(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 May 2018 (24.05.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/091680 A1

(51) International Patent Classification:
C12N 7/00 (2006.01) *A61K 35/768* (2015.01)
C12N 15/863 (2006.01)

(21) International Application Number:
PCT/EP2017/079656

(22) International Filing Date:
17 November 2017 (17.11.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16306513.9 18 November 2016 (18.11.2016) EP
17306016.1 28 July 2017 (28.07.2017) EP

(71) Applicant: **TRANSGENE SA** [FR/FR]; Parc d'innovation Boulevard Gonthier d'Andernach, 67400 ILLKIRCH GRAFFENSTADEN (FR).

(72) Inventors: **RICORDEL, Marine**; 6 rue des boutons d'or, 67650 Dambach-la-ville (FR). **ERBS, Philippe**; 4 rue de la Source, 67120 Molsheim (FR). **FOLOPPE, Johann**; 71 rue du Rhin, 67115 Plobsheim (FR).

(74) Agent: **REGIMBEAU**; 20, rue de Chazelles, 75847 PARIS CEDEX 17 (FR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*

(54) Title: COWPOX-BASED ONCOLYTIC VECTORS

(57) Abstract: The present invention relates to a cowpox virus comprising at least a defective CPXV105 CDS gene, to a composition comprising it and a process for preparing such a cowpox virus. The present invention also provides a cowpox virus and composition thereof for use as an oncolytic virus for prophylactic or therapeutic purposes, and more particularly for the treatment of cancer.

WO 2018/091680 A1

COWPOX-BASED ONCOLYTIC VECTORS

TECHNICAL FIELD OF THE INVENTION

The present invention is in the field of oncolytic viruses. The invention provides new oncolytic viruses which are cowpox viruses. More precisely, the invention provides an alternative to the existing oncolytic virus vectors which are largely used for the vaccination. These alternative oncolytic vectors are used for prophylaxis or treatment of proliferative diseases, like cancers, tumors or restenosis, and for prophylaxis or treatment of diseases associated to an increased osteoclast activity, like rheumatoid arthritis or osteoporosis.

BACKGROUND ART

Oncolytic viruses are a class of therapeutic agents that have the unique property of tumor-dependent self-perpetuation (Hermiston et al., 2006, Curr. Opin. Mol. Ther., 8:322-30). The benefit of using these viruses is that as they replicate, they lyse their host cells. Oncolytic viruses are capable of selective replication in dividing cells (e.g. cancer cells) while leaving non- dividing cells (e.g. normal cells) unharmed. As the infected dividing cells are destroyed by lysis, they release new infectious particles to infect the surrounding dividing cells. Therefore, oncolytic viruses offer new area for treating cancer, optionally in association with conventional treatments for cancer (Fisher et al., 2006, Curr. Opin. Mol. Ther., vol.8(4)301-13). 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., 2006, British Med. J., 332:170-2). A number of viruses including adenovirus, reovirus, measles, herpes simplex, Newcastle disease virus and vaccinia virus have now been clinically tested as oncolytic agents.

Some naturally oncolytic viruses have been widely used for human vaccination. For example, vaccinia virus (VACV) was employed for almost two centuries, to provide cross-protection against variola virus, the causative agent of smallpox, until the disease was eradicated in the late 1970s.

Some viruses are naturally oncolytic and have an innate ability to selectively infect and kill tumor cells. However, oncolytic viruses can also be engineered by modifying naturally occurring viruses. For this purpose, the main strategies used currently to modify the viruses include: functional deletions in viral genes, the use of tumor- or tissue-specific promoters to control the expression of these viral genes, tropism modification to redirect virus to the cancer cell surface, among many other possibilities.

Viral modifications of the virus can be practiced in order to enhance the ability of viruses to infect and lyse 100% of the tumor cells which is difficult to achieve in *in vivo* context. Therefore, oncolytic viruses are often "armed" with enzyme-prodrug system which enhance the oncolytic efficacy of the virus therapy by exerting a strong bystander effect and thus permitting elimination of neighboring uninfected tumor cells. For example, armament with the so-called *FCU1* suicide gene, encoding a bifunctional chimeric protein that combines the enzymatic activities of FCY1 and FUR1, efficiently catalyzed the direct conversion of 5-fluorocytosine (5-FC), a nontoxic antifungal agent, into the toxic metabolites 5-fluorouracil (5-FU) and 5-fluorouridine-5'monophosphate (5-FUMP), thus bypassing the natural resistance of certain human tumor cells to 5-fluorouracil (Erbs et al., 2000, Cancer Res., 60(14):3813-22).

Virus modifications can also be used to increase safety. In this regard, thymidine kinase (*TK*) deleted virus was shown to have decreased pathogenicity compared with wild type virus, but replication in tumor cells was preserved (Buller et al., 1985, Nature, 317(6040):813-5). Foloppe et al. showed that a *TK* gene-deleted VACV expressing the *FCU1* gene has potent anti-tumor effect both *in vitro* and *in vivo* in a murine model of a human colon tumor (Foloppe et al., 2008, Gene Ther., 15:1361–71)

There is clearly an important need to develop effective approaches for the treatment of cancer. Various oncolytic viral platforms are being evaluated in clinical studies. The number of current and clinical trials based on oncolytic vaccinia viruses (VACV) reflects their interesting therapeutic potential because of their ability to efficiently replicate, lyse host cell and spread across a broad mammalian host range while providing an excellent safety profile. Indeed, VACV viruses have been widely used during vaccination campaigns aimed at preventing world population against smallpox. For this reason, VACV may have a limited

clinical utility by the fact that the majority of patients more than 35 years old have performed immune response against this virus as a result of smallpox vaccination. Systemic delivery of VACV would thus be limited by neutralizing pre-formed antibodies.

As a large part of the population has antibodies which can confer a resistance to the widely used oncolytic VACV, there is a need of an efficient and secure alternative for treating patients over 35 years, which are also the most impacted by cancer.

The inventors surprisingly discovered that Cowpox virus (CPXV) has oncolytic properties which make it particularly appropriate for anti-cancer oncolytic virotherapy considering its limited pathogenicity in humans: CPXV replicates very poorly in human normal tissues as illustrated herein. Moreover, the inventors discovered that CPXV can be modified, and that modifications aimed to inactivate *CPXV105 CDS* gene lead to an increased efficacy and safety compared to wild type CPXV. Moreover, a recombinant and *CPXV105 CDS*-defective cowpox virus engineered to express the suicide *FCU-1* gene was shown particularly effective to replicate and lyse human tumor cells.

Based on these results, one may anticipate that CPXV may be successfully used as an alternative oncolytic to VACV therapy virus for treating or preventing proliferative diseases such as cancer in smallpox vaccinated people as well as in non-vaccinated ones. CPXV can also be exploited in combination with additional anticancer therapy/ies.

SUMMARY OF THE INVENTION

One aspect of the invention relates to a cowpox virus comprising a defective *CPXV105 CDS* gene as well as to a cowpox virus for use as an oncolytic virus for the prophylaxis or the treatment of a disease such as a proliferative disease or a disease associated with an increased osteoclast activity.

Another aspect relates to a cowpox virus comprising defective *CPXV083 CDS* and/or *CPXV051 CDS* gene(s). In one embodiment, the cowpox virus is further defective for *CPXV105 CDS* gene.

In another embodiment, the cowpox virus is further defective for *CPXV049 CDS* gene.

In still another embodiment, the CPXV of the present invention further comprises a truncated *CPXV032 CDS* gene.

In still another embodiment, said CPXV further comprises at least a nucleic acid of interest, in particular a suicide gene, a gene coding for an immunostimulatory polypeptide, a gene coding for an antigen, a gene coding for a permease, or a gene coding for other molecules of interest.

In another aspect, the present invention further provides a composition comprising the CPXV as described herein. In one embodiment, the CPXV is preferably formulated for intra-venous or intra-tumoral administration.

In a further aspect, the present invention also concerns a process for preparing the CPXV, which comprises at least the steps of introducing said CPXV into a producer cell, culturing the producer cell under conditions that are appropriate for enabling said CPXV to be produced and recovering the produced CPXV from the cell culture. Optionally, the recovered CPXV can be purified at least partially.

In still a further aspect, the present invention provides a CPXV (e.g. wild type, or modified derivative CPXV, or recombinant CPXV), or a composition thereof, for use for the prophylaxis and/or the treatment of a disease. In one embodiment, said disease is a proliferative disease such as cancers, tumors and restenosis. Said cancer is preferably selected from the group consisting of renal cancer, bladder cancer, prostate cancer, breast cancer, colorectal cancer, lung cancer, hepatic cancer, gastric cancer, pancreatic cancer, melanoma, ovarian cancer and glioblastoma, and especially metastatic ones. In another embodiment, said disease is a disease associated with an increased osteoclast activity, like rheumatoid arthritis and osteoporosis.

In yet a further aspect of the present invention is provided a method of treatment of a disease which comprises the administration into a host organism in need thereof of a therapeutically effective amount of a CPXV (e.g. a wild type, or modified derivative CPXV, or a recombinant CPXV) or a composition thereof. Said method of treatment may be used in conjunction with one or more additional therapies such as ones selected from the group consisting of surgery, radiotherapy, chemotherapy, cryotherapy, hormonal therapy, toxin therapy, immunotherapy or cytokine therapy. In a particular embodiment where said CPXV is engineered to express a suicide gene, said recombinant virus may be used in conjunction with a pharmaceutically acceptable amount of the corresponding prodrug.

DESCRIPTION OF THE FIGURES

Figure 1: Generation of Cowpox virus (CPXV) expressing the *GFP::FCU1* fusion gene and evaluation of the GFP-FCU1 protein expression.

(a) Schematic representation of CPXVwt and CPXV*tk-/gfp::fcu1* used in this study. CPXV*tk-/gfp::fcu1* contains a deletion of *CPXV105 CDS* gene replaced by a fusion between GFP and *FCU1* genes driven by the synthetic p11K7.5 early late promoter. (b) Specific detection of the GFP-FCU1 protein on western blot by monoclonal antibody 3H1 directed against FCU1. Lane 1 (left to the right), mock-infected LoVo cells; Lane 2, LoVo cells infected with CPXVwt; Lane 3, LoVo cells infected with CPXV*tk-/gfp::fcu1*. Molecular weight standards are shown in kDa on the left. The presence of GFP-FCU protein (M_r 72 000) is indicated (arrow).

Figure 2: Specific CDase and UPRTase activities in LoVo cell lines.

Abbreviations: CDase, cytosine deaminase; ND, not detectable; UPRTase, uracil phosphoribosyltransferase.

CDase and UPRTase activities are expressed as the number of nmoles of 5-FC deaminated per min per mg of protein and the number of nmoles 5-FU phosphorylated per min per mg of protein, respectively. The indicated enzymatic activities were measured as described in Materials and Methods. Each value represents the average of three independent experiments ± standard deviation.

Figure 3: Release of 5-Fluorouracil (5-FU) in the cell culture supernatant.

LoVo cells were infected with the indicated vectors at a multiplicity of infection (MOI) of 0.001 and then incubated with 0.1 mM 5-FC from day 2 to day 5 post infection, the relative concentration of 5-FU in the media was measured by HPLC. The data are expressed as the percentage of 5-FU in media relative to the total amount of 5-FC + 5-FU.

Figure 4: characterization of CPXV*tk-/gfp::fcu1*.

a) Transduction efficiency: a panel of human tumoral cells were infected with CPXV*tk-/gfp::fcu1* at MOI 0.01 and 0.1. Sixteen hours after infection, cells were harvested and washed with PBS and GPF fluorescence was evaluated by flow cytometry. b) Fold amplification between input viral titers and viral titers produced at 72 hpi was calculated in a panel of human tumoral cells. c) Viability by trypan blue exclusion: 3.10^5 cells/well were

infected with CPXV*tk-/gfp::fcu1* at various MOI (MOI 0.00001 to 0.1). Five days after, cells were counted by ViCell automate based on trypan blue exclusion method.

Figure 5: Combination oncolytic and prodrug activation cytotoxicity.

Human tumor cells were infected with the wild type CPXV (CPXwt), the recombinant CPXV vector (CPX*tk-/gfp::fcu1*) at MOI 0.01 on LoVo cells (a) and 0.000001 on A549 cells (b). In both cases, mock was used as control. After 48h, 5-FC was added at increasing concentrations varying from 0 to 1000 µmol and cell survival was determined 3 days later as described in Materials and Methods section. Results were standardized against values for wells lacking virus and drug, which represented 100 % viability.

Figure 6: Virulence studies in CPXV-infected mice.

Immunocompetent BalB/c mice were infected (Day 0) intravenously with purified CPXwt or CPX*tk-/gfp::fcu1* virus or were mock infected with formulation buffer. Cowpox virus wild type and the recombinant CPX*tk*$^-$*/gfp::fcu1* were used at a range of 1 x 10^4 (a), 1 x 10^5 (b), 1 x 10^6 (c) and 1 x 10^7 (d) pfu/mouse (n = 10 per group). Survival data presented as Kaplan-Meier plots, *P* values obtained by statistical analysis (log-rank). (e) Animals were individually weighed and monitored for signs of disease for 83 days. Mice were euthanized if 20% of their initial weight loss occurred. Mean group weight on each day, expressed relative to the mean weight for that group at the day of infection according to the dose of virus injected.

Figure 7: Safety in normal human primary cells.

Human normal hepatocytes and HepG2 were infected with 100pfu/well of CPXV (CPXwt and CPX*tk-/gfp::fcu1* virus), and VACV (wild type VACV of Copenhagen strain designated VVCop wt) and a recombinant *TK*- VACV expressing the suicide *FCU1* gene (VV*tk-/fcu1*)). 72h after, cells on supernatant were harvested and sonicated. Fold increase was determined by plaque assay on Vero cells.

Figure 8: Organ distribution of CPXV in mice.

Swiss Nude mice were injected i.v. with 10^6 pfu CPXV*tk-/gfp::fcu1*. At day 2 and day 7, mice were euthanized and the indicated organs were collected and homogenized, and the viral titer (pfu/mg) contained in the lysate was measured on Vero cells.

Figure 9: *In vivo* CPXV activity in glioblastoma and colorectal tumor.

Immuno-deficient Swiss Nude mice were implanted subcutaneously with U87-MG glioblastoma cells (a) or LoVo colorectal cells (b). Mice (n=10/group) were treated i.t. with CPX*tk-/gfp::fcu1* (10^6 pfu) or with placebo control (buffer) on day 18 after tumor cell transplantation, when tumors were approximately 100 mm^3. 5 days after the viral injection, half of the mice in each group were gavaged with soluble 5-FC in an amount of 100mg/kg (05mL 5-FC 0.5% in water) twice a day. Tumor size was measured twice a week using calipers. Tumor volumes were calculated in mm^3 using the formula (Π/6) (length x $width^2$).

Figure 10: Replication of CPXwt and CPX*tk-/gfp::fcu1* virus on 3D skin model.

3D Phenion® FT skin models were infected with 8.10^4 pfu of CPXwt and CPX*tk-/gfp::fcu1* by scarification. Seven days post infection, 3D skin and supernatant were collected and sonicated, viral titers were determined by plaque assay. Results are expressed as viral fold increased (corresponding to output/input ratio).

Figure 11: Infection and replication of CPXwt and CPX*tk-/gfp::fcu1* on hPBMC.

Fresh human PBMC were infected by CPX*tk-/gfp::fcu1* at different MOI. Sixteen hours post infection, eGFP level was measured on flow cytometry. Four days post infection, cells and supernatants were harvested and sonicated. Viral titers were determined by plaque assay on Vero cells. Results are expressed as viral fold increased (corresponding to output/input ratio).

Figure 12 : In vivo CPXV activity in pancreatic tumor.

Immuno-deficient Swiss Nude mice were implanted subcutaneously with MIA-Paca-2 human pancreatic cancer cells lines. Mice (n=10/group) were treated i.t. with CPX*tk-/gfp::fcu1* (1 x 10^6 pfu) or with placebo control (buffer) when tumors reached a diameter of 100-300 mm^3. Tumor size was measured twice a week using calipers. Tumor volumes were calculated in mm^3 using the formula (Π/6) (length x $width^2$).

Figure 13 : *In vitro* evaluation of CPXV toxicity on human pancreatic islets.

Fresh pancreatic islets (InSphero®) (human primary cells) were infected with 100 pfu of CPXwt and CPX*tk-/gfp::fcu1*. Seven days post infection, islets were collected and sonicated, viral titers were determined by plaque assay. Results are expressed as viral fold increased (corresponding to output/input ratio).

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

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.

The term “one or more” refers to either one or a number above one (e.g. 2, 3, 4, etc.).

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".

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.

As used herein, when used to define products and compositions, the terms "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are open-ended and do not exclude additional, un-recited elements or method steps. The expression "consisting essentially of" means excluding other components or steps of any essential significance. Thus, a composition consisting essentially of the recited components would not exclude traces, contaminants and pharmaceutically acceptable carriers. "Consisting of" shall mean excluding more than trace elements of other components or steps.

The terms “polypeptide”, “peptide” and “protein” refer to polymers of amino acid residues which comprise at least nine or more amino acids bonded via peptide bonds. The polymer can be linear, branched or cyclic and may comprise naturally occurring and/or amino acid analogues and it may be interrupted by non-amino acids. As a general indication, if the amino acid polymer is more than 50 amino acid residues, it is preferably referred to as a polypeptide or a protein whereas if it is 50 amino acids long or less, it is referred to as a “peptide”.

Within the context of the present invention, the terms “nucleic acid”, “nucleic acid molecule”, “polynucleotide” and “nucleotide sequence” are used interchangeably and define a polymer of any length of either polydeoxyribonucleotides (DNA) (e.g. cDNA, genomic DNA, plasmids, vectors, viral genomes, isolated DNA, probes, primers and any mixture thereof) or polyribonucleotides (RNA) (e.g. mRNA, antisense RNA, SiRNA) or mixed polyribo-polydeoxyribonucleotides. They encompass single or double-stranded, linear or circular, natural or synthetic, modified or unmodified polynucleotides. Moreover, a polynucleotide may comprise non-naturally occurring nucleotides and may be interrupted by non-nucleotide components.

The term "analogue" as used herein refers to a molecule (polypeptide or nucleic acid) exhibiting one or more modification(s) with respect to the native counterpart. Any modification(s) can be envisaged, including substitution, insertion and/or deletion of one or more nucleotide/amino acid residue(s). Preferred are analogues that retain a degree of sequence identity of at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, and even more preferably at least 98% identity with the sequence of the native counterpart. For illustrative purposes, “at least 80% identity” means 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. In a general manner, the term “identity” refers to an amino acid to amino acid or nucleotide to nucleotide correspondence between two polypeptides or nucleic acid sequences. The percentage of identity between two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps which need to be introduced for optimal global alignment and the length of each gap. Various computer programs and mathematical algorithms are available in the art to determine the percentage of identity between amino acid sequences after optimal global alignment, such as for example the algorithm of Needleman et Wunsch. J.Mol. Biol. 48,443-453, 1970, the Blast program available at NCBI or ALIGN in Atlas of Protein Sequence and Structure (Dayhoffed, 1981, Suppl., 3: 482-9). Programs for determining identity between nucleotide sequences are also available in specialized data base (e.g. Genbank, the Wisconsin Sequence Analysis Package, BESTFIT, FASTA and GAP programs, and the needle software available from ebi.ac.uk worldwide under the name « Align »).

As used herein, the term “host cell” should be understood broadly without any limitation concerning particular organization in tissue, organ, or isolated cells. Such cells may be of a unique type of cells or a group of different types of cells such as cultured cell lines, primary cells and dividing cells. In the context of the invention, the term “host cells” include prokaryotic cells, lower eukaryotic cells such as yeast, and other eukaryotic cells such as insect cells, plant and mammalian (e.g. human or non-human) cells as well as cells capable of producing the cowpox virus of the invention. This term also includes cells which can be or has been the recipient of the virus described herein as well as progeny of such cells.

The terms “virus”, ‘viral particle”, “viral vector” and virion” are used interchangeably and are to be understood broadly as meaning a vehicle comprising at least one element of a wild-type virus genome and may be packaged into a viral particle or to a viral particle. Although, viral particles may or may not contain nucleic acid (i.e. the viral genome) it is preferred that a virus comprises a DNA or RNA viral genome packaged into a viral particle (or virion) and is infectious (i.e. capable of infecting and entering into a host cell or subject). Desirably, the virus according to this invention is associated with a DNA genome, and most preferably a double-stranded DNA genome. In the context of the present disclosure, a "virus” includes wild-type and engineered viruses.

The term “naturally occurring” or “wild-type” or “native” is used to describe a biological molecule or organism that can be found in nature as distinct from being artificially produced by man. For example, a virus which can be isolated from a source in nature is wild-type. The present invention also encompasses wild-type viruses that can be obtained from specific collections (e.g. ECCAC, ATCC, CNCM, etc.). A biological molecule or an organism which has been intentionally modified by man in the laboratory is not naturally occurring. Representative examples of non-naturally occurring viruses include, among many others, recombinant viruses engineered by insertion of one or more gene(s) of interest in the viral genome and mutated viruses engineered by total or partial deletion of a viral gene to make the modified virus defective for the encoded gene product (e.g. *TK*- virus) as well as chimeric viruses containing genomic fragments obtained from different virus origins.

The term "obtained from", “originating” or “originate” is used to identify the original source of a component (e.g. polypeptide, nucleic acid molecule, virus, etc.) but is not meant

to limit the method by which the component is made which can be, for example, by chemical synthesis or recombinant means.

As used herein, the term "oncolytic virus" refers to a virus capable of selectively replicating in dividing cells (e.g. a proliferative cell such as a cancer cell) with the aim of slowing the growth and/or lysing said dividing cell, either *in vitro* or *in vivo*, while showing no or minimal replication in non-dividing cells (e.g. primary cells).

The terms "replication" and "propagation" are used interchangeably and refer to the ability of a virus to reproduce and proliferate. Virus replication can be quantified at the level of nucleic acid or at the level of infectious viral particle using assays standard in the art and described herein such as a virus titer assay, plaque assay, absorbance, fluorescence detection, mass spectrometry, etc.

The term "treatment" (and any form of treatment such as "treating", "treat") as used herein encompasses prophylaxis (e.g. preventive measure in a subject at risk of having the pathological condition to be treated) and/or therapy (e.g. in a subject diagnosed as having the pathological condition), optionally in association with conventional therapeutic modalities. The result of the treatment is to slow down, cure, ameliorate or control the progression of the targeted pathological condition. For example, a subject is successfully treated for a cancer if after administration of a cowpox virus as described herein, alone or in combination with other therapy/ies, the subject shows an observable improvement of its clinical status.

The term "administering" (or any form of administration, such as "administered") as used herein refers to the delivery to a subject of a therapeutic agent such as the cowpox virus described herein.

As used herein, the term "proliferative disease" encompasses any disease or condition resulting from uncontrolled cell growth and spread including cancers, tumors and some cardiovascular diseases (restenosis that results from the proliferation of the smooth muscle cells of the blood vessel wall, etc.). The term "cancer" may be used interchangeably with any of the terms "tumor", "malignancy", "neoplasm", etc. These terms are meant to include any type of tissue, organ or cell, any stage of malignancy (e.g. from a pre-lesion to stage IV).

As used herein, the term “disease associated with an increased osteoclast activity” encompasses any disease or condition resulting in bone resorption or destruction (e.g. rheumatoid arthritis, osteoporosis, etc.).

The term “subject” generally refers to an organism for whom any product and method of the invention is needed or may be beneficial. Typically, the organism is a mammal, particularly a mammal selected from the group consisting of domestic animals, farm animals, sport animals, and primates. Preferably, the subject is a human who has been diagnosed as having or at risk of having a proliferative disease such as a cancer. The terms “subject” and “patients” may be used interchangeably when referring to a human organism and encompasses male and female. The subject to be treated may be a new-born, an infant, a young adult, an adult or an elderly.

The terms “combination treatment”,"combination therapy", "combined treatment" or "combinatorial treatment", may be used interchangeably and refer to a treatment of a subject with at least two different therapeutic agents. According to the invention, one of therapeutic agent is a cowpox virus as described herein. The second therapeutic agent may be any clinically established therapeutic agent, in particular one selected from the group consisting of surgery, radiotherapy, chemotherapy, cryotherapy, hormonal therapy, toxin therapy, immunotherapy, cytokine therapy, targeted cancer therapy, gene therapy, photodynamic therapy, transplantation etc. A combinatorial treatment may include a third or even further therapeutic agent(s).

COWPOX VIRUS

As used herein, the term “cowpox virus” or “CPXV” refers to a group of viruses belonging to the Poxviridae family, more precisely to the Chordopoxvirinae subfamily, and even more precisely to the Orthopoxvirus genus.

In 1790s, Edward Jenner provided the first exhaustive descriptions of human cowpox in the publication of “An Inquiry into the causes and effects of the Variolae Vaccinae or Cow-Pox” (1798). In 1796, Jenner introduced the concept of vaccination with cowpox virus in order to elicit cross protective immunity against related orthopoxviruses, including smallpox virus (variola virus). Over time, vaccinia virus replaced cowpox virus as the smallpox vaccine,

and vaccination efforts eventually led to the successful global eradication of smallpox in 1979 (Verardi et al., 2012, Hum. Vaccin. Immunother., 8:961–70). Cowpox virus has a broad host range and is believed to persist in a reservoir comprising various rodents indigenous to parts of Europe and adjoining Asia (Chantrey et al., 1999, Epidemiol. Infect., 122:455-60.). Very often domestic cats cause intermediates CPXV infected hosts, which may transmit the virus to humans (Essbauer et al., 2002, Revue Méd. Vét., 153, 10:635-42). However, *in vitro* and *in vivo* studies performed with laboratory strains of cowpox virus strongly support that compounds like cidofovir and ST-246 will be active against genetically diverse CPXV isolates.

Poxviruses are DNA viruses that replicate in the cytoplasm of infected cells. Recent genotypic data pointed to a much higher genomic diversity among CPXV as compared to isolates from other Orthopoxvirus species. In particular, they have the largest genome (more than 220 kbp), about 30kbp larger than the VACV genome (Carroll et al., 2011, PLoS One 6:e23086). 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 ribonucleotide reductase and deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase).

CPXV can be divided into two major monophyletic clades (Cowpox-like and Vaccinia-like). The cowpox-like clade can be further grouped into four distinct monophyletic clusters, clusters 1, 2, 3 and 4, respectively and Vaccinia-like clade comprises cluster 5.

In the context of the present invention, the cowpox virus for use herein is any virus of the Cowpox phylogeny belonging to any clade, cluster and strain described in the art encompassing clinical, laboratory and vaccine isolates (see e.g. Carroll et al., 2011, PLoS One 6:e23086; Duraffour et al., 2013, PLoS One 8(2):1-8). Moreover, it may be isolated from any host organism including domestic and zoological garden animals (e.g. elephants, cats, pet rodents, etc.) as well as primate (e.g. humans), and may be pathogenic or have a reduced virulence or be avirulent at least with respect to the subject to be treated.

In one embodiment, the CPXV of the invention belongs to (wild-type virus) or is obtained from (e.g. engineered or recombinant virus) the Cowpox-like clade and more particularly to any of clusters 1, 2, 3 and 4. Exemplary CPXV of Cluster 1 comprises CPXV_GER1980_EP4 (Genbank HQ420895) and CPXV_GER2002_MKY (Genbank HQ420898).

Exemplary CPXV of cluster 2 comprises CPXV_GER1991_3 (Genbank DQ 437593). Exemplary CPXV of cluster 3 comprises CPXV_FRA2001_NANCY (Genbank HQ420894), CPXV_GER1990_2 (Genbank HQ420896), CPXV_UK2000_K2984 (Genbank HQ420900), CPXV_BR (Genbank AF482758.2 or NC 003663) and CPXV_NOR1994-MAN (Genbank HQ420899). Exemplary CPXV of cluster 4 comprises CPXV_GER1998_2 (Genbank HQ420897). CPXV of vaccinia-like clade and cluster 5 of this clade or any derivative thereof are suitable as well in the context of this invention, and in particular CPXV_gri (Genbank X94355), CPXV_FIN2000_MAN (Genbank HQ420893) and CPXV_AUS1999_867 (Genbank HQ407377). A preferred embodiment is directed to a CPXV of clade 3 and especially to the Brighton red strain (CPXV_BR).

As mentioned before, wild-type cowpox may be engineered in the context of the present invention. In one embodiment, the cowpox virus of the invention may be altered and may comprise one or more mutation(s) in its genome, i.e. deletion, substitution or addition of one or more nucleotides or any combination of these possibilities. When several modifications are contemplated, they may be consecutive or not. Desirably, said modifications lead to the inability for the virus to produce a protein having the activity of the protein produced by the unmodified gene, resulting in a defective virus for this particular activity. Although modification(s) may occur in the promoter region or in the coding sequence or both, preferred modification(s) comprises deletion of a whole gene sequence (i.e. at least 70% of the gene including promoter and coding sequences). Methods for modifying the genome of a poxvirus can be used to modify the genome of cowpox virus of the invention, for example, the methods disclosed in McCart et al., 2001, Cancer Res., 61:8751-57, Kim et al., 2006, Mol. Ther., 14:361-70, WO 2004/014314 in view of the information and sequence data given in the present application and those available in Genbank. The Example section also illustrates appropriate methods to produce cowpox viruses according to the invention.

In a preferred embodiment, the cowpox virus of the invention comprises a defective *CPXV105 CDS* gene (UniprotKB Q8QMX0), resulting in a defective thymidine kinase (TK) activity. In the natural context, the TK enzyme is involved in the synthesis of deoxyribonucleotides. TK is needed for viral replication in normal cells as these cells have

generally low concentration of nucleotides whereas it is dispensable in dividing cells which contain high nucleotide concentration. The reaction catalyzed by TK involves the transfer of a γ-phosphoryl moiety from ATP to 2'deoxy-thymidine (dThd) to produce thymidine 5'-monophosphate (dTMP). Cowpox viruses' 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., 2006, BMC Struct. Biol., 6:22). It is within the reach of the skilled in the art to generate a cowpox defective for *CPXV105 CDS* gene, based on the information given herein and the available CPXV genome sequence, using conventional molecular biology techniques (PCR, gene targeting, use of restriction enzymes, ligations, molecular cloning, CRISPR/Cas9, etc.).

Alternatively, or in combination, the CPXV of the present invention is modified by altering at least one gene or both genes encoding ribonucleotide reductase (RR). In the natural context, this enzyme catalyzes the reduction of ribonucleotides to deoxyribonucleotides that represents a crucial step in DNA biosynthesis. The viral enzyme is similar in subunit structure to the mammalian enzyme, being composed of two heterologous subunits, designed R1 and R2 encoded respectively by the *CPXV083 CDS* (corresponding to Vaccinia virus I4L) and *CPXV051 CDS* (corresponding to vaccinia virus F4L locus). In the context of the invention, either the *CPXV083 CDS* gene (encoding the R1 large subunit) or the *CPXV051 CDS* gene (encoding the R2 small subunit) or both may be inactivated.

Alternatively, or in combination, the CPXV of the present invention comprises a defective *CPXV049 CDS* gene, corresponding to Vaccinia Virus F2L, and encoding deoxyuridine triphosphatase (dUTPase). In the natural context, this enzyme catalyzes the conversion 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 providing the precursor to produce TMP by thymidylate synthase.

Alternatively, or in combination, the CPXV of the present invention comprises a truncated *CPXV032 CDS* gene, the homologue of *C5L* of Vaccinia Virus. In accordance with

the present invention, a truncated gene means may be defined as lacking <80% of the amino acid length as compared to the wild-type gene. Truncation may be at the N or the C terminus or internally.

Alternatively or in combination, the CPXV of the present invention is modified by altering one or more of the following genes (named using VACV nomenclature) : genes encoding interferon-modulating polypeptide (including, but not limited to, *B8R*, *B18R*, *B19R* and/or *vC12L*) that results in the virus lacking at least an interferon-modulating function; genes encoding a complement control polypeptide (e.g. vaccinia virus complement control protein : VCP) that results in the virus lacking at least one complement control function; genes encoding a TNF-modulating polypeptide (including, but not limited to, *A53R* and *B28R*) that results in the virus lacking at least one TNF-modulating function; genes encoding a serine protease inhibitor (including, but not limited to *B13R*, *B22R*, and/or *K2L*) that results in the virus lacking at least one serine protease inhibitor function; genes encoding an IL-1β modulator polypeptide (e.g. *B15R*, *B16R*) that results in the virus lacking at least one IL-1β modulator function; genes encoding inhibitor of IL-1 and TLR signal (e.g. *A46R* and *A52R*); genes encoding NF-κB inhibitor (e.g. *N1L*, *K7L, M2L*) ; genes encoding IRF3/7 inhibitor (e.g. *C6L*, *N2L*) ; genes encoding chemokine binding protein (e.g. *C23L*, *A41L, vCKBP, vCCI*) ; genes encoding antiapoptotic proteins (e.g. *F1L*); genes encoding proteins involved in nucleotide metabolism (e.g. *A48R*, *A57R*) ; other examples are *A26L*, *A56R*, *C4L*, *D4R*, *O1L*, *B7R*, and *A44L*.

The CPXV of the present invention may also be modified by altering *CPXV021 CDS* gene (also called *CPXV VGF* gene, or *C11R* in vaccinia virus) which encodes proteins expressed early after cell infection and which function seems important for virus spread in normal cells; gene encoding ubiquitine ligase (e.g. *CPXV023 CDS*); gene encoding soluble IL-18 binding proteins (e.g. *CPXV024 CDS*) and A-type inclusion body ATI gene (e.g. *CPXV158 CDS*).

RECOMBINANT CPXV

In one embodiment, the cowpox virus of the invention is recombinant (i.e. engineered to express a nucleic acid of interest) and comprises inserted in its genome at least one nucleic acid of interest. According to the invention, the nucleic acid of interest can be homologous

or heterologous to the host organism into which it is introduced. More specifically, it can be of human origin or not (e.g. of bacterial, yeast or viral origin). Advantageously, said nucleic acid of interest encodes a therapeutic molecule and, notably, all or part of a polypeptide. 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.

In one embodiment, the nucleic acid of interest encodes a therapeutic molecule of therapeutic or prophylactic interest which is capable of providing a biological activity when administered appropriately to a subject, which is expected to cause a beneficial effect on the course or a symptom of the pathological condition to be treated. A vast number of therapeutic genes may be envisaged in the context of the invention such as those encoding therapeutic molecules that can compensate for defective or deficient proteins in the subject, or those that act through toxic effects to limit or remove harmful cells from the body or those that encode immunity conferring polypeptides. They may be native genes or genes obtained from the latter by mutation, deletion, substitution and/or addition of one or more nucleotides. Representative examples of suitable molecule of therapeutic interest include, without limitation, polypeptides encoded by suicide genes which are capable of reinforcing the oncolytic nature of the cowpox virus of the present invention, as well as polypeptides capable of potentiating anti-tumor efficacy such as immunostimulatory polypeptides and antigens (for inducing or activating an immune humoral and/or cellular response).

Suicide gene product

The term “suicide gene” refers to a gene coding for a polypeptide able to convert a precursor of a drug, also named “prodrug”, into a cytotoxic compound. Examples of suicide genes and corresponding prodrugs comprising one nucleobase moiety are disclosed in the following table:

Table 1

Suicide gene	Prodrug
Thymidine Kinase	Ganciclovir; Ganciclovir elaidic acid ester; penciclovir; Acyclovir; Valacyclovir; (E)-5-(2-

Suicide gene	Prodrug
	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
Thymidylate kinase	Azidothymidine

Desirably, the cowpox of the invention carries in its genome a suicide gene encoding a polypeptide having at least cytosine deaminase (CDase) activity. In the prokaryotes and lower eukaryotes (it is not present in mammals), CDase is involved in the pyrimidine metabolic pathway by which exogenous cytosine is transformed into uracil by means of a hydrolytic deamination. CDase also deaminates an analogue of cytosine, i.e. 5-fluorocytosine (5-FC), thereby forming 5-fluorouracil (5-FU), a compound which is cytotoxic by itself but even more when it is converted into 5-fluoro-UMP (5-FUMP). CDase encoding nucleic acid molecule can be obtained from any prokaryotes and lower eukaryotes such as *Saccharomyces cerevisiae* (*FCY1* gene), *Candida Albicans* (*FCA1* gene) and *Escherichia coli* (*CodA* gene). The gene sequences and encoded CDase proteins have been published and are available in specialized data banks (SWISSPROT EMBL, Genbank, Medline and the like). Functional analogues of these genes may also be used. Such analogues preferably have a degree of identity of at least 80%, preferably of at least 90%, and most preferably of at least 95% with the amino acid sequence of the native gene.

Alternatively, or in combination, the cowpox virus of the invention carries in its viral genome a suicide gene encoding a polypeptide having uracil phosphoribosyl transferase (UPRTase) activity. In prokaryotes and lower eukaryotes, uracil is transformed into UMP by the action of UPRTase. This enzyme converts 5-FU into 5-FUMP. By way of illustration, the nucleic acid sequences encoding the UPRTases from *E. coli* (Andersen et al., 1992, European J. Biochem. 204: 51-56), from *Lactococcus lactis* (Martinussen et al., 1994, J. Bacteriol. 176: 6457-63), from *Mycobacterium bovis* (Kim et al., 1997, Biochem. Mol. Biol. Internat. 41: 1117-

24) and from *Bacillus subtilis* (Martinussen et al., 1995, J. Bacteriol. 177: 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 is disclosed in Kern et al. (1990, Gene, 88: 149-57). Functional UPRTase analogues may also be used such as the N-terminally truncated FUR1 mutant described in EP998568 (with a deletion of the 35 first residues up to the second Met residue present at position 36 in the native protein) which exhibits a higher UPRTase activity than that of the native enzyme.

Preferably, the suicide gene inserted in the viral genome of the cowpox virus of the present invention encodes a polypeptide having CDase and UPRTase activities. Such a polypeptide can be engineered by fusion of two enzymatic domains, one having the CDase activity and the second having the UPRTase activity. Exemplary polypeptides include without limitation fusion polypeptides codA::upp, FCY1::FUR1 and FCY1::FUR1[Delta] 105 (FCU1) and FCU1-8 described in WO96/16183, EP998568 and WO2005/07857. Of particular interest is the *FCU1* suicide gene (or FCY1::FUR1[Delta] 105 fusion) encoding a polypeptide comprising the amino acid sequence represented in the sequence identifier SEQ ID NO: 1 of WO2009/065546. The present invention encompasses analogues of such polypeptides providing they retain the CDase and/or UPRTase activities. 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 phase the polypeptides with CDase and UPRTase activity, and consequently all or part of the corresponding genes.

In a particular embodiment, wherein the cowpox virus of the invention encodes suicide gene product(s), it might be relevant to associate suicide gene product with a permease so-as to reinforce prodrug uptake in the infected cells and neighbouring cells. Typically, a “permease” is a trans-membranous protein involved in the transfer of a drug comprising one nucleobase moiety, or a precursor thereof through the cell membrane. The one skilled in the art is able to choose the permease appropriate for the selected suicide gene and its associated drug/prodrug. Permeases comprise purine permeases, cytosine permeases and nucleoside transporters. In a preferred embodiment of the invention, the

permease is a purine or a cytosine permease of *S. Cerevisiae* with a specific preference for the purine-cytosine permease, known as FCY2, and the uracil permease, known as FUR4 or any analogue thereof (i.e. at least 80% identity with the wild-type genes). For general information, FCY2 and Fur4 are preferably associated with 5-Fluorocytosine (5-FC).

The purine-cytosine permease, FCY2 mediates symport of protons and adenine, guanine, hypoxanthine and cytosine across the yeast plasma membrane. FCY2 protein mediates also the transport of 5-fluorocytosine, an analogue of cytosine (Grenson et al., 1970, J. Bacteriol., 103(3):770-7). Uracil uptake into *S. cerevisiae* is mediated by the uracil permease, FUR4 which is an uracil-proton symporter. FUR4 protein can also mediates the transport of 5-fluorouracil, an analogue of uracil (Jund and Lacroute, 1970, J. Bacteriol., 102(3):607–15). Amino acid sequences of FCY2 and Fur4 are available in the swissprot database (accession number P17064 and P05316 respectively). Preferred permeases appropriate for expression in the cowpox virus of the invention, especially a CDase and/or UPRTase -encoding CPXV (e.g. FCU1) are disclosed in WO 2006/048768 (see e.g. the amino acid sequence SEQ ID NO: 1 and SEQ ID NO: 2).

Immunostimulatory polypeptide

As used herein, the term “immunostimulatory polypeptide” refers to a polypeptide or protein, which has the ability to stimulate the immune system, in a specific or non-specific way. A vast number of proteins are known in the art for their ability to exert an immunostimulatory effect. Examples of suitable immunostimulatory polypeptides in the context of the invention include, without limitation, agents such as, e.g. alpha, beta or gamma interferon, interleukin (in particular IL-2, IL-6, IL-10 or IL-12) and tumor necrosis factor (TNF); 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); agents that affect angiogenesis such as, e.g. inhibitor of Vascular Endothelial Growth Factor (in particular bevacizumab or ranibizumab) ; agents that stimulates stem cells to produce granulocytes (neutrophils,

eosinophils, and basophils) and macrophages such as, e.g. granulocyte macrophage - colony stimulating factor (GM-CSF).

Antigens

The term "antigen" generally refers to a substance that is recognized and selectively bound by an antibody or by a T cell antigen receptor, in order to trigger an immune response. It is contemplated that the term antigen encompasses native antigen as well as fragment (e.g. epitopes, immunogenic domains, etc.) and derivative thereof, provided that such fragment or derivative is capable of being the target of an immune response. Suitable antigens in the context of the invention are preferably polypeptides (e.g. peptides, polypeptides, post translationally modified polypeptides, etc.) including one or more B cell epitope(s) or one or more T cell epitope(s) or both B and T cell epitope(s) and capable of raising an immune response, preferably, a humoral or cell response that can be specific for that antigen. Typically, the one or more antigen(s) is selected in connection with the disease to treat. Preferred antigens for use herein are cancer antigens and antigens of pathogens.

In certain embodiments, the antigen(s) contained in or encoded by the cowpox virus is/are cancer antigen(s) (also called tumor-associated antigens) that is associated with and/or serve as markers for cancers. Cancer antigens encompass various categories of polypeptides, e.g. those which are normally silent (i.e. not expressed) in normal cells, those that are expressed only at low levels or at certain stages of differentiation and those that are temporally expressed such as embryonic and foetal antigens as well as those resulting from mutation of cellular genes, such as oncogenes (e.g. activated ras oncogene), proto-oncogenes (e.g. ErbB family), or proteins resulting from chromosomal translocations. The cancer antigens also encompass antigens encoded by pathogenic organisms (bacteria, viruses, parasites, fungi, viroids or prions) that are capable of inducing a malignant condition in a subject (especially chronically infected subject) such as RNA and DNA tumor viruses (e.g. HPV, HCV, EBV, etc.) and bacteria (e.g. *Helicobacter pilori*).

Some non-limiting examples of cancer antigens include, without limitation, MART-1/Melan-A, gp100, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-C017-1A/GA733,

Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family (e.g. MUC1, MUC16, etc. ; see e.g. US6,054,438; WO98/04727; or WO98/37095), HER2/neu, p21ras, RCAS1, alpha-fetoprotein, E-cadherin, alpha-catenin, beta-catenin and gamma-catenin, p120ctn, gp100.sup.Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, Smad family of cancer antigens brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, and c-erbB-2 and viral antigens such as the HPV-16 and HPV-18 E6 and E7 antigens and the EBV-encoded nuclear antigen (EBNA)-1.

Other antigens suitable for use in this invention are marker antigens (beta-galactosidase, luciferase, green fluorescent proteins, etc.).

The present invention also encompasses cowpox virus expressing two or more polypeptides of interest as described herein, e.g. at least two antigens, at least one antigen and one cytokine, at least two antigens and one cytokine, etc.

<u>Other molecules of therapeutic interest:</u>

In one embodiment, the cowpox virus of the invention further comprises at least one nucleic acid of interest including, but not limited to:

- apoptotic genes, including pro-apoptotic genes (e.g. Bax, Bak, Bok, Bad, Bid et Bim), inhibitors of pro-apoptotic genes (e.g. Bax inhibitor, Bak inhibitor, Bok inhibitor, Bad inhibitor, Bid inhibitor, Bim inhibitor), anti-apoptotic genes (e.g. Bcl-2, Bcl-xL, Bcl-w,

Nr13) and inhibitors of anti-apoptotic genes (e.g. Bcl2 inhibitor, Bcl-xL-inhibitor, Bcl-w inhibitor, Nr13 inhibitor),

- nucleic acid coding for endonuclease, like restriction enzymes (e.g. restriction enzymes of type I, II, III, IV or V, artificial restriction enzymes like Transcription activator-like effector nucleases (TALEN) or zinc finger nuclease), CRISPR/Cas9
- nucleic acid coding for immune checkpoint inhibitors, including, but not limited to anti-PD1, anti-PDL1, anti-PDL-2, anti-CTLA4, anti-Tim3, anti-LAG3, anti-BTLA,
- RNA, including but not limited to miRNA, targets of miRNA, shRNA, siRNA.

Expression of the nucleic acid(s) of interest

The nucleic acid sequences may be easily obtained by cloning, by PCR or by chemical synthesis according to the conventional techniques. 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. In addition, the nucleic acid(s) of interest can be optimized for providing high level expression in a particular host cell or subject. It has been indeed observed that, the codon usage patterns of organisms are highly non-random and the use of codons may be markedly different between different hosts. As the nucleic acid(s) of interest might be from bacterial or lower eukaryote origin (e.g. the suicide gene), it may have an inappropriate codon usage pattern for efficient expression in higher eukaryotic cells (e.g. human). Typically, codon optimization is performed by replacing one or more "native" (e.g. bacterial or yeast) codon corresponding to a codon infrequently used in the host organism of interest by one or more codon encoding the same amino acid which is more frequently used. It is not necessary to replace all native codons corresponding to infrequently used codons since increased expression can be achieved even with partial replacement.

Further to optimization of the codon usage, expression in the host cell or subject can further be improved through additional modifications of the gene sequence. For example, the sequence of the nucleic acid of interest can be modified so-as to prevent clustering of rare, non-optimal codons being present in concentrated areas and/or to suppress or modify "negative" sequence elements which are expected to negatively influence expression levels.

Such negative sequence elements include without limitation the regions having very high (>80%) or very low (<30%) GC content; AT-rich or GC-rich sequence stretches; unstable direct or inverted repeat sequences; R A secondary structures; and/or internal cryptic regulatory elements such as internal TATA-boxes, chi-sites, ribosome entry sites, and/or splicing donor/acceptor sites.

In accordance with the present invention, the cowpox virus comprises the elements necessary for the expression of the nucleic acid(s) of interest. More precisely, the nucleic acid(s) of interest inserted in the genome of the cowpox virus of the invention is/are operably linked to suitable regulatory elements for its/their expression in a host cell or subject. As used herein, the term "regulatory elements" or "regulatory sequence" refers to any element that allows, contributes or modulates the expression of the nucleic acid(s) of interest in a given host cell or subject, including replication, duplication, transcription, splicing, translation, stability and/or transport of the nucleic acid(s) or its derivative (i.e. mRNA). As used herein, “operably linked” means that the elements being linked are arranged so that they function in concert for their intended purposes. For example, a promoter is operably linked to a nucleic acid molecule if the promoter effects transcription from the transcription initiation to the terminator of said nucleic acid molecule in a permissive host cell.

It will be appreciated by those skilled in the art that the choice of the regulatory sequences can depend on such factors as the gene itself, the virus into which it is inserted, the host cell or subject, the level of expression desired, etc. The promoter is of special importance. In the context of the invention, it can be constitutive directing expression of the nucleic acid(s) of interest in many types of host cells or specific to certain host cells (e.g. liver-specific regulatory sequences) or regulated in response to specific events or exogenous factors (e.g. by temperature, nutrient additive, hormone, etc.) or according to the phase of a viral cycle (e.g. late or early). One may also use promoters that are repressed during the production step in response to specific events or exogenous factors, in order to optimize virus production and circumvent potential toxicity of the expressed polypeptide(s).

Although conventional promoters such as cytomegalovirus (CMV) immediate early promoter (US 5,168,062), the RSV promoter, the adenovirus major late promoter, the phosphoglycero kinase (PGK) promoter (Adra et al., 1987, Gene, 60: 65-74), the thymidine

kinase (TK) promoter of herpes simplex virus (HSV)-1 and the T7 polymerase promoter (WO98/10088) may be used in the context of the present invention. Poxvirus promoters appear to be particularly adapted for expression in cowpox viruses. Representative examples include without limitation the vaccinia 7.5K, H5R, 11K7.5 (Erbs et al., 2008, Cancer Gene Ther. 15(1): 18-28), TK, p28, p11K, Pr13.5 (WO2014/063832), pB8R, pF11L, pA44L, pC11R (WO2011/128704) and K1L promoter, as well as synthetic promoters such as those described in Chakrabarti et al. (1997, Biotechniques, 23: 1094-7; Hammond et al., 1997, J. Virol. Methods, 66: 135-8; and Kumar and Boyle, 1990, Virology, 179: 151-8) as well as early/late chimeric promoters (e.g. US8,394,385; US 8,772,023). Cowpox promoters are also suitable as well (e.g. the ATI promoter).

Those skilled in the art will appreciate that the regulatory elements controlling the expression of the nucleic acid(s) of interest may further comprise additional elements for proper initiation, regulation and/or termination of transcription (e.g. a transcription termination sequences), mRNA transport (e.g. nuclear localization signal sequences), processing (e.g. splicing signals), and stability (e.g. introns and non-coding 5' and 3' sequences), translation (e.g. an initiator Met, tripartite leader sequences, IRES ribosome binding sites, signal peptides), targeting sequences, transport sequences, secretion signal, and 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.

The nucleic acid(s) of interest can be inserted at any location of the viral genome, with a specific preference for a non-essential locus. For example, *CPXV105 CDS* gene, *CPXV083 CDS* gene, *CPXV051 CDS* gene or intergenic zones are particularly appropriated for insertion of the nucleic acid sequence of interest and appropriate regulatory sequences in cowpox virus. In a preferred embodiment, the cowpox virus is defective for the *CPXV105 CDS* gene and comprises inserted in place of the *CPXV105 CDS* gene a nucleic acid of interest (e.g. the *FCU-1* gene) under the transcriptional control of a synthetic promoter (e.g. the p11K7.5 promoter).

PROCESS FOR PREPARING A COWPOX VIRUS

The invention also relates to a process for preparing a cowpox virus according to the invention, in which process:

(i) a cowpox virus of the invention is introduced into a cell,

(ii) said cell is cultured under conditions which are appropriate for enabling said cowpox virus to be produced, and

(iii) said cowpox virus is recovered from the cell culture.

Typically, the cowpox virus of the present invention is produced into a suitable host cell line using conventional techniques including culturing the transfected or infected host cell under suitable conditions so-as to allow the production of infectious viral particles and recovering the produced infectious viral particles from the culture of said cell and optionally purifying said recovered infectious viral particles. Suitable host cells for production of the oncolytic virus include without limitation human cell lines such as HeLa (ATCC), Monkey cells such as Vero (ATCC CCL-081), 293 cells (Graham et al., 1997, J. Gen. Virol. 36: 59-72), HER96, PER-C6 (Fallaux et al., 1998, Human Gene Ther., 9: 1909-17), CV1 (ATCC CCL-70) and BSC1 (ATCC CCL-26) cell lines, avian cells such as those described in WO2005/042728, WO2006/108846, WO2008/129058, WO2010/130756, WO2012/001075, etc.), hamster cell lines such as BHK-21 (ATCC CCL-10) as well as primary chicken embryo fibroblasts (CEF) prepared from chicken embryos obtained from fertilized eggs. Host cells are preferably cultivated in a chemically defined medium with no product of animal or human origin. Culturing is carried out at a temperature, pH and oxygen content appropriate for the producer cell. Such culturing conditions are within the expertise of one of ordinary skill in the art. If growth factors are present, they are preferably recombinantly produced and not purified from animal material. Suitable animal-free media are commercially available, for example VP-SFM medium (Invitrogen) for culturing CEF producer cells. Producer cells are preferably cultivated at a temperature comprised between +30°C and +38°C (more preferably at about +37°C) for between 1 and 8 days (preferably for 1 to 5 days for CEF and 2 to 7 days for immortalized cells) before infection. If needed, several passages of 1 to 8 days may be made in order to increase the total number of cells.

Producer host cells are infected by the CPXV with an appropriate multiplicity of infection (MOI) to permit productive infection, which can be as low as 0.001 (more preferably between 0.05 and 5).

In step ii), infected producer cells are cultured under appropriate conditions well known to those skilled in the art until progeny viral vector (e.g. infectious CPXV particles) is produced. Culture of infected producer cells is also preferably performed in a chemically defined medium (which may be the same as or different from the medium used for culture of producer cells and/or for infection step) free of animal- or human-derived products at a temperature between +30°C and +37°C, for 1 to 5 days.

In step iii), the viral CPXV particles may be collected from the culture supernatant and/or the producer cells. Recovery from producer cells (and optionally also from culture supernatant), may require a step allowing the disruption of the producer cell membrane to allow the liberation of the virus from producer cells. The disruption of the producer cell membrane can be induced by various techniques well known to those skilled in the art, including but not limited to, freeze/thaw, hypotonic lysis, sonication, microfluidization, or high speed homogenization.

The recovered Cowpox virus can be at least partially purified before being used according to the present invention. Various purification steps can be envisaged, including clarification, enzymatic treatment (e.g. endonuclease such as benzonase, protease), ultracentrifugation (e.g. sucrose gradient or cesium chloride gradient), chromatographic and filtration steps. Appropriate methods are described in the art (e.g. WO2007/147528; WO2008/138533, WO2009/100521, WO2010/130753, WO2013/022764).

COWPOX VIRUS COMPOSITION

The invention also relates to a composition which comprises a therapeutically effective amount of a cowpox virus as described herein (e.g. wild type, modified derivative thereof such as a *CPXV105 CDS*-defective cowpox, or recombinant cowpox), or prepared according to the process described herein. In one embodiment, the composition further comprises a pharmaceutically acceptable vehicle.

The composition of the present invention is more specifically intended for the preventive or curative treatment of proliferative diseases (cancers, tumors, restenosis, etc.) or diseases associated to an increased osteoclast activity (e.g. rheumatoid arthritis, osteoporosis). A preferred composition comprises a therapeutically effective amount of a *CPXV105 CDS*-defective cowpox (e.g. a BR cowpox) and notably a recombinant *CPXV105 CDS*-defective cowpox encoding a suicide gene product such as FCU-1.

A "therapeutically effective amount" corresponds to the amount of cowpox virus that is sufficient for producing one or more beneficial results. Such a therapeutically effective amount may vary as a function of various parameters, in particular the mode of administration; the disease state; the age and weight of the subject; the ability of the subject to respond to the treatment; kind of concurrent treatment; the frequency of treatment; and/or the need for prevention or therapy. When prophylactic use is concerned, the composition of the invention is administered at a dose sufficient to prevent or to delay the onset and/or establishment and/or relapse of a pathologic condition (e.g. a proliferative disease such as cancer), especially in a subject at risk. For "therapeutic" use, the composition of the invention is administered to a subject diagnosed as having a pathological condition (e.g. a proliferative disease such as cancer) with the goal of treating the disease, optionally in association with one or more conventional therapeutic modalities. In particular, a therapeutically effective amount could be that amount necessary to cause an observable improvement of the clinical status over the baseline status or over the expected status if not treated as described hereinafter. An improvement of the clinical status can be easily assessed by any relevant clinical measurement typically used by physicians and skilled healthcare staff. For example, techniques routinely used in laboratories (e.g. flow cytometry, histology) may be used to perform tumor surveillance. A therapeutically effective amount could also be the amount necessary to cause the development of an effective non-specific (innate) and/or specific anti-tumor response. Typically, development of an immune response in particular T cell response can be evaluated *in vitro*, in suitable animal models or using biological samples collected from the subject. One may also use various available antibodies so-as to identify different immune cell populations involved in anti-tumor response that are present in the

treated subjects, such as cytotoxic T cells, activated cytotoxic T cells, natural killer cells and activated natural killer cells.

The term "pharmaceutically acceptable vehicle" is intended to include any and all carriers, solvents, diluents, excipients, adjuvants, dispersion media, coatings, antibacterial and antifungal agents, absorption agents and the like compatible with administration in mammals and in particular human subjects.

The cowpox virus of the invention can independently be placed in a solvent or diluent appropriate for human or animal use. The solvent or diluent is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength. Representative examples include sterile water, physiological saline (e.g. sodium chloride), Ringer's solution, glucose, trehalose or saccharose solutions, Hank's solution, and other aqueous physiologically balanced salt solutions (see for example the most current edition of Remington: The Science and Practice of Pharmacy, A. Gennaro, Lippincott, Williams&Wilkins).

In other embodiments, the cowpox virus is suitably buffered for human use. Suitable buffers include without limitation phosphate buffer (e.g. PBS), bicarbonate buffer and/or Tris buffer capable of maintaining a physiological or slightly basic pH (e.g. from approximately pH 7 to approximately pH 9).

The composition of the invention may also contain other pharmaceutically acceptable excipients for providing desirable pharmaceutical or pharmacodynamic properties, including for example osmolarity, viscosity, clarity, colour, sterility, stability, rate of dissolution of the formulation, modifying or maintaining release or absorption into a human or animal subject, promoting transport across the blood barrier or penetration in a particular organ.

In a further embodiment, the composition of the invention may be adjuvanted to further enhance immunity (especially a T cell-mediated immunity) or facilitate infection of tumor cells upon administration. Representative examples of suitable adjuvants include, without limitation, alum, mineral oil emulsion such as, Freunds complete and incomplete (IFA), lipopolysaccharide or a derivative thereof (Ribi et al., 1986, Plenum Publ. Corp.,407-419), saponins such as QS21 (Sumino et al., 1998, J.Virol. 72: 4931; WO98/56415), imidazo-quinoline compounds such as Imiquimod (Suader, 2000, J. Am Acad Dermatol. 43:S6), S-27609 (Smorlesi, 2005, Gene Ther. 12: 1324) and related compounds such as those described

in WO2007/147529, polysaccharides such as Adjuvax and squalenes, oil in water emulsions such as MF59, double-stranded RNA analogs such as poly(I:C), single stranded cytosine phosphate guanosine oligodeoxynucleotides (CpG) (Chu et al., 1997, J. Exp. Med., 186: 1623; Tritel et al., 2003, J. Immunol., 171: 2358) and cationic peptides such as IC-31 (Kritsch et al., 2005, J. Chromatogr. Anal. Technol. Biomed. Life Sci., 822: 263-70).

In one embodiment, the composition of the invention may be formulated with the goal of improving its stability in particular under the conditions of manufacture and long-term storage (i.e. for at least 6 months, with a preference for at least two years) at freezing (e.g. -70°C, -20°C), refrigerated (e.g. 4°C) or ambient temperatures. Various virus formulations are available in the art either in frozen, liquid form or lyophilized form (e.g. WO98/02522, WO01/66137, WO03/053463, WO2007/056847 and WO2008/114021, etc.). Solid (e.g. dry powdered or lyophilized) compositions can be obtained by a process involving vacuum drying and freeze-drying. For illustrative purposes, buffered formulations including NaCl and/or sugar are particularly adapted to the preservation of viruses (e.g. Tris 10 mM pH 8 with saccharose 5 % (W/V), sodium glutamate 10 mM, and NaCl 50 mM or phosphate-buffered saline with glycerol (10%) and NaCl).

The cowpox virus composition is preferably formulated in a way adapted to the mode of administration to ensure proper distribution and release *in vivo*. For example, gastro-resistant capsules and granules are particularly appropriate for oral administration, suppositories for rectal or vaginal administration, optionally in combination with absorption enhancers useful to increase the pore size of the mucosal membranes. Such absorption enhancers are typically substances having structural similarities to the phospholipid domains of the mucosal membranes (such as sodium deoxycholate, sodium glycocholate, dimethyl-beta-cyclodextrin, lauryl-1-lysophosphatidylcholine). Another and particularly appropriate example is a formulation adapted to the administration through microneedle means (e.g. transcutaneous or intradermal patches). Such a formulation may comprise resuspension of the immunotherapeutic product in endotoxin-free phosphate-buffered saline (PBS).

The appropriate dosage of cowpox virus can be adapted as a function of various parameters and may be routinely determined by a practitioner in the light of the relevant circumstances. Suitably, individual doses for the cowpox virus may vary within a range

extending from approximately 10^3 to approximately 10^{12} vp (viral particles), iu (infectious unit) or pfu (plaque-forming units) depending on the virus and the quantitative technique used. Cowpox virus suitable doses are selected preferably between approximately 10^4 pfu to approximately 10^{11} pfu, more preferably between 10^5 pfu to approximately 10^{10} pfu; doses of approximately 10^6 pfu to approximately $5x10^9$ pfu being particularly adapted (e.g. dose of 10^6, $2x10^6$, $3x10^6$, $4x10^6$, $5x10^6$, $6x10^6$, $7x10^6$, $8x10^6$, $9x10^6$, 10^7, $2x10^7$, $3x10^7$, $4x10^7$, $5x10^7$, $6x10^7$, $7x10^7$, $8x10^7$, $9x10^7$, 10^8, $2x10^8$, $3x10^8$, $4x10^8$, $5x10^8$, $6x10^8$, $7x10^8$, $8x10^8$, $9x10^8$, 10^9, $2x10^9$, $3x10^9$, $4x10^9$ or $5x10^9$ pfu) to human use. The quantity of virus present in a sample can be determined by routine titration techniques, e.g. by counting the number of plaques following infection of permissive cells (e.g. BHK-21 or CEF), immunostaining (e.g. using anti-virus antibodies; Caroll et al., 1997, Virology 238: 198-211), by measuring the A260 absorbance (vp titers), or still by quantitative immunofluorescence (iu titers).

ADMINISTRATION

The cowpox virus or the composition of the invention may be administered in a single dose or multiple doses. If multiples doses are contemplated, administrations may be performed by the same or different routes and may take place at the same site or at alternative sites. Intervals between each administration can be from several hours to 8 weeks (e.g. 24h, 48h, 72h, weekly, every two or three weeks, monthly, etc.). Intervals can also be irregular. It is also possible to proceed via sequential cycles of administrations that are repeated after a rest period (e.g. cycles of 3 to 6 weekly administrations followed by a rest period of 3 to 6 weeks). The dose can vary for each administration within the range described above.

Any of the conventional administration routes are applicable in the context of the invention including parenteral, topical or mucosal routes. Parenteral routes are intended for administration as an injection or infusion and encompass systemic as well as locoregional routes. Locoregional administrations are restricted to a localized region of the body (e.g. intraperitoneal or intrapleural administration). Common parenteral injection types are intravenous (into a vein), intra-arterial (into an artery), intradermal (into the dermis), subcutaneous (under the skin), intramuscular (into muscle) and intratumoral (into a tumor

or at its proximity). Infusions typically are given by intravenous route. Topical administration can be performed using transdermal means (e.g. patch and the like). Mucosal administrations include without limitation oral/alimentary, intranasal, intratracheal, intrapulmonary, intravaginal or intra-rectal route. Preferred routes of administration for the oncolytic CPXV of the invention include intravenous and intratumoral routes.

Administrations may use conventional syringes and needles (e.g. Quadrafuse injection needles) or any compound or device available in the art capable of facilitating or improving delivery in the subject. Transdermal systems are also appropriate, e.g. using solid, hollow, coated or dissolvable microneedles (see e.g., Van der Maaden et al., 2012, J. Control release 161: 645-55) and preferred are silicon and sucrose microneedle patches (see, e.g., Carrey et al., 2014, Sci Rep 4: 6154 doi 10.1038; and Carrey et al., 2011, PLoS ONE, 6(7) e22442).

According to an advantageous embodiment, especially when the cowpox virus is armed with a suicide gene, the cowpox virus or composition is administered to the subject in combination with a pharmaceutically acceptable quantity of prodrug(s). In the context of the present invention, the cowpox virus and the prodrug can be administered concurrently (within the same time period), sequentially (e.g., the cowpox virus being administered first and the prodrug given second, or vice-versa), in an interspersed manner or in any combination of these types of administration. 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.

By way of illustration, it is possible to use a dose of prodrug comprised between 50 and 500 mg/kg/day, preferably between 50 mg/kg/day and 200 mg/kg/day, and more preferably of 100 mg/kg/day. Within the context of the present invention, the prodrug is administered in accordance with standard practice. The oral or intravenous route is preferred. A preferred embodiment relates to intravenous or intratumoral administration(s) of the cowpox virus (e.g. a FCU-1 expressing and *CPXV105 CDS*-defective cowpox) advantageously combined with oral or intravenous administration of the corresponding prodrug in a sequential schedule of administration with a specific preference for the prodrug therapy starting after the cowpox virus therapy, preferably at least 3 days, more preferably

at least 4 days and even more preferably at least 7 days after the first administration of the virus. In a preferred embodiment, especially when the cowpox virus carries a suicide gene encoding a CDase activity, the prodrug is advantageously an analogue of cytosine, in particular 5-FC or 5-FU.

A particularly preferred composition comprises 10^6 pfu to $5x10^9$ pfu of a *CPXV105 CDS*-defective cowpox (e.g. a BR cowpox) and, notably, a recombinant *CPXV105 CDS*-defective cowpox encoding a suicide gene product such as FCU-1 formulated for intravenous or intratumoral administration, optionally in association with 5-FC or 5-FU prodrug.

METHODS AND USE

In another aspect, the present invention provides a cowpox virus or a composition thereof for use as an oncolytic virus to treat or prevent a disease or a pathologic condition in a subject in need thereof. The present invention also relates to a method of treatment comprising administering such a cowpox virus or composition thereof in an amount sufficient for treating or preventing a disease or a pathologic condition in a subject in need thereof.

In one embodiment, the cowpox virus is as described herein (e.g. wild type, modified derivative thereof such as a *CPXV105 CDS*-defective cowpox, or recombinant cowpox), or prepared according to the process described herein, or comprised in a composition as described herein. In a preferred embodiment, the cowpox virus is a *CPXV105 CDS*-defective cowpox (e.g. CPXV_BR) and, notably, a recombinant *CPXV105 CDS*-defective cowpox encoding a suicide gene product such as FCU-1. In another preferred embodiment, the cowpox virus is wild type.

A “disease” (and any form of disease such as “disorder” or “pathological condition”) is typically characterized by identifiable symptoms.

A preferred use consists in treating or preventing a proliferative disease and a disease associated to an increased osteoclast activity. Examples of proliferative diseases that may be prevented or treated using the CPXV of the invention or composition thereof include cancers, tumors or restenosis; examples of diseases associated to an increased osteoclast activity that may be prevented or treated using the combination and methods of the invention include rheumatoid arthritis and osteoporosis.

The present invention is particularly suited for treating or preventing cancers and particularly Adrenocortical Carcinoma, Adrenal Cortex Cancer, Anal Cancer, Gastrointestinal Carcinoid Tumors (for example Appendix Cancer and Carcinoid Tumor), Bile Duct Cancer (for example Cholangiocarcinoma), Bladder Cancer, Bone Cancer (for example Ewing Sarcoma, Malignant Fibrous Histiocytoma of Bone and Osteosarcoma), Brain Tumors (for example Astrocytomas, Embryonal Tumors, Germ Cell Tumors, Central Nervous System Atypical Teratoid/Rhabdoid Tumor, Craniopharyngioma, Ependymoma, Gliomas and Glioblastoma), Breast Cancer (for example Ductal Carcinoma In Situ), Bronchial Tumors, Carcinoma of Unknown Primary, Cardiac (Heart) Tumors, Cervical Cancer, Chordoma, Chronic Myeloproliferative Neoplasms, Colorectal Cancer (for example Rectal Cancer), Esthesioneuroblastoma, Extracranial Germ Cell Tumor, Extragonadal Germ Cell Tumor, Retinoblastoma, Gallbladder Cancer, Gastrointestinal Carcinoid Tumor, Testicular Cancer, Gestational Trophoblastic Disease, Head and Neck Cancer (for example Hypopharyngeal Cancer, pharyngeal Cancer, Laryngeal Cancer, Lip and Oral Cavity Cancer, Metastatic Squamous Neck Cancer with Occult Primary, Mouth Cancer, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Salivary Gland Cancer, Throat Cancer, Esophageal Cancer), Hepatocellular (Liver) Cancer, Histiocytosis, Langerhans Cell, Kidney cancer (for example Wilms Tumor, Renal Cell Cancer, Transitional Cell Cancer of the Renal Pelvis and Ureter), Langerhans Cell Histiocytosis, Laryngeal Cancer and Papillomatosis, Leukemia (for example Hairy Cell Leukemia, Chronic Lymphocytic Leukemia (CLL), Chronic Myelogenous Leukemia (CML), Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL)), Liver Cancer, Lung Cancer (Small Cell Lung Cancer and Non-Small Cell Lung Cancer), Lymphoma (for example AIDS-Related Lymphoma, Primary CNS Lymphoma, Cutaneous T-Cell Lymphoma, Hodgkin Lymphoma, Burkitt Lymphoma, Primary Lymphoma, Mycosis Fungoides, Non-Hodgkin Lymphoma, Macroglobulinemia, Waldenström, Primary Central Nervous System (CNS) Lymphoma, Sézary Syndrome, T-Cell Lymphoma), Intraocular Melanoma, Mesothelioma, Midline Tract Carcinoma Involving *NUT* Gene, Multiple Endocrine Neoplasia Syndromes, Multiple Myeloma/Plasma Cell Neoplasms Myelodysplastic Syndromes, Chronic Myeloproliferative Neoplasms, Neuroblastoma, Ovarian Cancer (for example Primary Peritoneal Cancer and Fallopian Tube Cancer), Pancreatic Cancer and Pancreatic

Neuroendocrine Tumors (Islet Cell Tumors), Papillomatosis, Paraganglioma, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pituitary Tumor, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Prostate Cancer, Retinoblastoma, Vascular Tumors, Skin Cancer (for example Basal Cell Carcinoma, Melanoma, Squamous Cell Carcinoma and Merkel Cell Carcinoma), Small Intestine Cancer, Soft Tissue Sarcoma (for example Gastrointestinal Stromal Tumors (GIST), AIDS-Related Cancers Kaposi Sarcoma, Kaposi Sarcoma and Rhabdomyosarcoma), Stomach (Gastric) Cancer, Testicular Cancer, Thymoma and Thymic Carcinoma, Thyroid Cancer, Urethral Cancer, Endometrial and Uterine Sarcoma, Vaginal Cancer and Vulvar Cancer. The present invention is also useful for treatment of metastatic cancers.

In a preferred embodiment, the cowpox virus or a composition according to the invention is used for treating glioblastoma, lung cancer, liver cancer, colorectal cancer, pancreatic cancer and cervical cancer, and it is preferably administered either intratumorally or intravenously. A particularly preferred method comprises 1 to 6 intravenous or intratumoral administrations of the cowpox virus of the invention or composition thereof given at weekly to monthly intervals with a specific preference for 3 bi-weekly administrations of a composition comprising 10^6-$5x10^9$ pfu of a *CPXV105 CDS*-defective cowpox (e.g. at approximately D1, D14 and D29) or 10^6-$5x10^9$ pfu of a *CPXV105 CDS*-defective cowpox armed with the *FCU-1* suicide gene inserted in place of the *CPXV105 CDS* (e.g. placed under the control of the p11K7.5 promoter).

The beneficial effects provided by the methods of the present invention can be evidenced by an observable improvement of the clinical status over the baseline status or over the expected status if not treated according to the modalities described herein. An improvement of the clinical status can be easily assessed by any relevant clinical measurement typically used by physicians and skilled healthcare staff. In the context of the invention, the therapeutic benefit can be transient (for one or a couple of months after cessation of administration) or sustained (for several months or years). As the natural course of clinical status which may vary considerably from a subject to another, it is not required that the therapeutic benefit be observed in each subject treated but in a significant number of subjects (e.g. statistically significant differences between two groups can be determined

by any statistical test known in the art, such as a Tukey parametric test, the Kruskal-Wallis test the U test according to Mann and Whitney, the Student's t-test, the Wilcoxon test, etc.).

In a particular embodiment, as the methods according to the present invention are particularly appropriate for treating cancer, such methods can be correlated with one or more of the followings: inhibiting or slowing tumor growth, proliferation and metastasis, preventing or delaying tumor invasion (spread of tumor cells in neighbouring tissues), reducing the tumor number; reducing the tumor size, reducing the number or extent of metastases, providing a prolonged overall survival rate (OS), increasing progression free survival (PFS), increasing the length of remission, stabilizing (i.e. not worsening) the state of disease, providing a better response to the standard treatment, improving quality of life and/or inducing an anti-tumor response (e.g. non-specific (innate) and/or specific such as a cytotoxic T cell response) in the subject treated in accordance with the present invention.

The appropriate measurements that can be used to assess a clinical benefit such as blood tests, analysis of biological fluids and biopsies as well as medical imaging techniques are evaluated routinely in medical laboratories and hospitals and a large number of kits is available commercially. They can be performed before the administration (baseline) and at various time points during treatment and after cessation of the treatment.

The present invention also relates to a method for treating a disease or a pathologic condition in a subject in need thereof comprising administering the cowpox virus described herein (wild type, or modified derivative CPXV, or recombinant), or prepared according to the process described herein, or comprised in the composition described herein, or a cowpox for use described herein. More precisely, the present invention related to a method for inhibiting tumor cell growth *in vivo* comprising administering a cowpox virus or a composition thereof in a subject in need thereof so-as to inhibit the growth of a tumor. For general guidance, inhibition of tumor cell growth can be evaluated routinely, for example by radiography means. The administration(s) of the cowpox virus or a composition thereof desirably result(s) in at least a 10% decrease of the tumor mass.

The present invention also relates to a method of decreasing lytic activity of a cowpox virus or a composition thereof in a non-dividing cell (e.g. as compared to a method relying on the use of a vaccinia virus such as a Copenhagen VV). The administration(s) of the cowpox

virus or a composition thereof desirably result(s) in at least a 10% decrease of lytic activity in a primary cell (e.g. administration of the cowpox virus of the invention is at least 15%, at least 20%, et least 25%, at least 30%, at least 40%, or at least 50% less cytotoxic in primary cells than administration of a vaccinia virus under the same experimental conditions). Advantageously, the cowpox virus of the present invention or the cowpox virus for use according to this invention does not replicate in a primary cell, meaning that the output to input ratio is 2 or less. Preferably, said primary cell is hepatocyte.

In specific embodiments, as mentioned before, any of the methods of the present invention may be implemented with a suicide gene expressing cowpox virus or composition thereof in association with a pharmaceutically acceptable quantity of a prodrug corresponding to the encoded suicide gene product. A preferred association is directed to a CDase-encoding cowpox virus or a UPRTase-encoding cowpox virus (or both CDase and UPRTase) and 5-FC or 5-FU.

Furthermore, the CPXV, composition thereof or method according to the invention can be combined with one or more substances which potentiate the cytotoxic effect of the compounds obtained after conversion of the prodrug (e.g. 5-FU or 5-FUMP). 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., 18:685-92), 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, 1135-7) 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. 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, 3317-21), Leflunomide, A771726 (active metabolite of Leflunomide; Davis et al., 1996, Biochem. 35, 1270-1273) and Brequinar (Chen et al., 1992, Cancer Res. 52, 3251-7).

COMBINATION THERAPIES

In any of the methods according to this aspect of the invention, the cowpox virus of the present invention can be administered in association with any conventional therapeutic modalities which are available for treating or preventing the targeted disease or pathological condition. Representative examples of conventional therapy include, without limitation, surgery, radiotherapy, chemotherapy, cryotherapy, hormonal therapy, toxin therapy, immunotherapy, cytokine therapy, transplantation (e.g. stem cell), hyperthermia, photodynamic therapy.

In one embodiment, the CPXV, composition or method according to the invention can also be used in association with radiotherapy. Those skilled in the art can readily formulate appropriate radiation therapy protocols and parameters (see for example Perez and Brady, 1992, Principles and Practice of Radiation Oncology, 2nd Ed. JB Lippincott Co; using appropriate adaptations and modifications as will be readily apparent to those skilled in the field). The types of radiation that may be used in cancer treatment are well known in the art and include electron beams, high-energy photons from a linear accelerator or from radioactive sources such as cobalt or cesium, protons, and neutrons. Dosage ranges for radio-isotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells. Regular X-rays doses for prolonged periods of time (3 to 6 weeks), or high single doses are contemplated by the present invention.

In other embodiments, the methods may also be used in conjunction with surgery. For example, CPXV or composition thereof may be administered upon excision of the tumor (e.g. by local application within the excised zone for example).

In further embodiments of any of the methods of the invention, the CPXV may be used in combination with one or more substances effective in anticancer therapy, like chemotherapeutic drugs or immunotherapeutic products.

In a specific embodiment, the CPXV may be used in conjunction with chemotherapeutic drugs currently used for treating cancer. Although any chemotherapy drug conventionally used in anti-cancer therapy may be used in combination with the cowpox virus of the present invention or the composition thereof, there may be mentioned

more specifically alkylating agents, topoisomerase I inhibitors, topoisomerase II inhibitors, platinum derivatives, inhibitors of tyrosine kinase receptors, antimetabolites and antimitotic agents.

In still further embodiments, the CPXV may be used in conjunction with immunotherapy, and especially with anti-neoplastic antibodies as well as siRNA and antisense polynucleotides. Representative examples include, among others, monoclonal antibodies blocking immune checkpoint (e.g. Ipilimumab, tremelimumab pembrolizumab, nivolumab, pidilizumab, AMP-224MEDI4736, MPDL3280A, BMS-936559, etc.), monoclonal antibodies blocking Epidermal Growth Factor Receptor (in particular cetuximab, panitumumab, zalutumumab, nimotuzumab, matuzumab, trastuzumab (Herceptin™), etc.) and monoclonal antibodies blocking Vascular Endothelial Growth Factor (in particular bevacizumab and ranibizumab). It is also possible to immunotherapeutic product such as a vector or virus which is not based on CPXV, either oncolytic or replication-defective.

In a specific embodiment, the cowpox virus or composition thereof is administered in combination with an immunotherapeutic product, like for example a vaccine. Representative examples of such immunotherapeutic vaccines suitable for use in the present invention are plasmid DNA vector, or viral vectors such as vaccinia virus (e.g. Copenhagen, WR, Wyeth, MVA, etc.), adenovirus, lentivirus, herpes virus, recombinant polypeptides, among many others. A preferred vaccine is different from the cowpox virus of the present invention, e.g. preferably an oncolytic virus based-vaccine, and more preferably a Vaccinia Virus based-vaccine (e.g. wild-type, attenuated e.g. by TK defectiveness and/or recombinant VACV).

One may provide the subject with the oncolytic cowpox virus and the additional anti-cancer therapy sequentially (e.g. the cowpox therapy may be started first before other therapies or vice versa) or in an interspersed way but concomitant administrations of both therapies within the same period of time are also contemplated. The course of treatment may be routinely determined by a practitioner and various protocols are encompassed by the present invention. For example, 1 to 6 administrations of the CPXV (e.g. 3 bi-weekly injections) may be given during one cycle of chemotherapy. Moreover, after a course of treatment, it is contemplated that there is a period of time at which no anti-cancer treatment is administered before repetition of treatment cycle(s).

All of the above cited disclosures of patents, publications and database entries are specifically incorporated herein by reference in their entirety. Other features, objects, and advantages of the invention will be apparent from the description and drawings and from the claims. The following examples are incorporated to demonstrate preferred embodiments of the invention. However, in light of the present disclosure, those skilled in the art should appreciate that changes can be made in the specific embodiments that are disclosed without departing from the spirit and scope of the invention.

EXAMPLES

MATERIALS & METHODS

Cell lines

Human colon cancer cell lines LoVo (ATCC® CCL-229™), HCT 116 (ATCC® CCL-247™), human lung cancer cell line A549 (ATCC® CCL-185™), hepatocarcinoma human cell line HepG2 (ATCC®HB 8065™), glioblastoma human cancer cell line U-87 MG (ATCC® HTB-14), cervical human cancer cell line HeLa (ATCC® CCL-2™), pancreatic human cancer cell line MIA-Paca-2 (ATCC® CRL-1420™) and Vero cell line (ATCC® CCL-81) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Human esophagus cancer cell line OE-19 (ECACC n°96071721) was obtained from European Collection of Cell Culture (ECACC). All cell lines were grown in recommended media supplemented with 10% fetal calf serum (FCS). Fresh human hepatocytes were purchased from Kalycell (Plobsheim, France) and maintained in hepatocyte medium (Kalycell, France).

Viruses

CPXV (CPXV*wt*) (ATCC® VR-302™) used in this study was obtained from ATCC. Recombinant CPXV expressing the enhanced green fluorescent protein fused to FCU1 (*gfp::fcu1)* were generated in Vero cells infected with CPXV*wt* at a MOI of 0.01. After being incubated at 37°C for 3 hours, the cells were then transfected with a shuttle plasmid containing the fusion gene *GFP::FCU1* positioned under the control of the synthetic p11K7.5 promoter and surrounded by the flanking sequence of the *TK* gene.

The cells were incubated for 48 hours at 37°C. Double recombination occurs between *TK* homologous regions in the shuttle plasmid and the virus, resulting in the insertion of the gene fusion *GFP::FCU1* into the *TK* locus of the CPXV. Virus was isolated from GFP-fluorescent plaques and submitted to additional plaque purification cycles in Vero cells. Virus structure was confirmed by multiple PCRs and DNA sequencing and the resulting virus is named CPXV*tk-/gfp::fcu1*. CPXV*wt* and CPXV*tk*$^-$*/gfp::fcu1* were amplified in Hela cells and purified by sucrose gradient. Virus stocks were titrated on Vero cells by plaque assay.

Western blotting

LoVo tumor cells were infected by CPXV*wt* and CPXV*tk⁻/gfp::fcu1* at an MOI of 0.1 and incubated for 24 h. Cell lysate proteins (30 µg) (determined using a Bio-Rad protein assay) were run on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions and transferred onto a nitrocellulose membrane. The membrane was incubated with mouse monoclonal antibody 3H1 directed against FCU1 (Foloppe et al., 2008, Gene Ther., 15:1361–71), washed and incubated with secondary antibody coupled horseradish peroxidase (Amersham, Les Ulis, France). Blots were developed using enhanced chemiluminescence (Amersham).

Enzymatic assays

CDase activity and UPRTase activity were determined in LoVo cells using 5-FC (Toronto Research Chemicals Inc., North York, Canada) and 5-FU (Sigma) as substrates. Lovo human tumor cells (3 x 10^6 cells) were infected with each CPXV vector at a MOI of 0.001. Fourty- eight hours later, enzymatic assays were determined as previously described (Erbs et al., 2000, Cancer Res., 60(14):3813-22). 5-FC, 5-FU and 5-FUMP were separated isocratically using HPLC (supelcosil LC-18-S column and UV detection at 260 nm and 280 nm). For CDase activity, the mobile phase was 50 mM phosphoric acid adjust to pH 2.1 with ammonium hydroxide. For UPRTase activity, the mobile phase was 20 mM KH_2PO_4, 5mM tetrabutylammoniumsulfate, 5% methanol adjusted to pH5 with potassium hydroxyde.

CDase activity was also measured indirectly by measuring 5-FU released in the culture media. LoVo cells were infected with the different vectors at a MOI of 0.0001 and plated in 6-well culture dish (10^6 cells/well). After 48 h, 1 mM 5-FC was added to the cultures. Every day during 1 week**,** the concentrations of 5-FC and 5-FU in the media were measured using HPLC. Fifty µl of media were quenched with 1 ml of ethyl acetate/2-propanol/0.5M acetic acid solution (84:15:1). The samples were vortexed and centrifuged. The organic supernatant was evaporated to dryness under a stream of nitrogen at 60°C and reconstituted in 50 µl of water and analyzed by HPLC using a mobile phase of 50 mM phosphoric acid adjusted to pH 2.1. The data are expressed as the percent of 5-FU in the media for various incubation times with 5-FC.

In vitro cell sensitivity to 5-FC

Human LoVo and A549 tumor cells were transduced in suspension at a MOI of 0.01 and 0.000001, respectively. A total of 5 x 10^5 cells/well were plated in 6-well culture dishes in 2 ml of medium supplemented with 10 % FCS. At 48 h after infection, cells were exposed to various concentrations of 5-FC for 3 days, before determination of cell viability by trypan blue exclusion using a Vi-Cell Cell Counter.

In vitro viability assay

Human tumor cells were infected in suspension by CPXV*wt* and CPXV*tk*$^-$*/gfp::fcu1* at a MOI of 0.1, 0.01, 0.001 and 0.0001. A total of 3 x 10^5 cells/well were plated in 6-well culture dishes in 2 ml of medium supplemented with 10 % FCS. Cells were then cultured at 37°C for 5 days and the viable cells were counted by trypan blue exclusion using a Vi-Cell Cell Counter (Beckmann Coulter, California).

In vitro permeability assay

Human tumor cells were infected with CPXV*tk*$^-$*/gfp::fcu1* at MOI 0.0001 to 1. A total of 5 x 10^5 cells/well were plated in 6-well culture dishes in 2 ml of medium supplemented with 10 % FCS. At 16 hours post infection, cells were harvested, washed with PBS, and GFP signal was measured by flow cytometry using Navios™ flow cytometer.

In vitro virus yield

Growing human tumor cells were seeded onto 6-well plates at 5 x 10^5 cells/well. Twenty four hours later, cells were infected with CPXV*tk*$^-$*/gfp::fcu1* at MOI of 0.001 and were incubated in fresh growth medium supplemented with 10 % FCS. Supernatants and cells collected 72 hours post-infection were submitted to a quick freeze-thaw cycle and sonication to release intracellular viral particles and viral progeny were quantified on Vero cells by plaque assay.

To evaluate viral replication between human tumor cells and human primary cells, human hepatocarcinoma cells Hep G2 and human primary hepatocytes were infected in 6-

well plates (1 x 10^6 cells/well) by CPXV*wt* and CPXV*tk⁻/gfp::fcu1* at a MOI of 0.0001 (100 PFU/well). Cells were incubated in fresh growth medium supplemented with 10 % FCS until harvesting. At 72h post-infection, supernatant and cells were collected, freeze-thawed and sonicated and viral progeny were quantified on Vero cells by plaque assay.

In vivo viral pathogenicity and biodistribution experiment

Viral pathogenicity was assessed by survival studies in immunocompetent BALB/c mice (female, 6 weeks old from Charles Rivers Laboratories). Increasing dose ranging from 1 x 10^4 PFU to 1 x 10^7 PFU of CPXVwt and CPXV*tk⁻/gfp::fcu1* were injected intravenously by tail vein injection. The animals were followed daily throughout the course of the experiment for sign of illness, examining weight loss, general appearance, lesion formation.

Subcutaneous tumor models

Female Swiss nude mice were obtained from Charles River Laboratories. Animals used in the studies were uniform in age (6 weeks) and body weight (20-23 g).

To evaluate biodistribution, therapeutic activity of CPXV in human xenograft tumor model, 5 x 10^6 human cancer cells (LoVo or U-87 MG) were injected subcutaneously (s.c.) into the flank of the mice. When tumors reached a diameter of 70-100 mm^3, the mice were randomized in a blinded manner and treated with the recombinant CPXV.

Biodistribution of the recombinant CPXV

The presence of CPXV*tk⁻/gfp::fcu1* in tumors and organ samples was evaluated by virus titration. The virus at 1 x 10^6 PFU was injected intravenously (i.v.) by tail vein injection into female nude mice bearing established s.c. U-87 MG. Mice were sacrificed at indicated time points. Before collecting the different organs, mice were perfused intracardially with an exsanguinating solution (0.9 % NaCl with heparin 50 UI/ml) until all the blood was removed. Tumors and other organs were collected and weighted, homogenized in PBS, sonicated and titers were determined on Vero cells by plaque assay. Viral titers were standardized to milligram of tissue.

In vivo antitumor activity of the recombinant CPXV

CPXV*tk*$^-$*/gfp::fcu1* at 1 x 10^6 pfu (in 100 µl PBS) was injected once intratumorally in established s.c U-87 MG or LoVo model. A control group was injected in the same manner with PBS. Starting on day 5 post-virus injection, 5-FC was given by oral gavage for 3 weeks at 100 mg/kg (0.5 ml 5-FC 0.5% in water) twice a day. Tumor size was measured twice a week using calipers. Tumor volumes were calculated in mm^3 using the formula (*П*/6) (length x width^2).

In vivo antitumor activity of the recombinant CPXV without 5-FC

CPXVtk-*/gfp::fcu1* at 1 x 10^6 pfu (in 100 µl PBS) was injected once intratumorally in nude mice bearing subcutaneous MIA-Paca-2 tumors. A control group was injected in the same manner with PBS. In this experience, no 5-FC was administrated to the mice. Tumor size was measured twice a week using calipers. Tumor volumes were calculated in mm^3 using the formula (П/6) (length x width^2).

Statistical analysis

Statistical analyses of tumor volume were performed using the nonparametric Mann-Whitney *U* test and the log-rank test was applied for statistical survival analysis (Statistica 7.1 software, StatSoft, Inc.). A *P*-values < 0.05 was considered to be statistically significant.

RESULTS

Virus engineering

The engineered virus is shown in Figure 1a. The coding sequence of fusion green fluorescent protein-FCU1 (GFP-FCU1) was introduced into the *tk* locus under transcriptional control of the synthetic vaccinia promoter p11k7.5, as described in the Materials and Methods. The chimeric *GFP::FCU1* gene was generated by directly fusing in frame the coding sequences of *GFP* and *FCU1*, followed by a precise deletion of the translation stop and start codons of *GFP* and *FCU1*, respectively. GFP-FCU1 fusion protein exhibits CDase and UPRTase activities similar to the FCU1 protein and this chimeric protein displays a fluorescent signal intensity

equivalent to the native GFP protein. Virus structures were confirmed by PCR and sequencing.

Expression of the FCU1 protein in the cowpox virus was confirmed by Western blot using the mouse monoclonal antibody directed against FCU1 (Figure 1b). Western blot shows that CPXV*tk*$^-$*/gfp::fcu1* expressed the expected 72kDa GFP::FCU1 protein.

Analysis of the FCU1 enzymatic assays and bystander effect

Confirmation of expression of functional FCU1 by CPXV*tk-/gfp::fcu1* was next examined by measuring the enzymatic activities of FCU1 as described previously (Erbs et al., 2008, Cancer Gene Ther. 15(1): 18-28). The CDase and UPRTase activity were determined 48h post infection by the analysis of the enzymatic conversions of 5-FC to 5-FU and 5-FU to 5-FUMP, respectively. This was determined using lysates prepared from LoVo cells infected at a MOI of 0.001.

As shown in Figure 2, elevated CDase activity was found in cells infected with CPXV*tk-/gfp::fcu1*, while no CDase activity was detectable in mock infected or CPXVwt infected cells. In the same way as the CDase activity, UPRTase activity was found in cells infected by the recombinant CPXV but no endogenous activity was detected in control cells. These *in vitro* enzymatic activities demonstrate that CPXV deleted in the *TK* gene can express a functional therapeutic gene.

A major strength of any prodrug activation model is the potential to extend the cytotoxic therapeutic effect to untransfected cells. In the case of FCU1/5-FC, an efficient bystander effect has been reported as 5-FU can reach neighboring cells by simple diffusion. An analysis of supernatant by high performance liquid chromatography (HPLC) revealed a progressive amount of 5-FU in the extracellular medium of LoVo cells transduced with CPXV*tk-/gfp::fcu1* at MOI 0.001 and incubated with 0.1mM 5-FC (Figure 3).

CPXV infects, replicates and kills human tumor cells *in vitro*

To determine permissiveness of the virus, flow cytometric assay was performed based on GFP fluorescence (Figure 4a). We confirm that, at 16h post infection, the recombinant cowpox virus is able to correctly penetrate different cell lines, and that the viral

expression is sufficient. More specifically, HCT116 and HEP-G2 cells were efficiently transduced by CPXV*tk-/gfp::fcu1* reaching a transduction efficiency per population of 89 and 45 at MOI 0.1, and 25 and 8 at MOI 0.01, respectively.

To evaluate the ability of CPXV to enter and replicate in human tumor cell lines, we next measured the increase of viral particles between input viral titer and virus production at 72hpi (Figure 4b). The CPXV*tk⁻/gfp::fcu1* virus was able to productively infect and replicate in 83% of cell lines tested (10 of the 12 human tumor cell lines tested). Most resulted in CPXV titers that were well above input virus. More precisely, in HCT116, Cal33, Hela, A549 and LoVo cells, CPXV*tk⁻/gfp::fcu1* reached a fold increase of, respectively, 100 000, 70 000, 62 000, 32 000 and 25 000, demonstrating the high capacity of CPXV*tk-/gfp::fcu1* to kill tumor cell lines *in vitro*. New experiments (not presented) showed that the CPXVtk-/gfp::fcu1 virus could also infect and replicate in the U87MG cell line.

Oncolytic activity was evaluated by determining the percentage of viability of 8 cell lines upon CPXV*tk⁻/gfp::fcu1* infection according to different MOI (from 0.1 to 0.0001). Cells were infected with the virus at different MOI and counted five days later. Trypan blue exclusion test demonstrates that CPXV*tk⁻/gfp::fcu1* infection rapidly kills the majority of cells (Figure 4c). We observed an interesting and encouraging dose-response of the virus in all cell lines tested. Indeed, 75-80% of A549 (lung carcinoma) and U87MG (glioblastoma) cell lines were killed even at the lowest virus dose (MOI 0.0001). Nevertheless, oncolytic activity was observed after virus infection at MOI of 0.1 in all cell lines, varying from 90-100% lysis in Hep-G2, HCT116, HeLa, U87-MG and A549 to approximately 60% lysis in LoVo, OE19 and Mia-Paca2.

<u>Cell killing by combination of prodrug activation with viral oncolysis</u>

We next evaluated the combined oncolytic efficiency of CPX*tk⁻/gfp::fcu1* and 5-FC. CPX*tk⁻/gfp::fcu1* or CPXwt were used to infect LoVo and A549 cancer cells at MOI of respectively 0.01 and 0.000001. After 48h, 5-FC was added to the cultures at a range of concentrations from 0,1 to 1000µM, and cell viability was determined 3 days later by trypan blue exclusion. As shown in Figure 5, the oncolytic effect of CPXV in the absence of prodrug resulted in approximately 15% reduction in viable cell number. CPX*tk⁻/gfp::fcu1* in absence

of prodrug showed no difference in cytotoxicity at these low MOI. The addition of 5-FC did not increase toxicity in mock and in tumoral cells infected with CPXwt. Conversely, 5-FC conferred increased toxicity to human tumor cells infected by CPX*tk*$^-$*/gfp::fcu1* in a prodrug dose-dependent manner (Figure 5). Thus the enhanced cell killing by the combination of CPX*tk*$^-$*/gfp::fcu1* and 5-FC is due to the expression of FCU1 able to activate the prodrug.

Safety on human primary hepatocytes and on immunocompetent mice

We next assessed the virulence of cowpox virus in Balb/c mice, which are immunocompetent. Mice were infected i.v. with CPXwt or CPX*tk*$^-$*/gfp::fcu1* at a range of 1 x 10^4 to 1 x 10^7 pfu/mouse and formulation buffer was used as control (Figure 6). Weight loss and mortality were monitored for 83 days. All CPX*tk*$^-$*/gfp::fcu1* infected group mice were healthy and no clinical signs of illness were observed in mice. CPXwt at 1 x10^7 pfu/dose killed 9/10 mice at day 1 and one died later (Figure 6d). At 1 x10^6 pfu/dose, 9/10 mice died between day 10 and 25 (Figure 6c). Clinical signs appeared approximately at 9 days after infection. Infected mice exhibit pox lesions around the genital area, the footpad and the head. At lower doses, none of the mice challenged with CPXwt died. Pox lesions appears on the tail and the face of mice at day 9, however they heal after 29 days. In this model system, and except the two lethal doses, virus infected mice don't lose weight. All mice that lost more than 20% of their body weight were euthanized according to the established animal protocol, though surviving mice start to regain weight at 15 days post-challenge (Figure 6e).

Anticipating potential clinical tests, we next evaluated the safety of CPXV on human fresh hepatocytes as control for normal cells and HepG2 hepatocarcinoma cells as control for cancer cells. Fresh hepatocytes were obtained by sampling on healthy liver during abdominal surgery.

Human primary cells and HepG2 cells were plated on 96-well plates. They both were infected with CPXwt and CPX*tk*$^-$*/gfp::fcu1* during 72h with 100pfu/well. Viral fold amplification was next determined by standard plaque assay on Vero cells. Vaccinia virus strain Copenhagen and *tk*-deleted vaccinia virus were also used in this study as control. As shown in Figure 7, Vaccinia viruses can strongly replicate in hepatocarcinoma but also in human primary hepatocytes although at a lesser extent. Conversely, neither CPXwt nor

CPX*tk*-*/gfp::fcu1* replicate in primary hepatocytes. Again, we demonstrate that CPX*tk*-*/gfp::fcu1* selectively replicate in tumoral cells.

Biodistribution of CPXV*tk*-*/gfp::fcu1* and Tumor tissue staining

A biodistribution assay was performed in U87-MG glioblastoma tumor-bearing immunodeficient mice. A dose of 10^6 pfu CPXV*tk*-*/gfp::fcu1* was injected by the i.v. route. At 2 and 7 days p.i., 3 mice were euthanized by exsanguination and the organs were collected. CPXV*tk*-*/gfp::fcu1* virus titers were determined by a standard plaque assay on Vero cells.

Two days after inoculation of the virus, it was only detected in tumors. As shown in Figure 8, by 7 days after injection, low amounts of virus were detected in several organs: it spread into skin and lymph nodes and to a smaller extent in lung (less than 1 pfu/mg). There was no detectable infection of ovary, spleen, liver, kidney, bone marrow blood and brain. The highest level of virus in normal cells was quantified into the skin. This is not surprising result considering the natural tropism of cowpox and more generally the predisposition of orthopoxviridae to infect, replicate and form skin lesions (Chantrey et al., 1999, Epidemiol. Infect., 122:455-60) (McCollum et al., 2012 JID 2012:206). The important point is that CPXV*tk*-*/gfp::fcu1* specifically targets tumors and thus from early time point.

CPXV*tk*-*/gfp::fcu1* therapy reduces tumor in a glioblastoma and colorectal xenograft model of cancer

To evaluate the clinical potential of the virus, *in vivo* growth curve assay was performed on two human cancer cells lines: U87-MG for glioblastoma adenocarcinoma model (Figure 9a) and LoVo as model for colorectal cancer (Figure 9b).

Tumor cells were implanted subcutaneously into the right flank of 7 weeks old Swiss Nude mice. 17 days later, when tumor size reached approximately $100mm^3$, one single dose of CPXwt or CPX*tk*-*/gfp::fcu1* was injected into the tumor. Control groups were injected with buffer. After 5 days, to allow for the viruses to replicate, 5-FC treatment was started. Mice were treated twice a day with soluble 5-FC at a dose of 200mg/kg/day for 2 weeks.

After 5 days of treatment, mice showed classical signs of illness due to 5-FU toxicity, mainly diarrhea and loss of weight. This discomfort was controlled by a recovery time of two days between two phases of 5-FC treatment.

As shown in Figure 9a, CPXV infection resulted in an impressive inhibition of the U87-MG tumors growth of approximately 77%. Treatment with 200 mg/kg/day 5-FC subsequently increases CPXV anti-tumor activity to more than 88% inhibition of tumor size progression.

As shown in Figure 9b, the injection of CPXV shows a slight effect on the progression of tumor volume compared to control group. Combination of 5-FC administration and CPXV action resulted in 59% of tumor regression after 60 days.

CPX*tk-/gfp::fcu1* does not replicate in 3D skin models

To evaluate the safety improvement of the TK-deleted CPXV, the replication of both CPXwt and the TK-deleted virus was studied. As shown in Figure 10, after 7 days of infection, CPX*tk-/gfp::fcu1* presents a viral fold amplification less than 30 compared to the CPXwt which presents a viral fold amplification of 833. These results confirmed the benefit of the *TK gene* deletion for safety improvement.

Safety of CPX*tk-/gfp::fcu1* on PBMC

In peripheral blood mononuclear cells (PBMCs), the deleted cowpox demonstrated to be able to infect but not to replicate. In this study, PBMCs were used for infection and viral replication assay in vitro. As shown in Figure 11, after 16 hours of infection, CPX*tk-/gfp::fcu1* penetrate poorly into PBMCs, with less than 5% of cells infected at MOI 1. Furthermore, no viral amplification was observed 4 days post infection. The replication of the recombinant CPXV was totally abortive into these blood cells, showing that CPX*tk-/gfp::fcu1* has a negligible impact on these cells.

CPXV*tk*$^-$*/gfp::fcu1* therapy reduces tumor in a pancreatic model of cancer

As shown in Figure 12, CPXtk-/gfp::fcu1 treatment resulted in the stabilization of the tumor growth with a reduction of more than 90% of tumoral mass as compared to the control group ($p<0.0005$).

Pox lesions appeared 26 days after virus injection, with 3 to 10 pox/mouse allocated on tail, footpad, back and face.

CPXtk-/gfp::fcu1 does not replicate in human pancreatic islets

To evaluate the safety improvement of the TK-deleted CPXV, the replication of both CPXwt and the TK-deleted virus was studied in human primary cells. As shown in Figure 13, 7 days after infection of the human pancreatic islets, CPX*tk-/gfp::fcu1* presents a viral fold amplification of 11 compared to the CPXwt which presents a viral fold amplification of 55. These results confirmed the benefit of the TK gene deletion for safety improvement.

Altogether, these results show that cowpox viruses can be modified in order to express recombinant polypeptides. They show that *in vivo* and *in vitro*, wild type and recombinant CPXV can efficiently infect, replicate and lyse a large panel of human tumour cells without any impact on its therapeutic index Indeed the recombinant cowpox viruses demonstrated a high safety, as they do not replicate in primary cells, showing the high specificity of CPXV for tumoral cells. We also have demonstrated that the expression of the *FCU1* gene with addition of 5-FC prodrug can increase the antitumoral activity of CPX*tk-/gfp::fcu1* vector in the infected tumor cells. These results were confirmed *in vivo* where the intravenous administered recombinants CPXV selectively target the tumor. Moreover, the intra tumoral injection with and without prodrug, in immuno-deficient mice, resulted in a significant reduction of the tumor progression. Our data showed a clear benefit in combining the oncolytic virotherapy using CPX*tk-/gfp::fcu1* and the prodrug 5-FC for treatment of resistant tumor models.

BIBLIOGRAPHIC REFERENCES

Adra et al., 1987, Gene, 60: 65-74

Andersen et al., 1992, European J. Biochem. 204: 51-56

Buller et al., 1985, Nature, 317(6040):813-815

Cadman et al., 1979, Science 250, 1135-1137

Caroll et al., 1997, Virology 238: 198-211

Carroll et al., 2011, PLoS ONE 6(8): e23086

Chakrabarti et al. (1997, Biotechniques, 23: 1094-7

Chantrey et al., 1999, Epidemiol. Infect., 122:455-460

Chen et al., 1992, Cancer Res. 52, 3251-3257

Chernajovsky et al., 2006, British Med. J., 332:170-2

Chu et al., 1997, J. Exp. Med., 186: 1623

Davis et al., 1996, Biochem. 35, 1270-1273

Dayhoffed, 1981, Suppl., 3: 482-9

Duraffour et al., 2013, PLoS One 8(2):1-8

El Omari et al., 2006, BMC Struct. Biol., 6:22

Erbs et al., 2000, Cancer Res., 60(14):3813-22

Erbs et al., 2008, Cancer Gene Ther. 15(1): 18-28

Essbauer et al., 2002, Revue Méd. Vét., 153, 10, 635-642

Fallaux et al., 1998, Human Gene Ther., 9: 1909-17

Fisher et al., 2006, Curr. Opin. Mol. Ther., vol.8, no.4, p.301-13

Foloppe et al., 2008, Gene Ther., 15:1361–1371

Graham et al., 1997, J. Gen. Virol. 36: 59-72

Grenson et al., 1970, J. Bacteriol., 103(3):770-777

Hammond et al., 1997, J. Virol. Methods, 66: 135-8

Hermiston et al., 2006, Curr. Opin. Mol. Ther., 8:322-30

Jund and Lacroute, 1970, J. Bacteriol., 102(3):607–615

Kern et al., 1990, Gene, 88: 149-57

Kim et al., 1997, Biochem. Mol. Biol. Internat. 41: 1117-24

Kim et al., 2006, Mol. Ther., 14:361-70

Kritsch et al., 2005, J. Chromatogr. Anal. Technol. Biomed. Life Sci., 822: 263-70

Kumar and Boyle, 1990, Virology, 179: 151-8

Martinussen et al., 1994, J. Bacteriol. 176: 6457-63

Martinussen et al., 1995, J. Bacteriol. 177: 271-4

McCart et al., 2001, Cancer res., 61:8751-57

McCollum et al., 2012, Journal of Infectious Disease 2012 :206

Moore et al., 1982, Biochem. Pharmacol. 31, 3317-3321

Needleman et Wunsch. J.Mol. Biol. 48,443-453, 1970

Perez and Brady, 1992, Principles and Practice of Radiation Oncology, 2nd Ed. JB Lippincott Co

Ribi et al., 1986, Plenum Publ. Corp.,407-419

Smorlesi, 2005, Gene Ther. 12: 1324

Suader, 2000, J. Am Acad Dermatol. 43:S6

Sumino et al., 1998, J.Virol. 72: 4931; WO98/56415

Tritel et al., 2003, J. Immunol., 171: 2358

Verardi et al., 2012, Hum. Vaccin. Immunother., 8:961–970

Waxman et al., 1982, Eur. J. Cancer Clin. Oncol., 18:685-692

US 5,168,062

WO96/16183

WO98/02522

WO98/10088

EP998568

WO01/66137

WO03/053463

WO 2004/014314

WO2005/042728

WO2005/07857

WO 2006/048768

WO2006/108846

WO2007/056847

WO2007/147528

WO2007/147529

WO2008/114021

WO2008/129058

WO2008/138533

WO2009/065546

WO2009/100521

WO2010/130756

WO2012/001075

WO2013/022764

CLAIMS

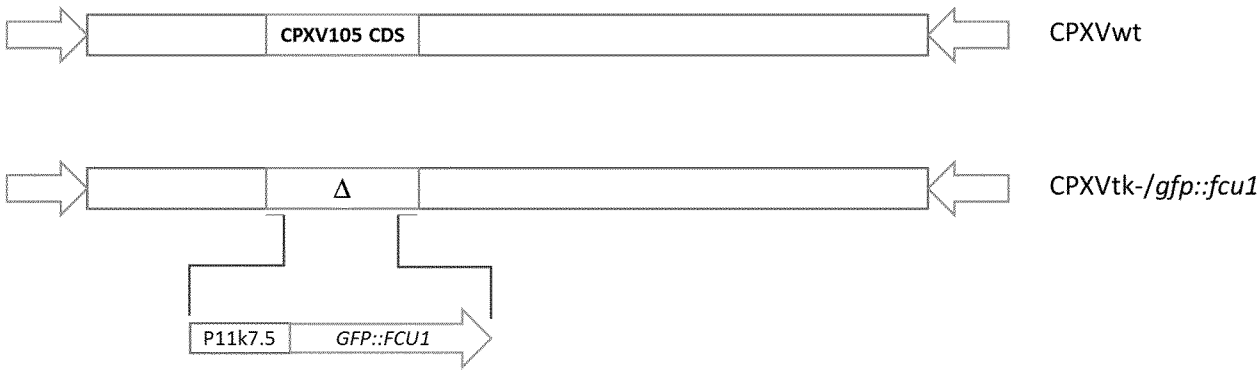
1. A cowpox virus comprising a defective *CPXV105 CDS* gene.
2. The cowpox virus of claim 1, wherein said cowpox virus further comprises a defective *CPXV083 CDS* gene and/or *CPXV051 CDS* gene.
3. The cowpox virus of claim 1 and 2, wherein said cowpox virus further comprises a defective *CPXV049 CDS* gene.
4. The cowpox virus of any one of claims 1 to 3, wherein said cowpox further comprises a truncated *CPXV032 CDS* gene.
5. The cowpox virus of any one of claims 1 to 4, wherein said cowpox virus is engineered to express a nucleic acid of interest.
6. The cowpox virus of claim 5, wherein said nucleic acid of interest encodes one or more polypeptides selected from the group consisting of suicide gene products, immunostimulatory polypeptides, antigens, permease and other molecules of therapeutic interest.
7. The cowpox virus of claim 6, wherein said suicide gene product has at least a cytosine deaminase activity.
8. The cowpox virus of claim 7, wherein said cytosine deaminase-encoding suicide gene comprises *FCY1, FCA1 or CodA* gene or an analogue thereof.
9. The cowpox virus of claim 6 wherein said suicide gene encodes a protein having cytosine deaminase and uracil phosphoribosyl transferase activities.
10. The cowpox virus of claim 6, wherein said permease is a purine or a cytosine permease of *S. Cerevisiae*, and preferably selected from the group consisting of FCY2 and Fur4 and analogues thereof.
11. The cowpox virus of any one of claims 5 to 10, wherein said cowpox virus further comprises the elements necessary for the expression of the nucleic acid(s) of interest.
12. A process for preparing a cowpox virus, in which process:
    - (i) a cowpox virus of anyone of claims 1 to 11 is introduced into a cell;
    - (ii) said cell is cultured under conditions which are appropriate for enabling said cowpox virus to be produced, and;
    - (iii) said cowpox virus is recovered from the cell culture.

13. A composition comprising a therapeutically effective amount of the cowpox virus of any one of claims 1 to 11 or the cowpox virus prepared according to the process of claim 12 and a pharmaceutically acceptable vehicle.
14. A cowpox virus or a composition thereof for use as an oncolytic virus for the prophylaxis or the treatment of a proliferative disease or a disease associated with an increased osteoclast activity.
15. The cowpox virus for use according to claim 14, wherein said cowpox virus is as defined in anyone of claims 1 to 11, or is prepared according to claim 12, or is comprised in a composition as defined in claim 13.
16. The cowpox virus for use according to claim 14 and 15, wherein said proliferative disease is cancer, tumor or restenosis.
17. The cowpox virus for use according to claim 16, wherein said cancer is selected from the group consisting of glioblastoma, lung cancer, liver cancer, colorectal cancer, pancreatic cancer and cervical cancer.
18. The cowpox virus for use according to claims 14 and 15, wherein said disease associated to an increased osteoclast activity is rheumatoid arthritis or osteoporosis.
19. The cowpox virus for use according to any one of claims 14 to 18, wherein said cowpox virus is administered via intravenous or intratumoral route.
20. The cowpox virus for use according to any one of claims 14 to 19, wherein said cowpox virus is administered as a single dose or multiple doses of 10^6 to $5x10^9$ pfu.
21. The cowpox virus for use according to any one of claims 14 to 20, wherein said cowpox virus is administered in association with a pharmaceutically acceptable quantity of a prodrug.
22. The cowpox virus for use according to claim 21, wherein said cowpox virus is defined in any one of claims 7 to 9 and said prodrug is 5-FC.
23. The cowpox virus for use according to claim 21 and 22, wherein said cowpox virus is administered in association with one or more substances which potentiate the cytotoxic effect of compounds obtained after conversion of the prodrug.

24. The cowpox virus for use according to claim 23, wherein said substances which potentiate the cytotoxic effect of the 5-Fluorocytosine are PALA, Leflunomide, A771726 and methotrexate.
25. The cowpox virus for use according to any one of claims 14 to 24, wherein said cowpox virus is administered in combination with one or more substances effective in anticancer therapy.
26. The cowpox virus for use according to claim 25, wherein said one or more substances effective in anticancer therapy is a vaccine.
27. The cowpox virus for use according to claim 26, wherein said vaccine is a viral-based vaccine, preferably an oncolytic virus based-vaccine, and more preferably a Vaccinia Virus based-vaccine.
28. A method for treating a disease or a pathologic condition in a subject in need thereof comprising administering the cowpox virus of anyone of claims 1 to 11, or prepared according to the process of claim 12, or the composition of claim 13, or a cowpox for use according to any one of claims 14 to 27.

Figure 1

1A



1B

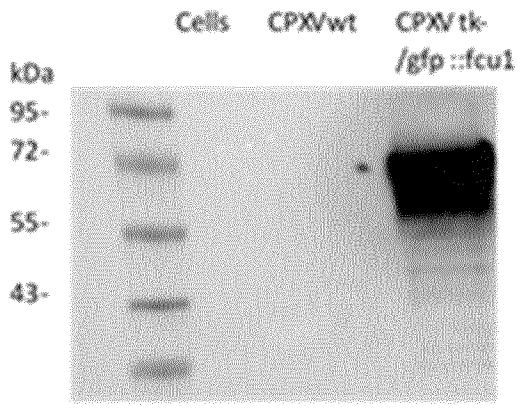


Figure 2

Vector	CDase 5-FC → 5-FU	UPRTase 5-FU → 5-FUMP
Mock	ND	ND
CPXVwt	ND	ND
CPXVtk$^-$/*gfp ::fcu1*	106 ± 17	5,65 ± 0,3

Figure 3

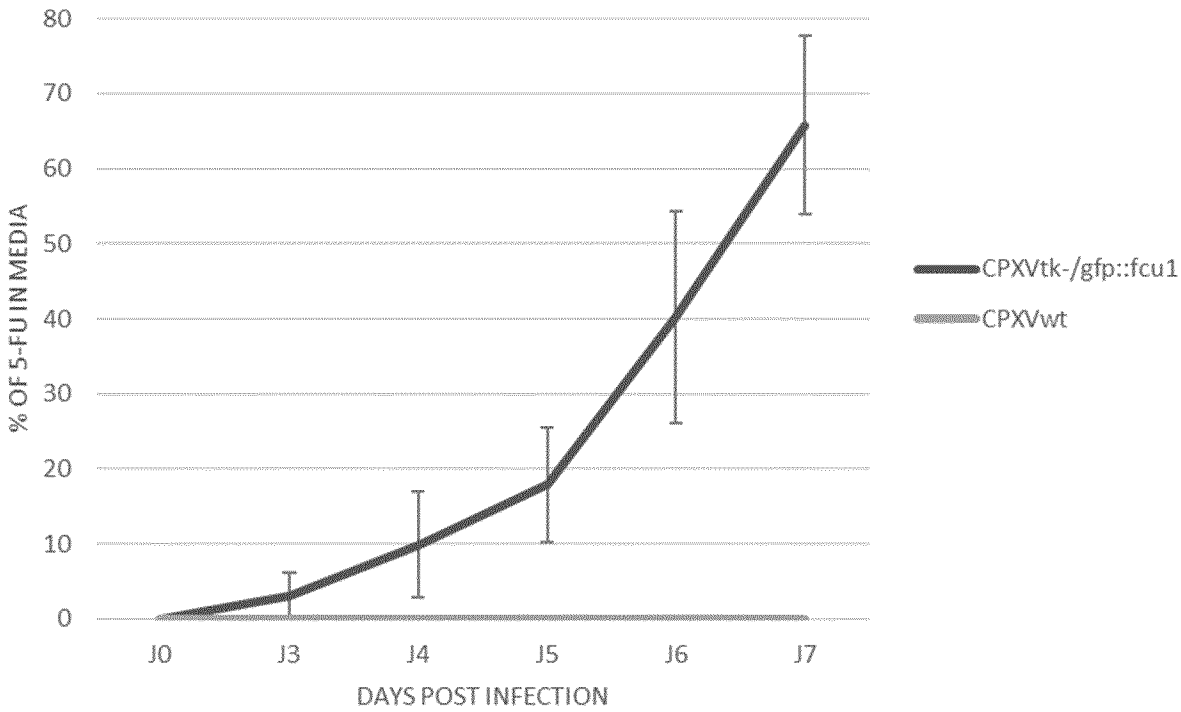


Figure 4

4A

cell line	MOI	Transduction efficiency
HeLa	0,01	0.9 ± 0.1
	0,1	10 ± 0.2
LoVo	0,01	0.4 ± 0.3
	0,1	4 ± 1
U87-MG	0,01	0.3 ± 0.1
	0,1	2 ± 0.1
A549	0,01	2 ± 0.2
	0,1	16 ± 1.2
MIA-Paca 2	0,01	1 ± 0.2
	0,1	14 ± 0.5
HCT116	0,01	25 ± 1.2
	0,1	89 ± 0.5
HEP-G2	0,01	8 ± 0.3
	0,1	45 ± 0.7

4B

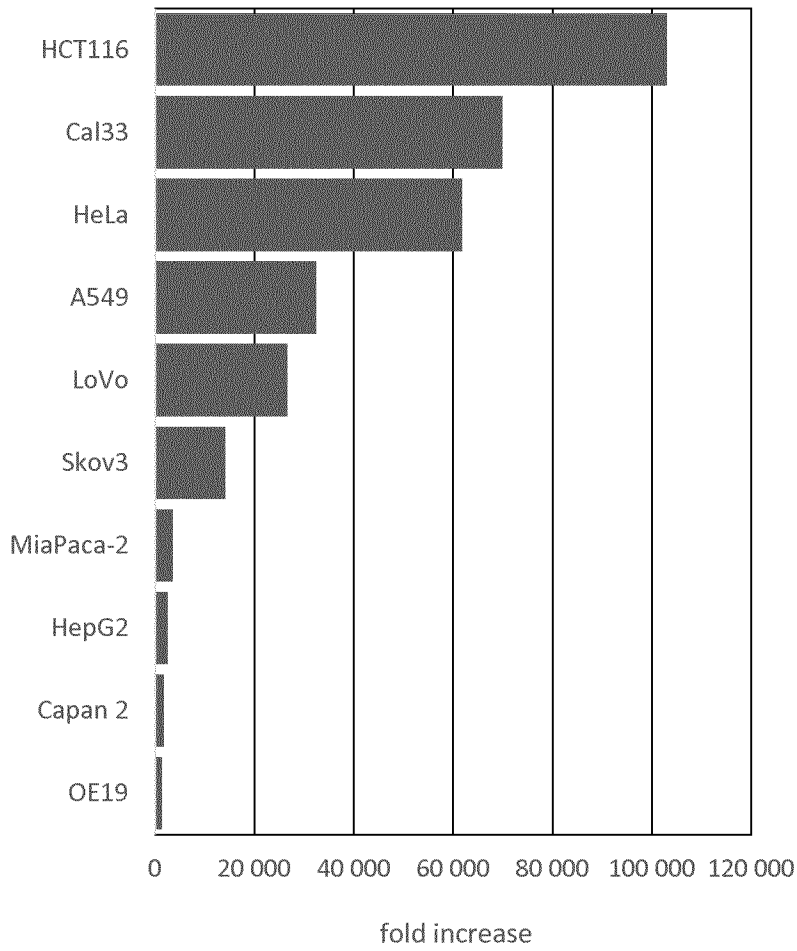


Figure 4

4C

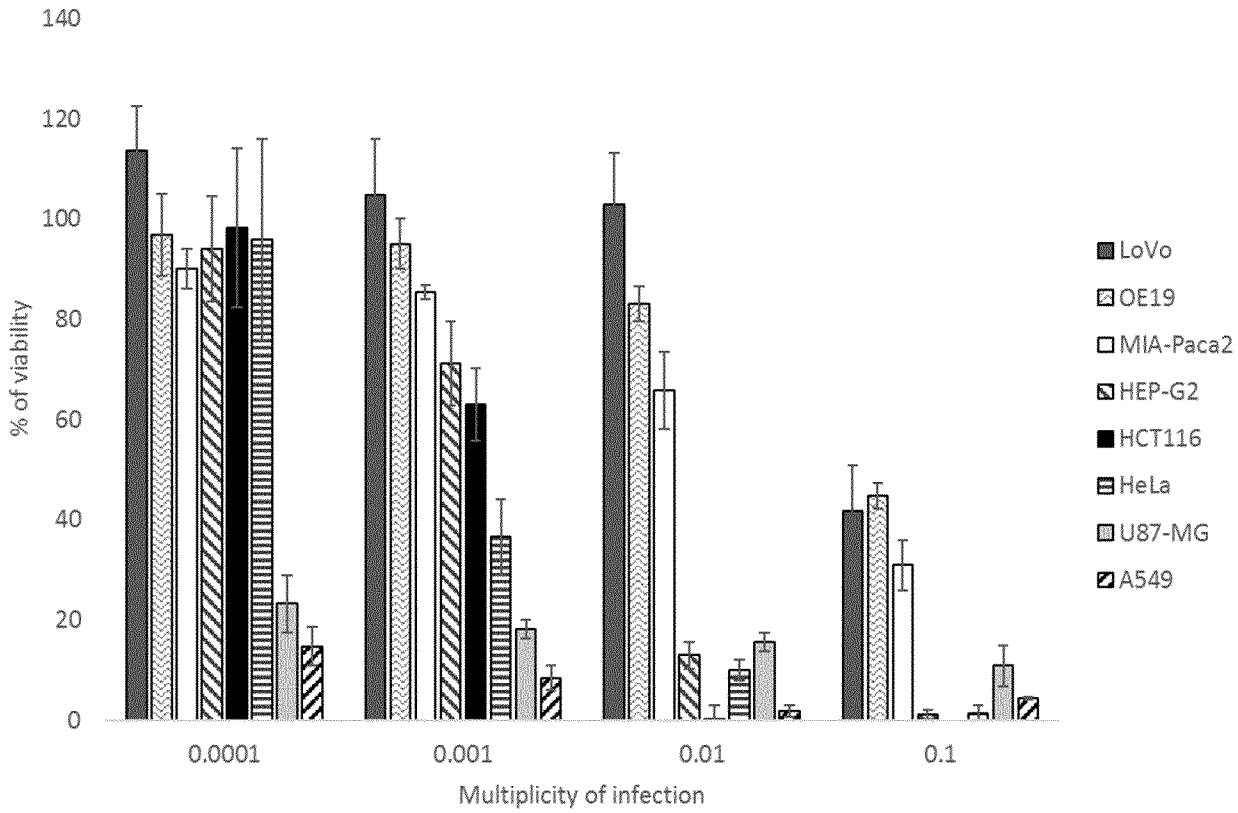
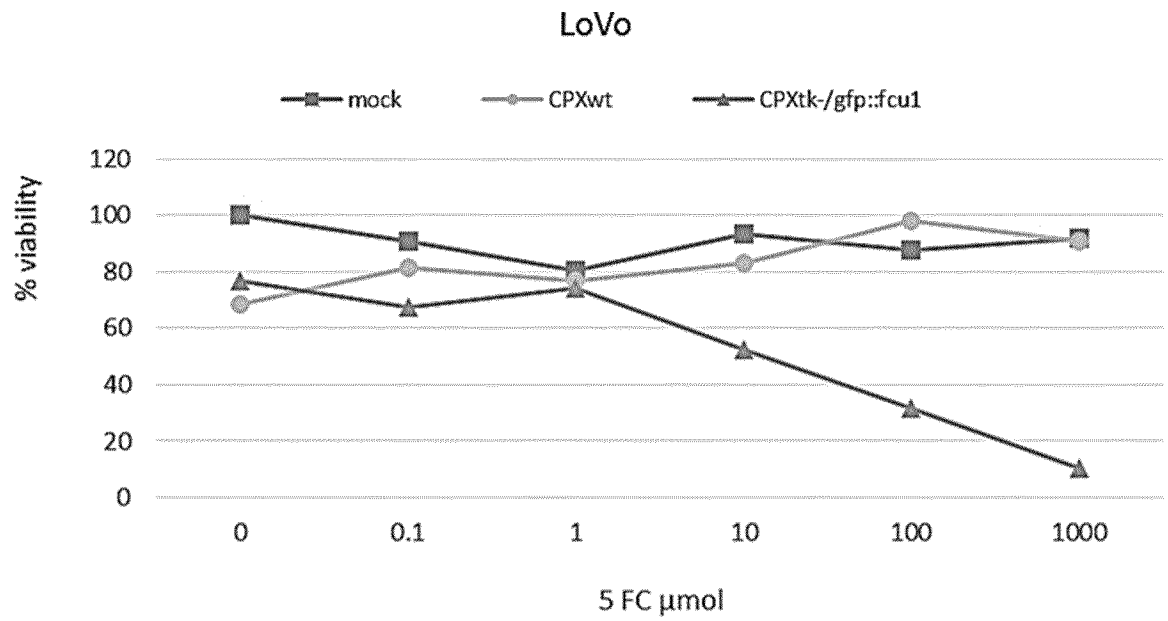

140
120
100
80
60
40
20
0
% of viability
0.0001
0.001
0.01
0.1
Multiplicity of infection
LoVo
OE19
MIA-Paca2
HEP-G2
HCT116
HeLa
U87-MG
A549

Figure 5

5A



5B

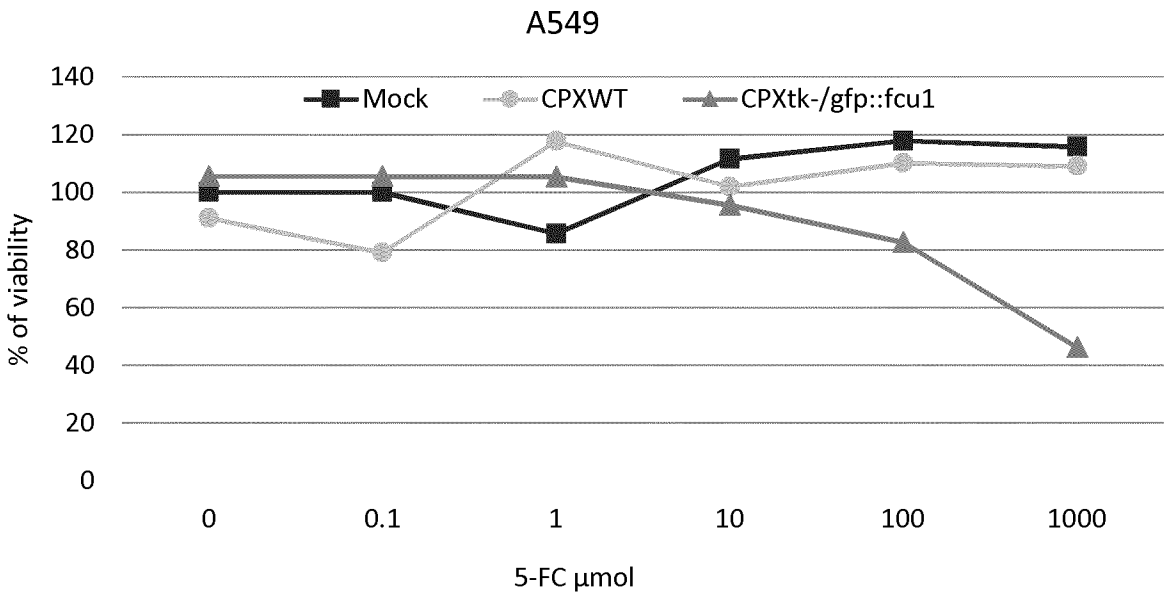
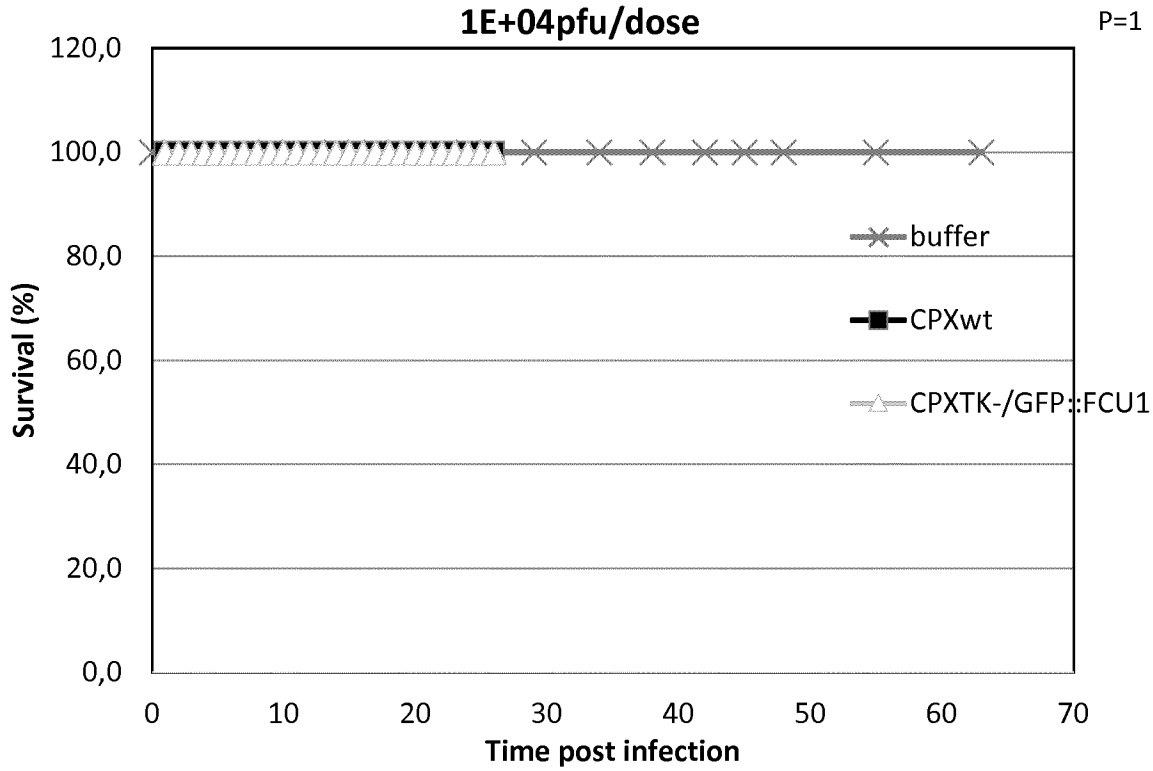


Figure 6

6A



6B

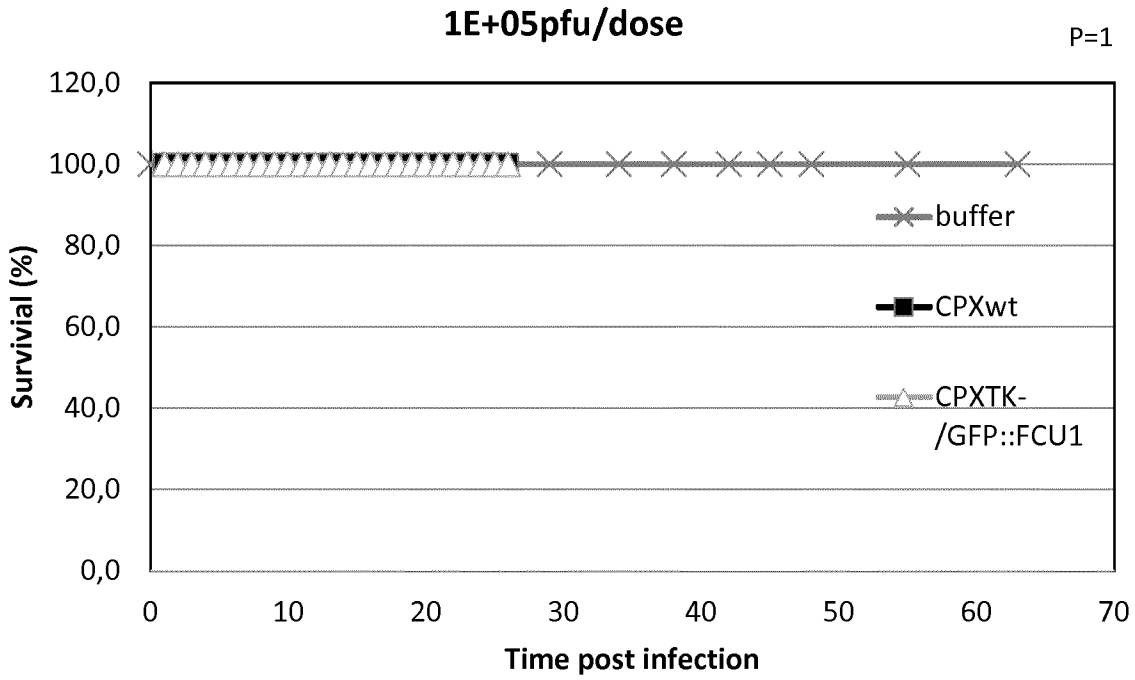
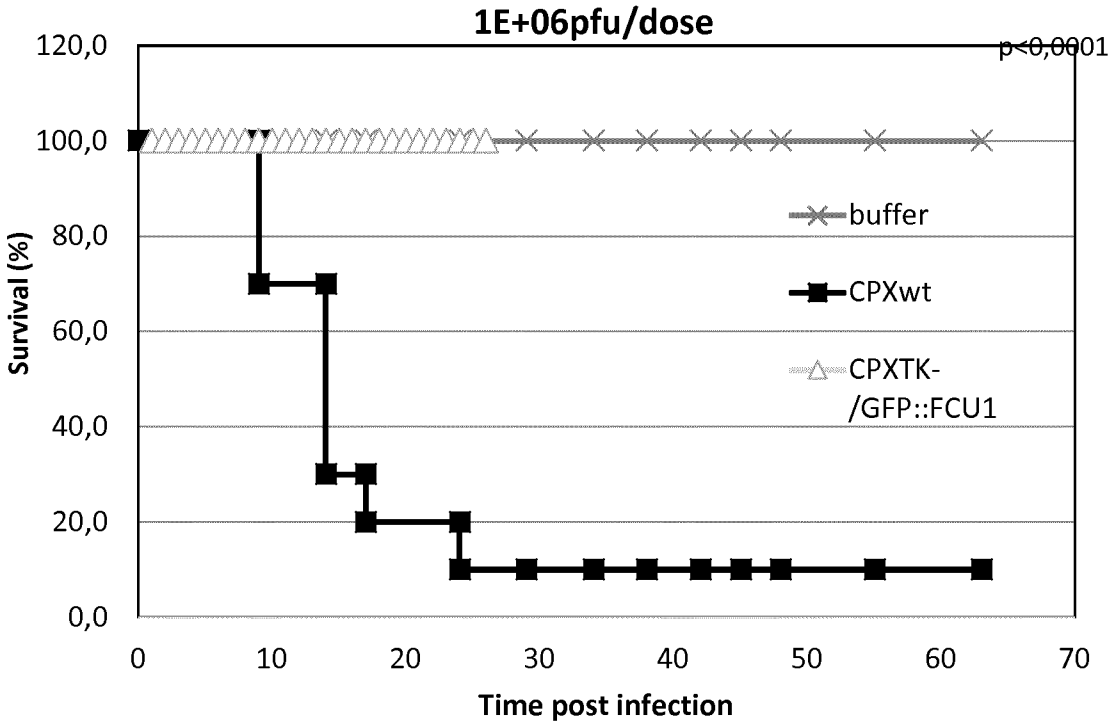


Figure 6

6C



6D

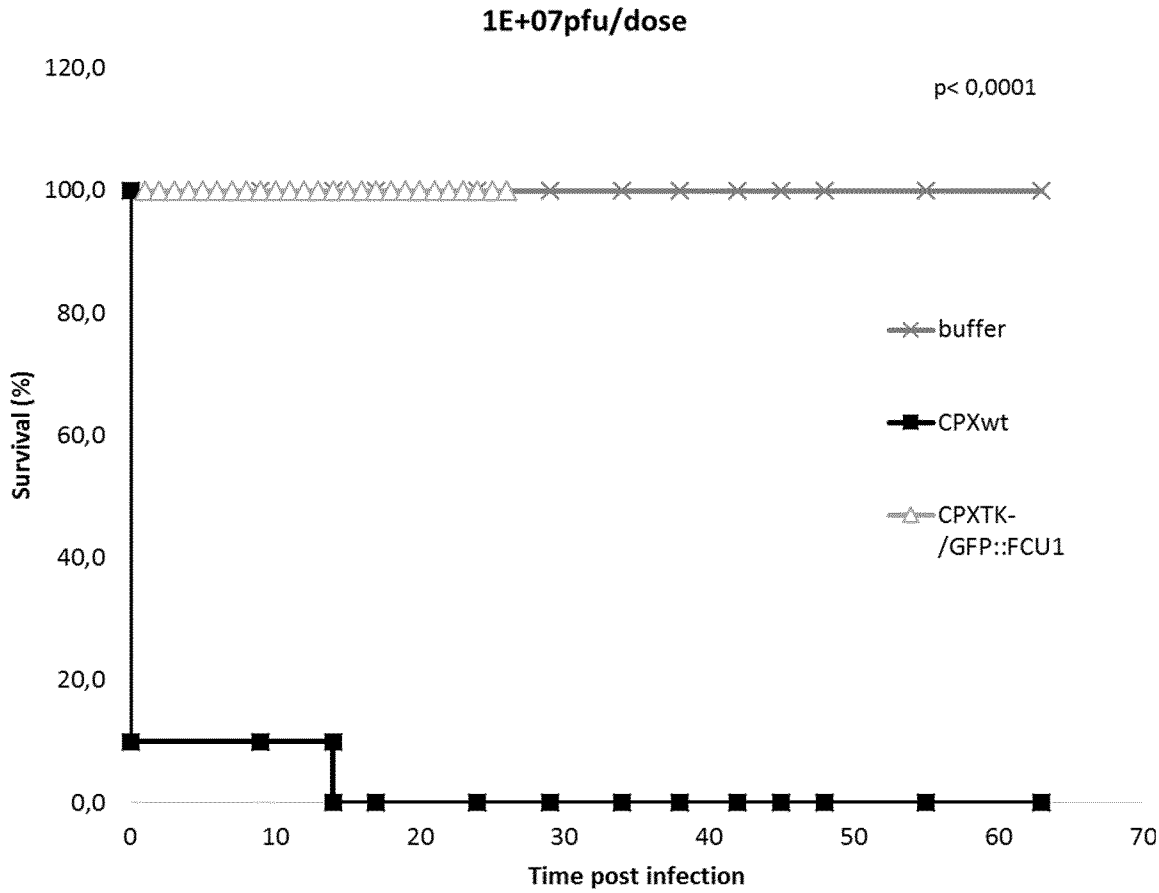


Figure 6

6E

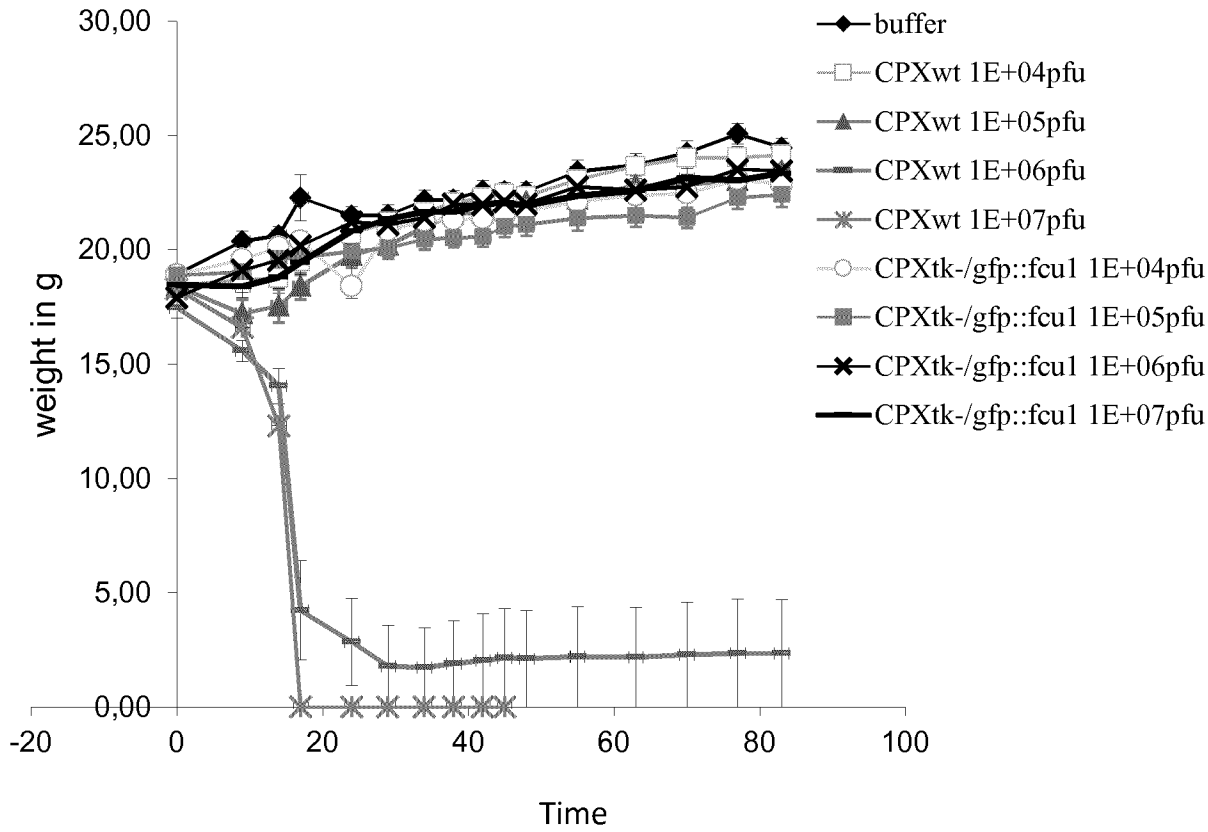


Figure 7

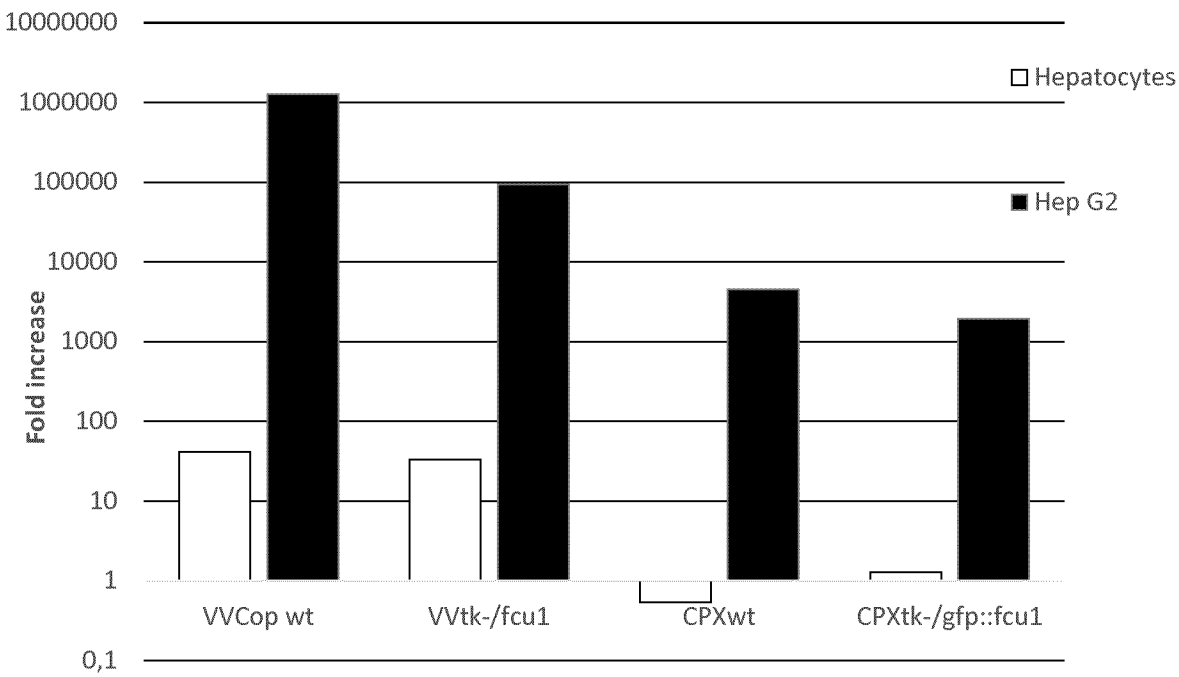


Figure 8

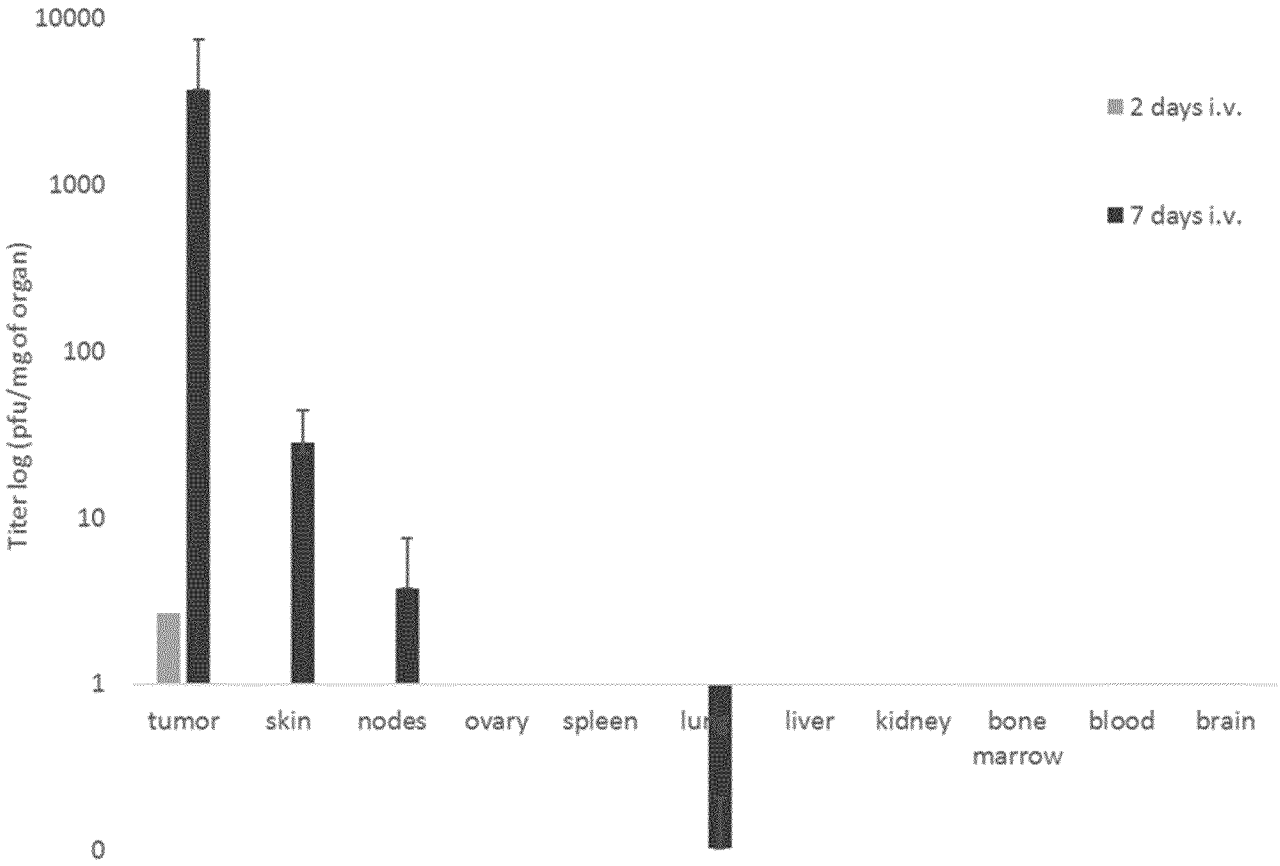
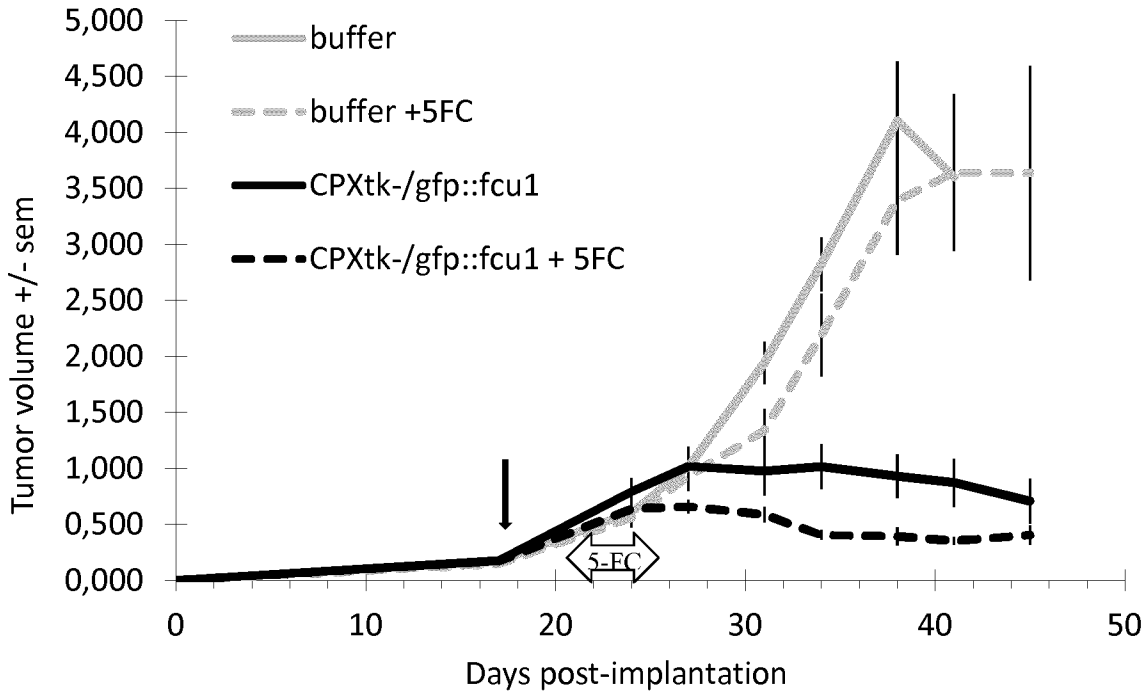

10000
1000
100
10
1
0
Titer log (pfu/mg of organ)
2 days i.v.
7 days i.v.
tumor
skin
nodes
ovary
spleen
liver
kidney
bone marrow
blood
brain

Figure 9

9A



9B

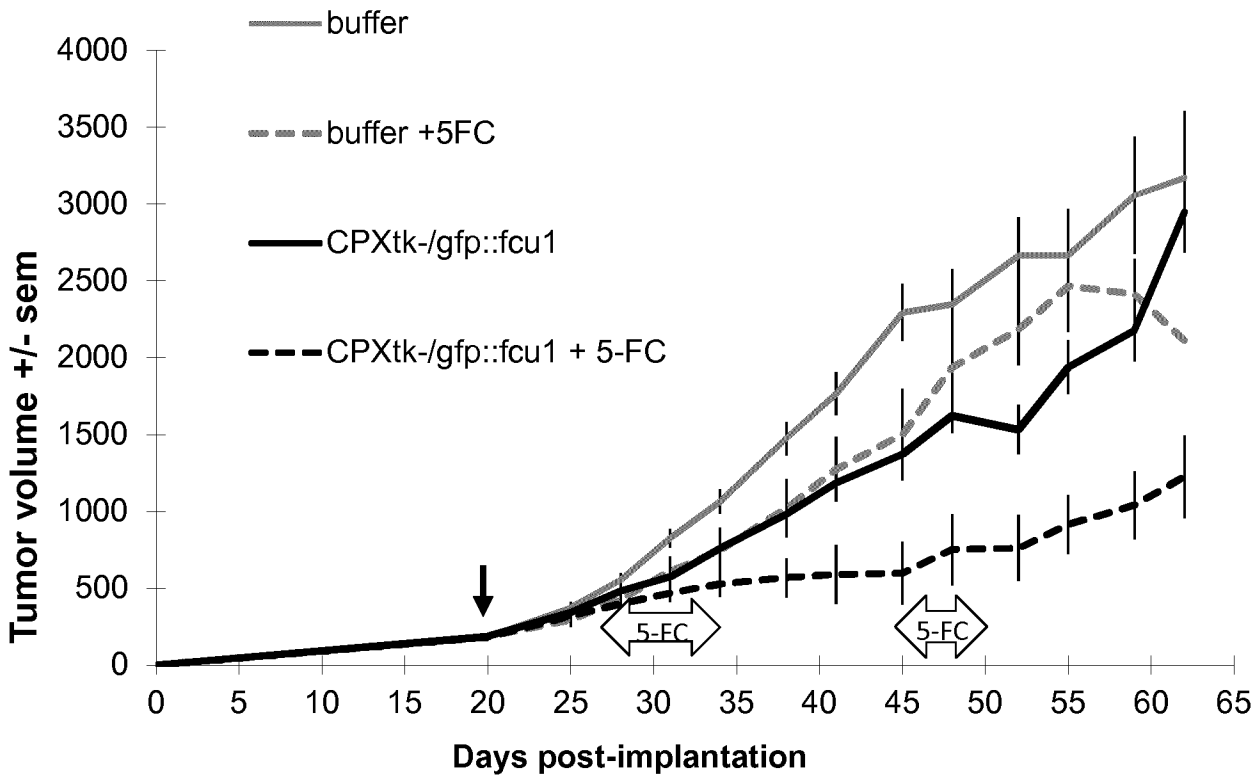


Figure 10

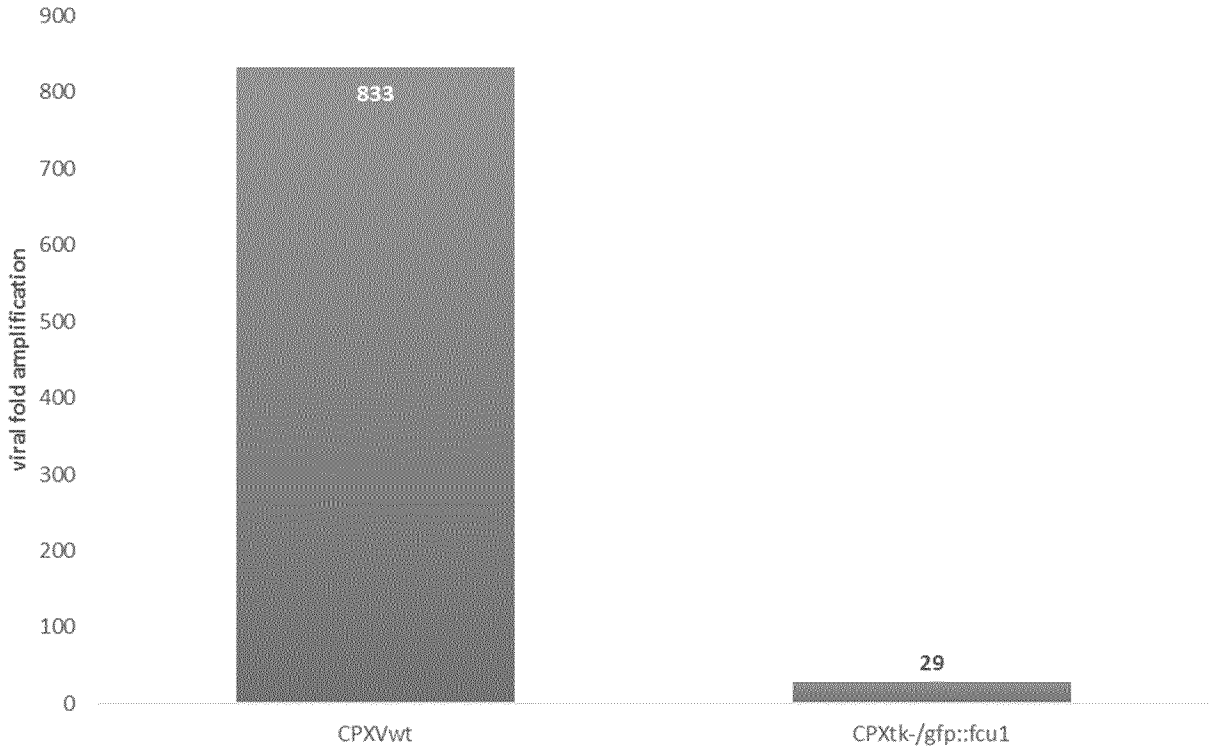


Figure 11

MOI	GFP + (% gated)	Fold amplification 96h p.i.
0.01	0,24	0
0.1	0,52	0,05
1	3,27	0,13

Figure 12

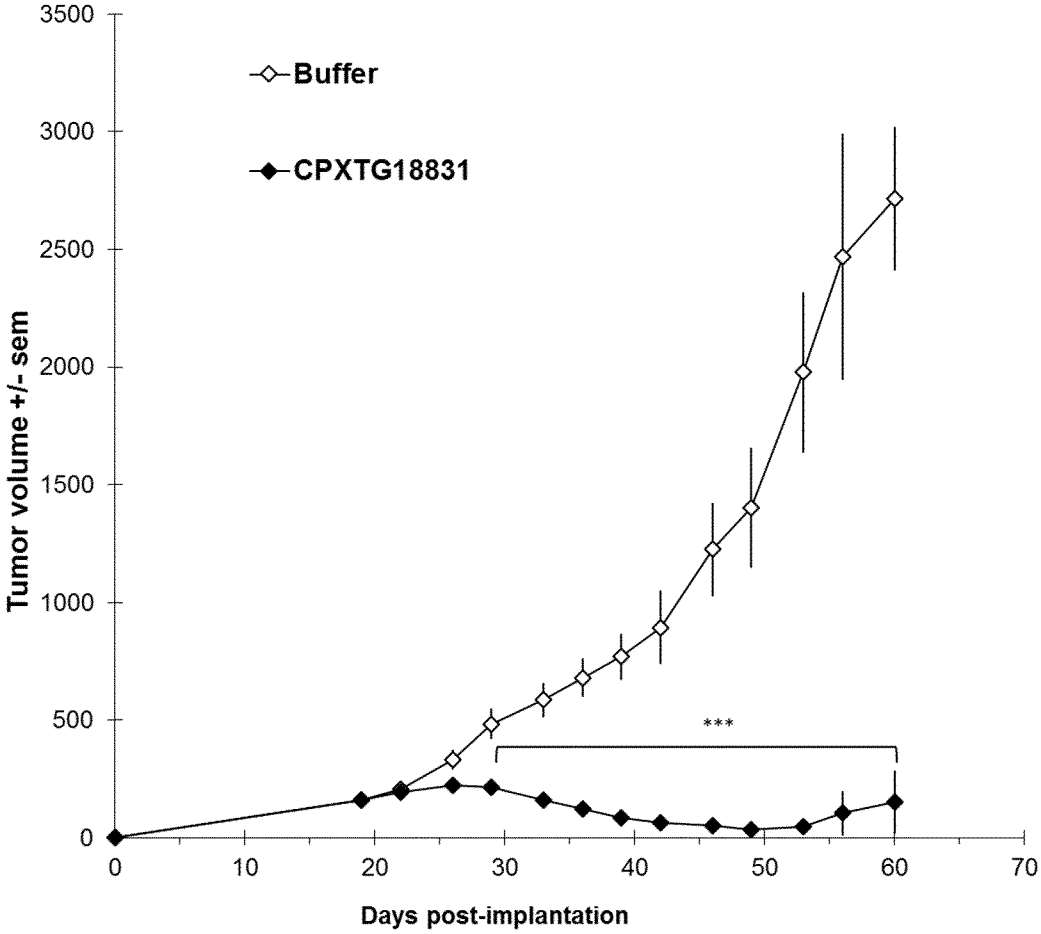
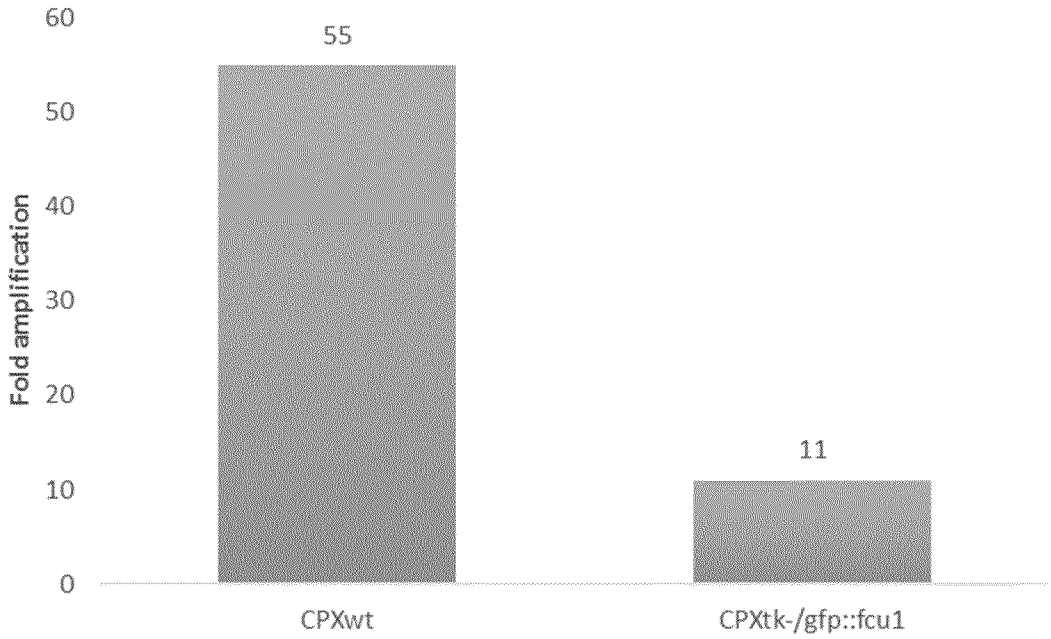


Figure 13



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/079656

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/00 C12N15/863 A61K35/768
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROTH SWAANTJE J ET AL: "Recovery of infectious virus from full-length cowpox virus (CPXV) DNA cloned as a bacterial artificial chromosome (BAC)", VETERINARY RESEARCH, BIOMED CENTRAL LTD, LONDON, UK, vol. 42, no. 1, 11 January 2011 (2011-01-11), page 3, XP021085706, ISSN: 1297-9716, DOI: 10.1186/1297-9716-42-3 see in particular the section "Generation of recombinant CPXV BR.TK- harboring mini-F vector sequence Generation of an infectious full-length clone of CPXV BR (pBRf) and reconstitution of recombinant virus (vBRf) from cloned DNA" and the other cited passages; figure 1; compound vBRf -/--	1,5,6, 11-13

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 2017	20/12/2017

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

3

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Z. XU ET AL: "Generation of a Complete Single-Gene Knockout Bacterial Artificial Chromosome Library of Cowpox Virus and Identification of Its Essential Genes", JOURNAL OF VIROLOGY., vol. 88, no. 1, 1 January 2014 (2014-01-01), pages 490-502, XP055431033, US ISSN: 0022-538X, DOI: 10.1128/JVI.02385-13 see sections "Generation of the dual marker CPXV-BR BAC clone pBRFseR.", "Targeted knockout of all unique CPXV-BR ORFs" and "Virus reconstitution", as well as the cited passages; table 1	1-6, 11-13
X	WO 2015/155263 A1 (TRANSGENE SA [FR]) 15 October 2015 (2015-10-15) paragraphs [0013], [0014], [0019], [0025], [0026], [0028], [0029], [0034] - [0040], [0050] - [0056], [0061] - [0064], [0066] - [0068], [0073], [0075]	1-3, 5-25,28
X	Edward Jenner: "The Three Original Publications on Vaccination Against Smallpox, by Edward Jenner : I. An Inquiry Into the Causes and Effects of the Variolae Vaccinae, Or Cow-Pox. 1798", The Harvard Classics Vol. XXXVIII, Part 4, 1909-14, 25 April 2001 (2001-04-25), XP055431236, New York Retrieved from the Internet: URL:http://www.bartleby.com/38/4/1.html [retrieved on 2017-12-01] see e.g. CASE XVII	28
A	FEND LAETITIA ET AL: "Oncolytic virotherapy with an armed vaccinia virus in an orthotopic model of renal carcinoma is associated with modification of the tumor microenvironment", ONCOIMMUNOLOGY, LANDES BIOSCIENCE, US, vol. 5, no. 2, 1 January 2016 (2016-01-01) , pages e1080414-1, XP008180456, ISSN: 2162-402X the whole document	1-28

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CODY J J ET AL: "Expression of osteoprotegerin from a replicating adenovirus inhibits the progression of prostate cancer bone metastases in a murine model", LABORATORY INVESTIGATION, NATURE PUBLISHING GROUP, THE UNITED STATES AND CANADIAN ACADEMY OF PATHOLOGY, INC, vol. 93, no. 3, 1 March 2013 (2013-03-01), pages 268-278, XP002742297, ISSN: 0023-6837, DOI: 10.1038/LABINVEST.2012.179 [retrieved on 2013-01-28] abstract -----	14
A	W XU ET AL: "The systemic delivery of an oncolytic adenovirus expressing decorin inhibits bone metastasis in a mouse model of human prostate cancer", GENE THERAPY, vol. 22, no. 3, 1 March 2015 (2015-03-01), pages 31-40, XP055431016, GB ISSN: 0969-7128, DOI: 10.1038/gt.2014.110 abstract -----	14
X,P	MARINE RICORDEL ET AL: "Cowpox Virus: A New and Armed Oncolytic Poxvirus", MOLECULAR THERAPY - ONCOLYTICS, vol. 7, 24 August 2017 (2017-08-24), pages 1-11, XP055430885, ISSN: 2372-7705, DOI: 10.1016/j.omto.2017.08.003 the whole document -----	1-17, 19-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/079656

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-28

Cowpox virus and its uses

1.1. claims: 1-13(completely); 14-28(partially)

A cowpox virus comprising a defective CPXV105 CDS gene; A process for preparing said cowpox virus; A composition comprising a therapeutically effective amount of said cowpox virus; Said cowpox virus or said composition for use as an oncolytic virus for the prophylaxis or the treatment of a proliferative disease or a disease associated with an increased osteoclast activity; Method for treating a disease or a pathologic condition in a subject comprising administering said cowpox virus or said composition. Cowpox virus or composition thereof for use as an oncolytic virus for the prophylaxis or the treatment of a proliferative disease or a disease associated with an increased osteoclast activity; Method for treating a disease or a pathologic condition in a subject comprising administering said cowpox virus or said composition.

1.2. claims: 14-28(partially)

Cowpox virus or composition thereof for use as an oncolytic virus for the prophylaxis or the treatment of a proliferative disease or a disease associated with an increased osteoclast activity; Method for treating a disease or a pathologic condition in a subject comprising administering said cowpox virus or said composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/079656

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015155263 A1	15-10-2015	AU 2015243297 A1	29-09-2016
		CA 2943871 A1	15-10-2015
		CN 106413726 A	15-02-2017
		EP 3129037 A1	15-02-2017
		JP 2017515806 A	15-06-2017
		KR 20160145058 A	19-12-2016
		WO 2015155263 A1	15-10-2015