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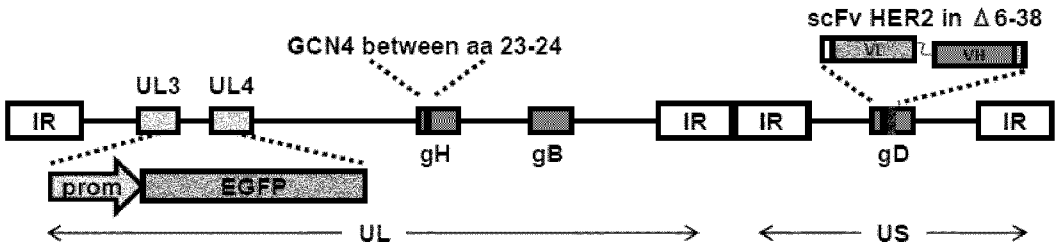
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(54) Title: HERPESVIRUS WITH MODIFIED GLYCOPROTEIN H FOR PROPAGATION IN A CELL

Fig. 3



(57) Abstract: The present invention is directed to a recombinant herpesvirus which comprises the GCN4 yeast transcription factor or a part thereof fused to or inserted into glycoprotein H and is capable of binding to a target molecule present on a cell for propagation and production of the herpesvirus. The herpesvirus may comprise additional modification in glycoprotein D and/or glycoprotein B for retargeting the herpesvirus to a diseased cell. The present invention is further directed to a nucleic acid and a vector coding for the gH, a polypeptide comprising the gH, and a cell comprising the herpesvirus, nucleic acid, vector or polypeptide. Moreover, the present invention is directed to a cell having accessible on the surface a target molecule for the GCN4 yeast transcription factor or part thereof and to a method for producing the herpesvirus in said cell.

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Herpesvirus with modified glycoprotein H for propagation in a cell

BACKGROUND OF THE INVENTION

The work leading to this invention has received funding from the European Research Council under the European Union's Seventh Framework Program (FP7/2007-2013) / ERC grant agreement n° 340060.

Despite a steady development in healthcare, the burden of diseases and pathologies that cannot be treated or cannot be sufficiently treated, remains elevated. Eminent among these are numerous forms of tumors, in particular metastatic forms of tumors that are treated with chemo-radio-therapy or biological medicaments, or combinations thereof, however, with limited success.

An alternative approach of tumor treatment is oncolytic virotherapy, whereby a replication competent virus infects the tumor cells, spreads from cell to cell of the tumor and destroys them.

Herpes simplex virus (HSV) is a pathogen virus for humans. In culture, it infects a large number of mammalian cells. It is an enveloped virus which enters the cell by membrane fusion, either at the plasma membrane or through endocytosis, depending on the target cell type. Entry of HSV into a target cell is a multistep process, requiring complex interactions and conformational changes of viral glycoproteins gD, gH/gL, gC and gB. These glycoproteins constitute the virus envelope which is the most extemal structure of the HSV particle and consists of a membrane. For cell entry, gC and gB mediate the first attachment of the HSV particle to cell surface heparan sulphate. Thereafter, gD binds to at least two alternative cellular receptors, being Nectin-1 and HVEM or HVEA, causing conformational changes in gD that initiates a cascade of events leading to virion-cell membrane fusion. Thereby, the intermediate protein gH/gL (a heterodimer) is activated which triggers gB to catalyze membrane fusion. Thereby, gB is membrane bound and functions as a viral fusogen.

Oncolytic HSVs (o-HSV) have been used in recent years as oncolytic agents. As wild-type HSV viruses are highly virulent, there is a requirement that the o-HSVs are attenuated. T-VEC/Imlygic and the viruses that have reached clinical trials carry deletion of one or more HSV genes, including the gamma $\gamma_1$34.5 gene, which encodes the ICP34.5 protein whose role is to preclude the shut off of protein synthesis in infected cells, and the UL39 gene, which encodes the large subunit of ribonucleotide reductase. In addition to some disadvantages which are shown by these viruses, such as the failure to produce high yield of progeny viruses, they furthermore have the preserved ability to bind to any cell bearing their natural receptors. Thus, the therapeutic effect of tumor cell killing is diminished and the viruses may have limitations in medical use.

One approach to overcome these limits has been genetic engineering of o-HSVs which exhibit a highly specific tropism for the tumor cells, and are otherwise not attenuated. This approach has been defined as retargeting of HSV tropism to tumor-specific receptors.

The retargeting of HSV to cancer-specific receptors entails the genetic modifications of gD, such that it harbors heterologous sequences which encode a specific ligand. Upon infection with the recombinant virus, progeny viruses are formed which carry in their envelope the chimeric gD-ligand glycoprotein, in place of wildtype gD. The ligand interacts with a molecule specifically expressed on the selected cell and enables entry of the recombinant o-HSV into the selected cell. Examples of ligands that have been successfully used for retargeting of HSV are IL13α, uPaR, a single chain antibody to HER2 and a single chain antibody to EGFR.

The retargeting through modification of glycoproteins has also been attempted with gC. The inserted ligands were EPO and IL13. The virus carrying the gC-EPO polypeptide attached to cells expressing the EPO receptor. However, this attachment did not lead to infectious entry. In addition, the gC-IL13 polypeptide was present in a virus that carried a second copy of IL13 in the gD gene. Therefore, it cannot be inferred from those studies whether the gC-IL13 contributed or not to the retargeting to the IL13 alpha2 receptor.

The retargeting through genetic modification of gH has also been achieved. The inserted ligand was a single-chain antibody (scFv) directed to HER2, without or with deletions within the gH gene. The virus was successfully retargeted to a cell carrying the HER2 receptor (Gatta et al., 2015). In addition, a recombinant virus was constructed which contained the scFv directed to HER2 in gH and an scFv directed to EGFR in the mature gD protein. This resulted in double retargeting to the cells carrying the receptors. Further, a recombinant virus was constructed which contained the scFv directed to HER2 in gH and the scFv directed to HER2 in the mature gD protein. This resulted in double retargeting to the HER2 receptors (Abstract No. P-28, 9th International conference on Oncolytic virus Therapeutics, Boston 2015).

While the art knows methods for retargeting of HSV to disease-specific receptors, these HSVs with the capability of being retargeted need to be propagated so that they can be produced in high amounts and are available as pharmaceuticals for treating diseases. In view of the fact that, for reasons of safety, the cells for propagation and production of the HSVs should not be diseased cells, so as to avoid the introduction of material such as DNA, RNA and/or protein of the diseased cells such as tumor cells in humans, the HSVs need to comprise additional modifications for enabling the HSVs of infecting “safe” cells which do not produce components which are harmful to humans for propagation and production of the HSVs. However, the prior art has not disclosed so far methods which enable the propagation and production of herpesviruses with the capability of being retargeted to disease-specific receptors in safe cells.

Thus, there is a need in the art to provide retargeting strategies for retargeting herpesvirus with the capability of being retargeted to disease-specific receptors and to cells which can be safely used for the propagation and production of the herpesvirus.

The present invention describes a recombinant HSV with a modified gH protein which retargets the herpesvirus to receptors of cells which are able to safely propagate and produce the herpesvirus.

The present inventors have shown that it is possible to construct a recombinant HSV which comprises a part of the GCN4 yeast transcription factor as a fusion

protein with gH, whereby due to the presence of the part of the GCN4 yeast transcription factor, the HSV is retargeted to cells carrying a receptor of the part of the GCN4 yeast transcription factor. Furthermore, the HSV has been shown to maintain infectivity, resulting in the entry into the cells carrying the receptor and propagation and production of the HSV.

DETAILED DESCRIPTION OF THE INVENTION

In the following, the present invention is described in detail. The features of the present invention are described in individual paragraphs. This, however, does not mean that a feature described in a paragraph stands isolated from a feature or features described in other paragraphs. Rather, a feature described in a paragraph can be combined with a feature or features described in other paragraphs.

The term “comprise/es/ing”, as used herein, is meant to “include or encompass” the disclosed features and further features which are not specifically mentioned. The term “comprise/es/ing” is also meant in the sense of “consist/s/ing of” the indicated features, thus not including further features except the indicated features. Thus, the product of the present invention may be characterized by additional features in addition to the features as indicated.

In a first aspect, the present invention provides a recombinant herpesvirus comprising a peptide having a length of 5 to 274 amino acids, fused to or inserted into glycoprotein H (gH) present in the envelope of the herpesvirus.

In an embodiment thereof, the peptide has a length of 5 to 200 amino acids, preferably of 11 to 29, 31 to 39, 41 to 49 or 51 to 200 amino acids, more preferably of 12 to 20 amino acids.

In an embodiment thereof, the peptide comprises a part of the GCN4 yeast transcription factor, preferably the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, most preferably the peptide is the peptide identified by SEQ ID NO: 13.

In an embodiment thereof, the peptide is inserted within the N-terminal region starting at any one of amino acids 19 to 23 and ending at any one of amino acids 48 to 88 or starting at amino acid 116 and ending at amino acid 136 of the gH according to SEQ ID NO: 1 or a corresponding region of a homologous gH.

In an embodiment thereof, the peptide is inserted N-terminally of the H1A domain of gH.

In an embodiment thereof, one or more gH amino acids of the N-terminal region are deleted.

In an embodiment thereof, the herpesvirus has the capability of binding to a cell expressing or binding a target molecule via the peptide, preferably of fusing with the cell membrane, more preferably of entering the cell, most preferably of propagating within the cell.

In an embodiment thereof, the target molecule is the scFv as comprised by SEQ ID NO: 5, most preferably the molecule identified by the sequence of SEQ ID NO: 7.

In an embodiment thereof, the herpesvirus comprises a gD which is modified to retarget the herpesvirus to a diseased cell and/or a gB which is modified to retarget the herpesvirus to a diseased cell.

In an embodiment thereof, the herpesvirus encodes one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell.

The recombinant herpesvirus of the present invention serves the purpose of infecting and killing diseased cells in humans. This requires the provision of the herpesvirus and, therefore, its propagation and production. As propagation of the herpesvirus shall be avoided in diseased cells, so as to avoid the introduction of material such as DNA, RNA and/or protein of the diseased cells such as tumor cells into humans, the recombinant herpesvirus has to be engineered to be capable of infecting cells which are useful for the production of the herpesvirus and do not produce material which may be harmful to humans. Such cells are also referred to herein as “safe” cells. This requires the retargeting of the recombinant herpesvirus of the present invention to such cells for propagation and production. To achieve this, glycoprotein H of the recombinant herpesvirus is modified to include a peptide, namely a peptide of 5 to 274 amino acids, preferably of 5 to 200 amino acids, more preferably of 11 to 29, 31 to 39, 41 to 49 or 51 to 200 amino acids, such as 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 amino acids, still more preferably of 12 to 20 amino acids, still more preferably a part of the GCN4 yeast transcription factor, still more preferably the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, most preferably the peptide identified by SEQ ID NO: 13, which allows for binding to a

target molecule which is accessible on the surface of a cell which can be safely used for the production of the herpesvirus. The use of the peptide as a ligand for binding to a target molecule requires the accessibility of such target molecule on a cell which can be safely used for propagating and producing the recombinant herpesvirus. This in turn requires the modification of cells which are capable of safely producing the recombinant herpesvirus of the present invention to comprise target molecules capable of binding to the peptide. Preferred target molecules are antibodies or antibody derivatives such as scFvs such as the scFv as comprised by SEQ ID NO: 5, which are specifically generated as target molecules to fit to the ligand.

The recombinant herpesvirus of the present invention may additionally comprise heterologous polypeptide ligand(s) in other glycoprotein(s) such as gD and/or gB involved in herpesvirus entry into a cell for retargeting the herpesvirus to target molecules present in unwanted such as diseased cells. Thus, while the modification of gH serves the purpose of retargeting the herpesvirus to a cell for production, further modifications of other glycoproteins serve the purpose of retargeting the herpesvirus to target molecules on unwanted cells for killing them.

Glycoprotein H (gH) is a 110 kDa virion envelope glycoprotein that plays a role in herpesvirus infectivity. It forms a heterodimer with herpesvirus glycoprotein L. Upon entry of herpesvirus into a cell, the heterodimer gH/gL interacts with the profusion domain of glycoprotein D (gD) which profusion domain is dislodged upon interaction of gD with one of its receptors, Nectin-1, HVEM, and modified heparan sulfates during cell entry. When a herpesvirus does not comprise a gD molecule, gH/gL interacts with analogous proteins having the same function as gD such as gp42 encoded by Epstein Barr virus. This interaction is the critical event in the activation cascade of the four glycoproteins gD, gH, gL, and gB, which are involved in herpesvirus entry into a cell. The activation cascade starts with the binding of gD to one of its receptors and results in the fusion of the herpesvirus with the target cell membrane mediated by gB. Among at least human and monkey herpesviruses, gH is conserved. Crystal structures of the extracellular portion of three gH proteins are known: one from the alphaherpesvirus HSV-2 gH (Chowdary et al., 2010), one from the swine PrV (Backovic et al., 2012), also an alphaherpesvirus, and one from Epstein-Barr virus (Matsuura et al., 2010), a

gamma herpesvirus. They are substantially similar, for example, an organization in structurally similar domains is present in all crystal structures. The nucleotide and amino acid sequences of a variety of gHs of different herpesviruses are known in the art. For illustrative purposes only, without being limited thereto, reference is made to the amino acid sequence of gH of human herpesvirus 1 disclosed herein as SEQ ID NO: 1. The corresponding nucleotide sequence and the amino acid sequence are available from the NCBI (National Centre for Biotechnology Information; National Library of Medicine, Bethesda, MD20894, USA; www.ncbi.nlm.nih.gov) under the accession number “Genome”, GU734771.1, coordinates from 43741 to 46498.

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1   MGNGLWFVGV IILGVAWGQV HDWTEQTDPW FLDGLGMDRM YWRDTNTGRL WLPNTPDPQK
61  PPRGFLAPPD ELNLTTASLP LLRWYEERFC FVLVTTAEFP RDPGQLLYIP KTYLLGRPPN
121 ASLPAPTTVE PTAQPPPSVA PLKGLLYNPV ASVLLRSRAW VTFSAVPDPE ALTFPRGDNV
181 ATASHPSGPR DTPPPRPPVG ARRHPTTELD ITHLHNASTT WLATRGLLRS PGRYVYFSPS
241 ASTWPVGIWT TGELVLGCDA ALVRARYGRE FMGLVISMHD SPPVEVMVVP AGQTLDRVGD
301 PADENPPGAL PGPPGGPRYR VFVLGSLTRA DNGSALDALR RVGGYPEEGT NYAQFLSRAY
361 AEFFSGDAGA EQGPRPPLFW RLTGLLATSG FAFVNAAHAN GAVCLSDLLG FLAHSRALAG
421 LAARGAAGCA ADSVFFNVSV LDPTARLQLE ARLQHLVAEI LEREQSLALH ALGYQLAFVL
481 DSPSAYDAVA PSAAHLIDAL YAEFLGGRVL TTPVVHRALF YASAVLRQPF LAGVPSAVQR
541 ERARRSLLIA SALCTSDVAA ATNADLRTAL ARADHQKTLF WLPDHFSPCA ASLRFDLDES
601 VFILDALAQA TRSETPVEVL AQQTHGLAST LTRWAHYNAL IRAFVPEASH RCGGQSANVE
661 PRILVPITHN ASYVVTHSPL PRGIGYKLTG VDVRRPLFLT YLTATCEGST RDIESKRLVR
721 TQNQRDLGLV GAVFMRYTPA GEVMSVLLVD TDNTQQQIAA GPTEGAPSVF SSDVPSTALL
781 LFPNGTVIHL LAFDTQPVAA IAPGFLAASA LGVVMITAAL AGILKVLRTS VPFFWRRE*
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SEQ ID NO: 1

gH homologs are found in all members of the *Herpesviridae*. Therefore, the term “glycoprotein H”, as referred to herein, refers to any gH homolog found in *Herpesviridae*. Alternatively, gH, as referred to herein, refers to any gH which has an amino acid identity to the sequence of SEQ ID NO: 1 of at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%. Alternatively, the gH, as referred to herein, refers to any gH which has an amino acid homology to SEQ ID NO: 1 of at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, or 100%. The gH, as referred to herein, also includes a fragment of gH. Preferably, gH, as referred to herein, including any gH found in *Herpesviridae*, any gH having an amino acid identity to the sequence of SEQ ID NO: 1, as defined above, and any fragment of a gH, has

the same activity of the gH according to SEQ ID NO: 1. More preferably, a gH homolog plays a critical role in herpesvirus entry into a cell. Namely, during the entry process of the virus into a cell, the heterodimer gH/gL interacts with the profusion domain of gD, or analogous proteins, e.g. gp42 encoded by Epstein Barr virus, or with cellular receptors to gH/gL, including but not limited to integrins. These events lead to an activation cascade of the four glycoproteins gD or an analogous protein, gH, gL, and gB, involved in herpesvirus entry.

The percentage of “sequence identity," as used herein, refers to the percentage of amino acid residues which are identical in corresponding positions in two optimally aligned sequences. It is determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence, SEQ ID NO: 1 (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and *Waterman,* 1981, by the homology alignment algorithm of Needleman and Wunsch, 1970, by the search for similarity method of Pearson and Lipman, 1988, by the algorithm of Karlin and Altschul, 1990, modified by Karlin and Altschul, 1993, or by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection. GAP and BESTFIT are preferably employed to determine the optimal alignment. Typically, the default values of 5.00 for gap weight and 0.30 for gap weight length are used.

The "percentage of homology", as used herein, refers to the percentage of amino acid residues which are homologous in corresponding positions in two optimally aligned sequences. The "percentage of homology" between two sequences is established in a manner substantially identical to what has been described above

with reference to the determination of the "percentage of identity" except for the fact that in the calculation also homologous positions and not only identical positions are considered. Two homologous amino acids have two identical or homologous amino acids. Homologous amino acid residues have similar chemical-physical properties, for example, amino acids belonging to a same group: aromatic (Phe, Trp, Tyr) , acid (Glu, Asp), polar (Gln, Asn) , basic (Lys, Arg, His), aliphatic (Ala, Leu, lie, Val), with a hydroxyl group (Ser, Thr), or with a short lateral chain (Gly, Ala, Ser, Thr, Met). It is expected that substitutions between such homologous amino acids do not change a protein phenotype (conservative substitutions).

A gH is “homologous” or a “homolog” if it has an identity to SEQ ID NO: 1 of at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%, if it has an amino acid homology to SEQ ID NO: 1 of at least 50 %, 60%, 70%, 80%, 85%, 90%, 95%, or 100%, or if it has the same activity as the gH according to SEQ ID NO: 1. Preferably, “same activity” may be understood in the sense that the heterodimer gH/gL interacts with the profusion domain of gD or an analogous protein, thus playing a critical role in the activation cascade of the four glycoproteins gD or an analogous protein, gH, gL, and gB, involved in herpesvirus entry. A homolog may also be a fragment of a full length gH having the activity as indicated above.

The chimeric gH of the present invention (as exemplified by SEQ ID NO: 3) carries the peptide and thereby confers a new activity on the virus, in addition to the activity that the gH portion carries out for the wildtype (wt) virus. The chimeric gH, once it is part of the envelope of the recombinant virus, enables the binding of the recombinant virus to a target molecule, which can be bound by the peptide, and retargets the tropism of recombinant virus to a cell carrying the target molecule. Preferably, the heterodimer gH/gL interacts with the profusion domain of gD or an analogous protein, which is a critical event in the activation cascade of the four glycoproteins gD or an analogous protein, gH, gL, and gB, involved in herpesvirus entry. After fusion with a cell carrying the target molecule of the peptide, the recombinant herpesvirus enters the cell, and the cell infected by the recombinant herpesvirus produces proteins encoded by the viral genome, including the chimeric gH harboring the peptide. The infected cell produces progeny virus which

is released from the cell by lysis of the cell. The herpesvirus thus produced can be isolated and used for intended purposes, e.g. as a pharmaceutical. .

The indication of a specific amino acid number or region of gH, as used herein, refers to the "precursor" form of gH, as exemplified in SEQ ID NO: 1 that includes the N-terminal signal sequence comprising the first 18 amino acids. The "mature" form of gH starts with amino acid 19 of SEQ ID NO: 1 and extends until amino acid 838. As gH glycoproteins with amino acid sequences different from SEQ ID NO: 1 are also comprised by the present invention, the indication of a specific amino acid number or of a specific amino acid region which relates to SEQ ID NO: 1 means also the amino acid number or region of a homologous gH, which corresponds to the respective amino acid number or region of SEQ ID NO: 1.

The term "chimeric glycoprotein H" or "chimeric gH", as used herein, means a gH having fused to or inserted into the gH the peptide. The chimeric gH is encoded by the recombinant virus, is synthesized with the cell that produces the recombinant virus, and becomes incorporated in the envelope of the virion. Methods to produce the recombinant viruses by genetic engineering are known in the art. Methods for producing chimeric glycoprotein H are known in the art.

The term "retargeting", as used herein, means that the recombinant herpesvirus of the present invention is targeted to the target molecule which is bound by the ligands introduced into the herpesvirus. However, the recombinant herpesvirus is still capable of being targeted to the natural receptor of the unmodified herpesvirus. Retargeting is different form "detargeting", which means that the recombinant herpesvirus is no longer capable of being targeted to the natural receptor of the unmodified herpesvirus. "Detargeting" means that the recombinant virus is only targeted to the target molecule of the ligand.

The GCN4 yeast transcription factor is state of the art (see e.g. Arndt and Fin, 1986; Hope and Struhl, 1987). An exemplary GCN4 yeast transcription factor is one identified by SEQ ID NO: 20 (UniProtKB - P03069) encoded by the gene identified in SEQ ID NO: 19 (GenBank accession No. AJ585687.1). The term "GCN4 yeast transcription factor", as referred to herein, refers to any GCN4 yeast transcription factor present in nature. Alternatively, GCN4 yeast transcription factor, as referred to herein, refers to any GCN4 yeast transcription factor which has an

amino acid identity to the sequence of SEQ ID NO: 20 of at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%. Alternatively, the GCN4 yeast transcription factor, as referred to herein, refers to any GCN4 yeast transcription factor which has an amino acid homology to SEQ ID NO: 20 of at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, or 100%. A GCN4 yeast transcription factor is "homologous" or a "homolog" if it has an identity to SEQ ID NO: 1 of at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%, if it has an amino acid homology to SEQ ID NO: 1 of at least 50 %, 60%, 70%, 80%, 85%, 90%, 95%, or 100%, or if it has the same activity as the GCN4 yeast transcription factor according to SEQ ID NO: 20. Preferably, "same activity" may be understood in the sense that GCN4 yeast transcription factor works as a transcription factor in the same way as the GCN4 yeast transcription factor according to SEQ ID NO: 20. The term "a part thereof", as used herein, comprises any part of the GCN4 yeast transcription factor against which a target molecule can be generated to which the "part thereof" is capable of binding. Preferably, the length of "the part thereof" is such that a peptide length of 5 to 274 amino acids, preferably 5 to 200 amino acids, more preferably 11 to 29, 31 to 39, 41 to 49 or 51 to 200 amino acids, such as 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 amino acids, still more preferably 12 to 20 amino acids results, whereby the peptide may include additional amino acids such as linker sequences. Most preferably, the length of the "the part thereof" is 12 amino acids. The most preferred "part thereof" is the epitope YHLENEVARLKK (SEQ ID NO: 14) of GCN4 yeast transcription factor. For fusion to or insertion into gH, the epitope YHLENEVARLKK may further comprise two flanking wt (wildtype) GCN4 residues on each side and two GS linkers. This construct is herein named GCN4 peptide. This 20 amino acid peptide confers to the herpesvirus the ability to infect and replicate in a cell line bearing a target molecule to which the "part thereof" binds.

The present invention discloses a recombinant herpesvirus comprising the GCN4 yeast transcription factor, fused to or inserted into glycoprotein H (gH) present in the envelope of the herpesvirus.

The term "recombinant" herpesvirus, as referred to herein, refers to a herpesvirus that has been genetically engineered by genetic recombination to include additional nucleic acid sequences which encode the peptide. Methods of

producing recombinant herpesviruses are well known in the art (see for example Sandri-Goldin et al., 2006). However, the present invention is not limited to genetic engineering methods. Also other methods may be used for producing a herpesvirus having fused or inserted a peptide to or into gH, respectively.

The term "herpesvirus", as referred to herein, refers to a member of the *Herpesviridae* family of double-stranded DNA viruses, which cause latent or lytic infections. Herpesviruses all share a common structure in that their genomes consist of relatively large (about from 100.000 to 200.000 base pairs), double-stranded, linear DNA encoding 80 to 200 genes, encased within an icosahedral protein cage called the capsid which is itself wrapped by a protein layer called the tegument containing both viral proteins and viral mRNAs and a lipid bilayer membrane called the envelope. This whole particle is also known as a virion. The term "herpesvirus" also refers to members of the *Herpesviridae* family which are mutated comprising one or more mutated genes, such as, e.g., herpesviruses which were modified in a laboratory.

In a preferred embodiment, the herpesvirus is selected from the group consisting of Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Varicella Zoster Virus (human herpesvirus 3 (HHV-3)), swine alphaherpesvirus Pseudorabievirus (PRV), chimpanzee alpha1 herpesvirus (ChHV), Papiine herpesvirus 2 (HVP2), Cercopithecine herpesvirus 2 (CeHV2), Macacine herpesvirus 1 (MHV1), Saimiriine herpesvirus 1 (HVS1), Callitrichine herpesvirus 3 (CalHV3), Saimiriine herpesvirus 2 (HVS2), Bovine herpesvirus 1 (BoHV-1), Bovine Herpesvirus 5 (BoHV-5), Equine herpesvirus 1 (EHV-1), Equine herpesvirus 2 (EHV-2), Equine herpesvirus 5 (EHV-5), Canine herpesvirus 1 (CHV), Feline herpesvirus 1 (FHV-1), Duck enteritis virus (DEV), Fruit bat alphaherpesvirus 1 (FBAHV1), Bovine herpesvirus 2 (BoHV-2), Leporid herpesvirus 4 (LHV-4), Equine herpesvirus 3 (EHV-3), Equine herpesvirus 4 (EHV-4), Equine herpesvirus 8 (EHV-8), Equid herpesvirus 9 (EHV-9), Cercopithecine herpesvirus 9 (CeHV-9), Suid herpesvirus 1 (SuHV-1), Marek's disease virus (MDV), Marek's disease virus serotype 2 (MDV2), Falconid herpesvirus type 1 (FaHV-1), Gallid herpesvirus 3 (GaHV-3), Gallid herpesvirus 2 (GaHV-2), Lung-eye-trachea disease-associated herpesvirus (LETV), Gallid herpesvirus 1 (GaHV-1), Psittacid herpesvirus 1 (PsHV-1), Human herpesvirus 8 (HHV-8), Human

herpesvirus 4 (HHV-4), Chelonid herpesvirus 5 (ChHV5), Ateline herpesvirus 3 (AtHV3) or Meleagrid herpesvirus 1 (MeHV-1). In a more preferred embodiment, the herpesvirus is HSV-1 or HSV-2, most preferably HSV-1.

The term “peptide”, as used herein, is a continuous and unbranched peptide chain consisting of amino acids connected by peptide bonds. The length of the peptide chain is 5 to 274 amino acids, preferably 5 to 200 amino acids, more preferably 11 to 29, 31 to 39, 41 to 49 or 51 to 200 amino acids, still more preferably 12 to 20 amino acids, still more preferably the peptide comprises a part of the GCN4 yeast transcription factor, still more preferably the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, most preferably the peptide is the peptide identified by SEQ ID NO: 13. In the present invention, the peptide is used as a fusion to or insertion into gH. The peptide, if not specifically defined, may be any peptide to which a target molecule which is present on a target cell is capable of binding. Thus, the peptide may be a part of a natural polypeptide. The natural polypeptide may be derived from any organism, preferably from an organism which is not harmful to human. For example, the natural polypeptide is a fungal or bacterial polypeptide, such as a polypeptide from the genus Saccharomyces such as Saccharomyces cerevisiae. As the peptide is capable of binding to a target molecule present on a cell, the peptide represents a ligand. The term “ligand” is generally used herein as binding to or being capable of binding to a target molecule accessible on the surface of a cell.

The term “polypeptide”, as used herein, is a continuous and unbranched peptide chain consisting of amino acids connected by peptide bonds. The length of the polypeptide chain is unlimited and may range from some amino acids such as 5 amino acids to some hundreds or thousands amino acids. More than one polypeptide chains may assemble to a complex such as an antibody. The term “polypeptide”, as used herein, also comprises an assembly of polypeptide chains. While the term “peptide” is used herein for a ligand which is inserted into or fused to gH, the term “polypeptide” is used herein for ligands inserted into gD or gB which serve to target diseased cells, for the gH polypeptide having fused to or inserted the peptide or for specific polypeptides as indicated.

The term "corresponding region of a homologous gH" refers to a region of a gH which aligns with a given region of the gH according to SEQ ID NO: 1 when using

the Smith-Waterman algorithm and the following alignment parameters: MATRIX: BLOSUM62, GAP OPEN: 10, GAP EXTEND: 0.5. This algorithm is generally known and used in the art if performing pairwise sequence comparisons and the skilled person knows how to apply it. In case only a part or parts of the given region of SEQ ID NO: 1 aligns with the sequence of a homologous gH using above algorithm and parameters, the term "corresponding region" refers to the region which aligns with the part(s) of the given region of SEQ ID NO: 1. In this case, the region in the homologous gH, in which the peptide is inserted, comprises only the amino acids which align with the part(s) of the given region of SEQ ID NO: 1. The term "corresponding region" may also refer to a region which is flanked by corresponding flanking sequences, wherein the flanking sequences align, using above algorithm and parameters, with sequences flanking the region of SEQ ID NO: 1. These flanking sequences are at least 5, 6, 7, 8, 9, 10, 15, 20, 30, 40 or 50 amino acids long. Other algorithms which may be used are the algorithms of Needleman and Wunsch, 1970, the similarity method of Pearson and Lipman, 1988, or the algorithm of Karlin and Altschul, 1990, modified by Karlin and Altschul, 1993, or computerized implementations of these algorithms.

The term “corresponding amino acid” refers to an amino acid which is present within a corresponding region and which is the counterpart of a given amino acid of SEQ ID NO: 1 in the alignment. A corresponding amino acid must not be identical to its counterpart in SEQ ID NO: 1 in the alignment, as far as it is present within a corresponding region.

In the recombinant herpesvirus of the present invention, the peptide may be fused to or inserted into gH. In this context, the term "fused" or "fusion", as referred to herein, refers to the addition of the peptide to the N-terminal amino acid of gH by peptide bonds, either directly or indirectly via a peptide linker. "Fused" or "fusion" to the N-terminal region is different from “insertion” insofar as "fused" or "fusion" means addition to the terminus of gH, whereas “insertion” means incorporation into the gH.

A peptide linker, as referred to herein, serves to connect amino acid sequences derived from different sources. Such a linker serves to connect and to enable proper folding of the peptide with glycoprotein H sequences. It may also serve to

connect peptide sequences with glycoprotein sequences other than gH. A linker has typically a length between 1 and 30 amino acids, preferably 2 to 25 amino acids, more preferably 2 to 10 amino acids, most preferably 2 amino acids and may comprise any amino acids. Preferably, it comprises the amino acid(s) Gly and/or Ser and/or Thr, more preferably it comprises at least 1, 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 or 30 amino acids selected from the group consisting of Gly, Ser and/or Thr. Still more preferably, it consists of the amino acids Gly and/or Ser. Linkers based on Gly and/or Ser provide flexibility, good solubility and resistance to proteolysis. Alternatively, the linker may not predominantly comprise glycine, serine and/or threonine, but glycine, serine and/or threonine may not be present or only to a minor extent. The most preferred linker to connect the peptide with gH sequences is the linker GS. In case of insertion, it is present on both sides of the peptide.

In the recombinant herpesvirus of the present invention, the peptide is fused to or inserted into the gH glycoprotein. Preferably, the peptide is inserted within the N-terminal region of gH starting at any one of amino acids 19 to 23 (preferably 19) and ending at any one of amino acids 48 to 88 (preferably 88), preferably starting at amino acid 19 and ending at amino acid 88, starting at amino acid 61 and ending at amino acid 65, starting at amino acid 69 and ending at amino acid 72, or starting at amino acid 74 and ending at amino acid 80; or is inserted within a region starting at amino acid 116 and ending at amino acid 136 of the gH according to SEQ ID NO: 1 or a corresponding region of a homologous gH. The ranges 61-65, 69-72 and 74-80 are thought to be particularly useful since they represent exposed loop regions of the gH H1A domain and therefore represent insertion points that retain the structural integrity of the gH H1A domain. In a more preferred embodiment, it is inserted within the N-terminal region of gH starting at amino acid 19 and ending at amino acid 50 of the gH according to SEQ ID NO: 1 or a corresponding region of a homologous gH. In an even more preferred embodiment, it is inserted within the N-terminal region of gH starting at amino acid 19 and ending at amino acid 48 of the gH according to SEQ ID NO: 1 or a corresponding region of a homologous gH. In another more preferred embodiment, it is inserted within the N-terminal region of gH starting at amino acid 23 and ending at amino acid 48 of the gH according to SEQ ID NO: 1 or a

corresponding region of a homologous gH. In all these embodiments, the amino acids defining start and end of a region are included in the region, i.e. the insertion may by either N-terminal or C-terminal of the start or end amino acid. In the most preferred embodiment, the peptide is inserted between amino acid 23 and amino acid 24 of the gH according to SEQ ID NO: 1 or a corresponding region (in this case corresponding to said amino acids 23 and 24) of a homologous gH. In a particular embodiment, one or more gH amino acids of the N-terminal region as specified above are deleted. In a related embodiment, gH is truncated.

In another embodiment, the peptide is inserted N-terminally of the H1A domain of gH. N-terminally inserted in this respect does not mean adjacent to the H1A domain on the N-terminal side, but anywhere on the N-terminal side of the H1A domain. The H1A domain of gH is a subdomain of the Hl domain of gH. The H1 domain extends from amino acid 49 to 327 of the gH protein according to SEQ ID NO: 1, and the H1A domain extends from amino acid 49 to 115 of the gH protein according to SEQ ID NO: 1 (Chowdary et al., 2010). Many gH proteins have a H1A domain, which can be identified by sequence alignment with SEQ ID NO: 1 or by structural similarity within the H1 domain as is the case for gH from Varicella Zoster Virus (human herpesvirus 3). Not every herpesvirus may have a gH with a region corresponding to amino acids 1 to 48 of the gH protein according to SEQ ID NO: 1. However, every mature gH has at least some, e.g. 1, 2 or 3 amino acids N-terminally of the H1A domain. An example is EBV, wherein only 1 residue precedes the H1A domain in the mature peptide (assuming that the H1A domain starts at the first residue visible in the X-ray structure, i.e. for EBV position 19 of the gH precursor). In case of a gH in which this preceding region is very short, for example 10 or less, 5 or less, or 3 or less amino acids, it is envisaged that the insertion is behind (i.e. C-terminally of) these residues and, that, optionally, these residues are duplicated behind the insertion, i.e. between the insertion and the H1A domain.

The term "inserted" or "insertion", as referred to herein in the sense that the peptide is inserted into gH, refers to the incorporation into the gH, wherein the incorporated peptide is introduced between two amino acids of the gH by peptide bonds, either directly or indirectly via one or more peptide linkers, more specifically via an upstream and/or downstream located peptide linker with

respect to the insert. The linker is directly connected to the peptide. The fusion of the peptide to gH can also be seen as an insertion of the peptide sequence into the gH precursor, exemplified by SEQ ID NO: 1 or a homologous gH, directly before amino acid 1 of the gH; such an insertion is herein termed as fusion. The gH carrying the fused, or inserted peptide is herein referred to chimeric gH. The chimeric gH is part of the virion envelope. The definition of "linker" is, as described above.

The insertion and fusion are preferably carried out by genetic engineering of the gH gene, in the genome of HSV. The genetic engineering of HSV genomes is known in the art, exemplified by, but not limited to, BAC technologies

The peptide which is present in the envelope of the recombinant herpesvirus of the present invention enables the recombinant herpesvirus to enter into any cell which expresses or binds a target molecule to which the peptide is capable of binding. Consequently, as used herein, the target molecule may be any molecule which is accessible on the surface of a cell and which can be bound by the peptide. Preferably, the target molecule is an artificial molecule which is not naturally produced by the target cell which is used for propagation and production of the recombinant herpesvirus. Thus, the term "artificial target molecule", as referred to herein, may be a natural molecule which is not naturally produced by the target cell such as an antibody or a molecule which does not naturally occur, i. e. that has a non-natural amino acid sequence such as an antibody derivative. Such artificial molecule may be constructed to be expressed by a cell on its surface, as e.g. described in Douglas et al., 1999; and Nakamura et al., 2005, or it may be bound by a cell surface. The artificial target molecule is specifically designed so that it can be bound by the peptide. Examples of artificial target molecules bound by the peptide are antibodies or antibody derivatives. Preferred artificial target molecules are scFvs, more preferably an scFv capable of binding to a part of the GCN4 yeast transcription factor, still more preferably an scFv capable of binding to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, still more preferably the scFv as comprised by SEQ ID NO: 5 (Zahnd et al., 2004), most preferably the molecule identified by the sequence of SEQ ID NO: 7. Methods for producing antibodies or derivatives thereof are known in the art and can be used to generate target molecules which are bound by the peptide.

The most preferred peptide-target molecule pair of the present invention is the peptide identified by SEQ ID NO: 13 and the target molecule identified by the sequence of SEQ ID NO: 7.

The recombinant herpesvirus of the present invention may, in addition to the chimeric gH, comprise a modified gD glycoprotein, as disclosed in WO 2009/144755, herein incorporated by reference, but not limited to those types of modifications. A modified gD carries a modification for retargeting the recombinant herpesvirus to unwanted cells such as diseased cells such as tumor cells for elimination of such cells. Thus, gD may comprise additional polypeptide sequences that readdress the tropism of the herpesvirus to selected receptors of choice, e.g to receptors on diseased cells such as the HER2 receptor. In addition, the modified gD may carry a deletion of the amino acid portion 6 to 38 that detarget herpesvirus tropism from the natural receptors Nectin-1 and HVEM. Alternatively, a modified gD may carry other modifications for detargeting. Modification of gD occurs by fusing to or inserting into gD heterologous polypeptide ligands that are capable of binding to a target molecule naturally present on a diseased cell which should be eliminated. A preferred ligand is an scFv directed to HER2 for eliminating tumor cells which express HER2. The recombinant herpesvirus of the present invention may, in addition to the chimeric gH, comprise a modified gB glycoprotein which is modified to comprise a heterologous polypeptide ligand and to retarget the recombinant herpesvirus to unwanted cells such as diseased cells such as tumor cells for elimination of such cells. The recombinant herpesvirus of the present invention may, in addition to the chimeric gH, comprise a modified gD and/or a modified gB glycoprotein. Modification of gH serves for the propagation and production of the recombinant herpesvirus in vitro in cell culture via binding of the recombinant herpesvirus to a target molecule present on the cell in cell culture, whereas modification of the gD and/or gB serves for the killing of unwanted cells such as diseased cells such as tumor cells via binding of the recombinant herpesvirus to a target molecule present on the unwanted cells.

The term “diseased cell”, as used herein, refers to a cell which negatively influences an organism and is, therefore, not wanted. The eradication of such a cell is desired, as its killing may be live-saving or enhances the health of an

organism. In a preferred embodiment, the diseased cell is characterized by an abnormal growth, more preferably the cell is a tumor cell. In an alternative preferred embodiment, the cell is an infected cell such as a chronically infected cell, a degenerative disorder-associated cell or a senescent cell.

In case of a tumor cell, the underlying disease is a tumor, preferably selected from the group consisting of adrenal cancer, anal cancer, bile duct cancer, bladder cancer, bone cancer, brain/CNS tumors, breast cancer, cancer of unknown primary treatment, Castleman disease, cervical cancer, colon/rectum cancer, endometrial cancer, esophagus cancer, Ewing family of tumors, eye cancer, gallbladder cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (gist), gestational trophoblastic disease, Hodgkin disease, Kaposi sarcoma, kidney cancer, laryngeal and hypopharyngeal cancer, leukemia, liver cancer, lung cancer, lymphoma, lymphoma of the skin, malignant mesothelioma, multiple myeloma, myelodysplastic syndrome, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, non-Hodgkin lymphoma, oral cavity and oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, penile cancer, pituitary tumors, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, sarcoma - adult soft tissue cancer, skin cancer, small intestine cancer, stomach cancer, testicular cancer, thymus cancer, thyroid cancer, uterine sarcoma, vaginal cancer, vulvar cancer, Waldnstrom macroglobulinemia, and Wilms tumor. Preferred tumor diseases are HER2-positive cancers (like breast cancer, ovary cancer, stomach cancer, lung cancer, head and neck cancer, osteosarcoma and glioblastoma multiforme), EGFR-positive cancers (like head and neck cancer, glioblastoma multiforme, non-small cell lung cancer, breast cancer, colorectal and pancreatic cancer), EGFR-vIII-positive cancers (like glioblastoma multiforme), PSMA-positive cancers (like prostate cancer), CD20+ positive lymphoma, and EBV related tumors such as B-cell lymphoproliferative disorders such as Burkitt's lymphoma, classic Hodgkin's lymphoma, and lymphomas arising in immunocompromised individuals (post-transplant and HIV-associated lymphoproliferative disorders), T-cell lymphoproliferative disorders, angioimmunoblastic T-cell lymphoma, extranodal nasal type natural killer/T-cell lymphoma.

In case of an infected cell, the underlying disease is an infectious disease, such as a chronic infectious disease, wherein the infectious agent may be a virus, a bacterium or a parasite. Examples are tuberculosis, malaria, chronic viral hepatitis (HBV, Hepatitis D virus and HCV), acquired immune deficiency syndrome (AIDS, caused by HIV, human immunodeficiency virus), EBV related disorders, or HCMV related disorders.

In case of a degenerative disorder-associated cell, the underlying disease may be Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Lou Gehrig's Disease, osteoarthritis, atherosclerosis, Charcot Marie Tooth disease (CMT), chronic obstructive pulmonary disease (COPD), chronic traumatic encephalopathy, diabetes, ehlers-danlos syndrome, essential tremor, Friedreich's ataxia, huntington's disease, inflammatory bowel disease (IBD), keratoconus, keratoglobus, macular degeneration, marfan's syndrome, multiple sclerosis, multiple system atrophy, muscular dystrophy, Niemann Pick disease, osteoporosis, Parkinson's Disease, progressive supranuclear palsy, prostatitis, retinitis pigmentosa, rheumatoid arthritis, or Tay-Sachs disease. The term “degenerative disorder-associated cell” refers to a cell which is in relationship with the disorder, meaning that an alteration of the cell contributes to the development of the disease or the cell is altered as a consequence of the disease. Destroying the cell results in the treatment of the disease.

In case of a senescent cell, the underlying disease is a senescence-associated disease, such as (i) rare genetic diseases called progeroid syndromes, characterized by pre-mature aging: Werner syndrome (WS), Bloom syndrome (BS), Rothmund-Thomson syndrome (RTS), Cockayne syndrome (CS), xeroderma pigmentosum (XP), trichothiodystrophy or Hutchinson-Gilford Progeria syndrome (HGPS) or (ii) common age related disorders, such as obesity, type 2 diabetes, sarcopenia, osteoarthritis, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, cataracts, neurodegenerative diseases, systemic autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, or Sjögren syndrome), or multiple sclerosis.

The recombinant herpesvirus of the present invention may be attenuated, for example by deletions in or alterations of genes known to attenuate virus virulence,

such as the viral genes $y_1$34.5, UL39, and/or ICP47. The term "attenuated" refers to a weakened or less virulent herpesvirus. Preferred is a conditional attenuation, wherein the attenuation affects only non-diseased cells. More preferred, only the diseased cells such as tumor cells are affected by the full virulence of the herpesvirus. A conditional attenuation can be achieved, for example, by the substitution of the promoter region of the $y_1$34.5, UL39 and/or ICP47 gene with a promoter of a human gene that is exclusively expressed in diseased cells (e.g. the survivin promoter in tumor cells). Further modifications for a conditional attenuation may include the substitution of regulatory regions responsible for the transcription of IE genes (immediate early genes) like the ICP-4 promoter region with promoter regions of genes exclusively expressed in diseased cells (e.g. the survivin promoter). This change will result in a replication conditional HSV, which is able to replicate in diseased cells but not in normal cells. Additional modification of the virus may include the insertion of sequence elements responsive to microRNAs (miRs), which are abundant in normal but not tumor cells, into the 3´ untranslated region of essential HSV genes like ICP4. The result will be again a virus that is replication incompetent only in normal cells.

The recombinant herpesvirus of the present invention may, furthermore, encode one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell, as defined above. A molecule that stimulates the host immune response is also termed “immunotherapy molecule”. Thus, the recombinant herpesvirus of the present invention may be a combined oncolytic and immunotherapeutic virus. An immunotherapeutic virus is a virus that encodes molecules that boost the host immune response to a cell, i.e. that stimulate the host immune response so as to be directed against a cell. An example of such a virus is T-VEC (Liu et al., 2003).

Immunotherapy molecules enable the recombinant virus, besides the modification of glycoproteins for retargeting the herpesvirus to diseased cells for killing them, to stimulate a subject`s immune system in a specific or unspecific manner. Expression of immunotherapy molecules by the recombinant virus in a subject can induce an immune response which finally results in the killing of diseased cells. Immunotherapy may act specifically wherein the immunotherapy molecules stimulate the subject`s immune system against one or some specific antigen(s)

present on (a) cell(s). For example, an immunotherapy molecule may be an antibody which is directed against a specific cell surface receptor, e.g. CD20, CD274, and CD279. Once bound to an antigen, antibodies can induce antibody-dependent cell-mediated cytotoxicity, activate the complement system, or prevent a receptor from interacting with its ligand. All that can lead to cell death. Preferred cells are tumor cells. This technique is known and approved in the art. There are multiple antibodies which are approved to treat cancer, including Alemtuzumab, Ipilimumab, Nivolumab, Ofatumumab, and Rituximab. Alternatively, the immunotherapy molecule can act non-specifically by stimulating the subject`s immune system. Examples of immunotherapy molecules are inter alias cytokines, chemokines or immune checkpoint regulators. For example, some cytokines have the ability to enhance anti-tumor activity and can be used as passive cancer treatments. The use of cytokines as immunotherapy molecules is known in the art. Examples of cytokines are GM-CSF, interleukin-2, interleukin-12, or interferon-α. GM-CSF is used, for example in the treatment of hormone-refractory prostate cancer or leukemia. Interleukin-2 is used, for example, in the treatment of malignant melanoma and renal cell carcinoma.IL-12 is used in the experimental treatment of glioblastoma. Interferon-α is, for example, used in the treatment of hairy-cell leukemia, AIDS-related Kaposi's sarcoma, follicular lymphoma, chronic myeloid leukemia and malignant melanoma.

In a second aspect, the present invention provides a pharmaceutical composition comprising the herpesvirus of the present invention and a pharmaceutically acceptable carrier, optionally additionally comprising one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell, as defined above. The recombinant herpesvirus of the present invention can be used as a medicament. For the production of the medicament the herpesvirus has to be in a pharmaceutical dosage form comprising the recombinant herpesvirus of the present invention and a mixture of ingredients such as pharmaceutically acceptable carriers which provide desirable characteristics. The pharmaceutical composition comprises one or more suitable pharmaceutically acceptable carrier which is/are known to those skilled in the art. The pharmaceutical composition may additionally comprise one or more molecule(s) that stimulate(s) the host immune response against a cell. The definition of a molecule that stimulates the host

immune response against a cell is referred to above under the first aspect of the present invention.

The pharmaceutical composition can be manufactured for systemic, nasal, parenteral, vaginal, topic, vaginal, intratumoral administration. Parental administration includes subcutaneous, intracutaneous, intramuscular, intravenous or intraperitoneal administration.

The pharmaceutical composition can be formulated as various dosage forms including solid dosage forms for oral administration such as capsules, tablets, pills, powders and granules, liquid dosage forms for oral administration such as pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs, injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions, compositions for rectal or vaginal administration, preferably suppositories, and dosage forms for topical or transdermal administration such as ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants or patches.

The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the activity of the recombinant herpesvirus of the present invention, the dosage form, the age, body weight and sex of the subject, the duration of the treatment and like factors well known in the medical arts.

The total dose of the compounds of this invention administered to a subject in single or in multiple doses may be in amounts, for example, from 10^3 to 10^{10}. Single dose compositions may contain such amounts or submultiples thereof to make up the daily dose. The dosages of the recombinant herpesvirus may be defined as the number of plaque forming unit (pfu). Examples of dosages include 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, or 10^{10}.

The recombinant herpesvirus of the present invention may serve to treat diseases in which diseased cells express specific target molecules on their surface, so that they are accessible from the outside of the cell, which target molecules are not produced by a normal cell or are produced by the normal cell to a lower degree. The normal cell may be the respective normal cell. “Respective” means that the

diseased and normal cells are of the same origin, however, cells develop into diseased cells due to disease-generating influences, whereas other cells of same origin remain healthy.

In a third aspect, the present invention provides the herpesvirus of the present invention, optionally in combination with one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell, for use in the treatment of a tumor, infection, degenerative disorder or senescence-associated disease. The recombinant herpesvirus of the present invention and the molecule(s) that stimulate(s) the host immune response against a cell can be present within the same pharmaceutical composition or within different pharmaceutical compositions. If they are present in different pharmaceutical compositions, they may be administered simultaneously, or subsequently, either the herpesvirus before the molecule or the molecule before the herpesvirus. The herpesvirus or the molecule may be administered at different frequencies and/or time points. However, a combined treatment comprises that the herpesvirus and the molecule are administered at time intervals and/or time points that allow the simultaneous treatment of the disease.

The present invention also discloses a method of treating a subject having a tumor, infection, degenerative disorder or senescence-associated disorder by administering a pharmaceutically effective amount of the recombinant herpesvirus of the present invention.

The recombinant herpesvirus of the present invention may be administered to a subject in combination with further treatments which stimulate the host immune response against a cell, preferably a diseased cell, and/or serve to treat the specific disease of the subject. Such further treatments may include other drugs, chemotherapy, radiotherapy, immunotherapy, combined virotherapy etc.

The present invention also discloses the use of the herpesvirus of the present invention, optionally in combination with one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell, for the preparation of a pharmaceutical composition for the treatment of a tumor, infection, degenerative disorder or senescence-associated disease.

The subjects which are treated by the recombinant herpesvirus of the present invention are preferably humans.

In a forth aspect, the present invention provides a nucleic acid molecule comprising a nucleic acid coding for the chimeric gH of the present invention having fused or inserted the peptide, preferably the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, more preferably the sequence of SEQ ID NO: 13. The nucleic acid molecule may be the genome of the recombinant herpesvirus of the present invention or a part thereof. Preferably, the nucleic acid molecule encodes the precursor form of the chimeric gH including the signal sequence of the gH glycoprotein. If the chimeric gH was engineered to harbor the peptide to its N-terminal amino acid, the corresponding nucleic acid has the nucleic acid sequence of the peptide inserted between the last amino acid of the signal sequence and the first amino acid of the mature protein.

In a fifth aspect, the present invention provides a vector comprising the nucleic acid molecule. Suitable vectors are known in the art and include plasmids, cosmids, artificial chromosomes (e.g. bacterial, yeast or human), bacteriophages, viral vectors (retroviruses, lentiviruses, adenoviruses, adeno-associated viruses), in particular baculovirus vector, or nano-engineered substances (e.g. ormosils). In one embodiment, the vector is modified, in particular by a deletion, insertion and/or mutation of one or more nucleic acid bases, such that its virulence is attenuated, preferably in case of a viral vector, or that it replicates conditionally in diseased cells but not in non-diseased cells. For example, deletion of one or both copies of the $\gamma_1$34.5 gene, the UL39 gene, the ICP47 gene results in attenuation of the virus. Attenuation or attenuated refers to weakened or less virulent virus.

Moreover, the substitution of the promoter region of the $\gamma_1$34.5 gene with a promoter of a human gene that is exclusively expressed in diseased cells, e.g. tumor cells (e.g. survivin promoter in tumor cells), which will result in an attenuated phenotype in non-diseased cells and non-attenuated phenotype in diseased cells, is included. Further modifications may include the substitution of regulatory regions responsible for the transcription of IE genes like the ICP-4 promoter region with promoters of genes exclusively expressed in diseased cells (e.g. survivin promoter). This change will produce a replication conditional

herpesvirus, able to replicate in diseased cells but not in normal cells. Cell culture cells for propagation of the virus progeny will provide high levels of specific promoter activating proteins to allow for the production of high virus yields.

In a sixth aspect, the present invention provides a polypeptide comprising the chimeric gH having fused or inserted the peptide, preferably the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, more preferably the sequence of SEQ ID NO: 13.

In a seventh aspect, the present invention provides a cell comprising the recombinant herpesvirus of the present invention, the nucleic acid molecule of the present invention, the vector of the present invention, or the polypeptide of the present invention.

In an embodiment thereof, the cell is a cultured cell suitable for growth of herpesvirus, more preferably a cell line approved for growth of herpesvirus, still more preferably a Vero, 293, 293T, HEp-2, HeLa, BHK, or RS cell, most preferably a Vero cell.

In an eighth aspect or in an embodiment of the seventh aspect, the present invention provides a cell, wherein the cell comprises an artificial molecule capable of binding to the peptide comprised by the recombinant herpesvirus of the present invention, preferably to a part of the GCN4 yeast transcription factor, most preferably to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, accessible on the surface of the cell, preferably wherein the artificial molecule is an antibody, more preferably an antibody derivative, still more preferably an scFv, still more preferably an scFv capable of binding a part of the GCN4 yeast transcription factor, still more preferably an scFv capable of binding to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, still more preferably the scFv as comprised by SEQ ID NO: 5, most preferably the molecule identified by the sequence of SEQ ID NO: 7.

The term “cell”, as referred to herein, is any cell which carries the target molecule, which can be infected by the recombinant herpesvirus of the present invention and which can produce the herpesvirus. As propagation of the herpesvirus shall be avoided in diseased cells, so as to avoid the introduction of

material such as DNA, RNA and/or protein of diseased cells such as tumor cells in humans, the cell for producing the herpesvirus is a safe cell which does not produce material which may be harmful if present in humans, e.g. a non-diseased cell. The cell may be present as a cell line. Preferably, the cell is a cultured cell suitable for growth of herpesvirus, still more preferably the cell is a cell line approved for herpesvirus growth and still more preferably the cell is Vero, 293, 293T, HEp-2, HeLa, BHK, or RS cell, whereby the Vero cell is particularly preferred. The cell may be modified to express an artificial target molecule or to bind an artificial target molecule. More preferably, the cell comprises as the target molecule an antibody derivative, still more preferably an scFv, still more preferably an scFv capable of binding to a part of the GCN4 yeast transcription factor, still more preferably an scFv capable of binding to a part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, still more preferably an scFv as comprised by SEQ ID NO: 5, and most preferably the molecule identified by the sequence of SEQ ID NO: 7.

A “cultured” cell is a cell which is present in an in vitro cell culture which is maintained and propagated, as known in the art. Cultured cells are grown under controlled conditions, generally outside of their natural environment. Usually, cultured cells are derived from multicellular eukaryotes, especially animal cells. “A cell line approved for growth of herpesvirus” is meant to include any cell line which has been already shown that it can be infected by a herpesvirus, i. e. the virus enters the cell and is able to propagate and produce the virus. A cell line is a population of cells descended from a single cell and containing the same genetic composition. Preferred cells for propagation and production of the recombinant herpesvirus are Vero, 293, 293T, HEp-2, HeLa, BHK, or RS cells.

In a ninth aspect, the present invention provides an in-vitro method for producing a recombinant herpesvirus in a cell using the herpesvirus of the present invention, wherein the cell comprises an artificial molecule capable of binding to the peptide comprised by the recombinant herpesvirus of the present invention, preferably to a part of the GCN4 yeast transcription factor, most preferably to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, accessible on the surface of the cell, preferably wherein the artificial molecule is an antibody, more preferably an antibody derivative, still more preferably an scFv, still more

preferably an scFv capable of binding a part of the GCN4 yeast transcription factor, still more preferably an scFv capable of binding to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, still more preferably the scFv as comprised by SEQ ID NO: 5, most preferably the molecule identified by the sequence of SEQ ID NO: 7..

The most preferred cell of the present invention is the Vero-GCN4 cell line which expresses as target molecule the molecule with the sequence of SEQ ID NO: 7 comprising the scFv capable of binding to the GCN4 peptide as identified by SEQ ID NO: 13. The Vero-GCN4 cell line serves inter alia the purpose of enabling the cultivation of herpesvirus recombinants retargeted to HER2-positive cells, and detargeted from natural herpesvirus receptors. Because HER2 is an oncogene, and the HER2-positive cells are cancer cells, it is advisable to avoid the growth of oncolytic herpesvirus recombinants destined to human use in cancer cells, in order to avoid the possible, accidental introduction of tumor-derived material (DNA, RNA, proteins) in humans. The rationale for the construction of the Vero-GCN4 cell line, and the companion HER2-retargeted herpesvirus was as follows. Vero-GCN4 cells express an artificial receptor made of a scFv to the peptide GCN4, fused to extracellular domains 2 and 3, transmembrane (TM), and C-tail of nectin1. Conversely, the HER2 retargeted herpesvirus expresses the GCN4 peptide in one of the envelope glycoproteins. In view of this, the recombinant virus is simultaneously retargeted to HER2 (in order to infect cancer cells) and to GCN4 peptide (in order to infect the Vero-GCN4 cell line, for virus growth and production). In the example system described below, the recombinant HSV named R-VG213 carries the scFv to HER2 fused in gD, in place of AA 6-38 (which are deleted from the final virus) and also carries the GCN4 peptide fused to gH, between AA 23 and 24.

Suitable techniques and conditions for growing herpesvirus in a cell are well known in the art (Florence et al., 1992; Peterson and Goyal, 1988) and include incubating the herpesvirus with the cell and recovering the herpesvirus from the medium of the infected cell culture.

FIGURES

Fig. 1 Schematic drawing of the chimeric scFv to GCN4 – Nectin receptor. The receptor presents N-terminal leader peptide and HA tag sequence, followed by the scFv to GCN4, placed between two short linker, GA and GSGA linker. The second part of the molecule corresponds to human Nectin-1 (PVRL1) residues Met143 to Val517 comprising the Nectin-1 extracellular domains 2 and 3, the TM segment and the intracellular cytoplasmic tail.

Fig. 2 Stability of Vero-GCN4 positive cells. The expression of the scFv GCN4-Nectin receptor was analyzed by FACS by means of Mab to HA tag. Diagrams show the percentage positive cells from Vero-GCN4 clone 11.2 cells at passages 10, 15, 30, 40. Result: the expression of the artificial receptor remained stable after 40 consecutive passages.

Fig. 3 Genome organization of R-VG213. Sequence arrangement of HSV-1 genome shows the inverted repeat sequences as rectangular boxes. The scFv-HER2 sequence (VL-linker-VH) is inserted in position Δ6-38 of gD, bracketed by upstream and downstream Gly-Ser linkers. LOX-P-bracketed p-Belo-BAC and EGFP sequences are inserted between UL3-UL4 region. The sequence encoding the GCN4 peptide is engineered in gH, at AA position 23-24.

Fig. 4 Tropism of R-VG213 *vs* R-LM113. J cells express no receptor for wt-HSV. J-HER2, J-Nectin1, J-HVEM only express the indicated receptor. The indicated cells were infected with R-VG213 (panel a-k) or R-LM113 (panel l-v) and monitored for EGFP by fluorescence microscopy. Cells in panel b,d,f,h, and m,o,q,s were infected in presence of Herceptin/Trastuzumab at neutralizing dose (28 µg/ml). R-VG213 infects both the Vero-GCN4 cells (c), and the HER2-positive cancer cell line SK-OV-3 (e), in addition to the J-HER2 cells (g); it also infects wt-Vero cells, which express a simian ortholog of HER2 (a). Herceptin inhibits R-VG213 infection of wt-Vero, SK-OV-3 and J-HER2 cells (b, f, h), but not of Vero-GCN4 cells (d). R-VG213 fails to infect J-nectin1, J-HVEM and wt-J cells (i, j, k). The parental R-LM113, which is retargeted to HER2, but not to GCN4 peptide, infects HER2-positive cells (l, n, p, r), and fails to infect Vero-GCN4 cells treated with Herceptin (o).

Fig. 5 Yield of R-VG213 and R-LM5 in Vero-GCN4 cells. The extent of R-VG213 replication in Vero-GCN4 cells was compared to that of R-LM5 virus. Vero-GCN4

cell were infected at MOI 0.1 PFU/cell with R-VG213 or R-LM5 (inoculum titrated in Vero-GCN4). Samples were collected at 0, 24 and 48 hours post infection and progeny virus was titrated in Vero GCN4 cells.

Fig. 6 Yield of R-VG213, R-LM113, R-LM5 in SK-OV-3 cells, and extent of progeny R-VG213 release in extracellular medium of SK-OV-3 cells. (A, B) The extent of R-VG213 replication in SK-OV-3 cells was compared to that of R-LM113 and wt-R-LM5 viruses. SK-OV-3 cells were infected at MOI 0.1 PFU/cell (panel A) or MOI 0.01 PFU/cell (panel B) (inocula were titrated in SK-OV-3 cells). Samples were collected at 0, 24 and 48 hours post infection and progeny virus was titrated in SK-OV-3 cells. (C) SK-OV-3 cells were infected with R-VG213 or R-LM113 at MOI 0.1 PFU/cell as in panel A (inoculum was titrated in SK-OV-3 cells). Samples were collected at 48 hours post infection and progeny virions released in the extracellular medium, present in the cell-associated fraction, or cell-associated plus medium were titrated.

Fig. 7. Plating efficiency of R-VG213 in different cell lines. (A) Replicate aliquots of R-VG213 were plated in Vero-GCN4, wt-Vero, SK-OV-3 and J-HER2 cells and plaques were scored 3 days later. (B) Relative plaque size of R-VG213 in different cell lines. Replicate aliquots of R-VG213, R-LM113 and R-LM5 were plated in Vero-GCN4, Wt-Vero and SK-OV-3. Plaques were analyzed at fluorescence microscope 3 days post infection.

SEQUENCES

SEQ ID NO: 1: Amino acid sequence of gH wild type, precursor from HSV-1 (Human Herpesvirus 1 strain F, GenBank accession number: GU734771.1; gH encoded by positions 43741 to 46498).

SEQ ID NO: 2: Nucleotide sequence of chimeric gH-GCN4.

SEQ ID NO: 3: Amino acid sequence of gH precursor (SEQ ID NO: 1) having inserted the GCN4 peptide between amino acids 23 and 24, as encoded by the construct R-VG213. The GCN4 peptide is flanked by a Gly-Ser linker.

SEQ ID NO: 4: Nucleotide sequence of scFv to GCN4 peptide, optimized for human codon usage, and preceded by 96 nucleotide that form the signal sequence and the HA tag.

SEQ ID NO: 5: Amino acid sequence of scFv to GCN4 peptide (GenBank 1P4B), preceded by 32 AA that constitute the signal sequence and the HA tag. The sequence of the scFv to GCN4 peptide starts at amino acid 33.

SEQ ID NO: 6: Nucleotide sequence of scFv-GCN4 Nectin1 chimera.

SEQ ID NO: 7: Amino acid sequence of scFv-GCN4 Nectin1 chimera.

SEQ ID NO: 8: Primer gH5_galK_r

SEQ ID NO: 9: Primer gH6_galK_f

SEQ ID NO: 10: Primer galK_129_f

SEQ ID NO: 11: Primer galK_417_r

SEQ ID NO: 12: GCN4 peptide cassette - Nucleotide sequence of GCN4 peptide, bracketed by upstream and downstream GS linkers.

SEQ ID NO: 13: GCN4 peptide - Amino acid sequence of GCN4 peptide, bracketed by upstream and downstream GS linkers.

SEQ ID NO: 14: GCN4 epitope derived from Saccharomyces cerevisiae GCN4 mRNA (http://www.ncbi.nlm.nih.gov/nuccore/15811626/).

SEQ ID NO: 15: Oligonucleotide GCN4gH_23_42_fB

SEQ ID NO: 16: Oligonucleotide GCN4gH_23_24_rB

SEQ ID NO: 17: Primer gH_ext_r pallino

SEQ ID NO: 18: Primer gH_2176_2200_f

SEQ ID NO: 19: GenBank accession number AJ585687.1 (gene encoding the GCN4 transcription factor)

SEQ ID NO: 20: amino acid sequence of GCN4 yeast transcription factor UniProtKB - P03069 (GCN4_YEAST)

EXAMPLES

Example 1: Generation of Vero-GCN4 cell line

The Vero-GCN4 cell line expresses an artificial chimeric receptor, made of a scFv to the GCN4 peptide (Zahnd et al., 2004), fused to Nectin-1. More in detail, a N-terminal signal peptide and HA tag sequence is present like in the pDISPLAY (Invitrogen) vector. This should ensure efficient and proper processing of the leader peptide. After the HA tag a short GA linker is present upstream of the scFv. The nucleotide and amino acid sequences of the scFv to GCN4, with sequence optimized for human codon usage, are reported in SEQ ID NOs: 4 and 5; included in those sequences are the signal peptide sequence and the sequence of the HA tag, which precede the sequences of the scFv. C-terminal to the scFv a short GSGA linker is present. The rest of the molecule corresponds to human Nectin-1 (PVRL1) residues Met143 to Val517 comprising the Nectin-1 extracellular domains 2 and 3, the TM segment and the intracellular cytoplasmic tail (Fig. 1). The chimera was synthesized in vitro by Gene Art, and cloned into pcDNA3.1 – Hygro(+), resulting in plasmid scFv_GCN4_Nectin1 chimera, whose insert has the nucleotide sequence reported as SEQ ID NO: 6, and the amino acid sequence identified as SEQ ID NO: 7.

The DNA from plasmid scFv_GCN4_Nectin1 chimera was transfected into Vero cells (ATCC CCL-81™) by means of Lipofectamine 2000. Vero cells expressing the artificial receptor to GCN4 peptide were selected by means of hygromycin (200 ug/ml), and subsequently sorted by means of magnetic beads (Miltenyi), in combination with MAb to HA tag. The sorted cells were subjected to single cell cloning in 96 well (0.5 cell/well).

Single clones were analysed by FACS for detection of expression of the scFv to GCN4 peptide by means of MAb to HA tag. The selected clone was 11.2.

Example 2: Stability of Vero-GCN4 cell line

The inventors ascertained that during serial passages of the Vero-GCN4 cell line, the expression of the artificial receptor remained stable after 40 consecutive passages (Fig. 2).

Example 3: Description of the HSV recombinant named R-VG213 (Fig. 3), which expresses a genetically modified gH carrying the GCN4 peptide, a gD carrying a single chain antibody (scFv) directed to HER2 (scFv-HER2), and eGFP as reporter gene.

Below is a description of the insertion of the sequence encoding the GCN4 peptide, between AA 23 and 24 of HSV gH. The insertion was carried out in the HSV recombinant named R-LM113, which expresses a scFv-HER2 in gD, in place of the deleted sequences AA 6-38. Specifically, the sequence encoding the GCN4 peptide was inserted between AA 23 and 24 of immature gH, corresponding to AA 5 and 6 of mature gH, after cleavage of the signal sequence, which encompasses AA 1-18. The starting genome was the BAC LM113, which carries scFv-HER2 in place of AA 6 to 38 of gD, LOX-P-bracketed pBeloBAC11 and eGFP sequences inserted between U_L3 and U_L4 of HSV-1 genome (Menotti et al., 2008). The engineering was performed by means of galK recombineering. In order to insert the GCN4 peptide in gH, the galK cassette with homology arms to gH was amplified by means of primers gH5_galK_r TCGTGGGGGTTATTATTTTGGGCGTTGCGTGGGGTCAGGTCCACGACTGGTC AGCACTGTCCTGCTCCTT (SEQ ID NO: 8) and gH6_galK_f ATGCGGTCCATGCCCAGGCCATCCAAAAACCATGGGTCTGTCTGCTCAGTCC TGTTGACAATTAATCATCGGCA (SEQ ID NO: 9) using pGalK as template. This cassette was electroporated in SW102 bacteria carrying the BAC LM113. The recombinant clones carrying the galK cassette were selected on plates containing M63 medium (15 mM $(NH_4)_2SO_4$, 100 mM KH_2PO_4, 1.8 µg $FeSO_4 \cdot H_2O$, adjusted to pH7) supplemented with 1 mg/L D-biotin, 0.2 % galactose, 45 mg/L L-leucine, 1 mM $MgSO_4 \cdot 7H_2O$ and 12 µg/ml chloramphenicol. In order to exclude galK false positive bacterial colonies, they were streaked also on MacConkey agar base plates supplemented with 1% galactose and 12 µg/ml chloramphenicol and checked by colony-PCR with primer galK_129_f ACAATCTCTGTTTGCCAACGCATTTGG (SEQ ID NO: 10) and galK_417_r CATTGCCGCTGATCACCATGTCCACGC (SEQ ID NO: 11). Next, the DNA

fragment encoding the GCN4 peptide cassette, with nucleotide sequence identified as SEQ ID NO: 12, encoding the GCN4 peptide having the AA sequence identified as SEQ ID NO: 13, bracketed by upstream and downstream Gly-Ser linkers, and by homology arms to gH, was generated through the annealing and extension of synthetic oligonucleotides GCN4gH_23_42_fB TCGTGGGGGTTATTATTTTGGGCGTTGCGTGGGGTCAGGTCCACGACTGGG GATCCAAGAACTACCACCTGGAGAACGAGGTGGCCAGACTGAAGAAGCTGG TGGGCAGC (SEQ ID NO: 15) and GCN4gH_23_24_rB ATGCGGTCCATGCCCAGGCCATCCAAAAACCATGGGTCTGTCTGCTCAGTGC TGCCCACCAGCTTCTTCAGTCTGGCCACCTCGTTCTCCAGGTGGTAGTTCTT GGATCC (SEQ ID NO: 16), which introduce a silent restriction site for the BamHI endonuclease, useful for screening of colonies by means of restriction analysis. The recombinant BAC R-VG-213 encodes the chimeric gH, whose nucleotide sequence is identified as SEQ ID NO: 2, and whose amino acid sequence is identified as SEQ ID NO: 3. The recombinant BAC R-VG213 bacterial clones were selected on plates containing M63 medium (see above) supplemented with 1 mg/L D-biotin, 0.2% deoxy-2-galactose, 0.2% glycerol, 45 mg/L L-leucine, 1 mM $MgSO4 \cdot 7H_2O$ and 12 µg/ml chloramphenicol. Bacterial colonies were checked for the presence of sequence of choice by means of colony PCR with primers gH_ext_r pallino GTTTCTTCCTTTTCCCCACCCCACCCC (SEQ ID NO: 17) and gH_2176_2200_f CAGGTAGGTCTTCGGGATGTAAAGC (SEQ ID NO: 18).

To reconstitute the recombinant virus R-VG213, 500 ng of recombinant BAC DNA was transfected into the Vero-GCN4 cell line by means of Lipofectamine 2000 (Life Technologies), and then grown in these cells. Virus growth was monitored by green fluorescence. The authenticity of the recombinants was verified by sequencing the entire gH and gD ORFs. Virus stocks were generated in Vero-GCN4 cells and titrated in Vero-GCN4 and SK-OV-3 cells.

Example 4: Double tropism of R-VG213 for Vero-GCN4 and for HER2-positive J-HER2 and SK-OV-3 cells.

It has previously been shown that the insertion of scFv-HER2 in gD confers to the recombinant virus R-LM113 the ability to enter cells through the HER2 receptor, and that R-LM113 is detargeted from the natural gD receptors Nectin1 and HVEM,

because of the deletion of the gD region between AA 6-38. To verify whether the insertion of the GCN4 peptide enables R-VG213 to infected the Vero-GCN4 cells, the inventors made use of Vero-GCN4 cell line and its wt counterpart, wt Vero. To verify that R-VG213 is still capable to infect through the HER2 receptor, the inventors made us of the J-HER2 cells, which express HER2 as the sole receptor, and of the HER2-positive cancer cells, SK-OV-3 cells. In addition, to verify that R-VG213 maintains the detargeting from nectin1 and HVEM, the inventors made us of J-Nectin1 and J-HVEM, which express only the indicated receptor. Cells were infected with R-LM213 (Fig. 4 panel A) and with R-LM113 (Fig. 4 panel B). Where indicated, infection was carried out in the presence of MAb to HER2, named herceptin, at the concentration of 28μg/ml. Infection was carried out at 1 PFU/cell, and was monitored 24 hours later by fluorescence microscopy. As shown in Fig. 4 A, R-VG213 infected Vero-GCN4 cells; this infection was not inhibited by herceptin, indicating that it occurred through the GCN4 receptor. The infection seen in wt Vero was inhibited by herceptin, indication that it occurs through the simian ortholog of HER2. R-VG213 infected SK-OV-3 and J-HER2 cells, in a fashion inhibited by herceptin, i.e. HER2-dependent. As expected, R-VG213 did not infect J-nectin1, J-HVEM, and wt-J cells indicating that it preserved the detargeted phenotype. The infection of Vero-GCN4 cells with R-VG213 is in sharp contrast with that of R-LM113, which infects cells solely through HER2 (Fig. 4 B).

Example 5: Extent of R-VG213 replication in Vero-GCN4 cells, as compared to that of the wt-virus R-LM5.

The inventors compared the extent of replication in Vero-GCN4 cells of R-VG213 to that of R-LM5, a virus carrying wt gH and wt-gD. Vero-GCN4 cell were infected at MOI 0.1 PFU/cell with R-VG213 or R-LM5 (inoculum titrated in VERO-GCN4 cells), for 90 min at 37°C. Unabsorbed virus was inactivated by means of an acidic wash (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3]). Replicate cultures were frozen at the indicated times (0, 24 and 48 h) after infection and the progeny was titrated in VERO-GCN4 cells. It can be seen from Fig. 5 that R.VG213 grew about one log less than R-LM5 at 24 h. At 48 h the extent of replication was undistinguishable.

Example 6: Replication of R-VG213 in SK-OV-3 cells, in comparison to R-LM113 and R-LM5, and extent of progeny R-VG213 release in extracellular medium of SK-OV-3 cells.

(A, B) The inventors compared the extent of replication of R-VG213 to that of the recombinant R-LM113, also retargeted to HER2 through the insertion of scFv-HER2 in gD, and of the wt R-LM5. Replication was measured in SK-OV-3 cells, which express HER2 and Nectin-1/HVEM as receptors. Replication was carried out at input MOI of 0.1 (panel A) or 0.01 (panel B) PFU/cell. Unabsorbed virus was inactivated by means of an acidic wash (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3]). Replicate cultures were frozen at the indicated times (0, 24 and 48 h) after infection and the progeny was titrated in SK-OV-3 cells. It can be seen from Fig. 6 A and B that R-VG213 replication could not be differentiated from that of its parent R-LM113, and about half log less than the wt R-LM5. (C). The inventors compared R-VG213 and R-LM113 with respect to the extent of progeny virus release to the extracellular medium of SK-OV-3 cells infected at 0.1 PFU/cell (experiment shown in panel A). At 48 h after infection, replicate cultures were either frozen as whole lysates plus medium (cell-associated + medium), or medium and cell-associated fractions were separated and frozen. Progeny virus was titrated in SK-OV-3 cells. It can be seen that the efficiency of progeny release in the extracellular medium was very similar.

Example 7: Plating efficiency of R-VG213 in different cell lines.

The inventors compared the ability of R-VG213 to form plaques in different cell lines, with respect to number of plaques (A), and to plaque size (B). (A) Replicate aliquots of R-VG213 were plated in Vero-GCN4, Wt-Vero, SK-OV-3 and J-HER2 cells and the number of plaques were scored 3 days later. It can be seen that the highest plating efficiency is reached in Vero-GCN4 cells. (B) Typical examples of relative plaque size of R-VG213 in different cells. Even by this parameter R-VG213 exhibits a large plaque phenotype in Vero-GCN4 cells.

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Claims

1. A recombinant herpesvirus comprising a peptide having a length of 5 to 274 amino acids, fused to or inserted into glycoprotein H (gH) present in the envelope of the herpesvirus.

2. The herpesvirus according to claim 1, wherein the peptide has a length of 5 to 200 amino acids, preferably of 11 to 29, 31 to 39, 41 to 49 or 51 to 200 amino acids, more preferably of 12 to 20 amino acids.

3. The herpesvirus according to claim 1 or 2, wherein the peptide comprises a part of the GCN4 yeast transcription factor, preferably the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, most preferably the peptide is the peptide identified by SEQ ID NO: 13.

4. The herpesvirus according to any one of claims 1 to 3, wherein the peptide is inserted within the N-terminal region starting at any one of amino acids 19 to 23 and ending at any one of amino acids 48 to 88 or starting at amino acid 116 and ending at amino acid 136 of the gH according to SEQ ID NO: 1 or a corresponding region of a homologous gH.

5. The herpesvirus according to any one of claims 1 to 4, wherein the peptide is inserted N-terminally of the H1A domain of gH.

6. The herpesvirus according to any one of claims 1 to 5, wherein one or more gH amino acids of the N-terminal region are deleted.

7. The herpesvirus according to any one of claims 1 to 6, wherein the herpesvirus has the capability of binding to a cell expressing or binding a target molecule via the peptide, preferably of fusing with the cell membrane, more preferably of entering the cell, most preferably of propagating within the cell.

8. The herpesvirus according to claim 7, wherein the target molecule is the scFv as comprised by SEQ ID NO: 5, most preferably the molecule identified by the sequence of SEQ ID NO: 7.

9. The herpesvirus according to any one of claims 1 to 8, wherein the herpesvirus comprises a gD which is modified to retarget the herpesvirus to a diseased cell and/or a gB which is modified to retarget the herpesvirus to a diseased cell.

10. The herpesvirus according to any one of claims 1 to 9, wherein the herpesvirus encodes one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell.

11. A pharmaceutical composition comprising the herpesvirus according to any one of claims 1 to 10 and a pharmaceutically acceptable carrier, optionally additionally comprising one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell.

12. The herpesvirus according to any one of claims 1 to 10, optionally in combination with one or more molecule(s) that stimulate(s) the host immune response against a cell, preferably a diseased cell, for use in the treatment of a tumor, infection, degenerative disorder or senescence-associated disease.

13. A nucleic acid molecule comprising a nucleic acid coding for the gH, as defined in any one of claims 1 to 8, having fused or inserted the peptide.

14. A vector comprising the nucleic acid molecule according to claim 13.

15. A polypeptide comprising the gH, as defined in any one of claims 1 to 8, having fused or inserted the peptide.

16. A cell comprising the herpesvirus according to any one of claims 1 to 10, the nucleic acid molecule according to claim 13, the vector according to claim 14, or the polypeptide according to claim 15.

17. The cell according to claim 16, wherein the cell is a cultured cell suitable for growth of herpesvirus, more preferably a cell line approved for growth of herpesvirus, still more preferably a Vero, 293, 293T, HEp-2, HeLa, BHK, or RS cell, most preferably a Vero cell.

18. A cell or the cell according to claim 16 or 17, wherein the cell comprises an artificial molecule capable of binding to the peptide comprised by the recombinant herpesvirus according to any one of claims 1 to 10, preferably to a part of the GCN4 yeast transcription factor, most preferably to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, accessible on the surface of the cell, preferably wherein the artificial molecule is an antibody, more preferably an antibody derivative, still more preferably an scFv, still more preferably an scFv capable of binding a part of the GCN4 yeast transcription factor, still more preferably an scFv capable of binding to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, still more preferably the scFv as comprised by SEQ ID NO: 5, most preferably the molecule identified by the sequence of SEQ ID NO: 7.

19. An in-vitro method for producing a recombinant herpesvirus in a cell using the herpesvirus according to any one of claims 1 to 10, wherein the cell comprises an artificial molecule capable of binding to the peptide comprised by the recombinant herpesvirus according to any one of claims 1 to 9, preferably to a part of the GCN4 yeast transcription factor, most preferably to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, accessible on the surface of the cell, preferably wherein the artificial molecule is an antibody, more preferably an antibody derivative, still more preferably an scFv, still more preferably an scFv capable of binding a part of the GCN4 yeast transcription factor,

still more preferably an scFv capable of binding to the part of the GCN4 yeast transcription factor as comprised by SEQ ID NO: 13, still more preferably the scFv as comprised by SEQ ID NO: 5, most preferably the molecule identified by the sequence of SEQ ID NO: 7.

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Fig. 1

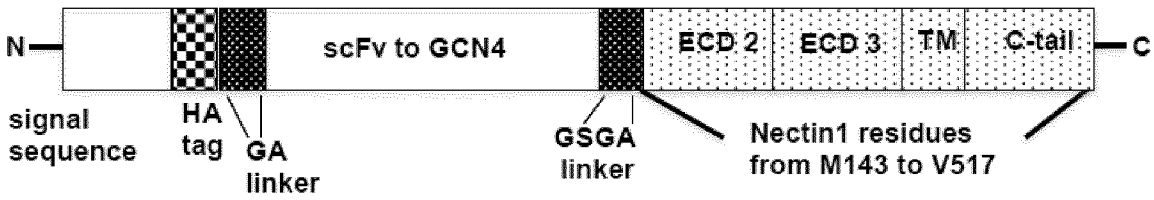

N
scFv to GCN4
ECD 2
ECD 3
TM
C-tail
C
signal sequence
HA tag
GA linker
GSGA linker
Nectin1 residues from M143 to V517

Fig. 2

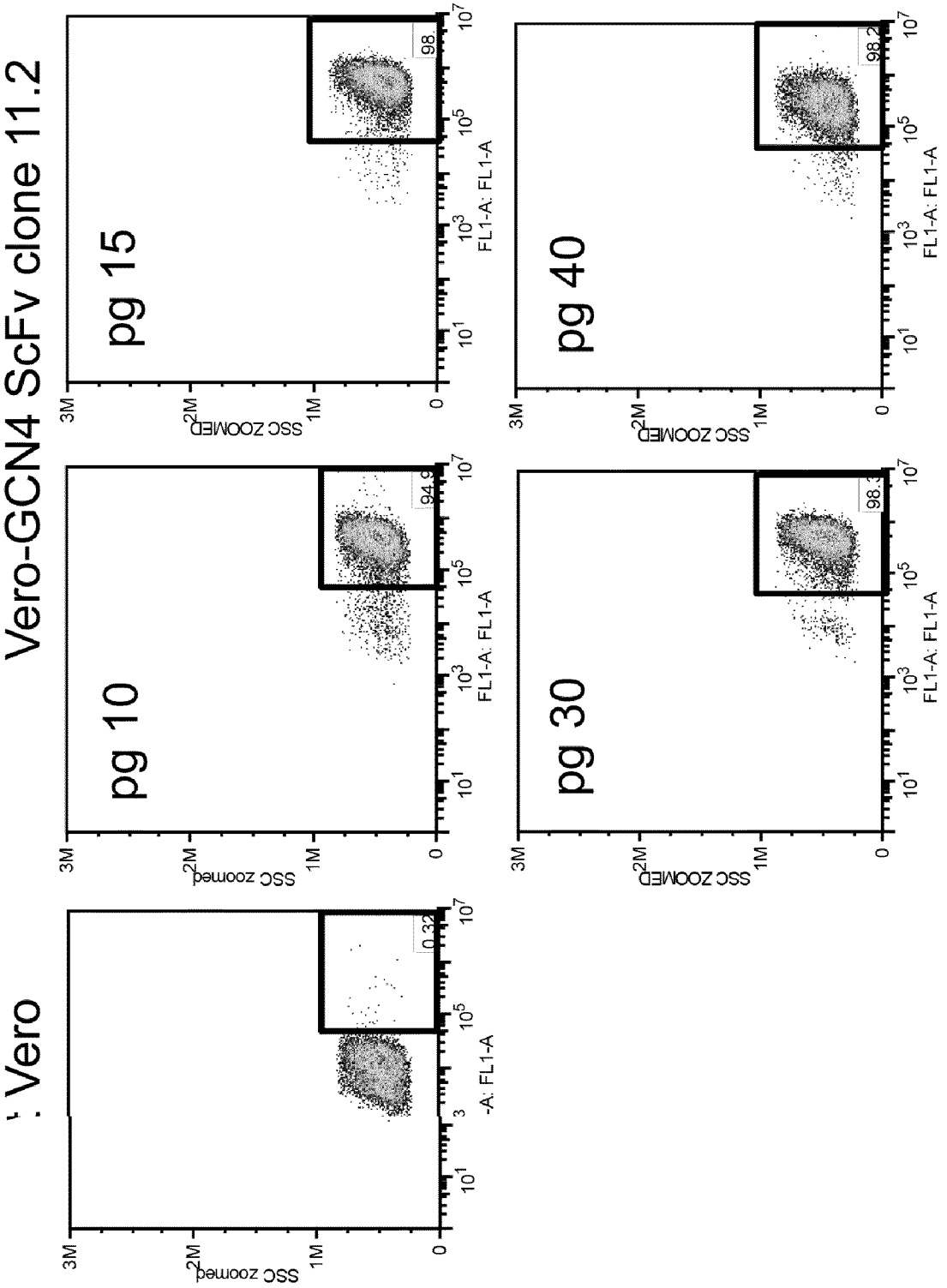

Vero-GCN4 ScFv clone 11.2
pg 15
pg 40
pg 10
pg 30
Vero
SSC ZOOMED
SSC zoomed
FL1-A: FL1-A
-A: FL1-A
98.
98.2
94.9
98.3
0.32

Fig. 3

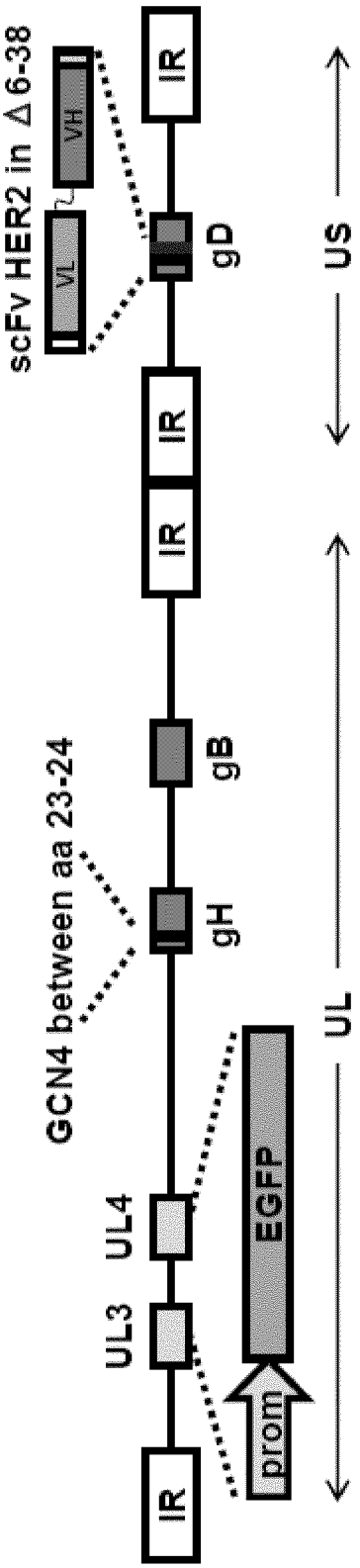

scFv HER2 in Δ 6-38
VH
VL
IR
gD
US
IR
IR
gB
GCN4 between aa 23-24
gH
UL
EGFP
UL4
UL3
prom
IR

Fig. 4

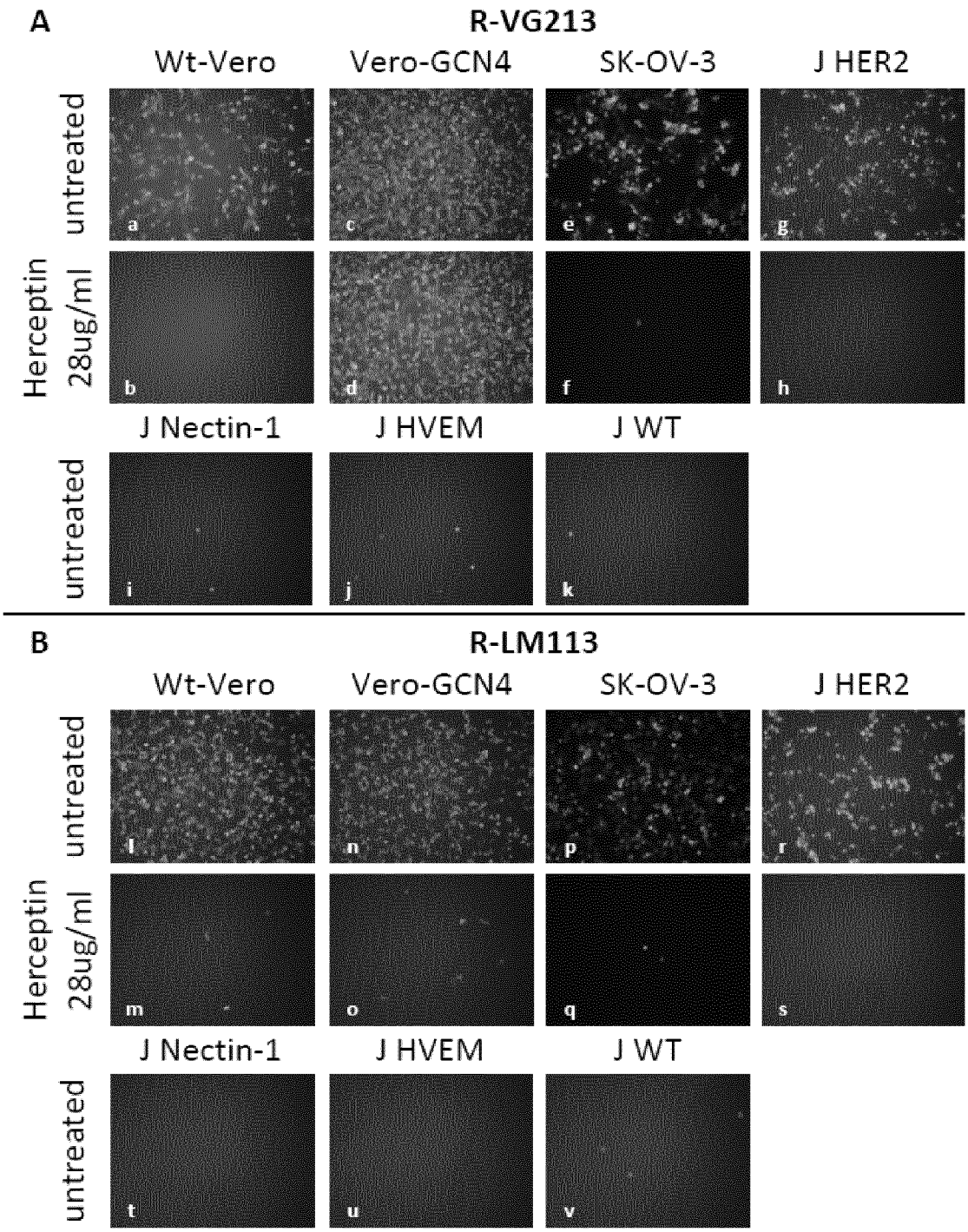

A
R-VG213
Wt-Vero
Vero-GCN4
SK-OV-3
J HER2
untreated
Herceptin 28ug/ml
a
b
c
d
e
f
g
h
J Nectin-1
J HVEM
J WT
untreated
i
j
k
B
R-LM113
Wt-Vero
Vero-GCN4
SK-OV-3
J HER2
untreated
Herceptin 28ug/ml
l
m
n
o
p
q
r
s
J Nectin-1
J HVEM
J WT
untreated
t
u
v

Fig. 5

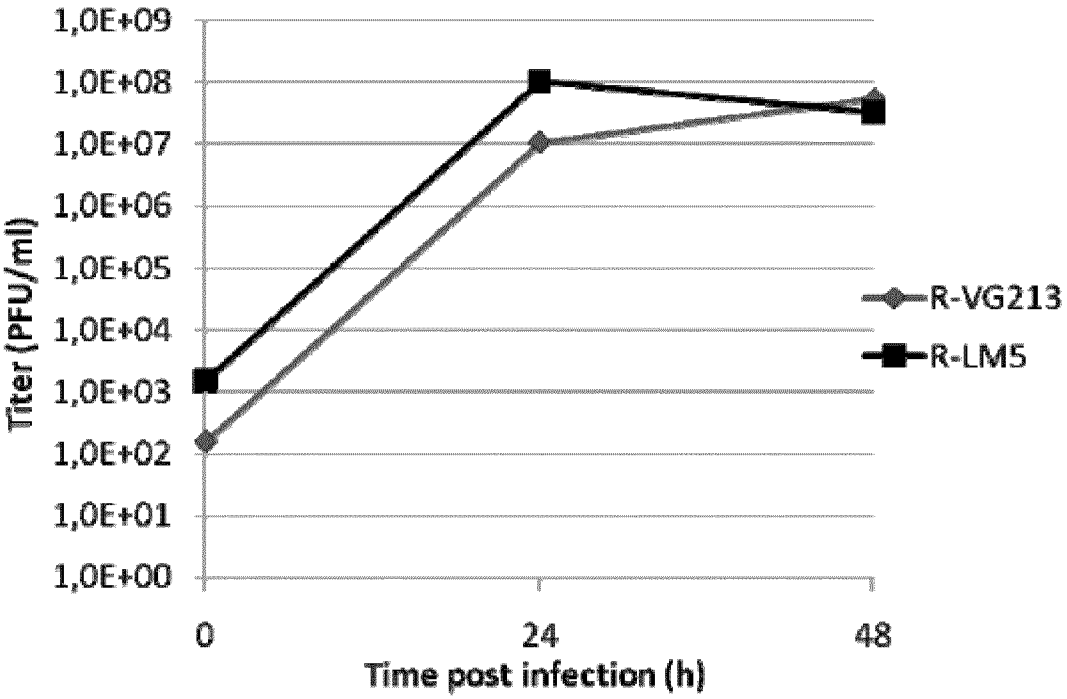

1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
1,0E+00
Titer (PFU/ml)
0
24
48
Time post infection (h)
R-VG213
R-LM5

Fig. 6

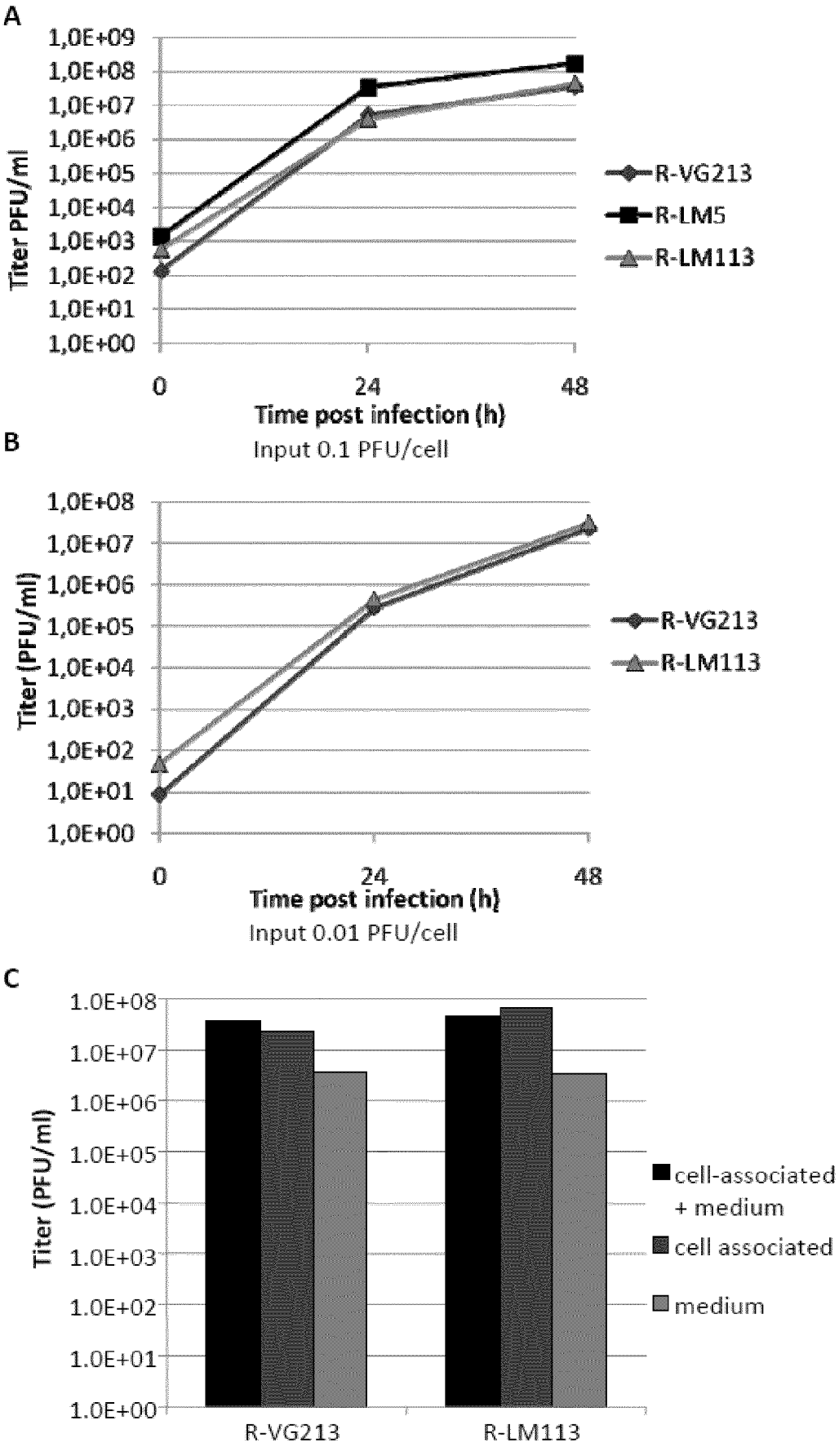
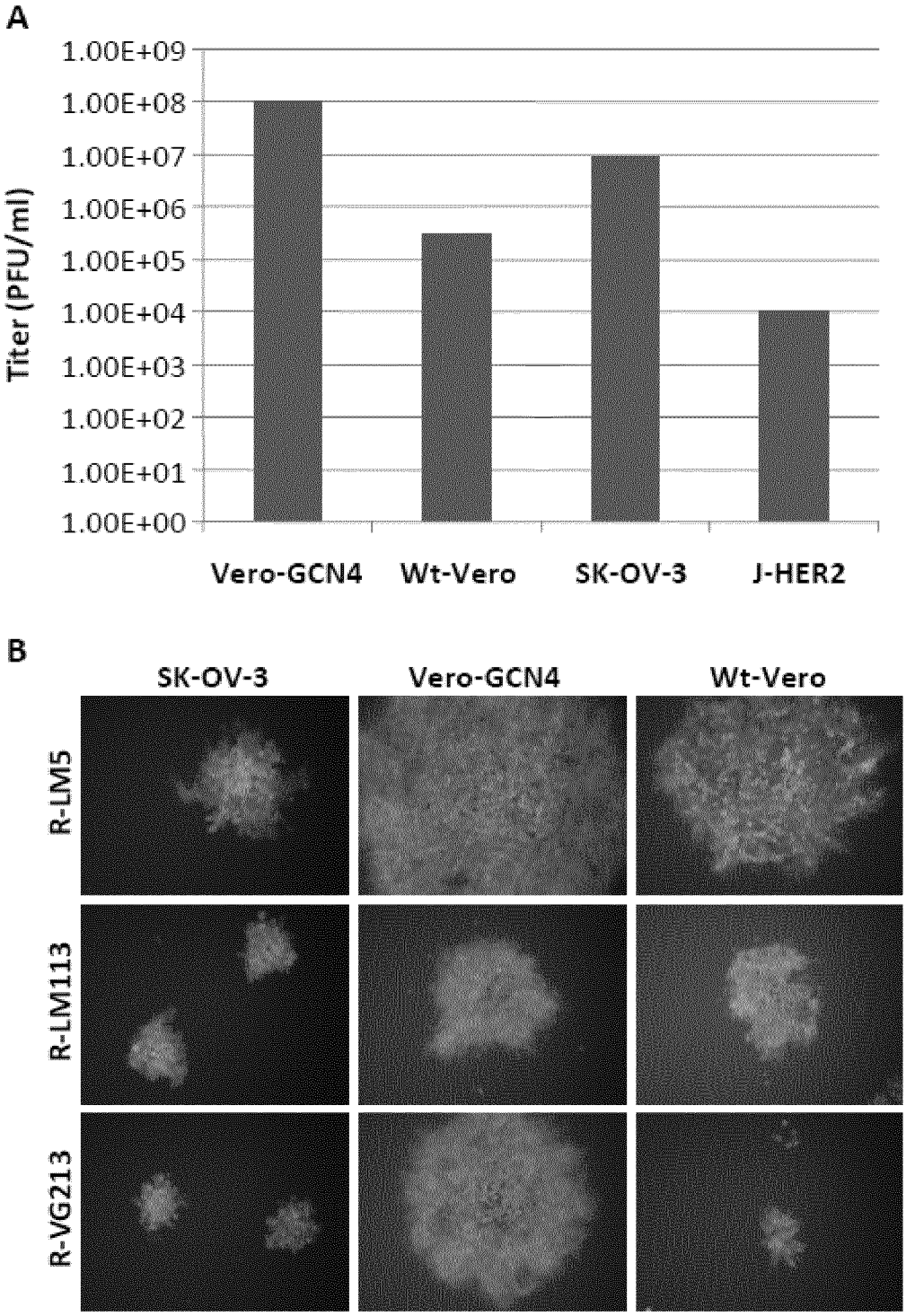

A
1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
1,0E+00
Titer PFU/ml
0
24
48
Time post infection (h)
R-VG213
R-LM5
R-LM113
Input 0.1 PFU/cell
B
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
1,0E+00
Titer (PFU/ml)
0
24
48
Time post infection (h)
R-VG213
R-LM113
Input 0.01 PFU/cell
C
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Titer (PFU/ml)
R-VG213
R-LM113
cell-associated + medium
cell associated
medium

Fig. 7


A
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Titer (PFU/ml)
Vero-GCN4
Wt-Vero
SK-OV-3
J-HER2
B
SK-OV-3
Vero-GCN4
Wt-Vero
R-LM5
R-LM113
R-VG213

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/063949

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/00 A61K35/763 C12N15/38 C07K14/03 C12N15/62
C12N5/10
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)
C12N C07K A61K

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, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	E. AVITABILE ET AL: "Complexes between Herpes Simplex Virus Glycoproteins gD, gB, and gH Detected in Cells by Complementation of Split Enhanced Green Fluorescent Protein", JOURNAL OF VIROLOGY., vol. 81, no. 20, 1 August 2007 (2007-08-01), pages 11532-11537, XP055319537, US ISSN: 0022-538X, DOI: 10.1128/JVI.01343-07 see fusion proteins gHN and gHC expressed in COS or 293T cells ----- -/--	13-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
8 September 2017	25/10/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brenz Verca, Stefano

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LORENTZEN E U ET AL: "Replication-competent herpes simplex virus type 1 mutant expressing an autofluorescent glycoprotein H fusion protein", INTERVIROLOGY, KARGER, CH, vol. 44, no. 4, 1 January 2001 (2001-01-01), pages 232-242, XP009185040, ISSN: 0300-5526 see the whole document, in particular Figure 1	1,2,5, 13-17
X	VALENTINA GATTA ET AL: "The Engineering of a Novel Ligand in gH Confers to HSV an Expanded Tropism Independent of gD Activation by Its Receptors", PLOS PATHOGENS, vol. 11, no. 5, 21 May 2015 (2015-05-21), page e1004907, XP055198421, DOI: 10.1371/journal.ppat.1004907 page 12, lines 14-17; figure 1	1,2,4,5, 7,11-17
X	T. M. CAIRNS ET AL: "Structure-Function Analysis of Herpes Simplex Virus Type 1 gD and gH-gL: Clues from gDgH Chimeras", JOURNAL OF VIROLOGY, vol. 77, no. 12, 15 June 2003 (2003-06-15) , pages 6731-6742, XP055198259, ISSN: 0022-538X, DOI: 10.1128/JVI.77.12.6731-6742.2003 see the whole document, in particular the cited passages; page 6735, left-hand column, paragraph 3; figures 1,5	1,2,5,6, 13-17
A	WO 2009/013448 A2 (CRUSADE LAB LTD [GB]; CONNER JOE [GB]) 29 January 2009 (2009-01-29) page 23, line 17 - page 30, last line; claims 16-19,22-25,27,29,30-34; figures 6, 20b page 59, paragraph 3 - page 60, paragraph 1	1-19
A	DOUGLAS J T ET AL: "A SYSTEM FOR THE PROPAGATION OF ADENOVIRAL VECTORS WITH GENETICALLYMODIFIED RECEPTOR SPECIFICITIES", NATURE BIOTECHNOLOGY, GALE GROUP INC, US, vol. 17, 1 May 1999 (1999-05-01), pages 470-475, XP002935956, ISSN: 1087-0156, DOI: 10.1038/8647 cited in the application the whole document	18,19

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/063949

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	VALERIO LEONI ET AL: "A Strategy for Cultivation of Retargeted Oncolytic Herpes Simplex Viruses in Non-cancer Cells", JOURNAL OF VIROLOGY., vol. 91, no. 10, 1 March 2017 (2017-03-01) , pages e00067-17, XP055388945, US ISSN: 0022-538X, DOI: 10.1128/JVI.00067-17 the whole document -----	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/063949

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009013448 A2	29-01-2009	US 2010272691 A1	28-10-2010
		WO 2009013448 A2	29-01-2009