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(54) Title: VIRAL VECTORS ENCODING A DNA POLYMERASE WITH DECREASED PROOF-READING CAPACITY AND USES THEREOF

(57) Abstract: There is provided a self-attenuating double-stranded DNA viral vector having genetically lowered DNA replication fidelity.



VIRAL VECTORS ENCODING A DNA POLYMERASE WITH DECREASED
PROOF-READING CAPACITY AND USES THEREOF

The present invention relates generally to viral vectors and particularly to double-stranded DNA virus-based vectors. In some aspects and embodiments the present invention relates to herpesvirus-based
5 viral vectors and particularly, although not exclusively, to cytomegalovirus (CMV) - based vectors.

Cytomegalovirus (CMV) -based vaccines, as well as other herpesvirus-based vaccines, are on the horizon as a promising addition to our arsenal against infectious disease and cancer.

10 CMV vectors are unique, not only in the high level of immunity they induce against their heterologous encoded pathogen (or cancer) target antigen, but also in the durability of the immunity and, for T cells, in its 'immediate-effector' quality. A CMV-based vaccine against the monkey version of HIV (simian immunodeficiency virus, SIV) was recently shown to induce protection against systemic infection – a level of protection never observed before for any SIV vaccination regimen. CMV-based vaccines have
15 also recently been shown to protect against such diverse pathogens and diseases as Ebola virus, tetanus and prostate cancer in other animal model systems.

A major hurdle to the clinical application of CMV vectors is their replication-competent nature. Although generally benign, CMV causes a persistent, life-long infection, and can cause disease in
20 immunosuppressed individuals. There is also a possibility for uncontrolled environmental spread of genetically-modified CMV vectors if replication competency is maintained. Remarkably, replication-defective versions of CMV have been shown to maintain their immunogenicity, at least in mouse systems. However, replication-defective vaccines are extremely expensive to make, and may also be less immunogenic than a fully replication-competent vector. A replication-defective CMV vector would also
25 not be suitable for development as 'disseminating' vaccine to target diseases in inaccessible animal populations, for example Ebola virus in wild great apes.

For a virus, CMV's genome is huge consisting of >230,000 DNA base pairs encoding >200 proteins. Approximately 20% of these proteins are critical for replication of the virus. The remaining proteins are

deemed as 'non-essential', but presumably play important roles for the virus during its replication in the mammalian host.

The CMV-encoded enzyme that copies the viral DNA during virus replication is called DNA
5 polymerase. A polymerase is an enzyme that synthesizes long chains or polymers of nucleic acids. DNA polymerase is used to assemble DNA by copying a DNA template strand using base-pairing interactions. The polymerase moves along a single strand of DNA, building the complementary strand as it goes.

The function of CMV DNA polymerase is not quite perfect, with the enzyme making about one mistake
10 for every billion base pairs copied. Given the large size of the genome, CMV and other large DNA viruses (including other herpesviruses) have 'proof-reading' to prevent accumulation of too many mutations per replication cycle by correcting mistakes in newly synthesized DNA.

DNA replication is believed to involve the two stranded DNA molecule passing through the DNA
15 polymerase molecule after synthesis is complete. If the wrong base is inserted then the bond is unstable. Because the double strand is passing through the DNA polymerase the missing base can be detected and replaced.

When an incorrect base pair is recognized, DNA polymerase excises the mismatched base. Following
20 base excision, the polymerase can re-insert the correct base and replication can continue. The replacement is done by a different part of the enzyme in a process known as 3' to 5' exonuclease excision.

The accumulation of too many mutations during genome replication can have dire consequences for
25 viruses. This has been shown for RNA viruses such as poliovirus, which lack 'proof-reading'. For these viruses, only a small increase in the mutation rate (by addition of a chemical that increases the mutation rate) can result in accumulation of too many mutations per replication cycle, which pushes the virus into 'genomic catastrophe' and virus 'death' (severe loss of fitness). This is believed to be similar to how larger multicellular organisms age due to the accumulation of mutations resulting from environmental
30 insults such as free-radicals and ionizing radiation that eventually result in the death of the individual.

According to an aspect of the present invention there is provided a low-fidelity double stranded DNA virus-based vector.

- 5 A further aspect provides a self-attenuating double-stranded DNA viral vector having genetically lowered DNA replication fidelity.

The vector may comprise a nucleic acid sequence encoding a DNA polymerase with modified, decreased or inactivated proof-reading capacity.

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The vector may comprise a nucleic acid sequence encoding a DNA polymerase with modified exonuclease activity.

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The vector may comprise a mutation, deletion or substitution in the DNA polymerase exonuclease region.

The vector may be a recombinant herpesvirus-based vector; for example a cytomegalovirus (CMV)-based vector.

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The herpesvirus-based vector may, for example, be a CMV-based vector. The CMV-based vector may non-exclusively be selected from: Human CMV (HCMV), Simian CMV (SCCMV), Rhesus CMV (RhCMV), Chimpanzee CMV (CCMV) Murine CMV (MCMV) and Gorilla CMV (GCMV).

25

The vector may be an adenovirus-based vector.

According to a further aspect of the present invention there is provided a recombinant herpesvirus-based vector comprising a nucleic acid sequence encoding a DNA polymerase with inactivated proof-reading capacity.

A further aspect provides a replication competent, low-fidelity herpesvirus-based vector comprising a nucleic acid sequence encoding a DNA polymerase with inactivated exonuclease activity.

5 The present invention relates to a virus vector that has essentially been given a life span, after which it cannot maintain itself as a replication-competent organism, by genetic inactivation of the 'proof-reading' capacity of its DNA polymerase.

This results in a vector that gradually accumulates increased numbers of mutations per replication cycle that eventually is unsustainable and leads to 'genomic catastrophe' and death of the virus.

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This approach will also enable environmental control of 'disseminating', for example, CMV-based vectors, as these vectors will only be able to persist for a defined period in the environment before their eventual demise.

15 This novel method of vector control solves a major problem concerning the safety of this potentially important new vector system, which will be applicable to viral vector-based vaccines.

The vector may comprise a mutation, deletion or substitution in the exonuclease region of the DNA polymerase encoding sequence.

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The vector may be a replication competent or a replication attenuated vector.

The present invention also provides a vaccine consisting of, comprising or including a vector as described herein.

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The present invention also provides a composition comprising the vaccine or vector of any preceding claim and a pharmaceutically acceptable carrier.

The present invention also provides a method of treating a subject with an infectious disease, or at risk of becoming infected with an infectious disease comprising selecting a subject in need of treatment and administering to the subject the recombinant vector, vaccine or composition of any preceding claim.

- 5 The present invention also provides a method of treating a subject with cancer, or at risk of developing cancer, comprising selecting a subject in need of treatment and administering to the subject the recombinant vector, vaccine or composition as described herein.

- The present invention also provides use of a vaccine, vector or composition as claimed in any preceding
10 claim for the prevention or treatment of a disease.

The present invention also provides use of a vaccine, vector or composition as described herein for the manufacture of a medicament for the prevention or treatment of a disease.

- 15 The present invention also provides an intrinsic biocontrol mechanism for a viral vector, comprising genetically modified proof reading functionality whereby to confer upon the vector a finite number of replication cycles before its biological fitness demise (inability to replicate or compete with wild-type vector in the host or environment).

- 20 The present invention also provides an intrinsic biocontrol mechanism for a double stranded DNA vector, comprising genetically modified DNA replication fidelity.

Different aspects and embodiments of the invention may be used separately or together.

- 25 Further particular and preferred aspects of the present invention are set out in the accompanying independent and dependent claims. Features of the dependent claims may be combined with the features of the independent claims as appropriate, and in combination other than those explicitly set out in the claims.

- 30 The present invention will now be more particularly described, by way of example.

Example embodiments are described below in sufficient detail to enable those of ordinary skill in the art to embody and implement the systems and processes herein described. It is important to understand that embodiments can be provided in many alternate forms and should not be construed as limited to the examples set forth herein.

Accordingly, while embodiments can be modified in various ways and take on various alternative forms, specific embodiments thereof are shown in the drawings and described in detail below as examples. There is no intent to limit to the particular forms disclosed. On the contrary, all modifications, equivalents, and alternatives falling within the scope of the appended claims should be included. Elements of the example embodiments are consistently denoted by the same reference numerals throughout the drawings and detailed description where appropriate.

The terminology used herein to describe embodiments is not intended to limit the scope. The articles “a,” “an,” and “the” are singular in that they have a single referent, however the use of the singular form in the present document should not preclude the presence of more than one referent. In other words, elements referred to in the singular can number one or more, unless the context clearly indicates otherwise. It will be further understood that the terms “comprises,” “comprising,” “includes,” and/or “including,” when used herein, specify the presence of stated features, items, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, items, steps, operations, elements, components, and/or groups thereof.

Unless otherwise defined, all terms (including technical and scientific terms) used herein are to be interpreted as is customary in the art. It will be further understood that terms in common usage should also be interpreted as is customary in the relevant art and not in an idealized or overly formal sense unless expressly so defined herein.

In one embodiment the present invention may be based on studies to *in vitro* characterize a low-fidelity ‘mutator’ CMV expressing a DNA polymerase inactivated for ‘proof-reading’ as an added level of environmental control of CMV vaccines.

High species specificity of CMV serves to reduce environmental concerns regarding CMV-based vectors.

Proposed studies using a 'mutator' (low-fidelity) CMV is provided as an exploratory and highly
5 innovative strategy to provide an additional level of control of CMV-based vectors.

Studies may use HCMV, as this is the most fully characterized CMV in terms of DNA polymerase
function. Sequence comparison shows CMV DNA polymerase to be highly conserved, enabling
translation of results from these studies into CMVs of mouse and rhesus, which are amenable to *in vivo*
10 studies.

The HCMV DNA polymerase exonuclease region involved in 'proof-reading' is comprised of three
domains: I, II and III.

15 Our strategy consists of two components.

First, we determine whether an HCMV genetically designed for low fidelity based on mutation of the
exonuclease region exhibits an increased mutation rate using a molecular cloning-based strategy. Viral
DNA from passage 1, 5 and 15 of this recombinant HCMV, an HCMV control that possesses UL54
20 mutation not affecting exonuclease, and WT HCMV will be collected. Two independent 1kb regions
within genes non-essential for *in vitro* replication in human fibroblasts (e.g., US28 and UL10) will then be
amplified and cloned into a TOPO cloning vector. Single colonies (150 from each sample) will be
sequenced, and mutation rate determined. Next generation sequencing of the UL54 gene from these
viral DNA samples will identify low-fidelity reversion or other compensatory second site mutations.
25 UL54 sequence results will be filtered to identify variants corresponding to >0.5% of the total
population, and any observed UL54 mutations that are absent from WT HCMV will be regarded as
putatively compensatory, and cloned back into the original low-fidelity UL54 background for analysis.

The second component examines whether an observed mutator phenotype of the low-fidelity HCMV is
30 associated with decreased virus fitness. Multiple studies have shown that for RNA viruses a mutator

phenotype may not significantly impact *in vitro* 'bulk virus' replication. In contrast, fitness costs of low fidelity become much more apparent during 'bottlenecks' associated with either small virus populations under defined *in vitro* conditions, or during virus replication *in vivo*. This characteristic is believed to result from smaller populations being more sensitive to extinction from the presence of a deleterious mutation in a member of the population.

Consistent with this model, an *in vitro* assay based on plaque-to-plaque serial passaging of coxsackie B3 mutator viruses revealed a substantial drop in fitness (using plaque size as a surrogate marker of fitness), with complete extinction of the lowest fidelity viruses. Many of these viruses were unable to establish their normally persistent infection in mice. We will use a similar *in vitro* plaque-to-plaque assay to assess fitness of the low fidelity HCMV. In addition to complete fitness failure (i.e., virus extinction), plaque surface area will be used as a surrogate marker of fitness. These studies will also be performed in the presence of DNA/RNA mutagens (5-fluorouracil and 5-azacytidine), which have been shown to further increase the fitness cost of the mutator phenotype of RNA viruses *in vitro*.

Together, these studies are expected to confirm the mutator phenotype of a low-fidelity HCMV, and provide initial *in vitro* evidence for a loss of fitness resulting from low-fidelity. These studies therefore will provide the initial necessary groundwork that will be critical for translation into other CMV vectors.

Assessment of mutation rates may be used to assess efficacy. For example lowered fidelity and wild type could be serially passaged and then sequenced. The lowered fidelity virus should accumulate mutations faster.

The design, construction and characterization of a genetically stable 'low fidelity' is described below. This virus contains a deletion of the codon encoding aspartic acid (D) residue at amino acid position 413 in the exonuclease domain II region of the viral polymerase (virus designated, T4138). The use of a complete codon deletion in this low-fidelity virus is a critical design feature that has prevented reversion to a wild type fidelity phenotype, as was seen earlier for a D413A mutant that contained only a point mutation (aspartic acid to alanine) for inactivation of the proof-reading function [Chou and Marousek, 2008. J Virol. 82: 246].

The virus exhibits increased evolution of drug resistance consistent with a 'low fidelity' phenotype; this phenotype is similar to the D4I3A prior to reversion. Although not quantitative, an impact on fitness is suggested by the attenuation phenotype of the virus.

5

Deleting the entire codon in frame has been very effective at preventing reversion to wild type and should be featured as a design decision for this strategy. The mutant is proven viable and has been used for the rapid selection of drug resistance mutations.

10 T4I38 was used to increase the rate of acquisition of drug resistance CMV mutations. Letermovir (LMV) is an experimental cytomegalovirus terminase inhibitor undergoing Phase 3 clinical trials. Viral mutations have been described at UL56 codons 231-369 that confer widely variable levels of LMV resistance. In this study, 15 independent experiments propagating the exonuclease mutant T4I38 viral strain under escalating LMV concentrations replicated 6 of the 7 published UL56 mutations and commonly elicited
15 additional resistance-conferring mutations at UL56 codons 231, 236, 237, 244, 257, 261, 325, and 329. Mutations were first detected earlier under LMV (median 3 passages) than in 8 parallel experiments under foscarnet (median 15 passages). As LMV concentrations increased, the typical initial UL56 change F261L that confers low-grade resistance combined or was replaced with mutations conferring higher grade resistance, eventually enabling normal viral growth under 30 μ M LMV (>5000-fold EC₅₀ for wild
20 type). At high LMV concentrations, UL56 changes C325F/R were commonly detected, or a combination of changes at codons 236, 257, 329 and/or 369. Recombinant viruses containing individual UL56 mutations and combinations were constructed to confirm their resistance phenotypes and normal growth in cell culture. Several double and triple mutants showed much higher LMV resistance than the respective single mutants, particularly those including changes at both codons 236 and 257. The
25 multiplicity of pathways to high grade LMV resistance with minimal viral growth impact suggests a low viral genetic barrier and the need for close monitoring during treatment of active infection.

The prevention and treatment of human cytomegalovirus (CMV) infection and disease is an important aspect of the medical care of immunosuppressed individuals. Viral DNA polymerase inhibitors
30 ganciclovir, its oral prodrug valganciclovir, foscarnet and cidofovir have long been used for this purpose,

with generally satisfactory outcomes but also well known limitations of toxicity, intravenous treatment complexity, and risk of drug resistance after prolonged therapy (1). Cross-resistance among current drugs may develop because of the same DNA polymerase target (2). Therefore, priority has been given to the development of alternative CMV drug targets. The viral terminase complex, including components encoded by CMV genes UL56, UL89 and UL51, acting in concert with UL104 and others, is responsible for the cleavage of concatameric DNA formed during viral replication into unit length genomes and packaging them into preformed viral capsids (3). This essential process is an attractive target for specific viral inhibition. Drug discovery programs have identified diverse chemical structures that turned out to be CMV terminase inhibitors. Earlier candidates, a benzimidazole pyranoside 275175X (4) and a chemically unrelated compound Bay38-4766 (tomeglovir) (5) were tested in Phase I trials but were not advanced to later stage clinical development. Resistance mutations were mapped to genes UL89 or UL56 consistent with the viral target. More recently, letermovir (LMV, formerly AIC246) has advanced to Phase III clinical trial for prevention of CMV infection in stem cell transplant recipients after a successful dose-ranging Phase II study (6). Published in vitro studies of LMV resistance after exposure to drug concentrations 10-fold higher than baseline EC50 identified mutations at UL56 codons 231, 236, 241, 325 and 369, distinct from those reported for the older terminase inhibitors (7). These UL56 mutations were found to confer diverse levels of LMV resistance ranging from 5-fold increased EC50 for V231L to >5000-fold for C325Y.

The objective of the present study was to conduct a series of in vitro selection experiments under increasing LMV concentrations starting near the baseline EC50, to monitor the evolution and phenotypes of resistance mutations selected under escalating drug concentrations. As with previous work on the experimental CMV antivirals maribavir (8) and cyclopropavir (9), an error-prone UL54 exonuclease domain II (codon 413) mutant was used as the baseline strain to accelerate the evolution of drug resistance mutations by 5 to 10 passages as compared with wild type virus. When this approach was first used for maribavir, the specific mutations evolved accurately predicted those subsequently observed in treated subjects (10, 11). As an additional comparator for the current experiments, parallel selection experiments were conducted using the standard DNA polymerase inhibitor foscarnet.

Materials and Methods

Antiviral compounds. Letermovir was obtained from MedChemExpress (Princeton, NJ, Cat. HY-15233, MW 572.55) as a chiral product of >99% purity, as documented by NMR, mass spectrometry and high-pressure liquid chromatography. A 100 mM stock solution was made in DMSO for further serial dilution into culture media. Foscarnet was obtained as the pharmaceutical 125 mM aqueous solution (Foscavir, Astra).

Viral clones, strains and cells. CMV laboratory strain AD169 was modified by the insertion at US3 of a secreted alkaline phosphatase (SEAP) reporter gene driven by the CMV immediate-early promoter (12). The resulting strain T2211 was subsequently cloned as a bacterial artificial chromosome (BAC) BAI (13). Using established recombineering technology as described (14, 15), the conserved UL54 exonuclease domain II residue D413 was deleted in frame, yielding BAC clone BA308, which was transfected into human foreskin fibroblast (HFF) cells to generate live recombinant virus (strain T4138) that was slightly attenuated in growth and had a foscarnet susceptible, ganciclovir-cidofovir resistant phenotype as previously noted for the error-prone UL54 D413A mutant (8). The D413 deletion mutant was chosen in the current study to prevent spontaneous reversion of residue 413 to wild type after prolonged passage. All CMV cultures and antiviral assays were performed in HFF culture monolayers maintained under Minimal Essential Medium (Earle's salts, Gibco), supplemented with 1x Glutamax-I (Gibco) and fetal bovine serum (8% when subconfluent and 3% when confluent).

Viral propagation under letermovir. Exonuclease mutant T4138 was inoculated into 25 cm² HFF monolayers initially at MOI ~0.1 and incubated for a week at a time under antiviral drug concentrations starting near the EC50 value (5 nM for LMV and 50 µM for foscarnet). At the end of the week, if viral growth was effectively inhibited (single enlarged cells, no spreading CPE), the same drug concentration was maintained, otherwise the drug concentration was increased (1.5 to 4-fold depending on the extent of spreading CPE), and the culture was propagated by trypsinization and passage of 30% of the cell suspension to a new subconfluent HFF monolayer. This was continued for up to 30 passages and/or a maximum LMV concentration of 30 µM (threshold for cytotoxicity). Every few passages as prompted by the emergence of uninhibited CPE under drug, DNA extracts of infected cell suspension were prepared, amplified by PCR and sequenced using conventional dideoxy sequencing (BigDye v3.1, Applied Biosystems) to screen for mutations at UL56 codons 1-500. At the final passage, DNA extracts were

sequenced in the entire CMV coding sequences of UL51 (158 codons), UL56 (851 codons) and UL89 (675 codons), which represent the 3 well characterized components of the CMV terminase complex (16, 17). The control foscarnet selection experiments were monitored for mutation in the UL54 sequence (codons 94-1000 for intermediate passages and the full 1243 codons at the final passage). For all mutations detected at any stage, additional stored infected cell samples from intermediate passages were extracted and sequenced to determine the timing of earliest detection of the respective mutations, tracing back to the timepoint where the mutation was not detected.

Recombinant virus construction. Novel mutations and selected combinations were phenotyped by transfer to a baseline BAC-cloned CMV strain and testing the derived recombinant live virus strain for LMV susceptibility using a standardized SEAP reporter yield reduction assay (12, 13). Similar to other BAC clones of CMV strain AD169 (18, 19), clone BAI was discovered to have a compensatory deletion of US7-US16 dating back to strain T2211 (12) prior to cloning. Therefore a new baseline BAC clone BDI was derived from BAI, by markerless “en passant” (20) replacement of the missing US7-US16 coding sequence PCR-amplified from strain AD169 (ATCC VR-538) and deletion of the redundant IRL1-13 sequences to enable the viral genome to accommodate the BAC vector and SEAP expression cassette. To reduce the possibility of residual wild type sequences in recombinant virus progeny, a derivative of BDI with the entire UL56 sequence deleted and replaced with an ampicillin-selectable marker (bLac) was constructed as clone BD2. This clone was used as the base for introduction of UL56 mutations by means of a transfer vector containing a Frt-Kan selectable marker, using the same strategy as previously used to construct large series of UL97 and UL54 mutants (13, 14). In this case, the Frt-Kan marker was introduced at the natural Bsu36I restriction site upstream of the UL56 coding sequence and incorporated into a transfer vector consisting of a plasmid clone of the EcoRI fragment of the AD169 genome that includes UL56. The residual 34-bp Frt sequence did not impair viral growth and the baseline virus incorporating this motif was used as the reference wild type strain for phenotyping. Desired UL56 mutations were first introduced into the transfer vector between the BamHI and BsrGI unique sites, using a suitable PCR product. Sequence-verified transfer vectors were then digested with EcoRI and the mutant UL56 sequence was recombined into the BAC clone BD2 in heat-induced *E. coli* host SW105 as described (13, 14). Resulting kanamycin-resistant, ampicillin-sensitive colonies were checked for the expected BAC EcoRI digest pattern. The Kan selection marker was removed from a

qualifying clone by arabinose-induced Flp recombinase activity in the SW105 host. The final recombinant BAC was sequenced throughout UL56 to confirm the intended mutation(s), and transfected into HFF monolayers using transfection reagent XtremeGene-HP (Roche) according to manufacturer instructions. The resulting live virus was propagated at low multiplicity of infection to make cell-free virus stock for phenotypic assays.

Drug susceptibility phenotyping was performed by a SEAP-reporter based yield reduction assay as used for previous studies of various CMV antiviral compounds (8, 9, 12-14). A SEAP-calibrated low multiplicity viral inoculum (12) was inoculated into 6 wells (one row) of 24-well cluster plates containing fully confluent HFF monolayers, then incubated for one week under no drug and a series of 5 drug dilutions of 2-fold increasing concentration covering the anticipated EC50 endpoint. At one week, samples of supernatant medium were collected for SEAP activity assays, measured as relative light units (RLU) using a chemiluminescent substrate, to determine the drug concentration associated with a reduction of SEAP activity to 50% of its baseline value in absence of drug (EC50). At least 7 assays were performed for each mutant over at least 4 separate setup dates (to allow for variation in cell culture conditions), with simultaneous controls consisting of baseline and known UL56 mutant resistant strains. Results were reported as the mean and standard deviation EC50 values along with the number of replicates.

Growth curves of baseline and mutant viral strains were compared as previously described (14, 21). Viral inocula of MOI ~0.01 were calibrated based on comparable culture supernatant SEAP activity at 24 hours. Culture media were then sampled daily at days 4 through 8, frozen and subsequently simultaneously assayed for SEAP activity. Growth curves were plotted as mean and standard deviation RLU values based on 4 replicates.

25

Results

Overview of mutations selected under letermovir. Table 1 lists the observed UL56 amino acid substitutions (all resulting from single nucleotide mutations), their relative frequency and timing of detection. At baseline, there were no detectable UL56 sequence differences from the reference strain AD169. Among the 15 in vitro selection experiments, each evolved 2 to 8 (median 4) UL56 mutations

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during serial passage as the LMV concentration was escalated from the wild type EC50 value of 5 nM toward the 30 μ M range. The detected mutations included 6 of the 7 previously published (substitutions V231L, V236M, L241P, R369G/M/S) as well as 14 others, including the 3 most commonly detected (F261L, C325F and L257I). All of the newly recognized mutations are in the UL56 codon range 231-329, except for a single instance of L51M, which evolved in the same timing and subpopulation fraction as L257I and was suspected to be coincidental and not necessarily resistance-related. Sequencing of the complete coding regions for the UL51 and UL89 components of the terminase complex revealed no mutations relative to strain AD169 at the end of each of the 15 selection experiments. Eight parallel selection experiments performed using foscarnet for 25-30 passages eventually resulted in the selection of 1 to 3 (median 1.5) UL54 pol mutations per experiment.

Timing and order of appearance of UL56 mutations. Despite the insensitivity of standard dideoxy sequencing in detecting mutant subpopulations of <20% (22), mutation was detected in all 15 experiments within 5 passages under LMV (range 2-5, median 3), and at a median drug concentration of 8 nM (1.4-fold EC50). In the 8 selection experiments with foscarnet, UL54 pol mutations were first detected much later, at passages 11-20 (median 15) and at a median drug concentration of 650 μ M (13-fold EC50). There were differences in the relative timing of specific mutations under LMV (Table 1). For example, F261L was detected early in every experiment, while V236L and L257I were common at intermediate passages. Substitutions such as C325F, C325R, M329T, and R369G/S were usually observed at higher LMV concentrations, although C325F appeared as early as passage 3, and by passage 10 in 4 of 15 experiments, at LMV concentrations of <1 μ M.

Table 1. UL56 mutations detected in vitro under letermovir

Amino acid substitution	N ¹	Passage ²	[LMV] ³
<u>L51M</u>	1	5	8
<u>V231A</u>	1	17	240
V231L	5	14	320
<u>V236L</u>	4	8	160
V236M	5	13	200
<u>E237D</u>	1	13	80
L241P	1	10	50
<u>T244K</u>	2	8	34
<u>T244R</u>	1	15	40
<u>L257I</u>	7	8	20
<u>F261C</u>	2	26	30 μ M
<u>F261L</u>	15	3	8
<u>F261S</u>	1	20	2 μ M
<u>Y321C</u>	1	13	200
<u>C325F</u>	9	15	700
<u>C325R</u>	4	20	20 μ M
<u>M329T</u>	2	21	20 μ M
R369G	3	22	30 μ M
R369M	2	3	10
R369S	4	20	5.5 μ M

Underlined amino acid changes are newly recognized

1. Number of independent selection experiments where detected

2. Median first passage of detection of mutation

3. Median LMV concentration at first detection of mutation
(nM unless otherwise specified)

Sequential changes in viral genotype under escalating LMV concentrations for the individual experiments are detailed in Table 2, categorized by the range of drug concentrations under which they were detected. Despite the evolutionary complexity, some common patterns can be discerned. At low passages and LMV concentrations, F261L was dominant. At intermediate passages, this mutant was often overgrown by others, notably V236L/M and/or L257I. Changes at both codons 236 and 257 occurred

often (6 experiments), and this genotype was sustainable through a range of moderately high LMV concentrations. Finally, as LMV concentration increased into the highest (> 5 μ M) range, the most common pattern was for C325F/R to emerge (12 experiments) and displace other pre-existing mutants. The previously reported C325Y mutant (7) was not detected. In the minority of experiments where

5 C325F/R did not evolve, combinations of multiple mutations such as V236L/M, L257I, M329T and R369S also appeared to enable viral growth under high LMV concentrations. The passage number where normal appearing viral growth occurred at LMV concentrations of >100-fold EC50 ranged from 3 to 23 (median 13).

Table 2. Evolution of UL56 mutations in vitro under letermovir					
Expt	N	UL56 mutations detected under letermovir concentrations			
		5 - 50 nM	50 nM - 1 μ M	1 μ M - 5 μ M	> 5 μ M
M137	8	<u>L51M</u> (5) T244R (15) <u>L257I</u> (5) F261L(5)	<u>L51M</u> V231A(17) T244R <u>L257I</u>	<u>L51M</u> <u>V236L</u> (20) <u>L257I</u>	<u>L51M</u> V231A <u>V236L</u> <u>L257I</u> F261C(29) R369S(25)
M138	6	<u>T244K</u> (5) <u>F261L</u> (5)	<u>E237D</u> (13) <u>T244K</u> <u>F261L</u>	<u>E237D</u> <u>T244K</u> <u>F261L</u>	V231L(28) <u>E237D</u> <u>T244K</u> <u>F261L</u> <u>C325R</u> (25) R369S(25)
M142	5	V231L(5) L257I(5) F261L(3)	V231L <u>V236L</u> (8) <u>L257I</u> F261L		V231L <u>V236L</u> <u>L257I</u> <u>C325R</u> (13)
M143	3	F261L(2)	V231L(8) <u>C325F</u> (8)	<u>C325F</u>	
M144	2	F261L(2) <u>C325F</u> (3)	<u>C325F</u>		
M145	7	F261L(3)	V236L(8) V236M(8)	<u>V236L</u> <u>L257I</u> (14) F261L R369S(14)	<u>V236L</u> <u>F261L</u> <u>C325F</u> (15) <u>C325R</u> (17) R369S
M146	4	F261L(3) V236L (8)	<u>V236L</u> F261L	<u>V236L</u> <u>L257I</u> (13)	<u>V236L</u> <u>L257I</u> <u>C325F</u> (19)
M151	5	<u>L257I</u> (8) F261L(5)	<u>V236M</u> (13) <u>L257I</u> F261L Y321C(13)	<u>V236M</u> <u>L257I</u>	<u>V236M</u> <u>L257I</u> F261L <u>M329I</u> (19)
M152	6	<u>F261L</u> (3)	V231L(14) V236M(15) <u>F261L</u>	V231L V236M F261L F261S(20)	V231L V236M F261L F261S <u>C325F</u> (23) R369G(25)
M162	7	<u>V236M</u> (3) F261L(3) R369M(3)	<u>V236M</u>	<u>V236M</u> <u>L257I</u> (15) F261L	<u>V236M</u> <u>L257I</u> F261C <u>C325R</u> (22) M329T(22) R369G(22)
M163	4	F261L(3) R369M(3)	L241P(10) R369M	V231L(16) <u>L241P</u>	V231L <u>L241P</u> <u>C325F</u> (20)
M164	3	F261L(3) L257I(5)	<u>R369S</u> (10)	<u>L257I</u> <u>R369S</u>	<u>V236M</u> (18) <u>R369S</u>
M166	4	F261L(3)	<u>T244K</u> (10) <u>F261L</u> <u>C325F</u> (15) R369G(10)		<u>T244K</u> <u>F261L</u> <u>C325F</u>
M169	2	F261L(3)	<u>C325F</u> (10)		<u>C325F</u>
M170	2	F261L(3)	<u>C325F</u> (10)		<u>C325F</u>
N = Number of distinct mutations detected over duration of experiment					
Numbers in parentheses indicate first passage of detection of mutation (usually as a subpopulation)					
Underlined mutations were detected as completely mutant populations within the indicated drug concentration range					
Bold face mutations (codon 325) or combinations (V236M/L + L257I) are associated with growth under drug concentrations > 1 μ M					

10

Recombinant phenotyping of single and multiple UL56 mutations. Of the 7 previously reported UL56 mutations that confer LMV resistance, 3 were chosen as controls for the construction of BAC-cloned mutant CMV strains to compare the measured levels of LMV resistance to published data. The

15 substitutions V231L, L241P and C325Y represent those reported as conferring low (~5-fold), moderately high (~200-fold) and very high level (>5000-fold) LMV resistance respectively. LMV EC50 values are shown in Table 3, determined using a standardized reporter-based yield reduction assay. Published data derived using a different phenotyping assay reported a comparable baseline LMV EC50 of

3-5 nM and EC50 ratios of 5, 160-218 and >8000 for V231L, L241P and C325Y respectively (7, 23, 24). EC50 ratios of >3000 can be interpreted as absolute LMV resistance because viral yield reduction occurs at visibly cytotoxic LMV concentrations.

5 All of the individual amino acid substitutions listed as newly recognized in Table 1, except T244R and F261S (single instances considered similar to T244K and F261L/C), were transferred into recombinant BAC clones and the LMV susceptibility phenotype determined for the derived live CMV strains, as detailed in Table 3. The 12 strains containing single UL56 point mutations were noted to have widely divergent LMV susceptibilities. As surmised, L51M was found to confer no LMV resistance and its
10 appearance was the presumed result of a random mutation that was co-selected with L257I as the latter emerged to confer a resistant phenotype. The low-level LMV resistance conferred by F261L is consistent with the timing of its appearance in the selection experiments. A variety of single UL56 substitutions conferred a low to medium level of resistance (4-14 fold increased EC50), while C325F and C325R conferred the same absolute LMV resistance as C325Y. A set of double and triple mutants,
15 selected from among those observed in the individual selection experiments, was constructed and tested for LMV resistance. The results (Table 3) show a strong multiplier effect of double and triple mutations. For example, the combination of F261L and T244K conferred at least twice the LMV resistance (8.2-fold) of either change alone, and adding a third change E237D further increased the resistance to >100-fold, thus explaining the accumulation of mutations as LMV concentrations were
20 escalated. The frequent emergence of the V236L and L257I combination at higher LMV concentrations is also well explained by the strongly augmented level of resistance (>250-fold EC50 increase) conferred by the double mutant whereas the single mutants confer only a 5- to 14-fold increased EC50. The triple combination of mutations at codons 236, 257 and 329 as observed in experiments M151 and M162 conferred absolute LMV resistance comparable to the C325 mutants.

25

Normal growth in cell culture of UL56 mutants conferring LMV resistance. The present inventors have established the comparative growth curves of representative UL56 mutants exhibiting low, medium or high levels of resistance. Any difference in fitness of the mutant viruses as compared to baseline strains is not discernible among the growth curves. This is consistent with a previous report (7).

30

Discussion

The present study recapitulated almost all of the published LMV resistance mutations in the CMV UL56 component of the terminase complex and newly identified many others in the codon range 231-369 that confer varying levels of LMV resistance when present singly or in combination, as confirmed by recombinant phenotyping. LMV resistance mutations were selected rapidly at low drug concentrations relative to baseline EC50. Multiple mutations evolved to sustain normal viral growth under escalating drug concentrations, eventually conferring absolute resistance to LMV from single mutations C325F/R, or less commonly from combinations of mutations.

The rate of evolution of mutations under LMV can be compared with other CMV antiviral compounds tested using the same in vitro selection strategy (8). The first appearance of UL56 mutation F261L at a median of 3 passages is somewhat earlier than reported for the first mutations emerging under the UL97 inhibitor maribavir (median 5 passages) (8), notably earlier than with the nucleoside analog polymerase inhibitor cyclopropavir (median 7 passages) (9) and in this study with the polymerase inhibitor foscarnet (median 15 passages). These differences in timing likely result from the relative growth fitness of the elicited drug-resistant mutants, as foscarnet-resistant mutants have been consistently growth-attenuated (14, 21, 25). Together with UL54 pol mutations reported in clinical specimens that have a nonviable phenotype (26), this suggests a relatively higher genetic barrier to the development of foscarnet resistance. In contrast, critical UL56 residues involved in LMV binding to the terminase complex do not appear at all important for biological activity, even though LMV is remarkably potent at disrupting wild type terminase function (24). Although published (7) and current data (Fig. 1) show no growth impairment of highly LMV-resistant mutants, a pattern of initial selection of low-level resistant mutants such as F261L and progressive replacement by higher-grade resistant UL56 mutants suggests that there may be subtle differences in growth fitness that are not detectable in growth curve assays.

The variety of mutations detected per selection experiment under LMV as drug concentrations were escalated suggests many alternative genetic pathways for achieving a level of resistance matching any level of drug exposure. Selection experiments under other drugs as described above rarely select for more than 3 distinct mutations per experiment, but the median number of mutations under LMV was 4,

ranging up to 8 (Table 2). Point mutation at residue C325 appears to have the unique role of conferring absolute LMV resistance with preserved fitness, requiring no further viral genetic adaptation or diversification. More UL56 mutations evolved in the absence of C325 changes, but all remained in the codon range 231-369 as earlier noted (7), with some combinations of changes conferring high-level or absolute resistance. No sequence changes were noted in other terminase component genes UL51 and UL89, indicating that these are not preferential loci for LMV resistance. However, we are monitoring additional LMV selection experiments that did not evolve C325 changes to see if the diversity of selected variants occasionally includes changes in UL51, UL89 and UL104.

Recombinant phenotyping data confirmed that various levels of LMV resistance were conferred by the newly recognized UL56 mutations (Table 3), ranging from low level for F261L to absolute for C325F/R, but commonly in the 4- to 15-fold range for single mutations. Transfers of combinations of 2 or 3 mutations at codons as observed in specific selection experiments showed a strong multiplier effect on the level of LMV resistance, which was notable with combinations of mutations at codons 236, 257 and 329. Although not specifically phenotyped, Table 2 suggests that combinations of R369G/S and other mutations in the codon 236-257 range also have this effect. Moderate resistance mutations in the same gene combining to confer very high resistance to the same drug are not a feature of standard CMV polymerase inhibitors.

Clinical correlation is pending to assess the in vitro impression of a low genetic barrier to the development of LMV resistance. Available in vitro data predict that drug resistance may emerge during ongoing CMV replication more quickly with maribavir, and even more so with LMV, than with traditional polymerase inhibitors. Limited information is available at present from clinical trials and treatment studies. In the failed Phase III maribavir prophylaxis trials, genotypic drug resistance was not detected (27) but the study drug was stopped soon after detection of breakthrough viral infection. Two case reports exist of the relatively early onset of maribavir resistance after attempted salvage therapy (10, 11), along with 5 additional recent unpublished cases among 35 treated subjects (Alain S, 15th International CMV/beta Herpesvirus Workshop, Brisbane, April 2015, Abstract O59), all involving the same mutations at UL97 codons 409 and 411 originally observed in vitro. For LMV, one case of breakthrough infection during the Phase II prophylaxis trial was recently reported to develop UL56

V236M (28) at 7 weeks, a resistance mutation which was previously detected in vitro (7) and also well represented in Table I.

Translation of in vitro drug resistance data into clinical practice involves factors such as interstrain
5 sequence polymorphism, the cell culture assays for EC50 values, and the effective intracellular antiviral
concentrations achieved after therapeutic doses. For LMV, the UL56 codons 231-369 that include the
most common resistance mutations are distinct from the codon ranges (e.g. 425-476) where sequence
polymorphisms are prevalent (29), and rare polymorphisms noted at codons 242 and 327 actually confer
reduced LMV EC50s (30). Variation in cell culture conditions resulted in EC50 values for maribavir that
10 differed by 100-fold for the same viral strain (31), but this was tested for LMV without finding a
significant effect of cell type or viral inoculum (24). Comparison of in vitro EC50 values with therapeutic
LMV concentrations is limited by the paucity of published pharmacokinetic data (32). Better information
is needed relevant to the higher LMV doses used in the current Phase III trials to determine if the drug
levels achieved in vivo are sufficient to suppress the replication of some of the resistant mutants in Table
15 3.

Table 3. Recombinant phenotyping data for newly characterized UL56 mutations

Amino Acid Substitution(s)	Recombinant Virus Strain	Letermovir				
		EC50	Unit	SD	N	Fold change
Control strains						
None (wt)	T4190	5.7	nM	1.8	43	
V231L	T4194	29	nM	6.9	30	5.1
L241P	T4192	0.55	μM	0.20	15	96
C325Y	T4206	20	μM	2.0	8	>3000
Mutations conferring <4-fold change in LMV EC50						
L51M	T4195	4.3	nM	1.2	7	0.8
V231A	T4248	12	nM	2.9	9	2.1
T244K	T4213	19	nM	4.5	21	3.3
F261L	T4218	16	nM	4.3	15	2.8
Mutations conferring 4-15 fold change in LMV EC50						
V236L	T4185	80	nM	12	13	14
E237D	T4222	58	nM	13	8	10
T244K F261L	T4219	47	nM	16	8	8.2
L257I	T4214	28	nM	11	22	4.9
F261C	T4245	25	nM	5.4	9	4.4
Y321C	T4251	26	nM	4.1	8	4.6
M329T	T4271	25	nM	6.4	14	4.4
Mutations conferring 100-1000 fold change in LMV EC50						
E237D T244K F261L	T4217	0.59	μM	0.07	8	104
V236L L257I	T4237	1.5	μM	0.14	9	260
Mutations conferring >1000 fold change in LMV EC50						
V236M L257I M329T	T4270	18	μM	2.3	13	>3000
C325F	T4189	21	μM	3.5	9	>3000
C325R	T4257	20	μM	1.9	11	>3000
EC50 = Mean LMV concentration (nM or μM) reducing viral SEAP reporter growth by 50%						
SD = Standard deviation of EC50 values						
N = number of replicates (done on at least 4 separate dates)						
Fold change is that of EC50 compared with that of wild type control value						

In a prophylaxis setting, it is plausible that with the nanomolar in vitro antiviral potency of LMV, adequate doses may maintain complete viral suppression (28) without a high incidence of breakthrough

infection and consequent drug resistance. It is more probable that the treatment of high-grade viremia or CMV end-organ disease will result in the rapid selection of LMV resistance mutations. Genotypic resistance testing may be needed sooner than typically recommended for the standard polymerase inhibitors (I). Use of suitable antiviral drug combinations may be warranted to decrease the likelihood of LMV resistance in therapeutic use.

Although illustrative embodiments of the invention have been disclosed in detail herein, it is understood that the invention is not limited to the precise embodiments shown and that various changes and modifications can be effected therein by one skilled in the art without departing from the scope of the invention as defined by the appended claims and their equivalents.

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CLAIMS

1. A low-fidelity double stranded DNA virus-based vector.
- 5 2. A self-attenuating double-stranded DNA viral vector having genetically lowered DNA replication fidelity.
3. A vector as claimed in claim 1 or claim 2, in which the vector comprises a nucleic acid sequence encoding a DNA polymerase with modified, decreased or inactivated proof-reading capacity.
- 10 4. A vector as claimed in claim 3, comprising a nucleic acid sequence encoding a DNA polymerase with modified exonuclease activity.
5. A vector as claimed in claim 4, in which the vector comprises a mutation, deletion or
15 substitution in the DNA polymerase exonuclease region.
6. A vector as claimed in any preceding claim, in which the vector is a recombinant herpesvirus-based vector.
- 20 7. A vector as claimed in any preceding claim, in which the herpesvirus-based vector is a cytomegalovirus (CMV)-based vector.
8. A vector as claimed in claim 7, in which the CMV-based vector is selected from Human CMV (HCMV), Simian CMV (SCCMV), Rhesus CMV (RhCMV), Chimpanzee CMV (CCMV) Murine CMV
25 (MCMV) and Gorilla CMV (GCMV).
9. A vector as claimed in any of claims 1 to 5, in which the vector is an adenovirus-based vector.
10. A recombinant herpesvirus-based vector comprising a nucleic acid sequence encoding a DNA
30 polymerase with modified, decreased or inactivated proof-reading capacity.

11. A replication competent, low-fidelity herpesvirus-based vector comprising a nucleic acid sequence encoding a DNA polymerase with modified, decreased or inactivated exonuclease activity.
- 5 12. A vector as claimed in any preceding claim, in which the vector is a replication competent or a replication attenuated vector.
13. A vaccine consisting of, comprising or including a vector as claimed in any preceding claim.
- 10 14. A composition comprising the vaccine or vector of any preceding claim and a pharmaceutically acceptable carrier.
- 15 15. A method of treating a subject afflicted with an infectious disease, or at risk of becoming infected with an infectious disease comprising selecting a subject in need of treatment and administering to the subject the recombinant vector, vaccine or composition of any preceding claim.
16. A method of treating a subject with cancer, or at risk of developing cancer, comprising selecting a subject in need of treatment and administering to the subject the recombinant vector, vaccine or composition of any of claims 1 to 14.
- 20 17. Use of a vaccine, vector or composition as claimed in any of claims 1 to 14 for the prevention or treatment of a disease.
18. Use of a vaccine, vector or composition as claimed in any of claims 1 to 14 for the manufacture of a medicament for the prevention or treatment of a disease.
- 25 19. An intrinsic biocontrol mechanism for a viral vector, comprising genetically modified proof reading functionality whereby to confer upon the vector a finite number of replication cycles before its biological fitness demise through inability to replicate and/or compete with wild-type vector in the host or environment.
- 30

20. An intrinsic biocontrol mechanism for a double stranded DNA vector, comprising genetically modified DNA replication fidelity.
- 5 21. A method for providing a proofreading attenuation strategy substantially as hereinbefore described.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/050526

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/02 C12N7/00 C12N9/12 A61K31/517 C12Q1/70
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 C12Q

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, 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	T. G. UIL ET AL: "Directed adenovirus evolution using engineered mutator viral polymerases", NUCLEIC ACIDS RESEARCH, vol. 39, no. 5, 7 December 2010 (2010-12-07), pages e30-e30, XP055274385, GB ISSN: 0305-1048, DOI: 10.1093/nar/gkq1258 the whole document * see in particular abstract; pages 1-2; page 4, right-hand col.; page 6, left-hand col.; page 9; page 11-12 * -/-	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

25 May 2016

Date of mailing of the international search report

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Name and mailing address of the ISA/

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Sirim, Pinar

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/050526

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X	N. D. MYERS ET AL: "Directed Evolution of Mutator Adenoviruses Resistant to Antibody Neutralization", JOURNAL OF VIROLOGY., vol. 87, no. 10, 15 May 2013 (2013-05-15), pages 6047-6050, XP055274382, US ISSN: 0022-538X, DOI: 10.1128/JVI.00473-13 the whole document * see in particular pages 6047, 6049 * -----	1-21
X	WO 2009/150848 A1 (NEO MORGAN LAB INC [JP]; OGAWA MASANORI [JP]; HORIUCHI TAKAYUKI [JP];) 17 December 2009 (2009-12-17) the whole document * see in particular paragraphs [0003], [0010], examples 1, 2, 4, 7, 9, 10-12 * -----	1-8, 10-21
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International application No

PCT/GB2016/050526

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