfer into mammalian cells. For example, retroviral systems have been described (U.S. Patent No. 5,219,740; Miller et al, *Biotechniques* 7:980-990 (1989); Miller, *Human Gene Therapy* 1 :5-14, (1990); Scarpa et al, *Virology* 180:849-852 (1991); Burns et al, *Proc. Natl Acad. Sci. USA* 90:8033-8037 (1993); and, Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* 3: 102-109 (1993). A number of adenovirus vectors have also been described, see e.g., Haj-Ahmad et al., *J. Virol.* 57:267-274 (1986); Bett et al., *J. Virol.* 67:5911-5921 (1993); Mittereder et al, *Human Gene Therapy* 5:717-729 (1994); Seth et al., *J. Virol.* 68:933-940 (1994); Barr et al, *Gene Therapy* 1 :51-58 (1994); Berkner, *BioTechniques* 6:616-629 (1988); and, Rich et al, *Human Gene Therapy* 4:461-476 (1993). Adeno-associated virus (AAV) vector systems have also been developed for nucleic acid delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 and WO 93/03769; Lebkowski et al, *Molec. Cell Biol.* 8:3988-3996 (1988); Vincent et al, *Vaccines* 90 (Cold Spring Harbor Laboratory Press) (1990); Carter, *Current Opinion in Biotechnology* 3:533-539 (1992); Muzyczka, *Current Topics in Microbiol. And Immunol.* 158:97-129 (1992); Kotin, *Human Gene Therapy* 5:793-801 (1994); Shelling et al., *Gene Therapy* 1 : 165-169 (1994); and, Zhou et al., *J. Exp. Med.* 179: 1867-1875 (1994).

In some embodiments, the vector is an oncolytic virus. The oncolytic virus of the present invention can be obtained from any member of virus identified at present time provided that it is oncolytic by its propensity to selectively replicate and kill dividing cells as compared to non-dividing cells. It may be a native virus that is naturally oncolytic or may be engineered by modifying one or more viral genes so as to increase tumor selectivity and/or preferential replication in dividing cells, such as those involved in DNA replication, nucleic acid metabolism, host tropism, surface attachment, virulence, lysis and spread (see for example Kirn et al., 2001, Nat. Med. 7: 781; Wong et al., 2010, Viruses 2: 78-106). One may also envisage placing one or more viral gene(s) under the control of event or tissue-specific regulatory elements (e.g. promoter). Exemplary oncolytic viruses include without limitation reovirus, Seneca Valley virus (SVV), vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), herpes simplex virus (HSV), morbillivirus virus, retrovirus, influenza virus, Sin bis virus, poxvirus, adenovirus, or the like.

In some embodiments, the oncolytic virus of the present invention is obtained from a reovirus. A representative example includes Reolysin (under development by Oncolytics Biotech; NCT01166542).

In some embodiments, the oncolytic virus of the present invention is obtained from a Seneca Valley virus. A representative example includes NTX-010 (Rudin et al., 2011, Clin. Cancer Res. 17(4): 888-95).

In some embodiments, the oncolytic virus of the present invention is obtained from a vesicular stomatitis virus (VSV). Representative examples are described in the literature (e.g. Stojdl et al., 2000, Nat. Med. 6(7): 821-5; Stojdl et al., 2003, Cancer Cell 4(4): 263-75).

In some embodiments, the oncolytic virus of the present invention is obtained from a Newcastle disease virus. Representative examples include without limitation the 73-T PV701 and HDV-HUJ strains as well as those described in the literature (e.g. Phuangsab et al., 2001, Cancer Lett. 172(1): 27-36; Lorence et al., 2007, Curr. Cancer Drug Targets 7(2): 157-67; Freeman et al., 2006, Mol. Ther. 13(1): 221-8).

In some embodiments, the oncolytic virus of the present invention is obtained from a herpes virus. The Herpesviridae are a large family of DNA viruses that all share a common structure

and are composed of relatively large double-stranded, linear DNA genomes encoding 100-200 genes encapsided within an icosahedral capsid which is enveloped in a lipid bilayer membrane. Although the oncolytic herpes virus can be derived from different types of HSV, particularly preferred are HSV1 and HSV2. The herpes virus may be genetically modified so as to restrict viral replication in tumors or reduce its cytotoxicity in non-dividing cells. For example, any viral gene involved in nucleic acid metabolism may be inactivated, such as thymidine kinase (Martuza et al., 1991, Science 252: 854-6), ribonucleotide reductase (RR) (Boviatsis et al., Gene Ther. 1: 323-31; Mineta et al., 1994, Cancer Res. 54: 3363-66), or uracil-N-glycosylase (Pyles et al., 1994, J. Virol. 68: 4963-72). Another aspect involves viral mutants with defects in the function of genes encoding virulence factors such as the ICP34.5 gene (Chambers et al., 1995, Proc. Natl. Acad. Sci. USA 92: 1411-5). Representative examples of oncolytic herpes virus include NV1020 (e.g. Geevarghese et al., 2010, Hum. Gene Ther. 21(9): 1119-28) and T-VEC (Andtbacka et al., 2013, J. Clin. Oncol. 31, abstract number LBA9008).

In some embodiments, the oncolytic virus of the present invention is obtained from a morbillivirus which can be obtained from the paramyxoviridae family, with a specific preference for measles virus. Representative examples of oncolytic measles viruses include without limitation MV-Edm (McDonald et al., 2006; Breast Cancer Treat. 99(2): 177-84) and HMWMAA (Kaufmann et al., 2013, J. Invest. Dermatol. 133(4): 1034-42)

In some embodiments, the oncolytic virus of the present invention is obtained from an adenovirus. Methods are available in the art to engineer oncolytic adenoviruses. An advantageous strategy includes the replacement of viral promoters with tumor-selective promoters or modifications of the E1 adenoviral gene product(s) to inactivate its/their binding function with p53 or retinoblastoma (Rb) protein that are altered in tumor cells. In the natural context, the adenovirus E1B55 kDa gene cooperates with another adenoviral product to inactivate p53 (p53 is frequently dysregulated in cancer cells), thus preventing apoptosis. Representative examples of oncolytic adenovirus include ONYX-015 (e.g. Khuri et al., 2000, Nat. Med 6(8): 879-85) and H101 also named Oncorine (Xia et al., 2004, Ai Zheng 23(12): 1666-70).

In some embodiments, the oncolytic virus of the present invention is a poxvirus. As used herein the term “poxvirus” refers to a virus belonging to the Poxviridae family, with a specific preference for a poxvirus belonging to the Chordopoxviridae subfamily and more preferably to

the Orthopoxvirus genus. Sequences of the genome of various poxviruses, for example, the vaccinia virus, cowpox virus, Canarypox virus, Ectromelia virus, Myxoma virus genomes are available in the art and specialized databases such as Genbank (accession number NC_006998, NC_003663, NC_005309, NC_004105, NC_001132 respectively).

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In some embodiments the oncolytic poxvirus is an oncolytic vaccinia virus. Vaccinia viruses are members of the poxvirus family characterized by a 200 kb double-stranded DNA genome that encodes numerous viral enzymes and factors that enable the virus to replicate independently from the host cell machinery. The majority of vaccinia virus particles is intracellular (IMV for intracellular mature virion) with a single lipid envelop and remains in the cytosol of infected cells until lysis. The other infectious form is a double enveloped particle (EEV for extracellular enveloped virion) that buds out from the infected cell without lysing it.

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Pharmaceutical compositions of the present invention:

A further of the present invention relates to a pharmaceutical composition that comprises as active ingredient an amount of the peptide, conjugate, fusion protein, polynucleotide or vector of the present invention and a pharmaceutically acceptable carrier.

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A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to the subjects. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms. Typically, the pharmaceutical compositions contain vehicles, which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The

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pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists.

- 5 It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Solutions comprising compounds of the present invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils.
- 10 Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The active ingredient of the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such
- 15 organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example,
- 20 glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens,
- 25 chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate
- 30 solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-

drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

Methods of the present invention:

A further object of the present invention relates to the peptide, conjugate, fusion protein, polynucleotide or vector of the present invention for use as a drug.

In particular, a further object of the present invention relates to a method of treating cancer in a patient in need thereof comprising administering to the patient a therapeutically effective amount of the peptide, conjugate, fusion protein, polynucleotide or vector of the present invention.

In some embodiments, the peptide, conjugate, fusion protein, polynucleotide or vector of the present invention is used in combination with a chemotherapeutic agent. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065

(including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estrarnustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin (11 and calicheamicin 211, see, e.g., Agnew Chem Intl. Ed. Engl. 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, carabycin, canninomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptomicin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitestosterone, mepitosterone, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofiran; spirogennanin; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verrucurin A, roridin A and anguidine); urethane; vindesine; dacarbazine; mannomustine; mitobromtol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxorubicin (TAXOTERE®, Rhone-Poulenc Rorer, Antony,

France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are antihormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In some embodiments, the peptide, conjugate, fusion protein, polynucleotide or vector of the present invention in combination with a targeted cancer therapy. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules ("molecular targets") that are involved in the growth, progression, and spread of cancer. Targeted cancer therapies are sometimes called "molecularly targeted drugs," "molecularly targeted therapies," "precision medicines," or similar names. In some embodiments, the targeted therapy consists of administering the subject with a tyrosine kinase inhibitor. The term "tyrosine kinase inhibitor" refers to any of a variety of therapeutic agents or drugs that act as selective or non-selective inhibitors of receptor and/or non-receptor tyrosine kinases. Tyrosine kinase inhibitors and related compounds are well known in the art and described in U.S. Patent Publication 2007/0254295, which is incorporated by reference herein in its entirety. It will be appreciated by one of skill in the art that a compound related to a tyrosine kinase inhibitor will recapitulate the effect of the tyrosine kinase inhibitor, e.g., the related compound will act on a different member of the tyrosine kinase signaling pathway to produce the same effect as would a tyrosine kinase inhibitor of that tyrosine kinase. Examples of tyrosine kinase inhibitors and related compounds suitable for use in methods of embodiments of the present invention include, but are not limited to, dasatinib (BMS-354825), PP2, BEZ235, saracatinib, gefitinib (Iressa), sunitinib (Sutent; SU11248), erlotinib (Tarceva; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec; STI571), leflunomide (SU101), vandetanib (Zactima; ZD6474), MK-2206 (8-[4-aminocyclobutyl]phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one hydrochloride) derivatives thereof, analogs

thereof, and combinations thereof. Additional tyrosine kinase inhibitors and related compounds suitable for use in the present invention are described in, for example, U.S. Patent Publication 2007/0254295, U.S. Pat. Nos. 5,618,829, 5,639,757, 5,728,868, 5,804,396, 6,100,254, 6,127,374, 6,245,759, 6,306,874, 6,313,138, 6,316,444, 6,329,380, 6,344,459, 6,420,382, 6,479,512, 6,498,165, 6,544,988, 6,562,818, 6,586,423, 6,586,424, 6,740,665, 6,794,393, 6,875,767, 6,927,293, and 6,958,340, all of which are incorporated by reference herein in their entirety. In some embodiments, the tyrosine kinase inhibitor is a small molecule kinase inhibitor that has been orally administered and that has been the subject of at least one Phase I clinical trial, more preferably at least one Phase II clinical, even more preferably at least one Phase III clinical trial, and most preferably approved by the FDA for at least one hematological or oncological indication. Examples of such inhibitors include, but are not limited to, Gefitinib, Erlotinib, Lapatinib, Canertinib, BMS-599626 (AC-480), Neratinib, KRN-633, CEP-11981, Imatinib, Nilotinib, Dasatinib, AZM-475271, CP-724714, TAK-165, Sunitinib, Vatalanib, CP-547632, Vandetanib, Bosutinib, Lestaurtinib, Tandutinib, Midostaurin, Enzastaurin, AEE-788, Pazopanib, Axitinib, Motasenib, OSI-930, Cediranib, KRN-951, Dovitinib, Seliciclib, SNS-032, PD-0332991, MKC-I (Ro-317453; R-440), Sorafenib, ABT-869, Brivanib (BMS-582664), SU-14813, Telatinib, SU-6668, (TSU-68), L-21649, MLN-8054, AEW-541, and PD-0325901.

In some embodiments, the peptide, conjugate, fusion protein, polynucleotide or vector of the present invention is administered to the patient in combination with an immune checkpoint inhibitor. Preferred immune checkpoint inhibitors are antibodies that specifically recognize immune checkpoint proteins. A number of immune checkpoint inhibitors are known and in analogy of these known immune checkpoint protein inhibitors, alternative immune checkpoint inhibitors may be developed in the (near) future. The immune checkpoint inhibitors include peptides, antibodies, nucleic acid molecules and small molecules. Examples of immune checkpoint inhibitor include PD-1 antagonist, PD-L1 antagonist, PD-L2 antagonist CTLA-4 antagonist, VISTA antagonist, TIM-3 antagonist, LAG-3 antagonist, IDO antagonist, KIR2D antagonist, A2AR antagonist, B7-H3 antagonist, B7-H4 antagonist, and BTLA antagonist. In some embodiments, PD-1 (Programmed Death-1) axis antagonists include PD-1 antagonist (for example anti-PD-1 antibody), PD-L1 (Programmed Death Ligand-1) antagonist (for example anti-PD-L1 antibody) and PD-L2 (Programmed Death Ligand-2) antagonist (for example anti-PD-L2 antibody). In some embodiments, the anti-PD-1 antibody is selected from the group consisting of MDX-1106 (also known as Nivolumab, MDX-1106-04, ONO-4538, BMS-

936558, and Opdivo®), Merck 3475 (also known as Pembrolizumab, MK-3475, Lambrolizumab, Keytruda®, and SCH-900475), and CT-011 (also known as Pidilizumab, hBAT, and hBAT-1). In some embodiments, the PD-1 binding antagonist is AMP-224 (also known as B7-DCIg). In some embodiments, the anti-PD-L1 antibody is selected from the group consisting of YW243.55.S70, MPDL3280A, MDX-1105, and MEDI4736. MDX-1105, also known as BMS-936559, is an anti-PD-L1 antibody described in WO2007/005874. Antibody YW243.55.S70 is an anti-PD-L1 described in WO 2010/077634 A1. MEDI4736 is an anti-PD-L1 antibody described in WO2011/066389 and US2013/034559. MDX-1106, also known as MDX-1106-04, ONO-4538 or BMS-936558, is an anti-PD-1 antibody described in U.S. Pat. No. 8,008,449 and WO2006/121168. Merck 3745, also known as MK-3475 or SCH-900475, is an anti-PD-1 antibody described in U.S. Pat. No. 8,345,509 and WO2009/114335. CT-011 (Pidilizumab), also known as hBAT or hBAT-1, is an anti-PD-1 antibody described in WO2009/101611. AMP-224, also known as B7-DCIg, is a PD-L2-Fc fusion soluble receptor described in WO2010/027827 and WO2011/066342. Atezolimumab is an anti-PD-L1 antibody described in U.S. Pat. No. 8,217,149. Avelumab is an anti-PD-L1 antibody described in US 20140341917. CA-170 is a PD-1 antagonist described in WO2015033301 & WO2015033299. Other anti-PD-1 antibodies are disclosed in U.S. Pat. No. 8,609,089, US 2010028330, and/or US 20120114649. In some embodiments, the PD-1 inhibitor is an anti-PD-1 antibody chosen from Nivolumab, Pembrolizumab or Pidilizumab. In some embodiments, PD-L1 antagonist is selected from the group comprising of Avelumab, BMS-936559, CA-170, Durvalumab, MCLA-145, SP142, STI-A1011, STIA1012, STI-A1010, STI-A1014, A110, KY1003 and Atezolimumab and the preferred one is Avelumab, Durvalumab or Atezolimumab.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. Schematic representation of the GFP constructs assembled with flavivirus M oligopeptides. In (A), schematic representation of mature prM protein that is structured into a “pr” polypeptide followed by a 40 residues-long M ectodomain (ectoM) and the transmembrane anchoring region (TMDs). The sequences of ectoM from epidemic Brazilian ZIKV strain BeH819015 (NC_009227208), epidemic Indian ocean DENV-2 strain RUJUL (MN272404) and vaccine strain 17D of YFV (NC_776001.1) are listed. The last eleven

residues of ectoM are underlined. In (B), the GFP constructs include the signal peptide (SP) of ZIKV prM followed by a FLAG epitope at the N-terminus. The soluble sGFP constructs are ended by a glycine-serine spacer followed by the residues M-31/41 of ZIKV, DENV-2 and YFV or without viral sequence. The GFP^{ZIKV.M31/41} construct is lacking of prM signal peptide.

The sGFP construct is limited to a glycine-serine spacer at its C-terminus. The sGFP^{ZIKV.M31/41AAA} is an Ala-mutant of sGFP^{ZIKV.M31/41} construct (hereafter called mutGFP^{ZIKV.M31/41}) bearing the three Ala substitutions at positions M-33/35/38 which are underlined.

Figure 2. The ZIKV M oligopeptide associated to sGFP has an effect on cell integrity. Cells were transfected 24 h (left) or 48 h (right) with plasmids expressing sGFP, GFP^{ZIKV.M-31/41}, GFP^{DENV-2.M-31/41} and GFP^{YFV.M-31/41} or mock-transfected (control). LDH activity was measured and cell membrane permeability was expressed as signal intensity (O.D.). The results are the mean ($\pm$ SEM) of three or six independent experiments respectively. Pairwise comparisons with sGFP were performed and all significant comparisons are noted (** $p < 10^{-2}$; * $p < 10^{-1}$); any non-statistically significant comparisons are omitted.

Figure 3. ZIKV M oligopeptide has ability to induce caspase-3 activation in Huh7 cells. Cells were transfected 24 h with plasmids coding for sGFP^{ZIKV.M-31/41}, sGFP^{DENV-2.M-31/41} and sGFP^{YFV.M-31/41} or mock-transfected (control). Plasmid expressing sGFP served as a control. In (A), cell metabolic activity was measured using MTT assay. Viability was expressed as the signal intensity (O.D.). In (B), caspase 3/7 activity was expressed as the fold change of caspase activity in assay relative to that in sGFP. The results are the mean ($\pm$ SEM) of six independent assays. Pairwise comparisons with sGFP were performed and all statistically significant comparisons are noted (** $p < 10^{-4}$; * $p < 10^{-2}$).

Figure 4. The pro-apoptotic activity of ZIKV M oligopeptide involves the residues M-33/35/38 and requires its trafficking into the secretory pathway. Huh7 cells were transfected 24 h with plasmids expressing sGFP, sGFP^{ZIKV.M-31/41}, the GFP^{ZIKV.M-31/41} mutant that lacks of signal peptide, or the mutGFP^{ZIKV.M-31/41} mutant bearing the three Ala mutations. Caspase 3/7 activity is expressed as the fold change of caspase activity in assay relative to that in sGFP. The results are the mean ($\pm$ SEM) of three independent experiments. Pairwise comparisons with sGFP or sGFP^{ZIKV.M-31/41} were performed and all statistically significant comparisons are noted (** $p < 10^{-4}$; * $p < 10^{-3}$).

Figure 5. Schematic representation of MPF constructs with ZAMP. In top, the organization of 69-kDa preprotein mesothelin (MSLN) that is processed into MPF (megakaryocyte-potentiating factor) and membrane-anchored MSLN by furin protease. The

signal peptide of MSLN (residues 1/37) is indicated as blue box. In bottom, the MPF constructs with at the C-terminus, a Gly-Ser spacer followed by a FLAG epitope or ZIKV M oligopeptide ZAMP. The mutant MPF-mutZAMP includes the three mutations Ala inserted into the ZAMP sequence. The amino-acid changes are underlined in the ZAMP sequence.

Figure 6. ZAMP conjugated to MPF has no effect on HEK-293T cells. Cells were transfected 24 h with plasmids encoding MPF-FLAG, MPF-ZAMP, MPF-mutZAMP, or mock-transfected (control). In (A), FACS analysis was performed on cells expressing MPF-FLAG using anti-FLAG antibody. The percentage and the mean of fluorescence intensity (MFI) of positive cells are shown. In (B), cell metabolic activity was measured at 48 h post-transfection using an MTT assay. Viability was expressed as signal intensity (O.D.) and the results are the mean ($\pm$ SEM) of four independent assays. Non-statistically differences were observed between the MPF constructs and control.

Figure 7. Expression of MPF-ZAMP induces apoptosis in A549 cells. Cells were transfected 24 h with plasmids expressing MPF-FLAG, MPF-ZAMP, MPF-mutZAMP or transfectant alone (vehicle) or mock-transfected (control). In (A), A549 cells were transfected with plasmids expressing MPF-ZAMP or MPF-mutZAMP. Cells in apoptotic state were quantified by staining with FITC-labeled Annexin V. The percentage of Annexin V-positive cells was determined by FACS analysis. The results are the mean ($\pm$ SEM) of two independent assays. In (B), cell metabolic activity was measured using an MTT assay. Viability was expressed as signal intensity (O.D.) and the results are the mean ($\pm$ SEM) of three independent assays. Pairwise comparisons with MPF-FLAG were performed and all statistically comparisons are noted (* $p < 10^{-1}$); any non-statistically significant comparisons are omitted. In (C), caspase 3/7 activity was measured as signal intensity (O.D). The results are the mean ($\pm$ SEM) of four independent assays. Pairwise comparisons with MPF-FLAG were performed and all statistically comparisons are noted (** $p < 10^{-2}$; * $p < 10^{-1}$); any non-statistically significant comparisons are omitted.

Figure 8. Expression of MPF-ZAMP induces caspase-3 activation in Huh7 cells. Cells were transfected with plasmids encoding MPF-FLAG, MPF-ZAMP, MPF-mutZAMP or transfectant alone (vehicle) or mock-transfected (control). In (A), cell metabolic activity was measured at 18 h post-transfection using an MTT assay. Viability was expressed as signal intensity (O.D.) and the results are the mean ($\pm$ SEM) of four independent assays. In (B), Caspase 3/7 activity was measured at 18 h post-transfection and was expressed as signal intensity (O.D). The results are the mean ($\pm$ SEM) of six independent assays. Pairwise

comparisons with MPF-FLAG were performed and all statistically comparisons are noted (** $p < 10^{-4}$; * $p < 10^{-2}$); any non-statistically significant comparisons are omitted.

EXAMPLE:

5 **Material & Methods**

Cell lines

Huh7 cells (from Nolvonn Jouvenet, Institut Pasteur), A549 cells (ATCC, CCL-185) and HEK-293T cells (ATCC, CRL-1573) were cultured in Minimum Essential Media (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and non-essential amino acids.

Plasmids

The coding sequences for the last eleven amino-acid residues of M ectodomain designated as M³¹⁻⁴¹ from YF-17D, DENV-2, Brazilian BeH819015 strains or its mutant, preceded in frame by the last amino-acid residues of ZIKV capsid protein which compose the signal peptide for translocation of prM, the FLAG_{tag} sequence, GFP sequence and glycine-serine spacer (**Fig. 1**) were synthesized and cloned into Nhe I and Not I restriction sites of the pcDNA 3.1 (+) Neo plasmid by Genecust (Luxembourg).

To generate MPF-ZAMP and its mutant, the coding sequence for M³¹⁻⁴¹ from Brazilian BeH819015 strain, preceded in frame by the soluble MPF region of human mesothelin (Genbank access number NM_001177355.1) which contains the signal peptide for the transport of MPF-ZAMP into secretory pathway (**Fig. 5**) were synthesized and cloned into Nhe I and Not I restriction sites of the pcDNA 3.1 (+) Neo plasmid (Genecust, Boynes, France).

The recombinant plasmids were verified by sequencing (Genecust, Boynes, France). Plasmids were transfected in Huh7, A549 and HEK-293T cells using LipofectaminTM 3000 according to the manufacturer's instructions (Thermo Fischer Scientific, Illkirch-Grafenstaden, France).

Flow cytometry analysis

Huh7 cells (1.5×10^5) or A549 cells (1×10^5) were fixed with 3.7% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 min and GFP expression was analyzed by flow cytometry using FlowJo software (BD Bioscience).

30 *Lactate dehydrogenase assay*

Huh7 cells were seeded in 12-well culture plate at a density of 1.5×10^5 cells per well. Cell damages were evaluated on supernatants of transfected-cells measuring lactate dehydrogenase (LDH) release using Cytotox 96® non-radioactive cytotoxicity assay (Promega) according to

manufacturer instructions. Absorbance of converted dye was measured at 490 nm (Tecan) with a background subtraction at 690 nm.

MTT assay

Huh7 cells (2×10^4), A549 cells (2×10^4) or HEK-293T cells (2×10^4) were cultured in 96-well culture plate. Cell monolayers were rinsed with PBS 1X and incubated with culture growth medium mixed with 5 mg.mL⁻¹ MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution for 1 h at 37°C. MTT medium was removed and the formazan crystals were solubilized with dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm with a background subtraction at 690 nm.

Caspase 3/7 activity

Huh7 cells (2×10^4) or A549 (2×10^4) were seeded in 96-well culture plate. Caspase 3/7 activity in raw cell lysates was measured using a Caspase Glo® 3/7 assay kit (Promega, Charbonnières-les-bains, France) according to the manufacturer's protocol. Caspase activity was quantified by luminescence using a FLUOstar Omega Microplate Reader (BMG LABTECH, Champigny-sur-Marne, France).

Early apoptosis analysis

A549 cells were seeded in 12-well culture plate at a density of 1×10^5 cells per well. Cells were fixed with 3.7% PFA in PBS for 10 min and stained with the Ca²⁺-dependent phosphatidylserine-binding protein FITC-labelled annexin V (BioLegend). FITC expression was analyzed by flow cytometry using Flowjo software (BD Bioscience).

Statistical analysis

An unpaired t-test was used to compare quantitative data. GraphPad Prism was used for all statistical analysis.

Results

Expression of a soluble GFP bearing the ZIKV residues M-31/41 triggers apoptosis in Huh7 cells

We investigated whether the last C-terminal amino-acids of ZIKV M ectodomain can induce apoptosis as previously observed with DENV and YFV (**Fig. 1A**). Consequently, we generated a soluble recombinant GFP (sGFP) protein in which ZIKV M oligopeptide representing the residues M-31/41 of epidemic ZIKV strain BeH819015 (ZIKV.M-31/41) was linked to its C-terminus (**Fig. 1B**). The sequence coding for ZIKV prM signal peptide which encompasses the last C-terminal residues of the adjacent C protein allowed the translocation of GFP^{ZIKV.M-31/41} into the secretory pathway. A same design of GFP-based constructs was applied for DENV-2 and

YFV M oligopeptides to serve as positive controls. A sGFP with only the glycine-serine spacer at the C-terminus was used as a negative control (**Fig. 1B**).

Given that human hepatoma cells were susceptible to death-promoting activity of flavivirus ectoM [6], transfection with plasmids expressing recombinant sGFP was performed in Huh7 cells. FACS analysis showed that GFP expression rate was similar between Huh7 cells expressing sGFP with or without flavivirus M oligopeptide, indicating that the different constructs were suitable for further characterization (not shown).

To determine whether expression of soluble GFP proteins with the C-terminal flavivirus residues M31/41 has an effect on cell membrane integrity, Huh7 cells were transfected with vector plasmids expressing either GFP^{ZIKV.M-31/41}, GFP^{DENV-2.M-31/41}, or GFP^{YFV.M-31/41} (**Fig. 2**). A recombinant sGFP without the C-terminal viral sequence was used as a negative control. Cell membrane integrity was evaluated by measuring LDH release (**Fig. 2**). At 24 h post-transfection, no significant change in LDH activity was observed in transfected Huh7 cells whatever recombinant sGFP constructs tested. At 48 h post-transfection, expression of sGFP resulted in a marked increase of LDH activity in Huh7 cells as compared to mock-transfected cells (**Fig. 2**). We observed that GFP^{ZIKV.M-31/41} and also GFP^{YFV.M-31/41} were more cytopathic as compared to sGFP. From these results, it has been estimated that the time-point of 24 h post-transfection was appropriated to evaluate the pro-apoptotic activity of GFP^{ZIKV.M-31/41} in Huh7 cells.

We evaluated whether GFP^{ZIKV.M31/41} expression has an effect on the metabolism of Huh7 cells by measuring MTT activity at 24 h post-transfection (**Fig. 3**). As expected, both GFP^{DENV-2.M31/41} and GFP^{YFV.M31/41} have a significant impact on cell metabolic activity as compared to control sGFP (**Fig. 3A**). A comparable reduction was observed with sGFP^{ZIKV.M31/41} indicating that ZIKV M oligopeptide has an effect on metabolic activity of Huh7 cells. To determine whether sGFP^{ZIKV.M31/41} can trigger apoptosis in Huh7 cells, activation of caspase-3 was measured using a caspase-3/7 assay kit (**Fig. 3B**). There was a 2.5-fold increase in caspase-3 activity in Huh7 cells expressing GFP^{DENV-2.M-31/41} and GFP^{YFV.M-31/41}, consistent with their death-promoting properties in human hepatoma cells[6]. A comparable caspase-3 activity was observed in Huh7 cells expressing sGFP^{ZIKV.M-31/41} (**Fig. 3B**). Like DENV-2 and YFV, ZIKV M oligopeptide representing the residues M-31/41 of viral strain BeH819015 is a potent inducer of apoptosis in human hepatoma cells through caspase-3 activation.

Apoptosis mediated by DENV ectoM involves translocation of the viral sequence into the secretory pathway [6]. To evaluate whether a same pre-requisite does exist for ZIKV M oligopeptide, the sGFP^{ZIKV.M-31/41} construct was deleted from its signal peptide to generate a mutant GFP^{ZIKV.M-31/41} (**Fig. 1B**). As shown in **Fig. 4**, lack of signal peptide resulted in severe reduction of caspase-3 activity as compared to sGFP^{ZIKV.M-31/41}. Thus, induction of apoptosis mediated by sGFP^{ZIKV.M-31/41} requires trafficking of ZIKV M oligopeptide into the secretory pathway.

We evaluated the possibility to generate a mutant of sGFP^{ZIKV.M-31/41} which has lost its death-promoting activity. We noted that the residues E-33/35/38 are identical among the M proteins of ZIKV, DENV-2 and YFV (**Fig. 1A**). Moreover, the residue Tryp at position M-35 is strictly conserved among flavivirus M sequences suggesting an important role in ectoM-mediated apoptosis. By direct mutagenesis, three amino-acid substitutions M-E33A/W35A/R38A were introduced into a plasmid expressing sGFP^{ZIKV.M-31/41} leading to a mutant mutGFP^{ZIKV.M-31/41} (**Fig. 1B**). As shown in **Fig. 4**, there was comparable increase in caspase-3 activity in Huh7 cells expressing mutGFP^{ZIKV.M-31/41} and sGFP control. Thus, introduction of Ala residues at three positions M-33/35/38 dramatically reduced the capacity of sGFP^{ZIKV.M-31/41} to trigger apoptosis. These results suggest a critical role for the three ectoM residues E33, W35, and R38 in apoptosis mediated by ZIKV M oligopeptide.

The ZIKV M oligopeptide confers pro-apoptotic properties to a soluble tumor-associated antigen

We next investigated whether ZIKV M oligopeptide has ability to confer death-promoting properties to a soluble tumor-associated antigen. Mesothelin (MSLN, 69 kDa) is a tumor-associated antigen which is overexpressed in many human cancers [8]. The megakaryocyte-potentiating factor (MPF, 32-kDa) is a N-terminal cleavage product from preprotein MSLN [9] (**Fig. 5**). As human tumor antigen, soluble MPF is released into the circulation serving as biomarker for the prognosis of lung cancers. The oligopeptide RVEWIFRNPG (hereafter referred to as ZAMP for Zika Apoptosis M Peptide) corresponding to ZIKV M residues M-31/41 was linked to the C-terminus of MPF. The gene coding for human MPF followed by a glycine-serine spacer and ZAMP at its C-terminus was inserted into an expression vector. The authentic signal peptide of MPF was used for translocation of MPF-ZAMP into the secretory pathway. A recombinant MPF protein tagged with a FLAG epitope in place of ZAMP was used as a control (**Fig. 5**). Given that amino-acid substitutions M-A33E, M-A35W and M-A38R

abrogate the death-promoting activity of ZAMP (**Fig. 4**), these three mutations were introduced into MPF-ZAMP leading to a mutant MPF-mutZAMP (**Fig. 5**).

To validate expression of our recombinant MPF in human cells, human embryonic kidney HEK-293T cells were transfected with a plasmid expressing MPF-FLAG protein. FACS analysis detected a high expression level of MPF-FLAG in HEK-293T cells using anti-FLAG antibody (**Fig. 6A**). The effects of MPF-ZAMP expression on cell viability were examined by measuring MTT activity. At 48 h of transfection, there was no change in cell metabolic activity in HEK-293T cells expressing MPF-ZAMP or MPF-mutZAMP as compared to MPF-FLAG (**Fig. 6B**). Thus, expression of a recombinant MPF bearing ZAMP or its defective mutant has no effect on viability of HEK-293T cells. The absence of sensitivity of HEK-293T cells to ZAMP expression correlates with a previous report showing that DENV ApoptoM does not mediate apoptosis in these cells [6].

Given that MPF is a biomarker of lung cancers [10], pulmonary adenocarcinoma A549 cells were tested with plasmids expressing MPF-ZAMP proteins. FACS analysis using anti-FLAG antibody showed that expression of MPF-FLAG protein was efficient in A549 cells as observed with HEK-293T cells (not shown). The death-promoting activity of MPF-ZAMP in A549 cells was assessed by measuring the rate of early apoptosis using the Annexin V affinity assay, which detects phosphatidylserine (PS) translocation to the outer layer of the cell membrane. At 24 h post-transfection, expression of MPF-ZAMP resulted in a significant increase of the rate of Annexin V-positive A549 cells by at least 14% as compared to control MPF-FLAG (**Fig. 7A**). No Annexin V-positive A549 cells were detected with MPF-mutZAMP mutant. We wondered whether the increase in Annexin V-positive A549 cells correlates with a decreased metabolic activity in cells expressing MPF-ZAMP (**Fig. 7B**). At 24 h post-transfection, a moderate but significant reduction in MTT activity was observed in A549 cells expressing MPF-ZAMP but not MPF-mutZAMP as compared to MPF-FLAG. We next evaluated whether activation of caspase-3 occurs in A549 cells expressing MPF-ZAMP in. At 24 h post-transfection, caspase-3/7 assay indicated that expression of MPF-ZAMP resulted in a higher level of caspase-3 activity as compared to MPF-mutZAMP and MPF-FLAG (**Fig. 7C**). Together, these results demonstrated that MPF-ZAMP can trigger apoptosis in pulmonary adenocarcinoma cells through caspase-3 activation.

We evaluated whether MPF-ZAMP has also ability to induce apoptosis in hepatoma cells such as Huh7 cells. By using MTT assay, we observed that expression of MPF-ZAMP results in a

marked decrease of cell metabolic activity at 24 h post-transfection. Consequently, the loss of cell metabolism mediated by MPF-ZAMP was examined in Huh7 cells at an earlier time in the transfection. At 18 h post-transfection, expression of MPF-ZAMP again resulted in a severe decrease in cell metabolism as compared to MPF-FLAG and MPF-mutZAMP (**Fig. 8A**). We next evaluated whether MPF-ZAMP activates caspase-3 in Huh7 cells at 18 h post-transfection (**Fig. 8B**). There was a high level of caspase-3 activity in Huh7 cells expressing MPF-ZAMP as compared to MPF-FLAG. Whereas introduction of Ala mutations in ZAMP resulted in a weak increase of caspase-3 activity. At 24 h post-transfection, there was a comparable level of caspase-3 activity in Huh7 cells expressing either MPF-mutZAMP or MPF-FLAG confirming that the Ala mutant of ZAMP has no pro-apoptotic activity (not shown). Taken together, these results showed that ZIKV M oligopeptide ZAMP conjugated to a tumor antigen has ability to trigger apoptosis in pulmonary adenocarcinoma and also hepatoma cells through caspase-3 activation.

Discussion:

Flaviviruses expose on their surface the structural proteins E and M, the latter being cleaved from its glycosylated precursor form prM into immature virus particles during their exocytic transit towards extracellular compartment [11]. The mature M protein comprises a 40-residues long ectodomain followed by a double-membrane anchor. Little is still known on the role of small integral membrane M protein in the biology and pathogenicity of flaviviruses. It has been proposed a role for M in flavivirus assembly [12-15]. Recently, a channel activity has been designated to ZIKV M protein promoting virus entry in the host-cell [16]. Historically, we identified a role for the M ectodomain in apoptotic cell death triggered by DENV and YFV [6]. It has been found that pro-apoptotic activity of DENV M ectodomain essentially relates to its last C-terminal amino-acid residues which compose a viral apoptosis inducer untitled ApoptoM [6]. Expression of ApoptoM results in mitochondrial membrane permeabilization leading to caspase-3 activation [6, 17].

ZIKV can trigger apoptotic cell death in vitro as well as in vivo [18-21]. The structural proteins prM and E have been associated with apoptosis mediated by ZIKV [22]. In the present study, we demonstrated that the last C-terminal residues M-31/41 of the ZIKV M ectodomain from Brazilian viral strain BeH819015 have death-promoting activity through caspase-3 activation. To our knowledge, this is the first time that a pro-apoptotic activity has been assigned to the M oligopeptide RVEWIFRNPG for which the acronym ZAMP (for Zika Apoptosis M Peptide) has been proposed. We noted that ZAMP sequence shares at least 60%

of identity to the counterpart from DENV-2 but less than 30% with YFV despite the fact that the death-promoting activity of the last C-terminal residues of the M ectodomain is conserved among the three flaviviruses. We identified the residues M-33/35/38 as playing a key role in the pro-apoptotic activity of ZAMP. Noteworthy, the residue Tryp to position M-35 is strictly conserved among flavivirus M proteins. Although it is highly likely that ZIKV cannot tolerate a complete change of the three residues M-33/35/38, it would be of great interest to assess the contribution of each the three residues in relation with the death-promoting activity of ZIKV. We reported that the amino-acid substitution M-L36F which differentiates wild-type to live attenuated YFV strains results in a lack of death-promoting activity for YFV M ectodomain [6]. A residue Ile has been identified at position ZIKV M-36. It has been recently demonstrated that Japanese encephalitis virus, a close relative to ZIKV, was attenuated in neurovirulence when amino acid change I36F was introduced in the M protein [23]. Experiments will be undertaken to determine whether a residue Phe at position M-36 causes attenuation for ZIKV. Such a study could be beneficial for the further development of live attenuated ZIKV strains as vaccine candidates against ZIKV-associated disease [24].

Mechanisms by which ZAMP activates apoptosis signaling pathway remain to be understood. It has been admitted that flavivirus infection causes a consistent increase in amount of the pro-apoptotic Bax [25, 26]. A dysregulation of MCL-1 expression associated with inhibition of anti-apoptotic BCLXL protein as well as caspase-8 activation contribute to apoptosis triggered by flaviviruses [27]. Our previous studies on DENV M ectodomain have shown that a correlation exists between the translocation of viral sequence in a post-endoplasmic reticulum (ER) compartment and induction of apoptosis pathway[6]. We herein report that ZAMP-mediated apoptosis involves the transport of the Zika M oligopeptide into the secretory pathway. In the ER, Sphingosine kinase SPHK2 can generate pro-apoptotic ceramide through the production of sphingosine 1-phosphate which acts as intracellular signaling molecule. Interestingly, SPHK2 has been shown to interact with DENV proteins leading to apoptotic cell death [28]. Whether the subcellular distribution of ZAMP leads to activation of apoptosis signaling pathway through a protein-protein interaction requires further investigation.

In the present study, we demonstrated that ZAMP association with MPF which relates to tumor-associated antigen mesothelin can trigger apoptosis in hepatoma and pulmonary adenocarcinoma cells. Expression of MPF with ZAMP protein results in PS translocation to the outer leaflet of the plasma membrane and caspase-3 activation leading to a loss of cell metabolic activity without affecting cell membrane permeability. A lack of apoptosis was observed in

HEK-293T cells expressing ZAMP as it has been already reported with the pro-apoptotic M sequence of DENV-2[6]. Transformed HEK-293T cells are defective in terms of p53 protein activation [29] that has been described to be the central component in the complex network of apoptosis signaling pathways [30]. The fact that ZAMP acts as caspase-3 activator in A549 cells having a functional p53 protein [31] as well as p53-deficient Huh7 cells [32] do not sustain a major role for p53-dependent signaling pathway in apoptosis mediated by ZAMP. The megakaryocyte-potentiating factor is part of the tumor-associated antigen (TAA) mesothelin. Over the past several decades, several useful TAAs have been identified and characterized for their biochemical properties. TAAs represent a group of normal non-mutant molecules and can be subdivided into four major categories according to expression pattern: (1) cancer-testis like antigens such as MAGE-1 are expressed in a wide range of different cancers including melanoma, blade cancer and neuroblastoma[33]; (2) Differentiation antigens including tyrosinase TRP-1 are expressed in differentiation stage-dependent and tissue-specific manners [34, 35]; (3) Oncofetal antigens including carcinoembryonic antigen (CEA) are found in embryonic and fetal tissues as well as certain cancers [36, 37]; (4) Overexpressed antigens such as mesothelin that are normal proteins whose expression is up-regulated in cancer cells [9].

Recently, ZIKV has gained attention in oncolytic virotherapy field as a potential oncolytic virus due to its neurotropism and its efficacy against glioblastoma multiforme and aggressive metastatic forms of human CNS tumors [38-40]. As aforementioned, association of ZAMP with MPF results in activation of apoptosis executioner caspase-3 in different tumor cells. The ability of ZIKV M oligopeptide RVENWIFRNPG to confer pro-apoptotic activity to TAA could open important perspective for ZAMP in cancer therapy strategy through tumor cell death and immune recognition of the TAA by antigen-presenting cells.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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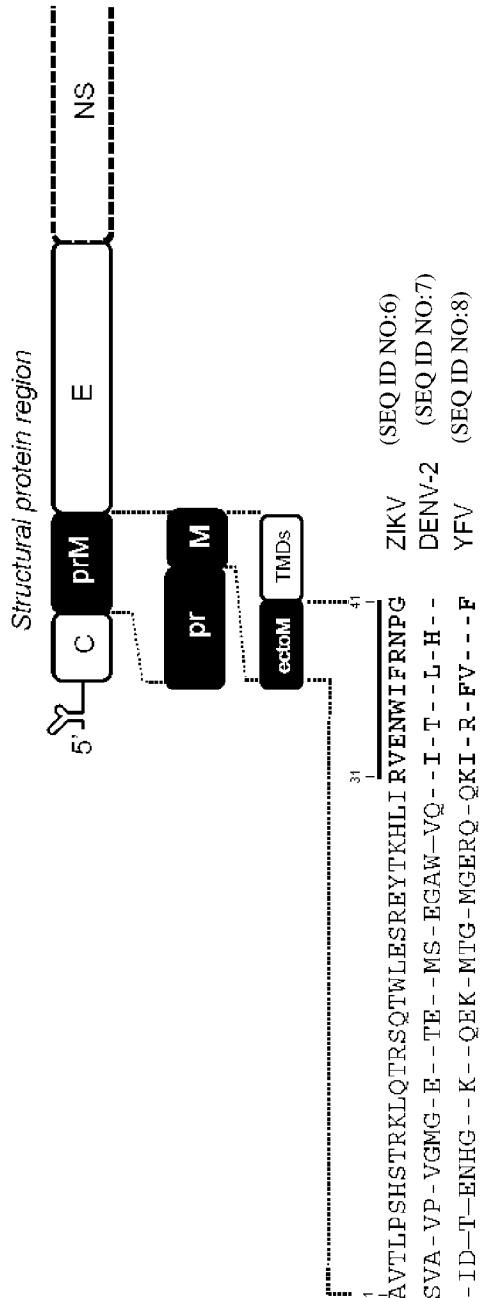
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CLAIMS:

1. The peptide that derives from the ZIKV M oligopeptide and having the amino acid sequence as set forth in SEQ ID NO:1 (RVENWIFRNPG).
2. A conjugate wherein a heterologous polypeptide is conjugated or fused to the peptide
5 of the peptide of claim 1.
3. The conjugate of claim 2 wherein the heterologous polypeptide is fused to the peptide of claim 1 to form a fusion protein.
4. The conjugate of claim 3 wherein the heterologous polypeptide can be fused to the N-terminus or C-terminus of the peptide of the present invention.
- 10 5. The conjugate of claim 2 wherein the heterologous polypeptide is a tumor antigen, in particular a tumor-associated antigen.
6. The conjugate of claim 5 wherein the tumor antigen is selected from the group consisting of epithelial cell adhesion molecule (Ep-CAM/TACSTD1), mesothelin, tumor-associated glycoprotein 72 (TAG-72), gp100, Melan-A, MART-1, KDR,
15 RCAS1, MDA7, cancer-associated viral vaccines (e.g., human papillomavirus antigens), prostate specific antigen (PSA, PSMA), RAGE (renal antigen), CAMEL (CTL-recognized antigen on melanoma), CT antigens (such as MAGE-B5, -B6, -C2, -C3, and D; Mage-12; CT10; NY-ESO-1, SSX-2, GAGE, BAGE, MAGE, and SAGE), mucin antigens (e.g., MUC1, mucin-CA125, etc.), cancer-associated ganglioside
20 antigens, tyrosinase, gp75, C-myc, Mart1, MelanA, MUM-1, MUM-2, MUM-3, HLA-B7, Ep-CAM, and tumor-derived heat shock proteins.
7. The conjugate of claim 5 wherein the heterologous polypeptide is mesothelin and more particularly the megakaryocyte-potentiating factor (MPF, 32-kDa) that derives from mesothelin.
- 25 8. The conjugate of claim 7 wherein the heterologous polypeptide consists of the amino acid sequence that has at least 80% of identity with the amino acid sequence that ranges from the amino acid residue at position 37 to the amino acid at position 286 in SEQ ID NO:2.

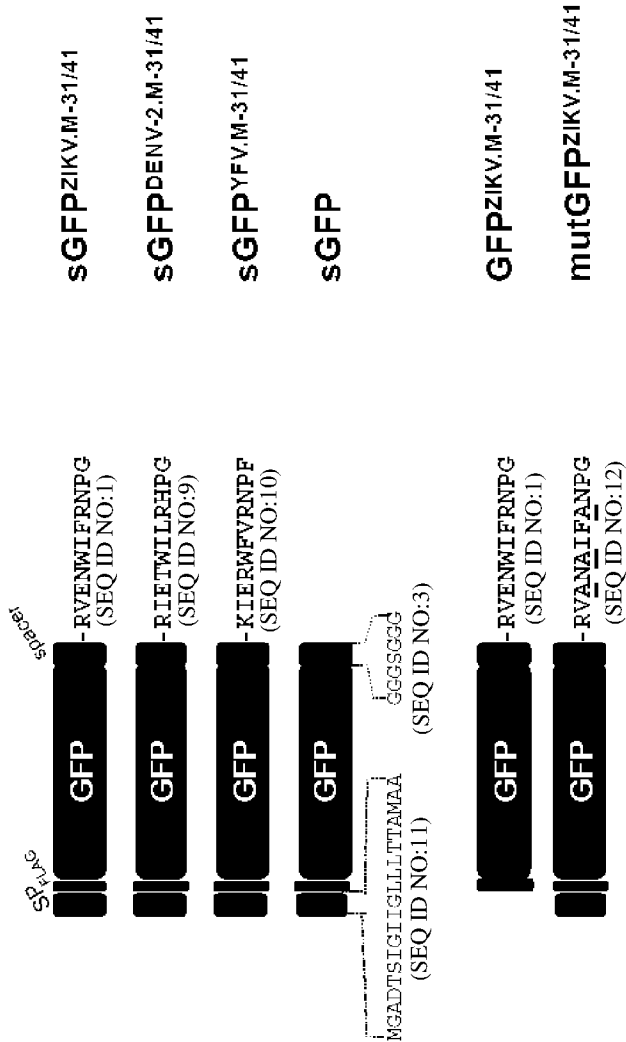
9. The conjugate of claim 3 wherein the peptide of claim is fused either directly or via a linker to the heterologous polypeptide.
10. The conjugate of claim 9 wherein the linker is a glycine-serine linker having the amino acid sequence as set forth in SEQ ID NO:3 (GGGSGGG).
- 5 11. A fusion protein that comprises the amino acid sequence as set forth in SEQ ID NO:4.
12. The fusion protein of claim 11 that comprises a signal peptide, preferably the mesothelin signal peptide.
13. The fusion protein of claim 11 that consists of the amino acid sequence as set forth in SEQ ID NO:5.
- 10 14. A polynucleotide that encodes for the peptide of claim 1 or the conjugate of claim 2 including the fusion proteins of claim 11.
15. A vector that comprises the polynucleotide of claim 14.
16. The vector of claim 15 that is an oncolytic virus.
- 15 17. A pharmaceutical composition that comprises as active ingredient an amount of the peptide of claim 1, or the conjugate of claim 2, or the fusion protein of claim 11, the polynucleotide of claim 14 or the vector of claim 15 and a pharmaceutically acceptable carrier.
- 20 18. A method of treating cancer in a patient in need thereof comprising administering to the patient a therapeutically effective amount of the peptide of claim 1, or the conjugate of claim 2, or the fusion protein of claim 11, the polynucleotide of claim 14 or the vector of claim 15.

A



B

Figure 1



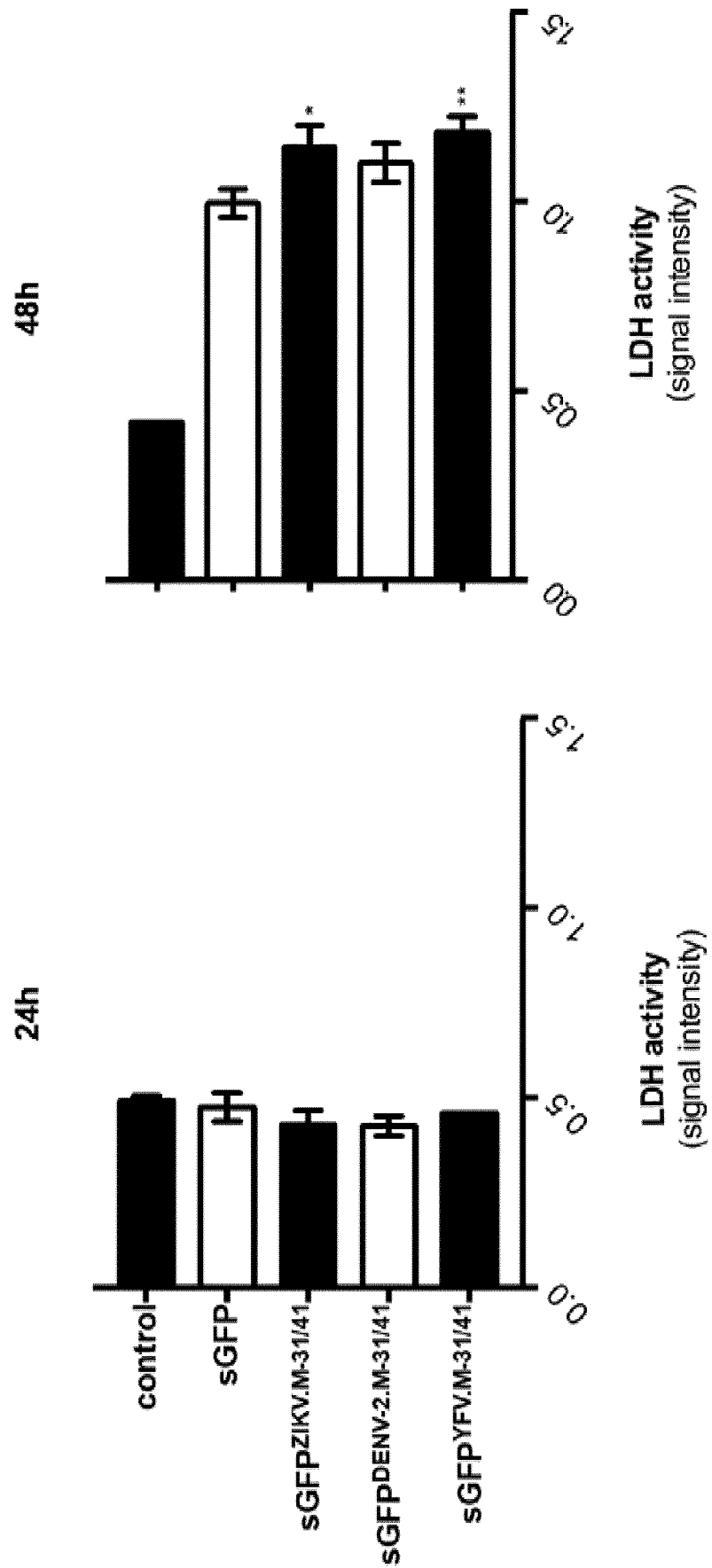


Figure 2

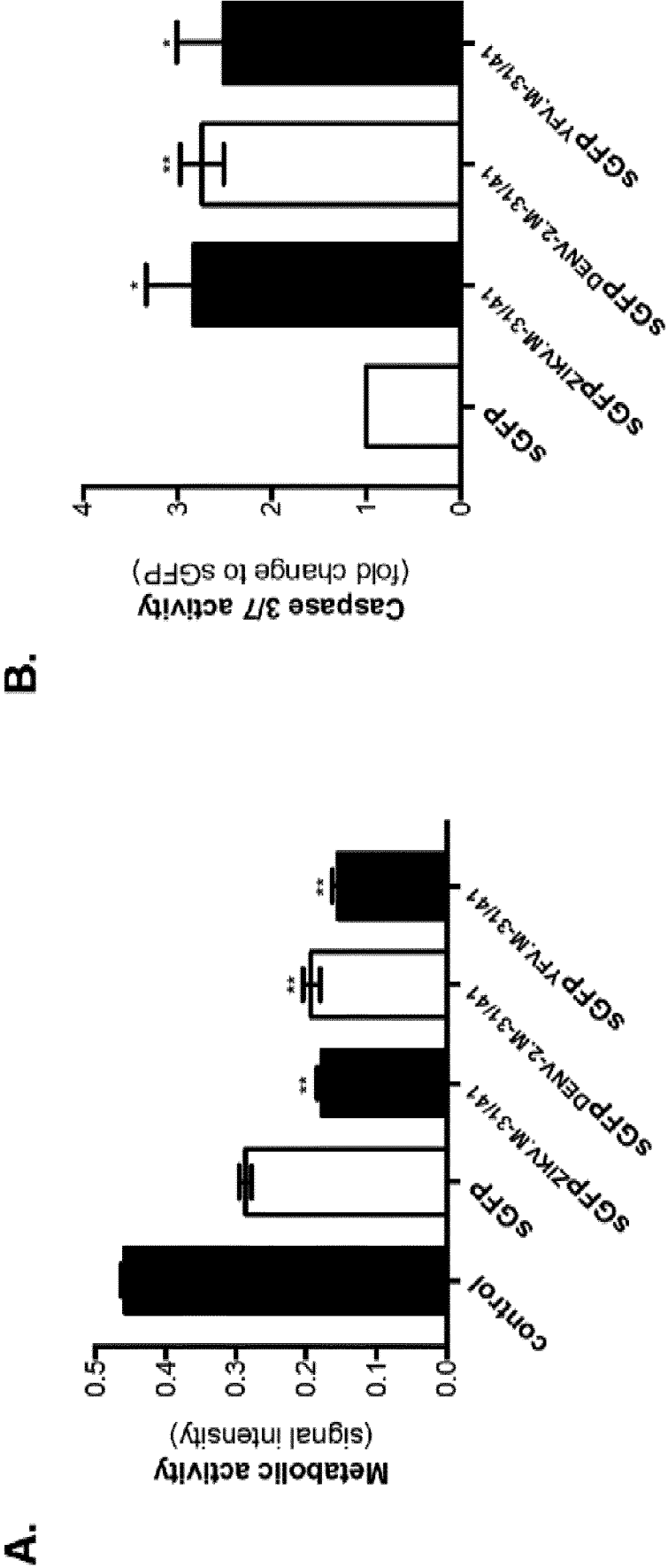


Figure 3

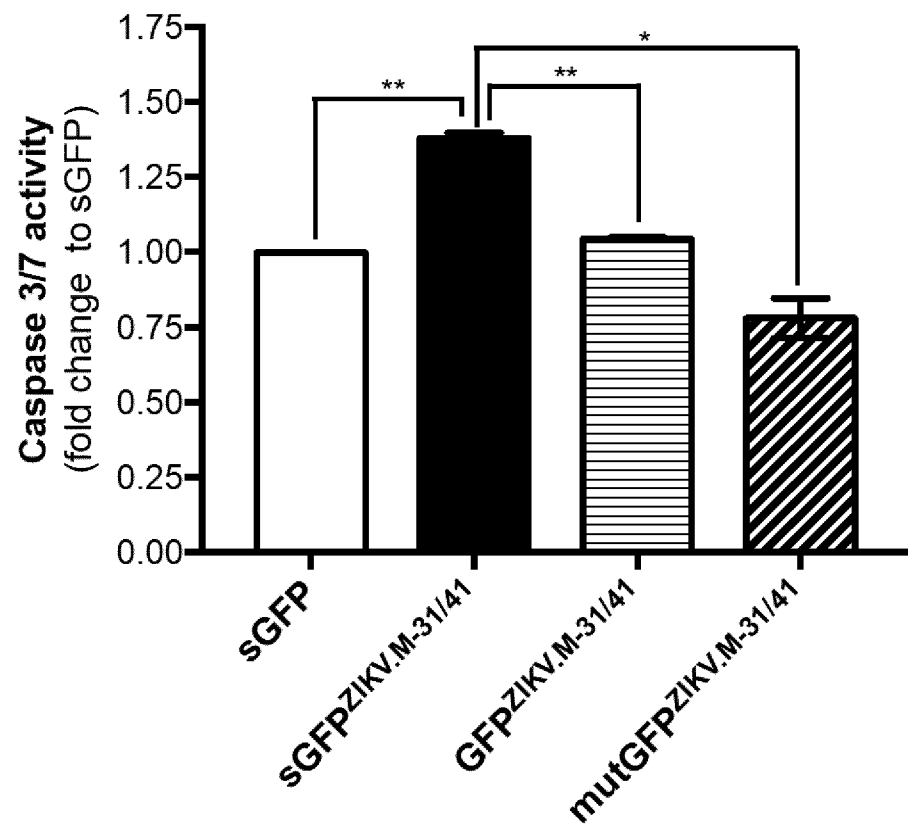


Figure 4

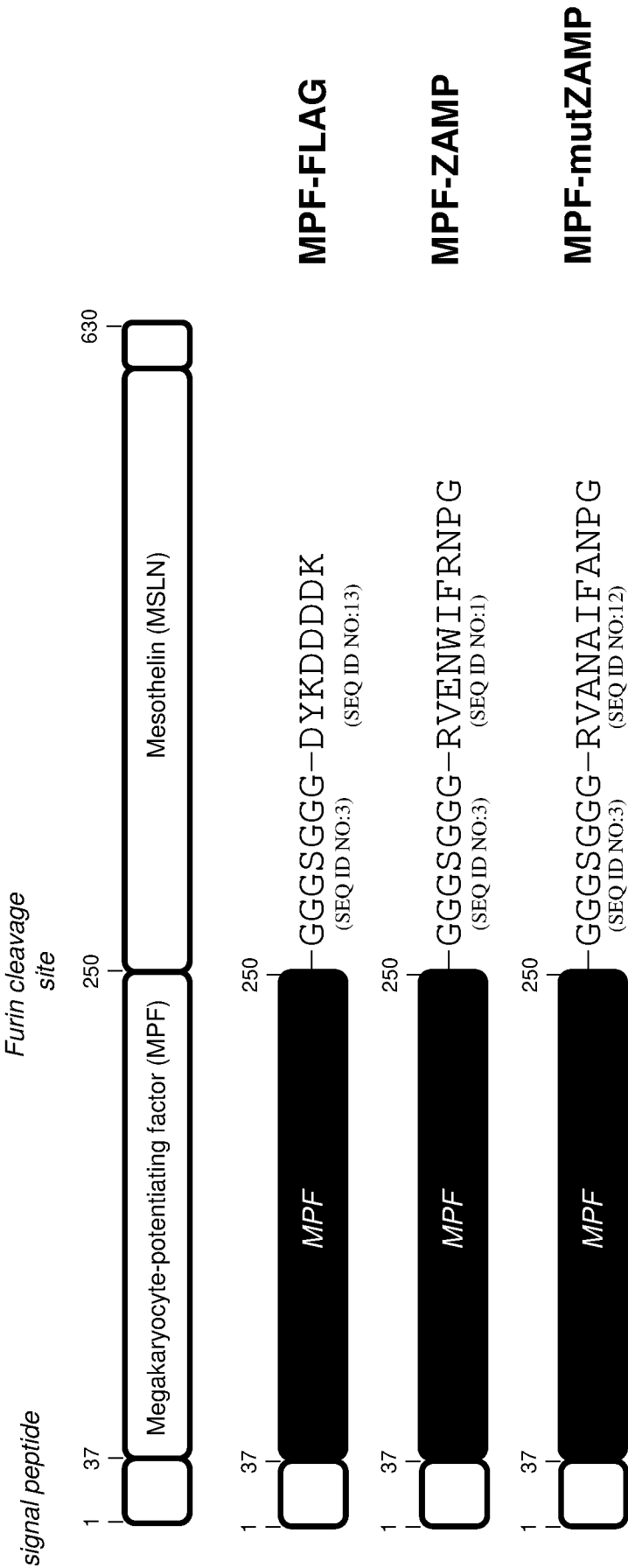


Figure 5

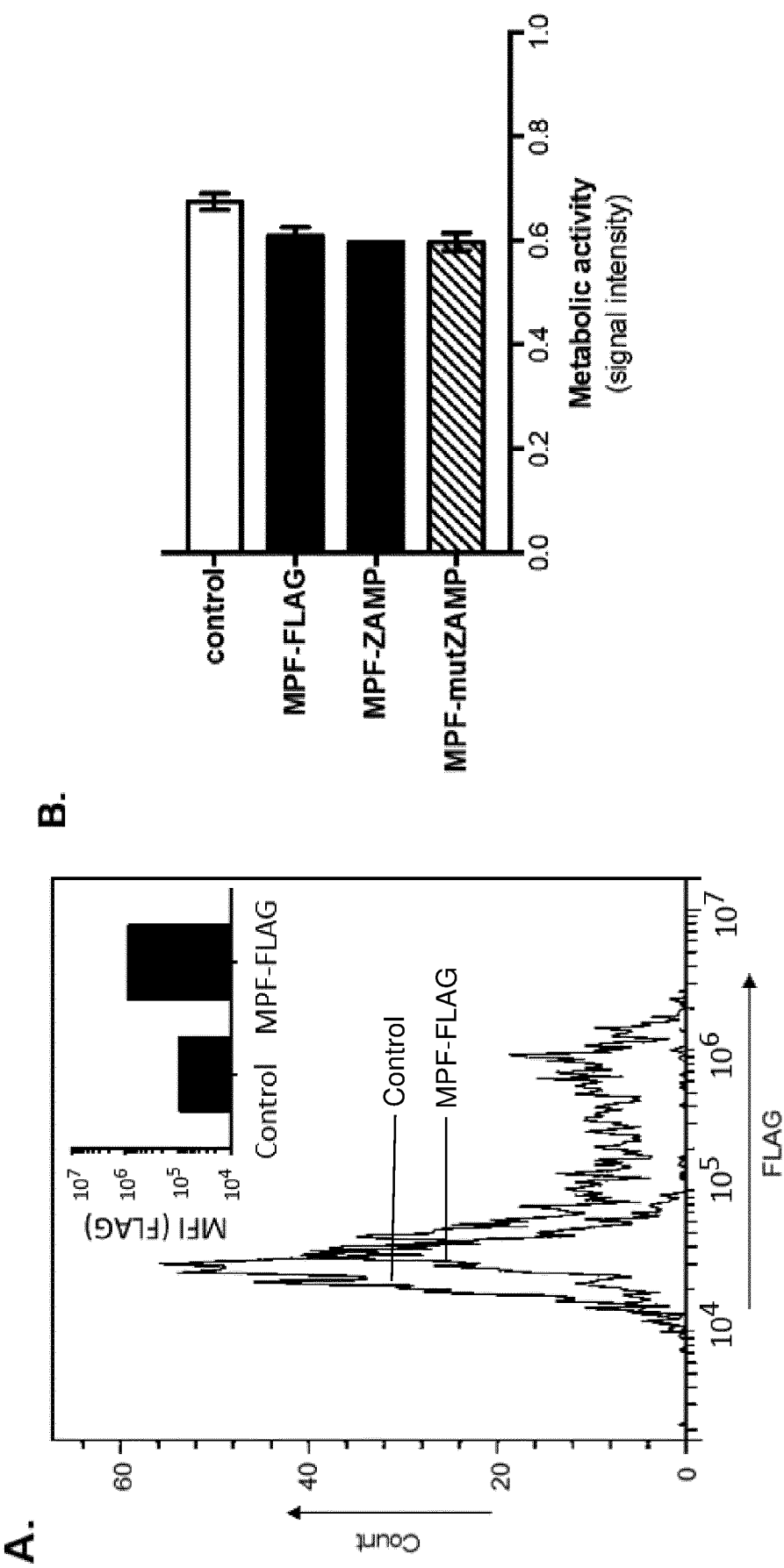


Figure 6

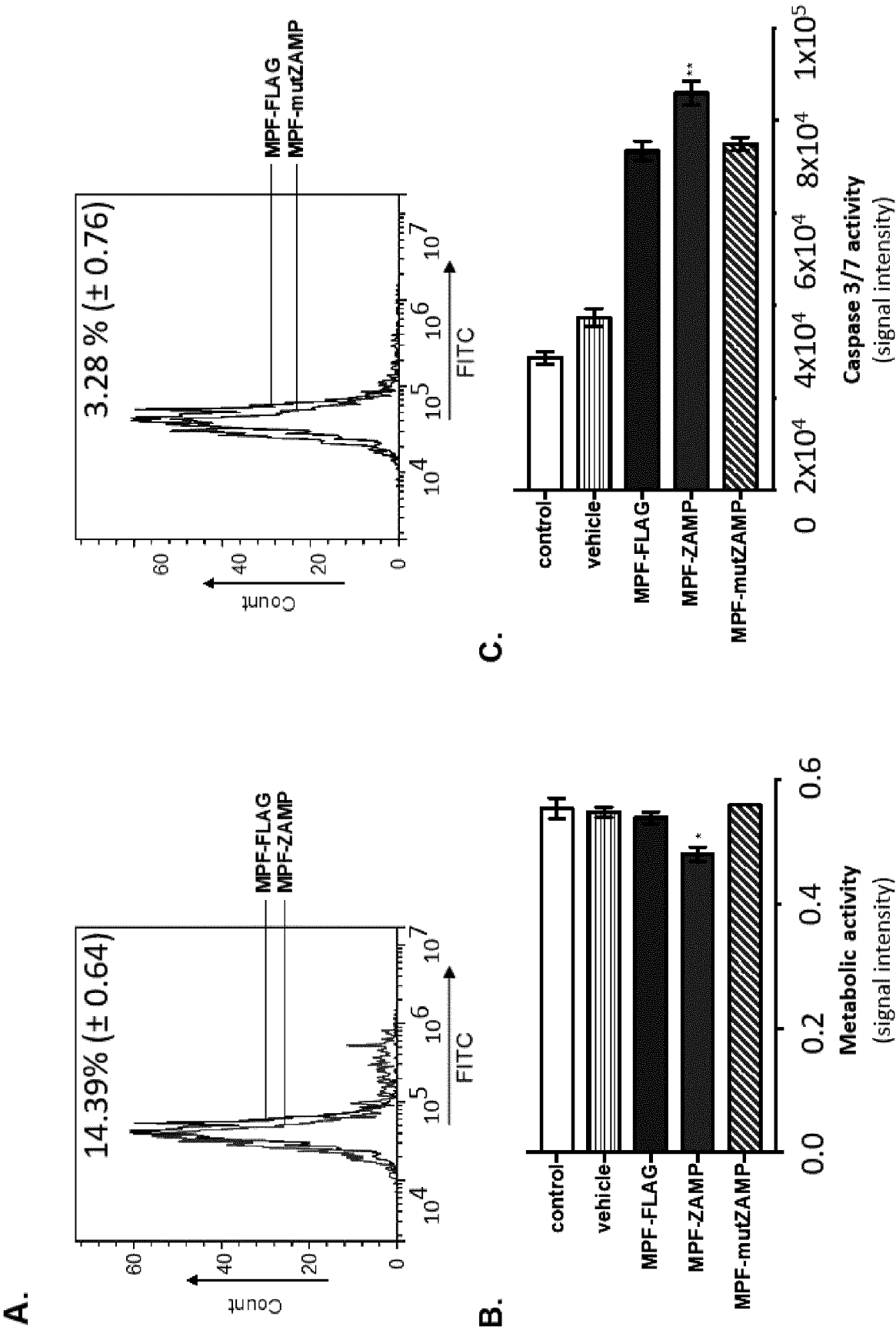


Figure 7

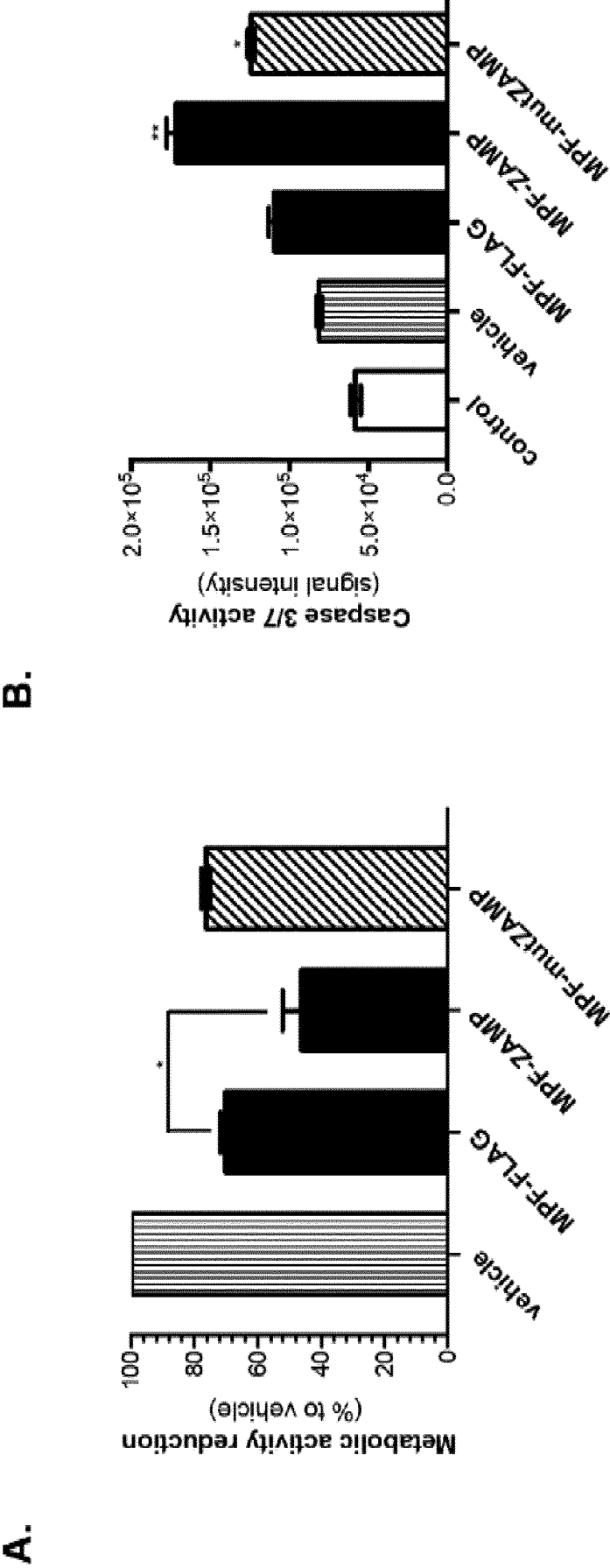


Figure 8

International application No
PCT/EP2021/081631

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K14/005 A61K38/08 C07K7/06 C07K19/00 C07K14/47 A61P35/00 ADD. According to International Patent Classification (IPC) or to both national classification and IPC																
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K 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, WPI Data, BIOSIS, Sequence Search																
C. DOCUMENTS CONSIDERED TO BE RELEVANT																
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.														
X	DATABASE Geneseq [Online] 1 October 2020 (2020-10-01), "Zika virus M protein TM1 ectodomain, SEQ ID 29.", XP055797248, retrieved from EBI accession no. GSP:BIJ53317 Database accession no. BIJ53317 the whole document ----- -/--	1														
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"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone															
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Date of the actual completion of the international search 27 January 2022	Date of mailing of the international search report 07/02/2022															
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Kools, Patrick															

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