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ONCOLYTIC HERPES SIMPLEX VIRUS ENCODING A HETEROLOGOUS NITROREDUCTASE

Für eine heterologe Nitroreduktase kodierender onkolytischer herpes simplex virus

Virus de l’herpes simplex oncolytique codant pour une nitroreductase heterologue

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Description

Field of the Invention

[0001] The present invention relates to mutant herpes simplex viruses wherein the herpes simplex virus genome comprises nucleic acid encoding a nitroreductase.

Background to the Invention

Herpes simplex virus

[0002] The herpes simplex virus (HSV) genome comprises two covalently linked segments, designated long (L) and short (S). Each segment contains a unique sequence flanked by a pair of inverted terminal repeat sequences. The long repeat (RL) and the short repeat (RS) are distinct.

[0003] The HSV ICP34.5 (also γ34.5) gene, which has been extensively studied\(^1\),\(^6\),\(^7\),\(^8\), has been sequenced in HSV-1 strains F\(^9\) and syn17\(^3\), and in HSV-2 strain HG529\(^4\). One copy of the ICP34.5 gene is located within each of the RL repeat regions. Mutants inactivating both copies of the ICP34.5 gene (i.e. null mutants), e.g. HSV-1 strain 17 mutant 1716\(^2\) or the mutants R3616 or R4009 in strain F\(^5\), are known to lack neurovirulence, i.e. be a virulent, and have utility as both gene delivery vectors or in the treatment of tumours by oncolysis. HSV-1 strain 17 mutant 1716 has a 759bp deletion in each copy of the ICP34.5 gene located within the BamHI S restriction fragment of each RL repeat.

[0004] ICP34.5 null mutants such as HSV1716 are, in effect, first-generation oncolytic viruses. Most tumours exhibit individual characteristics and the ability of a broad spectrum first generation oncolytic virus to replicate in or provide an effective treatment for all tumour types is not guaranteed.

[0005] HSV 1716 is described in EP 0571410 and WO 92/13943 and has been deposited on 28 January 1992 at the European Collection of Animal Cell Cultures, Vaccine Research and Production Laboratories, Public Health Laboratory Services, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number V92012803 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the 'Budapest Treaty').

Nitroreductase prodrug activation

[0006] Enzyme prodrug therapy is based on the enzymatic activation of a non toxic or low toxicity prodrug to a form that is considerably more cytotoxic. The activation may involve enzymatic reduction of the prodrug to a cytotoxic reduced form.

[0007] The E.coli nitroreductase enzyme (NTR) has been proposed for use in gene-directed enzyme prodrug therapy (GDEPT) as an activating enzyme for nitroaromatic prodrugs of the dinitrobenzamide class\(^16\). E.coli NTR is a homodimeric enzyme with two active sites and is the oxygen insensitive enzyme from E.coli (the nfsB gene product). It has the ability to reduce a wide range of nitro-containing compounds such as nitrofurazone (to the hydroxylamines) and quinones such as menadione (to the quinols). It is specifically inhibited by the irreversible inhibitor dicoumarol.

[0008] The ability of NTR to reduce aromatic nitro groups to the corresponding hydroxylamine (and possibly amine) derivatives has been proposed for cancer chemotherapy mainly with the dinitrobenzamide class of prodrugs. The 5-aziridin-1-yl-2,4-dinitrobenzamide CB1954 (CAS Registry number 21919-05-1) is one such prodrug which has been studied as a prodrug for GDEPT with NTR\(^16\).

[0009] Cyclic and acyclic nitroaryl phosphoramide mustard analogues have also been shown to be activated by E.coli NTR\(^\rightarrow\). The acyclic 4-nitrobenzyl phosphoramide mustard showed 167,500x selective cytotoxicity toward nitroreductase-expressing V79 cells with an IC\(_{50}\) as low as 0.4nM which is about 100x more active and 27x more selective than CB1954.

[0010] Recombinant adenovirus and recombinant retrovirus\(^\rightarrow\) expressing nitroreductase have been constructed for use with the prodrug CB1954 with the intention of providing a treatment for cancer. The recombinant virus is not oncolytic and relies on gene directed enzyme-prodrug therapy to achieve tumour cell kill.

Summary of the Invention

[0011] The inventors have determined that herpes simplex virus having an inactivating mutation in the RL1 locus, 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, can be used in the delivery to a cell of a gene encoding a gene product useful in targeted tumour therapy.

[0012] The inventors have provided a novel second generation oncolytic mutant HSV. The genome of this mutant HSV comprises the heterologous (i.e. non-HSV originating) E.coli nitroreductase protein coding sequence inserted at
The herpes simplex virus is a mutant of one of HSV-1 strains 17, F or HSV-2 strain HG52. The herpes simplex virus may be a further mutant of HSV-1 strain 17 mutant 1716. In certain arrangements the herpes simplex virus may be a gene specific null mutant, such as an ICP34.5 null mutant.

In other arrangements the herpes simplex virus may lack at least one expressible ICP34.5 gene.

In yet another arrangement the herpes simplex virus may lack only one expressible ICP34.5 gene.

In yet another arrangement the herpes simplex virus may be non-neurovirulent.

In herpes simplex viruses of the present invention the nucleic acid encoding the NTR may form part of a nucleic acid cassette permanently integrated in the herpes simplex virus genome, said cassette comprising nucleic acid encoding:
In need of treatment an effective amount of a mutant HSV or a medicament comprising or derived from such HSV are to the present invention for use in oncotherapy and methods of treating tumours comprising administering to a patient for the treatment of cancer is also provided.

The use of herpes simplex viruses according to the present invention in the manufacture of a medicament are provided for use in the treatment of cancer- Suitably they may be provided for use in the oncolytic treatment of cancer/a tumour. The use of herpes simplex viruses according to the present invention in the manufacture of a medicament for use in a method of medical treatment. Suitably they are provided for use in the treatment of disease-

In other aspects of the present invention herpes simplex viruses according to the present invention are provided are not limited to Herpes simplex viruses obtained in this way.

Mutant herpes simplex viruses of the present invention may be generated by site directed insertion of a nucleic acid cassette into the viral genome, more preferably by homologous recombination. However, the viruses of the invention is preferably located downstream (3') of the nucleic acid encoding the marker wherein the ribosome binding site is located between the cistrons.

In another arrangement, the nucleic acid encoding the NTR may be arranged upstream (i.e. 5') of a first regulatory nucleotide sequence and the first regulatory nucleotide sequence is arranged upstream (i.e. 5') of the marker. During transcription a single transcript may be produced from the cassette comprising a first cistron comprising nucleic acid encoding NTR (e.g. an mRNA transcript) and a second cistron comprising nucleic acid encoding the marker wherein the ribosome binding site is located between said first and second cistrons.

The cassette may disrupt a protein coding sequence of the herpes simplex virus genome resulting in inactivation of the respective gene product.

Preferably the marker is a defined nucleotide sequence coding for a polypeptide which can be expressed in a cell line (e.g. BHK cells) infected with mutant herpes simplex virus into which the cassette has been recombined. The function of the marker is to enable identification of virus plaques containing mutant virus transformed with the cassette.

The marker is preferably a detectable marker, more preferably an expressible marker polypeptide or protein comprising at least the coding sequence with a role in controlling transcription of the marker mRNA. Preferred markers include the Green Fluorescent Protein (GFP) protein coding sequence or gene, preferably the enhanced Green Fluorescent Protein (EGFP) protein coding sequence or gene.

In other arrangements the marker may comprise a defined nucleotide sequence which can be detected by hybridisation under high stringency conditions with a corresponding labelled nucleic acid probe, e.g. using a fluorescent- or radio-label.

The cassette may also comprise nucleic acid encoding a polyadenylation ("polyA") sequence, which sequence is preferably located downstream (3') of the nucleic acid encoding the marker. One preferred polyA sequence is the Simian Virus 40 (SV40) polyadenylation sequence. The preferred location of the polyA sequence within the cassette is immediately downstream (i.e. 3') of the marker.

Mutant herpes simplex viruses of the present invention may be generated by site directed insertion of a nucleic acid cassette into the viral genome, more preferably by homologous recombination. However, the viruses of the invention are not limited to Herpes simplex viruses obtained in this way.

In other aspects of the present invention herpes simplex viruses according to the present invention are provided for use in a method of medical treatment. Suitably they are provided for use in the treatment of disease- Preferably they are provided for use in the treatment of cancer- Suitably they may be provided for use in the oncolytic treatment of cancer/a tumour. The use of herpes simplex viruses according to the present invention in the manufacture of a medicament for the treatment of cancer is also provided.

In another aspect of the present invention medicaments comprising herpes simplex virus mutants according to the present invention for use in oncology and methods of treating tumours comprising administering to a patient in need of treatment an effective amount of a mutant HSV or a medicament comprising or derived from such HSV are

(a) said nucleic acid encoding NTR; and nucleic acid encoding:

(a) a ribosome binding site; and
(b) a marker,
also provided.

[0046] A medicament, pharmaceutical composition or vaccine comprising an Herpes simplex virus according to the present invention is also provided. The medicament, pharmaceutical composition or vaccine may further comprise a pharmaceutically acceptable carrier, adjuvant or diluent. Pharmaceutical compositions or vaccines may further comprise an NTR prodrug.

[0047] The present invention may also include the following aspects which may be provided in combination with any of the other aspects and features described.

[0048] According to another aspect of the present invention a herpes simplex virus as defined in the claims is provided, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase (NTR) in at least one of the long repeat regions (RL).

[0049] According to another aspect of the present invention a herpes simplex virus as defined in the claims is provided, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase (NTR) and wherein the herpes simplex virus is non-neurovirulent.

[0050] A compositions comprising a herpes simplex virus of the invention may be provided in combination with an NTR prodrug. The NTR prodrug may be CB1954.

[0051] According to another aspect of the present invention a herpes simplex virus as defined in the claims for use in the treatment of a tumour is provided, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase and wherein the herpes simplex virus is non-neurovirulent.

[0052] According to another aspect of the present invention a herpes simplex virus as defined in the claims for use in the treatment of a tumour is provided, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase and wherein the herpes simplex virus is non-neurovirulent.

[0053] According to another aspect of the present invention a herpes simplex virus as defined in the claims is provided, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (RL), for use, in combination with an NTR prodrug, in the treatment of a tumour.

[0054] According to another aspect of the present invention a herpes simplex virus as defined in the claims is provided, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase and wherein the herpes simplex virus is non-neurovirulent, for use, in combination with an NTR prodrug, in the treatment of a tumour.

[0055] According to another aspect of the present invention a kit of parts is provided comprising a first container having a quantity of an herpes simplex virus of the invention and a second container having a quantity of an NTR prodrug.

[0056] In another aspect the use of a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (RL), in the manufacture of a medicament for the treatment of a tumour is also provided.

[0057] In another aspect the use of a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase and wherein the herpes simplex virus is non-neurovirulent, in the manufacture of a medicament for the treatment of a tumour is also provided.

[0058] In another aspect the use in the manufacture of a medicament for the treatment of a tumour of a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (RL), and an NTR prodrug is also provided.

[0059] In another aspect the use in the manufacture of a medicament for the treatment of a tumour of a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase and wherein the herpes simplex virus is non-neurovirulent, and an NTR prodrug is also provided.

[0060] In another aspect the use of a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (RL) in the manufacture of a first medicament for administering sequentially or simultaneously with a second medicament comprising an NTR prodrug in the treatment of a tumour is also provided.

[0061] In another aspect the use of an NTR prodrug in the manufacture of a first medicament for administering sequentially or simultaneously with a second medicament comprising a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (RL), in the treatment of a tumour is also provided.

[0062] In another aspect the use of an NTR prodrug in the manufacture of a first medicament for administering sequentially or simultaneously with a second medicament comprising a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase and wherein the herpes simplex virus is non-neurovirulent, in the treatment of a tumour is also provided.

[0063] In another aspect the use of a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase and wherein the herpes simplex virus is non-neurovirulent, in the manufacture of a medicament for administering sequentially or simultaneously with a
second medicament comprising an NTR prodrug, in the treatment of a tumour is also provided.

[0064] The time period between sequential administrations may be such that the herpes simplex virus and NTR prodrug may interact in the body to produce an active pharmaceutical agent in situ. Preferred time periods may be less than 15 minutes, less than one hour, two hours, three hours, four hours, five hours or six hours, twelve hours, twenty four hours, forty eight hours, one week or two weeks. Either the herpes simplex virus or NTR. prodrug may be administered first.

[0065] Where the herpes simplex virus is used in methods of treatment said herpes simplex virus is preferably capable of killing tumour cells, e.g. by oncolysis.

[0066] In aspects of the invention involving an NTR prodrug, one preferred prodrug is CB1954.

[0067] In another aspect a method of expressing in vitro a nitroreductase is provided, said method comprising the step of infecting at least one cell tissue of interest with a herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding a heterologous nitroreductase in at least one of the long repeat regions (R_L), said nitroreductase operably linked to a transcription regulatory sequence.

[0068] In another aspect a method of expressing in vitro a nitroreductase is provided, said method comprising the step of infecting at least one cell or tissue of interest with a non-neurovirulent herpes simplex virus as defined in the claims, wherein the genome of said virus comprises a nucleic acid sequence encoding a heterologous nitroreductase, said nitroreductase operably linked to a transcription regulatory sequence.

[0069] Herpes simplex viruses of the invention having nucleic acid encoding an heterologous nitroreductase in at least one of the long repeat regions (R_L) of the HSV genome Preferably have said nucleic acid in each of the long repeat regions of the HSV genome. Two long repeat regions are usually present in the HSV genome.

[0070] The NTR nucleotide sequence may encode a full length transcript or polypeptide (i.e. comprise the complete NTR protein coding sequence). Alternatively, provided the polypeptide product retains nitroreductase activity, the NTR nucleotide sequence may comprise one or more fragments of the full length sequence respectively coding for a fragment of the full length transcript or a truncated polypeptide.

[0071] A fragment may comprise a nucleotide sequence encoding at least 10% of the corresponding full length sequence, more preferably the fragment comprises at least 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% of the corresponding full length sequence. Preferably, the fragment comprises at least, i.e. has a minimum length of, 20 nucleotides, more preferably at least 30, 40, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000 nucleotides. The fragment may have a maximum length, i.e. be no longer than, 20 nucleotides, more preferably no longer than 30, 40, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000 nucleotides. The fragment length may be anywhere between said minimum and maximum length.

[0072] In one preferred arrangement, the mutant HSV is HSV1716/CMV-NTR/GFP deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 05 November 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03110501 in accordance with the provisions of the Budapest Treaty.

[0073] Suitably, the administration of said herpes simplex virus and/or said NTR prodrug may comprise parenteral administration. Preferably administration of the herpes simplex virus is by injection, more preferably injection to the tumour which is to be treated. The NTR prodrug may also be administered by injection, which may also comprise direct injection to the site of the tumour. Alternatively injections may be intravenous.

[0074] Administration of the herpes simplex virus and NTR prodrug may be simultaneous, e.g. by combining virus and prodrug in a single composition, or be substantially simultaneous, e.g. one being administered immediately after the other. Alternatively, a predetermined time period may be provided between administration of the herpes simplex virus and the NTR prodrug. The invention is not limited by the order of administration.

[0075] The present invention also relates to in vitro methods for delivery of nucleic acid encoding a nitroreductase to at least one cell or to a tissue of interest said method comprising the step of infecting said cell(s) or tissue with a herpes simplex virus according to the invention.

[0076] In another aspect of the invention, a kit of parts is provided comprising, a first container in which a quantity of herpes simplex virus according to the invention is provided and a second container in which a quantity of NTR prodrug is provided. Instructions for the administration, optionally including information on suitable dosages of herpes simplex virus and/or the NTR prodrug, may also be provided with the kit.

[0077] The invention also relates to a method of making or producing a modified herpes simplex virus of the invention comprising the step of introducing a nucleic acid sequence encoding a nitroreductase at a selected and/or predetermined insertion site in the genome of a selected herpes simplex virus.

[0078] As described, the nucleic acid sequence encoding the nitroreductase may form part of a nucleic acid cassette which is inserted in the genome of a selected herpes simplex virus by homologous recombination. Whether part of a
cassette or not, the site of insertion may be in any genomic location selected. One preferred insertion site is in one or both of the long repeat regions (R_L), and one copy of the cassette is preferably inserted in each copy of the long repeat (D_L). More preferably the insertion site is in at least one (preferably both) RL1 locus and most preferably it is inserted in at least one (preferably both) of the ICP34.5 protein coding sequences of the HSV genomic DNA. It is preferred that the insertion occurs in identical or substantially similar positions in each of the two repeat regions, RL1 loci or ICP34.5 protein coding sequences.

[0079] Insertion may be such as to produce a modified virus which is a non-neurovirulent mutant capable of expressing the encoded nitroreductase polypeptide upon transfection into mammalian, more preferably human, cells in vivo and in vitro in a form which is functional to facilitate the uptake and/or activation of NTR prodrug. The non-neurovirulent mutant may be an ICP34.5 null mutant.

[0080] The nucleic acid cassette may be of any size, e.g. up to 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50kbp in length.

[0081] Preferably, the herpes simplex virus contains at least one copy of the nucleic acid encoding the nitroreductase in each long repeat region (R_L), i.e. in the terminal and internal long repeat (TR_L and IR_L) regions. In a preferred arrangement each exogenous sequence or cassette is located in an RL1 locus of the herpes simplex virus genome, more preferably in the DNA of the herpes simplex virus genome encoding the ICP34.5 gene or protein coding sequence. The herpes simplex virus thereby lacks neurovirulence.

[0082] The parent herpes simplex virus, from which a virus of the invention is derived may be of any kind, e.g. HSV-1 or HSV-2. In one preferred arrangement the herpes simplex virus is a variant of HSV-1 strain 17 and may be obtained by modification of the strain 17 genomic DNA. Suitable modifications include the insertion of the exogenous nitroreductase 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 of the exogenous nucleic acid sequence into the genome of the selected herpes simplex virus.

[0083] Although the non-neurovirulent phenotype of the herpes simplex virus of the invention may be the result of insertion of the exogenous nucleic acid sequence in the RL1 locus, herpes simplex viruses according to the present invention may be obtained by utilising a non-neurovirulent parent strain, e.g. HSV1716 deposited under the Budapest Treaty at the European Collection of Animal Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, United Kingdom under accession number V92012803, and inserting the exogenous nucleic acid sequence at another location of the genome by standard genetic engineering techniques, e.g. homologous recombination. In this aspect the location of the herpes simplex virus genome selected for insertion of the nitroreductase nucleic acid sequence or cassette containing said sequence may be a neutral location.

[0084] Herpes simplex viruses of the present invention may be variants of a known 'parent' strain from which the herpes simplex virus of the invention has been derived. A particularly preferred parent strain is HSV-1 strain 17. Other parent strains may include HSV-1 strain F or HSV-2 strain HG52. A variant comprises an HSV in which the genome substantially resembles that of the parent, contains the nitroreductase nucleic acid sequence or cassette containing said sequence and may contain a limited number of other modifications, e.g. one, two or three other specific mutations, which may be introduced to disable the pathogenic properties of the herpes simplex virus, for example a mutation in the ribonucleotide reductase (RR) gene, the 65K trans inducing factor (TIF) and/or a small number of mutations resulting from natural variation, which may be incorporated naturally during replication and selection in vitro or in vivo. Otherwise the genome of the variant will be that of the parent strain.

[0085] Herpes simplex viruses of the invention may be used alone, or in combination with an NTR prodrug in a method of medical treatment. This may involve treatment of diseases associated with or involving the proliferation of cells, or cancers or tumours of any kind. Treatment may involve the selective lysis of dividing cells. This may be oncolysis, i.e. lysis of tumour cells. Tumours to be treated may be of any kind, may comprise cancers, neoplasms or neoplastic tissue and may be in any animal or human patient.

[0086] Cancer/tumour types which may be treated may be primary or secondary (metastatic) tumours. 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 oligodendroglia, 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 mutants of the present invention may be used to treat metastatic tumours of the central or peripheral nervous system which originated in a non-nervous system tissue.

[0087] Herpes simplex viruses of the invention may be used in 'gene delivery' methods in vitro or in vivo. Non-neurovirulent herpes simplex viruses of the invention are expression vectors and may be used to infect selected cells or tissues in order to express the nitroreductase encoded by the herpes simplex virus genome.

[0088] In one arrangement, cells may be taken from a patient, a donor or from any other source, infected with a herpes simplex virus of the invention, optionally screened for expression and/or function of the encoded nitroreductase, and optionally returned/introduced to a patient's body, e.g. by injection.

[0089] Delivery of herpes simplex viruses of the invention to the selected cells may be performed using naked virus
or by encapsulation of the virus in a carrier, e.g. nanoparticles, liposomes or other vesicles.

[0090] In vitro cultured cells, preferably human or mammalian cells, transformed with viruses of the present invention and preferably cells expressing the nitroreductase protein as well as methods of transforming such cells in vitro with said viruses form further aspects of the present invention.

[0091] In this specification, a mutant herpes simplex virus is a non-wild type herpes simplex virus and may be a recombinant herpes simplex virus. Mutant herpes simplex viruses may comprise a genome containing modifications relative to the wild type. A modification may include at least one deletion, insertion, addition or substitution.

[0092] 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, parenteral, intravenous, intramuscular, intratumoural, oral and nasal. The medicaments and compositions may be formulated in fluid or solid (e.g. tablet) form. Fluid formulations may be formulated for administration by injection to a selected region of the human or animal body.

[0093] 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 ≥10^6 pfu.

[0094] Where all copies of the ICP34.5 gene present in the herpes simplex virus genome (two copies are normally present) are disrupted such that the herpes simplex virus is incapable of producing a functional ICP34.5 gene product, the virus is considered to be an ICP34.5 null mutant.

[0095] A regulatory sequence (e.g. promoter) that is operably linked to a nucleotide sequence may be located adjacent to that sequence or in close proximity such that the regulatory sequence can effect and/or control expression of a product of the nucleotide sequence. The encoded product of the nucleotide sequence may therefore be expressible from that regulatory sequence.

NTR prodrug

[0096] In this specification, "NTR prodrug" means any chemical compound or agent which is not toxic, or exhibits low toxicity, to a selected human or animal body, or to selected cells or tissues of the human or animal body, and which may be activated by a nitroreducase enzyme to a chemical compound or agent which is cytotoxic to the human or animal body or to those selected cells.

[0097] "Activation" may involve conversion of a non-toxic (or low toxicity) prodrug to an active cytotoxic form. That conversion may involve enzymatic reduction of the prodrug by NTR. The enzymatic reduction reaction may involve the prodrug as a substrate for NTR and may involve other cofactors.

[0098] Examples of NTR prodrugs may include compounds from the following classes of molecules:

1. dinitrobenzamides;
2. dinitroaziridinylbenzamides (e.g. CB1954);
3. dinitrobenzamide mustard derivatives (e.g. SN23862);
4. 4-nitrobenzylcarbamates;
5. nitroindolines;
6. nitroaromatics that are substrates of NTR and are activated to release a cytotoxic phosphoramid mustard or like-reactive species upon NTR-reduction (also called nitroaryl phosphoramides);
7. nitroaromatic prodrugs of the dinitrobenzamide class.

[0099] Examples of NTR prodrugs are disclosed in references 16 and 17 which are incorporated herein in their entirety by reference.

Nitroreductase (NTR)

[0100] Nitroreductase enzymes commonly catalyze the reduction of nitro compounds, quinones, and dyes. The enzymatic reduction may involve the co-factor NADPH.

[0101] In this specification nitroreductase (NTR) refers to an enzyme capable of activating an NTR prodrug to an active cytotoxic form.

[0102] Preferred NTR's may have the ability to reduce a wide range of nitro-containing compounds such as nitrofurazone (to the hydroxylamines) and quinones such as menadione (to the quinols).

[0103] Preferred NTR's may be specifically inhibited by the irreversible inhibitor dicoumarol.

[0104] One preferred NTR is the E.coli oxygen insensitive nitroreductase enzyme (the nfsB gene product). Sequence information for E.coli NTR can be found at the NCBI database (http://www.ncbi.nlm.nih.gov/) under accession numbers BA000007 (GI:47118301) - E.coli complete genome sequence - and BAB34039 (GI:13360074) - nitroreductase sequence information.
The amino acid sequence for the E.coli NTR protein (SEQ ID No. 1) and polynucleotide sequence for the E.coli NTR gene (SEQ ID No. 2) are reproduced at Figure 32 (A) and (B) respectively.

The nucleotide and amino acid sequences of suitable nitroreductase enzymes may be derived or obtained from any animal, insect or microorganism including humans, non-human mammals and bacteria and may be selected from those sequences which are publicly available. Many sequences for other nitroreductase genes are publicly available. Examples of other nitroreductase nucleic acid sequences which may form part of a herpes simplex virus according to the present invention include the following which are referred to by their accession number for the NCBI database (www.ncbi.nlm.nih.gov):

- BAB34039 (GI:13360074) - E.coli
- BAA35218.1 (GI:1651240) - E.coli
- AAB72053.1 (GI:2415385) - B.subtilis.

Hybridisation stringency

In accordance with the present invention, 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.

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):

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

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.

Sequences exhibiting 95-100% sequence complementarity may be considered to hybridise under very high stringency conditions, sequences exhibiting 85-95% complementarity may be considered to hybridise under high stringency conditions, sequences exhibiting 70-85% complementarity may be considered to hybridise under intermediate stringency conditions, sequences exhibiting 60-70% complementarity may be considered to hybridise under low stringency conditions and sequences exhibiting 50-60% complementarity may be considered to hybridise under very low
stringency conditions.

[0117] The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

[0118] 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.

Brief Description of the Figures

[0119]

**Figure 1.** Generation of plasmid RL1.dIRES-GFP from plasmids pNAT-IRESGFP and RL1.del.

**Figure 2.** Agarose gel electrophoresis of Hpal digested, CIP treated, RL1.del. RL1.del was digested with Hpal. The digested DNA was then treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A sample of the digested/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. Hpal linearises the vector at 8.6 Kbp.

**Figure 3.** Agarose gel electrophoresis of NsiI/SspI digested pNAT-IRESGFP (A) and purified/blunt-ended pCMV-NAT-IRESGFP-PolyA (B). Four NsiI/SspI digestions of pNAT-IRESGFP were electrophoresed, beside a 1Kbp DNA ladder (Promega) on a 1% agarose gel. The 5.4Kbp fragments (pNAT-IRESGFP-PolyA) were purified from the gel. The purified DNA was blunt ended using Klenow polymerase and a sample electrophoresed on an agarose gel to check its concentration.

**Figure 4.** Identification of RL1.del clones containing the pCMV-NAT-IRESGFP-PolyA insert. Ligation reactions were set up with the purified, blunt ended pCMV-NAT-IRESGFP-PolyA fragment and Hpal digested, CIP treated RL1.del. Bacteria were transformed with samples from the ligation reactions and plated out on LB0 (Amp') plates. Colonies were picked and plasmid DNA was extracted and digested with AflII. Digested samples were electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel.

*Clones 5 and 8 contained the pCMV-NAT-IRESGFP-PolyA insert as two fragments of the predicted size - 4.8Kbp and 9.2Kbp were generated from AflII digestion. Clones without inserts would not be digested with AflII as there is no AflII site in RL1.del.*

N.B. Inserts could have been cloned in two orientations, both of which were acceptable.

**Figure 5.** Determination of the orientation of pCMV-NAT-IRESGFP-PolyA in clone 5 (RL1.dCMV-NAT-IRESGFPb), pCMV-NAT-IRESGFP-PolyA (blunt ended) could have been cloned into the Hpal site of RL1.del in two orientations. To determine the orientation of the insert in clone 5, the plasmid was digested with XhoI and the digested DNA electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. If the insert had been cloned in the orientation shown in A, two fragments of 10.2Kbp and 3.8Kbp would be generated from XhoI digestion. If it had been cloned in the opposite orientation (B), two fragments of 12.4Kbp and 1.6Kbp would be generated. The presence of two fragments of 10.2Kbp and 3.8Kbp in the gel confirmed that the insert had been cloned in the orientation shown in A.

*This XhoI site was present in the initial cloning vector (RL1.del), upstream of the Hpal site into which pCMV-NAT-IRESGFP-PolyA was cloned.*

**Figure 6.** Removal of pCMV-NAT from clone 5 (A) and large scale plasmid preparation of RL1.dIRES-GFP (B). Four samples of clone 5 were digested with XhoI and electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel (A). The large fragment of DNA generated from this digestion (10.2Kbp) was purified from the gel and ligated back together, at the XhoI sites, to form a single XhoI site in a new plasmid, designated RL1.dIRES-GFP. A large-scale plasmid preparation was grown up and the preparation checked by digesting with XhoI. 1µl and 4µl of the digested DNA was electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel (B). The DNA should produce a single fragment of 10.2Kbp when digested with XhoI. The ClaI, BglII, NruI and XhoI sites of RL1.dIRES-GFP are all unique.

*Clone 5 is the RL1.del plasmid into which has been cloned the 5.4Kbp pCMV-NAT-IRESGFP-PolyA fragment from pNAT-IRESGFP.*

**Figure 7.** Generation, detection and purification of ICP34.5 null HSV-1 expressing a gene product of interest.

**Figure 8.** Strategy used to clone pCMV-NTR from pPS949 into RL1.dIRES-GFP. (1) Digest pPS949 with BamH1
and purify the 1.6Kbp pCMV-NTR fragment; (2) Digest RL1.dIRES-GFP with BglII and treat with Calf Intestinal Phosphatase (CIP); (3) Clone the pCMV-NTR fragment (BamHI ends) into the BglII site of RL1.dIRES-GFP.

* The pPS949 plasmid was a kind gift from Professor Lawrence Young (University of Birmingham) and contains the E.coli nitroreductase (NTR) gene downstream of the CMV-IE promoter (pCMV) in pLNCX (Clontech).

Figure 9. Agarose gel electrophoresis of BamHI digested pPS949 (A) and the purified pCMV-NTR fragment (B). Four samples of pPS949 were digested with BamHI and electrophoresed, beside a 1Kbp DNA ladder (L) (New England Biolabs), on a 1% agarose gel. The 1.6Kbp fragments, consisting of the E.coli nitroreductase (NTR) gene downstream of the CMV IE promoter (pCMV), were purified from the gel and a sample of the purified DNA was electrophoresed on an agarose gel to check its concentration.

Figure 10. Agarose gel electrophoresis of BglII digested, CIP treated RL1.dIRES-GFP. RL1.dIRES.GFP was digested with BglII. The digested plasmid was then treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A sample of the digested/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel to check its concentration. pCMV-NTR from pPS949 was subsequently cloned into this digested/CIP treated vector.

Figure 11. Determination of the orientation of pCMV-NTR in clone 4. pCMV-NTR (BamHI ends) could have been cloned into the BglII site of RL1.dIRES-GFP in two orientations. To determine the orientation, clone 4 was digested with BglII and XhoI and the digested DNA electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. If the insert was in the desired orientation (A), two fragments (11.5Kbp and 300bp) would be generated. If in the opposite orientation, two fragments of 10.5Kbp and 1.3Kbp would be generated. The presence of a band at ~300bp (and the absence of a band at 1.3Kbp) confirmed that the pCMV-NTR fragment had been cloned into the vector in the desired orientation.

Figure 12. Agarose gel electrophoresis of ScaI digested clone 4 (A) and HSV1716/CMV-NTR/GFP viral titre (B). Clone 4 (RL1.dCMV-NTR-GFP) was digested with Scal, the digested DNA purified and 5µl electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel, to check its concentration. 80% confluent BHK cells were then co-transfected with 10µl HSV17+ DNA and an appropriate volume of the remaining digested clone 4. The cells were incubated at 37°C for 3 days until cpe was evident. Recombinant viral plaques were picked under the fluorescent microscope, purified and a virus stock, named HSV1716/CMV-NTR/GFP, grown up. The cell-associated and cell-released fraction of the virus stock was titrated on BHK cells.

Figure 13. Growth kinetics of HSV17+, HSV1716 and HSV1716/CMV-NTR/GFP in confluent BHK and 3T6 cells. Confluent BHK and 3T6 cells were infected at a MOI of 0.1pfu/cell. Infected cells were harvested at 0, 4, 24, 48 and 72hrs post infection, sonicated and progeny virus titrated on BHK cell monolayers. All viruses replicated with similar kinetics in BHK cells (A); HSV1716 and HSV1716/CMV-NTR/GFP both failed to replicate efficiently in confluent 3T6 cells (B).

Figure 14. Western blot analysis of ICP34.5 expression in HSV17+ and HSV1716/CMV-NTR/GFP infected BHK cells. BHK cells were infected with HSV17+ and HSV1716/CMV-NTR/GFP at a MOI of 10pfu/cell. 16hrs post infection, the cells were harvested and protein extracts analysed using 10% SDS-PAGE in a Western blot using a polyclonal anti-ICP34.5 antibody. ICP34.5 was strongly expressed in HSV17+ infected cells but was not expressed in HSV1716/CMV-NTR/GFP infected cells.

Figure 15. Western blot analysis of NTR expression in HSV1716/CMV-NTR/GFP infected cell lines. BHK, C8161, VM and 3T6 cells were infected with 10pfu/cell HSV1716/CMV-NTR/GFP, HSV17+ or mock infected. 16hrs post infection, the cells were harvested and protein extracts analysed in a Western blot using a polyclonal NTR-specific antibody. Significant NTR expression was detected in all the HSV1716/CMV-NTR/GFP infected cells. No NTR expression was detected in the mock or HSV17+ infected cells.

Figure 16. Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP with or without CB1954 (50µM) on confluent 3T6 cells. Confluent 3T6 cells in three wells of a 96-well plate were mock infected, infected with 1 or 10pfu/cell HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of HSV1716-GFP. 45 minutes later, infected cells were overlaid with media containing 50µM CB1954 or with media alone and incubated at 37°C. 24, 48, 72, 96, and 120hrs later, % cell survival was determined relative to that of mock infected cells without prodrug using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Figures shown represent the mean of 3 values +/- standard error of the mean.
Figure 17. Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP with or without CB1954 (50µM) on confluent C8161 cells. Confluent C8161 cells in three wells of a 96-well plate were mock infected, infected with 1 or 10pfu/cell HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of HSV1716-GFP. 45 minutes later, infected cells were overlaid with media containing 50µM CB1954 or with media alone and incubated at 37°C. 24, 48 and 72hrs later, % cell survival was determined relative to that of mock infected cells without prodrug using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Figures shown represent the mean of 3 values +/- standard error of the mean.

Figure 18. Confluent 3T6 cells 72hrs post treatment with 10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell HSV1716/CMV-NTR/GFP with 50µM CB1954 (B). The extent of cell death is significantly more pronounced in HSV1716/CMV-NTR/GFP infected cells overlaid with media containing 50µM CB1954 than in HSV1716/CMV-NTR/GFP infected cells overlaid with normal media. The extent of cell death following infection of these cells with 10pfu/cell HSV1716, with or without CB1954, is comparable to that seen in A (data not shown). 50µM CB1954 alone has no effect on these cells.

Figure 19. Confluent C8161 cells 72hrs post treatment with 10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell HSV1716/CMV-NTR/GFP with 50µM CB1954 (B). The extent of cell death is significantly more pronounced in HSV1716/CMV-NTR/GFP infected cells overlaid with media containing 50µM CB1954 than in HSV1716/CMV-NTR/GFP infected cells overlaid with normal media. The extent of cell death following infection of these cells with 10pfu/cell HSV1716, with or without CB1954, is comparable to that seen in A (data not shown). 50µM CB1954 alone has no effect on these cells.

Figure 20. Weight change (as a guide to health) in athymic nude mice with subcutaneous A2780 (xenograft) tumours injected intratumourally with HSV 1790. Group size = 3 mice per dose. A2780 xenografts at date of intratumoural injection (Day 0) are between 0.5 - 1mm in diameter. The xenografts have reached this size 12 days after injection with 10 million A2780 cells subcutaneously on the flank of female athymic nude mice.

Figure 21. Change in tumour volume over time in athymic nude mice with A2780 xenografts after intratumoural injection of HSV 1790.

Figure 22. Starting tumour sizes of mice.

Figure 23. Alterations in weight after treatment with CMV-ntr, CB1954 or a combination of both.

Figure 24. Change in tumour volume after treatment with CMV-ntr, CB1954 or a combination of both.

Figure 25. Starting tumour volume of each treatment group (see Table 2).

Figure 26. Weight (as a measurement of health) in athymic nude mice with A2780 xenograft treated with either HSV 1790, HSV 1716, CB 1954 or a combination of them.

Figure 27. Change in tumour volume of xenografts treated with the prodrug CB1954.

Figure 28. Changes in tumour volume in xenograft treated with 10^5 PFU HSV 1790 and CB1954.

Figure 29. Changes in tumour volume in xenografts treated with 10^6 PFU HSV 1790 and CB1954.

Figure 30. Changes in tumour volume in xenografts treated with 10^5 PFU HSV 1716 and CB1954.

Figure 31. Comparison of 10^5 PFU, 10^6 PFU HSV 1790 and 10^5 PFU HSV 1716.

Figure 32. Sequence information for E.coli NTR. (A) Amino acid sequence of NTR polypeptide (SEQ ID No.1); (B) polynucleotide sequence for NTR gene (SEQ ID No.2).

Figure 33. Structure of two NTR prodrugs. (A) CB1954; (B) SN23862.
Vectors Useful for Generation of Herpes Simplex virus Mutants

[0121] Mutant herpes simplex viruses of the invention may be generated by use of nucleic acid vectors. One such vector useful for generation of mutant herpes simplex viruses according to the present invention is a nucleic acid vector comprising, consisting or consisting essentially of:

- first and second nucleotide sequences corresponding to nucleotide sequences flanking an insertion site in the genome of a selected herpes simplex virus; and
- a cassette located between said first and second nucleotide sequences comprising nucleic acid encoding:
  - a) one or a plurality of insertion sites; and
  - b) a ribosome binding site; and
  - c) a marker.

[0123] Another vector useful for generation of mutant herpes simplex viruses according to the present invention is a nucleic acid vector comprising, consisting or consisting essentially of:

- first and second nucleotide sequences corresponding to nucleotide sequences flanking an insertion site in the genome of a selected herpes simplex virus; and
- a cassette located between said first and second nucleotide sequences comprising nucleic acid encoding:
  - a) one or a plurality of insertion sites; and
  - b) a first regulatory nucleotide sequence; and
  - c) a marker.

[0124] The first and second nucleotide sequences may correspond to nucleotide sequences flanking an insertion site formed in, or comprising all or a part of, the ICP34.5 protein coding sequence of the genome of a selected herpes simplex virus.

[0125] The cassette may comprise a plurality of insertion sites, each insertion site preferably formed by nucleic acid encoding a specific restriction endonuclease site (‘restriction site’). Together the restriction sites may form a multiple cloning site (MCS) comprising a series of overlapping or distinct restriction sites, preferably a series of distinct restriction sites comprising one or more of the ClaI, BglII, NruI, XhoI restriction sites.

[0126] The encoded components of the cassette may be arranged in a predetermined order. In one arrangement, the one or plurality of insertion sites is/are arranged upstream (i.e. 5’) of the ribosome binding site/first regulatory sequence and the ribosome binding site/first regulatory sequence is arranged upstream (i.e. 5’) of the marker.

[0127] The first and second nucleotide sequences may comprise nucleotide sequences having identity to regions of the genome surrounding the insertion site in the selected herpes simplex virus (the ‘viral insertion site’). These sequences enable the cassette to be incorporated at the viral insertion site by homologous recombination between the first and second nucleotide sequences and their respective corresponding sequences in the viral genome.

[0128] Thus the first and second nucleotide sequences are flanking sequences for homologous recombination with corresponding sequences of a selected viral genome, such homologous recombination resulting in insertion of the cassette at the viral insertion site.

[0129] The first and second nucleotide sequences may correspond to nucleotide sequences flanking an insertion site in the RL1 locus of the HSV genome, more preferably in the ICP34.5 protein coding sequence of the HSV genome.

[0130] The first and second nucleotide sequences may each be at least 50bp in length, more preferably at least 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000bp in length. Each of the first and second nucleotide sequences may have at least 50% sequence identity to their corresponding sequence in the viral genome, more preferably at least 60%, 70%, 75%, 80%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% 99% or 100% identity. Identity of sequences is determined across the entire length of a given nucleotide sequence. Where sequences are of different length, sequence identity of the shorter sequence is determined over the entire length of the longer sequence.
The first and second nucleotide sequences may be characterised by the ability of one strand of a given sequence to hybridise with the corresponding single-stranded complement of the HSV genome under varying hybridisation stringency conditions. Suitably, the first and second nucleotide sequences will hybridise with their corresponding complement under very low, low or intermediate stringency conditions, more preferably at high or very high stringency conditions.

The viral insertion site is the position between the genomic nucleotide sequences corresponding to the first and second nucleotide sequences of the vector (the ‘genomic’ and ‘vector flanking sequences’ respectively) at which homologous recombination will occur and may be predetermined by selection of the vector flanking sequences. Where the genomic flanking sequences are immediately adjacent, the insertion site is the position between the peripheral and immediately adjacent bases of the two genomic flanking sequences, such that insertion of the cassette separates the genomic flanking sequences. Where the genomic flanking sequences are separated by one or a plurality of bases in the viral genome, the insertion site is formed by said one or a plurality of bases which are excised from the genome by the homologous recombination event.

The position of the viral insertion site may be accurately selected by careful selection and construction of the vector flanking sequences. Accordingly, the vector may be constructed such that homologous insertion of the cassette results in disruption of a chosen protein coding sequence and inactivation of the respective gene product or such that the cassette is inserted at a non-protein coding region of the viral genome. The complete genome sequences of several herpes simplex virus strains have been reported and are publicly available. The complete genome sequence for HSV-1 strain 17syn+ was reported by Dolan et al. (incorporated herein by reference) and the complete genome sequence of HSV-2 strain HG52 was reported by Dolan et al (incorporated herein by reference) and is available from the EMBL database under accession code Z86099. Using this information, the vector of the present invention may preferably be designed for use in generating mutant HSV-1 (e.g. in strain 17 or F) or mutant HSV-2 (e.g. in strain HG52).

The first and second nucleotide sequences (vector flanking sequences) may each comprise sequence corresponding to the RL terminal repeat region of the genome of the selected HSV (e.g. HSV-1 strains 17 or F or HSV-2 strain HG52). The vector flanking sequences may comprise, consist or consist essentially of nucleotide sequences of the RL repeat region which flank the ICP34.5 protein coding sequence. In flanking the ICP34.5 coding sequence, one or both of the selected sequences may, in the corresponding HSV genome, overlap, i.e. extend into, the ICP34.5 protein coding sequence or one or both sequences may be selected so as to not overlap the ICP34.5 protein coding sequence. In a similar manner, the selected sequences may be chosen to overlap completely or partially other important encoded signals, e.g. transcription initiation site, polyadenylation site, defined promoters or enhancers. In this preferred arrangement the insertion site will thus comprise all or a part of the ICP34.5 protein coding sequence and/or be such that the inserted cassette disrupts the ICP34.5 protein coding sequence.

The vectors described, comprising first and second nucleotide sequences corresponding to regions of the RL repeat region flanking and/or overlapping the ICP34.5 protein coding sequence, may be used in the generation of ICP34.5 null mutants wherein all or a portion of the ICP34.5 protein coding sequence is excised and replaced during the homologous recombination event such that both copies of the ICP34.5 coding sequence are disrupted. The recombination may result in an insertion of nucleic acid within the ICP34.5 protein coding sequence thereby disrupting that sequence. In that case, successfully transformed virus are thus mutants incapable of generating the ICP34.5 active gene product from at least one copy, and preferably from both copies, of the ICP34.5 gene.

Successfully transformed virus are thus mutants incapable of generating the ICP34.5 active gene product.

Each component of the cassette may be positioned substantially adjacent the neighbouring component such that a single bicistronic transcript comprising or consisting essentially of the mRNA encoding the nucleotide sequence of interest, ribosome binding site and marker is obtainable.

The vectors described may further comprise, consist, or consist essentially of a nucleic acid encoding a selectable marker such as a polypeptide or protein conferring antibiotic resistance e.g. kanamycin resistance or ampicillin resistance.

The vectors described are preferably DNA vectors, particularly dsDNA vectors. The vector may be provided as a linear or circular (plasmid) DNA vector. The vector preferably contains nucleotide sequences, e.g. restriction endonuclease site(s), permitting transition between the two forms by use of DNA ligation and restriction materials (e.g. enzymes) and techniques known to the person skilled in the art. To achieve homologous recombination with a selected HSV, the vector is preferably provided in linear form.

One such vector provided by the inventors is plasmid RL1.dIRES-GFP deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the ‘Budapest Treaty’).

RL1.dIRES-GFP provides a platform for generating a plurality of ‘shuttle vectors’ which can exploit the process of homologous recombination to transfer a nucleotide sequence of interest (downstream of a selected promoter) into
the disabling RL1 locus of HSV-1, generating easily identifiable, oncolytic, ICP34.5 null HSV-1 mutants expressing the products of the nucleotide sequence of interest, e.g., an RNA transcript or a polypeptide, and GFP. RL1.dIRES-GFP thus provides for ease of generation and purification of ICP34.5 null HSV.

[0142] RL1.dIRES-GFP is a useful vector for making second-generation oncolytic viruses having enhanced cytotoxic potential and which may express the product(s) of selected gene(s) to enhance the oncolytic and/or therapeutic effect of the administered virus.

[0143] The RL1.dIRES-GFP plasmid incorporates a multi-cloning sequence (MCS), upstream of the internal ribosome entry site (IRES), the GFP gene, and SV40 polyadenylation sequences flanked by HSV-1 RL1 sequences. Incorporation of the encephalomyocarditis virus IRES (EMCV IRES) permits translation of two open reading frames from a single transcribed mRNA.

[0144] Following generation of a specific shuttle vector by cloning of the nucleotide sequence of interest (and the selected promoter) into RL1.dIRES-GFP, recombinant HSV-1, expressing the desired nucleic acid transcript or protein, can be generated and purified within 2 weeks. This compares with 2-3 months using prior art protocols.

[0145] In the ICP34.5 null HSV generated using the RL1.dIRES-GFP plasmid provided by the inventors transcription of both the nucleotide sequence of interest and GFP as a single transcript is controlled by the same promoter upstream of the nucleotide sequence of interest, the transcribed IRES directing cap-independent translation of GFP. The generated ICP34.5 null HSV are non-neurovirulent. By modifying the RL1.dIRES-GFP plasmid to incorporate appropriate flanking sequences surrounding the cassette other gene-specific HSV null mutants expressing GFP can be generated.

[0146] RL1.dIRES-GFP is promoterless, thus enabling a promoter of choice to be incorporated in the homologously recombined shuttle vector for controlling expression of the nucleotide sequence of interest from the inserted cassette.

[0147] Plasmid RL1.dIRES-GFP or modified plasmid shuttle vectors thereof further comprising nucleotide sequence encoding a nucleic acid transcript or polypeptide of interest may be provided in isolated or purified form.

[0148] The vector may be a variant of plasmid RL1.dIRES-GFP.

[0149] As the plasmid RL1.dIRES-GFP is designed for tandem expression of a sequence of interest and the marker gene encoding green fluorescent protein (GFP). The sequence of interest is cloned into RL1.dIRES-GFP along with its promoter (e.g., CMV) such that the promoter drives transcription of an mRNA for the sequence of interest along with the IRES-GFP. Translation results in expression of the GFP from the internal ribosomal entry site and the gene of interest and promoter must be cloned into RL1.dIRES-GFP in the correct orientation to achieve this. There are a number of instances where this tandem expression arrangement may be unsuitable and a variation of the cassette design is favourable.

[0150] One example is the expression of siRNAs as short hairpin RNAs using RNA polIII promoters such as H1 or U6. These promoters are unable to drive the additional tandem expression of the IRES-GFP as the RNApolIII expression cassette is designed only to produce short transcripts. Additionally, sequences of interest derived from genomic DNA with strong mRNA shut-off signals in their 3' untranslated regions may not support IRES-GFP expression.

[0151] Thus in some cases a cassette may be provided in which the sequence of interest and marker are expressed separately from independent promoters.

[0152] One variant contains a cassette in which the ribosome binding site of plasmid RL1.dIRES-GFP is replaced with a regulatory nucleotide sequence, preferably a strong, constitutive promoter such as the Phosphoglycerokinase promoter. The marker is thereby expressed under the control of this (the 'first') regulatory sequence, The nucleotide sequence of interest (e.g., NTR, an antisense or siRNA) is expressed under the control of a second regulatory sequence upstream (5') of the nucleotide sequence of interest, e.g., the CMV promoter. This vector variant is particularly suitable for expression of siRNA where a weak promoter may be used for expression of the siRNA molecule or where the nucleic acid encoding the NTR may have a strong termination signal making it difficult to transcribe or translate a single bi- or poly-cistronic transcript encoding the NTR and marker sequence. In this arrangement the transformed virus containing the cassette integrated in the viral genome produces two separate transcripts under the control of the first and second promoters.

[0153] One such cassette was constructed in the following manner. The 1.3kbp blunt-end EcoRI/AflII fragment that contains the PGK promoter/GFP gene was obtained by restriction digestion followed by Klenow treatment from the vector pSNRG and cloned into the RL1-del vector cut with the restriction enzyme NruI that generates blunt ends. Successful insertion of the PGK/GFP DNA was confirmed by BamH1 digestion and the orientation of the inserted DNA identified using the unique Xhol site in RL1-del and the BsrGI site at the 3' end of PGK/GFP. Plasmids with PGK/GFP in both forward and reverse orientation were obtained and the plasmids were designated RL1-dPGK/GFPPhot and RL1-dPGK/GFPPrev. Expression of GFP was confirmed in BHK cells transfected with the forward and reverse orientation plasmids.

[0154] Thus, sequences of interest along with their own promoters (although it is preferred that the PGK promoter is not also used for this purpose) can then be cloned into either RL1-dPGK/GFPPhot or RL1-dPGK/GFPPrev in either orientation using the remaining unique BglII, Xhol or HpaI unique restriction enzyme sites. The resulting plasmid can be used to derive recombinant HSV in which the marker GFP gene and the gene of interest are expressed independently from their own promoters.
The vectors described may be constructed for use in generating engineered HSV-1 or HSV-2 by insertion of a nucleic acid cassette through a mechanism of homologous recombination between nucleotide sequences flanking the cassette and corresponding sequences in the selected herpes simplex virus genome.

The vectors described may comprise and have use as:

i) gene delivery (gene therapy) vectors for delivery of a selected nucleotide sequence, e.g. NTR, to a specific locus of the HSV genome; and/or

ii) expression vectors for expression of the delivered nucleotide sequence of i) from the HSV genome under the control of a selected regulatory element; and/or

iii) vectors for the generation of HSV gene-specific null mutants wherein the cassette is inserted at a selected genomic location to disrupt the protein coding sequence of a selected HSV gene such that the gene product is inactive in the resultant mutant virus.

The vectors described may be used in the manufacture of engineered gene specific HSV null mutants, i.e. HSV mutants incapable of expressing an active gene product of a selected gene. They may be used in the manufacture of engineered viruses which express a selected protein from only one gene copy the other gene copy being disrupted or modified such that it cannot express a functional gene product. Such vectors may also be used in the manufacture of a medicament, preferably comprising said gene specific HSV null mutant, for use in treating cancer and tumours, preferably by the oncolytic treatment of the tumour.

The vectors described may also be used in the manufacture of engineered HSV mutants wherein the genome of the mutant HSV comprises an exogenous or heterologous gene which may have been inserted in the HSV genome by homologous recombination of the cassette. Preferably, the exogenous/heterologous gene is expressed in the mutant HSV, which expression may be regulated by a regulatory element, e.g. promoter, forming part of the inserted cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

The vectors described may also be used in the manufacture of an engineered HSV mutant wherein the genome of the mutant HSV comprises a nucleotide sequence which has been inserted in a protein coding sequence of theHSV genome by homologous recombination of the cassette such that the mutant HSV is incapable of expressing the active gene encoded by said nucleotide sequence and wherein the inserted nucleotide sequence is expressed under the control of a regulatory element to produce a desired transcript. Preferably, the regulatory element forms part of the cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

The vectors described may be used to generate mutant HSV by inserting the cassette into the genome of a selected HSV, the method of generation may comprise providing a vector described above, where the vector is a plasmid, linearising the vector; and co-transfecting a cell culture with the linearised vector and genomic DNA from said HSV.

The co-transfection may be carried out under conditions effective for homologous recombination of said cassette into an insertion site of the viral genome.

The method may further comprise one or more of the steps of:

1) screening said co-transfected cell culture to detect mutant HSV expressing said marker; and/or
2) isolating said mutant HSV; and/or
3) screening said mutant HSV for expression of the nucleotide sequence of interest or the RNA or polypeptide thereby encoded; and/or
4) screening said mutant HSV for lack of an active gene product; and/or
5) testing the oncolytic ability of said mutant HSV to kill tumour cells in vitro.
Example 1

Construction of plasmid RL1.dIRES-GFP

General Approach

[0164] Plasmid RL1.dIRES-GFP was generated in three stages, illustrated in Figure 1.

1. The DNA sequences containing the CMV IE promoter (pCMV), the NAT gene, the internal ribosome entry site (IRES), the GFP reporter gene and the SV40 polyadenylation sequences were excised from pNAT-IRES-GFP using NsiI and SspI and purified.

2. The purified pCMV-NAT-IRES-GFP-PolyA DNA fragment was cloned into RL1.del to form a new plasmid designated RL1.dCMV-NAT-GFP.

3. The pCMV-NAT DNA sequences of RL1.dCMV-NAT-GFP were excised using XhoI and the remainder of the plasmid religated to form a novel plasmid designated RL1.dIRES-GFP. This novel plasmid contained a multi-cloning site (all sites shown are unique) upstream of an IRES, the GFP gene and the SV40 polyA sequences all within the HSV-1 RL1 flanking sequences. Recombinant ICP34.5 null HSV-1, expressing a gene of interest in the RL1 locus, can be generated by cloning the gene of interest (downstream of a suitable promoter) into the multi-cloning site and co-transfecting BHK cells with the plasmid and HSV-1 DNA. Recombinant virus expressing the target gene can be identified using GFP fluorescence.

[0165] Removal of the CMV promoter and noradrenaline transporter gene (pCMV-NAT) from RL1.dCMV-NAT-GFP, followed by religation of the remainder of the plasmid, resulted in a novel plasmid (RL1.dIRES-GFP) containing a multi-cloning site (MCS), upstream of the encephalomyocarditis virus internal ribosome entry site (EMCV IRES), the GFP reporter gene and the SV40 PolyA sequences, all within RL1 flanking sequences. This novel arrangement of DNA sequences or 'smart cassette' allows ICP34.5 null HSV-1, expressing a gene of interest in the RL1 locus, to be easily generated by simply inserting the desired transgene (downstream of a suitable promoter) into the MCS and co-transfecting BHK cells with the plasmid and HSV-1 DNA. The IRES situated between the GFP gene and the MCS permits expression of two genes from the same promoter and so recombinant virus expressing the gene of interest also expresses GFP and can therefore be easily identified under a fluorescence microscope and purified.

Materials and Methods

[0166] 1 µg of RL1.del was digested with 10 units HpaI (Promega) in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega) at 37°C for 16hrs. The digested plasmid was then purified using the Qiaquick PCR purification kit (Qiagen), treated with 10 units of Calf Intestinal Phosphatase (Promega), in a suitable volume of 10x CIP buffer and nuclease free water for 4hrs at 37°C, before being purified again using a Qiaquick PCR purification kit (Qiagen). The eluted DNA was electrophoresed on a 1% agarose gel to check its concentration (Figure 2).

[0167] 4 x 1 µg of pNAT-IRES-GFP was digested with 10 units of NsiI and 10 units of SspI in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega) at 37°C for 16hrs. The reaction mixture was electrophoresed in a 1% agarose gel for 1hr at 110 volts. The 5.4kbp DNA fragment consisting of the CMV IE promoter (pCMV), upstream of the noradrenaline transporter gene (NAT), the encephalomyocarditis virus internal ribosome entry site (IRES), the gene for green fluorescent protein (GFP) and the SV40 polyadenylation sequences (SV40 Poly A), was excised using a sterile scalpel and the DNA purified from the gel using a QiaQuick Gel Extraction kit (Qiagen). The eluted DNA was blunt ended using 3 units Klenow Polymerase (Promega) in accordance with the manufacturers instructions and the DNA purified using a QiaQuick PCR purification kit (Qiagen). 5 µl of the purified DNA fragment was electrophoresed on a 1% agarose gel to check its concentration (Figure 3).

[0168] Ligation reactions were carried out in small eppendorf tubes containing 5 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega), nuclease free water (Promega) and various volumes of the Hpal digested/CIP treated RL1.del and blunt ended pCMV-NAT-IRES-GFP-SV40 Poly A DNA, at 16°C overnight. Competent JM109 bacterial cells (Promega) were then transformed with various aliquots of the ligation reactions. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened for inserts using Af11 (New England Biolabs) restriction enzyme analysis. Plasmid DNA containing the insert would produce two fragments of 4.8kbp and 9.2kbp following digestion with Af11. Two clones (clone 5 and 8) contained the insert (Figure 4). The orientation of the insert in clone 5 (RL1.dCMV-NAT-GFP) was determined using XhoI restriction enzyme analysis (Figure 5).
To generate RL1.dIRES-GFP from clone 5, the CMV-NAT portion of the CMV-NAT-IRES-GFP-SV40 PolyA insert was removed by digesting 4 x 500ng of clone 5 with 10 units of XhoI in a suitable volume of buffer and water (Promega), overnight at 37°C. The digested DNA was electrophoresed on a 1% agarose gel at 110 volts for 1hr (Figure 6A). The 10.2Kbp fragment consisting of the IRES, the GFP gene, the SV40 PolyA sequences and RL1 flanking sequences in a pGEM32f(-) (Promega) backbone, was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit.

Ligation reactions were performed in small eppendorf tubes containing 100ng - 500ng purified DNA, 3 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega) and nuclease free water (Promega) overnight at 16°C. Competent JM109 bacterial cells (Promega) were then transformed with various aliquots of the ligation reactions***. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened using XhoI (Promega) restriction enzyme analysis. Colonies containing plasmid DNA from which CMV-NAT had been removed would produce one fragment of 10.2Kbp when digested with XhoI. Several positive clones were found, one was isolated, and a large-scale plasmid preparation undertaken using Promega’s Wizard Plus Maxiprep kit. The large-scale plasmid preparation was checked by digesting with XhoI (Figure 6B). This plasmid DNA was subsequently named ‘RL1.dIRES-GFP’.

Plasmid RL1.dIRES-GFP has been deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 OJG, United Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty.

**RL1.del was provided by Dr.E.McKie and is the pGEM-3Zf(-) plasmid (Promega) into which has been cloned an HSV-1 fragment (123459-129403) consisting of the RL1 gene and its flanking sequences. The 477bp PflMI-BstEII fragment of the RL1 gene (125292-125769) has been removed and replaced with a multi-cloning site (MCS) to form RL1.del.

pNAT-IRES-GFP

** pNAT-IRES-GFP was supplied by Dr. Marie Boyd (CRUK Beatson Laboratories) and is the pRES2-EGFP plasmid (BD Biosciences Clontech) into which she has cloned the bovine noradrenaline transporter (NAT) gene (3.2Kbp), at the Nhel and XhoI sites.

*** Transformation of Bacterial Cells

10μl of a glycerol E.coli stock was added to 10ml 2YT medium in a 20ml griener tube. This was placed in a 37°C shaking incubator for 16-24hrs until a saturated culture was obtained. 1ml of this culture was then added to 100ml of 2YT in a 500ml sterile glass bottle and placed in the 37°C shaking incubator for 3hrs. The bacterial cells were pelleted by centrifugation at 2,000rpm for 10 minutes (Beckman). The cells were then resuspended in 1/10th of 2YT in a 500ml sterile glass bottle and placed in the 37°C shaking incubator for 16-24hrs until a saturated culture was obtained. 1ml of this culture was then added to 100ml of L-Broth was added, the tube inverted 2-3 times, and the bacteria incubated for 1hr at 37°C. 10μl of DNA was mixed with 100μl of competent bacteria in eppendorf tubes, and the tubes placed on ice for 30 minutes. After this, the samples were 'heat shocked' by incubating the tubes in a 42°C water bath for exactly 45 seconds before placing them on ice for a further 2 minutes. 1ml of L-Broth was added, the tube inverted 2-3 times, and the bacteria incubated for 1hr at 37°C. 100μl of the transformed bacteria was plated out onto L-broth agar plates containing 100μg/ml of the appropriate antibiotic (usually ampicillin or kanamycin). Plates were allowed to dry at room temperature, before incubating in an inverted position at 37°C overnight.

Example 2

Generation of ICP34.5 null HSV-1 expressing a gene product of interest and GFP using plasmid RL1.dIRES-GFP.

General Approach

Generation of ICP34.5 null HSV-1 expressing a gene product of interest requires insertion of nucleotide sequence encoding the gene product (polypeptide) of interest, and often a desired promoter, at the MCS of RL1.dIRES.GFP followed by co-transfection of BHK cells with the linearised plasmid, containing the gene of interest, and HSV DNA.
Following homologous recombination viral plaques expressing GFP are identified. Figure 7 illustrates the method steps involved.

[0177] Referring to Figure 7A plasmid DNA, containing the gene of interest and the desired promoter (X), is digested with restriction endonucleases to release the promoter/gene fragment.

[0178] The promoter/gene fragment is purified and cloned into the multi-cloning site (MCS) of RL1.dIRES.GFP forming a shuttle vector suitable for generating oncolytic HSV-1 (Figure 7B). This vector contains HSV-1 sequences that flank the essential RL1 gene but does not contain the RL1 gene. The plasmid also contains the gene for Green Fluorescent Protein (GFP) downstream of an internal ribosome entry site (IRES). The IRES permits expression of both the gene of interest and the GFP gene from the same upstream promoter.

[0179] BHK cells are then co-transfected with linearised RL1.dIRES.GFP, now containing the gene of interest, and HSV-1 DNA (Figure 7C). Following homologous recombination, designer virus, expressing the gene of interest and GFP, is generated and can be distinguished from wild type virus (also generated but not expressing GFP) under a fluorescence microscope.

[0180] Viral plaques, expressing GFP (and hence the gene of interest), are picked under the fluorescence microscope and purified until all wild-type HSV-1 has been removed. The recombinant HSV-1 is considered 100% pure when all the viral plaques are expressing GFP (Figure 7D).

[0181] Once the recombinant virus is completely pure, an isolated plaque is picked and a highly concentrated stock is grown and titrated (Figure 7E). Oncolytic HSV-1, expressing a gene product of interest from a selected promoter, is then ready for characterisation and in vitro examination of its tumour killing potential.

Materials and Methods

[0182] To generate recombinant ICP34.5 null HSV-1 expressing a gene of interest and GFP, requires the gene of interest, and often a suitable promoter, to be cloned into the MCS of RL1.dIRES-GFP in the forward orientation with respect to the GFP gene in this plasmid. Once this has been achieved the plasmid is linearised (i.e. digested with a restriction enzyme that cuts only once, usually SspI or Scal) in an irrelevant region. 80% confluent BHK cells in 60 mm petri dishes are then co-transfected with HSV-1 DNA and linearised plasmid DNA as described below.

[0183] To generate replication restricted HSV-1, expressing the gene of interest and GFP, the gene of interest must be cloned into RL1.dIRES-GFP downstream of a suitable promoter (e.g. CMV IE). The promoter is required upstream of the gene of interest for the production of a bicistronic mRNA transcript. The IRES sequence between the two open reading frames in the transcript functions as a ribosome binding site for efficient cap-independent internal initiation of translation. The design enables coupled transcription of both the gene of interest and GFP, followed by cap-dependent initiation of translation of the first gene (gene of interest) and IRES-directed, cap-independent translation of GFP. Coordinate gene expression is thus ensured in this configuration.

Co-Transfection of Virus and Plasmid DNA by CaPO₄ and DMSO Boost

[0184] HSV-1 (17+) DNA and 0.1-1µg linearized SMART cassette containing the gene and promoter of interest is pipetted into 1.5ml eppendorf tubes containing 1µl of calf thymus DNA (10µg/ml) and an appropriate volume of distilled water to give a final volume of 165µl. The solutions are very gently mixed using a 200µl pipette tip. 388µl of HEBS, pH 7.5, (130mM NaCl, 4.9mM KCl, 1.6mM Na₂HPO₄ 5.5mM D-glucose, 21mM HEPES) is then added, the solution mixed, before adding 26.5µl of 2M CaCl₂ dropwise and flicking the eppendorf tube two or three times. The samples are left at room temperature for 10-15 minutes then added dropwise to 80% confluent BHK's in 60mm petri dishes from which the medium has been removed. Following incubation at 37°C for 45 minutes, the cells are overlaid with 5ml of ETC10 and incubated at 37°C. Three to four hours later, the media is removed and the plates washed with ETC10. For exactly 4 minutes, the cells are overlaid with 1ml 25% (v/v) DMSO in HEBS at room temperature. After the 4 minutes, the cells are immediately washed three times with 5ml ETC10 before overlaying with 5ml of ETC10 and returning to the incubator. The following day, fresh medium is added to the cells. Two days later, when cpe is evident, cells are scraped into the medium, transferred to small bijoux and sonicated thoroughly. The sample is then stored at -70°C until required (see section below on plaque purification).

[0185] N.B. The volume of virus DNA to add is determined by undertaking the above procedure without plasmid DNA, using a range of virus DNA volumes and choosing the volume that gives the greatest number of viral plaques on the BHK monolayer after 2 or 3 days.

Plaque Purification

[0186] Sonicated samples from co-transfection plates are thawed and serially diluted 10 fold in ETC10. 100µl from neat to the 10⁵ dilution is plated out on confluent BHK's in 60 mm petri dishes from which the media has been removed.
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After 45 minutes incubation at 37°C, the cells are overlaid with 5ml EMC10 and incubated at 37°C for 48hrs. The plates are then checked for the presence of viral plaques and those dishes with the fewest, most separated plaques are placed under a fluorescent stereomicroscope. Recombinant virus, designed to express the green fluorescent protein (GFP) in addition to the gene of interest, can clearly be distinguished from wild type virus using a GFP filter. Fluorescent plaques are picked using a 20μl pipette and placed (including the tip) into an eppendorf tube containing 1ml ETC10. The sample is thoroughly sonicated before making serial 10 fold dilutions in ETC10 and repeating the above purification procedure. The process is repeated typically 3-4 times until every plaque on the BHK monolayer is fluorescent. Once this has been achieved, 50μl of this sample is used to infect BHK cells in roller bottles, in 50ml ETC10, and a virus stock grown.

Tissue Culture Media

[0187] BHK21/C13 cells are grown in Eagle’s medium (Gibco) supplemented with 10% newborn calf serum (Gibco) and 10% (v/v) tryptose phosphate broth. This is referred to as ETC10. For virus titrations and plaque purification, EMC10 (Eagles medium containing 1.5% methylcellulose and 10% newborn calf serum) is used to overlay the cells.

Example 3

Construction of HSV1716/CMV-NTR/GFP

General Approach

[0188] HSV1716/CMV-NTR/GFP was generated by cloning a 1.6Kbp BamHI fragment from pPS94910, consisting of the E.coli nitroreductase (NTR) gene downstream of the CMV IE promoter (pCMV), into the MCS of the RL1.dIRES-GFP smart cassette, in the forward orientation with respect to the GFP gene in RL1.dIRES-GFP (Figure 8). The resultant plasmid, named RL1.dCMV-NTR-GFP, was then linearised and recombinant virus generated and purified as described above. The plasmid pPS949 (referred to as ‘pXNLC-ntr’ in Ref 10) containing the NTR gene downstream of the CMV IE promoter (pCMV-NTR) in a pLNCX (Clontech) backbone, was a kind gift from Professor Lawrence Young, University of Birmingham, UK.

Materials and Methods

[0189] 4 x 1μg of pPS949 was digested with 10 units of BamHI (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. The reaction mixture was electrophoresed in a 1% agarose gel for 1hr at 110 volts. The 1.6Kbp DNA fragment consisting of the CMV promoter upstream of the NTR gene (pCMV-NTR), was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit (Qiagen). 5μl of the purified DNA fragment was electrophoresed on a 1% agarose gel to check its concentration (Figure 9).

[0190] 2μg of the RL1.dIRES-GFP smart cassette was then digested with 15 units of BgIII (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. The digested plasmid was then purified using the QIAquick PCR purification kit (Qiagen), treated with 10 units of Calf Intestinal Phosphatase (Promega), in a suitable volume of 10x CIP buffer and nuclease free water for 4hrs at 37°C, before being purified again using the QIAquick PCR purification kit. 5μl of the purified DNA was electrophoresed on a 1% agarose gel to check its concentration (Figure 10).

[0191] Ligation reactions were carried out in small eppendorf tubes containing 5 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega), nuclease free water (Promega) and various volumes of the BgIII digested/CIP treated RL1.dIRES-GFP smart cassette and pCMV-NTR (BamHI ends), at 16°C overnight. Competent JM109 bacterial cells (Promega) were then transformed with various aliquots of the ligation reactions. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened for inserts using BgIII/XhoI (Promega) restriction enzyme analysis. RL1.dIRES-GFP plasmid DNA containing the pCMV-NTR insert in the correct orientation would produce two fragments of 11.5Kbp and 300bp following digestion with BgIII and XhoI. One clone (clone 4) was found to contain the insert in the correct orientation (Figure 11). This plasmid was named ‘RL1.dCMV-NTR-GFP’.

[0192] 0.1-1μg of RL1.dCMV-NTR-GFP was linearized by digesting with 10 units of Scal (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. A sample (5μl) of the digested DNA was electrophoresed on a 1% agarose gel for 1hr at 110 volts to check that it had been linearized. 80% confluent BHK cells were then co-transfected with a suitable volume of the remaining linearised DNA and HSV-1 DNA. Recombinant HSV-1, expressing GFP (and hence NTR), was identified and purified using a fluorescent microscope and a virus stock, named HSV1716/CMV-NTR/GFP, was grown and titrated on BHK cells (Figure 12).

[0193] HSV1716/CMV-NTR/GFP has been deposited in the name of Crusade Laboratories Limited having an address
HSV1716/CMV-NTR/GFP Cell Killing

[0194] HSV1716/CMV-NTR/GFP replicates with almost identical' kinetics to HSV1716 in BHK cells and 3T6 cells. BHK cells support the replication of ICP34.5 null HSV while confluent 3T6 cells do not. Figure 13 shows that HSV1716/CMV-NTR/GFP will replicate as well as HSV1716 in permissive cell lines and that the introduction of exogenous genes, e.g. NTR and GFP, has not reduced the oncolytic potential of the ICP34.5 null HSV. The fact that HSV1716/CMV-NTR/GFP fails to replicate in 3T6 cells also indicates that this recombinant HSV is an ICP34.5 null mutant.

[0195] Figure 14 is a Western blot demonstrating that no ICP34.5 polypeptide is expressed from HSV1716/CMV-NTR/GFP, and that the virus is thus useful as a gene therapy vector.

[0196] Figure 15 is another Western blot demonstrating expression of NTR in a variety of cell lines infected with HSV1716/CMV-NTR/GFP, including a human malignant melanoma cell line (C8161) and confluent 3T6 cells in which ICP34.5 null HSV does not replicate. Expression of NTR in confluent 3T6 cells, following infection with HSV1716/CMV-NTR/GFP, is encouraging as it demonstrates that replication of this ICP34.5 null mutant is not required for expression of the produg-activating gene (i.e. NTR). Some tumour cells in vivo will not support the replication of ICP34.5 null HSV and as such, will not be killed with HSV1716.

[0197] Figure 16 shows the results from a cytotoxicity assay performed in confluent 3T6 cells. Infecting confluent 3T6 cells with an ICP34.5 null mutant (HSV1716/CMV-NTR/GFP), at a multiplicity of infection (MOI) of 1 plaque forming units (pfu)/cell, does not result in any significant cell death, neither does separate incubation of the cells with 50μM CB1954. However, significant cell death is evident 72hrs post infection with 1pfu/cell HSV1716/CMV-NTR/GFP when 50μM CB1954 is included in the growth medium. This clearly demonstrates that when there is no replication of the virus, substantial cell death is still possible from virus directed enzyme prodrug therapy (VDEPT).

[0198] Infecting confluent 3T6 cells with an ICP34.5 null mutant at a MOI of 10pfu/cell will result in cell death, by a mechanism known as 'viral antigen overload'. However, the level of cell killing is even more pronounced (approximately 20% more), when 50μM CB1954 is included in the growth medium.

[0199] A similar cytotoxicity assay was performed in human C8161 melanoma cells, the results are set out in Figure 17. Unlike confluent 3T6 cells, C8161 cells do support the replication of ICP34.5 null HSV. Therefore, cell death will occur following infection of the cells with ICP34.5 null HSV, at 1pfu/cell. However, when CB1954 is included in the overlay of HSV1716/CMV-NTR/GFP infected cells, the cells are killed more efficiently and more quickly. No enhanced cell killing is evident when CB1954 is included in the overlay of cells infected with HSV1716-GFP. These results demonstrate that enhanced cell killing is possible in human tumour cells.

[0200] Cell culture images for the cytotoxicity assays performed in confluent 3T6 and human C8161 melanoma cells are shown in Figures 18 and 19.

Example 4 - In vivo evaluation of the anti-tumour activity of a selectively replication competent herpes simplex virus in combination with enzyme pro-drug therapy.

[0201] The anti-tumour activity of a selectively replication competent herpes simplex virus in combination with an enzyme prodrug therapy approach in appropriate animal models in vivo was investigated.

[0202] The parental virus, HSV 1716 is a selectively replication competent mutant of the herpes simplex virus 1 (HSV 1) which lacks both copies of the RL1 gene that encodes the protein ICP 34.5. This protein is a specific determinant of virulence. The function of this protein has been described at length elsewhere. The virus can grow only in cells that have a high level of functional PCNA. High levels of PCNA are found only in cells that are dividing such as tumour cells and not normal differentiated cells.

[0203] It has already been shown that HSV 1716 can achieve selective tumour cell killing with minimal toxicity and improved survival times in a number of animal models. Initial phase 1 clinical trials using HSV1716 virus in patients has also meet with some success.

[0204] Although HSV1716 selectivity replicates in tumour reducing the tumour bulk by cell lysis the inventors did not anticipate HSV1716 to lytically replicate in all cells in the tumour due to the heterogeneity of the cell type and growth state.

[0205] In order to enhance the efficacy of the tumour cell killing - hence kill the entire tumour - the inventors have constructed a derivative of HSV1716 designated HSV1716/CMV-NTR/GFP that expresses the E.Coli nitroreductase gene (ntr) under the control of a CMV early promoter (see example 3 above). In this example and the figures referred to HSV1716/CMV-NTR/GFP is called HSV1790.

[0206] The enzyme ntr converts the inactive prodrug CB1954 to a functional cytotoxic alkylating agent that kills both...
dividing and non-dividing cells by apoptosis. This active drug is diffusible and membrane permeable resulting in an efficient bystander effect, i.e. wherein the activated drug may have an effect on surrounding cells.

[0207] As the prodrug will only be converted to its active form in the tumour which has been infected with ntr expressing virus, toxicity to normal cells is avoided hence improving the therapeutic index following systemic delivery of this compound.

[0208] Initial in vitro experiments using this combination have already shown enhanced cell kill using this virus in combination with CB1954 in a number of cell lines.

[0209] This example further evaluates this combination approach in vivo in appropriate animal models.

Results

Months 1 - 3

[0210] Months 1 - 3 were taken up mainly by in vitro work. During this time period high titre, sterile virus stock was generated for use in the xenograft models.

[0211] Xenograft models were also generated in athymic nude mice using the cell line A2780, a human ovarian epithelial carcinoma line initially derived from a tumour sample from an untreated patient (European Collection of Cell Cultures (ECACC) CAMR, Porton Down, Salisbury, Wiltshire, SP4 OJG, United Kingdom, accession number 93112520).

[0212] Generation of a gliomal xenograft model was attempted using 2 gliomal lines that were available in house, LN-18 and U373MG. There are reports in the literature of both being successfully grown as xenografts in athymic mice.

[0213] However, as shown in the table below the inventors failed to see any xenograft growth up to 28 days after injection with 5 million cells subcutaneously.

### Table 1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No of cells injected per mice</th>
<th>Number of mice</th>
<th>Presence of xenograft 28 days after cell injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN 18</td>
<td>5 million</td>
<td>5</td>
<td>0/5</td>
</tr>
<tr>
<td>U373 MG</td>
<td>5 million</td>
<td>5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

A2780 tumour take

[0214] As reported previously A2780 have a Take Rate of approximately 50 % - that is 50 % of mice that are injected with 5 million cells per flank subcutaneously will develop xenografts. When the number of cells injected was increased to 10 million or more an increase in take rate of approximately 15 - 25 % was seen, giving an overall take rate of 65 - 75 %.

[0215] Thus increasing the number of gliomal cells injected may increase the take rate of these cell lines

Dose response to the HSV 1790 virus

[0216] Before the mice can be treated with a combination of virus and prodrug, first one must carry out experiments to make informed decisions about how much of the virus, and the prodrug to give.

[0217] A dose response experiment will allow one to find both the most appropriate does of the virus to use in the experiments and the maximum tolerated dose (MTD) of the virus, that is, the largest amount of the virus that can be given to a single mouse without adverse side effects. Small groups of tumour bearing mice are given a small dose of virus. Assuming they do not have any adverse effects another group is given a larger dose of virus. This continues until either the mice start to suffer ill effects or we reach a maximum dose.

[0218] The maximum amount of virus that can be intratumourally injected is 100 µl, hence the maximum dose from our current stock is 10^9 PFU per injection.

[0219] Figure 20 shows the weight change in the mice after injection with a variety of doses of virus. Weight is a good indicator of the animals overall health. Any loss of weight signifies that the treatment is not being well tolerated. Where an animal loses more than 20% of its initial body weight it was sacrificed immediately.

[0220] A dose of 10^9 PFU of the HSV 1790 virus is not tolerated by these mice, they rapidly lost body weight and were sacrificed at Day 3 post injection. Doses of 10^8 PFU or less were better tolerated, the mice initially lost weight in the days following injection but quickly recovered to approximately their initial body weights.

[0221] It should be pointed out that as the experiment progresses the animals appear to be increasing in weight. This is almost certainly due to the fact that it is total body weight that is measured, which includes the weight of any tumour that is forming.
Response of the tumour to HSV 1790 treatment

Tumour volume was measured daily after intratumoural injection of the HSV 1790 virus to look for any growth delay or regression of the tumours.

Figure 21 shows the change in tumour volume as measured over a period of 100 days. If the tumour was injected with PBS only as a control the tumour increased in size rapidly and by approximately Day 13 post injection the tumours had become too large and the animals had to be sacrificed.

Treatment with all doses of virus appeared to delay the growth of the tumour to some degree. Doses of $10^5$ PFU increased the longevity of the mice by approximately 12 days while mice injected with $10^6$ PFU virus tumours survived for an extra 23 days compared to the control group before the tumours became prohibitively large. Perhaps surprisingly the group of mice injected with $10^8$ PFU of virus survived only slightly longer than the control group. It is possible there were a large number of non infectious particles or that sheer number of particles caused the cells which the virus would have grown in to be killed.

The group of mice treated with $10^7$ PFU of virus survived the longest and indeed two out of three of the mice did not have any visible signs of tumour when sacrificed at day 100.

Naked DNA Experiments

In order to check the alterations in tumour growth are due to the virus itself and not a result of the CMV-ntr plasmid DNA that had been introduced to the HSV 1716 virus, an experiment was set up looking at the effect of the CMV-ntr plasmid DNA alone and in combination with the prodrug CB1954.

Mice were randomised into treatment groups of 6 animals each when tumour diameters are approximately 5mm (this is Day 0). Figure 22 shows the starting tumour diameters for the mice used in this experiment. Two groups of mice were administered CMV-ntr plasmid by direct intratumoural injection at a dose of 0.2mg DNA per injection. One of these groups was then administered with a single dose of 80mg/kg of CB1954 on Day 2 by infra-peritoneal injection. The third group of mice had a single administration of CB1954 (80mg/kg) by intra-peritoneal injection on Day 2 following intratumoural injection of saline control at Day 0. Animals were weighed daily (Figure 23) and daily caliper measurements performed until the tumour sizes were in the region of 20mm by 20mm. Tumour volumes were estimated from these measurements (volume = d³ x 6) (Figure 24). In addition any toxicity from these administered agents was determined.

On the basis of these experiments the inventors determined that neither the CMV-ntr alone, CB1954 alone or the combination of both CMV-ntr and CB1954, has any anti-tumour activity as determined by tumour regression in this model system (Figure 24).

Scheduling Experiment

Previous dose response experiments have shown that doses of less than $10^8$ PFU virus per mouse do not appear to have any adverse effect of the animals health.

A dose of $10^7$ PFU virus per mouse resulted in a great reduction in tumour growth, indeed after 100 days two out of three of the mice in the group had no visible tumour. This is very encouraging - the virus only at high doses may be enough to delay growth or cause tumour regression.

However to look at the effect of a combination of the virus and the prodrug CB 1954 a lower dose of the virus was studied - if the treatment of the virus alone results in growth delay for such a long period one would be unable to ascertain the addition or synergistic effects of the prodrug.

The inventors proceeded to investigate two doses of the virus in combination with CB1954. The doses selected were $10^5$ PFU and $10^6$ PFU. Both these doses caused some tumour growth delay in earlier experiments.

The prodrug is given as an 80mg/kg intra-peritoneal injection, after dissolving the powdered form in 10% acetone and then making up the volume with peanut oil.

Another factor that determines how well the drug will work - hence how much tumour growth delay or regression is seen - is when the drug is actually given. As the prodrug will only be converted to an active substrate in the presence of NTR it was considered that the virus containing the NTR will have to been given first. It was also considered that if the virus is given time to replicate and produce more NTR then the prodrug may have a more pronounced effect.

To discover the optimal doses of both the virus and the drug and the optimal times of these treatments a scheduling experiment was set up.

Mice were randomized into treatment groups (treatment regimes shown in Table 2) of 3 animals when tumour diameters were approximately 5mm (tumour volume 0.5 - 1.5 mm³). Figure 25 shows the starting tumour volumes of each of the groups.
The treatment groups were administered with a single direct intratumoural injection of the virus and dose determined for that group. The virus was diluted PBS + 10% serum. 'No virus' control groups received an intratumoural injection of 100 μl of PBS + 10% serum. This day was designated as Experimental Day 0.

The intratumoural injections did not appear to have any adverse effects on the mice. Some tumours bleed slightly after injection but not to a great degree. The animals did not lose body weight (Figure 26) and their behaviour did not appear to alter. In all the tumours that bleed slightly, the following day the healing process had begun and within 3 - 5 days there was little evidence of any puncture wound on any tumour.

Injections of CB1954 were given to the appropriate groups at days 2, 10 and 15. A dose of 80mg/kg - the equivalent of approx. 2mg per mouse - was given. The powdered form of the CB1954 drug (from Sigma) was dissolved in acetone to 10% of the final volume (10 μl per 2mg). The volume was then made up to 2mg CB1954 in 100 μl using peanut oil. A syringe was used to mix the drug as peanut oil is thick and viscous. The drug was made up fresh every time.

The appropriate groups were then injected intra-peritoneal with this solution. Control groups which were not receiving drug were injected intra-peritoneal with a 100 μl solution of 10% acetone in peanut oil.

No swelling or irritation at the site of injection was noted on any of the mice either at time of injection or at any later time point. The mice appeared slightly lethargic for a short period after the injection but did not lose any body weight (Figure 26) or show signs of lethargy the following day.

No virus + CB1954 prodrug

Groups 5,6,7,8, 13 & 14 looked at the effect of prodrug alone on tumour growth. Figure 27 shows that there is little effect on tumour growth when CB1954 is given alone.

10^5 PFU virus +/- CB1954 prodrug

10^5 PFU virus was given at Day 0 followed by either prodrug or vehicle at Days 2 and 10.

As can be seen from the graph in Figure 28 tumours treated with either virus only or virus and prodrug did not grow as large as the untreated tumour. The tumour treated with the virus grew only to approximately half the size of the untreated tumours.

### Table 2: Treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Injection Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10^5 HSV 1790 + drug (Day 2) + Drug (Day 10)</td>
</tr>
<tr>
<td>2</td>
<td>10^5 HSV 1790 + drug (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>3</td>
<td>10^5 HSV 1790 + vehicle (Day 2) + drug (Day 10)</td>
</tr>
<tr>
<td>4</td>
<td>10^5 HSV 1790 + vehicle (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>5</td>
<td>No virus + drug (Day 2) + drug (Day 10)</td>
</tr>
<tr>
<td>6</td>
<td>No virus + drug (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>7</td>
<td>No virus + vehicle (Day 2) + drug (Day 10)</td>
</tr>
<tr>
<td>8</td>
<td>No virus + vehicle (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>9</td>
<td>10^6 HSV 1790 + vehicle (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>10</td>
<td>10^6 HSV 1790 + drug (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>11</td>
<td>10^6 HSV 1790 + drug (Day 2) + drug (Day 10)</td>
</tr>
<tr>
<td>12</td>
<td>10^6 HSV 1790 + drug (Day 2) + drug (Day 10) + drug (Day 15)</td>
</tr>
<tr>
<td>13</td>
<td>No virus + drug (Day 2) + drug (Day 10)</td>
</tr>
<tr>
<td>14</td>
<td>No virus + vehicle (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>15</td>
<td>10^5 HSV 1716 + drug (Day 2) + drug (Day 10)</td>
</tr>
<tr>
<td>16</td>
<td>10^5 HSV 1716 + vehicle (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>17</td>
<td>10^5 HSV 1716 + drug (Day 2) + vehicle (Day 10)</td>
</tr>
<tr>
<td>18</td>
<td>10^5 HSV 1716 + vehicle (Day 2) + drug (Day 10)</td>
</tr>
</tbody>
</table>

No virus + CB1954 prodrug

Groups 5,6,7,8, 13 & 14 looked at the effect of prodrug alone on tumour growth. Figure 27 shows that there is little effect on tumour growth when CB1954 is given alone.
untreated control.

[0244] Treatment with virus and prodrug resulted in tumours which grew to only approx 2 - 3 mm$^3$ in volume. This is significantly less than the untreated tumours which grew in size to approx 20 mm$^3$.

10$^6$ PFU virus +/- CB1954 prodrug

[0245] Figure 29 shows the changes in tumour volume over time after treatment with a higher dose of virus, 10$^6$ PFU per injection, in combination with the prodrug, given as described in Table 2. As with the lower virus dose, treatment with either virus only, or in combination with CB1954, results in significantly smaller tumours compared to the untreated controls.

HSV 1716 virus in combination with CB1954 prodrug

[0246] The parental strain of the virus, which has not been engineered to contain the CMV-ntr DNA was examined for its effects on tumour growth delay. This virus does have an oncolytic effect, however it doesn’t contain the NTR gene needed to convert the inactive prodrug into its active metabolite. Therefore one would not expect any additional or synergistic effects when the prodrug is added in combination with the virus. Figure 30 shows the results of this experiment.

[0247] The combination of the virus and the prodrug appeared to produce some reduction in tumour growth compared to the untreated control tumours.

[0248] The groups used in these results contained only 2 or 3 animals. The animals used were also older and their tumours had taken longer to grow than those used in previous experiments. Hence it is possible that repeating the experiment with a larger number, with younger mice or quicker forming tumours may result in a more marked growth delay after treatment with the HSV 1716 virus.

Comparison of HSV 1790 (at 10$^5$ and 10$^6$) and HSV 1716 in combination with CB1954 prodrug

[0249] Figure 31 shows a comparison between the two doses of the HSV 1790 virus in combination with the prodrug and the HSV 1716 prodrug combination. The parental virus HSV 1716 shows some growth delay in comparison with the untreated control. We would assume that this growth delay is due to the oncolytic effect of the virus as the NTR gene is not present to alter the inactive prodrug into its active form.

[0250] Tumour growth is reduced further when the tumour is treated with the HSV 1790 virus containing the NTR gene. This appears to be dose dependent - the higher dose of the virus results in a greater growth delay than the lower dose.

[0251] In conclusion it would appear from these results that indeed the HSV 1790 virus used in combination with the prodrug CB1954 results in growth delay in the model system examined. Giving both virus and drug in combination has a greater effect than given either alone.

[0252] It appears that the timing at which the prodrug is given after virus treatment is important. When CB1954 was given soon after viral injection (Day 2 post viral injection) tumour growth was not delayed as much as if the drug was given at a later date (Day 10). It may be that given at Day 2 the drug killed the cells that were supporting viral growth and replication and actually reduced the oncolytic effect of the virus.

[0253] By day 10 the virus may have replicated and killed as many cells by oncolysis as possible. It is anticipated that due to heterogeneity of the cell type and growth state that all the cells within a tumour would not be susceptible to lysis by the virus. The drug then comes in and ‘mops up’ by killing any cells that are supporting viral growth (hence containing the NTR gene) but were not susceptible to oncolysis. As the active drug is diffusible and membrane permeable it may have a bystander effect - killing not only the cells infected with the virus but also its near neighbours.

References

[0254]

2. WO 92/13943

Claims

1. An oncolytic herpes simplex virus wherein the herpes simplex virus genome comprises nucleic acid encoding an heterologous nitroreductase (NTR), wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F or HSV-2 strain Hug52.
2. An herpes simplex virus according to claim 1, wherein the herpes simplex virus genome comprises nucleic acid encoding an heterologous NTR, wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the genome of the herpes simplex virus otherwise is the genome of HSV-1 strain 17 or F or HSV-2 strain HG52.
3. An herpes simplex virus according to claim 1, wherein the herpes simplex virus genome comprises a nucleic acid encoding an heterologous NTR, wherein the herpes simplex virus genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, wherein the herpes simplex virus genome has a mutation in the ribonucleotide reductase gene, and wherein the herpes simplex genome otherwise is the genome of HSV-1 strain 17 or F or HSV-2 strain HG52.
4. An herpes simplex virus as claimed in any one of claims 1 to 3, wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F.
5. An herpes simplex virus as claimed in any one of claims 1 to 4, wherein said NTR is E.coli NTR.
6. An herpes simplex virus as claimed in claim 5 wherein said nucleic acid comprises SEQ ID Neo. 2 or nucleic acid encoding the polypeptide of SEQ ID No. 1.
7. An herpes simplex virus as claimed in any one of claims 1 to 4, wherein said nucleic acid has at least 60% sequence identity to SEQ ID No. 2 or to a nucleic acid encoding the polypeptide of SEQ ID No. 1.
8. An herpes simplex virus as claimed in claim 7 wherein said degree of sequence identity is at least 70%.
9. An herpes simplex virus as claimed in any one of claims 1 to 4, wherein said nucleic acid hybridises to the nucleic acid of SEQ ID No. 2, to its complement or to a nucleic acid encoding the polypeptide of SEQ ID No. 1 under high stringency conditions.

10. An herpes simplex virus according to any one of claims 1 to 9 wherein said herpes simplex virus genome further comprises a regulatory nucleotide sequence operably linked to said nucleic acid encoding NTR, wherein said regulatory nucleotide sequence has a role in controlling transcription of said NTR.

11. An herpes simplex virus as claimed in any one of claims 1 to 10 wherein said nucleic acid, is located in at least one RL1 locus of the herpes simplex virus genome.

12. An herpes simplex virus as claimed in any one of claims 1 to 11 wherein said nucleic acid is, located in, or overlaps, at least one of the ICP34.5 protein coding sequences of the herpes simplex virus genome.

13. An herpes simplex virus as claimed in any one of claims 1 to 12 wherein the herpes simplex virus is a variant of HSV-1 strain 17 mutant 1716.

14. An herpes simplex virus as claimed in any one of claims 1 to 13 which is an ICP34.5 null mutant.

15. An herpes simplex virus as claimed in any one of claims 1 to 14 in which all copies of the ICP34.5 gene present in the herpes simplex virus genome are disrupted such that the herpes simplex virus is incapable of producing a functional ICP34.5 gene product.

16. An herpes simplex virus as claimed in any one of claims 1 to 13 which lacks at least one expressible ICP34.5 gene.

17. An herpes simplex virus as claimed in any one of claims 1 to 12 which lacks only one expressible ICP34.5 gene.

18. An herpes simplex virus as claimed in any one of claims 1 to 17 which is non-neurovirulent.

19. An herpes simplex virus as claimed in any one of claims 1 to 18 wherein said nucleic acid encoding the heterologous nitroreductase (NTR) forms part of a nucleic acid cassette integrated in the genome of said herpes simplex virus, said cassette encoding:

(a) said nucleic acid encoding NTR; and nucleic acid encoding
(b) a ribosome binding site; and
(c) a marker,

wherein the nucleic acid encoding NTR is arranged upstream (5’) of the ribosome binding site and the ribosome binding site is arranged upstream (5’) of the marker.

20. An herpes simplex virus according to claim 19 wherein a regulatory nucleotide sequence is located upstream (5’) of the nucleic acid encoding NTR, wherein the regulatory nucleotide sequence has a role in regulating transcription of said nucleic acid encoding NTR.

21. An herpes simplex virus according to claim 19 or 20 wherein the cassette disrupts a protein coding sequence resulting in inactivation of the respective gene product.

22. An herpes simplex virus as claimed in any one of claims 19 to 21 wherein a transcription product of the cassette is a bi- or poly-cistronic transcript comprising a first cistron encoding the NTR and a second cistron encoding the marker wherein the ribosome binding site is located between said first and second cistrons.

23. An herpes simplex virus as claimed in any one of claims 19 to 22 wherein the ribosome binding site comprises an internal ribosome entry site (IRES).

24. An herpes simplex virus as claimed in any one of claims 19 to 23 wherein the marker is a defined nucleotide sequence encoding a polypeptide.

25. An herpes simplex virus as claimed in claim 24 wherein the marker comprises the Green Fluorescent Protein (GFP)
protein coding sequence or the enhanced Green Fluorescent Protein (GFP) protein coding sequence.

26. An herpes simplex virus according to any one of claims 19 to 23 wherein the marker comprises a defined nucleotide sequence detectable by hybridisation under high stringency conditions with a corresponding labelled nucleic acid probe.

27. An herpes simplex virus as claimed in any one of claims 19 to 26 wherein the cassette further comprises nucleic acid encoding a polyadenylation sequence located downstream (3') of the nucleic acid encoding the marker.

28. An herpes simplex virus as claimed in claim 27 wherein the polyadenylation sequence comprises the Simian Virus 40 (SV40) polyadenylation sequence.


30. An herpes simplex virus as claimed in any one of claims 1 to 28 for use in the treatment of cancer.

31. An herpes simplex virus as claimed in any one of claims 1 to 28 for use in the oncolytic treatment of a tumour.

32. Use of an herpes simplex virus as claimed in any one of claims 1 to 28 in the manufacture of a medicament for the treatment of cancer.

33. A medicament, pharmaceutical composition or vaccine comprising an herpes simplex virus as claimed in any one of claims 1 to 28.

34. A medicament, pharmaceutical composition or vaccine as claimed in claim 33 further comprising a pharmaceutical acceptable carrier, adjuvant or diluent.

35. A composition comprising a herpes simplex virus according to any one of claims 1 to 28 and an NTR prodrug.

36. A composition as claimed in claim 35 wherein said NTR prodrug is Cub1954.

37. An oncolytic herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (R_L), for use, in combination with an NTR prodrug, in the treatment of a tumour, wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F or HSV-2 strain HG52.

38. A kit of parts comprising a first container having a quantity of herpes simplex virus according to any one of claims 1 to 28 and a second container having a quantity of an NTR prodrug.

39. Use in the manufacture of a medicament for the treatment of a tumour of an oncolytic herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (R_L), and an NTR prodrug, wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F or HSV-2 strain HG52.

40. Use of an oncolytic herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (R_L) in the manufacture of a first medicament for administering sequentially or simultaneously with a second medicament comprising an NTR prodrug in the treatment of a tumour, wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F or HSV-2 strain HG52.

41. Use of an NTR prodrug in the manufacture of a first medicament for administering sequentially or simultaneously with a second medicament comprising an oncolytic herpes simplex virus, wherein the genome of said virus comprises
a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (RL), in the treatment of a tumour, wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F or HSV-2 strain HG52.

42. Use according to any one of claims 39 to 41, wherein said treatment of a tumour is oncolytic treatment of a tumour.

43. The virus, kit, or use as claimed in any one of claims 37 to 42 wherein said NTR prodrug is CB1954.

44. A method of expressing in vitro a nitroreductase, said method comprising the step of infecting at least one cell with an oncolytic herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding an heterologous nitroreductase in at least one of the long repeat regions (RL), said nitroreductase operably linked to a transcription regulatory sequence, wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F or HSV-2 strain HG52.

45. The herpes simplex virus, use, or method, as claimed in any one of claims 37 or 39 to 44, wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F.

46. HSV1716/CMV-NTR/GFP (ECACC accession number 03110501).

47. An oncolytic herpes simplex virus for use in a method of treatment, wherein the method of treatment comprises the treatment of a tumour, wherein the herpes simplex virus genome comprises nucleic acid encoding an heterologous nitroreductase (NTR), wherein the genome has an inactivating mutation in the RL1 locus such that the herpes simplex virus does not produce a functional ICP34.5 gene product, and wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F or HSV-2 strain HG52.

48. The herpes simplex virus for use in a method of treatment according to claim 47, wherein the herpes simplex virus is a variant of one of HSV-1 strains 17 or F.

Patentansprüche

1. Onkolytisches Herpes-simplex-Virus, worin das Herpes-simplex-Virusgenom eine für eine heterologe Nitroreduktase (NTR) kodierende Nucleinsäure umfasst, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet, und worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F oder des HSV-2-Stammes HG52 ist.

2. Herpes-simplex-Virus nach Anspruch 1, worin das Herpes-simplex-Virusgenom eine für eine heterologe NTR kodierende Nucleinsäure umfasst, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet, und worin das Herpes-simplex-Virus andernfalls das Genom des HSV-1-Stammes 17 oder F oder des HSV-2-Stammes HG52 ist.

3. Herpes-simplex-Virus nach Anspruch 1, worin das Herpes-simplex-Virusgenom eine für eine heterologe NTR kodierende Nucleinsäure umfasst, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet, worin das Herpes-simplex-Virusgenom eine Mutation im Ribonucleotidreduktasegen aufweist und worin das Herpes-simplex-Virusgenom andernfalls das Genom des HSV-1-Stammes 17 oder F oder des HSV-2-Stammes HG52 ist.

4. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 3, worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F ist.

5. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 4, worin die NTR E.coli-NTR ist.
Herpes-simplex-Virus nach Anspruch 5, worin die Nucleinsäure Seq.-ID Nr. 2 oder eine für das Polypeptid von Seq.-ID Nr. 1 kodierende Nucleinsäure umfasst.

8. Herpes-simplex-Virus nach Anspruch 7, worin der Grad der Sequenzidentität zumindest 70 % beträgt.

9. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 4, worin die Nucleinsäure an die Nucleinsäure von Seq.-ID Nr. 2, ihr Komplement oder eine für das Polypeptid von Seq.-ID Nr. 1 kodierende Nucleinsäure unter Bedingungen hoher Stringenz hybridisiert.

10. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 9, worin das Herpes-simplex-Virusgenom weiters eine an die für NTR kodierende Nucleinsäure operabel gebundene regulatorische Nucleotidsequenz umfasst, worin die regulatorische Nucleotidsequenz eine Rolle bei der Steuerung der Transkription von NTR spielt.

11. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 10, worin die Nucleinsäure in zumindest einem RL1-Locus des Herpes-simplex-Virusgenoms vorliegt.

12. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 11, worin die Nucleinsäure in zumindest einer der ICP34.5-Protein-kodierenden Sequenzen des Herpes-simplex-Virusgenoms vorliegt oder diese überlappt.

13. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 12, worin das Herpes-simplex-Virus eine Variante der Mutante 1716 des HSV-1-Stamms 17 ist.

14. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 13, das eine ICP34.5-Nullmutante ist.

15. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 14, worin alle Kopien des in dem Herpes-simplex-Virusgenom vorliegenden ICP34.5-Gens so unterbrochen sind, dass das Herpes-simplex-Virus nicht in der Lage ist, ein funktionelles ICP34.5-Genprodukt zu bilden.

16. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 13, dem zumindest ein exprimierbares ICP34.5-Gen fehlt.

17. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 12, dem nur ein exprimierbares ICP34.5-Gen fehlt.

18. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 17, das nicht neurovirulent ist.

19. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 18, worin die für die heterologe Nitroreduktase (NTR) kodierende Nucleinsäure Teil einer Nucleinsäurekassette bildet, die in das Genom des Herpes-simplex-Virus integriert ist, wobei die Kassette für Folgendes kodiert:

(a) die für NTR kodierende Nucleinsäure; und eine Nucleinsäure, die für Folgendes kodiert:
(b) eine Ribosomenbindungsstelle; und
(c) einen Marker,

worin die für NTR kodierende Nucleinsäure stromauf (5') der Ribosomenbindungsstelle angeordnet ist und die Ribosomenbindungsstelle stromauf (5') des Markers angeordnet ist.

20. Herpes-simplex-Virus nach Anspruch 19, worin eine regulatorische Nucleotidsequenz stromauf (5') der für NTR kodierenden Nucleinsäure vorliegt, worin die regulatorische Nucleotidsequenz eine Rolle bei der Steuerung der Transkription der für NTR kodierenden Nucleinsäure spielt.

21. Herpes-simplex-Virus nach Anspruch 19 oder 20, worin die Kassette eine Proteinkodierungssequenz unterbricht, was zur Inaktivierung des jeweiligen Genprodukts führt.

22. Herpes-simplex-Virus nach einem der Ansprüche 19 bis 21, worin ein Transkriptionsprodukt der Kassette ein bi-oder polycistronisches Transkript ist, das ein für die NTR kodierendes erstes Cistron und ein für den Marker kodierendes zweites Cistron umfasst, worin die Ribosomenbindungsstelle zwischen dem ersten und dem zweiten Cistron
vorliegt.

23. Herpes-simplex-Virus nach einem der Ansprüche 19 bis 22, worin die Ribosomenbindungsstelle eine interne Eintrittsstelle für Ribosomen (IRES) umfasst.

24. Herpes-simplex-Virus nach einem der Ansprüche 19 bis 23, worin der Marker eine für ein Polypeptid kodierende definierte Nucleotidsequenz ist.

25. Herpes-simplex-Virus nach Anspruch 24, worin der Marker die Protein kodierungssequenz des grün fluoreszierenden Proteins (GFP) oder die Protein kodierungssequenz des verstärkt grün fluoreszierenden Proteins (EGFP) umfasst.


27. Herpes-simplex-Virus nach einem der Ansprüche 19 bis 26, worin die Kassette weiters eine Nucleinsäure umfasst, die für eine stromab (3') der für den Marker kodierenden Nucleinsäure vorliegende Polyadenylierungssequenz kodiert.


29. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 28 zur Verwendung in einem medizinischen Behandlungsverfahren.

30. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 28 zur Verwendung bei der Behandlung von Krebs.

31. Herpes-simplex-Virus nach einem der Ansprüche 1 bis 28 zur Verwendung in der onkolytischen Behandlung eines Tumors.

32. Verwendung eines Herpes-simplex-Virus nach einem der Ansprüche 1 bis 28 zur Herstellung eines Medikaments zur Behandlung von Krebs.


34. Medikament, pharmazeutische Zusammensetzung oder Vakzine nach Anspruch 33, weiters umfassend ein(en) pharmazeutisch annehmbaren/s Träger, Adjuvans oder Verdünner.

35. Zusammensetzung, umfassend ein Herpes-simplex-Virus nach einem der Ansprüche 1 bis 28 und ein NTR-Prodrug.


37. Onkolytisches Herpes-simplex-Virus, worin das Genom des Virus eine für eine heterologe Nitroreduktase kodierende Nucleinsäuresequenz in zumindest einer der langen Wiederholungsregionen (R_L) umfasst, zur Verwendung in Kombination mit einem NTR-Prodrug zur Behandlung eines Tumors, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet, und worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F oder des HSV-2-Stamms HG52 ist.

38. Set von Teilen, umfassend einen ersten Behälter mit einer Menge an Herpes-simplex-Virus nach einem der Ansprüche 1 bis 28 und einen zweiten Behälter mit einer Menge an einem NTR-Prodrug.

39. Verwendung eines onkolytischen Herpes-simplex-Virus, worin das Genom des Virus eine für eine heterologe Nitroreduktase kodierende Nucleinsäuresequenz in zumindest einer der langen Wiederholungsregionen (R_L) umfasst, und eines NTR-Prodrugs zur Herstellung eines Medikaments zur Behandlung eines Tumors, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein
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funktionelles ICP34.5-Genprodukt bildet,
und worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F oder des HSV-2-Stamms HG52 ist.

40. Verwendung eines onkolytischen Herpes-simplex-Virus, worin das Genom des Virus eine für eine heterologe Nitroreduktase kodierende Nucleinsäuresequenz in zumindest einer der langen Wiederholungsregionen ($R_L$) umfasst, zur Herstellung eines ersten Medikaments zur nacheinander oder gleichzeitig erfolgenden Verabreichung mit einem zweiten Medikament, das ein NTR-Prodrug umfasst, zur Behandlung eines Tumors, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet,
und worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F oder des HSV-2-Stamms HG52 ist.

41. Verwendung eines NTR-Prodrugs zur Herstellung eines ersten Medikaments zur nacheinander oder gleichzeitig erfolgenden Verabreichung mit einem zweiten Medikament, das ein onkolytisches Herpes-simplex-Virus umfasst, worin das Genom des Virus eine für eine heterologe Nitroreduktase kodierende Nucleinsäuresequenz in zumindest einer der langen Wiederholungsregionen ($R_L$) umfasst, zur Behandlung eines Tumors, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet,
und worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F oder des HSV-2-Stamms HG52 ist.

42. Verwendung nach einem der Ansprüche 39 bis 41, worin die Behandlung eines Tumors eine onkolytische Behandlung eines Tumors ist.

43. Virus, Set oder Verwendung nach einem der Ansprüche 37 bis 42, worin das NTR-Prodrug CB1954 ist.

44. Verfahren zur In-vitro-Expression einer Nitroreduktase, wobei das Verfahren den Schritt des Infizierens zumindest einer Zelle mit einem onkolytischen Herpes-simplex-Virus umfasst, worin das Genom des Virus eine für eine heterologe Nitroreduktase kodierende Nucleinsäuresequenz in zumindest einer der langen Wiederholungsregionen ($R_L$) umfasst, zur Behandlung eines Tumors, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet,
und worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F oder des HSV-2-Stamms HG52 ist.


46. HSV1716/CMV-NTR/GFP (ECACC-Zugriffsnummer 03110501).

47. Onkolytisches Herpes-simplex-Virus zur Verwendung in einem Behandlungsverfahren, worin das Behandlungsverfahren die Behandlung eines Tumors umfasst, worin das Herpes-simplex-Virusgenom eine für eine heterologe Nitroreduktase (NTR) kodierende Nucleinsäure umfasst, worin das Genom eine inaktivierende Mutation in dem RL1-Locus aufweist, sodass das Herpes-simplex-Virus kein funktionelles ICP34.5-Genprodukt bildet,
und worin das Herpes-simplex-Virus eine Variante einer der HSV-1-Stämme 17 oder F oder des HSV-2-Stamms HG52 ist.


Revendications

1. Virus de l’herpès simplex oncolytique, où le génome du virus de l’herpès simplex comprend un acide nucléique codant pour une nitroréductase hétérologue (NTR).
où le génome présente une mutation d’inactivation dans le RL1 locus de sorte que le virus de l’herpès simplex ne produis pas de produit de gène fonctionnel ICP34.5,
et où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1 ou de la souche HG52 de HSV-2.

2. Virus de l’herpès simplex selon la revendication 1, où le génome du virus de l’herpès simplex comprend un acide nucléique codant pour une NTR hétérologue, où le génome présente une mutation d’inactivation dans le locus RL1 de telle sorte que le virus de l’herpès simplex ne produit pas de produit de gène ICP34.5 fonctionnel, et où le génome du virus de l’herpès simplex est sinon le génome de la souche 17 ou F de HSV-1 ou de la souche HG52 de HSV-2.

3. Virus de l’herpès simplex selon la revendication 1, où le génome du virus de l’herpès simplex comprend un acide nucléique codant pour une NTR hétérologue, où le génome du virus de l’herpès simplex présente une mutation d’inactivation dans le locus RL1 de sorte que le virus de l’herpès simplex ne produit pas de produit de gène ICP34.5 fonctionnel, où le génome du virus de l’herpès simplex présente une mutation dans le gène de la ribonucléotide réductase, et où le génome de l’herpès simplex est sinon le génome de la souche 17 ou F de HSV-1 ou de la souche HG52 de HSV-2.

4. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 3, où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1.

5. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 4, où ladite NTR est E. coli NTR.

6. Virus de l’herpès simplex selon la revendication 5, où ledit acide nucléique comprend la SEQ ID No. 2 ou bien l’acide nucléique codant pour le polypeptide de SEQ ID No. 1.

7. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 4, où ledit acide nucléique possède au moins 60% d’identité de séquence avec SEQ ID No. 2 ou avec un acide nucléique codant pour le polypeptide de la SEQ ID No. 1.

8. Virus de l’herpès simplex selon la revendication 7, où ledit degré d’identité de séquence est au moins de 70%.

9. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 4, où ledit acide nucléique s’hybride à l’acide nucléique de SEQ ID No. 2, à son complément ou à un acide nucléique codant pour le polypeptide de SEQ ID No. 1 sous des conditions de stringence élevées.

10. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 9, où ledit génome du virus de l’herpès simplex comprend en outre une séquence de nucléotides régulatoire fonctionnellement liée audit acide nucléique codant pour la NTR, où ladite séquence de nucléotides régulatoire a un rôle dans le contrôle de la transcription de ladite NTR.


12. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 11, où ledit acide nucléique est situé dans, ou chevauche, au moins une des séquences codant pour la protéine ICP34.5 du génome du virus de l’herpès simplex.

13. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 12, où le virus de l’herpès simplex est une variante du mutant 1716 de la souche 17 de HSV-1.

14. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 13, qui est un mutant null de ICP34.5.

15. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 14, dans lequel toutes les copies du gène ICP34.5 présentes dans le génome du virus de l’herpès simplex sont dissociaées de sorte que le virus de l’herpès simplex est inapte à produire un produit de gène ICP34.5 fonctionnel.

16. Virus de l’herpès simplex selon l’une quelconque des revendications 1 à 13, qui est exempt d’au moins un gène ICP34.5 expressible.
17. Virus de l'herpès simplex selon l'une quelconque des revendications 1 à 12, qui est exempt de seulement un gène ICP34.5 expressible.

18. Virus de l'herpès simplex selon l'une quelconque des revendications 1 à 17, qui est non-neurovirulent.

19. Virus de l'herpès simplex selon l'une quelconque des revendications 1 à 18, où ledit acide nucléique codant pour la nitroréductase hétérologue (NTR) fait partie d'une cassette d'acide nucléique intégrée dans le génome dudit virus de l'herpès simplex, ladite cassette codant pour:

(a) ledit acide nucléique codant pour la NTR; et l'acide nucléique codant pour
(b) un site de liaison de ribosome; et
(c) un marqueur,

où l'acide nucléique codant pour la NTR est agencé en amont (5') du site de liaison de ribosome, et le site de liaison de ribosome est agencé en amont (5') du marqueur.

20. Virus de l'herpès simplex selon la revendication 19, où une séquence de nucléotides régulatoire est située en amont (5') de l'acide nucléique codant pour la NTR, où la séquence de nucléotides régulatoire a un rôle dans la régulation de la transcription dudit acide nucléique codant pour la NTR.

21. Virus de l'herpès simplex selon la revendication 19 ou 20, où la cassette interrompt une séquence de codage de protéines se traduisant par l'inactivation du produit de gène respectif.

22. Virus de l'herpès simplex selon l'une quelconque des revendications 19 à 21, où un produit de transcription de la cassette est un bi- ou poly-transcript cistronique comprenant un premier cistron codant pour la NTR et un deuxième cistron codant pour le marqueur, où le site de liaison de ribosome se situe entre lesdits premier et deuxième cistrons.

23. Virus de l'herpès simplex selon l'une quelconque des revendications 19 à 22, où le site de liaison de ribosome comprend un site d'entrée de ribosome interne (IRES).

24. Virus de l'herpès simplex selon l'une quelconque des revendications 19 à 23, où le marqueur est une séquence de nucléotides définie codant pour un polypeptide.

25. Virus de l'herpès simplex selon la revendication 24, où le marqueur comprend la séquence de codage de protéine fluorescente verte (GFP) ou bien la séquence de codage de protéine fluorescente verte activatrice (EGFP).

26. Virus de l'herpès simplex selon l'une quelconque des revendications 19 à 23, où le marqueur comprend une séquence de nucléotides définie détectable par hybridation sous des conditions de stringence élevées avec une zone d'acide nucléique marquée correspondante.

27. Virus de l'herpès simplex selon l'une quelconque des revendications 19 à 26, où la cassette comprend en outre un acide nucléide codant pour une séquence de polyadénylation située en aval (3') de l'acide nucléique codant pour le marqueur.

28. Virus de l'herpès simplex selon la revendication 27, où la séquence de polyadénylation comprend la séquence de polyadénylation de Virus Simian 40 (SV40).

29. Virus de l'herpès simplex selon l'une quelconque des revendications 1 à 28 pour utilisation dans une méthode de traitement médical.

30. Virus de l'herpès simplex selon l'une quelconque des revendications 1 à 28 pour utilisation dans le traitement du cancer.

31. Virus de l'herpès simplex selon l'une quelconque des revendications 1 à 28 pour utilisation dans le traitement oncolytique d'une tumeur.

32. Utilisation d’un virus de l’herpès simplex selon l’une quelconque des revendications 1 à 28 dans la fabrication d’un médicament pour le traitement du cancer.
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33. Médicament, composition pharmaceutique ou vaccin comprenant un virus de l’herpès simplex selon l’une quelconque des revendications 1 à 28.

34. Médicament, composition pharmaceutique ou vaccin selon la revendication 33, comprenant en outre un support, adjuvant ou diluant pharmaceutiquement acceptable.

35. Composition comprenant un virus de l’herpès simplex selon l’une quelconque des revendications 1 à 28 et un promédicament de NTR.

36. Composition selon la revendication 35, où ledit promédicament NTR est CB1954.

37. Virus de l’herpès simplex oncolytique, où le génome dudit virus comprend une séquence d’acides nucléiques codant pour une nitroréductase hétérologue dans au moins une des régions de répétition longues (RL1), pour utilisation, en combinaison avec un promédicament de NTR, dans le traitement d’une tumeur, où le génome possède une mutation d’inactivation dans le locus RL1 de telle sorte que le virus de l’herpès simplex ne produit pas un produit de gène ICP34.5 fonctionnel, et où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1 ou de la souche HG52 de HSV-2.

38. Kit de pièces comprenant un premier contenant ayant une quantité de virus de l’herpès simplex selon l’une quelconque des revendications 1 à 28 et un deuxième contenant ayant une quantité d’un promédicament de NTR.

39. Utilisation dans la fabrication d’un médicament pour le traitement d’une tumeur d’un virus de l’herpès simplex oncolytique, où le génome dudit virus comprend une séquence d’acides nucléiques codant pour une nitroréductase hétérologue dans au moins une des régions de répétition longues (RL1), et un promédicament de NTR, où le génome présente une mutation d’inactivation dans le locus RL1 de sorte que le virus de l’herpès simplex ne produit pas de produit de gène ICP34.5 fonctionnel, et où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1 ou de la souche HG52 de HSV-2.

40. Utilisation d’un virus de l’herpès simplex oncolytique, où le génome dudit virus comprend une séquence d’acides nucléiques codant pour une nitroréductase hétérologue dans au moins une des régions de répétition longues (RL1) dans la fabrication d’un premier médicament pour l’administration séquentielle ou simultanée avec un deuxième médicament comprenant un promédicament de NTR dans le traitement d’une tumeur, où le génome présente une mutation d’inactivation dans le locus RL1 de sorte que le virus de l’herpès simplex ne produit pas de produit de gène ICP34.5 fonctionnel, et où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1 ou de la souche HG52 de HSV-2.

41. Utilisation d’un promédicament de NTR dans la fabrication d’un premier médicament pour l’administration séquentielle ou simultanée avec un deuxième médicament comprenant un virus de l’herpès simplex oncolytique, où le génome dudit virus comprend une séquence d’acides nucléiques codant pour une nitroréductase hétérologue dans au moins une des régions de répétition longues (RL1), dans le traitement d’une tumeur, où le génome présente une mutation d’inactivation dans le locus RL1 de telle sorte que le virus de l’herpès simplex ne produit pas de produit de gène ICP34.5 fonctionnel, et où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1 ou de la souche HG52 de la souche HSV-2.

42. Utilisation selon l’une quelconque des revendications 39 à 41, où ledit traitement d’une tumeur est un traitement oncolytique d’une tumeur.

43. Virus, kit ou utilisation selon l’une quelconque des revendications 37 à 42, où ledit promédicament de NTR est CB1954.

44. Méthode d’expression in vitro d’une nitroréductase, ladite méthode comprenant l’étape consistant à infecter au moins une cellule avec un virus de l’herpès simplex oncolytique, où le génome dudit virus comprend une séquence d’acide nucléique codant pour une nitroréductase hétérologue dans au moins une des régions de répétition longues (RL1), ladite nitroréductase étant fonctionnellement liée à une séquence régulatrice de transcription,
où le génome présente une mutation d’inactivation dans le locus RL1 de sorte que le virus de l’herpès simplex ne
produit pas de produit de gène ICP34.5 fonctionnel,
et où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1 ou de la souche HG52 de
HSV-2.

45. Virus, utilisation ou méthode de l’herpès simplex selon l’une quelconque des revendications 37 ou 39 à 44, où le
virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1.

46. HSV1716/CMV-NTR/GFP (numéro d’accès 03110501 de ECACC).

47. Virus de l’herpès simplex oncolytique pour utilisation dans une méthode de traitement, où la méthode de traitement
comprend le traitement d’une tumeur, où le génome du virus de l’herpès simplex comprend un acide nucléique
codant pour une nitroréductase hétérologue (NTR),
où le génome présente une mutation d’inactivation dans le locus RL1 de sorte que le virus de l’herpès simplex ne
produit pas de produit de gène ICP34.5 fonctionnel,
et où le virus de l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1 ou de la souche HG52 de
HSV-2.

48. Virus de l’herpès simplex pour utilisation dans une méthode de traitement selon la revendication 47, où le virus de
l’herpès simplex est une variante d’une des souches 17 ou F de HSV-1.
Figure 1
**Figure 2**
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11
Recombinant viral plaque expressing GFP and NTR

Co-transfect BHK cells in 60 mm dish with HSV17+ DNA and Scal digested clone 4.

BHK cells

<table>
<thead>
<tr>
<th>HSV1716/CMV-NTR/GFP Fraction</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Released (CR)</td>
<td>$1.00 \times 10^9$ pfu/ml</td>
</tr>
<tr>
<td>Cell Associated (CA)</td>
<td>$2.38 \times 10^9$ pfu/ml</td>
</tr>
</tbody>
</table>

**Figure 12**
Figure 13
Figure 14
<table>
<thead>
<tr>
<th></th>
<th>BHK</th>
<th>C8161</th>
<th>VM</th>
<th>3T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1716/CM-NTR/GFP</td>
<td>17⁺</td>
<td>Mock</td>
<td>17⁺</td>
<td>Mock</td>
</tr>
</tbody>
</table>

![Western Blot Image]

- **NTR (24kDa)**
- **131kDa**
- **86kDa**
- **43kDa**
- **33kDa**
- **19kDa**
- **7kDa**

**Figure 15**
Figure 16
Figure 17
Figure 20
Fig. 21

Figure 21
Figure 23

![Graph showing weight as ratio to original weight over days post treatment with different treatments: Naked DNA, Naked DNA + CB1954, CB1954 alone, No treatment.](image)
Figure 24
Figure 25
Figure 26
Figure 27
Figure 28
Fig. 29

Figure 29
Figure 30
Figure 31

Fig. 31

Tumour volume

-3 2 7 12 17

Days post viral injection

- 10e5 HSV 1790 + drug (2) + drug (10)
- No virus (combined)
- 10e6 HSV 1790 + drug (2) + drug (10)
- 10e5 HSV 1716 + drug (2) + drug (10)
MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLQLQYSPSSTNSQ
PWHFIVASTEEGKARVAKSAAGNYVFNERKILDASHVVVFCAKTMDDAWLKLVVDQE
DADGRFATPEAKAANDKGRKFDAHDDAEWMAKQVYLNVGNFLLGVAALGLD
AVPIEGFDAAILDAEFGLKEKGYTLSVVVPVGHHSVEDFNAFLPKSRLPQNITLTEV

1 atggatatca tttctgtcgc cttaagcgtcattccacta aggcatttga tgccagcaga
61 aaacattccc cggaacagcgc agacagatcg aaactctcct tcgaatacag ccatccagc
121 accaactcccc agcgggtgcca tttaatgttt gccagcaggga aagaaggtta aacgcggttt
181 gcacaatcgc ctggccgccga tatgtgttc aagcaacgag aatactgta ggcctgccac
241 gctggtggtgt tctgtgcaaaa aacccgagatg gacagatgcc ggtgtaagct ggttgtgac
301 caggaagatgc tctggtggcc cttgccagcg ccggagcggaa gcataagggct
361 cgcaagtctct cgcgcggatgc gcaccggataa gatcgtcacag atggtgcagc ccggggcggca
421 aacaggttt attcacagtt cggtaatttc ctgctcgccgg ggcggccttc ggtctggac
481 gcgcgacccca tgcaaggttt ttgacgcgcgc atctcgtgatg cagaattttg tcgaaagag
541 aagggctaca ccagtctgtgt ggtagtctcg gtgggtcatc acagcttga agatttttaac
601 gctacgctgc cgaatttctgc tgcgacgcaaa acacattcct taacacgaagt gtaa

Figure 32
REFERENCES CITED IN THE DESCRIPTION

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