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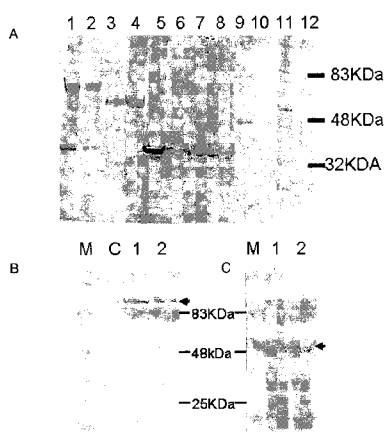


Figure 1

(57) Abstract: Herpes Simplex Viruses are disclosed having single-chain antibodies (scFv) embedded in the viral envelope via fusion with glycoprotein D and with glycoprotein H and L.

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Viruses

Field of the Invention

The present invention relates to Herpes Simplex Viruses, glycoproteins, including mutated glycoproteins and glycoprotein fusion molecules, and to HSV encoding such glycoproteins. More particularly, the invention relates to Herpes Simplex Viruses that are able to target specific cell types.

Background

Harnessing the cytopathic effects of virus infection is rapidly becoming established as an anti-cancer treatment and replication-competent mutants of the alpha-herpesvirus, herpes simplex virus type 1 (HSV-1), are showing great potential as oncolytic therapeutic agents.^{1, 2} The HSV-1 mutant 1716, lacking the ICP34.5 gene, has greatly reduced lethality in mice but replicates as efficiently as wild-type virus in actively dividing tissue culture cells.^{3, 4} The ICP34.5 ORF is a neurovirulence gene and its protein product has been proposed to condition post-mitotic cells for viral replication, probably via an interaction with proliferating cell nuclear antigen.^{5, 6} ICP34.5 deletion mutants cannot replicate in terminally differentiated cells but will lytically infect dividing cells and this has proved to be an effective tumour destruction strategy. In recent clinical trials, injection of HSV1716 has been shown to be safe in treating patients with recurrent glioma, metastatic melanoma and squamous cell carcinoma of the head and neck (Mace et al unpublished results) and proof of principle of selective replication within tumours has been obtained.⁷⁻¹⁰

Initiation of infection by HSV-1 requires cells to display the appropriate receptors to permit viral access, a process requiring the complex interplay of a number of cellular and viral membrane components.^{37, 38} Four virus membrane glycoproteins, gB, gD and the heterodimer comprising gH and gL

(gH/gL) have been shown to be necessary and sufficient for HSV-1 entry into cells. Initial contact is between gB and cellular heparan sulphate, gD then interacts specifically with the cellular receptors for HSV-1 entry which include herpesvirus entry mediator (HVEM), nectins-1 and -2 and 3-O-sulphated heparan sulphate. Membrane fusion requires the concerted activities of gB and gH/gL so that the nucleocapsid gains access to the cell and infection is initiated. Nectin-1 is probably the principal entry receptor for infection of central and peripheral nerve cells whereas HVEM expression is more restricted and limited to cells of lymphoid origin.³⁹⁻⁴³

An essential strategy for the improved effectiveness of oncolytic viral therapies depends upon systemic delivery of targeted viruses that seek out and destroy all cancerous cells.^{2, 11, 12} However, wide bioavailability of HSV-1 entry mediators will hinder the ability of systemically administered oncolytic HSV1716 to efficiently target the cells of interest.

Reprogramming viral tropisms has thus received much attention, an approach requiring redirection of the natural tropism from native receptors to a receptor of choice. Targeted infection by vaccinia virus, retrovirus and measles virus, displaying single chain antibody binding sites incorporated into their structure by fusion with viral envelope proteins has been described.^{13-21, 55} Bridging, bispecific targeting molecules comprising either an anti-adenovirus single chain antibody or the Coxsackievirus-adenovirus receptor linked to a targeting single chain antibody, have been used for targeting oncolytic adenoviruses.²²⁻²⁸

Results from a number of studies with HSV-1 have shown that it is possible to alter the tropism by incorporating ligands such as erythropoietin, IL13, human hepatitis B virus preS1 peptide, the N-terminal fragment of urokinase-type plasminogen activator or 6-His into the viral envelope as glycoprotein fusion proteins.^{29-34, 56, 57}

Targeting has also been achieved using a soluble adaptor molecule, in which the soluble adaptor molecule includes an EGFR scFv linked to the HSV-1 gD-binding domain of nectin-1. More recently, a scFv against HER2/neu, inserted within an N terminal region of gD, has been shown to redirect the tropism of HSV-1 to this mammary tumour receptor.^{35, 36} HSV capable of targeting cells and tissues is also described in WO 03/068809.

Summary of the invention

Systemic delivery of HSV oncolytic viral therapies requires that the oncolytic virus, once administered, is able to target tumour cells. If the systemically administered oncolytic virus shows only low specificity for the target tumour cells such treatment may not be optimally effective.

As far as the inventors are aware, studies to date have merely shown that the tropism of HSV may be redirected and have not provided any strategy for improving redirection. In view of the therapeutic potential of systemically delivered oncolytic viral therapies, there is a clear need for alternative oncolytic viral agents that are able to redirect tropism more efficiently towards tumour cells.

We have investigated the possibility of redesigning tropism of HSV by embedding single-chain antibodies (scFv) in the viral envelope via fusion with glycoprotein D and with glycoprotein H and L. We demonstrate here that scFv linked to the N-terminus of truncated glycoprotein D molecules is specifically able to influence the tropism of HSV towards cells displaying tumour markers. In particular, we have identified a short glycoprotein D truncate which efficiently redirected HSV infectivity, thereby providing a route for the development of effective tumour-targeted HSV for systemic delivery.

We investigated the ability of scFvs incorporated into the viral envelope to alter the tropism of herpes simplex virus

HSV1716. Fusion proteins were created by N-terminally linking a scFv that recognises the ganglioside GD3 to 15 sequentially deleted gD polypeptides and to both gH and gL in the gH/gL heterodimer. Vero cell lines stably expressing the scFv/glycoprotein fusions were infected with an HSV1716 variant expressing gfp and the efficiencies of the resultant viruses displaying the fusion proteins to infect non-permissive GD3-positive CHO cells were assessed by fluorescence microscopy and Western blotting. Viruses displaying scFv/gD or the scFv/gH/gL heterodimers were able to infect the normally non-permissive CHO cells. Further, our observations identified a gD fragment comprising amino acids 274-393 which was most efficient at redirecting viral tropism.

We subsequently created a number of HSV1716 variants that expressed different scFv targeting moieties linked to the gD fragments that were most efficient at redirecting viral tropism. Using non- or semi-permissive cell lines, we demonstrated that the tropism of these HSV1716 variants was modified such that infection was mediated via the cognate antigen.

We also performed in vivo experiments using mice in which subcutaneous tumours were established using the human epidermoid carcinoma cell line, A431. The mice were systemically injected with an HSV-1 variant which expressed anti-epidermal growth factor receptor (EGFR) scFv linked to the gD fragment comprising amino acids 274-393. We found that mice receiving this HSV-1 variant had more slowly growing tumours than those receiving HSV1716, indicating that the targeted HSV-1 variant had greater therapeutic benefit than the non-targeted HSV1716.

Thus, a redirection of HSV1716 tropism to specific cell types through the display of tumour targeting scFv moieties linked to particular glycoproteins and variants thereof identifies an efficient route for systemic delivery.

Accordingly, in a broad aspect, the invention provides a herpes simplex virus comprising an N-terminally truncated glycoprotein D, which truncated glycoprotein D is linked to a targeting agent.

The surprising discovery that N-terminally truncated glycoprotein D molecules linked to a targeting agent are able to efficiently redirect the tropism of HSV provides a new family of HSVs showing redirected tropism, and opens up a new route for the development of effective anti-tumour agents.

Structurally, HSV-1 glycoprotein D has an extracellular domain consisting of 315 amino acids, with binding to HVEM requiring residues 1 to 34 and binding to nectin-1 requiring residues 34 to 243. ⁴⁸⁻⁵¹

The inventors have demonstrated that a glycoprotein D lacking amino acids 1 to 36 and bound to a scFv is able to redirect HSV tropism. As binding to HVEM requires amino acids 1 to 34, this glycoprotein D will have an impaired ability to bind HVEM. The inventors have also demonstrated that a glycoprotein D lacking amino acids 1 to 259 and bound to a scFv is also able to redirect HSV tropism. As binding to nectin-1 requires amino acids 34 to 243, this glycoprotein D will have an impaired ability to bind nectin-1.

According to a first aspect of the invention, there is provided a HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain. Preferably, the glycoprotein D also does not include a functional nectin-1 binding domain.

In a further aspect of the invention, there is provided a HSV comprising nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-

terminally truncated glycoprotein D does not include a functional HVEM binding domain. Preferably the glycoprotein D also does not include a functional nectin-1 binding domain. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In lacking a functional HVEM binding domain and/or a nectin-1 binding domain, the truncated glycoprotein D has a reduced affinity, or no specific affinity, for HVEM and/or nectin-1. The affinity of the truncated glycoprotein D for HVEM and/or nectin-1 may be reduced such that the HSV comprising the truncated glycoprotein D is not capable of entering a cell by a mechanism which involves binding of glycoprotein D to HVEM and/or nectin-1. For example, the truncated glycoprotein D may not be able to mediate entry into cells expressing HVEM and/or nectin-1 as the sole receptor.

Binding of the truncated glycoprotein D to HVEM and/or nectin-1 may be measured, for example, by using ELISA. This may involve coating a surface with HVEM and/or nectin-1, adding the truncated glycoprotein D, and then detecting glycoprotein D bound to the surface. Preferably, the binding affinity of the truncated glycoprotein D for HVEM and/or nectin-1 is less than 50%, 40%, 30%, 20%, 10%, 5%, 3% or even less than 1% of the affinity of wild-type glycoprotein D, e.g. glycoprotein D having the sequence of SEQ ID NO: 1, for HVEM and/or nectin-1. Binding of the truncated glycoprotein D may be determined with or without the targeting agent linked to the truncated glycoprotein D. Preferably the truncated glycoprotein D has no specific affinity for HVEM and/or nectin-1.

The ability of the truncated glycoprotein D to mediate cell entry via binding to HVEM and/or nectin-1 may be determined by preparing a HSV comprising the truncated glycoprotein D, with or without the targeting agent linked to the glycoprotein D. The HSV may then be used to infect cells, e.g. CHO cells, that

express only HVEM and/or nectin-1 as a receptor for the glycoprotein D. The level of infection may be measured by fluorescence microscopy. Assays for determining infection of cells by HSV by fluorescence microscopy are described below. Preferably the ability of the truncated glycoprotein D to mediate cell entry via binding to HVEM and/or nectin-1 is less than 50%, 40%, 30%, 20%, 10%, 5%, 3% or even less than 1% of the ability of wild-type glycoprotein D, e.g. glycoprotein D having the sequence of SEQ ID NO: 1. Preferably, the truncated glycoprotein D has no ability to mediate cell entry via binding to HVEM and/or nectin-1.

Structural studies of glycoprotein D binding to HVEM have identified critical regions at the binding interface. In particular, three glycoprotein D mutations were identified that each resulted in the complete loss of HVEM binding to glycoprotein D and the failure to mediate HSV entry into cells expressing HVEM. These mutations were Q27A, T29A and D30A. Preferably the truncated glycoprotein D has an inactivating mutation at each of these amino acid positions, i.e. 27, 29, and 30, or more preferably, these residues are absent from the truncated gD.

The normal route by which HSV-1 infects a cell requires a complex interplay of a number of cellular and viral membrane components, including the specific interaction of glycoprotein D with HVEM and nectin-1. Nectin-1 is thought to be the principal entry receptor for infection of central and peripheral nerve cells whereas HVEM expression is more restricted and limited to cells of lymphoid origin. Thus, truncating glycoprotein D such that it does not include a functional HVEM and/or nectin-1 binding domain will reduce the ability of HSV to infect its normal target. This will increase the specificity of the HSV away from its normal target and towards cells expressing the surface antigens for which the targeting agent linked to the truncated glycoprotein D is specific, e.g. tumour cells.

In a further aspect of the invention, there is provided a HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a portion of amino acids corresponding to amino acids 1 to 34 of the full-length HSV-1 glycoprotein D.

In a further aspect of the invention, there is provided a HSV comprising nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a portion of amino acids corresponding to 1 to 34 of the full-length HSV-1 glycoprotein D. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

Preferably, the truncated glycoprotein D does not include amino acids 1 to 35, more preferably amino acids 1 to 36 of the full-length glycoprotein D.

Thus, the position of the truncation may be at least at a position corresponding to position 35 of the full-length HSV-1 glycoprotein D, for example at least at a position corresponding to position 36, or at least at a position corresponding to position 37. A truncation at least at a position corresponding to position 35, 36, or 37 means a truncation at a position corresponding to position 35, 36, 37, respectively, or at a position corresponding to a position in the full-length HSV-1 glycoprotein D that is closer to the C-terminus than position 35, 36, or 37, respectively. Preferably the position of the truncation is in the glycoprotein D extracellular domain.

An amino acid "position" refers to the position of the amino acid in the polypeptide chain, e.g. the polypeptide chain of

glycoprotein D. Unless otherwise indicated, amino acids are numbered such that the N-terminal amino acid is at the first position. For example, an amino acid at position 10 refers to the 10th amino acid in the polypeptide chain counting from the N-terminus.

The "N-terminal" amino acid is the amino acid at the amino-terminal end of the polypeptide of interest. Likewise, the "C-terminal" amino acid is the amino acid at the carboxyl-terminal end of the polypeptide of interest. Thus, an amino acid on the C-terminal side of a position of interest refers to an amino acid that has a position that is closer to the C-terminus of the polypeptide. Likewise, an amino acid on the N-terminal side of a position of interest refers to an amino acid that has a position that is closer to the N-terminus of the polypeptide.

An amino acid in an N-terminally truncated glycoprotein D that corresponds to an amino acid at a position of interest in the full-length HSV-1 glycoprotein D is an amino acid that occupies an equivalent position in the truncated glycoprotein D polypeptide chain compared to the full-length HSV-1 glycoprotein D polypeptide chain. Such corresponding amino acids may be identified by standard amino acid sequence alignments. Preferably corresponding amino acids are identified by aligning the extracellular domain of the N-terminally truncated glycoprotein D with the full-length HSV-1 glycoprotein D.

Methods of performing alignments are well known in the art, e.g. BLAST. The amino acid in the truncated polypeptide that is paired by the alignment with the amino acid at the position of interest in the full-length HSV-1 glycoprotein D sequence is the amino acid in the truncated glycoprotein D that corresponds to the amino acid position of interest.

A truncation at a position corresponding to amino acid position "X" in the full length HSV-1 glycoprotein D means that the amino acid corresponding to position X has been retained in the truncated glycoprotein D, and that all the amino acids on the N-terminal side of this amino acid have been deleted. For example, if the position of the truncation corresponds to amino acid position 37 of the full-length HSV-1 glycoprotein D, then the amino acid corresponding to position 37 is retained in the truncated polypeptide, whereas all amino acids on the N-terminal side of this amino acid are deleted, i.e. the amino acids corresponding to 1-36. For example, the amino acids corresponding to position 37 through to the C-terminal amino acid, e.g. position 393, are retained in the truncate.

Nucleic acid encoding any fusion protein of the invention is preferably inserted in the HSV genome such that it is capable of being transcribed. The nucleic acid may be operably linked to a suitable regulatory sequence (e.g. a promoter), the activity of which may be constitutive or may be controlled by the availability of a signal molecule. The promoter may be, for example, an endogenous HSV promoter such as the gC promoter, or a non endogenous promoter such as the CMV-IE promoter.

As reported here, we have also discovered that a truncated glycoprotein D truncated at position 274 and linked to a targeting agent retains the ability to redirect the tropism of HSV. Moreover, we found that, of the truncates tested, targeting agents linked to glycoprotein D truncated at position 260 and 274 were most efficient at redirecting viral tropism. For example, HSV comprising R24 scFv linked to these truncates was able to infect 60% and 50%, respectively, of normally non-permissive CHO cells.

Thus, the truncated glycoprotein D may have an extracellular domain comprising at least 35 amino acids. The 35 amino acids

are preferably the portion of amino acids in the extracellular domain of the full-length HSV-1 glycoprotein D that includes the C-terminal amino acid in the extracellular domain of the HSV-1 full-length glycoprotein D.

Preferably, the truncated glycoprotein D has an extracellular domain comprising at least 36, 37, 38, 39, 40 or 41 amino acids, most preferably at least 42 amino acids. The 36, 37, 38, 39, 40, 41, or 42 amino acids are preferably the portion of amino acids in the extracellular domain of the full-length HSV-1 glycoprotein D, which portion includes the C-terminal amino acid of the extracellular domain of the full-length HSV-1 glycoprotein D.

The HSV-1 glycoprotein D used in the experiments reported below has 315 amino acids in its extracellular domain. Thus, the glycoprotein D truncated at position 274 retained 42 amino acids in its extracellular domain.

Our discovery that a glycoprotein D truncated at position 274 was capable of efficiently redirecting tropism was particularly surprising. Previous studies have suggested that amino acids 261-305 form a distinct domain, the "profusion domain", which is necessary for mediating the membrane fusion event required for penetration of the HSV-1 nucleocapsid into the cell¹². The 274 truncate lacks a portion of the profusion domain, yet is still capable of mediating cell entry when linked to a targeting agent.

The truncated glycoprotein D may have an extracellular domain comprising a portion of amino acids corresponding to amino acids 280-315 of the full-length HSV-1 glycoprotein D. For example, the truncated glycoprotein D may have an extracellular domain comprising a portion of amino acids corresponding to amino acids 279-315, 278-315, 277-315, 276-315, or 275-315, preferably 274-315 of the full-length HSV-1 glycoprotein D. Preferably said portion of amino acids of the

truncated glycoprotein D includes an amino acid corresponding to the C-terminal amino acid of the extracellular domain of the full-length HSV-1 glycoprotein D.

The position of truncation may correspond to an amino acid position from 240-280 of the full-length HSV-1 glycoprotein D. For example, the position of truncation may correspond to amino acid position 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, or 280 of the full-length HSV-1 glycoprotein D. The position of the truncation may correspond to an amino acid position from 244-280, 244-279, 244-278, 244-277, 244-276, 244-275, or 244-274 of the full-length glycoprotein D. A glycoprotein D truncated at least at a position corresponding to position 244 will lack the entire HVEM and nectin-1 binding domains.

The position of the truncation may correspond to an amino acid position from 260-280, 260-279, 260-278, 260-277, 260-276, or 260-275 of the full-length HSV-1 glycoprotein D, for example from 250-274, 251-274, 252-274, 253-274, 254-274, 255-274, 256-274, 257-274, 258-274, or 259-274 of the full-length HSV-1 glycoprotein D, for example from 250-280, 253-279, 255-278, 257-277, 258-276, or 259-275 of the full-length HSV-1 glycoprotein D.

The position of the truncation may correspond to an amino acid position from 260-274 of the full-length HSV-1 glycoprotein D. Thus, the truncated glycoprotein D may not include amino acids corresponding to the amino acids at positions 1-259 of the full-length HSV-1 glycoprotein D. In particular, the position of the truncation may correspond to the amino acid at position 260 or 274 of the full-length HSV-1 glycoprotein D.

We also report here that truncating the glycoprotein D at position 37, 78, 92, and 128 also leads to redirected viral tropism.

Thus, the position of truncation may correspond to an amino acid position from 35-138 of the full-length HSV-1 glycoprotein D. For example, the position of truncation may correspond to amino acid position 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, or 138 of the full-length HSV-1 glycoprotein D. However, preferably the position of truncation does not correspond to position 58 or 105 of the full-length HSV-1 glycoprotein D.

The position of truncation may correspond to an amino acid position from 35-138, 35-137, 35-136, 35-135, 35-134, 35-133, 35-132, 35-131, 35-130, 35-129 or 35-128 of the full-length glycoprotein D; for example from 36-138, 36-137, 36-136, 36-135, 36-134, 36-133, 36-132, 36-131, 36-130, 36-129, or 36-128, of the full-length glycoprotein D; for example from 37-138, 37-137, 37-136, 37-135, 37-134, 37-133, 37-132, 37-131, 37-130, or 37-129 of the full-length HSV-1 glycoprotein D.

The position of the truncation may correspond to an amino acid position from 37 to 128 of the full-length polypeptide. In particular, the position of the truncation may correspond to the amino acid at position 37, 78, 92, or 128 of the full-length HSV-1 glycoprotein D.

The position of the N-terminal truncation is preferably not at an amino acid position corresponding to position 139, 164, 179, 191, 207, 231 and/or 239 of the full-length HSV-1

glycoprotein D. More preferably, the N-terminal truncation is not at an amino acid position corresponding to a position from 139-239 of the full-length HSV-1 glycoprotein D.

In addition, the position of the truncation may not correspond to an amino acid position from 129-138 and/or from 240-259 of the full-length HSV-1 glycoprotein D. For example, the position of truncation may not be at an amino acid position corresponding to position 138, 137, 136, 135, 134, 133, 132, 131, 130, or 129 of the full-length HSV-1 glycoprotein D. Likewise, the position of the truncation may not be at an amino acid position corresponding to position 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, or 259 of the full-length HSV-1 glycoprotein D. For example, the position of the truncation may not be at an amino acid position corresponding to a position from 137-243, 135-247, 133-251, 131-255, or 129-259 of the full-length HSV-1 glycoprotein D.

In all aspects and embodiments of the invention, the term "full-length HSV-1 glycoprotein D" refers to the polypeptide sequence of an endogenous HSV-1 glycoprotein D in the form in which it is presented in the viral envelope, e.g. without any N-terminal signal sequence or N-terminal methionine that is normally removed during post-translational modification. Preferably the term refers to the sequence of the HSV-1 strain 17 glycoprotein D, i.e. the glycoprotein D sequence having accession no. X14112.1 (GI:1944536), which is SEQ ID NO: 1.

However, the N-terminally truncated glycoprotein D molecules defined above are not limited to truncates of any particular glycoprotein D. Rather, the invention covers N-terminally truncated glycoprotein D molecules derived from any glycoprotein D of any strain of HSV. For example, the truncated glycoprotein may be derived from any of the following glycoprotein D NCBI database entries:

Human HSV-1: NC_001806.1 GI:9629378; AF487902.1 GI:19548970;
U03045.1 GI:436475; K02372.1 GI:330102; J02217.1 GI:330100;
AAL90884.1 GI:19548971, P36318 GI:549371; AAA45785.1
GI:330101; AF487902.1 GI:19548970; AF487901.1 GI:19548968;
AF487901.1 GI:19548968; J02217.1 GI:330100; AAL90883.1
GI:19548969; CAA38245.1 GI:60415; NP_044668.1 GI:9629447.

Human HSV-2: Z86099.2 GI:6572414, D00026.1 GI:221778;
NC_001798.1 GI:9629267; AY779754.1 GI:56698871; AY779753.1
GI:56698869; AY779752.1 GI:56698867; AY779751.1 GI:56698865;
AY779750.1 GI:56698863; E00394.1 GI:2168679; AAA98962.1
GI:409565; AAW23134.1 GI:56698872; AAW23133.1 GI:56698870;
AAW23132.1 GI:56698868; NP_044536.1 GI:9629336; NP_044537.1
GI:9629337

Equine HSV-1: AAS45960.1 GI:42795203; AY464052.1
GI:42795127.

Gallid HSV-2: DQ530348.1 GI:104303917.

Felid HSV-1: D42113.1 GI:893368.

Other glycoprotein D may be identified using the hybridisation and probe techniques as described below. However, it is preferred that the N-terminally truncated glycoprotein D molecules of the invention are truncates of any of the above glycoprotein D molecules for which a database accession number is provided. More preferably, the N-terminally truncated glycoprotein D molecules of the invention are truncates of glycoprotein D from human strains of HSV, in particular HSV-1 or HSV-2 glycoprotein D, more preferably HSV-1. Most preferably the truncate is derived from the glycoprotein D of HSV-1 strain 17 or F. The N-terminally truncated glycoprotein D is preferably an N-terminal truncate of a glycoprotein D having the amino acid sequence of SEQ ID NO: 1. Accordingly, the N-terminally truncated glycoprotein D may be derived from the glycoprotein D encoded by the nucleic acid of SEQ ID NO: 4. In this case, nucleic acid encoding the truncated glycoprotein D may comprise the portion of the nucleic acid

sequence of SEQ ID NO: 4 that encodes the truncated glycoprotein D.

Where, for example, the truncated glycoprotein D is derived from a glycoprotein D that has a different number of amino acids to the HSV-1 glycoprotein D, or a different amino acid sequence, corresponding amino acids may be identified by aligning the sequence with the full length HSV-1 glycoprotein D sequence using an alignment program, such as BLAST.

The N-terminally truncated glycoprotein D of the invention may also include amino acids corresponding to those of the transmembrane domain and/or the intracellular domain of the full-length HSV-1 glycoprotein D, i.e. amino acids corresponding to amino acids 316-393 of the full-length HSV-1 glycoprotein D. Preferably the truncated glycoprotein D comprises the transmembrane domain and intracellular domain of SEQ ID NO: 1, i.e. amino acids 316-393 of SEQ ID NO: 1.

In addition to the truncated glycoprotein D molecules of the invention, the HSV may, or may not, also comprise wild-type glycoprotein D, i.e. the full-length glycoprotein D without being linked to a targeting agent. In both cases the HSV will have a reduced affinity for its natural targets, e.g. HVEM and nectin-1. HSV that does not comprise wild-type glycoprotein D will have a restricted tropism, i.e. the absence of wild-type glycoprotein D means that the virus will have a reduced affinity, or no specific affinity, for the HVEM or nectin-1 binding receptors. The HSV would be dependent solely upon the truncated glycoprotein D linked to a targeting agent for infection. HSV comprising the truncated glycoprotein D molecules of the invention in addition to wild-type glycoprotein D will have an expanded tropism, i.e. an expanded repertoire of receptor binding capacity, due to the presence of the targeting agent linked to the truncated glycoprotein D. Any affinity of the HSV for HVEM and/or nectin-1 due to the presence of wild-type glycoprotein D will be diluted by

additional affinity for the ligand of the targeting agent. Also, assimilation of the truncated glycoprotein D into the viral envelope will compete with wild-type glycoprotein D incorporation leading to a reduction in the amount of wild-type glycoprotein D and reduced HVEM/nectin-1 binding capacity. This will reduce the affinity of the HSV for its natural target when the ligand of the targeting agent is present. The advantage of retaining the wild-type glycoprotein D in the HSV is that such a virus would be propagated readily in tissue culture whilst at the same time it would have greater specificity for a specific tumour type.

In a further aspect of the invention, there is provided a fusion protein comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain.

In a further aspect of the invention, there is provided a fusion protein comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D. Alternatively, the fusion protein may comprise any truncated glycoprotein D described above.

In a further aspect of the invention, there is provided a nucleic acid molecule comprising nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain.

In a further aspect of the invention, there is provided a nucleic acid molecule comprising nucleic acid encoding a

fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D. Alternatively, the fusion protein may comprise any truncated glycoprotein D described above.

Nucleic acid encoding a fusion protein may be arranged in the nucleic acid molecule such that it is operably linked to a suitable regulatory sequence (e.g. a promoter). A promoter may be constitutive or may be controlled by the availability of a signal molecule. The promoter may be, for example, an endogenous HSV promoter such as the gC promoter, or a non endogenous promoter such as the CMV-IE promoter. The nucleic acid molecule may also include nucleic acid for facilitating insertion of the nucleic acid encoding the fusion protein into the genome of a HSV. Said insertion may be, for example, by homologous recombination, or by using a site specific recombination system, such as the Gateway® Vector Conversion System (Invitrogen, Paisley, UK).

In a further aspect of the invention, there is provided a HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain, for use in a therapeutic method of treatment.

In a further aspect of the invention, there is provided a HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D, for use in a therapeutic method of treatment. Alternatively, the truncated glycoprotein D may be any truncated glycoprotein D described above.

In a further aspect of the invention, there is provided a HSV comprising nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain, for use in a therapeutic method of treatment. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In a further aspect of the invention, there is provided a HSV comprising nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D, for use in a therapeutic method of treatment. Alternatively, the truncated glycoprotein D may be any truncated glycoprotein D described above. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

Preferably the therapeutic method of treatment is a method for the treatment of a disease associated with the proliferation of cells, e.g. for treatment of tumour.

In a further aspect of the invention, there is provided use of a HSV in the manufacture of medicament for the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain,

In a further aspect of the invention, there is provided use of a HSV in the manufacture of a medicament for the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D. Alternatively, the truncated glycoprotein D may be any truncated glycoprotein D described above.

In a further aspect of the invention, there is provided use of a HSV in the manufacture of a medicament for the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In a further aspect of the invention, there is provided use of a HSV, in the manufacture of a medicament for the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises a nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D. Alternatively, the truncated glycoprotein D may be any truncated glycoprotein D described above. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In a further aspect of the invention, there is provided a method of treating a disease associated with the proliferation of cells, comprising administering a HSV, which HSV comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain.

In a further aspect of the invention, there is provided a method of treating a disease associated with the proliferation of cells, comprising administering a HSV, which comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D. Alternatively, the truncated glycoprotein D may be any truncated glycoprotein D described above.

In a further aspect of the invention, there is provided a method of treating a disease associated with the proliferation of cells, comprising administering a HSV, which HSV comprises nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain and may not include a functional nectin-1 binding domain. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In a further aspect of the invention, there is provided a method of treating a disease associated with the proliferation of cells, comprising administering a HSV, which HSV comprises nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include amino acids 1 to 34 of the full-length HSV-1 glycoprotein D. Alternatively, the

truncated glycoprotein D may be any truncated glycoprotein D described above. The nucleic acid is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In a further aspect of the invention, there is provided a method of producing a HSV, which HSV comprises a N-terminally truncated glycoprotein D linked to a targeting agent, wherein the targeting agent is linked to the N-terminally truncated glycoprotein D as a fusion protein, said method comprising infecting a cell line with said HSV that expresses the fusion protein. The truncated glycoprotein D may be any truncated glycoprotein D described above.

In a further aspect of the invention, there is provided a method of producing an HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the targeting agent is linked to the N-terminally truncated glycoprotein D as a fusion protein, said method comprising incorporating nucleic acid encoding said fusion protein into the viral genome of the modified HSV. The truncated glycoprotein D may be any truncated glycoprotein D described above.

In a further aspect of the invention, there is provided an HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent,

wherein the position of the truncation is at least at a position corresponding to position 37 of the full-length HSV-1 glycoprotein D,

wherein the extracellular domain of the N-terminally truncated glycoprotein D comprises a portion of amino acids corresponding to amino acids 274-315 of the full-length HSV-1 glycoprotein D,

and wherein the position of the truncation is not at an amino acid position corresponding to a position from 139-239 of the full-length HSV-1 glycoprotein D.

In a further aspect of the invention, there is provided a HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent,

wherein the position of truncation is at an amino acid position corresponding to a position from 37-128 or from 260-274 of the full-length HSV-1 glycoprotein D.

In all aspects of the invention, the term "N-terminally truncated glycoprotein D" refers to a glycoprotein D that does not include an amino acid corresponding to the N-terminal residue of the full-length HSV-1 glycoprotein D. For example, the N-terminally truncated glycoprotein D is derived from a non-N-terminally truncated glycoprotein D, e.g. the full-length HSV-1 glycoprotein D, by the deletion of at least the N-terminal amino acid. Usually a portion of amino acids will have been deleted, such that an N-terminal region of amino acids is missing compared to the parent glycoprotein D.

As discussed above, we also investigated the possibility of redesigning tropism of HSV by embedding single-chain antibodies (scFv) in the viral envelope via fusion with glycoproteins H and L. Glycoproteins H and L form a heterodimer with both proteins required for its correct folding and membrane presentation. We demonstrate here that linking an scFv to the N terminus of glycoprotein H and another scFv to the N-terminus of glycoprotein L redirects the tropism of HSV.

To the best of our knowledge, this is the first description of the construction of receptor-binding fusion proteins based on the glycoprotein H/glycoprotein L heterodimer. This discovery thus opens up an unexpected route for redirecting the tropism of HSV.

Accordingly, in a further aspect of the invention, there is provided a herpes simplex virus (HSV) comprising:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target.

In a further aspect of the invention, there is provided a HSV comprising:

- a. nucleic acid encoding a first fusion protein, which first fusion protein comprises a glycoprotein H linked to a first targeting agent, and
- b. nucleic acid encoding a second fusion protein, which second fusion protein comprises a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target. The nucleic acid encoding the first fusion protein and/or the nucleic acid encoding the second fusion protein is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

The first targeting agent is preferably linked to the N-terminus of the glycoprotein H polypeptide, optionally with one or more spacer molecules between the N-terminus and the first targeting agent. Likewise, the second targeting agent is preferably linked to the N-terminus of the glycoprotein L, optionally with one or more spacer molecules between the N-terminus and the second targeting agent. However, it is preferred that there is no spacer molecule between the glycoprotein H and the targeting agent. Without being bound by theory, it was thought that a spacer between the glycoprotein L and the scFv might provide a flexible arm allowing the scFv at the N terminus of glycoprotein L to interact and dimerise with the scFv at the N terminus of glycoprotein H.

The spacer molecule may be any molecule that includes appropriate chemical functional groups to allow its attachment to both the glycoprotein and a targeting agent. For example, a spacer molecule may be a peptide. A suitable peptide may be Gly-Gly-Gly-Gly-Ser. One or more of these molecules may be inserted between the glycoprotein N-terminus and a targeting agent.

Preferably a spacer molecule is between the N-terminus of the glycoprotein L polypeptide and the second targeting agent. The spacer molecule may be a 1-20 amino acid polypeptide, a 1-15 amino acid polypeptide, a 1-10 amino acid polypeptide, or even a 1-5 amino acid polypeptide. For example, the spacer molecule may be a 3-18 amino acid polypeptide, a 5-15 amino acid polypeptide, or a 5-10 amino acid polypeptide. The spacer molecule may be a polypeptide comprising or consisting of 1 or more units of the polypeptide Gly-Gly-Gly-Gly-Ser, for example, 1, 2, 3, or 4 units. Preferably the spacer molecule comprises or consists of 1 or 2 units of Gly-Gly-Gly-Gly-Ser, most preferably 1 unit.

Nucleic acid encoding any fusion protein of the invention may be inserted in the HSV genome such that it is capable of being transcribed. The nucleic acid may be operably linked to a suitable regulatory sequence (e.g. a promoter), the activity of which may be constitutive or may be controlled by the availability of a signal molecule. The promoter may be, for example, an endogenous HSV promoter such as the gC promoter, or a non endogenous promoter such as the CMV-IE promoter.

The glycoprotein H and glycoprotein L molecules are not limited to any particular glycoprotein H and glycoprotein L, although preferably they are capable of forming a heterodimer, e.g. they are derived from the same species of HSV. Rather, the invention covers any glycoprotein H and glycoprotein L, such as the following list of glycoprotein NCBI database entries:

Glycoprotein H:

Human HSV-1: NC_001806.1 GI:9629378; AAG17895.1 GI:10444402
 Human HSV-2: NC_001798.1 GI:9629267; Z86099.2 GI:6572414
 Human HSV-5: YP_081523.1 GI:52139248
 Human HSV-6: X83413.1 GI:853961; X64320.1 GI:296190;
 AB021506.1 GI:4995977
 Ovine HSV-2: NC_007646.1 GI:83642839; AY839756.1 GI:61970953
 Gallid HSV-2: DQ530348.1 GI:104303917
 Bovine HSV-1: Z78205.1 GI:1491620
 Equine HSV-1: AY464052.1 GI:42795127
 Murid HSV-1: NC_004065.1 GI:21716071
 Alcelaphine HSV-1: NC_002531.1 GI:10140926
 Suid HSV-1: DQ993360.1 GI:116272035
 Saimiriine HSV-2: D00400.1 GI:221845
 Cercopithecine HSV-9: AAB04139.1 GI:833831

Glycoprotein L:

Human HSV-1: NP_044602.1 GI:9629381;
 Human HSV-2: NP_044470.1 GI:9629270
 Human HSV-5: YP_081555.1 GI:52139280; AAR31659.1 GI:39842115
 Human HSV-6: NC_001664.1 GI:9628290
 Murid HSV-1: NC_004065.1 GI:21716071

Other glycoprotein H and L may be identified using the hybridisation and probe techniques as described below. However, it is preferred that the glycoprotein H and L of the invention are glycoprotein H and L for which a database accession number is provided. More preferably, the glycoprotein H and L of the invention are from a human strain of HSV, in particular HSV-1 or HSV-2, even more preferably HSV-1. Most preferably the glycoprotein H and L is derived from HSV-1 strain 17 or F, e.g. the strain F glycoprotein H and L under NCBI database accession number X14112.1 (GI:1944536), which are SEQ ID NO: 2 and SEQ ID NO: 3 respectively. Accordingly, nucleic acid encoding the glycoprotein H may comprise the nucleic acid sequence of SEQ

ID NO: 5, and nucleic acid encoding the glycoprotein L may comprise the nucleic acid sequence of SEQ ID NO: 6.

In a further aspect of the invention, there is provided a first fusion protein, which fusion protein comprises a glycoprotein H linked to a targeting agent. In a further aspect of the invention, there is provided a second fusion protein, which second fusion protein comprises a glycoprotein L linked to a targeting agent. The first fusion protein and the second fusion protein may have specific affinity for each other, for example they may form a heterodimer.

In a further aspect of the invention, there is provided a nucleic acid molecule encoding a fusion protein, which fusion protein comprises a glycoprotein H linked to a targeting agent. In a further aspect of the invention, there is provided a nucleic acid molecule comprising nucleic acid encoding a fusion protein, which fusion protein comprises a glycoprotein L linked to a targeting agent.

A single nucleic acid molecule may include a nucleic acid region encoding the first fusion protein and nucleic acid region encoding the second fusion protein. Alternatively the first fusion protein and second fusion protein may be encoded by separate nucleic acid molecules. The nucleic acid encoding the first fusion protein and/or the nucleic acid encoding the second fusion protein may be arranged in the nucleic acid molecule such that it is operably linked to a suitable regulatory sequence (e.g. a promoter). A promoter may be constitutive or may be controlled by the availability of a signal molecule. The promoter may be, for example, an endogenous HSV promoter such as the gC promoter, or a non endogenous promoter such as the CMV-IE promoter. The nucleic acid molecule may also include nucleic acid for facilitating insertion of the nucleic acid encoding the fusion protein into the genome of a HSV. Said insertion may be, for example, by homologous recombination, or by using a site specific

recombination system, such as the Gateway® Vector Conversion System (Invitrogen, Paisley, UK).

In a further aspect of the invention, there is provided a HSV comprising:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target, and wherein the HSV is for use in a therapeutic method of medical treatment.

In a further aspect of the invention, there is provided a HSV comprising:

- a. nucleic acid encoding a first fusion protein, which first fusion protein comprises a glycoprotein H linked to a first targeting agent, and
- b. nucleic acid encoding a second fusion protein, which second fusion protein comprises a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target, and wherein the HSV is for use in a therapeutic method of medical treatment. The nucleic acid encoding the first fusion protein and/or the nucleic acid encoding the second fusion protein is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

Preferably the therapeutic method of treatment is a method for the treatment of a disease associated with the proliferation of cells, e.g. for treatment of tumour.

In a further aspect of the invention, there is provided use of a herpes simplex virus in the manufacture of medicament for

the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target.

In a further aspect of the invention, there is provided use of a HSV in the manufacture of medicament for the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises:

- a. nucleic acid encoding a first fusion protein, which first fusion protein comprises a glycoprotein H linked to a first targeting agent, and
- b. nucleic acid encoding a second fusion protein, which second fusion protein comprises a glycoprotein L linked to a second targeting agent, and wherein the first targeting agent and the second targeting agent are specific for the same target. The nucleic acid encoding the first fusion protein and/or the nucleic acid encoding the second fusion protein is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In a further aspect of the invention, there is provided a method of treating a disease associated with the proliferation of cells, comprising administering a HSV, wherein the HSV comprises:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target.

In a further aspect of the invention, there is provided a method of treating a disease associated with the proliferation

of cells, comprising administering a HSV, wherein the HSV comprises:

a. nucleic acid encoding a first fusion protein, which first fusion protein comprises a glycoprotein H linked to a first targeting agent, and

b. nucleic acid encoding a second fusion protein, which second fusion protein comprises a glycoprotein L linked to a second targeting agent,

and wherein the first targeting agent and the second targeting agent are specific for the same target. The nucleic acid encoding the first fusion protein and/or the nucleic acid encoding the second fusion protein is preferably arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid encoding the fusion protein.

In a further aspect of the invention, there is provided a method of producing a HSV comprising:

a. a glycoprotein H linked to a first targeting agent, and

b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target,

said method comprising infecting a cell line with said HSV that expresses the fusion protein.

In a further aspect of the invention, there is provided a method of producing an HSV comprising:

a. a glycoprotein H linked to a first targeting agent, and

b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target,

said method comprising incorporating nucleic acid encoding said fusion protein into the viral genome of the modified HSV.

Truncated glycoprotein D variants

The N-terminally truncated glycoprotein D molecules of the invention may or may not also include one or more further amino acid deletions, and/or may or may not include one or more amino acid substitutions or insertions compared to the non-truncated glycoprotein D molecule from which it is derived. Thus, although the truncated glycoprotein D will usually contain fewer amino acids than the non-truncated glycoprotein D, in some cases it may contain the same number or more amino acids compared to the non-truncated glycoprotein D molecule. Preferably, such amino acid deletions, substitutions, or insertions do not result in any adverse functional dissimilarity compared to the glycoprotein D having the same N-terminal truncation and which does not contain the one or more deletions, substitutions or insertions.

Preferably, the identity of the amino acid sequence of the extracellular domain of the truncated glycoprotein D is substantially equivalent to the amino acid sequence of the non-truncated glycoprotein D from which the truncated glycoprotein D is derived. Preferably the truncated glycoprotein is derived from the glycoprotein D sequence under NCBI database accession number X14112.1 (GI:1944536), which is SEQ ID NO: 1. The truncated glycoprotein D may be derived from a glycoprotein D encoded by the nucleic acid sequence of SEQ ID NO: 4.

For example, the extracellular domain of the truncated glycoprotein D sequence may display a sequence identity of at least 70%. Alternatively, this identity may be any of 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% sequence identity. The region of the extracellular domain of the non-truncated glycoprotein D that has been deleted in the truncated glycoprotein D is not taken into account when determining sequence identity.

Alternatively, identity may be determined over the whole glycoprotein D sequence, i.e. not just the extracellular domain.

Thus, where the sequence identity of the glycoprotein D truncate is 100% compared to SEQ ID NO: 1 and the full-length glycoprotein D is SEQ ID NO: 1, as is preferred, the amino acid corresponding to a position of interest in the HSV-1 glycoprotein D full-length sequence will be identical to the corresponding amino acid in the full-length sequence. Likewise, amino acids in the truncate that correspond to a portion of amino acids in the full-length sequence will be identical to the portion of corresponding amino acids in the full-length sequence.

In case of doubt, the non-truncated glycoprotein D from which the truncated glycoprotein D is derived is the glycoprotein D listed above that has the closest sequence identity with the truncated glycoprotein D, not taking into account the truncated region.

Glycoprotein H and glycoprotein L variants

Likewise, the glycoprotein H and/or L of the invention may or may not include one or more amino acid deletions, substitutions or insertions compared to the wild-type glycoprotein H and/or L from which each is derived. Preferably, such amino acid deletions, substitutions or insertions do not result in any adverse functional dissimilarity compared to the glycoprotein H and/or L from which each is derived.

Preferably, the identity of the amino acid sequence of the glycoprotein H and/or L is substantially equivalent to the amino acid sequence of the glycoprotein H and/or L from which each is derived. For example, the glycoprotein H and/or L sequence may display a sequence identity of at least 70%. Alternatively, this identity may be any of 70, 71, 72, 73, 74,

75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% sequence identity.

Targeting agents

The targeting agent is conveniently an antibody or component of an antibody, e.g. an antibody binding domain such as a single chain variable fragment (scFv). The targeting agent is preferably capable of specifically binding to a cell surface protein present on the cell type targeted. This is discussed below. The targeting agent is preferably bound to the glycoprotein D, H, or L such that it forms a fusion protein with the glycoprotein. Alternatively, the targeting agent may be bound to the viral particle by chemical means, e.g. covalently, or by a binding agent. For example, the binding agent may bind directly to the glycoprotein D, H, or L of the invention. Alternatively, the binding agent may bind to a cognate ligand linked to the glycoprotein, e.g. avidin/streptavidin and biotin. Preferably the targeting agent is linked to the N-terminus of the glycoprotein, i.e. the N-terminally truncated glycoprotein D, glycoprotein H and/or glycoprotein L.

As an antibody binding domain forms a preferred embodiment of the invention, the following description will concentrate on the use of antibodies. However, it will be apparent to the skilled person that other targeting agents may be used, e.g. aptamers, or members of a specific binding pair such as a receptor and its ligand.

The antibody or antibody component incorporated into the viral envelope influences the selectivity of the virus by enhancing the efficiency of viral infection of a certain cell type or cell types. In other words, by displaying a targeting agent that is specific for a particular cell surface protein, the virus preferentially infects cells displaying that cell surface protein. The HSV is thereby targeted to that cell

type, in as much as it has increased binding affinity for the cell type, resulting in increased levels of infection of that cell type. Other cell types may still be infected by the HSV. The HSV infection process is initiated through contact between glycoproteins of the viral envelope and glycoproteins of the target cell membrane. In the present invention, antibodies with specific affinity for membrane proteins of the chosen cell type are attached to a truncated glycoprotein D, and/or a glycoprotein H and a glycoprotein L, and are thereby incorporated into the HSV viral envelope. This increases the affinity of the HSV for the surface of the chosen target cell through the additional interaction between the antibody and the cell surface protein. The binding of the antibody binding domain to its target antigen on the cell surface will bring both virion and cellular membranes into closer proximity and allow the viral envelope glycoproteins to initiate fusion of the membranes, leading to penetration of the cell.

Preferably, the antibody or antibody component is specific for a tumour cell surface antigen, i.e. an antigen found on the surface of a tumour cell and associated with that cell, being either unique to tumour cells or being more abundant on tumour cells than on most if not all non-tumour cells. Many novel or atypical forms of normal proteins are expressed by tumour cells, and antibodies directed against these provide tumour targeting strategies. For example, carcinoembryonic antigen (CEA) is an important marker on many tumour cells and engineered antibodies directed against CEA have undergone clinical trials (Mayer, A. *et al.*, *J. Immunol. Methods* **231** 261-273 (1999)). Engineered antibodies directed against the Her2/neu growth factor (Trastuzumab) and against CD20 (rituximab) have been licensed for the treatment of breast cancer and Non-Hodgkin's lymphoma respectively Holliger, P. and Hoogenboom, H. (1998), *Nature Biotechnology* 16, 1015. CD55 (decay accelerating factor) is over-expressed by tumour cells to block complement activation and antibodies directed against CD55 may have therapeutic potential (Li, L. *et al.* *B.*

J. Cancer **84** (1) 80-86 (2001)). CD38 is well represented on the cell surfaces of a variety of lymphoid tumours and is considered to be a promising target for antibody therapy (Stevenson, *Mol. Med.* (12) 345-346 (2006)). EGFR is also considered to be a valid target in the treatment of solid tumours and is overexpressed in many tumour types (Wujcik, *Seminars in Oncology Nursing*, (22) 5-9 (2006)). Thus, the antibody or antibody component may be specific for a target selected from the group consisting of GD3, CD38, CD55, CD20, EGRF, HER2/neu, CEA, squamous cell carcinoma antigens 1 and 2, ovarian carcinoma antigen CA125, Mucin 1, prostate-specific membrane antigen, melanoma-associated tumour antigen p97, 5T4 oncofoetal trophoblast glycoprotein, PLAC1, CA19-9, CA72-4 and CA195.

The antibody binding domain may have specific affinity for a cell surface protein found on the cell type from which the tumour originated, e.g. in the case of a breast cancer, the antibody or antibody component incorporated into the HSV viral envelope would be specific for an antigen commonly associated with breast cancer cells, e.g. Her2/Neu. The specificity of the avirulent HSV strain for infecting dividing cells would therefore be further modified so that breast cancer cells were preferentially infected by the virus more than other types of dividing cells. By targeting dividing breast cancer cells, the HSV should infect and lyse breast cancer cells more efficiently than any other cells. The use of antibodies or antibody components against particular cell types can also be used to extend the tropism of HSV to cell types that are not otherwise efficiently infected by HSV, e.g. the use of antibodies or antibody components specific for antigen found on B cells would be expected to extend the tropism of HSV to B cells. Antibodies or antibody components of different specificities may be included together in one HSV viral envelope. In view of the results reported here, the combination of these specificities would be expected to give greater specificity of targeting to the desired cell type.

HSV strains

Preferably the HSV is non-neurovirulent. The HSV is also preferably oncolytic. More preferably the HSV is modified in at least one of the long repeat regions (R_L) of the HSV genome, relative to the genome of the corresponding wild-type strain, such that the HSV lacks neurovirulence. The modification may be within the BamHI s restriction fragment of one or each R_L repeat. As such, the HSV genome may be modified within the Bam HI s region of the internal repeat R_L (0.81-0.83 mu) and within the counterpart region of the terminal R_L (0-0.02 mu) such that the variant lacks neurovirulence.

Such modification may take the form of at least one addition, deletion, substitution or insertion of one or more nucleotides.

In one arrangement the genome is modified in each said region, e.g. by a deletion of one or more nucleotides. The deletion may be of at least 50 or at least 100 nucleotides or from 0.5Kb to 3Kb or from 0.7Kb to 2.5Kb. In one arrangement the deletion is 759bp in length and is located between nucleotide positions 125213 and 125972 of the internal long repeat (IR_L) and in the counterpart region of the terminal long repeat (TR_L) of HSV-1 strain 17.

Suitable modifications may also include the insertion of an exogenous nucleic acid sequence or exogenous/ heterologous cassette comprising said sequence into the herpes simplex virus genomic DNA. The insertion may be performed by homologous recombination, or by site-specific recombination using an HSV genome with appropriate recombination sites, of the exogenous nucleic acid sequence into the genome of the selected herpes simplex virus. For example, the modification may take the form of insertion of a sequence of nucleotides encoding a gene product, such as a fusion protein of the invention, which may be operably linked to one or more control

sequences enabling expression of the gene product from the HSV vector.

Where a plurality of nucleotides are inserted, e.g. in the case of insertion of a gene sequence, the inserted nucleotides may be located entirely within, or may overlap, at least one of the ICP34.5 protein coding sequences of the HSV genome. The inserted nucleic acid may be located in both (this will usually be all) copies of the RL1 locus or ICP34.5 protein coding sequence.

The HSV may, therefore, have an inactivating mutation in the RL1 locus of the HSV genome, more specifically a mutation which inactivates the function of the ICP34.5 gene product, such that the herpes simplex virus does not produce a functional ICP34.5 gene product and is non-neurovirulent.

Accordingly, an inactivating mutation may be present in one or each ICP34.5 locus, disrupting the ICP34.5 protein coding sequence such that the ICP34.5 gene is non-functional and cannot express a functional ICP34.5 gene product.

Preferably, both copies of the ICP34.5 gene sequence contain inactivating mutations, which may be the result of one or more modifications of the HSV genome, as described above.

Preferably, the HSV is a null mutant and as such is not capable of producing a functional ICP34.5 protein. Such null mutants may contain modifications in both copies of the ICP34.5 gene present in the viral genome such that the virus is unable to express functional ICP34.5 protein. Preferably, the HSV does not comprise nucleic acid, e.g. an ICP34.5 gene, that encodes a functional ICP34.5 gene product.

The HSV may be a mutant of HSV-1 or HSV-2, more preferably of one of HSV-1 strains 17, F or HSV-2 strain HG52 and most preferably of HSV-1 strain 17. Non-neurovirulent ICP34.5 null

mutants of HSV-1 strain 17 are particularly preferred and suitable examples include:

- (a) HSV 1716 (ECACC accession number V92012803); and
- (b) HSV 1790 (ECACC accession number 03110501).

The HSV may be a further mutant of HSV 1716 or HSV 1790.

Suitable HSV may therefore be described as mutants or variants of the parent HSV strain from which they are derived or to which they correspond. For example, HSV 1716 and HSV 1790 are mutants of HSV-1 strain 17 and may be obtained by modification of the strain 17 genomic DNA. Suitable mutant HSV may be non-wild type and may be recombinant. Mutant herpes simplex viruses may comprise a genome containing modifications relative to the wild type, as described above.

In this specification, non-neurovirulence is defined by the ability to introduce a high titre of virus (approx 10^6 plaque forming units (pfu)) to an animal or patient without causing a lethal encephalitis such that the LD₅₀ in animals, e.g. mice, or human patients is in the approximate range of $\geq 10^6$ pfu.

Where the HSV comprises a truncated glycoprotein D linked to a targeting agent as described above, the HSV may be engineered, e.g. by mutation, such that it does not express a non-truncated glycoprotein D, e.g. the endogenous glycoprotein D. Likewise, where the HSV comprises a glycoprotein H and/or L linked to a targeting agent as described above, the HSV may be engineered, e.g. by mutation, such that it does not express the endogenous glycoprotein H and/or L.

Nucleic acid

In further aspects of the present invention a nucleic acid vector is provided which comprises, or encodes, a nucleic acid of the invention. The vector may preferably be a transcription vector from which a nucleic acid of the invention may be expressed. In preferred embodiments the

vector may be a Herpes Simplex Virus (HSV). The HSV genome may be modified to encode a nucleic acid of the invention which may be transcribed and expressed when the HSV infects a cell, preferably a mammalian cell. The HSV genome may further encode a polypeptide of interest capable of expression upon infection of a cell by the HSV. Alternatively the vector may be a vector for propagating the nucleic acid, e.g. in *E. coli*.

In this specification the term "operably linked" may include the situation where a nucleic acid and regulatory nucleotide sequence are covalently linked in such a way as to place the expression of the nucleic acid under the influence or control of the regulatory sequence. Thus a regulatory sequence is operably linked to a nucleic acid if the regulatory sequence is capable of effecting transcription of the nucleic acid of the invention. The regulatory sequence may, for example, be a component of a promoter system.

Where HSV is specified as comprising nucleic acid, the nucleic acid is preferably part of the HSV genome. The nucleic acid may be arranged in the HSV genome such that the HSV is capable of expressing the nucleic acid. Nucleic acid encoding a glycoprotein, truncate thereof, and/or targeting agent, preferably refers to an open reading frame, e.g. a gene, that encodes said glycoprotein, truncate thereof, or targeting agent. The nucleic acid is preferably DNA.

Fusion proteins

A fusion protein comprising a glycoprotein, or N-terminally truncated glycoprotein, and targeting agent of the invention comprises a polypeptide chain that incorporates the polypeptide chain of the glycoprotein, or truncate thereof, and the polypeptide chain of the targeting agent, e.g. they are contiguous. Normally the glycoprotein, or truncate thereof, and the targeting agent will be arranged in the fusion protein such that the glycoprotein, or truncate thereof, is on the C-terminal side of the targeting agent.

The fusion protein may or may not comprise one or more amino acids between the last amino acid (N-terminal amino acid) of the glycoprotein, or truncate thereof, and the first amino acid (C-terminal amino acid) of the targeting agent. For example, the N-terminal amino acid of the glycoprotein, or truncate thereof, may abut the C-terminal amino acid of the targeting agent, e.g. they are immediately adjacent. Alternatively the glycoprotein, or truncate thereof, may be separated by a spacer peptide or spacer polypeptide.

The nucleic acid encoding the fusion protein may be a single open reading frame, e.g. a single gene. Thus, the glycoprotein or truncate and the targeting agent may be transcribed and translated together, e.g. they may be transcribed onto the same mRNA molecule and translated such that they are provided on the same polypeptide chain.

In general, a fusion protein may be created via genetic engineering from two or more proteins/peptides. This may be achieved by creating a fusion gene, e.g. by removing the stop codon from a first gene encoding a first protein and appending it to a second gene encoding a second protein in frame. A linker region, e.g. nucleic acid encoding one or more additional peptides, may be inserted between the two genes to facilitate correct folding of the fusion protein.

The term "fusion protein" is herein used interchangeably with the term "fusion polypeptide".

Sequence identity

Percentage (%) sequence identity is defined as the percentage of amino acid residues in a candidate sequence that are identical with residues in the given listed sequence (referred to by the SEQ ID No.) after aligning the sequences and introducing gaps if necessary, to achieve the maximum sequence identity, and not considering any conservative substitutions as part of the sequence identity. Unless otherwise indicated,

sequence identity is preferably calculated over the entire length of the respective sequences.

Unless otherwise indicated, where the aligned sequences are of different length, sequence identity of the shorter comparison sequence may be determined over the entire length of the longer given sequence or, where the comparison sequence is longer than the given sequence, sequence identity of the comparison sequence may be determined over the entire length of the shorter given sequence.

For example, where a given sequence comprises 100 amino acids and the candidate sequence comprises 10 amino acids, the candidate sequence can only have a maximum identity of 10% to the entire length of the given sequence. This is further illustrated in the following example:

(A)

Given seq: XXXXXXXXXXXXXXXX (15 amino acids)

Comparison seq: XXXXXYYYYYYY (12 amino acids)

% sequence identity = the number of identically matching amino acid residues after alignment divided by the total number of amino acid residues in the longer given sequence, i.e. (5 divided by 15) x 100 = 33.3%

Where the comparison sequence is longer than the given sequence, sequence identity may be determined over the entire length of the given sequence. For example:

(B)

Given seq: XXXXXXXXXX (10 amino acids)

Comparison seq: XXXXXYYYYYYZZZZZZZZ (20 amino acids)

% sequence identity = number of identical amino acids after alignment divided by total number of amino acid residues in the given sequence, i.e. (5 divided by 10) x 100 = 50%.

Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways known to a person of skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software.

Hybridisation

In accordance with the present invention, glycoprotein D, H and L nucleic acid sequences may be identified by using hybridization and washing conditions of appropriate stringency.

Complementary nucleic acid sequences will hybridise to one another through Watson-Crick binding interactions. Sequences which are not 100% complementary may also hybridise but the strength of the hybridisation usually decreases with the decrease in complementarity. The strength of hybridisation can therefore be used to distinguish the degree of complementarity of sequences capable of binding to each other.

The "stringency" of a hybridization reaction can be readily determined by a person skilled in the art.

The stringency of a given reaction may depend upon factors such as probe length, washing temperature, and salt concentration. Higher temperatures are generally required for proper annealing of long probes, while shorter probes may be annealed at lower temperatures. The higher the degree of desired complementarity between the probe and hybridisable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so.

For example, hybridizations may be performed, according to the method of Sambrook et al., ("Molecular Cloning, A Laboratory

Manual, Cold Spring Harbor Laboratory Press, 1989) using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules is to calculate the melting temperature T_m (Sambrook et al., 1989):

$$T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/n$$

where n is the number of bases in the oligonucleotide.

As an illustration of the above formula, using $[\text{Na}^+] = [0.368]$ and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in sequence complementarity.

Accordingly, nucleotide sequences can be categorised by an ability to hybridise to a target sequence under different hybridisation and washing stringency conditions which can be selected by using the above equation. The T_m may be used to provide an indicator of the strength of the hybridisation.

The concept of distinguishing sequences based on the stringency of the conditions is well understood by the person skilled in the art and may be readily applied.

Treatment

The present invention provides HSV for use in a method of medical treatment. Preferably they are provided for use in the treatment of a disease associated with the proliferation of cells, such as a cancerous condition, i.e. in oncotherapy. This treatment may comprise the oncolytic treatment of the cancer, which may take the form of a tumour. Accordingly, the method of treatment may involve the killing of tumour cells by the HSV. Treatment may involve the selective infection and/or lysis of dividing cells. The use of HSV in the manufacture of a medicament, pharmaceutical composition or vaccine for the treatment of cancer is also provided. Such medicaments, pharmaceutical compositions or vaccines may comprise suitable HSV together with a pharmaceutically acceptable carrier, adjuvant or diluent.

In this specification a cancerous condition may be any unwanted cell proliferation (or any disease manifesting itself by unwanted cell proliferation), neoplasm or tumour. The cancerous condition may be a cancer and may be a benign or malignant cancer and may be primary or secondary (metastatic). A neoplasm or tumour may be any abnormal growth or proliferation of cells and may be located in any tissue. Examples of tissues include the colon, pancreas, lung, breast, uterus, stomach, kidney, testis, central nervous system (including the brain), peripheral nervous system, skin, blood or lymph.

Tumours to be treated may be nervous system tumours originating in the central or peripheral nervous system, e.g. glioma, medulloblastoma, meningioma, neurofibroma, ependymoma, Schwannoma, neurofibrosarcoma, astrocytoma and oligodendroglioma, or may be non-nervous system tumours originating in non-nervous system tissue e.g. melanoma, mesothelioma, lymphoma, hepatoma, epidermoid carcinoma, prostate carcinoma, breast cancer cells, lung cancer cells or

colon cancer cells. HSV of the present invention may be used to treat metastatic tumours occurring in the central or peripheral nervous system which originated in a non-nervous system tissue or metastatic tumours occurring outside the central or peripheral nervous system which originated in a central or peripheral nervous system tissue.

Formulation and administration

Medicaments and pharmaceutical compositions according to aspects of the present invention may be formulated for administration by a number of routes, including but not limited to, systemic, parenteral, intravenous, intra-arterial, intramuscular, intraperitoneal. The medicaments and compositions may be formulated in fluid or solid form. Fluid formulations may be formulated for administration by injection to a selected region of the human or animal body.

The HSV may be administered systemically, parenterally, intravenously, intra-arterially, intramuscularly, intrathecally, subcutaneously. Any one of these routes of administration may involve injection of the HSV. Injectable formulations may comprise the HSV in a sterile and/or isotonic medium.

The route of administration may be selected by the ability of that route to expose substantially the entire body to the HSV. This may be determined by the ability of the HSV to circulate throughout substantially all parts of the body via the selected route. Circulation throughout substantially all of the body may exclude exposure of the HSV to one or a small number of tissues. For example, where the HSV is circulated in the blood it may be excluded from the brain by the blood brain barrier.

In preferred embodiments of the invention the HSV is administered by injection to the circulating blood, e.g. by intravenous or intra-arterial injection.

The delivery of suitable HSV to cancerous cells that are to be treated may be performed using naked virus or by encapsulation of the virus in a carrier, e.g. in nanoparticles, liposomes or other vesicles.

Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the tumour being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins.

The HSV may be administered at any therapeutically effective dosage amount. Therapeutically effective dosages may comprise less than or equal to one of 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 or 10^9 plaque forming units (pfu).

The patient to be treated may be any animal or human. The patient may be a non-human mammal, but is more preferably a human patient. The patient may be male or female.

The invention includes the combination of the aspects and preferred features described, except where such a combination is clearly impermissible or expressly avoided. In particular, all aspects relating to glycoprotein D may be combined with each other, and all aspects relating to glycoprotein H and L may be combined with each other.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying Figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Brief Description of the Figures

Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying Figures in which:

Figure 1

Figure 1A shows a Western blot probed with anti-myc tag monoclonal antibody demonstrating expression of R24/gD fusion proteins in Vero cells (lanes 1, 4, 5, 7, 9, 11) and their presence in viruses derived from infection of the Vero cell line with HSV1716gfp (lanes 2, 3, 6, 8, 10, 12). Lanes 1 and 2 - R24/37gD, lanes 3 and 4 - R24/78gD, lanes 5 and 6 - R24/260gD, lanes 7 and 8 - R24/274gD, lanes 9 and 10 - R24/231gD, lanes 11 and 12 - R24/164gD and lane M molecular size markers. b) and c). Figures 1B and 1C show Western blot probed with anti-HA tag (B) and anti-myc tag (C) demonstrating expression of R24/gH (B, lane 1) and R24/gL3 (C, lane 1) in the same Vero cell extract and their combined incorporation into a virion preparation from the same cell line infected with HSV1716gfp (B and C, lane 2). Lane C is a control Vero cell extract and lane M is molecular size markers.

Figure 2

Figure 2 shows the results of immunofluorescence experiments with anti-myc tag (a-d, f, g) or anti-HA tag (e and h), demonstrating cellular localization of R24/gD and R24/gH/gL fusion proteins in the Vero cell lines expressing R24/37gD (a), R24/128gD (b), R24/191gD (c), R24/274gD (d), R24/gH (e) and R24/gL (f) Anti-myc tag (g) and anti-HA tag (h) monoclonal antibodies failed to stain normal Vero cells.

Figure 3

Figure 3 shows fluorescent micrographs of CHO cells infected with HSV1716gfp propagated on Vero (a) or BHK (b) cells or stable Vero cell lines expressing R24 linked to 37gD (c), 139gD (d), 78gD (e), 92gD (f), 128gD (g), 179gD (h), 260gD (i), 274gD (j), gH/gL (k), gH/gL1 (l), gH/gL2 (m) or gH/gL3 (n).

Figure 4

Figure 4 shows Western blots probed with antiserum 106 against HSV-1 R1 of whole cell extracts from CHO (upper panels a and b) or Vero (lower panels a and b) cells infected with HSV1716gfp viruses obtained from propagation on various Vero cell lines expressing R24/gD or R24gH/gL glycoprotein fusion protein. (a) shows HSV1716gfp propagated on BHK cells (lane 1) or viruses propagated on Vero cells expressing R24/37gD (lane 2), R24/78gD (lane 3), R24/179gD (lane 4), R24/128gD (lane 5), R24/92gD (lane 6), R24/191gD (lane 7), R24/207gD (lane 8), R24/260gD (lane 9), R24/274gD (lane 10) or mock infected cell extracts (lane 11). (b) shows HSV1716gfp propagated on Vero cells expressing R24/gH in tandem with R24/gL (lane 1), R24/gL1 (lane 2), R24/gL2 (lane 3) or R24/gL3 (lane 4), normal Vero cells (lane 5) or mock infected (lane 6).

Figure 5

Figure 5 shows the results of fluorescence microscopy experiments, showing levels of THP-1 cell infectivity by viruses derived from Vero cell lines expressing T/37gD (a, b, c), T/260gD (d, e, f), T/gH/gL2 (g, h, i), 5/37gD (j, k, l), 5/260gD (m, n, o) or 5/gH/gL2 (p, q, r). Prior to infection, THP-1 cells were preincubated in control medium (a, d, g, j, m, p), MabT (b, e, h, k, n, q) or Mab5 (c, f, i, l, o, r).

Figure 6

Figure 6 shows linear representations of key plasmids and genomic structures of viruses at RL1.

(a) shows plasmid sp73 with PGK-gfp and gCp-scFv/gD expression cassettes. (b) shows plasmid RL1-del used for homologous recombination reactions with flanking RL1 sequences and PGK-gfp and gCp-scFv/gD expression cassettes inserted within the ICP34.5 deleted region. Viruses derived from this plasmid by homologous recombination will have the same structure at RL1. (c) shows plasmid sp73 with PGK-gfp, gCp-scFv/gL and TKp-scFv/gH expression cassettes. (d) shows plasmid RL1-del used for homologous recombination reactions with flanking RL1 sequences and PGK-gfp, gCp-scFv/gL and TKp-scFv/gH expression cassettes inserted within the ICP34.5 deleted region. Viruses derived from this plasmid by homologous recombination will have the same structure at RL1. (e) shows structure at the RL1 loci of HSV1716GateRed with attR site-specific recombination sequences flanking a CMV-DsRed expression cassette. (f) shows plasmid pENTR1A-gfp with attL site-specific recombination sequences flanking PGK-gfp and CMV-scFv/gD expression cassettes. (g) shows product from site-specific recombination between (e) and (f) generates PGK-gfp and CMV-scFv/gD expression cassettes in the RL1 loci of the HSV1716 recombinant.

Figure 7

Figure 7 shows the results of immunofluorescence experiments, demonstrating localisation of scFv/gH fusion proteins (a-d) during infection of BHK cells with HSV1716 variants expressing scFvT (a and c) or scFv5 (c and d). HA-tag staining is shown in a and c and gfp fluorescence in d and e. Immunofluorescence with MabT (e and g) or Mab5 (f and h) demonstrating expression of CD38 but not CD20 by SupT cells (e and f) and CD38 and weak CD20 expression by TolB cells (g and h). Immunofluorescence with MabB (i and k) and a recombinant minibody version of MabB (j and l) demonstrating expression of DAF by CHO/DAF (i and j) but not by normal CHO (k and l) cells.

Figure 8

Figure 8 shows the results of fluorescence microscopy experiments, demonstrating levels of CHO (j) or CHO/DAF (a-i, k, l) cell infection by HSV1716gfp propagated on Vero (a) or BHK cells (b, h, i) or by Vero cell propagated HSV1716 variants expressing B/274gD (c-g, j), 5/260gD (k) or 5/274gD (l). CHO/DAF cells were preincubated in MabB (d), recombinant MabB (e, i), Mab5 (f, h) or MabT (g).

Figure 9

Figure 9 shows the results of fluorescence microscopy experiments, demonstrating levels of SupT (a, d, g, j, m), THP-1 (b, e, h, k, n) or TolB (c, f, i, l, o) cell infection by HSV1716gfp propagated on Vero (a-c) or BHK (d-f) or Vero propagated HSV1716 variants expressing T/260gD (g-i), T/274gD (j-l) or 5/260gD (m-o).

Figure 10

Figure 10 shows the results of fluorescence microscopy experiments, demonstrating levels of SupT (a-f), THP-1 (g-l) or TolB (m-x) cell infection by Vero propagated HSV1716 variants expressing 5/260gD (a-c, g-o, m-o), T/260gD (d-f, j-l, p-r), 5/274gD (s-u) or T/274gD (v-x). Cells were preincubated in Mab5 (a, d, g, j, m, p, s, v), MabT (b, e, h, k, n, q, t, w) or MabB (c, f, i, l, o, r, u, x).

Figure 11

Figure 11(a) shows RL1 PCR using DNA extracted from BHK cells infected with HSV1716 variants expressing 5/274gD (lane 1), 5/274gDopp (lane 2), T/274gD (lane 3), 8/274gD (lane 4), B/274gD (lane 5), HSV1716 (lane 7) and HSV-1 strain 17+ (lane 8). DNA extracted from uninfected BHK cells is shown in lane 6 and lane M is the 2-log DNA ladder. Figure 11(b) shows Western blotting using anti-myc-tag monoclonal antibody to probe infected whole cell extracts (lanes 1-5, 11, 12) or purified virions (lanes 6-10) from HSV1716 variants expressing 5/274gDopp (lanes 1 and 7), 5/274gD (lanes 2 and 6), B/274gD

(lanes 3 and 8), T/274gD (lanes 4 and 9) and 8/274gD (lanes 5 and 10). Mock- and HSV1716 infected cell extracts are shown in lanes 11 and 12 respectively and lane M is the molecular size markers. Immunofluorescence/fluorescence microscopy of BHK cells infected with HSV1716 variants expressing 5/274gD (c, d), B/274gD (e, f), T/274gD (g, h), 8/274gD (i, j) or 5/274gDopp (k, l). Mock infected BHK cells are shown in m and n. Staining with the anti-myc monoclonal antibody is shown in c, e, g, i, k and m and gfp is shown in d, f, h, j, l and n.

Figure 12

Figure 12 shows the results of immunofluorescence experiments with MabT (a-c) or Mab5 (d-f), demonstrating expression of CD38 but not CD20 by THP-1 (a, d) SupT cells (b, e) and CD38 and weak CD20 expression by TolB cells (c, f). The immunofluorescence with MabB (g, i) and a recombinant minibody version of MabB (h) demonstrates expression of DAF by CHO/DAF (g, h) but not by normal CHO (i) cells. The fluorescence microscopy also demonstrates levels of CHO (j-n) or CHO/DAF (o-x) cell infection by HSV1716gfp propagated on BHK cells (n, s, x) or by Vero cell propagated HSV1716 variants expressing 5/274gD (j, o), T/274gD (k, p), B/274gD (l, q, t-w) or 8/274gD (m, r). CHO/DAF cells were preincubated in MabB (v, x), recombinant MabB (w), Mab5 (t) or MabT (u).

Figure 13

Figure 13 shows a Western blot using an anti-HSV-1 R1 antiserum to probe whole CHO/DAF (lanes 1-10) or Vero (lanes 11-21) cell extracts after infection with either 1 (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19) or 5 (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20) pfu/cell of HSV1716 variants expressing 8/274gD (lanes 1, 2, 11, 12), B/274gD (lanes 3, 4, 13, 14), T/274gD (lanes 5, 6, 15, 16) or BHK- (lanes 7, 8, 17, 18) or Vero- (lanes 9, 10, 19, 20) propagated HSV1716. Mock infected Vero cell extract is shown in lane 21 and lane M indicates the molecular size markers.

Figure 14

Figure 14 shows the results of fluorescence microscopy experiments, demonstrating levels of SupT (a, d, g, j, m), THP-1 (b, e, h, k, n) or TolB (c, f, i, l, o) cell infection by Vero cell propagated HSV1716 variants expressing 5/274gD (a-c), 5/274gDopp (d-f), T/274gD (g-i) 8/274gD (j-l) or B/274gD (m-o).

Figure 15

Figure 15 shows the results of fluorescence microscopy experiments, demonstrating levels of SupT (a-h), THP-1 (i-p) or TolB (q-x) cell infection by Vero propagated HSV1716 variants expressing T/274gD (a-d, i-l, q-t), 8/274gD (e-h), B/274gD (m-p) or 5/274gD (u-x). Cells were preincubated in Mab5 (a, e, i, q), MabT (b, f, j, r), Mab8 (c, g, k, s) or MabB (d, h, l, t).

Figure 16

Figure 16 shows the results of fluorescence microscopy experiments, demonstrating presence of EGFR and infection by HSV1716EGFR1 and EGFR2 (two independently isolated variants of HSV1716 expressing 8/274gD) of A431 cells. Human squamous cell carcinoma (SCC) cells (A431) react with anti-EGFR Mab and are infected with viruses displaying anti-EGFR scFv.

Figure 17

Figure 17 shows the average tumour volumes of nude mice bearing subcutaneous A431 tumours after intravenous injection of PBS (no virus), HSV1716, HSV1716EGFR1 (a) or HSV1716EGFR2 (b). Intravenously injected EGFR-targeted HSV1716 viruses reduced average tumour volumes of subcutaneous SCC (A431) tumours compared to untargeted HSV1716.

Figure 18

Figure 18 shows survival data from nude mice bearing subcutaneous A431 tumours after intravenous injection of PBS (no virus), HSV1716, HSV1716EGFR1 or HSV1716EGFR2.

Intravenously injected EGFR-targeted HSV1716 viruses prolonged survival of mice with subcutaneous SCC (A431) tumours compared to untargeted HSV1716.

Figure 19

Figure 19 shows the results of immunohistochemistry experiments using an anti-HSV-1 antiserum of tumours from nude mice bearing subcutaneous A431 tumours after intravenous injection of PBS (no virus), HSV1716, HSV1716EGFR1 or HSV1716EGFR2. Intravenously injected EGFR-targeted HSV1716 viruses show improved tumour localisation to subcutaneous SCC (A431) tumours compared to untargeted HSV1716.

Figure 20

Figure 20 shows strategies for production of scFv-gD and scFv-gH/gL fusion proteins. (a)(i) shows a schematic diagram showing the construction of the gD truncates. The scFv were genetically fused to 15 sequentially deleted N-terminal truncations of gD generated by PCR from within the region spanning residues 37-274. (ii) shows a linear representation of gD showing the location of carbohydrate moieties (above), disulphide bridges (below) and the transmembrane region (in darker shading), all 15 N-terminal deletions were created between amino acids 37-274 as indicated. (iii) shows a linear representation of gD showing important functional regions of the extracellular domains. (b) shows linear representations of gH and gL (not to scale) and a cartoon depicting the gH/gL heterodimer formed by scFv/gH and scFv/gL fusion proteins.

Figure 21

Figure 21 shows the nucleic acid sequences and the amino acid sequences of the glycoproteins used in the experiments reported herein. (a) shows gD (SEQ ID NO: 1), (b) shows gH (SEQ ID NO: 2), (c) shows gL (SEQ ID NO: 3). (d) shows the gD nucleic acid sequence (SEQ ID NO: 4), (e) shows the gH complement nucleic acid sequence, (f) shows the gL nucleic

acid sequence. These sequences correspond to those under NCBI database accession number X14112.1 (GI:1944536).

Detailed Description of the Invention

Specific details of the best mode contemplated by the inventors for carrying out the invention are set forth below, by way of example. It will be apparent to one skilled in the art that the present invention may be practiced without limitation to these specific details.

Results

To analyse the effects of the scFv/gD or scFv/gH/gL glycoprotein fusion proteins on tropism, infection of non-permissive CHO cells by viruses propagated on stable Vero cell lines expressing the glycoprotein fusion proteins was assessed. Gangliosides are ubiquitous components of cell membranes and GD3 was present on CHO cells as determined by R24 binding (data not shown). All 15 gD deletions were linked to the R24 scFv and stable Vero cell lines expressing these scFv/gD fusion proteins created. Expression of the myc-tagged fusion proteins was demonstrated by immunofluorescence and Western blotting. (Figure 1, Table 2).

Figure 1A demonstrates expression of various R24/gD fusion proteins and their subsequent incorporation into virus preparations following infection of the cell line with HSV1716gfp. The results for all R24/gD fusion proteins are presented in Table 2. In Fig.1A, apart from R24/37gD (lane 1) and R24/260gD (lane 5) which were expressed at high levels, the other constructs, R24/78gD (lane 4), R24/164gD (lane 11), R24/231gD (lane 9) and R24/274gD (lane 7) all showed an intermediate level of expression. Two expressed constructs, R24/164gD (lane 12) and R24/231gD (lane 10) were not incorporated into virus whereas R24/37gD (lane 2), R24/78gD (lane 3), R24/260gD (lane 6) and R24/274gD (lane 8) were detected at intermediate levels in virus preparations. R24/58gD and R24/105gD were not expressed and R24/92gD,

R24/128gD, R24/139gD, R24/179gD, R24/191gD, R24/207gD and R24/239gD showed intermediate levels of expression. Of these, R24/92gD and R24/128gD demonstrated intermediate levels of incorporation into virus with the remainder incorporated at low levels (Table 2).

Combined transfection and dual selection with zeocin and hygromycin of Vero cells with R24/gH and each of the R24/gL, R24/gL1, R24/gL2 or R24/gL3 fusion protein constructs resulted in stable cell lines expressing both gH and gL fusion proteins. Following infection with HSV1716gfp, the R24/gH and R24/gL proteins were detected in purified virus preparations. The Western blots shown in Figure 1B (anti-HA tag) and C (anti-myc tag) demonstrate expression of R24/gH (Fig. 1B, lane 1) and R24/gL3 (1C, lane 1) in the same Vero cell extract and their combined incorporation into a virion preparation after infection with HSV1716gfp (Figs 1B and C, lane 2). Similar results were obtained with the R24/gH/gL, R24gH/gL1 and R24/gH/gL2 cell lines (data not shown).

The cellular localization of the R24/gD and R24/gH/gL fusion proteins was investigated in the Vero cell lines by immunofluorescence using either the anti-myc tag (gD, gL) or anti-HA tag (gH) monoclonal antibodies. All of the expressed R24/gD fusion proteins demonstrated similar perinuclear localizations (Table 2) as shown for R24/37gD (Fig. 2a), R24/128gD (Fig. 2b), R24/191gD (Fig. 2c) or R24/274gD (Fig. 2d). Similarly, Fig. 2e and f respectively show the same perinuclear localization for R24/gH and R24/gL in the same Vero cell line and this localization was identical for the other R24/gH/gL1-3 expressing cell lines (data not shown). Anti-myc tag and anti-HA tag monoclonal antibodies failed to stain normal Vero cells (Fig. 2g and h respectively) and no fluorescence was observed in cells that failed to express fusion protein constructs (data not shown). Thus, the cellular localization of the R24/gD and R24/gH/gL fusion proteins was

very similar and consistent with an accumulation in the nuclear membrane/endoplasmic reticulum/Golgi apparatus.

CHO cells, normally resistant to HSV1716 infection when the virus is propagated on Vero cells, were infected with 1 pfu/cell HSV1716gfp derived from infection of the R24/gD, R24/gH/gL fusion protein expressing cell lines and the percentages of infected cell determined by fluorescence microscopy (Fig. 3, Table 2). HSV1716gfp propagated on Vero cells failed to infect CHO cells (Fig. 3a) and this contrasts with HSV1716gfp propagated on BHK cells which infects approximately 30% of CHO cells (Fig. 3b).

A number of HSV1716gfp viruses propagated on Vero cell lines expressing R24/gD fusion proteins were able to infect CHO cells at levels similar to or greater than those obtained with HSV1716gfp propagated on BHK cells. Examples are shown in Fig. 3(c-j) and the results summarized in Table 2. R24/37gD (Fig. 3c), R24/77gD (Fig. 3e), R24/92gD (Fig. 3f), R24/128gD (Fig. 3k), R24/260gD (Fig. 3i) and R24/274gD (Fig. 3j) infected 30%, 10%, 20%, 20%, 60% and 50% respectively of CHO cells compared to only occasionally infected CHO cells (<1%) for the other R24/gD fusion proteins (Fig. 3d, example shown is R24/139gD) apart from R24/178gD (Fig. 3l) which consistently infected between 3-5%. All of the R24/gH/gL heterodimeric fusion proteins were able to infect CHO cells (Fig. 3k-n) with varying efficiencies. R24/gH/gL, with no spacer linking the scFv to the N terminus of gL, infected ~20% (Fig. 3k) compared to 40%, 30% and 10% respectively for R24/gH/gL1 (Fig. 3l), R24/gH/gL2 (Fig. 3m) and R24/gH/gL3 (Fig. 3n) with 1, 2 or 3 copies of the Gly-Gly-Gly-Gly-Ser spacer linking the scFv to the gL N terminus.

The fluorescence results were confirmed by Western blotting with extracts from CHO cells infected using the HSV1716gfp viruses obtained from propagation on the scFv/glycoprotein fusion protein expressing Vero cells and the anti-HSV-1 R1

antibody (Fig. 4) In Fig. 4a, the upper panel shows extracts from CHO cells infected with various HSV1716gfp viruses derived from R24/gD fusion protein expressing cell lines and the lower panels shows similarly infected normal Vero cells. R1 was readily detected in CHO cells after infection with viruses propagated on Vero cells expressing R24/37gD (lane 2), R24/128gD (lane 5), R24/92gD (lane 6), R24/260gD (lane 9), R24/274gD (lane 10) and HSV1716gfp propagated on BHK cells (lane 1). A weak R1 band was detected in CHO cells infected with viruses propagated on Vero cells expressing R24/77gD (lane 3) and R24/178gD (lane 4) but was undetectable in mock infected cell extracts (lane 11) or in extracts derived from CHO cells infected with viruses propagated on Vero cells expressing R24/191gD (lane 7) or R24/207gD (lane 8). The levels of R1 expression in CHO cells infected with the above viruses is in approximate agreement with the percentages of infected cells shown in Fig. 3. In the lower panel of Fig. 4a, all of these viruses efficiently infected normal Vero cells suggesting that there was no serious impairment to infectivity resulting from incorporation of the scFv/gD fusion protein into the virion membrane. R1 was readily detected in CHO cells following infection with viruses propagated on Vero cells expressing R24/gH in tandem with R24/gL, R24/gL1, R24/gL2 or R24/gL3 (Fig. 4b, upper panel lanes 1-4) but was not detected in CHO cells after infection with HSV1716gfp propagated on normal Vero cells (upper panel, lane 5). R1 was readily detected after infection of normal Vero cells with each of these viruses (Fig. 4b, lower panel, lanes 1-5) indicating that none had seriously impaired infectivity. No R1 was detected in mock infected CHO or Vero cells (lane 6).

The human monocytic cell line THP-1 expresses CD38 but not CD20 as shown by immunofluorescence with MabT but not Mab5 (see Fig. 12a and b respectively). Although HSV1716 was able to infect THP-1 cells, as with CHO cells, the number of cells infected showed some dependency on the cell line used to propagate the virus. At 1 pfu/cell, HSV1716 propagated on BHK

cells (HSV1716B) was able to infect approximately 20% of cells whereas, at a similar moi, HSV1716 propagated on Vero cells (HSV1716V) only infected 10% of cells (data not shown). The R24 scFv was exchanged for either scFvT or scFv5 in a number of gD or gH/gL fusion protein constructs and Vero cell lines expressing the scFv/glycoprotein fusion proteins were produced. The gD constructs all showed altered CHO cell tropism when linked with R24scFv and for the gH/gL heterodimer the scFvT or scFv5 was linked to gL via a 2 x Gly-Gly-Gly-Gly-Ser spacer. Expression of all of these fusion protein constructs was demonstrated by immunofluorescence using either anti-myc (gD, gL) or anti-HA (gH) monoclonals. Perinuclear localization of the fusion proteins in their respective cell lines was similar to that observed with R24scFv fusion proteins (Table 3).

Viruses derived from Vero cell lines expressing 5/37gD (Fig. 5j), 5/128gD (not shown), 5/260gD (Fig. 5m) and 5/274gD (not shown) infected between 10-15% of THP-1 cells and preincubation with either MabT or Mab5 did not alter infection levels of 5/37gD (Fig. 5k and l), 5/128gD (not shown), 5/260gD (Fig. 5n and o) and 5/274gD (not shown). In contrast, the viruses derived from cells expressing T/gD fusion proteins showed a higher level of THP-1 infectivity which was inhibited specifically by MabT but not Mab5. Viruses derived from Vero cell lines expressing T/37gD (Fig. 5a), T/128gD (not shown), T/260gD (Fig. 5d) or T/274gD (not shown) infected 30%, 20%, 60% or 40% respectively of THP-1 cells, preincubation of the THP-1 cells in Mab5 had no effect on the ability of T/37gD (Fig. 5c), T/128gD (not shown), T/260gD (Fig. 5f) or T/274gD (not shown) expressing viruses to infect whereas preincubation in MabT greatly reduced the infectivity of viruses derived from Vero cells expressing T/37gD (Fig. 5b), T/128gD (not shown), T/260gD (Fig. 5e) or T/274gD (not shown) to 10%, 10%, 20% or 10%, which is within the range of infectivity for other Vero cell derived viruses. Similarly, virus derived from Vero cells expressing T/gH and T/gL2 in tandem infected

approximately 30% THP-1 cells (Fig. 5g) and this higher level of infectivity was reduced to approximately 15% by preincubation of the THP-1 cells in MabT (Fig. 5h) but was unaffected by preincubation in Mab5 (Fig. 5i). In contrast, virus derived from Vero cells expressing 5/gH and 5/gL2 in tandem only infected approximately 10% of THP-1 cells (Fig. 5p) and this was unaffected by preincubation with either MabT (Fig. 5q) or Mab5 (Fig. 5r).

Similar data were obtained from several experiments with these viruses and the results, summarized in Table 3, taken together with the data from the R24 constructs and CHO cells, clearly demonstrate that the tropism of HSV1716 can be influenced by expression of specific scFv/gD and scFv/gH/scFvgL fusion proteins on the virion surface with optimum effects mediated by a small C-terminal fragment of gD.

An initial series of recombinant viruses based on HSV1716 which express scFv/gD or scFv/gH/gL heterodimers were created by homologous recombination with the RL1 shuttle vector pRL1-del containing the appropriate scFv/glycoprotein fusion protein expression construct. Linear representation showing the plasmids used and the resultant recombinant virus genomes at the RL1 loci are shown in Fig. 6. The structure of the viruses at the RL1 loci was confirmed either by Southern blotting using an ICP34.5 DNA probe or by PCR using primers that amplify across the deleted region (data not shown). In this first series of targeted viruses, expression of the scFv/glycoprotein fusion proteins was controlled by native HSV-1 promoters, scFv/gD and scFv/gL fusion protein expression was regulated by the HSV-1 gC promoter and scFv/gH fusion protein was controlled by the HSV-1 TK promoter. Although, the myc tags were absent from the gD and gL fusion proteins, gH retained its HA tag and expression of the scFv/gH fusion protein during infection was observed by immunofluorescence (Fig. 7). In Figure 7, both nuclear and cytoplasmic green fluorescent protein was observed in BHK cells infected with

HSV1716 variants expressing either TscFv or 5scFv fused to gH/gL (Fig. 7b and d respectively) and, using the anti-HA monoclonal antibody, both TscFv/gH and 5scFv/gH fusion proteins were observed (Fig. 7a and c) in the same cells, principally in a perinuclear location.

In addition to the human monocytic cell line THP-1, which we have demonstrated by immunofluorescence to express CD38 but not CD20, we also used two other human leukaemic cell lines, Supt, a T-cell line that expresses CD38 (Fig. 7e) but not CD20 (Fig. 7f) and TolB, a B-cell line that expresses both CD38 (Fig. 7g) and CD20 (Fig. 7h). The weak fluorescence observed with Mab5/TolB suggests that this protein is expressed at lower levels compared to CD38 expression in this and the other lines. We also created a CHO cell line (CHO/DAF) that constitutively expressed human DAF (CD55) as demonstrated by immunofluorescence with MabB (Fig. 7i) and a recombinant minibody version of MabB comprising two scFvs binding sites dimerised via a human IgG1 Fc region (Fig. 7j). Both of these antibodies identified intense membrane staining of CD55 in the modified CHO cells but neither stained normal CHO cells (Fig. 7k and l). The abilities of the targeted HSV1716 variants to infect these cell lines were analysed by fluorescent microscopy with the results summarized in Table 4. Examples of their infectivities in the presence or absence of various monoclonal antibodies are shown in Figures 8-10.

As previously reported (Conner et al 2005), HSV1716 propagated in Vero cells (HSV1716V) was unable to infect CHO/DAF cells compared with HSV1716 propagated in BHK cells (HSV1716B) which was able to infect approximately 20% of CHO/DAF cells (Fig. 8a and b respectively). Significantly, approximately 50% of CHO/DAF cells were infected by an HSV1716 variant expressing the B/274gD fusion protein (Fig. 8c) and this level of infectivity was reduced to approximately 5% by preincubating the CHO/DAF cells either in MabB (Fig. 8d) or the recombinant minibody form of MabB (Fig. 8e) but was unaffected by

preincubation of the cells in either Mab5 (Fig. 8f) or MabT (Fig. 8g). The ability of HSV1716B to infect CHO/DAF cells was unaffected by either Mab5 (Fig. 8h) or recombinant MabB (Fig. 8i). As the HSV1716 variant expressing B/274gD used in these experiments was propagated in Vero cells, it only infected approximately 5% of normal CHO cells (Fig. 8j). The HSV1716 variants expressing 5/260gD (Fig. 8k) and 5/274gD (Fig. 8l) and propagated on Vero cells were also able to infect only 1-5% of CHO/DAF cells.

Three human leukaemic cell lines, all of which display CD38 and one of which also displays CD20, were semi-permissive for HSV1716 infection as shown in Fig. 9. HSV1716V infected approximately 10% of both SupT and THP-1 cells (Fig. 9a and b) at 1pfu/ cell compared to approximately 20% of SupT or THP-1 cells infected with 1 pfu/cell HSV1716B (Fig. 9d and e). A higher proportion of TolB cells were infected with approximately 20% of cell infected by HSV1716V (Fig. 9c) compared with 50% infected by HSV1716B (Fig. 9f). Using 1pfu/cell of the Vero cell propagated HSV1716 variant expressing T/260gD, approximately 30% SupT (Fig. 9g), 50% THP-1 (Fig. 9h) and 50% TolB (Fig. 9i) cells were infected. Similarly, the Vero cell propagated HSV1716 variant expressing T/274gD infected 50% SupT (Fig. 9j), 50% THP-1 (Fig. 9k) and 70% TolB (Fig. 9l) which, when compared to the Vero cell propagated HSV1716 variant expressing 5/260gD that was only able to infect 5% SupT (Fig. 9m), 5% THP-1 (Fig. 9n) or 20% TolB (Fig. 9o), demonstrates that viruses displaying scFvT have enhanced infectivity for these three cell lines via CD38. This was confirmed using the same viruses to infect SupT, THP-1 and TolB cells that were preincubated with Mab5, MabB or MabT.

Infection of SupT cells by a Vero cell propagated HSV1716 variant expressing 5/260gD was unaffected by preincubation of the cells in Mab5 (Fig. 10a), MabT (Fig. 10b) or MabB (Fig. 10c) with approximately 20% of cells infected in each case,

which, in this experiment, was similar to HSV1716V infection of SupT cells preincubated with each of these monoclonal antibodies (data not shown). In contrast, a Vero cell propagated HSV1716 variant expressing T/260gD infected 40% or 50% of SupT cells when preincubated in Mab5 (Fig. 10d) or MabB (Fig. 10f) respectively and this higher level of infectivity was reduced to 5% when the SupT cells were preincubated in MabT (Fig. 10e). Similarly, the Vero cell propagated HSV1716 variant expressing T/274gD infected 50% and 40% of SupT cells preincubated with either Mab5 or MabB respectively and this was reduced to 10% by preincubation of the cells in MabT (data not shown). The Vero cell propagated HSV1716 variant expressing 5/274gD infected approximately 10% of SupT cells incubated in Mab5, MabT or MabB (data not shown).

THP-1 infection by a Vero cell propagated HSV1716 variant expressing 5/260gD was unaffected by preincubation of the cells in Mab5 (Fig. 10g), MabT (Fig. 10h) or MabB (Fig. 10i) with approximately 5% of cells infected in each case whereas HSV1716V infection of THP-1 cells preincubated with each of these monoclonal antibodies was approximately 10% (data not shown). Significantly, levels of THP-1 infection by a Vero cell-propagated HSV1716 variant expressing T/260gD were 50-60% when cells were preincubated in Mab5 (Fig. 10j) or MabB (Fig. 10l) respectively and this higher level of infectivity was reduced to 20% when the THP-1 cells were preincubated in MabT (Fig. 10k). Similarly, the Vero cell propagated HSV1716 variant expressing T/274gD infected 50-60% of THP-1 cells preincubated with either Mab5 or MabB respectively and this was reduced to approximately 20% by preincubation of the cells in MabT (data not shown). The Vero cell propagated HSV1716 variant expressing 5/274gD infected 10% of THP-1 cells irrespective of whether the cells were preincubated in Mab5, MabT or MabB (data not shown).

Infection of TolB cells by the Vero cell propagated HSV1716 variant expressing 5/260gD was unaffected by preincubation of

the cells in Mab5 (Fig. 10m), MabT (Fig. 10n) or MabB (Fig. 10o) with approximately 20-30% of cells infected in each case, which, in this experiment, was similar to HSV1716V infection of TolB cells preincubated with each of these monoclonal antibodies (data not shown). In contrast, the Vero cell propagated HSV1716 variant expressing T/260gD infected 50-60% of TolB cells when preincubated in Mab5 (Fig. 10p) or MabB (Fig. 10r) respectively and this higher level of infectivity was reduced to 10% when the TolB cells were preincubated in MabT (Fig. 10q). Similarly, the Vero cell propagated HSV1716 variant expressing T/274gD infected approximately 40% of TolB cells preincubated with either Mab5 (Fig. 10v) or MabB (Fig. 10x) and this was reduced to 20% by preincubation of the cells in MabT (Fig. 10w). In contrast, the Vero cell propagated HSV1716 variant expressing 5/274gD only infected 10-20% of TolB cells preincubated in Mab5 (Fig. 10s), MabT (Fig. 10t) or MabB (10u).

We also designed a series of targeted viruses which, under control of the constitutively active CMV-IE promoter, endogenously expressed scFvs 5, T, B or 8 fused to 274gD. These HSV1716 variants were generated using our novel HSV1716GateRed site-specific recombination system and their genomic structures at the RL1 loci were confirmed by PCR using primers that amplify across the HSV1716 ICP34.5 deleted region (Fig. 11a). PCR, using F13/R13 primers, with wild type HSV-1 17+ DNA as template generated an 880bp band (Fig. 11a, lane 8) compared to HSV1716 DNA which generated a 125bp product (Fig. 11a, lane 7). As the targeted HSV1716 variants contained both PGK-gfp and CMV-scFv/274gD expression cassettes in the RL1 loci, the DNAs from the HSV1716 variants expressing 5/274gD, T/274gD, 8/274gD and B/274gD generated 3500bp products (Fig. 11a, lanes 1 and 3-5 respectively). A 3,500bp product was also obtained from an HSV1716 variant in which the 5/274gD expression cassette was in the opposite orientation (Fig. 11a, lane 2) relative to PGK-gfp but no products were

obtained using DNA extracted from mock-infected BHK cells (Fig. 11a, lane 6).

Expression of the scFv/274gD fusion proteins following infection of BHK cells was confirmed by Western blotting of whole cell extracts using an anti-myc tag monoclonal antibody (Fig. 11b). A prominent 45kDa protein was detected in whole cell extracts obtained after infection of BHK cells with HSV1716 variants expressing 5/274gD (Fig. 11b, lane 2), B/274gD (Fig. 11b, lane 3), T/274gD (Fig. 11b, lane 4) and 8/274gD (Fig. 11b, lane 5) which was absent from mock-infected (Fig. 11b, lane 11) or BHK cells infected with HSV1716 (Fig. 11b, lane 12). The HSV1716 variant in which the 5/274gD expression cassette was in the opposite orientation (5/274gDopp) relative to PGK-gfp failed to express a detectable protein product (Fig. 11b, lane 1). SDS-PAGE/Western blotting was also performed using the anti-myc monoclonal antibody on 1×10^6 pfu HSV1716 variants expressing 5/274gD (Fig. 11b, lane 6), B/274gD (Fig. 11b, lane 8), T/274gD (Fig. 11b, lane 9) and 8/274gD (Fig. 11b, lane 10) and also 5/274gDopp (Fig. 11b, lane 7) with prominent 45kDa bands detected in all but 5/274gDopp.

Expression of the scFv/274gD protein in infected cells was also demonstrated by immunofluorescence with Fig. 11c-n clearly showing both gfp expression resulting from viral infection and, using the anti-myc tag monoclonal antibody, cytoplasmic/perinuclear distributions of 5/274gD (Fig. 11c and d), B/274gD (Fig. 11e and f), T/274gD (Fig. 11g and h) and 8/274gD (Fig. 11i and j). Only gfp expression was observed in cells infected with HSV1716gfp (data not shown) and with the variant 5/274gDopp (Fig. 11k and l) which failed to produce a protein product. Mock infected BHK cells are shown in Fig. 11m and n. These viruses, along with HSV1716B, at 1pfu/cell were used to infect CHO, CHO/DAF, SupT, THP-1 and TolB cells either in the presence or absence of Mab5, MabT, MabB or Mab8 and levels of infection were determined by fluorescence

microscopy. THP-1 and SupT are respectively monocyte and T-cell lines that express CD38 (Fig. 12a and b) but not CD20 (Fig. 12d and e) and TolB is a B-cell line that expresses both CD38 (Fig. 12c) and CD20 (Fig. 12f). The weak fluorescence observed with Mab5/TolB suggests that CD20 is expressed at lower levels compared to CD38 in this and the other lines. We also created a CHO cell line (CHO/DAF) that constitutively expressed human DAF (CD55) as demonstrated by immunofluorescence with MabB (Fig. 12g) and a recombinant minibody version of MabB (Fig. 12h) with intense membrane staining of CD55 in the modified CHO cells which was not detected in normal CHO cells (Fig. 12i).

Only a small number of CHO cells were infected by the HSV1716 variants expressing 5/274gD, T/274gD, B/274gD or 8/274gD (Fig. 12j-m respectively) compared to approximately 20% of CHO cells with HSV1716B (Fig. 12n). Compared to CHO cells, 5/274gD (Fig. 12o), T/274gD (Fig. 12p) and 8/274gD (Fig. 12r) were better able to infect CHO/DAF cells but the numbers of infected cells were still much lower (<5%) than the 30% of cells infected by HSV1716B (Fig. 12s). Significantly, the HSV1716 variant expressing B/274gD was able to infect approximately 50% of CHO/DAF cells (Fig. 12q) and, by preincubating the CHO/DAF cells in MabB (Fig. 12v) or the recombinant minibody B (Fig. 12w), this level of infectivity was reduced to approximately 5%. Preincubation of CHO/DAF cells in Mab5 (Fig. 12t), MabT (Fig. 12u) or Mab8 (not shown) had no effect on the infectivity of the HSV1716 variant expressing B/274gD with approximately 50-60% of cells infected and preincubation in MabB failed to compromise the infectivity of HSV1716B for CHO/DAF cells (Fig. 12x).

The fluorescence microscopy data was confirmed by Western blotting using an anti-HSV-1 R1 antiserum to probe whole cell extracts from CHO/DAF cells (Figure 13). R1 expression was only detected in CHO/DAF cells infected with 1 or 5pfu/cell of the HSV1716 variant expressing B/274gD (lanes 3, 4) or HSV1716

propagated on BHK cells (lanes 7, 8) with little or no expression detected in CHO/DAF cells infected with HSV1716 variants expressing 8/274gD (lanes 1, 2), T/274gD (lanes 5, 6), or HSV1716 propagated on Vero cells (lanes 9, 10). Prominent R1 bands were detected when all of these viruses were used to infect Vero cells (lanes 11-20) and no band was observed in mock-infected Vero cells (lane 21). Thus, viruses that display a targeting moiety against DAF are better able to infect CHO cells with membrane exposed DAF than viruses that do not display a targeting moiety or display targeting moieties directed against CD20, CD38 or EGFR not present on CHO or CHO/DAF cells.

The HSV1716 variant expressing 5/274gD was able to infect approximately 5% of SupT cells (Fig. 14a), 5% of THP-1 cells (Fig. 14b) and 10% of TolB (Fig. 14c) and these levels were similar to those obtained using 5/274gDopp which infected similar numbers of SupT (Fig. 14d), THP-1 (Fig. 14e) and TolB (Fig. 14f). Significantly, the HSV1716 variant expressing T/274gD was able to infect 20%, 50% or 50% respectively of SupT (Fig. 14g), THP-1 (Fig. 14h) or TolB (Fig. 14i) cells suggesting that the incorporation of the CD38 targeting moiety in this virus improves its ability to infect CD38 positive cells. In contrast the HSV1716 variants expressing either 8/274gD or B/274gD were respectively able to infect only 5% or 10% of SupT cells (Fig. 14j and m), 5% or 10% of THP-1 cells (Fig. 14k and n) or 20% or 10% of TolB cells (Fig. 14l and o). In this experiment, HSV1716V and HSV1716B respectively infected 10% or 20% SupT, 10% or 20% THP-1 or 20% or 40% TolB cells (data not shown). Further evidence that the presence of scFvT enhanced infectivity of CD38 positive cells was provided using the above viruses to infect SupT, THP-1 or TolB cells preincubated in Mab5, MabT, Mab8 or MabB with the results respectively summarized in Tables 5-7 and some examples shown in Fig. 15. The abilities of HSV1716 variants expressing 5/274gD, B/274gD or 8/274gD were all within the ranges obtained with the control viruses 5/274gDopp, HSV1716V or

HSV1716B for SupT, THP-1 or TolB cells and these low levels of infection were not affected by preincubating the cells in Mab5, MabT, MabB, Mab8 or control medium (Tables 5-7). Only the HSV1716 variant expressing T/274gD showed increased levels of infectivity for each of the SupT, THP-1 or TolB cells lines and, although this level of infectivity was unaffected by prior incubation of the cells in Mab5, MabB, Mab8 or control medium, it was compromised by preincubation in MabT. For example, in Fig. 15, approximately 25-30% of SupT cells were infected with the HSV1716 variant expressing T/274gD and this level of infection was unaffected by preincubation in Mab5 (Fig. 15a), Mab8 (Fig. 15c) or MabB (Fig. 15d) but was reduced to only 5% by preincubation in MabT (Fig. 15b). In contrast, approximately 10% of SupT cells were infected by the HSV1716 variant expressing 8/274gD and this level of infectivity was unaffected by preincubation in Mab5, MabT, Mab8 or MabB (Fig. 15e-h respectively). Similarly, approximately 60-80% of THP-1 cells were infected with the HSV1716 variant expressing T/274gD and this high infectivity was not compromised by preincubation in Mab5 (Fig. 15i), Mab8 (Fig. 15k) or MabB (Fig. 15l) but was reduced to approximately 20% by preincubation in MabT (Fig. 15j). In contrast, approximately 20-30% of THP-1 cells were infected by the HSV1716 variant expressing B/274gD and this level of infectivity was unaffected by preincubation in Mab5, MabT, Mab8 or MabB (Fig. 15m-p respectively). For TolB cells, approximately 50-60% were infected with the HSV1716 variant expressing T/274gD and this level of infection was unaffected by preincubation in Mab5 (Fig. 15q), Mab8 (Fig. 15s) or MabB (Fig. 15t) but was reduced to approximately 10% by preincubation in MabT (Fig. 15r). In contrast, approximately 20% of TolB cells were infected by the HSV1716 variant expressing 5/274gD and this level of infectivity was unaffected by preincubation in Mab5, MabT, Mab8 or MabB (Fig. 10u-x respectively). Thus, evidence that antibody binding sites displayed on the HSV1716 envelope significantly alter tropism is provided by the HSV1716 variant displaying scFvT which demonstrated enhanced infectivity for

CD38 positive cell lines with these augmented infection levels specifically inhibited by the corresponding monoclonal antibody.

The anti-EGFR scFv, derived from monoclonal antibody 8, was linked to 274gD and the resulting construct was used to create an HSV1716 variant that expressed the scFv8/274gD targeting moiety by site-specific recombination using HSV1716GateRed DNA. Two independent isolates, HSV1716EGFR1 and HSV1716EGFR2 were prepared.

The human epidermoid carcinoma cell line A431 expresses EGFR as shown by immunofluorescence with Mab 8 (Fig. 16) and was readily infected by HSV1716 (not shown) and by HSV1716EGFR1 and HSV1716EGFR2 (Fig. 16). The A431 cells were used to form subcutaneous tumours in nude mice and, once established, the mice were systemically injected in the tail vein with PBS or 1×10^6 pfu HSV1716, HSV1716EGFR1 or HSV1716EGFR2 on days 1 and 3. Tumours were measured daily and once they had reached 15mm diameter the mice were sacrificed and tumour tissue obtained for immunohistochemistry with an anti-HSV-1 antiserum.

Mice that received the control PBS injection had more rapidly growing tumours than mice that received 1×10^6 pfu HSV1716 (Fig. 17a and b) and were sacrificed sooner (Fig. 18). Significantly, mice that received 1×10^6 pfu HSV1716EGFR1 (Fig. 17a) or HSV1716EGFR2 (Fig 17b) had more slowly growing tumours than those which were injected with HSV1716 (Fig. 17a and b) and therefore survived longer (Fig. 18). The mean survival times for no virus, HSV1716, HSV1716EGFR1 and HSV1716EGFR2 were 10, 19, 25.5 and 23 days respectively indicating that the targeted viruses were better able to reduce tumour burdens and therefore enhance survival. Interestingly, after sacrifice and excision, the tumours on mice receiving intravenously injected HSV1716EGFR1 and EGFR2 were actually much smaller than measurements suggested as they formed a capsule-like structure, the majority of which was cellular debris,

presumably a consequence of rampant viral lysis. Consistent with this, large amounts of virus were extracted from tumours of mice injected with HSV1716EGFR1 compared to HSV1716-injected mice. Thus, 3.1×10^5 , 5.2×10^6 or 8.7×10^6 pfu were extracted from tumours of mice sacrificed on days 16, 23 or 24 respectively after HSV1716EGFR1 injection compared to 0, 0 or 4.1×10^3 pfu extracted from HSV1716-injected mouse tumours on days 16, 17 or 17 respectively. The failure to detect any virus in 2/3 HSV1716-injected tumour extracts is consistent with the small localized foci of virus replication observed immunohistochemically in these tumours (see below, Fig 19c) or, alternatively, after intravenous injection, little or no unmodified HSV1716 lodged in these tumours. No virus was extracted from the skin, heart, liver, gut, lung, brain, bone, spleen or kidney of several mice injected with HSV1716EGFR1 and this lack of virus in these organs was confirmed using an HSV-1 specific PCR with DNA extracted from these tissues as template.

Immunohistochemistry (Fig. 19) confirmed this by demonstrating exceedingly high levels of HSV1716EGFR1 and HSV1716EGFR2 replication within tumours when compared with HSV1716. Immunohistochemical analysis of excised tumours clearly demonstrated widespread virus replication following injection of HSV1716EGFR1 and 2 (Fig 19a and b) compared to the more localized foci of unmodified HSV1716 replication (Fig 19c) with no virus replication detected in the PBS-injected control (Fig 19d). Thus, systemic delivery of tumour targeted HSV1716 is more effective at reducing tumour burdens and enhancing survival than untargeted HSV1716.

Further evidence of the ability of 8/274gD to target HSV1716EGFR1 was provided by intraperitoneal injection of nude mice bearing subcutaneous A431 tumours. On days 1, 3, 5, 6 and 9 the mice were injected with PBS or 2×10^6 pfu HSV1716 or

HSV1716EGFR1. When injected by this route, HSV1716 was ineffective as the median survival time for both PBS- and HSV1716-injected mice was 18.5 days. At this time, the tumours on HSV1716EGFR1 mice were significantly smaller (e.g. on day 17; HSV1716 = 736 ± 156 mm vs HSV1716EGFR1 = 244 ± 60 mm, p value = 0.033 and on day 20; HSV1716 = 843 ± 142 mm vs HSV1716EGFR1 = 388 ± 107 mm, p value = 0.04) resulting in a prolonged median survival of 28 days for the HSV1716EGFR1-injected mice. Thus, by intraperitoneal injection, presumably, insufficient or no HSV1716 must reach the subcutaneous tumours to have any effect on tumour growth whereas targeting the virus to EGFR on the tumour cell surface promoted tumour uptake and prolonged survival.

Discussion

Recent Phase 1 clinical trials have demonstrated an excellent safety profile following direct intratumoral injection of oncolytic HSV1716 and the efficacy of this virus is being investigated currently in a Phase II/III trial for the treatment of recurrent glioma. It is clear, however, that the therapeutic usefulness of HSV1716 will be greatly facilitated if the virus can be administered via the circulation. In addition, the platform of tumour indications viz haematological tumours and metastatic disease, susceptible to oncolytic viral therapy could be greatly expanded. Incorporation of tumour-targeting moieties into the virus structure is a prerequisite for such strategies.

We have analysed the suitability of various glycoprotein fragments to direct the incorporation of N-terminally fused single chain antibodies into the HSV-1 envelope and assessed the abilities of incorporated fusion proteins to alter the tropism of the virus. Although scFv/glycoprotein fusion proteins have been used to redirect the tropism of various different viruses, including HSV-1 (Menotti et al 2006), these studies have used the scFv linked to almost full length glycoproteins close to the N terminus and optimization of

incorporation and targeting ability have not been analysed. We linked a scFv from the well-characterised anti-GD3 ganglioside monoclonal antibody, R24 to 15 sequentially deleted gD fragments and to full length gH and gL which form a heterodimer with both proteins required for its correct folding and membrane presentation.⁴⁴ Linking the scFvs to both gH and gL could result in the formation of dimeric scFvs with enhanced avidity and to promote this, the size of the linker between the scFv and the N terminus of gL was varied.

All of the constructs were created in mammalian cell expression vectors for stable cell line production with the plasmids used for tandem expression of gH and gL fusion proteins providing different antibiotic resistances. The resultant R24/glycoprotein fusion proteins were incorporated into virus by infecting the cell lines with HSV1716 expressing green fluorescent protein and epitope tagging of the fusion proteins allowed estimations of their levels of expression and incorporation into virus. The infectivity of the viruses derived from stable cell lines was assessed using fluorescence microscopy to monitor gfp expression in CHO cells which stained positively with R24. Since CHO cells are non-permissive for Vero cell propagated HSV1716 infection, they provided an excellent system to assess alterations to viral tropism.⁴⁵ HSV-1 is endocytosed by CHO cells but infection requires that the cells express an HSV-1 receptor such as nectin-1.^{46, 47} In normal CHO cells, endocytosed HSV-1 is destroyed but this process is prevented by the presence of the HSV-1 receptor and the virion glycoproteins gB, gD and gH/gL in the cells.

Using an anti-myc monoclonal antibody in Western blotting to probe extracts from stably expressing Vero cell lines, only 2/15 R24/gD fusion protein constructs failed to produce detectable amounts of protein. This is presumed to be due to scFv fusion to this gD residue resulting in unstable proteins that are rapidly degraded. Of the expressed R24/gD fusion

proteins, only two gD chimaeras were not detected in virus despite both incorporated and excluded glycoprotein fusion proteins having the same apparent cellular localizations. This is likely to be due to some structural defect in the non-incorporated proteins which prevented their assimilation into the viral envelope.

Both R24 and anti-CD38 scFvs linked to a number of gD deletions and also independently incorporated into gH/gL heterodimers were able to redirect HSV1716 infectivity. Out of 11 R24/gD fusion proteins which showed virus incorporation, six, in which R24 was linked to residues 37, 78, 92, 128, 260 and 274 were able to influence viral tropism with between 10% and 60% of normally non-permissive CHO cells infected compared to less than 1% with the remaining R24/gD. A greater influence on CHO cell infectivity was observed using viruses displaying R24 linked to gD residues 260 and 274. These smaller polypeptides may represent the minimum gD fragments required for optimum membrane incorporation and display of the receptor-binding scFv. However, as there was considerable variation in infection levels amongst the six R24/gD fusion proteins that mediated CHO cell penetration, other factors, such as scFv conformation and stability and antigen binding site display and accessibility, must also be involved. Attachment of the scFv to a number of different residues within gD region 139-239 resulted in fusion proteins which were poorly incorporated into virus and failed to significantly redirect HSV1716 tropism. Thus, either the low levels of fusion proteins with R24 linked to 139gD, 179gD, 191gD, 207gD or 239gD were insufficient to instigate receptor mediated penetration or scFv attachment to these amino acids resulted in structural destabilization and loss of antigen binding.

Although the ability of a subset of R24/gD fusion proteins to direct CHO cell infection strongly suggests that these chimaeric proteins alter HSV1716 tropism, conclusive proof was

not obtained as CHO cell infectivity could not be blocked specifically using the parental R24 monoclonal antibody. Although preincubation with the R24 Mab greatly reduced CHO cell infectivity mediated by the six R24/gD fusion proteins, it also reduced the levels of CHO cell infectivity by HSV1716 propagated on BHK cells and we were unable therefore to distinguish between a specific or non-specific inhibition of viral entry by R24 bound to GD3 ganglioside on the cell surface. However, in subsequent experiments using an anti-CD38 monoclonal antibody and CD38-expressing THP-1 cells a specific inhibition of infection was demonstrated.

It has been shown that the tropism of an oncolytic measles virus was redesigned using scFvs to CD38 or CD20 linked to the measles virus envelope glycoprotein H.²⁰⁻²¹ The human monocyte cell line THP-1 is CD38 positive but CD20 negative and is semi-permissive for HSV-1 infection with 10-20% of cells infected by 1pfu/cell HSV1716. ScFvs were derived from monoclonal antibodies that recognize CD20 (scFv5) or CD38 (scFvT) and viruses were propagated on stable Vero cell lines constitutively expressing either scFv5 or scFvT linked to gD residues 37, 128, 260 or 274. The number of THP-1 cells infected by viruses displaying 5/37gD, 5/128gD, 5/260gD or 5/274gD was similar to the numbers infected by HSV1716 propagated on Vero cells and these levels of infection were unaffected by preincubation of the cells in Mab5, MabT or control medium. Higher levels of THP-1 cell infection resulted from viruses displaying scFvT linked to gD residues 37, 128, 260 or 274 indicating that these viruses were able to use CD38 as a receptor for viral entry and, significantly, preincubation of THP-1 cells in MabT but not in Mab5 or control medium greatly reduced their infection rates. Thus, although HSV1716 can infect some THP-1 cells, indicating the presence of an HSV-1 receptor, most likely HVEM, receptor-binding sites must be limited either because of low levels of the receptor or poor accessibility on the cell surface. The presence of a CD38-binding moiety displayed on the virus

coupled with the cognate antigen on the cellular membrane, must increase the proportion of specific virus/cellular interactions which result in augmented infection. As with the R24/gD fusion proteins, T/260gD and T/274gD were better targeting moieties than T/37gD or T/128gD fusion proteins indicating that these small gD fragments are sufficient to ensure efficient membrane incorporation and appropriate scFv display.

Previous reports have identified gC and gD fusion proteins which have altered HSV-1 tropism but, to the best of our knowledge, this is the first description of the construction of receptor-binding fusion proteins based on the gH/gL heterodimer. By linking the scFv to the N terminus of full length gH and gL and by varying the size of the spacer arm linkage between the scFv and gL, fusion proteins that overcame the non- and semi-permissivity of CHO and THP-1 cells were produced. We postulated that during formation of the gH/gL complex, the N-terminal scFvs may form dimers that would increase the avidity for cognate antigen binding leading to enhanced infectivity. Certainly, there was some variability in the CHO cell infection levels of the R24/gL constructs with 0, 1, 2 or 3 copies of the Gly-Gly-Gly-Gly-Ser spacer and the highest levels of infection were obtained using the R24/gL fusion proteins with 1 or 2 copies of the spacer.

However, the 30-40% of CHO cells infected by viruses displaying either the R24/gH/gL1 or R24gH/gL2 fusion protein heterodimers was lower than the 50-60% of CHO cells infected by viruses displaying either R24/260gD or R24/274gD suggests that the gH/gL heterodimer is less efficient than the small gD fragments at scFv display/ presentation. Possibly, the R24/gH/gL heterodimers are present at lower concentrations in the viral envelope than the R24/260gD or R24/274gD fusion proteins or alternatively, the scFv stability or accessibility in the gH/gL heterodimer is sub-optimal. As with the R24/gD fusion proteins, a virus displaying scFvT in the gH/gL

heterodimer infected higher numbers of THP-1 cells compared to a similar virus displaying scFv5 and the level of infection was reduced by MabT but not by Mab5 or control medium and the T/gH/gL2 heterodimer was less efficient at directing THP-1 cell infection than either T/260gD or T/274gD. Despite this, scFv displayed as part of the gH/gL heterodimer were able to influence the tropism of HSV1716 and may provide an additional/alternative targeting route.

Our initial analysis using infection of Vero cell lines to achieve viral assimilation of targeting moieties identified an efficient scFv mediated route to influence HSV1716 tropism. However, although this cell-based system was a convenient method to analyse a large number of different fusion protein constructs, production of targeted viruses is restricted to propagation on the appropriate cell line. Recombinant viruses expressing the targeting moieties during infection will be required and we produced a series of recombinant HSV1716 viruses expressing scFvs 5, T, 8 and B linked to 274gD. These viruses were created using an efficient and rapid site-specific recombination protocol based on the Gateway cloning system. A single *in vitro* recombination reaction circumvented the need for any *in vivo* recombination in bacteria or tissue culture cells and variants were typically isolated in 5-7 days. Also the efficiency of the *in vitro* recombination reaction was not compromised by the large 3kbp inserts used in this study and other advantages include the use of very small amounts of viral DNA/entry plasmid and easy detection of contaminating non-recombinant DsRed viruses by fluorescence microscopy. PCR confirmed the genomic structure at the RL1 loci of the HSV1716 variants with inserted 5/274gD, T/274gD, B/274gD and 8/274gD transgenes and Western blotting confirmed both expression of the 45kDa fusion proteins and their incorporation into virus. The orientation of the scFv/274gD expression cassette relative to the PGK-gfp expression cassette was important as one virus, in which 5/274gD was in reverse orientation resulting in the close juxtaposition of

the CMV-IE and PGK promoters, failed to express the scFv/gD fusion protein. Elements of the PGK promoter immediately adjacent to the CMV-IE promoter must inhibit its transcriptional ability.

HSV1716 variants that express scFvB and scFvT, linked to 274gD, redirected the tropism of the virus allowing infection of non-permissive, DAF-positive CHO cells and semi-permissive CD38-positive SupT, THP-1 and TolB cells respectively. Viruses displaying an anti-EGFR scFv linked to 274gD were unable to infect EGFR negative CHO/DAF, SupT, THP-1 and TolB cells. Infection mediated by scFvB was dependent on specific antibody/antigen interactions as MabB or a recombinant MabB minibody specifically blocked infection of CHO/DAF cells by the HSV1716 variant expressing B/274gD whereas unrelated Mabs T, 8 or 5 had no effect. Similarly, increased infection levels of SupT, THP-1 and TolB cells by the HSV1716 variant expressing T/274gD were specifically inhibited by MabT but not by Mabs 5, 8 or B.

We attempted to produce other HSV1716 recombinants with redirected tropism using scFv 5, T, B or 8 each linked to gD residues 37, 92 and 128 but the resultant viruses had impaired replication and were difficult to plaque purify (data not shown). Structurally, the gD extracellular domain (amino acids 1-315) consists of N- and C- terminal extensions flanking a central Ig-folded core comprising residues 56-184 with HVEM or nectin-1 binding respectively requiring residues 1-34 or 34-243.⁴⁸⁻⁵¹ Therefore, apart possibly from scFv/37gD, none of these scFv/gD fusion proteins will be able to bind either HVEM or nectin-1 and their incorporation into virus at the expense of native gD or other essential membrane glycoproteins probably prevented the formation of infectious progeny. Propagation of the targeted viruses on BHK cells engineered to express the targeted antigen may circumvent this restriction. We were able to produce HSV1716 variants expressing scFvB and T linked to 260gD and these, similar to viruses expressing

B/274gD and T/274gD were able to infect efficiently CHO/DAF and SupT/THP-1/TolB cells respectively. We also attempted to produce HSV1716 variants expressing scFvs 5, T, 8 or B linked directly to the N-terminus of gH and to gL via 1, 2 or 3 copies of the linker but, again, these viruses replicated poorly and were difficult to purify (data not shown). Possibly, the addition of 12kbp to the viral genome by insertion of the PGK-gfp, scFv/gL and scFv/gH expression cassettes into both copies of RL1 reduced stability and packaging efficiency or, more likely, the expression/incorporation of scFv/gH and scFv/gL prevented normal assimilation of essential membrane glycoproteins and the resultant progeny were non-viable. Again, growth of the scFv/gH/gL viruses on modified BHK cells that express the targeted antigen may alleviate this restriction.

Using the EGFR-positive human squamous cell carcinoma cell line A431 to form subcutaneous tumours in nude mice, we were able to demonstrate that systemically delivered HSV1716 targeted to EGFR by an anti-EGFR scFv linked to 274gD was better able to destroy tumours and promote survival than unmodified HSV1716. Thus, two independently isolated variants of HSV1716 expressing 8/274gD reduced the tumour burden and prolonged survival compared to HSV1716. Indeed, the targeted viruses performed better than our data suggests as a number of mice which received HSV1716EGFR1 and HSV1716EGFR2 were killed prematurely because, although their apparent tumour diameters were 15mm, when the tumours were excised after sacrifice the bulk of the mass consisted of cellular detritus surrounding a much reduced tumour. Presumably, the EGFR binding capability of the targeted HSV1716 variants augmented greatly the number of viruses locating to the tumour compared to the untargeted HSV1716 and this initial higher dose of virus adsorbed from the circulation was responsible for greatly enhanced tumour destruction. Immunohistochemical and virus titration analysis of excised tumours confirmed this as levels of virus replication in mice injected intravenously with HSV1716

expressing 8/274gD were much higher than in mice receiving unmodified HSV1716. Further evidence of the efficacy of tumour targeting was provided using intraperitoneal injection. By this route of administration, HSV1716 had no discernable effect on tumour growth whereas HSV1716EGFR significantly reduced tumour growth and prolonged survival.

Although an HSV1716 variant expressing scFv5 linked to 274gD was produced, no data demonstrating its abilities to alter tropism was generated. The HSV1716 variant expressing 5/274gD did not enhance infection of the CD20 positive TolB cells although possibly, as Mab5 only reacted weakly with TolB cells, there was insufficient CD20 available to mediate viral penetration or, alternatively, as the recombinant minibody version of Mab5 did not react with TolB cells (data not shown), there was a loss of binding affinity or specificity during conversion to the single chain format.

These studies were performed to identify a reliable method for redesigning the tropism of oncolytic herpes simplex virus such that systemically delivered virus will be more effectively targeted to tumour cells. We have developed a plasmid-based system to link a monoclonal antibody-derived scFv to gD residue 274 and recombinant viruses which display the targeting scFv are produced rapidly using a novel *in vitro* site-specific recombination system. ScFvs have been used previously to target HSV-1 with an anti-EGFR scFv linked to the gD-binding domain of nectin-1 creating a soluble bridging molecule capable of attaching the virus to CHO cells displaying EGFR on their surface and insertion of an anti-HER2/neu scFv at gD residue 24 to target the virus to CHO cells expressing the HER2/neu receptor.^{35, 36} The soluble bridging molecule required gD to link virus and cell but the virus expressing the anti-HER2/neu scFv/gD fusion protein was gD negative. However, insertion of the scFv at residue 24 will only ablate HVEM binding and the chimaeric protein was still able to use nectin-1 as a receptor. Here, HSV1716 variants

that express an anti-DAF scFv or an anti-CD38 scFv linked to gD residue 274 mediated infection via cognate antigen binding although the recombinant viruses retained gD and were still able to infect cells using either HVEM or nectin-1. Possibly, the fusion proteins can function as the sole receptor-binding protein to mediate infection of targeted cells as the scFv will mimic the specific interaction required to tether the virus closely to the cellular membrane and membrane fusion will be elicited via the remaining membrane-proximal residues of gD which form a pro-fusion domain required for recruitment of gB and gH/gL.⁵² Currently, we are attempting to create a gD-negative HSV1716 variant but it is interesting to speculate whether a gD-negative virus is mandatory to achieve optimal tumour targeting. Certainly, the targeted HSV1716 expressing both full length gD and 8/274gD demonstrated enhanced tumour uptake and destruction compared to unmodified HSV1716 suggesting that adsorption by non tumour cells of the systemically injected HSV1716 variants is not a major issue. Neutralising antibodies that target gD may be a problem for such systemically administered HSV1716 variants and ablation of the gD ORF will prevent this. However, with such a virus, once a tumour has been infected, viral spread to other tumour cells in the mass will be totally dependent on a specific scFv/antigen interaction. As heterogeneity is a feature of many tumour types, not all cells will display the specific membrane protein and will therefore avoid infection. Perhaps, the presence of both scFv/gD fusion protein and gD in the virus envelope is a better configuration to allow efficient tumour targeting and optimum infection rates. Currently, the abilities of systemically delivered HSV1716 variants expressing various targeting scFvs to destroy tumour xenografts in mice are being assessed.

Materials and Methods

Cells and Viruses

BHK cells, CHO-K1 and Vero cells were grown as previously described.⁴⁵ Hybridomas MabR24, MabIF5 (Mab5), MabTHB-7 (MabT)

and Mab108 (Mab8), expressing monoclonal antibodies against the ganglioside GD3, CD20, CD38 and EGFR respectively and the human leukaemic SupT, THP-1 and TolB cell lines, were obtained from ATCC/LGCpromochem, London, UK and were cultured in RPMI1640 medium supplemented with 10% NCS. Hybridoma HD1B (MabB) which expresses a monoclonal antibody that recognizes human decay accelerating factor (DAF/CD55), a kind gift from Dr Claire Harris, Dept. of Medical Biochemistry, University of Wales College of Medicine, Cardiff, UK, was also maintained in RPMI1640 medium supplemented with 10% NCS. HSV1716gfp is described in Harland et al and production of virus stocks is described in Conner et al.^{6, 45}

Preparation of scFvs from hybridoma cells

Hybridoma cells were grown to high density and cell pellets obtained by centrifugation at 1000xg for 5 mins. Total RNA was extracted using the SV Total RNA kit (Promega, Southampton, UK) and cDNA prepared using the ImProm II Reverse Transcription System (Promega). Recombinant scFv variants of the monoclonal antibodies were derived from these cDNAs using the protocols and PCR primers described by Pope et al.⁵⁴ ScFv-encoding DNAs for each monoclonal antibody were prepared by RT-PCR amplification and linkage of antibody VH and VL sequences with the resultant scFv DNAs cloned into pGEM-Teasy (Promega) for DNA sequencing. The scFv-encoding DNAs were amplified using PCR primers that incorporate *SfiI* and *NotI* restriction enzymes sites at the 5' and 3' ends respectively for cloning in the phagemid vector pCANTAB6. Competent *E. coli* cells (strain HB2151) were transformed with the phagemid vectors and scFv expression by IPTG induction was confirmed by Western blotting (data not shown); scFvs expressed from pCANTAB6 have *myc* tags for detection.

Construction of pEL4, a vector for scFv/glycoprotein fusion protein production

Plasmid pEL4 for expression of scFv/glycoprotein fusion proteins was created to allow PCR-amplified scFv DNAs to be

cloned in-frame downstream of an IgG heavy chain leader sequence as *SfiI/NotI* fragments and in-frame upstream of a PCR-amplified HSV-1 glycoprotein DNA fragment. Initially, using pCANTAB6 with an R24 scFv insert as template, the scFv DNA was amplified using primers ELsfifor and R24notrev. Primer ELsfifor is a 99-base oligonucleotide [GAATTCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACT CCGCGGCCAGCCGCCGATGTGCAACTGGTGGAGTCT] (SEQ ID NO: 7) that has a 5' *EcoRI* site (in bold) then 63 bases (underlined) encode the R24 IgG heavy chain leader sequence followed by the R24 scFv 5' sequence with an intervening *SfiI* site (in bold and underlined). PCR with ELsfifor and R24notrev [GAGTCATTCTGCGGCCGCCGTTTATCTCCAGCTTGCT] (SEQ ID NO: 8), the anti-sense primer exactly matching the 3' end of the R24 scFv with a *NotI* site (in bold), generated the R24 scFv DNA for cloning into the *EcoRI/NotI* sites in the mammalian expression plasmid pCDNA4myc/HisA (Invitrogen, Paisley, UK) to generate pEL4. The presence of the IgG leader in this plasmid was confirmed by sequencing. The R24 scFv component in pEL4 can be readily exchanged with an alternative scFv, amplified by PCR using primers that provide flanking *SfiI/NotI* sites (Pope et al 1996).

Since each of the scFvs created in this study has a unique *RsaI* fingerprint, any scFv exchange was confirmed by PCR amplification of the scFv DNA and *RsaI* digestion (data not shown). Each fusion protein construct assembled in pEL4 consisted of a secretory leader sequence and N-terminal antibody domains for binding to a targeting ligand linked to C-terminal viral glycoprotein domains for virus incorporation.

Construction of vectors for the expression of scFv/glycoprotein fusion proteins

Three HSV-1 glycoproteins, gD, gH and gL were used for fusion protein construction and the DNA fragments were PCR amplified from HSV1716 DNA.

For HSV-1 gD which comprises 393 amino acids with the TM domain located between aa 342-364, 15 random deletions sequentially removing aa 1-273 were PCR amplified using the 15 forward and common reverse primers shown in Table 1. The gD forward primers are identified by the extent of the N-terminal deletion (e.g. 108gD amplified a gD DNA fragment which starts at nucleotide 109 and encodes a gD polypeptide with 36 amino acids deleted from the N terminus). An additional A nucleotide between the *NotI* site (in bold) and the gD sequences (underlined) ensured that the gD and scFv DNAs were cloned in frame; the *NotI* site plus the additional A inserts an Ala-Ala-Ala linker between the scFv and glycoprotein domains of the fusion protein. The gD reverse primer lacked a stop codon and the *XhoI* site used for cloning ensured that the inserted fragment was in-frame with the Myc/His tags provided by the pCDNA4 parent vector. After PCR amplification from viral DNA, the gD fragments were sequentially digested with *NotI* followed by *XhoI* and ligated into the likewise digested pEL4 containing 5, T, B or 8 scFv DNAs. In some instances, the gD PCR fragments were cloned into pGEMT-easy and sequenced to confirm their identity (data not shown).

HSV-1 gH (838 amino acids) and gL (224 amino acids) form a heterodimer in the virus envelope and the complete coding sequences minus signal peptides for both these proteins were PCR amplified for cloning into pEL4. HSV-1 gH was amplified using the forward primer GGCC**GGCGCCG**CAGTCCACGACTGGACTGAGCAGA (SEQ ID NO: 9), which amplifies from nucleotide 58 (gH sequence underlined) and incorporates a *NotI* site (bold) upstream for cloning in frame with the scFv of pEL4. The reverse primer, GGGCCCC**CTCGAGTTAGGCGTAGTCCGGGACATCATAGGGGTA**TTTCGCGTCTCCAAAAAACG GGAC (SEQ ID NO: 10), has a *XhoI* site (bold) for cloning and, in frame with the 3' gH sequence (underlined) minus stop codon, the primer incorporated an HA-tag sequence with stop codon (in bold and underlined). PCR amplified gH was sequentially digested with *NotI* followed by *XhoI* and ligated

into the likewise digested pEL4 containing scFv 5, T, B or 8. To allow the antibody domains in the gH/gL heterodimer to interact, the scFv was tethered to the gL N terminus directly or by a flexible linker comprising 1, 2, or 3 copies of a Gly-Gly-Gly-Gly-Ser spacer. HSV-1 gL downstream of nucleotide 60 was PCR amplified using the forward and reverse primers shown in Table 1 which incorporate 5' *NotI* and 3' *XbaI* restriction enzyme sites for cloning into pEL4. The forward primers gLfor0, gLfor1, gLfor2 and gLfor3 respectively incorporate 0, 1, 2 or 3 copies of Gly-Gly-Gly-Gly-Ser to the N terminus of the gL and gLrev has no stop codon but is in-frame with the pEL4 Myc/His tags. PCR amplified gL DNAs were sequentially digested with *NotI* followed by *XbaI* and ligated into the likewise digested pEL4 with 5, T, B or 8 scFv inserts. For dual expression in the same cell, scFv/gH constructs were transferred as *HindIII/XhoI* fragments into p3.1Hygro (Invitrogen), a vector which provides a CMV promoter and a hygromycin selection gene and the scFv/gL constructs were transferred as *HindIII/PmeI* fragments into pEF4Myc/His (Invitrogen), a vector with an EF-1a promoter and a zeocin selection gene.

For gD deletions, the scFv fusion proteins were designated according to the scFv used to make the construct with Mabs R24, 5, T, B and 8 providing the prefixes followed by the amino acid of gD to which it is linked. For example R24/128gD (amplified using primer 381gD) comprises the R24 scFv against GD3 linked to gD amino acid 128 with amino acids 1-127 deleted. For gH and gL fusion protein designation, 5gH/gL and 5gH/gL3 are the heterodimers comprising scFv5, against CD20, linked directly to gH and to gL either directly or with a 3x(Gly-Gly-Gly-Gly-Ser) spacer arm.

Production of stable cell lines expressing scFv/glycoprotein fusion proteins

Vero cells were transfected with 50ug scFv/gD fusion protein-encoding plasmids mixed with 10ul lipofectamine 2000

(Invitrogen) in 250ul of serum free DMEM/F12 medium. After 48-72 hours of transfection, cells were trypsinized and plated out with growth medium containing 1mg/ml zeocin (Invitrogen). Cells were selected with the zeocin antibiotic for 2-3 weeks after which time individual clones were clearly visible. Cells were trypsinized and cloned by limiting dilutions in 24-well plates. Clones were expanded and maintained in appropriate medium containing 1mg/ml zeocin. For Vero cells expressing scFv/gH and scFv/gL fusion proteins, 50ug of each plasmid was transfected in 20ul lipofectamine 2000 in 250ul serum free DMEM/F12 and, after trypsinisation and plating out, selection was achieved using 0.5mg/ml zeocin/hygromycin in combination. Expression of scFv/glycoprotein fusion proteins was confirmed either by Western blotting or by immunofluorescence using either anti-myc or anti-HA monoclonal antibodies (New England Biolabs, Hitchin, UK).

For production of virus stocks, confluent monolayers of stable cell lines were infected with 1×10^5 pfu HSV1716gfp and, after 3-4 days of infection, total virus was harvested, purified by high-speed centrifugation and titrated on BHK cells. All yields were within the range obtained for HSV1716gfp infection of normal Vero cells (data not shown). The presence of scFv/glycoprotein fusion proteins in the virion was confirmed by Western blotting using either anti-myc or anti-HA monoclonal antibodies.

Production of recombinant viruses expressing scFv/glycoprotein fusion proteins by homologous recombination

Previous analysis of results from stable cell lines identified potential tropism-altering constructs for recombinant virus production and the pEL4 constructs were used in two ways to generate HSV1716 variants expressing the relevant scFv/glycoprotein fusion proteins. In the first series of targeted viruses, plasmid sp73 (Promega) was used to assemble constructs prior to recombinant virus production. Initially, a gfp expression cassette was inserted into sp73 using the

1.3kbp blunt-ended *EcoRI*/*AflIII* fragment that contains the PGK promoter/gfp gene obtained by restriction digestion, followed by Klenow in-filling from the vector pSNRG (OligoEngine, Seattle, Washington, USA) and ligation into the sp73 vector cut with *EcoRV* then alkaline phosphatase treated. Successful insertion of the PGK/gfp DNA to create the vector sp73gfp was confirmed by *XhoI*/*BglIII* digestion and the orientation of the inserted DNA identified using the unique *XhoI* site in sp73 and the unique *BsrGI* site at the 3' end of PGK/gfp.

The gC promoter was amplified from HSV-1 DNA by PCR as a *SacI*/*KpnI* fragment using primers gCforsac (GT**GAGCTC**CCGAAGACCGCCGGTGTG (SEQ ID NO: 11), *SacI* in bold, gC promoter 5' sequence underlined) and gCrevkpn (AG**GGTACC**GGCAAAGCGAGACCGGG (SEQ ID NO: 12), *KpnI* site in bold, gC promoter 3' sequence underlined). After sequential *SacI*/*KpnI* digestion, the promoter DNA was ligated into *SacI*/*KpnI* digested sp73gfp to create the vector gCp73gfp. The TK promoter was amplified from HSV-1 DNA by PCR as a *Sall*/*PstI* fragment using primers TKforsal (AGA**AGTCG**ACTAT GATGACACAAACCCCGCC (SEQ ID NO: 13), *Sall* site in bold, TK promoter 5' sequence underlined) and TKrevpst (G**ACTGCAG**TGCGGCACGCTGTTGACGCT (SEQ ID NO: 14), *PstI* site in bold, TK promoter 3' sequence underlined). After sequential *Sall*/*PstI* digestion, the promoter DNA was ligated into the likewise digested gCp73gfp to create the dual promoter vector gC/TKp73gfp.

The gD DNA components from the selected scFv/gD fusion protein constructs were re-amplified by PCR from HSV-1 DNA using the appropriate forward primers (Table 1 and the reverse primer GGCC**CTCGAG**GCATGC**GCACCCATTAAGGGGGGGTAT**CTAGTAAAACAAGGGCTGGTGCGAG GA (SEQ ID NO: 15), which incorporates the gD stop codon and the 21 nucleotides immediately downstream of the gD stop codon in the HSV-1 genome (underlined in bold). The reverse primer also incorporates a *XhoI* site (in bold) and the *NotI*/*XhoI*-

digested gD DNA was ligated into the likewise digested appropriate scFv-containing pEL4 to recreate the scFv/gD fusion protein. This was excised from the pEL4 plasmid by *HindIII/XhoI* digestion and ligated into the likewise digested gCp73gfp such that expression of the scFv/gD fusion protein is controlled by the HSV-1 gC promoter.

To construct a plasmid suitable for generating HSV1716 variants expressing scFv/glycoprotein fusion proteins, a shuttle vector for the insertion of transgenes in the RL1 loci, termed RL1-del was used. RL1-del was designed as a promoterless cloning vector suitable for generating ICP34.5 null HSV-1 and consists of an HSV-1 DNA fragment containing the RL1 gene and its flanking sequences with the majority of the ICP34.5 open reading frame removed and replaced with a multi-cloning sequence (MCS). The transgene to be inserted into the RL1 loci was ligated into the MCS of RL1-del and homologous recombination with HSV-1 DNA, driven by the RL1 flanking sequences, results in concomitant deletion of the ICP34.5 gene and incorporation of the desired transgene. RL1-del contains the HSV-1 *BamHI* k DNA fragment (123459-129403) which includes the RL1 gene and its flanking sequences cloned into the *BamHI* site of plasmid pGem-3Zf (Promega). The 477bp *PflMI/BstEII* fragment from the RL1 ORF (125292-125769) has been removed to inactivate the ICP34.5 gene and replaced with a MCS providing unique restriction enzymes sites for *BglII*, *NruI* and *XhoI*.

The scFv/gD fusion protein and gfp expression cassettes were excised from their appropriate gCp73/gfp plasmids by *BglII/XhoI* digestion and ligated into RL1-del which was also *BglII/XhoI* digested. The *AseI* sites which flank the MCS of sp73 were used to excise the 6kbp DNA fragment containing the PGK/gfp, gCp/scFv/gL and TKp/scFv/gH expression cassettes from the relevant gC/TKp73gfp vectors and, after blunt ending with Klenow, the fragment was ligated into RL1-del digested with *NruI* and alkaline phosphatase treated. For any scFvs that

contained *AseI* sites, the alternative *DrdI* sites flanking the sp73 MCS were used to excise the PGK/gfp, gCp/scFv/gL and TKp/scFv/gH expression cassettes. Approximately 50ug of RL1-del plasmid with scFv/gD or scFv/gH/gL fusion protein inserts were linearized by *XmnI* digestion. After clean-up using a GFX kit (GE Healthcare, Little Chalfont, UK), the linearised plasmids were used in conjunction with HSV-1 DNA to cotransfect BHK cells.

RL1-del and viral DNA (100ug) were mixed with 20ul lipofectamine 2000 in 250ul DMEM/F12 serum-free medium and added to a 60mm plate which contained 50% confluent BHK cells. After 4 hours of incubation at 37°C, the medium was removed and the cells shocked for exactly 4 minutes with 25% DMSO. After 3 washes with 5ml culture medium the cells were returned to 37°C with 5ml BHK medium and left for 72 hours. Cells were scraped into the supernatant, the mixture sonicated for 2 minutes and stored at -70°C until required. Serial dilutions were plated out on Vero cells in 60mm plates, individual green fluorescent plaques were picked, added to 1ml culture medium and sonicated for 2 minutes before serial dilutions were again plated out on Vero cells. Plaque purification was repeated 6-10 times before stocks of HSV1716-scFv/gD or HSV1716-scFv/gL/gH were produced. The presence of either the scFv/gD or scFv/gL/gH expression cassettes in the RL1 loci of HSV1716 was confirmed by Southern blotting using the *AluI/RsaI* ICP34.5 fragment from plasmid pGEM34.5 (McKie et al 1994). Incorporation of these cassettes into RL1 alters the size of HSV-1 *BamHI* k fragment dependent on orientation of the insert relevant to the RL1 flanking sequences (scFv/gH/gL only) and/or the numbers of additional *BamHI* sites in the inserts (data not shown). In each of the viruses constructed, fusion protein expression was controlled by endogenous HSV-1 early (TK- scFv/gH) or late (gC-scFv/gD or -scFv/gL) promoters and although the expressed scFv/gH fusion protein retained its HA tag, the scFv/gD and scFv/gL fusion proteins lacked the vector-derived myc tags.

Production of viruses expressing scFv/gD fusion proteins by site-specific recombination

A series of targeted viruses was constructed using scFvs linked to myc-tagged 274gD using a novel *in vitro* site-specific recombination system. Central to this method was production of HSV1716GateRed, an HSV1716 variant, which contained Gateway destination sites located within each of the ICP34.5 deleted regions. The Gateway® Vector Conversion system (Invitrogen, Paisley, UK) provided DNA with attR site-specific recombination sequences for insertion into a vector of choice and was ligated into the *EcoRV*-digested and alkaline phosphatase-treated plasmid sp73 to create sp73gate. Once inserted in the plasmid, the DNA between the attR sites, encoding chloramphenicol resistance and the *ccdB* gene, was removed by *NotI/BstXI* digestion. The vector backbone was then blunt-ended with Klenow and alkaline phosphatase treated. The 1.3kbp CMV-DSred expression cassette was excised from the plasmid pCMV-DsRed-Express (BD Biosciences, Cowley, UK) by *AflIII/NsiI* digestion, blunt ended by Klenow and ligated into the sp73gate backbone to create the plasmid sp73gatered. The DNA consisting of the attR sites flanking the CMV-DSred expression cassette was excised from sp73gatered by *BglIII/XhoI* digestion, blunt-ended with Klenow and ligated into the blunt-ended, alkaline phosphatase-treated *BglIII* site in the plasmid RL1-del used for the production of HSV1716 variants by homologous recombination. RL1-del consists of the HSV-1 *BamHI* k DNA fragment (123459-129403) which includes the RL1 gene and its flanking sequences cloned into the *BamHI* site of plasmid pGem-3Zf (Promega). The 477bp *PflMI/BstEII* fragment from the RL1 ORF (125292-125769) has been removed to inactivate the ICP34.5 gene and replaced with a MCS providing unique restriction enzymes sites for *BglIII*, *NruI* and *XhoI*. The transgene to be inserted into the RL1 loci is ligated into the MCS of RL1-del and homologous recombination with HSV-1 DNA, driven by the RL1 flanking sequences, results in concomitant deletion of the ICP34.5 gene and incorporation of the desired transgene. Southern blotting confirmed that the Gateway

destination sites flanking the DsRed expression cassette was cloned into both RL1 loci (data not shown).

The Gateway entry vector, pENTR1A was modified for use in our HSV1716 site-specific recombination system as follows. The DNA between the attL sites in pENTR1A was removed by *EcoRI* digestion and the resulting vector backbone was blunt-ended and alkaline phosphatase treated. A green fluorescent protein expression cassette was inserted into the pENTR1A backbone by ligating it with the 1.3kbp blunt-ended *EcoRI/AflIII* fragment that contains the PGK promoter/gfp gene excised from the vector pSNRG (a kind gift from Dr B. Singh (MSKCC, New York, USA)). Although *EcoRI* digestion removed the *ccdB* gene from pENTR1A, a number of other restriction sites between the attL flanking sequences were retained for insertion of additional gene/DNA sequences of interest to be cloned into HSV1716 alongside the PGK-gfp expression cassette.

Novel HSV1716 variants were created *in vitro* by incubating HSV1716GateRed DNA with the appropriate gene/DNA sequence of interest inserted in pENTR1A-gfp. HSV1716GateRed DNA was obtained by phenol/chloroform extraction of BHK cells 24 hours after infection with the HSV1716GateRed virus and, after 70% isoprpropanol precipitation, the DNA was resuspended in 1ml nuclease free water. The expression cassettes for the scFv/274gD fusion proteins were excised directly from the appropriate pEL4 constructs as 2.5kbp *SspI/PvuII* fragments which contained the CMV-IE promoter, the scFv/gD encoding DNA and the BGH polyadenylation signal. These fragments were ligated directly into the plasmid pENT1A-gfp, which had been *BamHI* digested, blunt ended with Klenow and alkaline phosphatase treated. For site-specific recombination reactions using these pENTR1A-gfp constructs, 5ug plasmids and 3ug HSV1716GateRed DNA were incubated overnight with LR clonase. After enzyme inactivation by Proteinase K digestion, the entire reaction mix (11ul) was added to 250μl of serum free DMEM/F12 (Invitrogen) medium containing 10μl lipofectamine

2000 and used to transfect 50% confluent BHK cells in a 60mm dish. After 4-6 hours the cells were shocked in 25% DMSO/PBS, washed and cultured in 5ml of GMEM at 37°C for 48-72 hours. Cells were harvested by scraping into the medium and, after 2 minutes sonication, 5 sequential 10-fold dilutions were plated out on Vero cells. The *in vitro* site-specific recombination reaction was very efficient and converted up to 90% of viral DNA from DsRed to gfp expression such that, a single plaque picked from the serially diluted plates was 100% pure with no DsRed virus contamination and was used for HSV1716 recombinant stock production as described. All yields were within the range obtained for HSV1716gfp infection of normal Vero cells (data not shown).

PCR, with primers that amplify across the ICP34.5 deleted region was used to confirm the appropriate genomic structure at the RL1 loci of these viruses. Viral DNA was prepared using a Wizard SV genomic DNA kit from BHK cells 24 hours after infection with the relevant viruses at 5pfu/cell and 2ul extracted DNA was used as template for amplification by RL1 PCR. In addition to the viral DNA, the 50ul PCR mix contained 2uM primer R13, 14uM primer F3, 1mM Mg²⁺, 200uM each of dATP, dGTP, dCTP, dTTP, 200uM deazaGTP and 1.25U Platinum Pfx DNA polymerase (Invitrogen). The F3 primer sequence is CAGGCACGGCCCGATGACCGCCTC (SEQ ID NO: 16) corresponding to bases 125172-125195 and complementary to bases 1176-1199 of the HSV strain 17+ sequence. Primer R13 sequence is GGCCAGACGCCGAAAACG (SEQ ID NO: 17), complementary to bases 126035-126052 and corresponding to bases 319-336 of the HSV strain 17+ sequence. Primer F3 is positioned in the ICP34.5 coding region towards the 3'-end which is still present in HSV1716 and primer R13 lies outside the ICP34.5 ORF within the a sequence. The conditions for PCR were 94°C for 2 minutes then 35 cycles of 94°C for 15 seconds, 72°C for 1 minute and 72°C for 1 minute with a final extension of 72°C for 2 minutes. Samples were then analysed on 1% agarose gels.

Production of CHO cell lines expressing human CD55 and a recombinant MabB minibody

Plasmid pDAF, which contains a mammalian expression cassette for human CD55 with a hygromycin selectable marker, was a kind gift from Dr Claire Harris and was used to transfect CHO cells. Plasmid (50ug) was mixed with 0.25ml serum free DMEM/F12 medium containing 10ul lipofectamine 2000 was added to CHO cells and, after 48 hours in culture, cells were trypsinised and plated out in a T75 flask in growth medium supplemented with 0.5mg/ml hygromycin. After 14 days in culture individual colonies of cells were trypsinised and, after cloning by limiting dilution, a stable hygromycin-resistant CHO cell line, termed CHO/DAF, which expressed CD55, as assessed by Western blotting using MabB, was produced. The CHO/DAF cell line was maintained in DMEM/F12 supplemented with 10% NCS and 0.5mg/ml hygromycin.

Using the MabB scFv DNA cloned into pEL4, an expression cassette for a minibody construct comprising the scFv DNA linked to the human IgG1 CH2/CH3 (Fc) region DNA was prepared. The resultant expressed protein was able to dimerise via the IgG1 Fc region and was secreted into the medium from a stable CHO cell line isolated using the zeocin resistance gene of pEL4. The 5' and 3' primers for the amplification of the human IgG1 Fc DNA, which incorporate *Not1* and *Xho1* restriction sites (underlined), were respectively

ACCTTGCAGCGGCCGCAAGACCCAAATCTTGTGACAAAATC (SEQ ID NO: 18)

and GATCACGTCTCGAGTTATCATTTACCCGGAGACAGGGAGAGGCTCTTTCTG (SEQ ID NO: 19) and these were used to amplify the 700bp IgG1 Fc region from peripheral blood mononuclear cell total RNA by RT-PCR. The amplified fragment was cloned directly after RT-PCR into the PCR cloning vector pGEM-Teasy (Promega) and was sequenced to confirm its identity. The PCR fragment was digested sequentially using *Not1* followed by *Xho1* and ligated into the pEL4 plasmid containing scFvB to generate a plasmid that expresses the scFvB linked to the human IgG1 Fc region. CHO cells were transfected and selected as described above

except that the growth medium contained 1mg/ml zeocin for selection rather than hygromycin. Medium from zeocin-resistant stable cell lines was harvested after 5 days in culture and expression of the MabB minibody was confirmed by denaturing and non-denaturing PAGE/Western blotting using an anti-human IgG Fc antibody which identified respective 55kDa and 110kDa proteins (data not shown).

Fluorescence Microscopy/ Western Blotting/Immunofluorescence

As HSV1716 variants used in this study expressed gfp, the infectivity of progeny viruses was assessed by fluorescence microscopy as described in Conner et al.⁴⁵ For Western blotting, the stable cell lines expressing scFv/glycoprotein fusion proteins were plated out in 35mm plates and incubated for 24 hours at 37°C in 5% CO₂. Cell lines to be infected with the various HSV1716 variants expressing scFv/274gD fusion proteins were plated out in 35mm plates and incubated for 24 hours at 37°C in 5% CO₂ before infection. For harvesting, cells were washed once with 1ml PBS and whole cell extracts obtained by the direct addition of 0.2ml SDS PAGE sample buffer. After SDS PAGE and transfer to nitrocellulose membranes, blots were probed with either anti-myc or anti-HA monoclonal antibodies diluted 1:1000. To determine the presence of scFv/glycoprotein fusion proteins in virions, 1x10⁶ pfu purified virus were mixed with SDS PAGE sample buffer and analysed as described above. To assess infectivity of targeted viruses, confluent monolayers of cells were infected with 1x10⁷pfu virus and incubated for 8-24 hours before cells were harvested as described above. Blots were probed with a polyclonal antibody to the R1 subunit of the viral ribonucleotide reductase.⁵⁵ For immunofluorescence to detect scFv/glycoprotein fusion protein expression, the stable cell lines were plated out on glass coverslips and, after 18 hours in culture, cells were fixed in 4% paraformaldehyde. For immunofluorescence to detect scFv/274gD fusion protein expression by HSV1716 variants, BHK cells were plated out on glass coverslips and, cells were infected with the appropriate virus at 1pfu/cell for 24 hours

and fixed as described above. After blocking for 1 hour in 2% normal horse serum, cells were incubated overnight in either anti-myc or anti-HA monoclonal antibodies diluted 1:1,000, washed 4 times in PBS with 0.05% Tween (PBST) and incubated for 2-4 hours in anti-mouse IgG/FITC or IgG/Texas Red conjugates (Vector Laboratories Ltd) diluted 1:200. For recombinant minibody B, an anti-human Fc/FITC conjugate at 1:1000 was used. After 4 washes in PBST, coverslips were mounted and analysed by fluorescence microscopy.

In vivo tumour reduction studies

Female 6-8 week old athymic nude mice (Charles River Labs, UK) were maintained under specific pathogen free conditions. Actively growing A431 cells were harvested and after resuspension in PBS, 1×10^6 cells per mouse were injected subcutaneously. When tumours reached 5mm in diameter, the mice were injected either intravenously via the tail vein or intraperitoneally with PBS, HSV1716 or HSV1716EGFR1 or 2. Mice were inspected daily, when tumour volumes reached 15mm they were sacrificed and the tumour xenograft removed for immunohistochemistry/viral extraction. The viral load in tumours and various organs was assessed by plaque forming assay on BHK cells. Extracted intact tumours/organs were frozen immediately at -70°C and, after thawing, the tissues were mechanically homogenized in a Retsch homogenizer in 1ml PBS prior to titration.

Immunohistochemistry for HSV-1

Prior to immunohistochemistry tumour samples were fixed for at least 24 hours in 4% paraformaldehyde before embedding and sectioning using standard protocols. Briefly, paraffin embedded sections were dewaxed, dehydrated and endogenous peroxidase activity quenched. Sections were blocked with 10% normal goat serum before incubation in an anti-HSV-1 polyclonal primary antibody (DakoCytomation, Ely, UK) diluted 1:1000. A biotinylated secondary antibody at 1:500 dilution, an avidin/biotin complex solution and the chromagen DAB (all

from Vector Labs, Peterborough, UK) were then used to detect the primary antibody binding to tissue. The slides were counterstained, dehydrated mounted and analysed using light microscopy.

Table 1

Table 1 shows sequences of primers used for the PCR amplification of sequentially deleted gD fragments and for amplification of gL with 0, 1, 2 or 3 copies of the Gly-Gly-Gly-Gly-Ser spacer.

Restriction sites are in bold and HSV-1 glycoprotein sequences are underlined.

gD forward primers

108gD GGATCC**GCGGCCGC**AGCCGACCCCAATCGCTTTCGCGGC (SEQ ID NO: 20)
 171gD GGATCC**GCGG CCGC**AGGGGTCCGGCGCGTGTACCACATC (SEQ ID NO: 21)
 231gD GGATCC**GCGGCCGC**ACTCCCATCACGGTTTACTACGCC (SEQ ID NO: 22)
 273gD GGATCC**GCGG CCGC**ACGCAGCGTGCTCCTAAACGCACCG (SEQ ID NO: 23)
 312gD GGATCC**GCGGCCGC**AATTGT CCGCGGGGCC TCCGAAGAC (SEQ ID NO: 24)
 381gD GGATCC**GCGGCCGC**AGGAGGCAACTGTGCTATCCCCATC (SEQ ID NO: 25)
 414gD GGATCC**GCGGCCGC**AGAGTA CACCGAATGC TCCTACAAC (SEQ ID NO: 26)
 489gD GGATCC**GCGGCCGC**CAGACAGCTTCAGCGCCGTCAGCGAG (SEQ ID NO: 27)
 534gD GGATCC**GCGGCCGC**CACAGCCCCCGCGTTTGAGACCGCC (SEQ ID NO: 28)
 570gD GGATCC**GCGGCCGC**ACGGCTCGTGAAGATAAACGACTGG (SEQ ID NO: 29)
 618gD GGATCC**GCGGCCGC**AGAGCACCGAGCCAAGGGCTCCTGT (SEQ ID NO: 30)
 690gD GGATCC**GCGGCCGC**CAGGCCTACCAGCAGGGGTGACG (SEQ ID NO: 31)
 714gD GGATCC**GCGGCCGC**AGTGGACAGCATCGGGATGCTGCCC (SEQ ID NO: 32)
 777gD GGATCC**GCGGCCGC**AAGCTTGAAGATCGCCGGGTGGCAC (SEQ ID NO: 33)
 819gD GGATCC**GCGGCCGC**AACGAG CACCCTGCTG CCCCCGAG (SEQ ID NO: 34)

gD reverse primer

GGCCAAGCTT**CTCGAG**TCTAGAGTAAACAAGGGCTGGTG CGAGGA (SEQ ID NO: 35)

gL forward primers

gLfor0 GGCC**GCGGCCGC**ATTGTCTTCAACCGAATATGTTAT (SEQ ID NO: 36)
 gLfor1 GGATCC**GCGGCCGC**AGGTGGAGGCGGTTTCATTGTCTTCAA CCGAATATGTTATT
 (SEQ ID NO: 37)
 gLfor2 GGATCC**GCGGCCGC**AGGTGGAGGCGGTTTCAGGTGGAGGCGGTTTC
ATTGTCTTCAACCGAATATGTTATT (SEQ ID NO: 38)

gLfor3 GGATCCGCGGCCGCAGGTGGAGGCGGTTTCAGGTGGAGGCGGTTTC

AGGTGGAGGCGGTTTCATTGTCTTCAACCGAATATGTTATT (SEQ ID NO: 39)

gL reverse primer

GGCCAAGCTTGGGCCCCTCTAGAGAGGCGCCGGGAGTGGGGTCGTCG (SEQ ID NO: 40)

Table 2

Table 2 shows expression and CHO cell infection data from R24 scFv linked to 15 N-terminal deletions of HSV-1 gD.

Construct	Approx Mr (KDa) *	Vero cell expression **	Virus incorp **	Localization in Vero cells	CHO cell infectivity ***
R24/37gD†	70	++	++	perinuclear	30%
R24/58gD	67	n.d.	n.d.	n.d.	<1
R24/78gD†	65	++	++	perinuclear	10%
R24/92gD	64	++	++	perinuclear	20%
R24/105gD	61	n.d.	n.d.	n.d.	<1
R24/128gD	60	++	++	perinuclear	20%
R24/139gD	59	++	+	perinuclear	<1
R24/164gD†	56	++	n.d.	perinuclear	<1
R24/179gD	55	++	+	perinuclear	3
R24/191gD	54	++	+	perinuclear	<1
R24/207gD	52	++	+	perinuclear	<1
R24/231gD†	49	++	n.d.	perinuclear	<1
R24/239gD	48	++	+	perinuclear	<1
R24/260gD†	45	+++	++	perinuclear	60%
R24/274gD†	43	++	++	perinuclear	50%

*Fusion protein Mr = scFv (30kDa) + gD amino acids,

**Band intensity from Western blots; high = +++, intermediate = ++, low = +, n.d. = not detected, † indicates fusion protein shown in Fig. 1b,

*** Percentage of CHO cells infected

Table 3

Table 3 shows infection of THP-1 cells by HSV1716 variants that display scFv either against CD20 or CD38 glycoprotein fusion proteins.

Protein/ virus	Localization in Vero cells***	THP-1 infectivity	THP-1 infectivity +MabT	THP-1 infectivity + Mab5
5/37gD	perinuclear	10%	10%	10%
5/128gD	perinuclear	15%	10%	10%
5/260gD	perinuclear	15%	15%	15%
5/274gD	perinuclear	10%	10%	15%
T/37gD	perinuclear	30%	10%	40%
T/128gD	perinuclear	20%	15%	15%
T/260gD	perinuclear	60%	20%	50%
T/274gD	perinuclear	40%	10%	30%
5gH/gL2	perinuclear	10%	10%	10%
TgHgL2	perinuclear	30%	15%	30%
1716V*	n/a	5%	10%	10%
1716B**	n/a	20%	30%	20%

* HSV1716 propagated on Vero cells,

** HSV1716 propagated on BHK cells,

***immunofluorescence on stably transformed cell lines with anti-myc (gC, gD, gL) or anti-HA (gH) monoclonal antibodies.

Table 4

Table 4 shows the ability of various HSV1716 variants with targeting moieties to infect a variety of different permissive, semi-permissive and non-permissive cell lines.

Virus (1)	Titre (2)	BHK inf (3)	Vero inf (3)	CHO inf (4)	CHODAF inf (4)	THP- 1 inf (5)	SupT inf (5)	TolB inf (5)
T/260gD	1.8×10^{10} pfu	100	100	0	3	30	50	50
T/274gD	1.6×10^{10} pfu	100	100	1	5	50	50	70
B/274gD	6.6×10^9 pfu	100	100	5	50	10	20	10
1716V(6)	4.0×10^{10} pfu	100	100	0	0	10	10	30
1716B(7)	9.8×10^9 pfu	100	100	20	20	20	20	50

Notes on Table 4

(1) all HSV1716 variants with targeting moieties were propagated on Vero cells

(2) all viruses were titrated on BHK cells, and total yield from 4xT175 flasks is reported

(3) approximate percentage of cells infected as determined by fluorescence microscopy from a number of different experiments at 1pfu/cell, BHK and Vero cells are fully permissive for HSV1716 infection.

(4) CHO and CHO/DAF cells are non-permissive for HSV1716 infection when the virus is propagated on Vero cells but is semi-permissive when HSV1716 is propagated on BHK cells, CHO/DAF are CHO cells modified to constitutively express human CD55 (DAF)

(5) Cell lines which are semi-permissive for HSV1716 infection with the level of infectivity dependent on whether the cells are propagated on Vero or BHK cells. THP-1 is a human monocytic cell line which is CD38 positive and CD20 negative as determined by IF using MabT and Mab5. SupT is a human T-cell line which is CD38 positive and CD20 negative as determined by IF using MabT or Mab5, TolB is a human B-cell

line which is positive for CD38 and weakly positive for CD20 as determined by IF using MabT or Mab5

(6) HSV1716 propagated on Vero cells is the control virus for the targeted variants

(7) HSV1716 propagated on BHK cells is better able to infect CHO, CHODAF, THP-1, SupT and TolB cells

Table 5

Table 5 shows effects of monoclonal antibodies on the abilities of targeted HSV1716 variants to infect SupT cells. Percentages of cells infected by the various viruses at 1pfu/cell are reported

Virus	Mab5	MabT	Mab8	MabB	Medium
5/274gD	10	20	10	10	5
5/274gDopp	5	10	5	5	5
T/274gD	25	5	25	30	30
B/274gD	10	10	10	10	10
8/274gD	10	10	10	10	10
HSV1716V	5	15	5	5	5
HSV1716B	10	20	10	10	10

Table 6

Table 6 shows effects of monoclonal antibodies on the abilities of targeted HSV1716 variants to infect THP-1 cells. Percentages of cells infected by the various viruses at 1pfu/cell are reported

Virus	Mab5	MabT	Mab8	MabB	Medium
5/274gD	20	20	20	10	20
5/274gDopp	20	20	20	20	10
T/274gD	60	20	80	80	60
B/274gD	20	30	20	30	30
8/274gD	10	20	20	10	20
HSV1716V	10	20	20	20	15
HSV1716B	15	30	25	10	30

Table 7

Table 7 shows effects of monoclonal antibodies on the abilities of targeted HSV1716 variants to infect TolB cells. Percentages of cells infected by the various viruses at 1pfu/cell are reported

Virus	Mab5	MabT	Mab8	MabB	Medium
5/274gD	20	20	20	20	20
5/274gDopp	10	10	10	15	20
T/274gD	60	10	60	65	65
B/274gD	20	30	20	30	30
8/274gD	30	20	20	20	30
HSV1716V	20	30	30	30	20
HSV1716B	40	30	40	30	30

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Claims:

1. A herpes simplex virus (HSV) comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a functional HVEM binding domain.
2. A HSV according to claim 1, wherein the N-terminally truncated glycoprotein D does not include a functional nectin-1 binding domain.
3. A HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D does not include a portion of amino acids corresponding to amino acids 1 to 34 of the full-length HSV-1 glycoprotein D.
4. A HSV according to any one of claims 1 to 3, wherein the N-terminally truncated glycoprotein D has an extracellular domain comprising at least 42 amino acids.
5. A HSV according to any one of claim 1 to 4, wherein the N-terminally truncated glycoprotein D has an extracellular domain comprising a portion of amino acids corresponding to amino acids 274-315 of the full-length HSV-1 glycoprotein D.
6. A HSV according to any one of claims 1 to 5, wherein the position of the truncation corresponds to an amino acid position from 260-274 of the full-length HSV-1 glycoprotein D.
7. A HSV according to any one of claims 1 to 5, wherein the position of the truncation corresponds to an amino acid position from 37-128 of the full-length HSV-1 glycoprotein D.
8. A HSV according to any one of claims 1 to 7, wherein the position of the truncation is not at an amino acid position

corresponding to a position from 139-239 of the full-length HSV-1 glycoprotein D.

9. A HSV according to any one of claims 1 to 8, wherein the full-length HSV-1 glycoprotein D has the amino acid sequence of SEQ ID NO: 1.

10. A HSV according to any one of claims 1 to 9, wherein the N-terminally truncated glycoprotein D is an N-terminal truncate of a glycoprotein D having the amino acid sequence of SEQ ID NO: 1.

11. A fusion protein comprising an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D is as defined in any one of claims 1 to 10.

12. A nucleic acid molecule comprising nucleic acid encoding a fusion protein, which fusion protein comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D is as defined in any one of claims 1 to 10.

13. A HSV comprising an N-terminally truncated glycoprotein D linked to a targeting agent for use in a therapeutic method of treatment, wherein the N-terminally truncated glycoprotein D is as defined in any one of claims 1 to 10.

14. Use of a HSV in the manufacture of medicament for the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D is as defined in any one of claims 1 to 10.

15. A method of treating a disease associated with the proliferation of cells, comprising administering a HSV, which

HSV comprises an N-terminally truncated glycoprotein D linked to a targeting agent, wherein the N-terminally truncated glycoprotein D is as defined in any one of claims 1 to 10.

16. A herpes simplex virus (HSV) comprising:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target.

17. A HSV comprising:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target, and wherein the HSV is for use in a therapeutic method of medical treatment.

18. Use of a herpes simplex virus in the manufacture of medicament for the treatment of a disease associated with the proliferation of cells, wherein the HSV comprises:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target.

19. A method of treating a disease associated with the proliferation of cells, comprising administering a HSV, wherein the HSV comprises:

- a. a glycoprotein H linked to a first targeting agent, and
- b. a glycoprotein L linked to a second targeting agent, wherein the first targeting agent and the second targeting agent are specific for the same target.

20. A fusion protein comprising a glycoprotein L linked to a targeting agent.

21. A nucleic acid molecule encoding a fusion protein, which fusion protein comprises a glycoprotein L linked to a targeting agent.

22. A fusion protein comprising a glycoprotein H linked to a targeting agent.

23. A nucleic acid molecule encoding a fusion protein, which fusion protein comprises a glycoprotein H linked to a targeting agent.

24. A HSV, use, method, fusion protein, or nucleic acid according to any one of claims 16 to 21, wherein one or more spacer molecules are between the N-terminus of glycoprotein L and the targeting agent.

25. A HSV, use, method, fusion protein, or nucleic acid according to claim 24, wherein the spacer molecule is Gly-Gly-Gly-Gly-Ser.

26. A HSV, use, method, fusion protein, or nucleic acid according to claim 25, wherein one Gly-Gly-Gly-Gly-Ser molecule is between the N-terminus of glycoprotein L and the targeting agent.

27. A HSV, use, method, fusion protein, or nucleic acid according to any one of claims 16 to 19 and 22 to 26, wherein the glycoprotein H has the amino acid sequence of SEQ ID NO: 2.

28. A HSV, use, method, fusion protein, or nucleic acid according to any one of claims 15 to 20 and 22 to 25, wherein

the glycoprotein L has the amino acid sequence of SEQ ID NO: 3.

29. A HSV, use, method, fusion protein, or nucleic acid according to any one of claims 1 to 28, wherein the targeting agent is an antibody binding domain.

30. A HSV, use, method, fusion protein, or nucleic acid according to any one of claims 1 to 29, wherein the targeting agent is specific for a target selected from the group consisting of GD3, CD38, CD55, CD20, EGRF, HER2/neu CEA, squamous cell carcinoma antigens 1 and 2, ovarian carcinoma antigen CA125, Mucin 1, prostate-specific membrane antigen, melanoma-associated tumour antigen p97, 5T4 oncofoetal trophoblast glycoprotein, CA19-9, CA72-4 and CA195.

31. A HSV, use, or method according to any one of the preceding claims wherein the HSV is HSV-1.

32. A HSV, use, or method according to any one of the preceding claims wherein the HSV is non-neurovirulent.

33. A HSV, use, or method according to claim 32 wherein the HSV does not comprise an ICP34.5 gene that encodes a functional ICP34.5 gene product.

34. A HSV, use, or method according to claim 33, wherein the HSV is HSV1716.

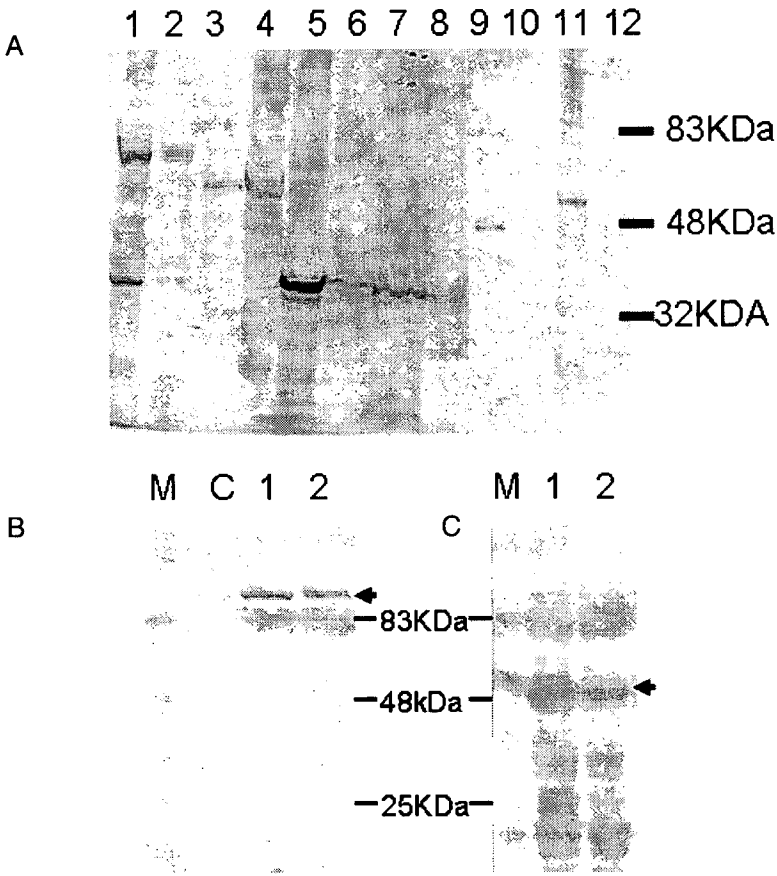


Figure 1

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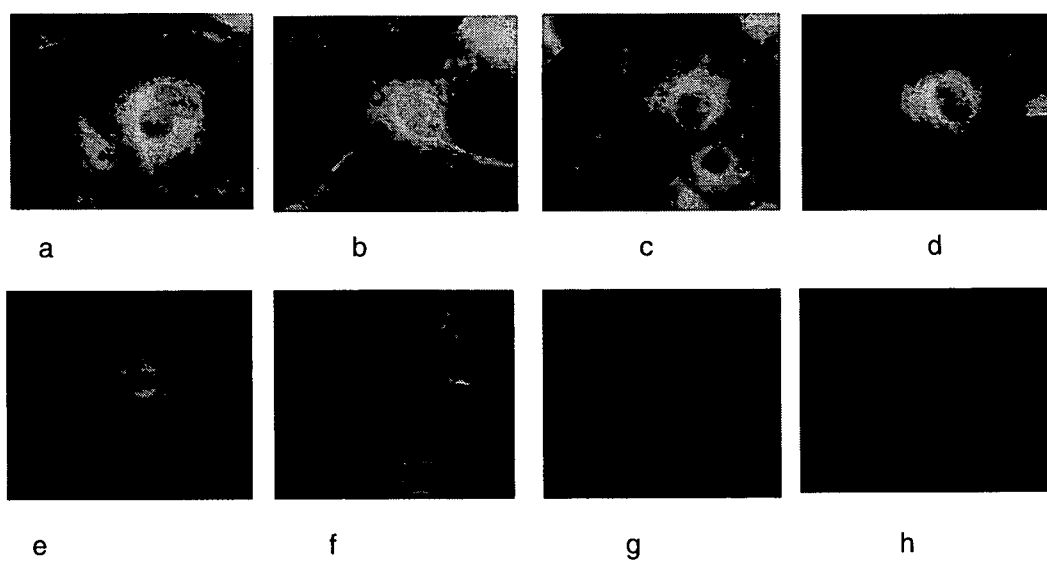


Figure 2

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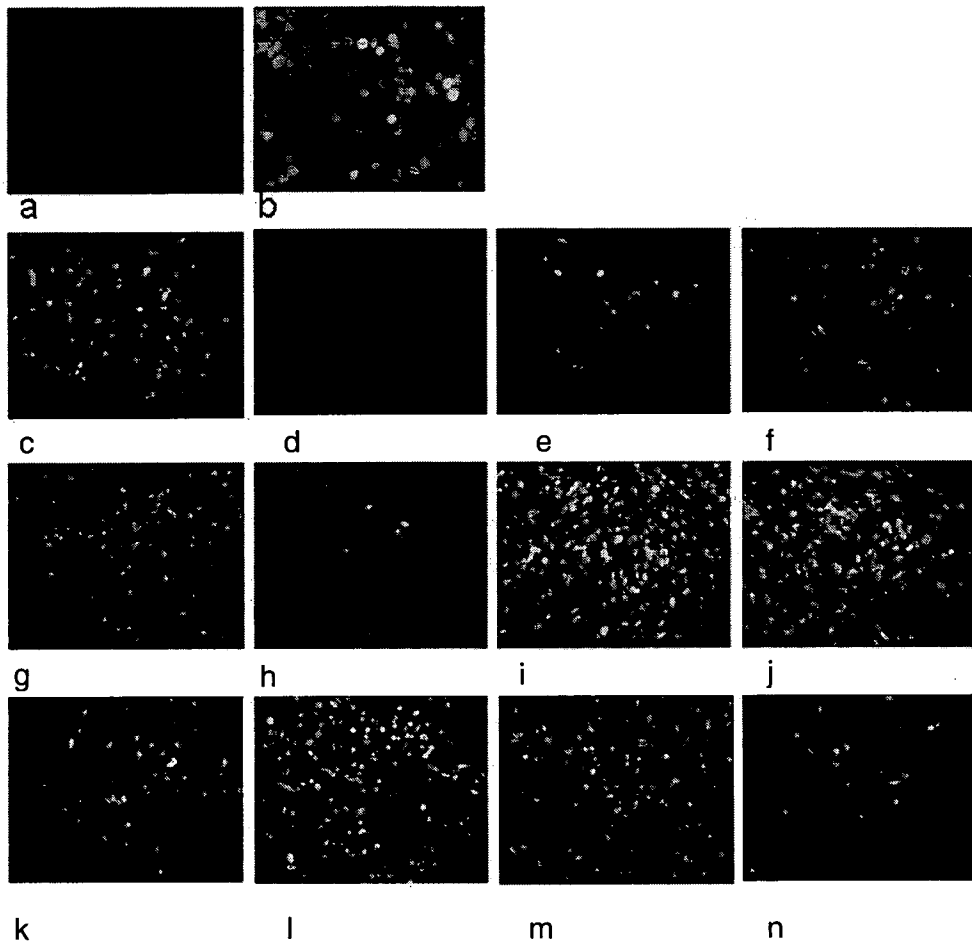


Figure 3

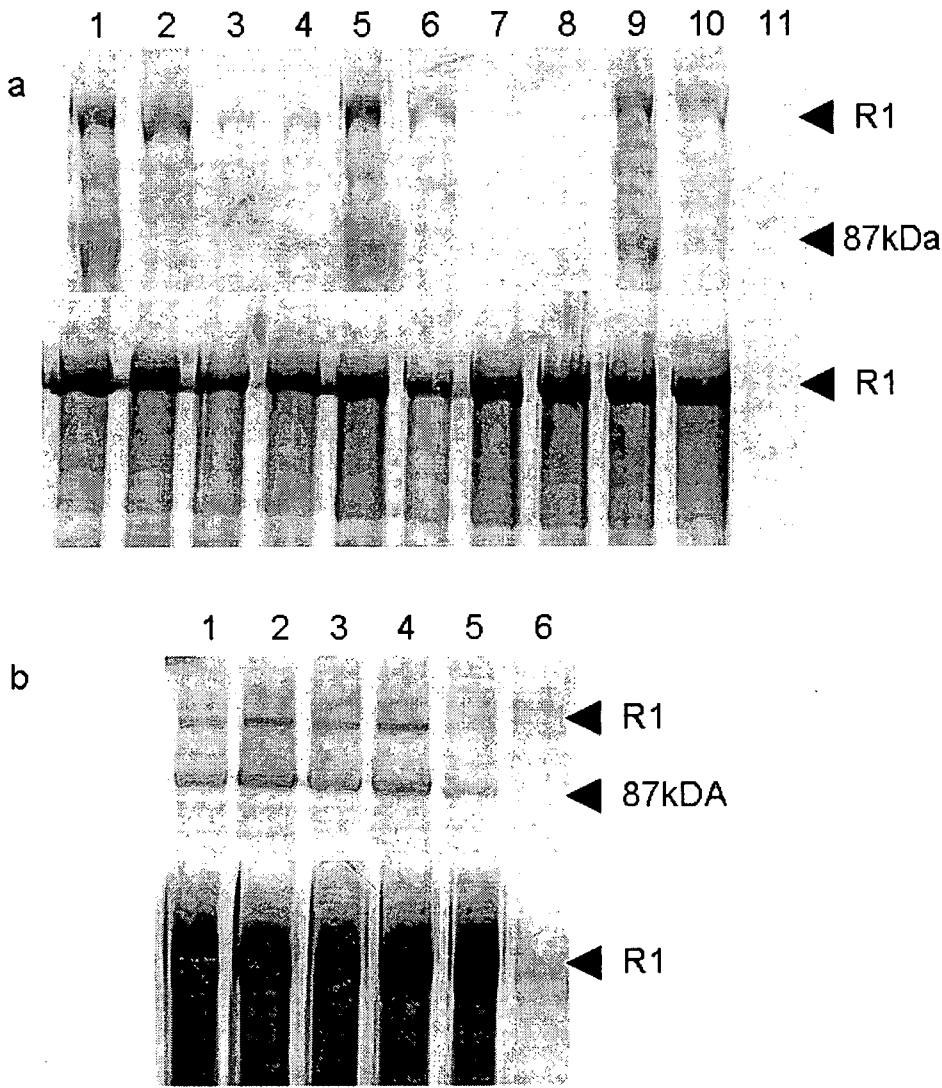


Figure 4

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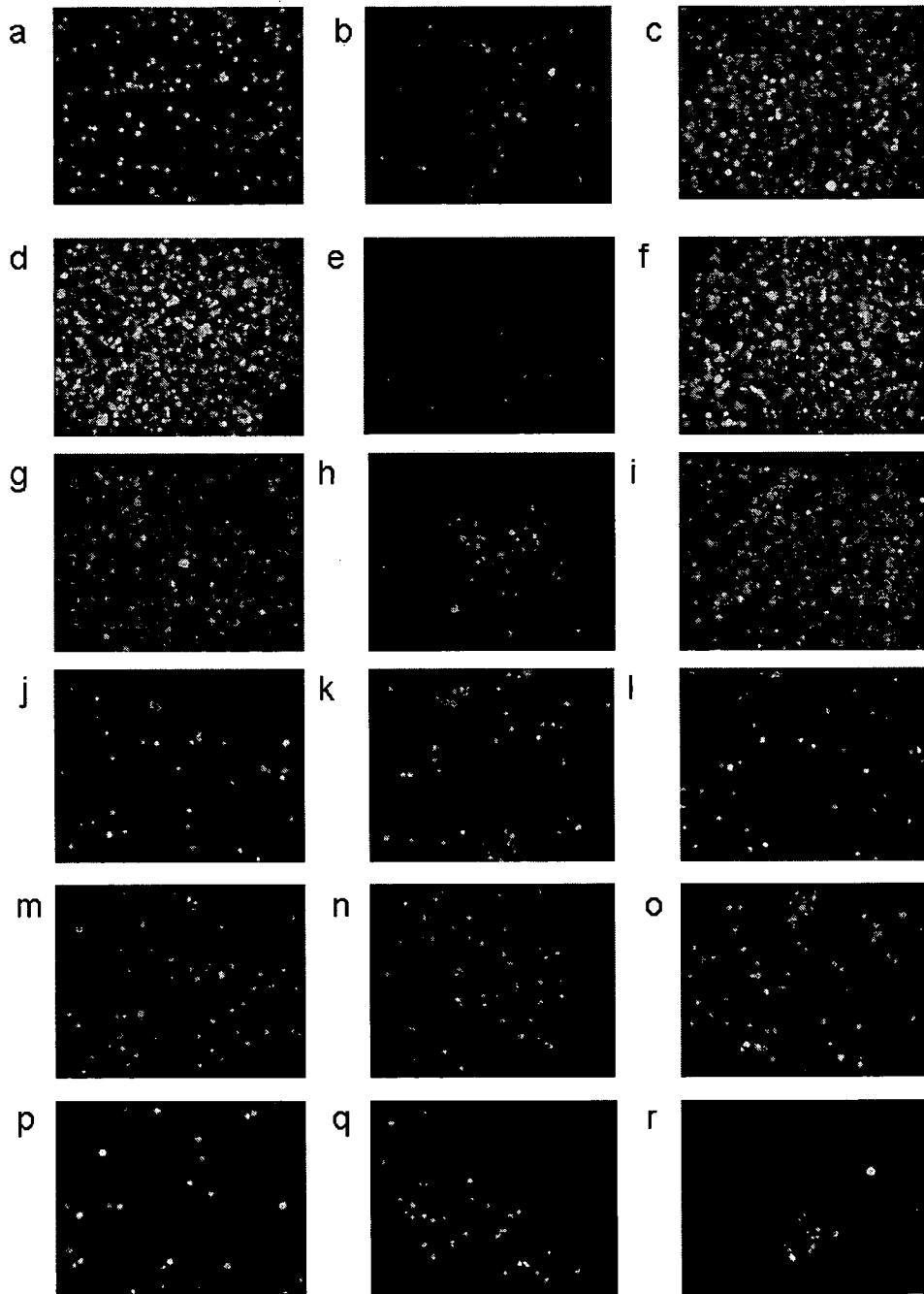


Figure 5

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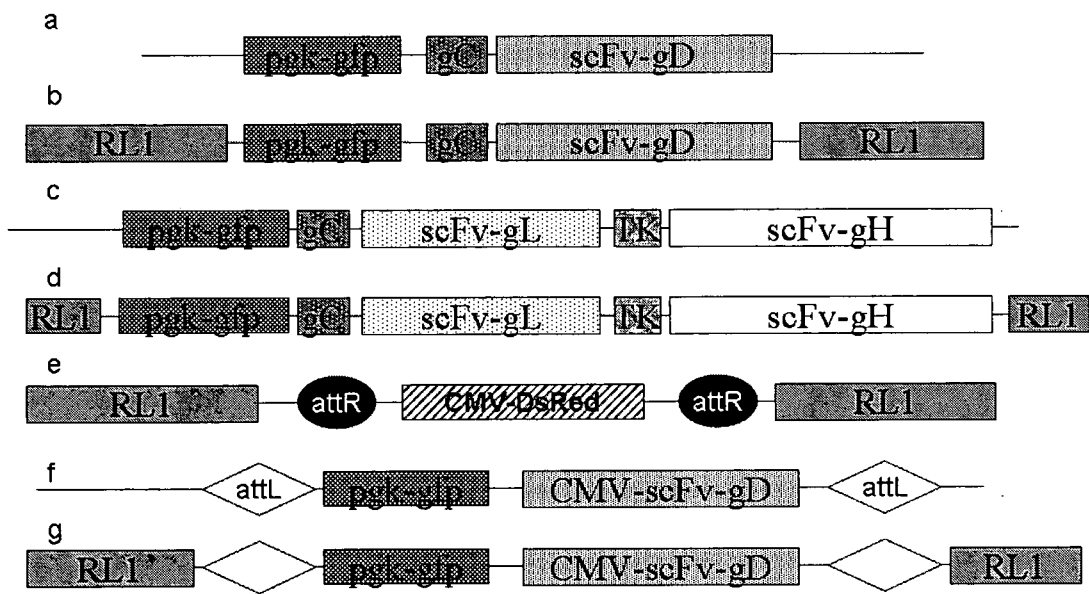


Figure 6

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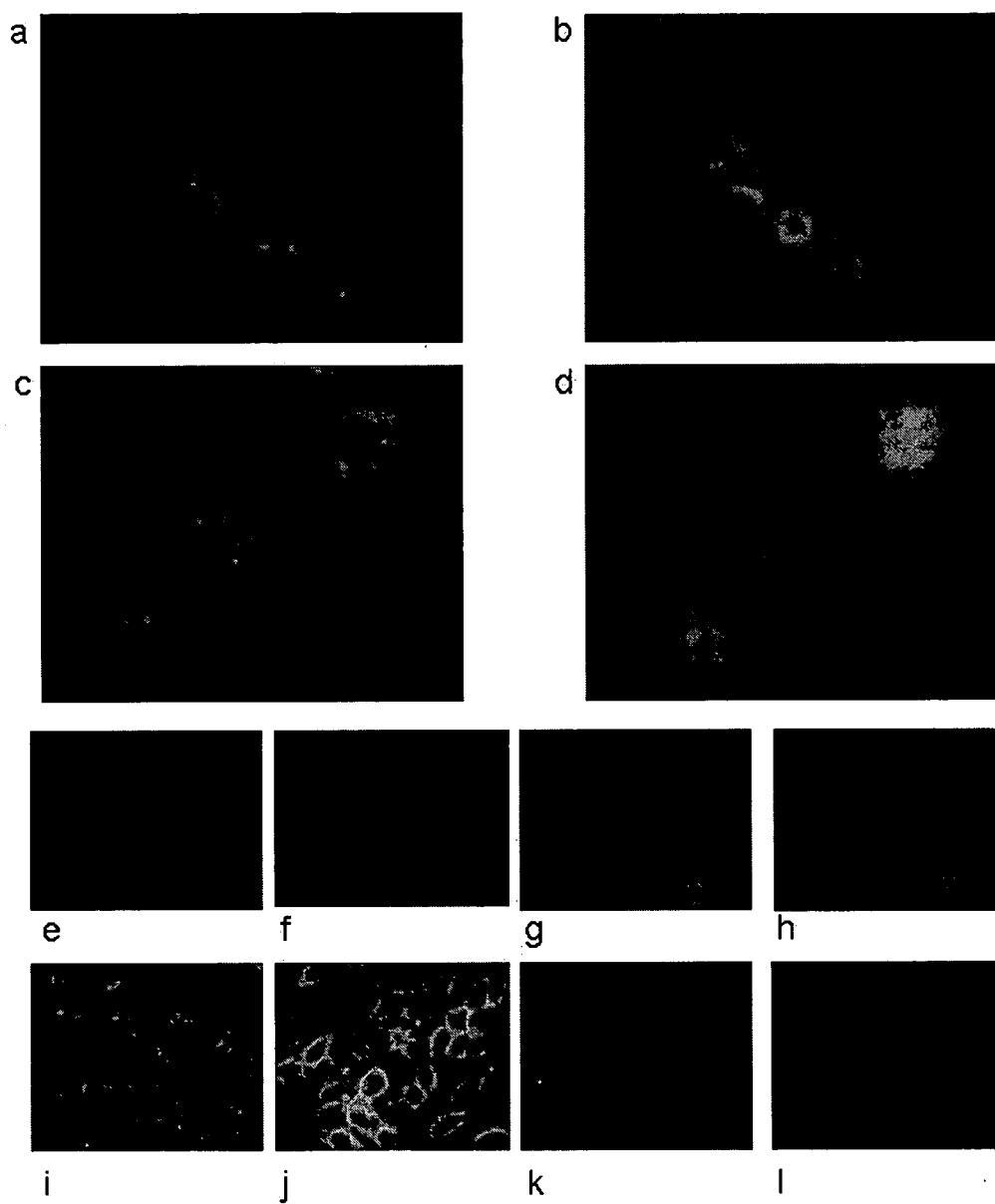


Figure 7

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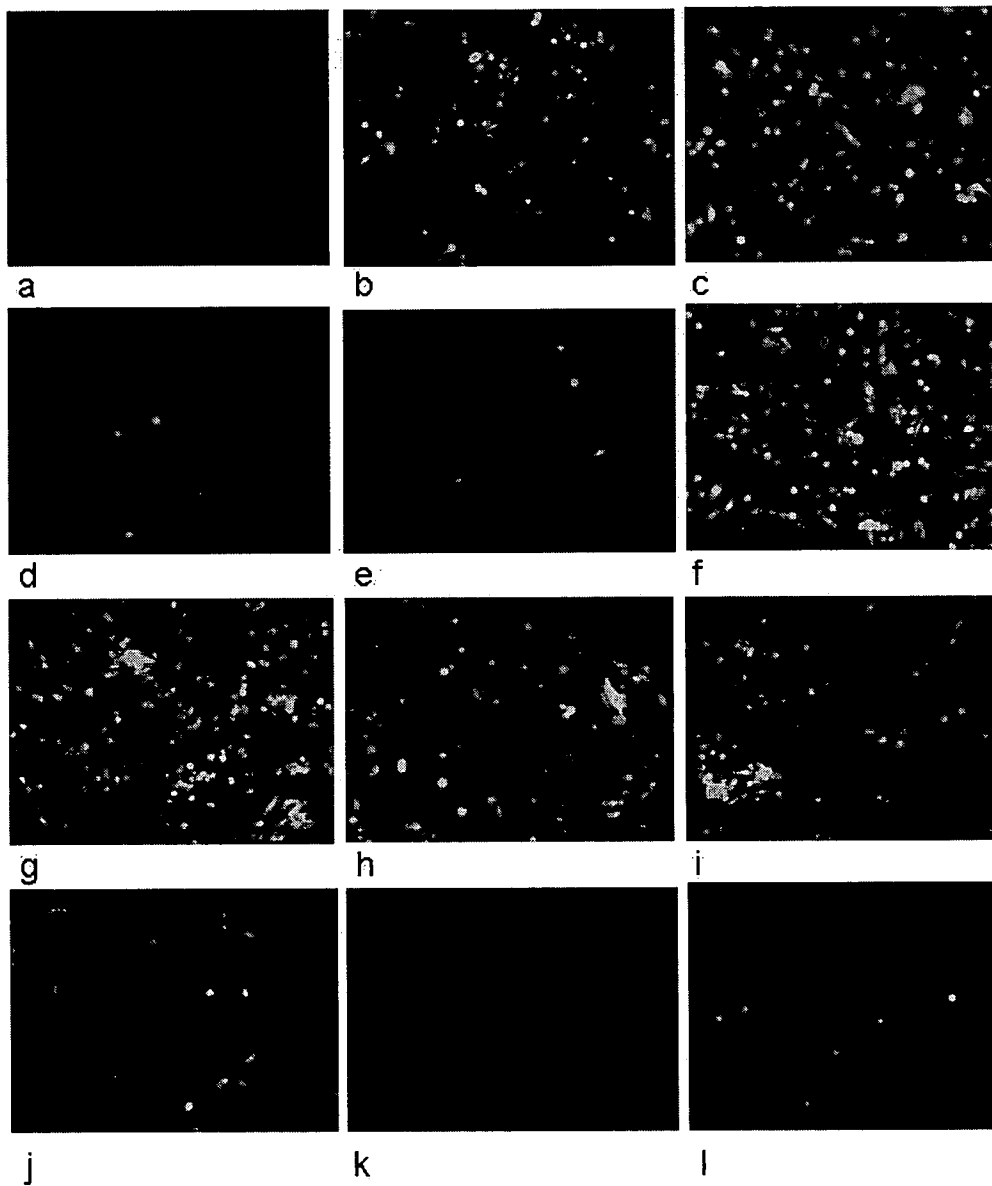


Figure 8

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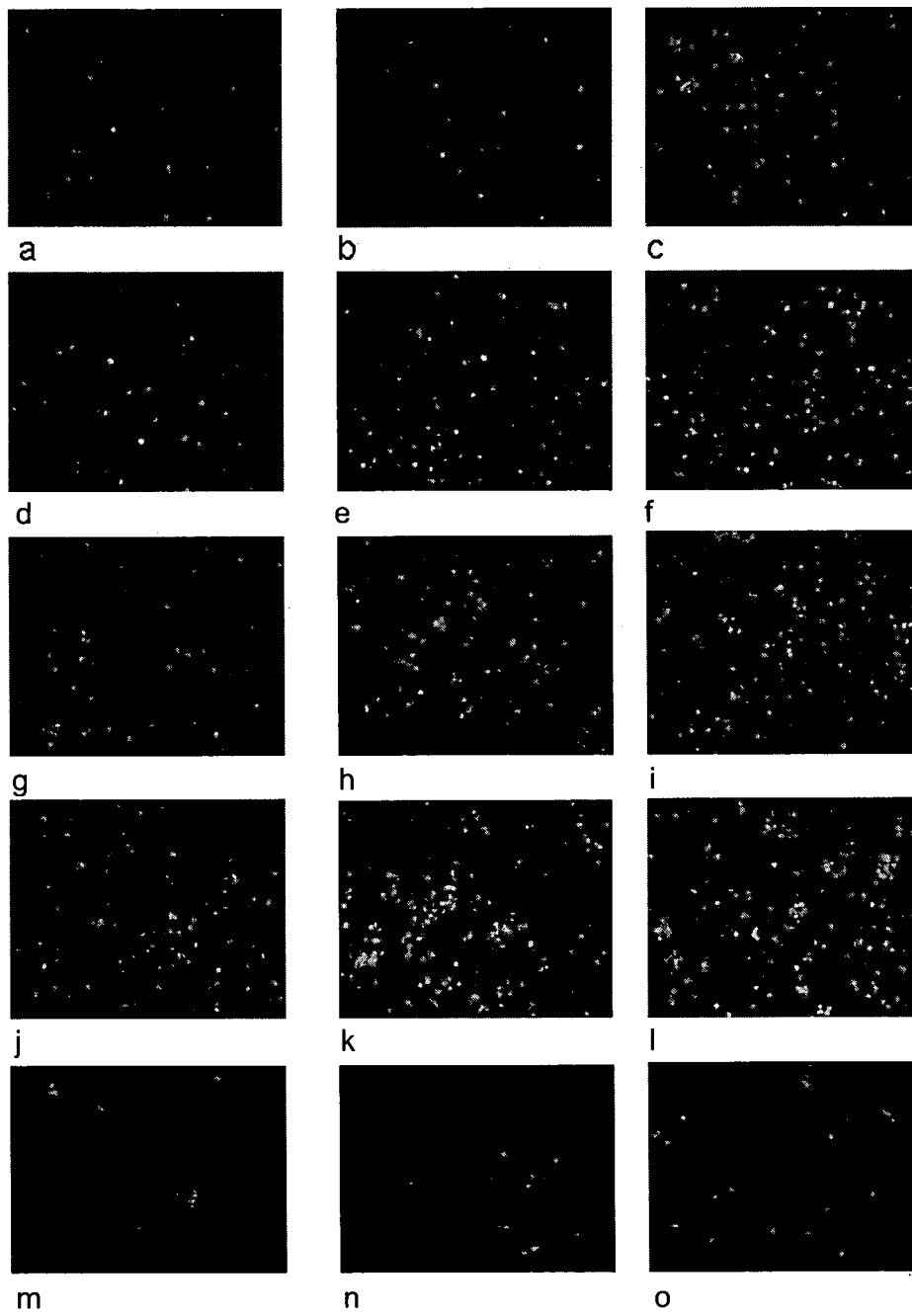


Figure 9

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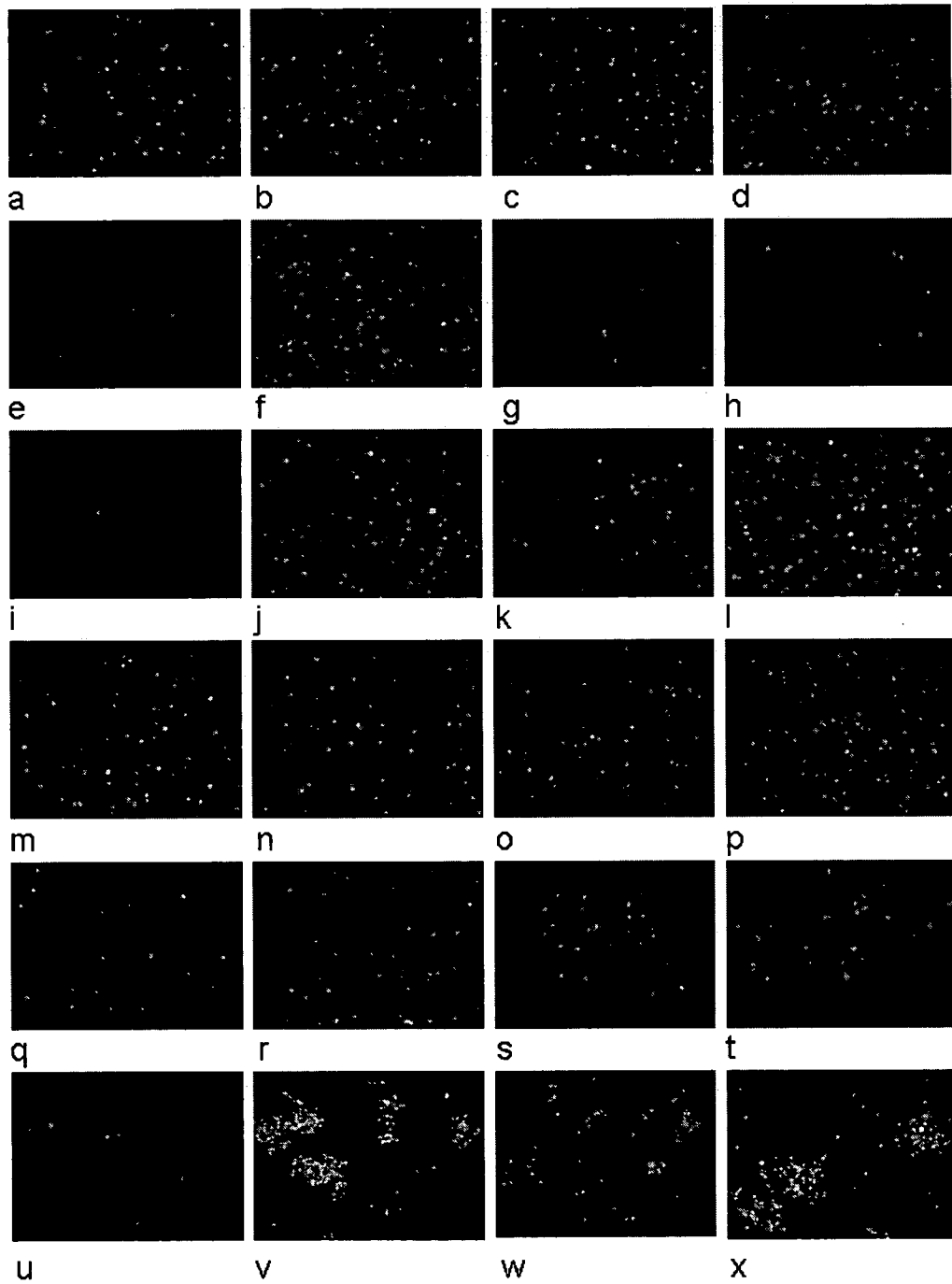


Figure 10

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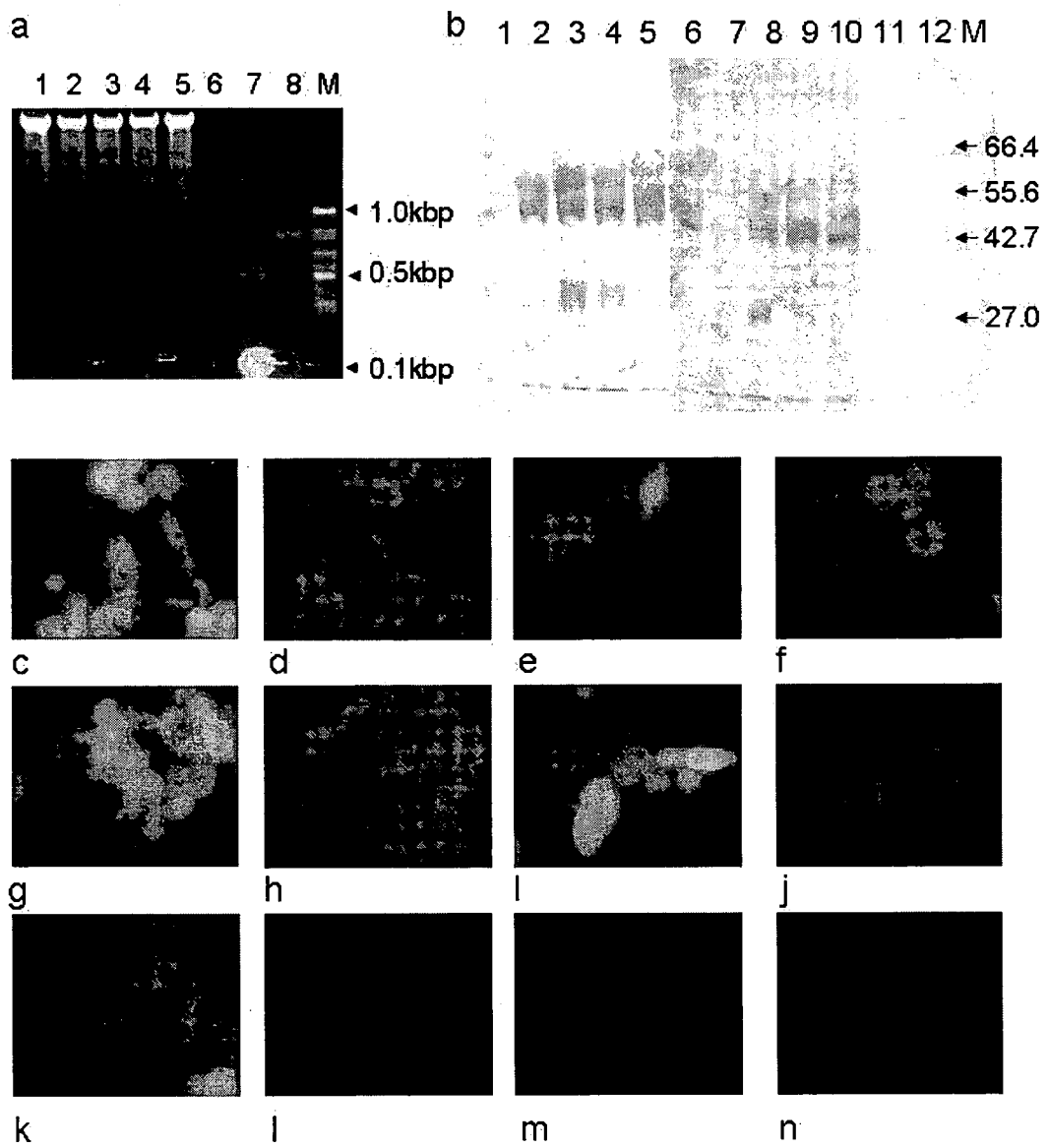


Figure 11

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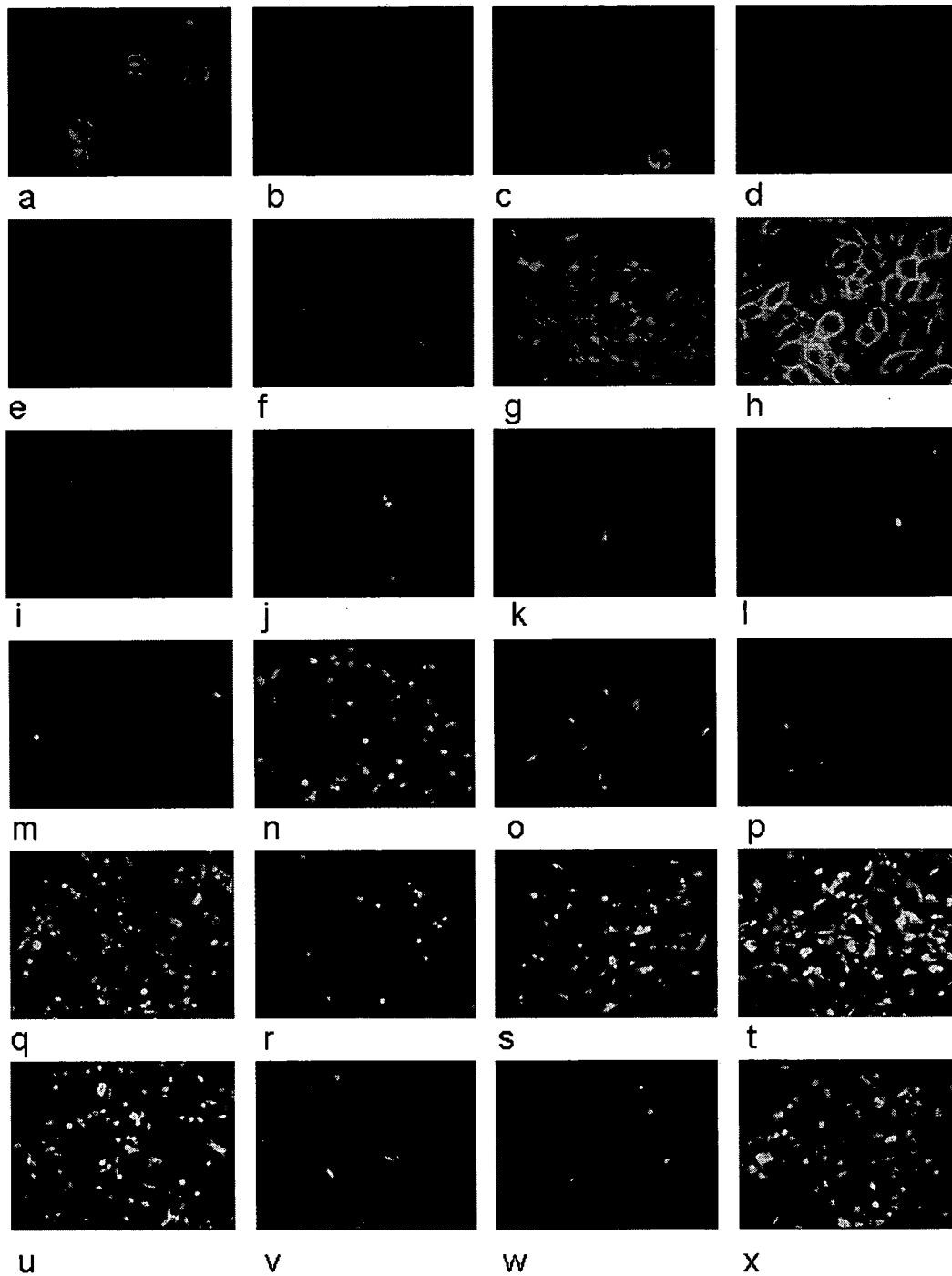


Figure 12

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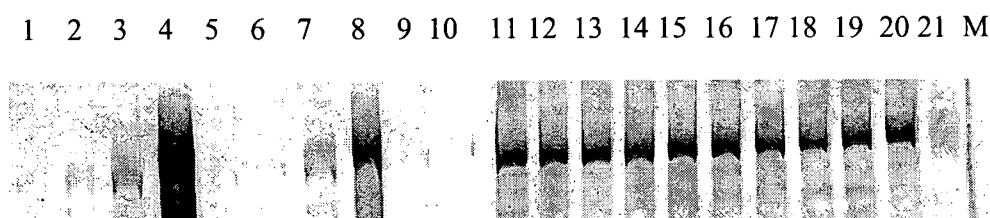


Figure 13

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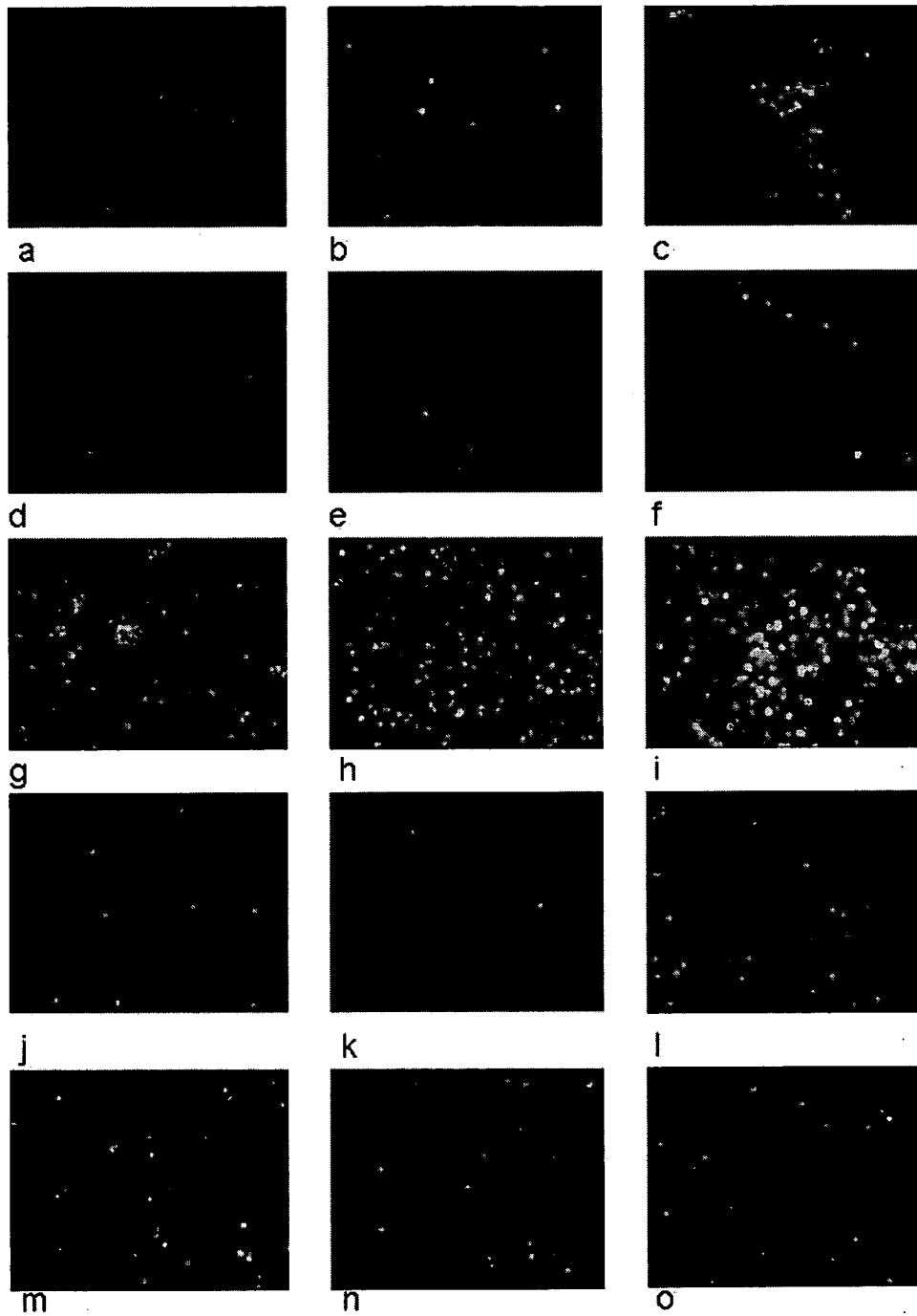


Figure 14

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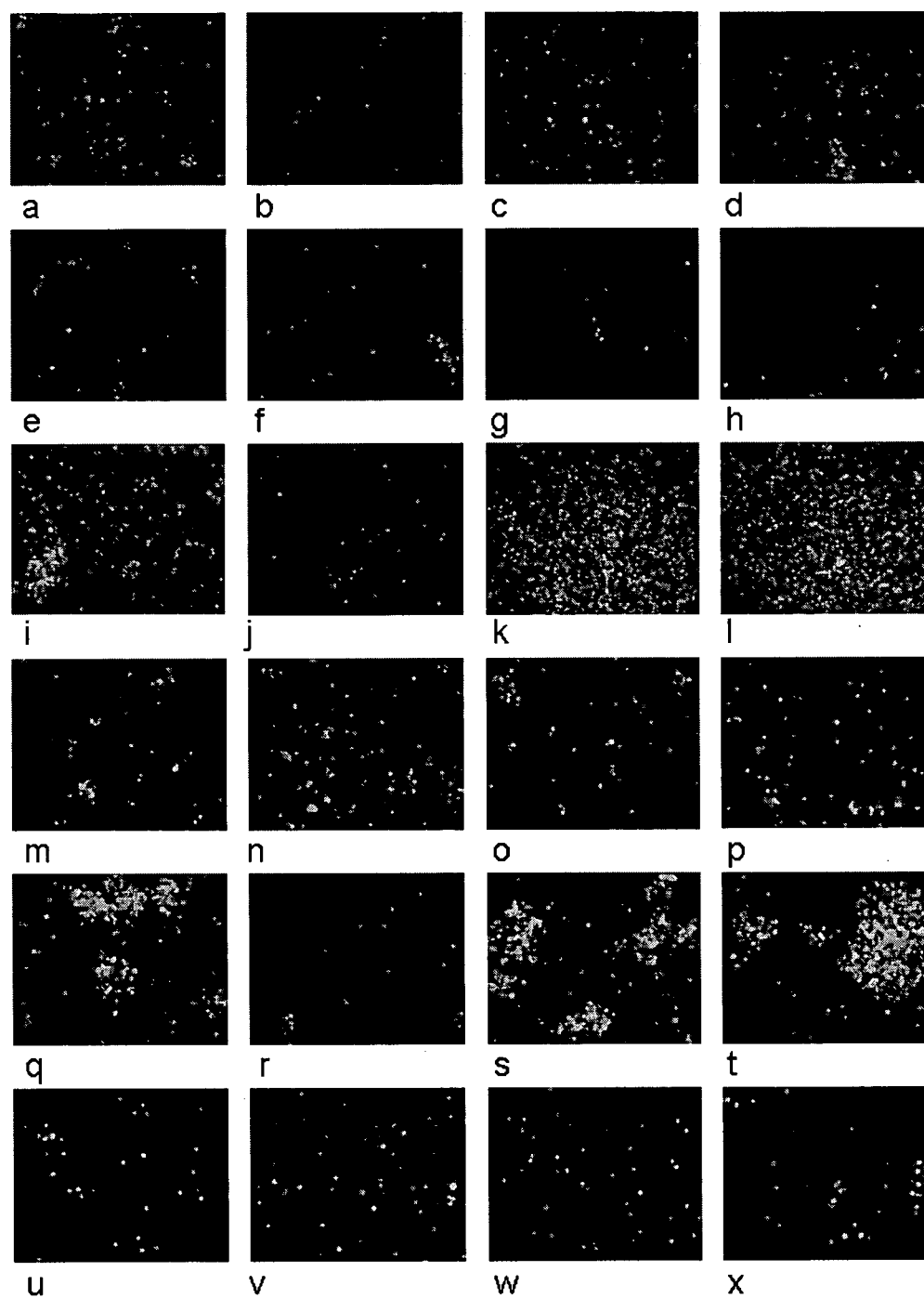


Figure 15

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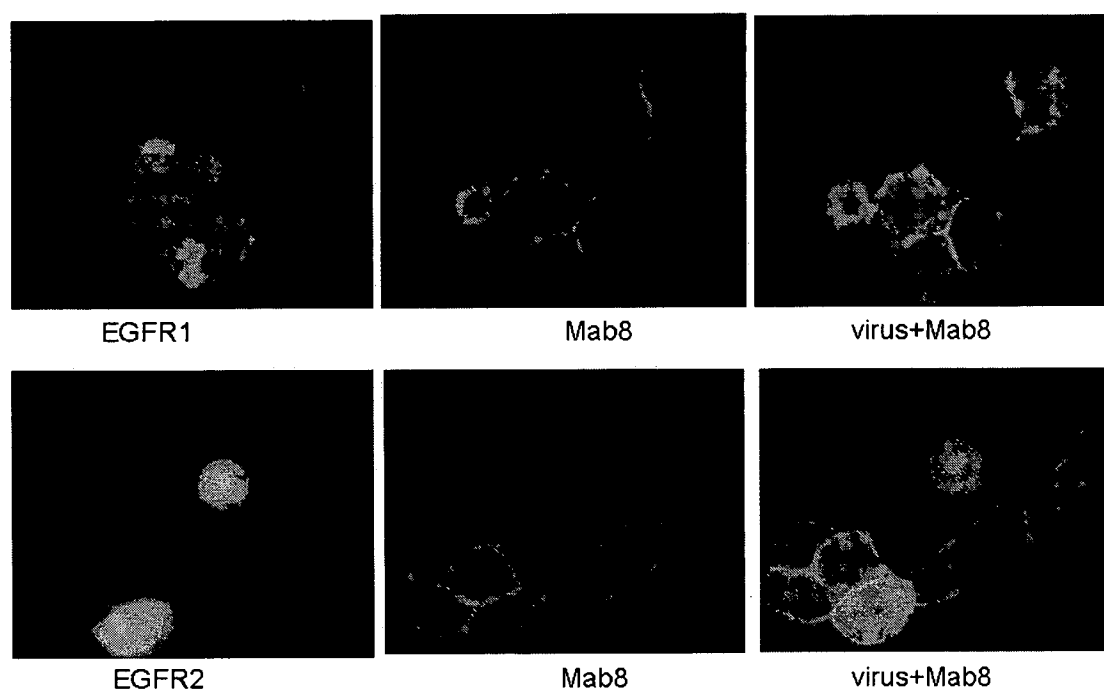


Figure 16

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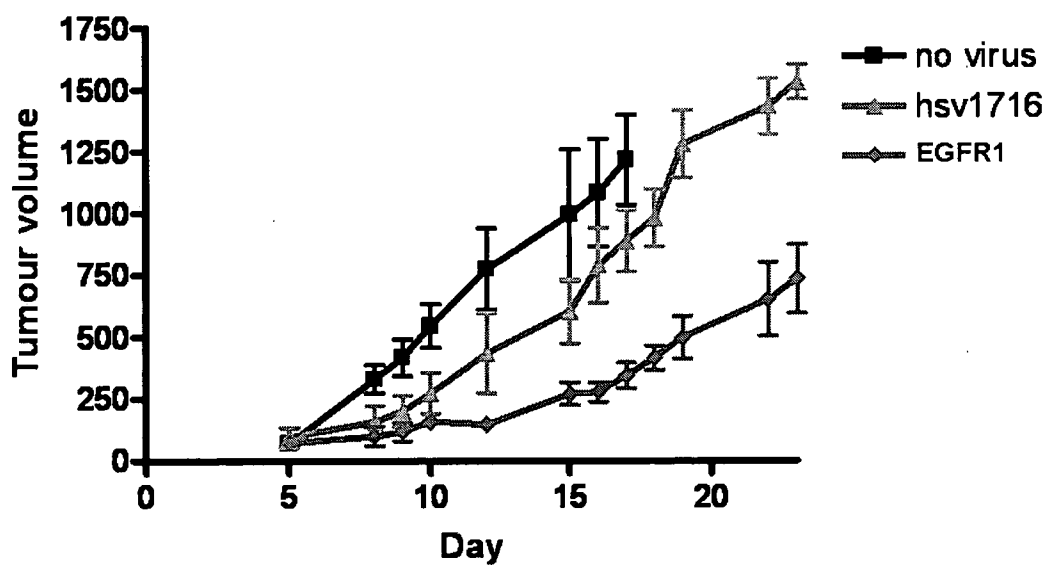
EGFR1

Figure 17a

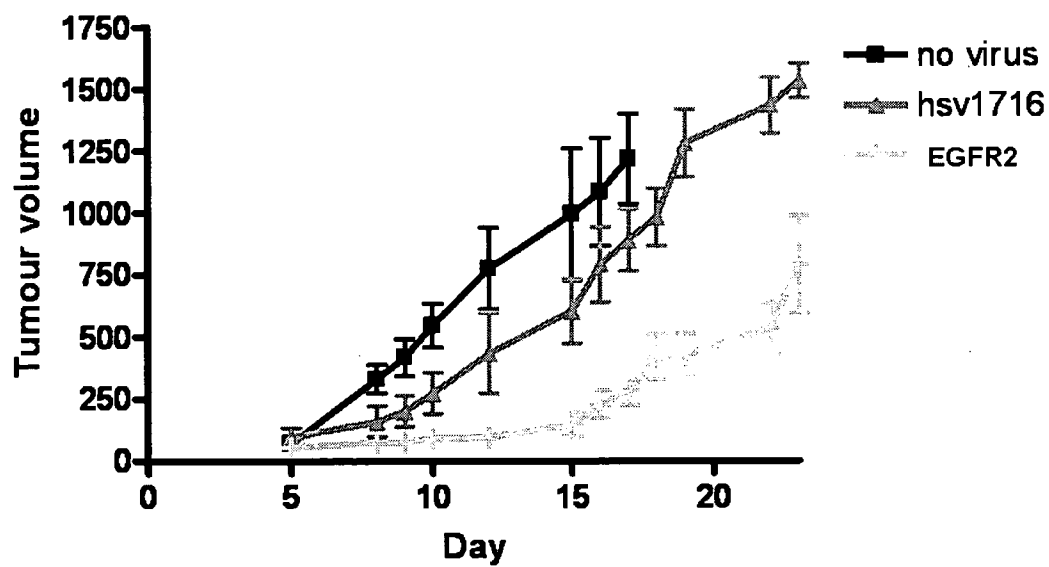
EGFR2

Figure 17b

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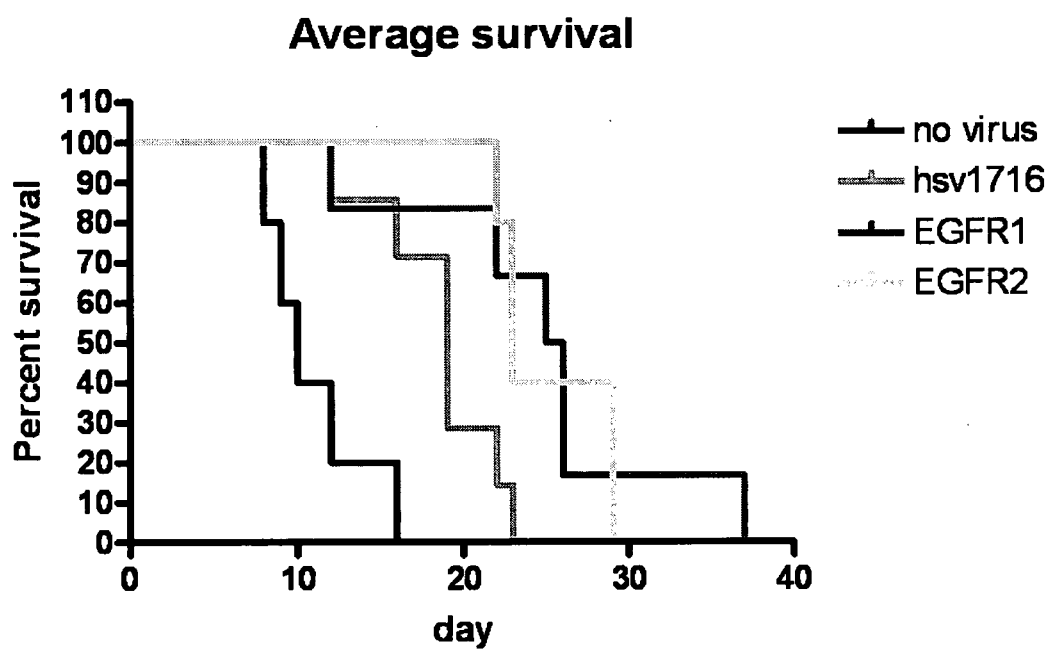


Figure 18

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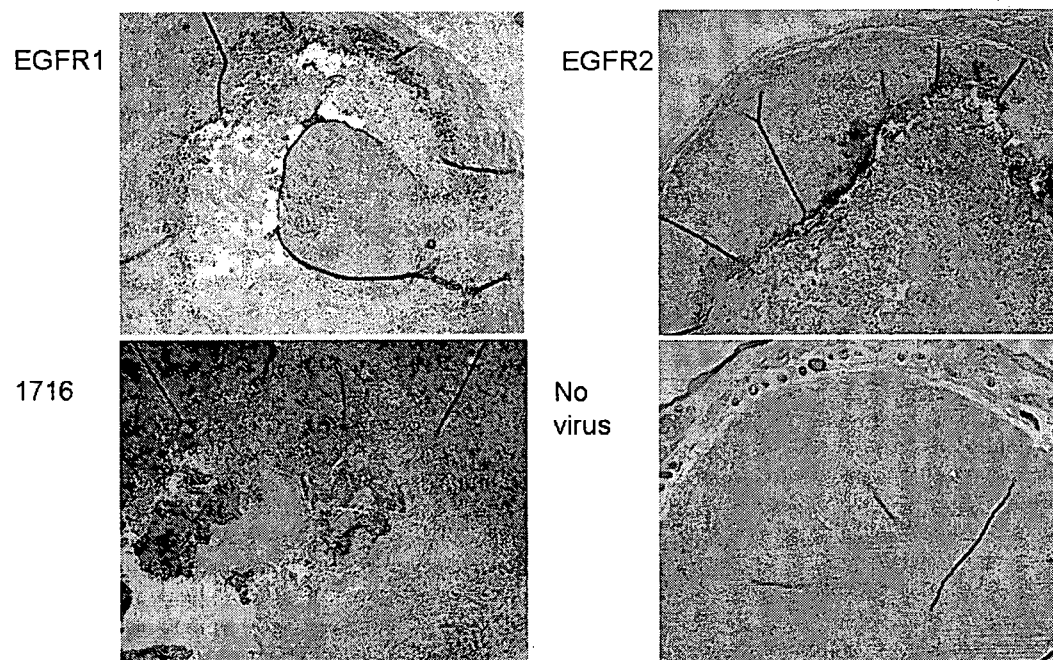


Figure 19

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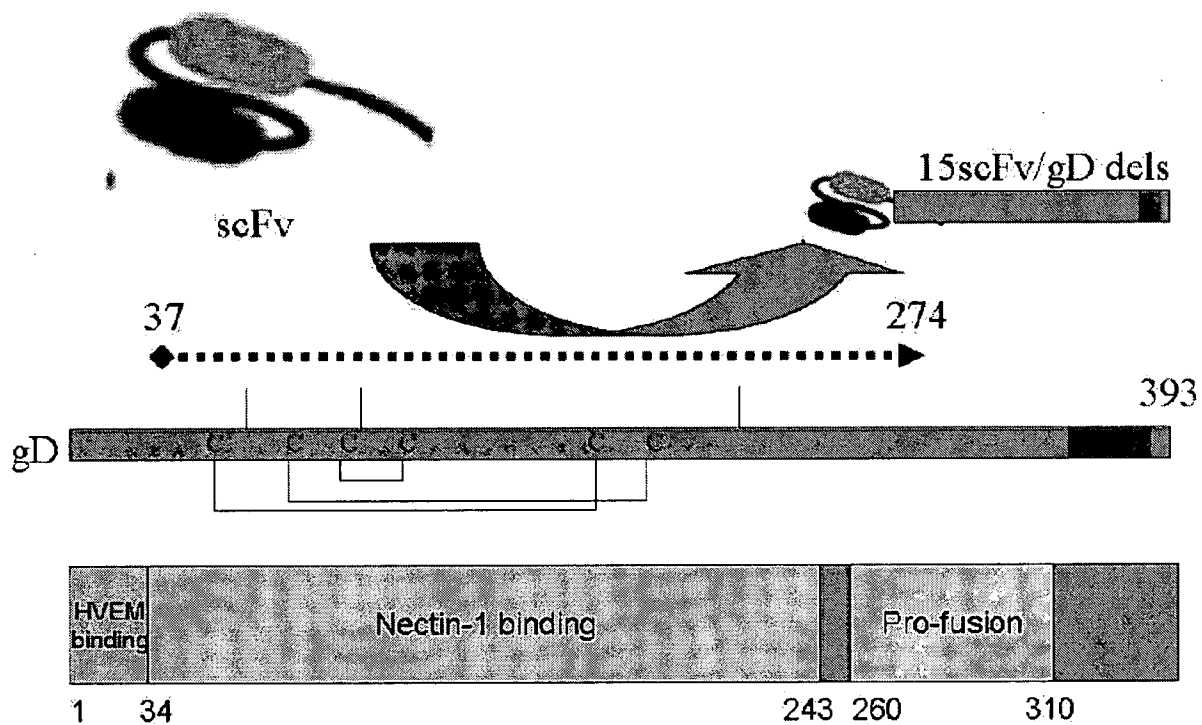


Figure 20a

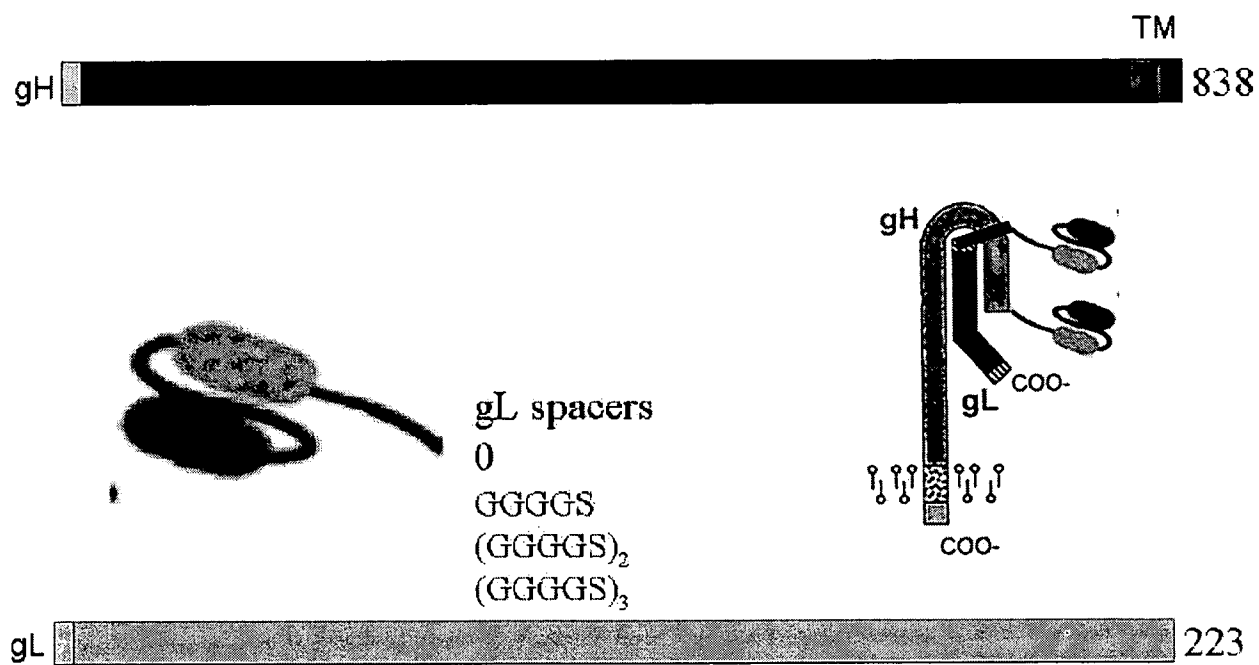


Figure 20b

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FY

Figure 21a

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MGNGLWFGVVIILGVAWGQVHDWTEQTDPWFLDGLGMDRMYWRD
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RTALARADHQKTLFWLPDHFSPCAASLRFDLDES VFILDALAQATRSETPVEVLAQQT
HGLASTLTRWAHYNALIRAFVPEASHRCGGQSANVEPRILVPITHNASYVVTHSPLPR
GIGYKLTGVDVRRPLFLTYLTATCEGSTRDIESKRLVRTQNQRDLGLVGAVFMRYTPA
GEVMSVLLVDTDNTQQQIAAGPTEGAPSVFSSDVPSTALLLFNGTVIHLLAFDTPQV
AAIAPGFLAASALGVVMITAALAGILKVLRTSVPPFFWRRE

Figure 21b

MGILGWVGLIAGVGLCVRGGLPSTEYVIRSRVAREVGDILKVPC
VPLPSDDLDWRYETPSAINYALIDGIFLRYHCPGLDTVLWDRHAQKAYWVNPFLFVAG
FLEDLSYPAFPANTQETETRLALYKEIRQALDSRKQAASHTPVKAGCVNFDYSRTRRC
VGRQDLGPTNGTSGRTPVLPPDDEAGLQPKPLTTPPPIIATSDPTPRRDAATKSRRRR
PHSRRL

Figure 21c

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138421 ggggggggct gccgccaggt tggggggcgt gattttgttt gtcgtcatag tgggcctcca
138481 tgggggtccgc agcaaatatg ccttggtgga tgcctctctc aagatggccg accccaatcg
138541 ctttcgcggc aaagaccttc cgttcctgga ccagctgacc gaccctccgg gggtcggcg
138601 cgtgtaccac atccaggcgg gcctaccgga ccggttccag ccccccagcc tcccgatcac
138661 ggttttactac gccgtgttgg agcgcgcctg ccgcagcgtg ctctaaacg caccgtcggg
138721 ggccccccag attgtccgcg gggcctccga agacgtccgg aaacaaccct acaacctgac
138781 catcgcttgg ttctggatgg gaggcaactg tgctatcccc atcacggtca tggagtacac
138841 cgaatgctcc tacaacaagt ctctgggggc ctgtcccatc cgaacgcagc cccgctggaa
138901 ctactatgac agcttcagcg ccgtcagcga ggataacctg gggttcctga tgcacgcccc
138961 cgcgttttgag accgccggca cgtacctgcg gctcgtgaag ataaacgact ggacggagat
139021 tacacagttt atcctggagc accgagccaa gggctcctgt aagtagcccc tcccgtgcg
139081 catccccccg tcagcctgcc tctcccccca ggcctaccag caggggggtga cgggtggacag
139141 catcgggatg ctgccccgct tcctccccga gaaccagcgc accgtcgccg tatacagctt
139201 gaagatcgcc ggggtggcacg ggcccaaggc ccatacacg agcacctgc tgcctccgga
139261 gctgtccgag accccaacg ccacgcagcc agaactcgcc ccggaagacc ccgaggattc
139321 ggccctcttg gaggaccccg tggggacggt ggcgccgcaa atccaccaa actggcacat
139381 accgtcgatc caggacgccg cgacgcctta ccatcccccg gccaccccg acaacatggg
139441 cctgatcgcc ggcgcggtgg gcggcagctt cctggcagcc ctggtcattt gcggaattgt
139501 gtactggatg cgccgccaca ctcaaaaagc cccaaagcgc atacgcctcc cccacatccg
139561 ggaagacgac cagccgtcct cgcaccagcc cttgttttac tag

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Figure 21d

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          ttatt cgcgtctcca aaaaaacggg acacttgtcc ggagaacctt taggatgccca
43921 gccaggggcgg cggtaatcat aaccacgccc agcgagagg cgccagaaaa cccgggcgca
43981 attgcggcca cgggctgcgt gtcaaaggct agcaaatgaa tgacggttcc gtttggaat
44041 agcaacaagg ccgtggacgg cacgtcgctc gaaaacacgc ttggggcgcc ctccgtcggc
44101 ccggcggcga tttgctgctg tgtgtgtgcc gtatccacca gcaacacaga catgacctcc
44161 ccggccgggg tgtagcgcat aaacacggcc cccacgagcc ccaggtcgcg ctggttttgg
44221 gtgcgcacca gccgcttgga ctcgatatcc cgggtggagc cttcgcatgt cgcggtgagg
44281 taggttagga acagtgggcg tcggacgtcg acgcccgtga gcttgtagcc gatcccccg
44341 ggcagagggg agtgggtgac gacgtagctg gcgttgtggg tgatgggtac caggatccgt
44401 ggctcgacgt tggcagactg cccccgcac cgatgtgagg cctcaggggac gaaggcgcg
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44521 gccaggactt cgaccggggt ctccggtcgg gtggcttgag ccagcgcgct caggataaac
44581 acgctctcgt ctagatcaaa gcgcaggagg gccgcgcatg gcgaaaagtg gtccggaagc
44641 caaaagaggg ttttctggtg gtcggcccg gccagcgcg tccggaggtc ggcgttggtc
44701 gctcggcgca cgtcggacgt acacaggggc gaggctatca gaaggctccg gcgggcgct
44761 tcccgtgca ccgcccagg gacgccagcc aagaacggct gccggaggac agccgagcg
44821 taaaatagcg cccggtggac gaccgggggt gtcagcacgc ggccccctag aaactcgcca
44881 tacagggcgt cgatgagatg ggctgcgctg ggcgcactg cgtcgtacgc cgaggggcta
44941 tccagcacga aggccagctg atagcccagc gcgtgtaatg ccaagctctg ttcgctcc
45001 agaactcctg ccaccagggt ctggagccga gcctctagct gcaggcgggc cgtgggatcc
45061 aagactgaca cattaataaa cacagaatcc gcggcacagc ccgcggcccc gcgggcggcc
45121 aaccggcaa gcgcgcgca gtggccaaa aagcctagca ggtcggagag gcagaccgcg
45181 ccgtttgctg gggcggcgtt cacgaaagca aaaccgcagc tcgcgagcag ccccgttagg
45241 cgccagaaga gagggggcg cgggccctgc tcggcgccc cgccccga gaaaaactcc
45301 cgttatgccc gcgacaggaa ctgggcgtag ttcgtgccct cctccgggta gccgccacg
45361 cgccggaggg cgtccagcgc ggagccgttg tcggcccgcg tcagggaccc taggacaaa
45421 accgataacc gggggccgccc cgggggccc ggaagagccc ccggggggtt ttcgtcccg
45481 gggccccga cccgatctag cgtctggccc gcggggacca ccatcacttc caccggagg
45541 ctgtcgtgca tggatatcac gagccccatg aattcccgcg ctagcgcgcc gcgcaccagc
45601 gcggcatcgc acccgagcac cagctcccc gtogtccaga tgcccacgg ccacgtcgag
45661 gccgacgggg agaaatacac gtacctacct ggggatctca acaggcccc ggtggccaac
45721 caggtcgtgg acgctgtgtg caggtgcgtg atgtccagct ccgtcgtcgg gtgcccggg
45781 gcccacaccg gcggtcgggg gggcggtgta tcacgcggcc cgctcgggtg gtcgcgcgtc
45841 gccacgttgt ctccccgcgg gaacgtcagg gcctcgggg cagggacggc cgaaaacgtt
45901 acccaggccc gggaacgcag caacacggag gcggctggat tgtgcaagag acccttaagg
45961 gggcgacccg aggggggagg ctggcggttc ggctcgaccg tgggtggggc gggcaggctc
46021 gcgttcgggg gccggccgag caggtaggctc ttcgggatgt aaagcagctg gccggggtcc
46081 cgcgaaact cgccgtggt gaccaataca aaacaaaagc gtcctcgtg ccagcgaaga
46141 aggggcagag atgccgtagt caggtttagt tcgtccggcg gcgcccagaa tccgcgcgtg
46201 ggtttttggg ggtcgggggt gtttggcagc cacagacgcc cggtgttcgt gtcgcgccag
46261 tacatgcggt ccatgccag gccatccaaa aacctgggt ctgtctgctc agtccagtcg
46321 tggacctgac cccacgcaac gcccaataa ataaccacca cgaaccataa accattcccc
46381 at

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Figure 21e

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          atgg ggattttggg ttgggtcggg
9361 cttattgccg ttggggtttt gtgtgtgcgg gggggcttgc cttcaaccga atatgttatt
9421 cggagtcggg tggctcgaga ggtgggggat atattaaagg tgcttgtgt gccgctccc
9481 tctgacgatc ttgattggcg ttacgagacc ccctcggcta taaactatgc tttgatagac
9541 ggtatatttt tgcgttatca ctgtcccga ttggacacgg tcttgtggga taggcatgcc
9601 cagaaggcat attgggttaa ccccttttta tttgtggcgg gttttttgga ggacttgagt
9661 taccgccgct ttcttgccta caccaggaa acagaaacgc gcttggccct ttataaagag
9721 atacgccagg cgctggacag tcgcaagcag gccgccagcc acacacctgt gaaggctggg
9781 tgtgtgaact ttgactatc gcgcacccgc cgctgtgtag ggcgacagga tttgggacct
9841 accaacggaa cgtctggacg gaccocggtt ctgccgccc acgatgaagc gggcctgcag
9901 ccgaagcccc tcaccacgcc gccgccatc atcgccacgt cggacccac cccgcgacgg
9961 gacgccgcca caaaaagcag acgcccagca cccactccc ggcgcctcta a

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Figure 21f