Description

Poxviral oncolytic vectors

Technical Field

[0001] Oncolytic viruses are a class of novel therapeutic agents used for the treatment of cancer that have the unique property of tumor-dependent self-perpetuation (HERMISTON. A demand for next-generation oncolytic adenoviruses. *Current opinion in molecular therapeutics.* 2006, vol.8, no.4, p.322-30.). Oncolytic viruses are capable of selective replication in malignant cells and therefore offer levels of potency and specificity that are potentially far higher than conventional treatments for cancer (FISHER. Striking out at disseminated metastases: the systemic delivery of oncolytic viruses. *Current opinion in molecular therapeutics .* 2006, vol.8, no.4, p.301-13.). The benefit of using these viruses is that as they replicate, they lyse their host cells. Cancer cells are ideal hosts for many viruses because they have the antiviral interferon pathway inactivated or have mutated tumour suppressor genes that enable viral replication to proceed unhindered (CHERNAJOVSKY, et al. Fighting cancer with oncolytic viruses. *British medical journal.* 2006, vol.332, no.7534, p.170-2.).

[0002] Some viruses are naturally able to selectively replicate in tumoral cells but oncolytic viruses can also be obtained by modifying naturally occurring viruses. For this purpose, the main strategies used currently to modify the viruses include: functional deletions in essential viral genes; tumor- or tissue-specific promoters used to control the expression of these viral genes; and tropism modification to redirect adenovirus to the cancer cell surface. In the near future, oncolytic adenoviruses need to be optimized to fully realize their potential as critical anticancer tools and, thus, improve the prognosis for patients with malignant gliomas (JIANG, et al. Oncolytic adenoviruses as antiglioma agents. *Expert review of anticancer therapy.* 2006, vol.6, no.5, p.697-708.).

[0003] For example, ONYX-015, an adenovirus modified selectively to replicate in and kill cells that harbor p53 mutations, is under development by Onyx Pharmaceuticals for the potential treatment of various solid tumors, including head and neck, gastrointestinal and pancreatic tumors. It is a

recombinant adenovirus that carries a loss-of-function mutation at the E1B locus, the product of which is a 55 kDa protein that binds to and inactivates the p53 tumor suppressor protein. Thus, the ONYX-015 adenovirus is supposed to leave normal cells unaffected. Mutations in the p53 tumor suppressor gene are the most common type of genetic abnormality in cancer, occurring in more than half of all major cancer types. Thus, these cells are susceptible to the virus, which will readily replicate and cause cell death. ONYX-015 is in ongoing phase III trials for the treatment of recurrent head and neck cancer, phase II trials for colorectal, ovary, pancreas and mouth tumors, and phase I trials for digestive disease, esophagus and liver tumors (COHEN, et al. ONYX-015. Onyx Pharmaceuticals. *Current opinion in investigational drugs.* 2001, vol.2, no.12, p.1770-5.).

[0004] Naturally oncolytic viruses are replication-competent viruses that have an innate ability to selectively infect and kill tumor cells. Despite being used in the original attempts to treat cancer with live viruses five decades ago, interest in naturally oncolytic viruses has lagged behind the support for engineered adenoviruses and herpesviruses as cancer therapeutics. Recently, however, there has been renewed interest in the high potency and selectivity of these naturally occurring agents (ROBERTS, et al. Naturally oncolytic viruses. *Current opinion in molecular therapeutics .* 2006, vol.8, no.4, p.314-21.).

[0005] Among naturally oncolytic viruses, Vaccinia viruses (a Poxviridae) possess many of the key attributes necessary for an ideal viral backbone for use in oncolytic virotherapy. These include a short lifecycle, with rapid cell-to-cell spread. strong lytic ability, a large cloning capacity and well-defined molecular biology. In addition, although capable of replicating in human cells, they are not considered a natural health problem and are especially well characterized. having been delivered to millions of individuals during the campaign to eradicate smallpox. Early clinical results using either vaccine strains or genetically modified vaccinia strains have demonstrated antitumor effects (THORNE, et al. Vaccinia virus and oncolytic

virotherapy of cancer. *Current opinion in molecular therapeutics.* 2005, vol.7, no.4, p.359-65.).

[0006] In contrast, the poxvirus myxoma virus is a novel oncolytic candidate that has no history of use in humans directly, as it has a distinct and absolute host species tropism to lagomorphs (rabbits). Myxoma virus has been recently shown to be able to also selectively infect and kill human tumor cells, a unique tropism that is linked to dysregulated intracellular signalling pathways found in the majority of human cancers. This review outlines the existing knowledge on the tropism of myxoma virus for human cancer cells, as well as preclinical data exhibiting its ability to infect and clear tumors in animal models of cancer (STANFORD, et al. Myxoma virus and oncolytic virotherapy: a new biologic weapon in the war against cancer. *Expert opinion on biological therapy.* 2007, vol.7, no.9, p.1415-25.).

[0007]

Technical Problem

[0008] The injection of high doses of Poxviruses necessary to achieve an antitumoral effect raised toxicity issues. The majority of adverse events are minor, adverse reactions that are usually linked to Vaccinia virus are self-limited and include fever, headache, fatigue, myalgia, chills, local skin reactions, nonspecific rashes, erythema multiforme, lymphadenopathy, and pain at the vaccination site. Other reactions might require additional therapies (e.g., VIG, a first-line therapy and cidofovir, a second-line therapy). Adverse reactions that might require further evaluation or therapy include inadvertent inoculation, generalized vaccinia (GV), eczema vaccinatum (EV), progressive vaccinia (PV), postvaccinial central nervous system disease, and fetal vaccinia (CONO, et al. Smallpox vaccination and adverse reactions. Guidance for clinicians. *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control.* 2003, vol.52, no.RR-4, p.1-28.).

[0009] Thus, there is need for safer Poxviruses with an oncolytic activity as good as to their natural counterparts.

Background Art

[0010] US 5364773 (VIROGENETICS CORPORATION (TROY, NY)) 15/11/1994 describes a modified recombinant poxvirus, more particularly a vaccinia virus having inactivated nonessential virus-encoded encoded genetic functions so that the recombinant poxvirus has attenuated virulence and enhanced safety. In particular, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor or by insertional inactivation of an open reading frame encoding a virulence factor. More particularly, this patent describes a vaccinia virus in which the open reading frame of for J2R, B13R+B14R, A26L, A56R, C7L - K1L, and I4L has been inactivated. This virus (NYVAC) can be engineered as a vector for a foreign nucleic acid and used as a vaccine for inducing an immunological response in a host animal. However, N YVAC is unable to efficiently replicate in most mammalian cels and can not be used as an oncolytic virus (**XIANGZHI, et al.** Vaccinia virus K1L protein supports viral replication in human and rabbit cells through a cell-type-specific set of its ankyrin repeat residues that are distinct from its binding site for ACAP2. *Journal of virology.* 2006, vol.353, no.1, p.220-233.).

[0011] WO 2004/014314 (KIRN DAVID (US)) 19/02/2004 describes an altered vaccinia virus that comprises one or more mutations in its viral genome. Described mutations are in one or more of the following classes of polypeptides: 1) interferon-modulating polypeptide; 2) complement control polypeptide ; 3) TNF or chemokine-modulating polypeptide; 4) serine protease inhibitor; 5) IL- Ip modulating polypeptide; 6) non-infectious EEV form polypeptides; and, 7) viral polypeptide that act to inhibit release of infectious virus from cells (anti-infectious virus form polypeptide). In addition, mutations in A41L or C11R of vaccinia virus are also disclosed.

[0012] Vaccinia genome regions such as A34R, A41L, A53R,B5R, B7R,B8R, B13R, B15R,B18R, B22R, B28R, B29R, CUR, E3L,K2L, N1L,vC12L, and vCKBP are more particularly described in this application. Methods of the invention involve using any of the poxviruses discussed herein. The inventors also disclose methods to treat cancer by administering to the cancer cell or patient an effective amount of this altered vaccinia virus.

Disclosure of Invention

[0013] The inventors have surprisingly discovered that poxviruses comprising a defective F2L gene have an improved safety profile but kept an equivalent oncolytic activity (compared to their natural counterpart).

[0014] The present invention relates to a poxvirus comprising a defective F2L gene.

[0015] As used throughout the entire application, the terms "a" and "an" are used in the sense that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced components or steps, unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

[0016] The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

[0017] The term "about" or "approximately" as used herein means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

[0018] As used herein, the terms "comprising" and “comprise” are intended to mean that the products, compositions and methods include the referenced components or steps, but not excluding others. "Consisting essentially of" when used to define products, compositions and methods, shall mean excluding other components or steps of any essential significance. Thus, a composition consisting essentially of the recited components would not exclude trace contaminants and pharmaceutically acceptable carriers. "Consisting of" shall mean excluding more than trace elements of other components or steps.

[0019] As used herein, the term “poxvirus comprising a defective gene“ refers to a poxvirus comprising a deletion, substitution or addition in one or more nucleic acid of the defective gene, or any combination of these possibilities wherein said modifications lead to the inability for the virus to produce a protein having the activity of the protein produced by the unmodified gene. In a preferred embodiment of the invention, a poxvirus comprising a defective gene refers to a poxvirus in which the whole gene sequence has been deleted. Mutation can be made in a number of ways known to those

skilled in the art using recombinant techniques. Methods for modifying the genome of a poxvirus are available in the art. For example the methods disclosed in MCCART, et al. Systemic cancer therapy with a tumor selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes.. *Cancer res.*. 2001, no.61, p.8751-57. , KIM, et al. Systemic armed oncolytic ans immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Molecular Therapeutic.* 2006, no.14, p.361-70. , WO 2004/014314 (KIRN DAVID (US)) 19/02/2004 and US 5364773 (VIROGENETICS CORPORATION (TROY, NY)) 15/11/1994 can be used to produce the poxvirus of the invention. The methods disclosed in the example of the present application are particularly relevant to produce a poxvirus according to the invention. Sequences of the genome of various poxviruses are available in the art, for example, the vaccinia virus, cowpox virus, Canarypox virus, Ectromelia virus, Myxoma virus genomes are available in Genbank (accession number NC_006998, NC_003663, NC_005309, NC_004105, NC_001132 respectively)

[0020] As used herein the term "poxvirus" refers to a virus belonging to the Poxviridae family. According to a preferred embodiment, the poxvirus according to the invention belongs to the Chordopoxvirinae subfamily, more preferably to the Orthopoxvirus genus and even more preferably to the Vaccinia virus specie.

[0021] For example, Vaccinia virus strains Dairen I, IHD-J, L-IPV, LC16M8, LC16MO, Lister, LIVP, Tashkent, WR 65-16, Wyeth, Ankara, Copenhagen, Tian Tan and WR can be used. According to a particularly preferred embodiment, the poxvirus according to the invention is a Vaccinia virus strains Copenhagen.

[0022] The poxvirus vaccinia contains a large duplex DNA genome (187 kilobase pairs) and is a member of the only known family of DNA viruses that replicates in the cytoplasm of infected cells. Because the infected cell must deliver large amounts of DNA precursors to cytoplasmic replication sites, the virus encodes and expresses many enzymatic activities required for

DNA metabolism and synthesis, including deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase).

[0023] Deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase, EC 3.6.1.23) catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate in the presence of Mg(2+) ions. dUTPase, in removing dUTP from the dNTP pool and generating dUMP, is involved in both maintaining the fidelity of DNA replication and in proviging the precursor for the production of TMP by thymidylate synthase. Vaccinia dUTPase is a 15 kDa protein encoded by the F2L gene (MCGEOGH. . *Nucleic Acids Research.* 1990, no.18, p.4105-10. ; BROYLES. . *Virology.* 1993, no.195, p.863-5.). Sequence of the F2L gene of the vaccinia virus is available in genbank via accession number M25392, sequences and locations of the F2L gene in various poxviruses genomes are also available in genbank, for example, via accession number NC_006998, DQ121394, NC_001611, AY689436, AY689437, NC_008291, DQ437594, DQ437593, AY313847, AY313848, NC_006966, NC_005309, NC_003391, NC_003389, NC_001132, NC_003310, NC_002188, M35027, AY243312, AF170726, DQ011157, DQ011156, DQ011155, DQ011154, DQ011153, X94355, Y16780, AY318871, U94848, AF198100 and M34368.

[0024] The gene nomenclature used herein is that of Copenhagen vaccinia strain and is used also for the homologous genes of other poxviridae unless otherwise indicated. However, gene nomenclature may be different according to the pox strain. For information, correspondance between Copenhagen and MVA genes can be found in Table I of ANTOINE. . *Virology.* 1998, no.244, p.365-396. .

[0025] According to a preferred embodiment, the poxvirus of the invention further comprises a defective J2R gene.

[0026] The J2R gene encodes a Thymidine kinase (TK) which form part of the salvage pathway for pyrimidine deoxyribonucleotide synthesis. The reaction catalysed by TK involves the transfer of a γ-phosphoryl moiety from ATP to 2'deoxy-thymidine (dThd) to produce thymidine 5'-monophosphate (dTMP). Vaccinia virus' TK is of type 2. Type 2 TKs have a smaller polypeptide chain compared to type 1, being of ~25 KDa but

form homotetramers. They are sensitive to the feedback inhibitors dTDP or dTTP, which are generated at the end of the metabolic pathway. Type 2 TKs have a much narrower substrate specificity compared to type 1 TKs and only phosphorylate 2'deoxyuridine (dU) and/or dThd (EL OMARI, et al. Structure of vaccinia virus thymidine kinase in complex with dTTP: insights for drug design. *BMC structural biology.* 2006, no.6, p.22.).

[0027] Poxviruses defective for the J2R region and methods to obtain them are available in the art. For example, the teaching of MCCART, et al. Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *cancer research.* 2001, vol.61, no.24, p.8751-7. , PUHLMANN, et al. Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. *Cancer gene therapy.* 2000, vol.7, no.1, p.66-73. , GNANT, et al. Systemic administration of a recombinant vaccinia virus expressing the cytosine deaminase gene and subsequent treatment with 5-fluorocytosine leads to tumor-specific gene expression and prolongation of survival in mice. *Cancer Research .* 1999, vol.59, no.14, p.3396-403. can be used to produced a poxviruses deleted for the J2R region.

[0028] According to a preferred embodiment, the poxvirus according to the invention further comprises a nucleic acid of interest.

[0029] In a preferred embodiment, the nucleic acid of interest contains at least one sequence of interest encoding a gene product which is a therapeutic molecule (i.e. a therapeutic gene). A "therapeutic molecule" is one which has a pharmacological or protective activity when administered appropriately to a patient, especially patient suffering from a disease or illness condition or who should be protected against this disease or condition. Such a pharmacological or protective activity is one which is expected to be related to a beneficial effect on the course or a symptom of said disease or said condition. When the skilled man selects in the course of the present invention a gene encoding a therapeutic molecule, he generally relates his choice to results previously obtained and can reasonably expect, without undue experiment other than practicing the invention as claimed, to obtain such pharmacological property. According

to the invention, the sequence of interest can be homologous or heterologous to the target cells into which it is introduced. Advantageously said sequence of interest encodes all or part of a polypeptide, especially a therapeutic or prophylactic polypeptide giving a therapeutic or prophylactic property. A polypeptide is understood to be any translational product of a polynucleotide regardless of size, and whether glycosylated or not, and includes peptides and proteins. Therapeutic polypeptides include as a primary example those polypeptides that can compensate for defective or deficient proteins in an animal or human organism, or those that act through toxic effects to limit or remove harmful cells from the body. They can also be immunity conferring polypeptides which act as endogenous antigen to provoke a humoral or cellular response, or both.

[0030] Examples of polypeptides encoded by a therapeutic gene include genes coding for a cytokine (alpha, beta or gamma interferon, interleukin, in particular IL-2, IL-6, IL-10 or IL-12, a tumor necrosis factor (TNF), a colony stimulating factor GM-CSF, C-CSF, M-CSF...), a immunostimulatory polypeptide (B7.1, B7.2 and the like), a coagulation factor (FVIII, FIX...), a growth factor (Transforming Growth Factor TGF, Fibroblast Growth Factor FGF and the like), an enzyme (urease, renin, thrombin, metalloproteinase, nitric oxide synthase NOS, SOD, catalase...), an enzyme inhibitor (alpha1-antitrypsin, antithrombin III, viral protease inhibitor, plasminogen activator inhibitor PAI-1), the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein, insulin, dystrophin, a MHC antigen of class I or II, a polypeptide that can modulate/regulate expression of cellular genes, a polypeptide capable of inhibiting a bacterial, parasitic or viral infection or its development (antigenic polypeptides, antigenic epitopes, transdominant variants inhibiting the action of a native protein by competition....), an apoptosis inducer or inhibitor (Bax, Bcl2, BclX...), a cytostatic agent (p21, p 16, Rb...), an apolipoprotein (ApoAI, ApoAIV, ApoE...), an inhibitor of angiogenesis (angiostatin, endostatin...), an angiogenic polypeptide (family of Vascular Endothelial Growth Factors VEGF, FGF family, CCN family including CTGF, Cyr61 and Nov), an oxygen radical scaveyer, a polypeptide having an anti-tumor effect, an antibody, a toxin, an

immunotoxin and a marker (beta-galactosidase, luciferase....) or any other genes of interest that are recognized in the art as being useful for the treatment or prevention of a clinical condition.

[0031] Suitable anti-tumor genes include but are not limited to those encoding tumor suppressor genes (e.g. Rb, p53, DCC, NF-1, Wilm's tumor, NM23, BRUSH-1, p16, p21, p56, p73 as well as their repective mutants), suicide gene products, antibodies, olypeptides inhibiting cellular division or transduction signals.

[0032] According to a particularly preferred embodiment, the poxvirus of the invention further comprises a suicide gene.

[0033] Suicide gene refers to a gene coding a protein able to convert a precursor of a drug into a cytoxic compound.

[0034] Suicide genes comprised but are not limited to genes coding protein having a cytosine deaminase activity, a thymidine kinase activity, an uracil phosphoribosyl transferase activity, a purine nucleoside phosphorylase activity and/or a thymidylate kinase activity.

[0035] Examples of Suicide genes and corresponding precursors of a drug comprising one nucleobase moiety are disclosed in the following table :

[0036]

Table 1

Suicide gene	predrug
Thymidine Kinase	Ganciclovir; Ganciclovir elaidic acid ester; penciclovir; Acyclovir; Valacyclovir; (E)-5-(2-bromovinyl)-2'-deoxyuridine; zidovudine; 2'-Exo-methanocarbathymidine
Cytosine deaminase	5-Fluorocytosine
Purine nucleoside phosphorylase	6-Methylpurine deoxyriboside; Fludarabine
uracil phosphoribosyl transferase	5-Fluorocytosine; 5-Fluorouracil

Suicide gene	predrug
thymidylate kinase.	Azidothymidine

[0037] According to a preferred embodiment of the invention, the suicide gene codes a protein having at least a CDase activity. CDase is involved in the pyrimidine metabolic pathway by which exogenous cytosine is transformed into uracil by means of a hydrolytic deamination. While CDase activities have been demonstrated in prokaryotes and lower eukaryotes (JUND, et al. . *Journal of Bacteriology.* 1970, no.102, p.607-15. ; BECK, et al. . *Journal of Bacteriology.* 1972, no.110, p.219-28. ; HOEPRICH, et al. . *Journal of Infectious Diseases.* 1974, no.130, p.112-18. ; ESDERS, et al. . *J. biol. chem..* 1985, no.260, p.3915-22.), they are not present in mammals (KOECHLIN, et al. . *Biochemical pharmacology.* 1966, no.15, p.435-46. ; POLAK, et al. . *Chemotherapy.* 1976, no.22, p.137-53.).

[0038] CDase also deaminates an analogue of cytosine, i.e. 5-fluorocytosine (5-FC), thereby forming 5-fluorouracil (5-FU), which is a compound which is highly cytotoxic when it is converted into 5-fluoro-UMP (5-FUMP). Cells which lack CDase activity, either because of a mutation which inactivates the gene encoding the enzyme or because they are naturally deficient in this enzyme, as are mammalian cells, are resistant to 5-FC (JUND, et al. . *Journal of Bacteriology.* 1970, no.102, p.607-15. ; KILLSTRUP, et al. . *Journal of Bacteriology.* 1989, no.171, p.2124-2127.). By contrast, mammalian cells into which the sequences encoding CDase activity were transferred became sensitive to 5-FC (HUBER, et al. . *Cancer Research.* 1993, no.53, p.4619-4626. ; MULLEN, et al. . *Proceedings of the National Academy of Sciences of the United States of America.* 1992, no.89, p.33-37. ; WO 93/01281 (US HEALTH)). In addition, the neighboring, untransformed cells also become sensitive to 5-FC (HUBER, et al. . *Proceedings of the National Academy of Sciences of the United States of America.* 1994, no.91, p.8302-6.). This phenomenon, which is termed a bystander effect, is due to the cells which are expressing the CDase activity secreting 5-FU, which then intoxicates the neighboring cells by straightforward diffusion across the plasma membrane. This property of 5-FU in diffusing passively represents an advantage as compared with the

tk/GCV reference system, where the bystander effect requires there to be contact with the cells which are expressing tk (MESNIL, et al. . *Proceedings of the National Academy of Sciences of the United States of America.* 1996, no.93, p.1831-35.). All the advantages which CDase offers within the context of gene therapy, in particular anticancer gene therapy, can therefore be readily understood.

[0039] The Saccharomyces cerevisiae (S. cerevisiae) FCY1, Candida Albicans FCA1 and the E. coli codA genes, which respectively encode the CDase of these two organisms, are known and their sequences have been published (SEQ ID N°:4; SEQ ID N°:5; SEQ ID N°:6 respectively).

[0040] With this respect, according to a more preferred embodiment of the invention, the gene coding a protein having a CDase activity is FCY1, FCA1 or CodA or an analogue thereof. Analogues of these genes refers to a gene having an nucleic acid sequence which have at least a degree of identity greater than 70%, advantageously greater than 80%, preferably greater than 90%, and most preferably greater than 95% with the nucleic acid sequence of the parent gene.

[0041] Patent WO 2005/007857 discloses a gene coding a protein having an improved CDase activity. This polypeptides derived from a native CDase by addition of an amino acid sequence. According to another preferred embodiment of the invention, the protein having a CDase activity is a polypeptide disclosed WO 2005/007857 and more preferably the FCU1-8 polypeptide represented in the sequence identifier SEQ ID N°:2 and analogues thereof.

[0042] In prokaryotes and lower eukaryotes, uracil is transformed into UMP by the action of uracil phosphoribosyl transferase (UPRTase). This enzyme converts 5-FU into 5-FUMP. According to another preferred embodiment of the invention, the suicide gene codes a protein having an UPRTase activity.

[0043] The UPRTase in question may be of any origin, in particular of prokaryotic, fungal or yeast origin. By way of illustration, the nucleic acid sequences encoding the UPRTases from *E. coli* (ANDERSEN, et al. Characterization of the upp gene encoding uracil phosphoribosyltransferase of Escherichia

coli K12. *European Journal of Biochemistry.* 1992, no.204, p.51-56.), from *Lactococcus lactis* (MARTINUSSEN, et al. Cloning and characterization of upp, a gene encoding uracil phosphoribosyltransferase from Lactococcus lactis. *Journal of Bacteriology.* 1994, vol.176, no.21, p.6457-63.), from *Mycobacterium bovis* (KIM, et al. Complete sequence of the UPP gene encoding uracil phosphoribosyltransferase from Mycobacterium bovis BCG. *Biochemistry and molecular biology international.* 1997, vol.41, no.6, p.1117-24.) and from *Bacillus subtilis* (MARTINUSSEN, et al. Two genes encoding uracil phosphoribosyltransferase are present in Bacillus subtilis. *Journal of Bacteriology.* 1995, vol.177, no.1, p.271-4.) may be used in the context of the invention. However, it is most particularly preferred to use a yeast UPRTase and in particular that encoded by the *S. cerevisiae FUR1* gene whose sequence disclosed in KERN, et al. The FUR1 gene of Saccharomyces cerevisiae: cloning, structure and expression of wild-type and mutant alleles. *Gene.* 1990, vol.88, no.2, p.149-57. is introduced here by way of reference. As a guide, the sequences of the genes and those of the corresponding UPRTases may be found in the literature and the specialist databanks (SWISSPROT, EMBL, Genbank, Medline and the like).

[0044] Application EP 0998568 A describes an *FUR1* gene lacking 105 nucleotides in 5' of the coding part allowing the synthesis of a UPRTase from which the 35 first residues have been deleted at the N-terminal position and starting with the methionine at position 36 in the native protein. The product of expression of the mutant gene, designated *FUR1Δ105*, is capable of complementing an *fur1* mutant of *S. cerevisiae*. In addition, the truncated mutant exhibits a higher UPRTase activity than that of the native enzyme. Thus, according to a particularly advantageous embodiment of the invention, the suicide gene codes a deletion mutant of a native UPRTase. The deletion is preferably located in the N-terminal region of the original UPRTase. It may be complete (affecting all the residues of said N-terminal region) or partial (affecting one or more continuous or discontinuous residues in the primary structure). In general, a polypeptide consists of N-terminal, central and C-terminal parts, each

representing about a third of the molecule. For example, since the *S. cerevisiae* UPRTase has 251 amino acids, its N-terminal part consists of the first 83 residues starting with the so-called initiator methionine situated at the first position of the native form. As for the *E. coli* UPRTase, its N-terminal part covers positions 1 to 69.

[0045] A preferred protein having an UPRTase activity comprises an amino acid sequence substantially as represented in the sequence identifier SEQ ID N°: 1 of EP 0998568 A , starting with the Met residue at position 1 and ending with the Val residue at position 216. The term "substantially" refers to a degree of identity with said sequence SEQ ID N°: 1 EP 0998568 A greater than 70%, advantageously greater than 80%, preferably greater than 90%, and most preferably greater than 95%. More preferably still, it comprises the amino acid sequence represented in the sequence identifier SEQ ID N°: 1 EP 0998568 A . As mentioned above, it may comprise additional mutations. There may be mentioned in particular the substitution of the serine residue at position 2 (position 37 in the native UPRTase) by an alanine residue.

[0046] According to another preferred embodiment of the invention, the suicide gene codes a protein having at least one CDase and one UPRTase activity. Patent applications WO 96/16183 and EP 0998568 A describe the use of a fusion protein encoding an enzyme with two domains having the CDase and UPRTase activities and demonstrate that the transfer of a hybrid gene *codA::upp* or *FCY1::FUR1* or *FCY1::FUR1Δ105 (i.e. FCU1)* carried by an expression plasmid increases the sensitivity of the transfected B16 cells to 5-FC. According to a more preferred embodiment of the invention, the suicide gene codes a polypeptide comprising an amino acid sequence substantially as represented in the sequence identifier SEQ ID N°:3 (coda::upp), SEQ ID N°:1 (FCU1) or FCY1::FUR1. The term "substantially" refers to a degree of identity with said sequence greater than 70%, advantageously greater than 80%, preferably greater than 90%, and most preferably greater than 95%. More preferably still, it comprises the amino acid sequence as represented in the sequence

identifier SEQ ID N°:3 (coda::upp), SEQ ID N°:1 (FCU1) or FCY1::FUR1. As mentioned above, it may comprise additional mutations.

[0047] The nucleic acid sequences may be easily obtained by cloning, by PCR or by chemical synthesis according to the conventional techniques in use. They may be native genes or genes derived from the latter by mutation, deletion, substitution and/or addition of one or more nucleotides. Moreover, their sequences are widely described in the literature which can be consulted by persons skilled in the art.

[0048] Persons skilled in the art are capable of cloning the CDase or UPRTase sequences from the published data and of carrying out possible mutations, of testing the enzymatic activity of the mutant forms in an acellular or cellular system according to the prior art technology or based on the protocol indicated in application EP 0998568 A , and of fusing, in particular in phase, the polypeptides with CDase and UPRTase activity, and consequently all or part of the corresponding genes.

[0049] According to a more preferred embodiment, the poxvirus of the invention further comprises a nucleic acid sequence comprising a gene coding a permease.

[0050] Permease refers to transmembraneous protein involved in the transfer of a drug comprising one nucleobase moiety, or a precursor thereof through the cell membrane.

[0051] Permease comprises but are limited to purine permease, cytosine permease and nucleoside transporters.

[0052] According to a preferred embodiment of the invention, permease is a purine or a cytosine permease of *S. Cerevisiae*. The nucleobase transporters of *S. cerevisiae* consist of the purine-cytosine permease, known as FCY2, and the uracil permease, known as FUR4 . The purine-cytosine permease, FCY2 mediates symport of protons and adenine, guanine, hypoxanthine and cytosine across the yeast plasma membrane (Grenson 1969, Jund and Lacroute 1970, Polak and Grenson 1973, Chevallier et al. 1975, Hopkins et al. 1988). FCY2 protein mediates also the transport of 5-fluorocytosine, an analogue of cytosine (Grenson 1969, Jund and Lacroute 1970). FCY2 gene encodes a protein of 533 amino

acids (58 kDa) initially predicted to have 10-12 transmembrane–spanning domains (**Weber et al.** 1990), with nine now favoured (**Ferreira et al.** 1999). FCY2 exhibits similar affinities for the purine nucleobases and cytosine (**Brethes et al.** 1992).Uracil uptake into *S. cerevisiae* is mediated by the uracil permease, FUR4 (**Jund and Lacroute** 1970, **Jund et al.** 1977). FUR4 is a uracil-proton symporter (**Hopkins et al.** 1988) predicted to be a protein of 633 amino acids (71.7 kDa) with 10 transmembrane domains and long cytoplasmic hydrophylic N- and C-terminal tails (**Jund et al.** 1988, **Garnier et al.** 1996). FUR4 protein can also mediates the transport of 5-fluorouracil, an analogue of uracil (**Jund and Lacroute** 1970).

[0053] Amino acid sequences of FCY2 and Fur4 are notably available in the swissprot database (accession number P17064 and P05316 respectively). Preferably, permease has an amino acid sequence chosen from the group comprising amino acid sequence SEQ ID N0:1 and SEQ ID N0:2 as disclosed in patent application **WO 2006/048768**.

[0054] With this respect, according to a preferred embodiment of the invention, the permease is chosen from the group comprising FCY2 and Fur4 and analogues thereof. Analogues of Fur4 and FCY2 refers to polypeptide having an amino acid sequence which have at least a degree of identity greater than 70%, advantageously greater than 80%, preferably greater than 90%, and most preferably greater than 95% with the amino acid sequence of the parent protein as described here above and which retains the ability to transport a drug comprising one nucleobase moiety through the cell membrane.

[0055] The one skilled in the art is able to choose the permease which will be associated with the drug or the precursor of the drug comprising one nucleobase moiety. For example, FCY2 and Fur4 are preferably associated with 5-Fluorocytosine (5-FC).

[0056] According to a more preferred embodiment, the poxvirus of the invention may further comprise the elements necessary for the expression of the nucleic acid of interest.

[0057] According to a more preferred embodiment, the poxvirus of the invention may further comprise the elements necessary for the expression of the nucleic acid sequence comprising a gene coding a permease.

[0058] These elements necessary for the expression of the nucleic acid of interest and/or the nucleic acid sequence comprising a gene coding a permease comprised the elements required for transcription of said DNA into mRNA and, if necessary, for translation of mRNA into polypeptide. Transcriptional promoters suitable for use in various vertebrate systems are widely described in literature. For example, suitable promoters include viral promoters like RSV, MPSV, SV40, CMV or 7.5k, vaccinia promoter, inducible promoters, etc. Preferred promoters are isolated from poxviruses e.g. 7.5K, H5R, TK, p28, p11 or K1L of vaccinia virus. Alternatively, one may use a synthetic promoter such as those described in CHAKRABARTI. . *Biotechniques.* 1997, no.23, p.1094-97. , HAMMOND, et al. . *Journal of Virological Methods.* 1997, no.66, p.135-38. and KUMAR. . *Virology.* 1990, no.179, p.151-8. as well as chimeric promoters between early and late poxviral promoters.

[0059] The nucleic acid sequence of interest and the nucleic acid sequence comprising a gene coding a permease may further include additional functional elements, such as intron sequences, targeting sequences, transport sequences, secretion signal, nuclear localization signal, IRES, poly A transcription termination sequences, tripartite leader sequences, sequences involved in replication or integration. Said sequences have been reported in the literature and can be readily obtained by those skilled in the art.

[0060] The invention also relates to a process for preparing a poxvirus according to the invention, in which process:

[0061] (i) a poxvirus according to the invention is introduced into a cell,

[0062] (ii) said cell is cultured under conditions which are appropriate for enabling said poxvirus to be produced, and

[0063] (iii) said poxvirus is recovered from the cell culture.

[0064] While the poxvirus can of course be recovered from the culture supernatant, it can also be recovered from the cells. One of the commonly

employed methods consists in lysing the cells by means of consecutive freezing/thawing cycles in order to collect the virions in the lysis supernatant. The virions can then be amplified and purified using the techniques of the art (chromatographic method, method of ultra-centrifugation, in particular through a cesium chloride gradient, etc.).

[0065] The present invention, also relates to a composition which comprises a poxvirus according to the invention in combination with a pharmaceutically acceptable excipient.

[0066] A composition according to the invention is more specifically intended for the preventive or curative treatment of diseases by means of gene therapy and is more specifically aimed at proliferative diseases (cancers, tumors, restenosis, etc.) or aimed at diseases associated to an increased osteoclast activity (e.g. rheumatoid arthritis, osteoporosis).

[0067] A composition according to the invention can be made conventionally with a view to administering it locally, parenterally or by the digestive route. In particular, a therapeutically effective quantity of the recombinant vector or poxvirus of the invention is combined with a pharmaceutically acceptable excipient. It is possible to envisage a large number of routes of administration. Examples which may be mentioned are the intragastric, subcutaneous, intracardiac, intramuscular, intravenous, intraperitoneal, intratumor, intranasal, intrapulmonary and intratracheal routes. In the case of these three latter embodiments, it is advantageous for administration to take place by means of an aerosol or by means of instillation. The administration can take place as a single dose or as a dose which is repeated on one or more occasions after a particular time interval. The appropriate route of administration and dosage vary depending on a variety of parameters, for example the individual, the disease to be treated or the gene(s) of interest to be transferred. The preparations based on viral particles according to the invention can be formulated in the form of doses of between 10^4 and 10^{14} pfu (plaque-forming units), advantageously 10^5 and 10^{13} pfu, preferably 10^6 and 10^{12} pfu, more preferably 10^6 and 10^7.

[0068] The composition can also include a diluent, an adjuvant or an excipient which is acceptable from the pharmaceutical point of view, as well as

solubilizing, stabilizing and preserving agents. In the case of an injectable administration, preference is given to a formulation in an aqueous, non-aqueous or isotonic solution. It can be presented as a single dose or as a multidose, in liquid or dry (powder, lyophilizate, etc.) form which can be reconstituted at the time of use using an appropriate diluent.

[0069] The present invention also relates to the use of a poxvirus or a composition according to the invention for preparing a medicament which is intended for treating the human or animal body by gene therapy. The medicament can be administered directly *in vivo* (for example by intravenous injection, into an accessible tumor, into the lungs by means of an aerosol, into the vascular system using an appropriate catheter, etc.). A preferred use consists in treating or preventing cancers, tumors and diseases which result from unwanted cell proliferation. Conceivable applications which may be mentioned are cancers of the breast, of the uterus (in particular those induced by papilloma viruses), of the prostate, of the lung, of the bladder, of the liver, of the colon, of the pancreas, of the stomach, of the oesophagus, of the larynx, of the central nervous system (e.g. glioblastoma) and of the blood (lymphomas, leukemia, etc.). An other preferred use consists in treating or preventing rheumatoid arthritis, osteoporosis and other diseases associated to an increased osteoclast activity. It can also be used in the context of cardiovascular diseases, for example in order to inhibit or retard the proliferation of the smooth muscle cells of the blood vessel wall (restenosis). Finally, in the case of infectious diseases, it is possible to conceive of the medicament being applied to AIDS.

[0070] When the poxvirus, composition or method of the invention is used for the treatment of cancer, the preferred route of administration is the systemic route since the poxvirus according to the invention is able to specifically target the tumoral cells.

[0071] The invention also extends to a method for treating diseases characterized in that a poxvirus, a composition according to the invention is administered to an host organism or cell which is in need of such treatment.

[0072] According to an advantageous embodiment, the therapeutic use or the treatment method also comprises an additional step in which pharmaceutically acceptable quantities of a prodrug, advantageously an analog of cytosine, in particular 5-FC, are administered to the host organism or cell. By way of illustration, it is possible to use a dose of from 50 to 500 mg/kg/day, with a dose of 200 mg/kg/day or of 100 mg/kg/day being preferred. Within the context of the present invention, the prodrug is administered in accordance with standard practice (e.g. per os, systematically).

[0073] Preferably, the administration taking place subsequent to the administration of the poxvirus or composition according to the invention, preferably at least 3 days, more preferably at least 4 days and even more preferably at least 5 days after the administration of the poxvirus or composition according to the invention. According to an even more preferred embodiment of the invention, the administration of the prodrug takes place 7 days after the administration of the therapeutic agent. The oral route is preferred. It is possible to administer a single dose of prodrug or doses which are repeated for a time which is sufficiently long to enable the toxic metabolite to be produced within the host organism or cell.

[0074] Furthermore, the composition or method according to the invention can be combined with one or more substances which potentiate the cytotoxic effect of the 5-FU. Mention may in particular be made of drugs which inhibit the enzymes of the pathway for the de novo biosynthesis of the pyrimidines (for example those mentioned below), drugs such as Leucovorin (Waxman et al., 1982, Eur. J. Cancer Clin. Oncol. 18, 685-692), which, in the presence of the product of the metabolism of 5-FU (5-FdUMP), increases the inhibition of thymidylate synthase, resulting in a decrease in the pool of dTMP, which is required for replication, and finally drugs such as methotrexate (Cadman et al., 1979, Science 250, 1135-1137) which, by inhibiting dihydrofolate reductase and increasing the pool of PRPP (phosphoribosylpyrophosphate), brings about an increase in the incorporation of 5-FU into the cellular RNA.

[0075] According to the present invention, the drugs which inhibit the enzymes of the pathway for the *de novo* biosynthesis of the pyrimidines are preferably selected from the group consisting of PALA (N-(phosphonoacetyl)-L-aspartate; **Moore et al.**, 1982, Biochem. Pharmacol. 31, 3317-3321), Leflunomide, A771726 (active metabolite of Leflunomide; **Davis et al.**, 1996, Biochem. 35, 1270-1273) and Brequinar (**Chen et al.**, 1992, Cancer Res. 52, 3251-3257).

[0076] The composition or method according to the invention can be combined with one or more substances effective in anticancer therapy. Among pharmaceutical substances effective in anticancer therapy which may be used in association or in combination with the compositions according to the invention, there may be mentioned alkylating agents such as, e.g., mitomycin C, cyclophosphamide, busulfan, ifosfamide, isosfamide, melphalan, hexamethylmelamine, thiotepa, chlorambucil, or dacarbazine; antimetabolites such as, e.g., gemcitabine, capecitabine, 5-fluorouracil, cytarabine, 2-fluorodeoxy cytidine, methotrexate, idatrexate, tomudex or trimetrexate; topoisomerase II inhibitors such as, e.g., doxorubicin, epirubicin, etoposide, teniposide or mitoxantrone; topoisomerase I inhibitors such as, e.g., irinotecan (CPT-11), 7-ethyl-10-hydroxy-camptothecin (SN-38) or topotecan; antimitotic drugs such as, e.g., paclitaxel, docetaxel, vinblastine, vincristine or vinorelbine; and platinum derivatives such as, e.g., cisplatin, oxaliplatin, spiroplatinum or carboplatinum.

[0077] The compositions or methods according to the invention can also be use in combination with radiotherapy.

[0078] The compositions or methods according to the invention may also be use in combination with one or more other agents including but not limited to immunomodulatory agents such as, e.g. alpha, beta or gamma interferon, interleukin (in particular IL-2, IL-6, IL-10 or IL-12) or tumor necrosis factor; agents that affect the regulation of cell surface receptors such as, e.g. inhibitors of Epidermal Growth Factor Receptor (in particular cetuximab, panitumumab, zalutumumab, nimotuzumab, matuzumab, gefitinib, erlotinib or lapatinib) or inhibitors of Human Epidermal Growth Factor Receptor-2

(in particular trastuzumab); and agents that affect angiogenesis such as, e.g. inhibitor of Vascular Endothelial Growth Factor (in particular bevacizumab or ranibizumab).

Brief Description of Figures in the Drawings

[0079] Figure 1. In vitro Sensitivities to 5-FC of vaccinia viruses infected human colorectal tumor cells (LoVo). LoVo cells, infected at a MOI of 0.0001 with the indicated viruses (mock (•) VVTK-/FCU1 (■) or VVTK-F2L-/FCU1 (◇)) were exposed to various concentration of 5-FC. Cell survival was measured at 5 days post-infection. Results were expressed in percentage of cellular viability in the presence or not of drugs. Values are represented in mean ± SD of three individual determinations without the cell mortality due to the replication of the viruses.

[0080] Figure 2. In vitro replication efficacy of viruses in LoVo infected at a MOI of 0:0001 with the indicated viruses at day 5 post infection. Values are represented in mean ± SD of three individual determinations.

[0081] Figure 3. Mean tumor volume ± SEM of s.c LoVo in Swiss nude mice after i.v injection of virus. 7 days after inoculation with tumor (palpable tumor), mice were treated by 10^7 pfu of buffer + saline (◊), buffer + 5-FC (♦), VVTK-F2L-/FCU1 + saline (□) or VVTK-F2L-/FCU1 + 5-FC (■). The animals were treated by saline or 5-FC at 100 mg/kg/j twice a day by oral gavage, 7 days after virus injection during 3 weeks. Tumor volume was measured twice a week.

[0082] Figure 4. Mean volume ± SEM of s.c HepG2 tumors in Swiss nude mice after i.v injection of virus. 14 days after inoculation with tumors (palpable tumor), mice were treated by buffer + water (◊), or buffer + 5-FC (♦), or 10^6 pfu of VVTK-/FCU1 + water (o), or 10^6 pfu of VVTK-/FCU1 + 5-FC (•) (A); or buffer + water (◊), or buffer + 5-FC (♦) or 10^6 pfu of VVTK-F2L-/FCU1 + water (◊), or 10^6 pfu of VVTK-F2L-/FCU1 + 5-FC (♦) (B). The animals were treated by 5-FC at 100 mg/kg twice a day by oral gavage, 7 days after virus injection and during 3 weeks. Tumor volume was measured twice a week.

[0083] Figure 5. Ratio of virus yield in dividing cells versus in confluent cells. PANC1 (pancreatic human tumor), H1299 (Lungs human tumor) or

U118MG (glioma human tumor) cells are infected with 100 pfu of (▮) VVTK-/FCU1 or (▯) VVTK-F2L-/FCU1. 48h post-infection, viral titers were determined. Values are the ratio between yields of virus in dividing cells versus in confluent cells.

[0084] Figure 6. Viral titers (pfu/mg of tissue) in organs or tumors at day 6 and day 21 after i.v. infection into Swiss nude mice bearing subcutaneous human tumors with $1x10^6$ PFU of VVTK-/FCU1 (▮) or VVTK-F2L-/FCU1 (▯).

[0085] Figure 7. Survival of Swiss nude mice after treatment with $1x10^8$ pfu of VVTK-/FCU1 (■) or VVTK-F2L-/FCU1 (◇) by i.v injection.

[0086] Figure 8. Survival of immunocompetent B6D2 mice after treatment with $1x10^7$ pfu (A) or $1x10^8$ pfu (B) of VVTK-/FCU1 (■) or VVTK-F2L-/FCU1 (◇) by i.v injection.

[0087] Figure 9. Average quantity of pocks on tails after i.v injection of $1x10^6$ pfu VVTK-/FCU1 or VVTK-F2L-/FCU1 in Swiss nude mice at day 13 post-infection and at day 34 post-infection.

[0088] Figure 10. Average quantity of pocks on tails after i.v injection of $1x10^7$ pfu VVTK-/FCU1 or VVTK-F2L-/FCU1 in Swiss nude mice at day 15 post-infection and at day 31 post-infection.

Mode(s) for Carrying Out the Invention

Examples

Construction of vector plasmids

[0089] A shuttle plasmid for deleting F2L was constructed using the DNA of vaccinia virus strain Copenhagen (accession number M35027). The DNA flanking regions of F2L were amplified by PCR. Primers of the upstream flanking region of F2L were 5'- CGC GGA TCC GAA AGC GAT GAA CTA AAT GTT C - 3' (SEQ ID N°:7; *BamHI* site underlined) and 5'- TCC CCC GGG GTT AGT TTC CTT AAC AAA TCT AAC -3' (SEQ ID N°:8; *SmaI* site underlined). Primers for the downstream region were 5'– GCC TGG CCA ACA AAT AGA GGA GAT CAA GGG T – 3' (SEQ ID N°:9; *MscI* site underlined) and 5' – GCC CAG CTG ACC ACT ACA TCA ATT TTA CAA AAG – 3' (SEQ ID N°:10; *PvuII* site underlined). The amplified DNA fragment were digested with restriction enzyme *SmaI/BamHI* or *MscI/PvuII*

and ligated into the corresponding sites in *Ppolylll* plasmid. A repeat region of the downstream flanking region of F2L was amplified by PCR using the primers 5'- GCC GCA TGC TCC AGA ATT GAT CAT AGT GGA TA - 3' (SEQ ID N°:11; *Sphl* site underlined) and 5' – GCT CTA GAG TTA GTT TCC TTA ACA AAT CTA AC – 3' (SEQ ID N°:12; *Xbal* site underlined) and inserted in *Ppolylll* plasmid. The repeat region is used to eliminate the selection cassette during the production of deleted viruses. The selection cassette, corresponding to the GFP/GPT fusion gene under the control of pH5R vaccinia promoter, was inserted into the *SmaI/SphI* site in *Ppolylll* plasmid. The obtained plasmid is the recombinant shuttle plasmid named pΔF2L for deletion of F2L gene.

The generation of recombinant vaccinia viruses.

[0090] CEF cells were infected with VVTK-FCU1(Vaccinia virus, defective for the J2R gene, expressing *FCU1* gene under the control of synthetic promoter p11k7.5) strain Copenhagen at a MOI of 0.1 and incubated at 37°C for 2 h, then transfected with a $CaCl_2$ coprecipitate of the recombinant shuttle plasmid (0.2 µg). The cells were incubated for 48 h at 37°C. Dilutions of virus emerging were then used to infect the CEF cells in selection medium containing Hypoxanthine at final concentration of 15 µg/ml xanthine at final concentration of 250 µg/ml and mycophenolic acide at final concentration of 250 µg/ml. Fluorescent (GFP) and positive (GPT selection) plaques were isolated and selected for a several round of selection in CEF cells in presence of GPT selection medium. The presence or not of VVTk-FCU1 was determined by 40 cycles of PCR with primers inside the deletion region. After the elimination of parental virus, the double deleted virus was used to infect CEF without GPT selection medium to eliminate the selection cassette. Non-fluorescent plaques were isolated and selected for 2 cycles in CEF. Final recombinant VV viruses were amplified in CEF, purified and virus stocks were titrated on CEF by plaque assay.

In vitro cell sensitivity to 5-FC.

[0091] Human tumor cells were transduced by the respective recombinant VV at a MOI of 0.0001. A total of 3×10^5 cells/well were plated in 6-well culture dishes in 2 ml of medium containing various concentrations of 5-FC. Cells

were then cultured at 37°C for 5 days, and the viable cells were counted by trypan blue exclusion. Results depicted in figure 1 and figure 2 shows that the FCU1 activity is equivalent in viruses defective for the J2R gene than in virus defective for the J2R and F2L gene.

In vitro replication in cultured cells.

[0092] Dividing or confluent cells were infected, in 6-wells plaques, at 100 PFU of viruses (nearly MOI 0.0005). 2 mL of medium supplemented with 10% FCS for dividing cells and no supplemented for confluent cells were added. The cells were harvested at 48 hours post-infection. The cells were stored at -20°C and sonicated to release the virus, virus was also quantified by plaque titration on CEF cells. The ratio between replication in dividing cells and confluent cells are similar in all cells. Both viruses VVTK-/FCU1 and VVTK-F2L-/FCU1 replicate more in dividing cells than in confluent cells.

[0093] As an indirect mean to assay for replication virus specificity, the yield of virus produced in dividing versus confluent tumor cells (pancreatic human tumor PANC1; lung human tumor H1299; glioma human tumor U118MG) was determined. Confluent cells were plated at $1x10^6$ cells/well and cultured in complete media for 7 days then 1 day before infection the cells were washed and cultured in media without serum. Dividing cells were plated at $3x10^5$ cells/well one day before infection. To evaluate the level of cell division, the amount of titrated thymidine incorporated into nucleic acid was measured 5 hours, 24 hours and 48 hours after plating cells. During this period thymidine incorporation was relatively constant in confluent cells whereas in dividing cells an increase in incorporation was seen over time. Then the cells were infected with 100 pfu of viruses, and 48h post infection the ratio between the yield of virus produced in dividing tumor cells and in confluent tumor cells was determined by plaque titration on CEF. Results depicted in figure 5 show that both viruses VVTK-/FCU1 and VVTK-F2L-/FCU1 replicate more in dividing cells than in confluent cells.

Subcutaneous tumor model.

[0094] Female Swiss nude mice were obtained from Charles River Laboratories. Animals used in the studies were uniform in age (6 weeks) and body

weights ranged from 20-23 g. Swiss nude mice were injected subcutaneously (s.c.) into the flank with $5x10^6$ LoVo cells/100 µL. When tumors reached a diameter of 50-70 mm^3, the mice were randomized in a blinded manner and treated with the indicated vectors for the *in vivo* experiments.

Biodistribution of the virus.

[0095] The presence of VV-FCU1 and VVTK-F2L-/FCU1 was evaluated by virus titration in tumors and organ samples. $1x10^6$ PFU of the viruses was injected intravenously (i.v.) by tail vein injection into nude mice bearing established s.c. LoVo tumors. Mice were sacrificed at day 14 post-infection, and the tumors and other organs were collected and weighted. Tumors and organs were homogenized in PBS and titers were determined on CEF as described previously. Viral titers were standardized to milligram of tissue. Results depicted in Table 2 and 3 (The range of virus titers is presented in pfu/mg of tissue) show that after 14 days the virus according to the invention is mostly found in the tumor. In a second set of experimentations according to the same conditions as described above, mice were sacrificed at day 6 and 21 post-infection. Results depicted in figure 6 show that both viruses VVTK-/FCU1 and VVTK-F2L-/FCU1 target the tumor with about 1 000 to 10 000 fold more virus in the tumor than in the other organs analyzed except for tails in the case of VVTK-/FCU1. A small amount of VVTK-/FCU1 is detected in lungs, spleen, kidney and lymph nodes (less than 10 pfu/mg) and more in skin, tail and bone marrow at day 6, and skin and tail at day 21. In contrast, VVTK-F2L-/FCU1 has higher tumor specificity with only a small amount in lungs, spleen, kidney, lymph nodes and skin at day 6, and in skin and tail at day 21.

[0096]

Table 2

	Tumor	Lungs	Spleen	Kidney	Heart	L.Nodes
VVTK-/FCU1	(0,2-3,3)$x10^5$	0.1-2	0-2.2	0-1.8	0-0.3	0-61
VVTK-F2L/FCU1	0-8.1$x10^4$	0-5.7	0-9.3	0.2-3	0,2	0-83

[0097]

Table 3

	Ovaries	Skin	Tail	Bone Marrow	Brain	Muscles	Heart
VVTK-/FCU1	2.2-74	0.1-24	13.5-7.10^4	0-800	0-1.8	0-22	0-0.3
VVTK-F2L/FCU1	0-108	0-88	0.8-16.3	n.d	0-0.2	0-1.8	0,2

[0098]

Antitumor activity of the poxvirus of the invention in s.c. tumor model.

[0099] Nude mice bearing established s.c. LoVo tumors (50-70 mm^3) were treated one time intratumorally or two times intravenously (by tail vein) with the indicated vectors at dose of 1.10^4 PFU, 1.10^6 or 1.10^7 PFU, respectively. Starting day 7 following viral injection, 5-FC was given by oral gavage at 100 mg/kg (0.5 ml 5-FC 0.5% in water) twice a day for 3 weeks. Tumor size was measured twice weekly using calipers. Tumor volume were calculated in mm^3 using the formula (π/6) (length x $width^2$). The results depicted in figure 3 show that the 2 viruses have a similar efficacy with an oncolytic activity ($p<0.05$) able to control the growth of tumor, and a combined activity (oncolytic of the virus and therapeutic of FCU1 gene) with administration of 5-FC which can further improve the control of the tumor growth ($p<0.01$).

[00100] Nude mice bearing established s.c. HepG2 (Human hepatocellular liver carcinoma cells) were treated intravenously (by tail vein) with the indicated vectors at dose of 1.10^6 PFU according to the followings: 14 days after inoculation with tumors (palpable tumor), mice were treated by buffer + water, or buffer + 5-FC, or 10^6 pfu of VVTK-/FCU1 + water, or 10^6 pfu of VVTK-/FCU1 + 5-FC, or 10^6 pfu of VVTK-F2L-/FCU1 + water, or 10^6 pfu of VVTK-F2L-/FCU1 + 5-FC. The animals were treated by 5-FC at 100 mg/kg twice a day by oral gavage, 7 days after virus injection and during 3 weeks. Tumor size was measured twice weekly using calipers. Tumor volume were calculated in mm^3 using the formula (π/6) (length x $width^2$). The results depicted in figure 4 show that tumor progression is controlled

after VVTK-/FCU1 and VVTK-F2L-/FCU1 injection ($p<0.0005$) compared to buffer. The activity is not enhanced with administration of 5-FC. The virus oncolytic activity alone is very potent with only $1x10^6$ pfu of viruses injected.

Viral pathogenicity.

[00101] Viral pathogenicity was assessed with survival studies done on both Swiss nude mice (figure 7) and immunocompetents B6D2 mice (6 weeks from Charles Rivers) (figure 8). Mice were injected I.V. with 1.10^7 or 1.10^8 PFU of all viruses in 100 µL of Buffer per mouse. Mice were observed daily throughout the course of the experiment. In Swiss nude mice (figure 7), the injection of $1x10^8$ PFU of VVTK-/FCU1 results in the death of 40% of the animals 3 days after infection. The remaining mice died between day 50 and day 80 after infection. The administration of VVTK-F2L-/FCU1 was less pathogenic, the majority of the animals died between day 65 to 115 ($p<0.05$). No evidence of toxicity has been observed with both viruses at 10^7 pfu. All mice died after i.v injection of 10^8 pfu of VVTK-/FCU1. The group with treatment of VVTK-F2L-/FCU1 had significantly prolonged survival to 92% ($p<0.00005$) compared with the VVTK-/FCU1 infected mice. Therefore, this result demonstrates the decrease of toxicity with the double-deleted virus VVTK-F2L-/FCU1.

Pocks tail lesion model.

[00102] Swiss nude mice were injected I.V. with 1.10^6 (figure 9) or 1.10^7 PFU (figure 10) of each virus. Tail lesions were enumerated once a week. Mice injected with 1.10^6 PFU of VVTK-F2L-/FCU1 have less than 1 pocks/mice compared with mice injected with VVTK-/FCU1 with a average of 8 pocks by mice in day 13 post-infection ($p<0.001$) as shown in figure 9 (A). The results are similar at day 34 post-injection with an average of 4 pocks with VVTK-/FCU1 compared to nearly 1 for VVTK-F2L-/FCU1 ($p<0.0001$) as shown in figure 9 (B). Mice injected with 1.10^7 PFU of VVTK-F2L-/FCU1 have respectively an average of 3,5 pocks/mice compared to mice injected with 1.10^7 PFU of VVTK-/FCU1 having an average of 10 pocks/mice at day 15 post-infection (figure 10 (A)). At day 31 post-infection mice injected with VVTK-F2L-/FCU1 have respectively an average of 3,5 pock/mice

compared to mice injected with VVTK-/FCU1 having an average of 7 pocks/mice (figure 10 (B)). The difference in pock number between VVTK-/FCU1 and and VVTK-F2L-/FCU1 is statistically significant ($p<0.05$). The pocks formation is correlated with the replication of virus in the tail and so with virulence and toxicity. Injection in i.v of VVTK-F2L-/FCU1 is less toxic than with the single deleted TK virus.

Statistical analysis.

[00103] Statistical analyses were performed using the nonparametric Mann-Whitney *U* test and STATISTICA 7.1 software (StatSoft, Inc.). A $P < 0.05$ was considered to be statistically significant.

References

[00104]

- US 5364773 (VIROGENETICS CORPORATION (TROY, NY)) 15.11.1994
- WO 2004/014314 (KIRN DAVID (US)) 19.02.2004
- WO 2004/014314 (KIRN DAVID (US)) 19.02.2004
- US 5364773 (VIROGENETICS CORPORATION (TROY, NY)) 15.11.1994
- WO 93/01281 (US HEALTH)
- WO 2005/007857
- WO 2005/007857
- EP 0998568 A
- EP 0998568 A
- EP 0998568 A
- EP 0998568 A
- WO 96/16183
- EP 0998568 A
- EP 0998568 A
- WO 2006/048768
- HERMISTON. A demand for next-generation oncolytic adenoviruses. *Current opinion in molecular therapeutics.* 2006, vol.8, no.4, p.322-30.

- FISHER. Striking out at disseminated metastases: the systemic delivery of oncolytic viruses. *Current opinion in molecular therapeutics* 2006, vol.8, no.4, p.301-13.
- CHERNAJOVSKY, et al. Fighting cancer with oncolytic viruses. *British medical journal.* 2006, vol.332, no.7534, p.170-2.
- JIANG, et al. Oncolytic adenoviruses as antiglioma agents. *Expert review of anticancer therapy.* 2006, vol.6, no.5, p.697-708.
- COHEN, et al. ONYX-015. Onyx Pharmaceuticals. *Current opinion in investigational drugs.* 2001, vol.2, no.12, p.1770-5.
- ROBERTS, et al. Naturally oncolytic viruses. *Current opinion in molecular therapeutics .* 2006, vol.8, no.4, p.314-21.
- THORNE, et al. Vaccinia virus and oncolytic virotherapy of cancer. *Current opinion in molecular therapeutics.* 2005, vol.7, no.4, p.359-65.
- STANFORD, et al. Myxoma virus and oncolytic virotherapy: a new biologic weapon in the war against cancer. *Expert opinion on biological therapy.* 2007, vol.7, no.9, p.1415-25.
- CONO, et al. Smallpox vaccination and adverse reactions. Guidance for clinicians. *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control.* 2003, vol.52, no.RR-4, p.1-28.
- XIANGZHI, et al. Vaccinia virus K1L protein supports viral replication in human and rabbit cells through a cell-type-specific set of its ankyrin repeat residues that are distinct from its binding site for ACAP2. *Journal of virology.* 2006, vol.353, no.1, p.220-233.
- MCCART, et al. Systemic cancer therapy with a tumor selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes.. *Cancer res..* 2001, no.61, p.8751-57.
- KIM, et al. Systemic armed oncolytic ans immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Molecular Therapeutic.* 2006, no.14, p.361-70.
- SLABAUGH, et al. . *Journal of virology.* 1988, vol.62, p.519-27.
- TENGELSEN, et al. . *Virology.* 1988, no.164, p.121-31.
- SCHMITT, et al. . *Journal of virology.* 1988, no.62, p.1889-97.

- SLABAUGH, et al. . *Journal of virology.* 1984, no.52, p.507-14.
- SLABAUGH, et al. . *Journal of virology.* 1984, no.52, p.501-6.
- HOWELL, et al. . *Journal of Biological Chemistry.* 1992, no.267, p.1705-11.
- MCGEOGH. . *Nucleic Acids Research.* 1990, no.18, p.4105-10.
- BROYLES. . *Virology.* 1993, no.195, p.863-5.
- ANTOINE. . *Virology.* 1998, no.244, p.365-396.
- EL OMARI, et al. Structure of vaccinia virus thymidine kinase in complex with dTTP: insights for drug design. *BMC structural biology.* 2006, no.6, p.22.
- MCCART, et al. Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *cancer research.* 2001, vol.61, no.24, p.8751-7.
- PUHLMANN, et al. Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. *Cancer gene therapy.* 2000, vol.7, no.1, p.66-73.
- GNANT, et al. Systemic administration of a recombinant vaccinia virus expressing the cytosine deaminase gene and subsequent treatment with 5-fluorocytosine leads to tumor-specific gene expression and prolongation of survival in mice. *Cancer Research .* 1999, vol.59, no.14, p.3396-403.
- JUND, et al. . *Journal of Bacteriology.* 1970, no.102, p.607-15.
- BECK, et al. . *Journal of Bacteriology.* 1972, no.110, p.219-28.
- HOEPRICH, et al. . *Journal of Infectious Diseases.* 1974, no.130, p.112-18.
- ESDERS, et al. . *J. biol. chem..* 1985, no.260, p.3915-22.
- KOECHLIN, et al. . *Biochemical pharmacology.* 1966, no.15, p.435-46.
- POLAK, et al. . *Chemotherapy.* 1976, no.22, p.137-53.
- JUND, et al. . *Journal of Bacteriology.* 1970, no.102, p.607-15.
- KILLSTRUP, et al. . *Journal of Bacteriology.* 1989, no.171, p.2124-2127.
- HUBER, et al. . *Cancer Research.* 1993, no.53, p.4619-4626.

- MULLEN, et al. . *Proceedings of the National Academy of Sciences of the United States of America.* 1992, no.89, p.33-37.
- HUBER, et al. . *Proceedings of the National Academy of Sciences of the United States of America.* 1994, no.91, p.8302-6.
- MESNIL, et al. . *Proceedings of the National Academy of Sciences of the United States of America.* 1996, no.93, p.1831-35.
- ANDERSEN, et al. Characterization of the upp gene encoding uracil phosphoribosyltransferase of Escherichia coli K12. *European Journal of Biochemistry.* 1992, no.204, p.51-56.
- MARTINUSSEN, et al. Cloning and characterization of upp, a gene encoding uracil phosphoribosyltransferase from Lactococcus lactis. *Journal of Bacteriology.* 1994, vol.176, no.21, p.6457-63.
- KIM, et al. Complete sequence of the UPP gene encoding uracil phosphoribosyltransferase from Mycobacterium bovis BCG. *Biochemistry and molecular biology international.* 1997, vol.41, no.6, p.1117-24.
- MARTINUSSEN, et al. Two genes encoding uracil phosphoribosyltransferase are present in Bacillus subtilis. *Journal of Bacteriology.* 1995, vol.177, no.1, p.271-4.
- KERN, et al. The FUR1 gene of Saccharomyces cerevisiae: cloning, structure and expression of wild-type and mutant alleles. *Gene.* 1990, vol.88, no.2, p.149-57.
- CHAKRABARTI. . *Biotechniques.* 1997, no.23, p.1094-97.
- HAMMOND, et al. . *Journal of Virological Methods.* 1997, no.66, p.135-38.
- KUMAR. . *Virology.* 1990, no.179, p.151-8.

Claims

1. A poxvirus comprising a defective F2L gene.
2. The poxvirus of claim 1 wherein said poxvirus further comprises a defective J2R gene.
3. The poxvirus according to claim 1 or 2 wherein said poxvirus belongs to the Chordopoxvirinae subfamily,
4. The poxvirus according to claim 3 wherein said poxvirus belongs to the Vaccinia virus specie.
5. The poxvirus according to claim 4 wherein said poxvirus is a Vaccinia virus strains Copenhagen.
6. The poxvirus according to any one of claims 1 to 5 wherein said poxvirus further comprises a nucleic acid of interest.
7. The poxvirus according to claim 6 wherein said the nucleic acid of interest contains at least one sequence of interest encoding a therapeutic molecule.
8. The poxvirus according to claim 7 wherein said nucleic acid of interest comprises a suicide gene.
9. The poxvirus according to claim 8 wherein said suicide gene codes a protein having at least a cytosine deaminase activity.
10. The poxvirus according to claim 9 wherein said suicide gene is FCY1, FCA1 or CodA or an analogue thereof.
11. The poxvirus according to claim 9 wherein said protein having at least a cytosine deaminase activity is the FCU1-8 polypeptide represented in the sequence identifier SEQ ID N°:2 and analogues thereof.
12. The poxvirus according to claim 8 wherein said suicide gene codes a protein having at least one cytosine deaminase and one uracil phosphoribosyl transferase activity.
13. The poxvirus according to claim 12 wherein said suicide gene codes a polypeptide comprising an amino acid sequence substantially as represented in the sequence identifier SEQ ID N°:3 (coda::upp), SEQ ID N°:1 (FCU1) or the amino acid sequence of FCY1::FUR1.
14. The poxvirus according to any one of claims 1 to 13 wherein said poxvirus further comprises a nucleic acid sequence comprising a gene coding a permease.

15. The poxvirus according to claim 14 wherein permease is a purine or a cytosine peremease of *S. Cerevisiae*.
16. The poxvirus according to claim 15 wherein permease is chosen from the group comprising FCY2 and Fur4 and analogues thereof.
17. The poxvirus according to any one of claims 6 to 13 wherein said poxvirus further comprises the elements necessary for the expression of the nucleic acid of interest.
18. The poxvirus according to any one of claims 14 to 16 wherein said poxvirus further comprises the elements necessary for the expression of the nucleic acid sequence comprising a gene coding a permease.
19. A process for preparing a poxvirus according to anyone of claims 1 to 18, in which process:
    (i) a poxvirus according to anyone of claims 1 to 18 is introduced into a cell;
    (ii) said cell is cultured under conditions which are appropriate for enabling said poxvirus to be produced, and;
    (iii) said poxvirus is recovered from the cell culture.
20. A composition which comprises a poxvirus according to anyone of claims 1 to 18 in combination with a pharmaceutically acceptable excipient.
21. The composition according to claim 20 in combination with one or more substances which potentiate the cytotoxic effect of the 5-Fluorocytosine.
22. The composition according to claim 21 wherein said substances which potentiate the cytotoxic effect of the 5-Fluorocytosine are drugs which inhibit the enzymes of the pathway for the *de novo* biosynthesis of the pyrimidines, preferably selected from the group consisting of PALA, Leflunomide and A771726.
23. The composition according to claim 21 wherein said substance which potentiate the cytotoxic effect of the 5-Fluorocytosine is methotrexate.
24. Use of a poxvirus according to anyone of claims 1 to 18 or a composition according to any one of claims 20 to 23 for preparing a medicament.
25. The use according to claim 24 for the preparation of a medicament for the treatment of cancer.

26. A method for treating diseases characterized in that a poxvirus according to anyone of claims 1 to 18 or a composition according to any one of claims 20 to 23 is administered to an host organism or cell which is in need of such treatment.
27. The method according to claim 26 wherein said poxvirus or composition is administered via the systemic route.
28. The method according to claim 26 or 27 further comprising an additional step in which a pharmaceutically acceptable quantities of a prodrug are administered to said host organism or cell.
29. The method according to claim 28 wherein the administration of said prodrug takes place preferably at least 3 days, more preferably at least 4 days and even more preferably at least 5 days after the administration of said poxvirus or composition.
30. The method according to claim 29 wherein the administration of said prodrug takes place 7 days after the administration of said poxvirus or composition.