

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
3 November 2005 (03.11.2005)

PCT

(10) International Publication Number
WO 2005/103272 A2

(51) International Patent Classification⁷: **C12N 15/861**,
7/00

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(21) International Application Number:
PCT/US2005/008094

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(22) International Filing Date: 11 March 2005 (11.03.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/556,549 25 March 2004 (25.03.2004) US
11/074,694 9 March 2005 (09.03.2005) US

(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ,
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA,
ZM, ZW.

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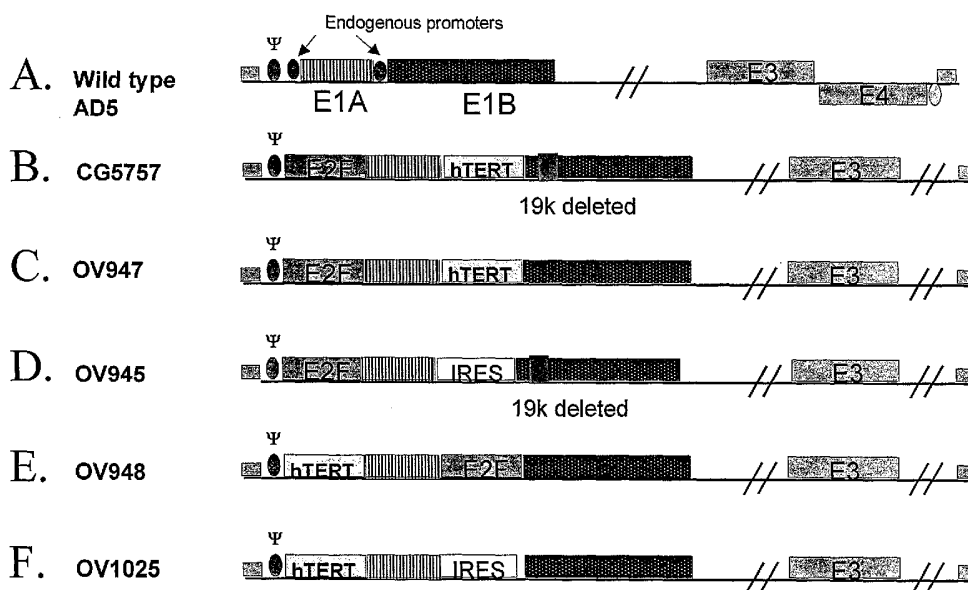
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(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO,

[Continued on next page]

(54) Title: PAN CANCER ONCOLYTIC VECTORS AND METHODS OF USE THEREOF



(57) Abstract: Replication-competent adenoviral vectors which selectively replicate in cancer cells are provided. The replication-competent viral vectors comprise an E2F responsive promoter and/or a telomerase promoter operatively linked to an adenoviral coding region. The replication-competent adenoviral vectors effectively replicate in a variety of types of cancer cells and find broad utility in the treatment of cancer.



SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

— *without international search report and to be republished upon receipt of that report*

TITLE OF THE INVENTION

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**PAN CANCER ONCOLYTIC VECTORS AND
METHODS OF USE THEREOF**

10 This application claims benefit U.S. Patent Application number 60/556,549
with a filing date of March 25, 2004, entitled "Pan Cancer Oncolytic Vectors And
Methods Of Use Thereof". The entirety of this application is incorporated herein by
reference.

Field of the Invention

15 The present invention relates to replication-competent viral vector constructs
and their use in the treatment of cancer.

BACKGROUND OF THE INVENTION

Adenoviruses form the basis of some of the most innovative and potentially
20 powerful disease-fighting tools. One such tool is gene therapy, in which an exogenous
nucleotide sequence provided to a cell. This approach holds great potential in treating
not only cancer, but many other diseases as well, including cystic fibrosis, anemia,
hemophilia, diabetes, Huntington's disease, AIDS, abnormally high serum
cholesterol levels, certain immune deficiencies, and many forms of cancer. Gene
25 therapy generally relies upon a delivery vehicle, such as a viral vector in order to
provide the exogenous sequence to a cell. Recombinant adenovirus has shown some
therapeutic efficacy against these diseases. For reviews, see Kim et al. (1996) *Mol.*

Med. Today 12:519-527 and Smith et al. (1996) *Gene Therapy* 3:496-502.

Adenoviruses that replicate selectively in target cells are being developed as therapeutic agents for treatment of cancer.

In an alternate approach applicable to cancer treatment, specific attenuated
5 replication-competent viral vectors have been developed for which selective
replication in cancer cells preferentially destroys those cells. Various cell-specific
replication-competent adenovirus constructs, which preferentially replicate in (and
thus destroy) certain cell types, are described in, for example, WO 95/19434, WO
96/17053, WO 98/39464, WO 98/39465, WO 98/39467, WO 98/39466, WO
10 99/06576, WO 99/25860, WO 00/15820, WO 00/46355, WO 02/067861, WO
02/06862, U.S. Patent application publication US20010053352 and U.S. Patent Nos.
5,698,443, 5,871,726, 5,998,205, and 6,432,700. Replication-competent adenovirus
vectors have been designed to selectively replicate in tumor cells.

Although replication-competent adenoviruses are able to achieve selective
15 targeting and amplification for the treatment of local and disseminated cancers, there
remains a need for improvement in both the adenovirus vectors themselves and
methods for their use dependent upon the type of cancer under treatment.

SUMMARY OF THE INVENTION

The present invention provides a recombinant adenoviral vector comprising an adenoviral nucleic acid backbone comprising in sequential order: a left ITR, an adenoviral packaging signal, an E2F responsive promoter or a TERT promoter
5 operatively linked to an E1a coding region, an E2F responsive promoter or a TERT promoter operatively linked to an E1b coding region, and a right ITR.

In another aspect, the transcriptional regulatory element operatively linked to the E1b coding region is an E2F responsive promoter such as the human E2F-1 promoter which comprises SEQ ID NO:1 or a TERT promoter, e.g., a human TERT
10 promoter, such as a human TERT promoter which comprises SEQ ID NO:2 or SEQ ID NO:3.

In another aspect, the recombinant viral vector comprises a mutation in the E1b 19k coding region.

In yet another aspect, the transcriptional element operatively linked to the E1b
15 coding region is an IRES, a TERT promoter, e.g., a human TERT promoter, such as a human TERT promoter which comprises SEQ ID NO:2 or SEQ ID NO:3 or an E2F responsive promoter, such as the human E2F-1 promoter which comprises SEQ ID NO:1.

A recombinant adenoviral vector of the invention may also comprise a
20 mutation or deletion in an E3 coding region, such as E3-6.7, KDa, gp19KDa, 11.6KDa (ADP), 10.4 KDa (RID α), 14.5 KDa (RID β), and E3-14.7Kda or a deletion in the E1b gene such as a deletion in the gene which encodes the E1b 19kD protein, e.g. the deletion presented as SEQ ID NO:12. The recombinant adenoviral vector may further comprise a transgene.

Exemplary recombinant adenoviral vectors comprise the nucleotide sequences presented as SEQ ID NO: 4 or SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

The invention further provides pharmaceutical compositions comprising the adenoviral vectors of the invention and their use in treating a host organism having a
5 neoplastic condition, such as lung, breast, prostate, or colon cancer. In one aspect, the pharmaceutical composition is administered by intratumoral injection.

The invention also provides a method for selective cytolysis of a cancer cell, comprising contacting a cell population with an effective amount of an adenoviral vector of the invention (described above), under conditions wherein the adenoviral
10 vector infects the cells of a cell population resulting in selectively cytolysis of cancer cells within the cell population.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-F provide a schematic depiction of the structure of exemplary pan
15 cancer replication-competent viral vector constructs.

Figure 1A depicts the structure of wild-type adenovirus serotype 5.

Figure 1B depicts the structure of CG5757, which comprises in sequential order a left ITR, an adenoviral packaging signal, a human E2F-1 promoter operatively linked to an E1a coding region, a human telomerase (hTERT) promoter operatively
20 linked to an E1b coding region wherein the E1b coding region comprises a deletion in the E1b19k coding region, and a right ITR.

Figure 1C depicts the structure of OV947, which comprises in sequential order a left ITR, an adenoviral packaging signal, a human E2F-1 promoter operatively linked to an E1a coding region, an hTERT promoter operatively linked to an E1b
25 coding region, and a right ITR.

Figure 1D depicts the structure of OV945, which comprises in sequential order a left ITR, an adenoviral packaging signal, a human E2F-1 responsive promoter operatively linked to an E1a coding region, an internal ribosome entry site (IRES) operatively linked to an E1b coding region wherein the E1b coding region comprises a mutation in the E1b19k coding region, and a right ITR.

Figure 1E depicts the structure of OV948, which comprises in sequential order a left ITR, an adenoviral packaging signal, an hTERT promoter operatively linked to an E1a coding region, a human E2F-1 promoter operatively linked to an E1b coding region, and a right ITR.

Figure 1F depicts the structure of OV1025, which comprises in sequential order a left ITR, an adenoviral packaging signal, an hTERT promoter operatively linked to an E1a coding region, an internal ribosome entry site (IRES) operatively linked to an E1b coding region, and a right ITR.

Figure 2 shows tumor growth inhibition of an A549 xenograft model infected with OV945. Tumors were injected with OV945 on the following days after tumor implantation: Regimen #1: 20, 21, 22, 23 (Triangles); Regimen #2: 20, 23, 26, 29 (Stars); Regimen #3: 20, 27, 34, 41 (Diamonds). Another set of tumors was treated with a PBS-glycerol control (Squares) by regimen 2.

Figures 3A-D shows the results of a MTT cytotoxicity assay performed with CG5757 compared to wild-type adenovirus type 5 (OAV802) on Hep3B and HRE cells. Cells were infected with viruses at different doses and subjected to MTT assay at selected times. The viral cytotoxicity is represented as percentage of the uninfected cell control. OAV802 is graphed as squares and CG5757 is graphed as diamonds. The graphs show cytotoxicity after infection as measured by MTT assay and graphed as a percentage compared to the uninfected cells. Figs. 3A and 3B show cytotoxicity at

8 days after infection with various MOIs for the cell lines Hep3B and HRE, respectively. Figures 3C and 3D show cytotoxicity after infection with an MOI of 1 for different days after infection for the cell lines Hep3B and HRE, respectively.

Figure 4 shows selective production of CG5757 on different cell lines. Various cell lines were infected with CG5757 (white bars) or OV802 (black bars) at an MOI of 2 (pfu/cell) for 72 hours. The cell lysates were harvested and plaque titrated on 293 cells.

Figure 5 shows the results of a growth kinetics study. CG5757 (triangles) and OV802 (squares) were infected on Hep3B cells (Fig. 5A) or HRE cells (Fig. 5B) at an MOI of 2 (pfu/cell) for selected times. Infected cells were harvested and titered for the corresponding time points on 293 cells

Figure 6 shows selective production of OV945 (gray bars), OV947 (white bars) and CG5757 (slanted striped bars) as compared to OV802 (black bars) on different cells.

Figure 7 shows selective production of OV945 (white bars) and OV947 (gray bars) compared to OV802 (black bars) on different cells.

Figure 8 shows results from an MTT assay with the viruses OV945 (squares), OV947 (diamonds) and OV802 (triangles) tested on the Hep3B cell line (A) and BSM cell line (B). Cells were harvested on the 10th day after infection for the MTT assay.

Figure 9 shows tumor growth inhibition of an A549 xenograft model infected with CG5757. Tumors were injected with CG5757 (circles) on days 20, 21, 22 and 23 after tumor implantation or were injected with PBS-glycerol control (squares).

Figure 10 shows tumor growth inhibition of a 253J B-V xenograft model infected with CG5757. Tumors were injected with CG5757 on the following days after tumor implantation: Regimen #1: 20, 21, 22, 23, 24 (Circles); Regimen #2: 20,

24, 27, 30 (diamonds); Regimen #3: 20, 27, 34, 41 (Triangles). Another set of tumors was treated with a PBS-glycerol control (Stars) according to Regimen #2.

Figure 11 shows tumor growth inhibition of a 253J B-V xenograft model infected with OV945. Tumors were injected with OV945 on the following days after tumor implantation: Regimen #1: 20, 21, 22, 23, 24 (Triangles); Regimen #2: 20, 24, 27, 30 (Circles); Regimen #3: 20, 27, 34, 41 (Diamonds). Another set of tumors was treated with a PBS-glycerol control (Squares) following regimen #2.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art and the practice of the present invention will employ, conventional techniques of microbiology and recombinant DNA technology.

The publications and other materials including all patents, patent applications, publications (including published patent applications), and database accession numbers referred to in this specification are used herein to illuminate the background of the invention and in particular, cases to provide additional details respecting the practice. The publications and other materials including all patents, patent applications, publications (including published patent applications), and database accession numbers referred to in this specification are each individually incorporated herein by reference in its entirety.

In describing the present invention, the following terms are employed and are intended to be defined as indicated below.

The abbreviation "pfu" stands for plaque forming units.

The terms "virus," "viral particle," "vector particle," "viral vector particle," and "virion" are used interchangeably and are to be understood broadly as meaning

infectious viral particles that are formed when, *e.g.*, a viral vector of the invention is transduced into an appropriate cell or cell line for the generation of infectious particles. Viral particles according to the invention may be utilized for the purpose of transferring DNA into cells either *in vitro* or *in vivo*. For purposes of the present invention, these terms refer to adenoviruses, including recombinant adenoviruses formed when an adenoviral vector of the invention is encapsulated in an adenovirus capsid.

An “adenovirus vector” or “adenoviral vector” (used interchangeably) as referred to herein is a polynucleotide construct, which is replication competent, exhibits preferential replication in cancer cells and contains a tissue-specific transcriptional regulatory sequence linked to an adenoviral gene. In some embodiments, an adenoviral vector of the invention includes a therapeutic gene sequence, *e.g.*, a cytokine gene sequence. Exemplary adenoviral vectors of the invention include, but are not limited to, DNA, DNA encapsulated in an adenovirus coat, adenoviral DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), adenoviral DNA encapsulated in liposomes, adenoviral DNA complexed with polylysine, adenoviral DNA complexed with synthetic polycationic molecules, conjugated with transferrin, or complexed with compounds such as PEG to immunologically “mask” the antigenicity and/or increase half-life, or conjugated to a nonviral protein. Hence, the terms “adenovirus vector” or “adenoviral vector” as used herein include adenovirus or adenoviral particles.

The terms “adenovirus” and “adenoviral particle” are used to include any and all viruses that may be categorized as an adenovirus, including any adenovirus that infects a human or an animal, including all groups, subgroups, and serotypes. Thus, as used herein, “adenovirus” and “adenovirus particle” refer to the virus itself or

derivatives thereof and cover all serotypes and subtypes and both naturally occurring and recombinant forms, except where indicated otherwise (For examples, see Table 1). In one embodiment, such adenoviruses infect human cells. Such adenoviruses may be wild-type or may be modified in various ways known in the art or as disclosed herein.

5 Such modifications include modifications to the adenovirus genome that is packaged in the particle in order to make an infectious virus. Such modifications include deletions known in the art, such as deletions in one or more of the E1a, E1b, E2a, E2b, E3, or E4 coding regions.

The term as used herein “replication-competent” as used herein relative to the

10 adenoviral vectors of the invention means the adenoviral vectors and particles of the invention preferentially replicate in certain types of cells or tissues but to a lesser degree or not at all in other types. In one embodiment of the invention, the adenoviral vector and/or particle selectively replicates in tumor cells and or abnormally proliferating tissue, such as solid tumors and other neoplasms. These include the

15 viruses disclosed in U.S. Patent Nos. 5,677,178, 5,698,443, 5,871,726, 5,801,029, 5,998,205, and 6,432,700, the disclosures of which are incorporated herein by reference in their entirety. Such viruses may be referred to as “oncolytic viruses” or “oncolytic vectors” and may be considered to be “cytolytic” or “cytopathic” and to effect “selective cytolysis” of target cells.

20 By “pan-cancer” is meant that the replication-competent adenoviral vectors of the invention selectively replicate in tumor cells and or abnormally proliferating tissue in general and replication is not necessarily limited to a particular type of cancer.

As used herein, the term “viral vector” is used according to its art-recognized meaning. It refers to a nucleic acid vector construct that includes at least one element

25 of viral origin and may be packaged into a viral vector particle. The adenoviral vector

and/or particle may be utilized for the purpose of transferring DNA, RNA or other nucleic acid derivatives into cells either *in vitro* or *in vivo*. Numerous forms of viral vectors including adenoviral vectors are known in the art.

The terms “vector,” “polynucleotide vector,” “polynucleotide vector construct,” “nucleic acid vector construct,” and “vector construct” are used
5 interchangeably herein to mean any nucleic acid construct for gene transfer, as understood by one skilled in the art.

The term “gene essential for replication” refers to a nucleotide sequence whose transcription is required for a viral vector to replicate in a target cell. For example, in
10 an adenoviral vector of the invention, a gene essential for replication may be selected from the group consisting of the E1a, E1b, E2a, E2b, and E4 genes.

As used herein, a “packaging cell” is a cell that is able to package adenoviral genomes or modified genomes to produce viral particles. It can provide a missing gene product or its equivalent. Thus, packaging cells can provide complementing functions
15 for the genes deleted in an adenoviral genome and are able to package the adenoviral genomes into the adenovirus particle. The production of such particles requires that the genome be replicated and that those proteins necessary for assembling an infectious virus are produced. The particles also can require certain proteins necessary for the maturation of the viral particle. Such proteins can be provided by the vector or
20 by the packaging cell.

The term “HeLa-S3” means the human cervical tumor-derived cell line available from American Type Culture Collection (ATCC, Manassas, VA) and designated as ATCC number CCL-2.2. HeLa-S3 is a clonal derivative of the parent HeLa line (ATCC CCL-2). HeLa-S3 was cloned in 1955 by T.T. Puck et al. (*J. Exp.*
25 *Med.* 103: 273-284 (1956)).

In the context of adenoviral vectors, the term “5’ ” is used interchangeably with “upstream” and means in the direction of the left inverted terminal repeat (ITR).

In the context of adenoviral vectors, the term “3’ ” is used interchangeably with “downstream” and means in the direction of the right ITR.

5 The term “nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof (“polynucleotides”) in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless
10 otherwise indicated, a particular nucleic acid molecule/polynucleotide also implicitly encompasses conservatively modified variants thereof (*e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is
15 substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, Nucleic Acid Res. 19: 5081 (1991); Ohtsuka *et al.*, J. Biol. Chem. 260: 2605-2608 (1985); Rossolini *et al.*, Mol. Cell. Probes 8: 91-98 (1994)). Nucleotides are indicated by their bases by the following standard abbreviations: adenine (A), cytosine (C), thymine (T), and guanine (G).

20 A nucleotide sequence is “operatively linked” when it is placed into a functional relationship with another nucleotide sequence. For example, a promoter or regulatory DNA sequence is said to be “operatively linked” to a DNA sequence that codes for an RNA and/or a protein if the two sequences are operatively linked, or situated such that the promoter or regulatory DNA sequence affects the expression

level of the coding or structural DNA sequence. Operatively linked DNA sequences are typically, but not necessarily, contiguous.

The terms “coding sequence” and “coding region” refer to a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense
5 RNA or antisense RNA. In one embodiment, the RNA is then translated in a cell to produce a protein.

The term “ORF” means Open Reading Frame.

The term “gene” refers to a defined region that is located within a genome and that, in addition to the aforementioned coding sequence, comprises other, primarily
10 regulatory, nucleic acid sequences responsible for the control of expression, *i.e.*, transcription and translation of the coding portion. A gene may also comprise other 5’ and 3’ untranslated sequences and termination sequences. Depending on the source of the gene, further elements that may be present are, for example, introns.

The terms “heterologous” and “exogenous” as used herein with reference to
15 nucleic acid molecules such as promoters and gene coding sequences, refer to sequences that originate from a source foreign to a particular virus or host cell or, if from the same source, are modified from their original form. Thus, a heterologous gene in a virus or cell includes a gene that is endogenous to the particular virus or cell but has been modified through, for example, codon optimization. The terms also
20 include non-naturally occurring multiple copies of a naturally occurring nucleic acid sequence. Thus, the terms refer to a nucleic acid segment that is foreign or heterologous to the virus or cell, or homologous to the virus or cell but in a position within the host viral or cellular genome in which it is not ordinarily found.

The terms “complement” and “complementary” refer to two nucleotide
25 sequences that comprise antiparallel nucleotide sequences capable of pairing with one

another upon formation of hydrogen bonds between the complementary base residues in the antiparallel nucleotide sequences.

The term “native” refers to a gene that is present in the genome of the wildtype virus or cell.

5 The term “naturally occurring” or “wildtype” is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a protein or nucleotide sequence present in an organism (including a virus), which can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory, is naturally occurring.

10 The term “recombinant” as used herein with reference to nucleic acid molecules refers to a combination of nucleic acid molecules that are joined together using recombinant DNA technology into a progeny nucleic acid molecule. As used herein with reference to viruses, cells, and organisms, the terms “recombinant,” “transformed,” and “transgenic” refer to a host virus, cell, or organism into which a
15 heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host or the nucleic acid molecule can also be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating. Recombinant viruses, cells, and organisms are understood to encompass not only the end product of a transformation process, but
20 also recombinant progeny thereof. A “non-transformed,” “non-transgenic,” or “non-recombinant” host refers to a wildtype virus, cell, or organism that does not contain the heterologous nucleic acid molecule.

“Regulatory elements” are nucleotide sequences involved in controlling the expression of a nucleic acid molecule. Regulatory elements include promoters,

enhancers, and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

The term “promoter” refers to an untranslated DNA sequence usually located upstream of the coding region that contains the binding site for RNA polymerase II and initiates transcription of the DNA. The promoter region may also include other elements that act as regulators of gene expression. The term “minimal promoter” refers to a promoter element, particularly a TATA element that is inactive or has greatly reduced promoter activity in the absence of upstream activation elements.

The term “enhancer” within the meaning of the invention may be any genetic element, *e.g.*, a nucleotide sequence that increases transcription of a coding sequence operatively linked to a promoter to an extent greater than the transcription activation effected by the promoter itself when operatively linked to the coding sequence, *i.e.* it increases transcription from the promoter.

A “termination signal sequence” within the meaning of the invention may be any genetic element that causes RNA polymerase to terminate transcription, such as for example a polyadenylation signal sequence. A polyadenylation signal sequence is a recognition region necessary for endonuclease cleavage of an RNA transcript that is followed by the polyadenylation consensus sequence AATAAA (SEQ ID NO: 13). A polyadenylation signal sequence provides a “polyA site”, *i.e.* a site on a RNA transcript to which adenine residues will be added by post-transcriptional polyadenylation. Polyadenylation signal sequences are useful insulating sequences for transcription units within eukaryotic cells and eukaryotic viruses. Generally, the polyadenylation signal sequence includes a core poly(A) signal that consists of two recognition elements flanking a cleavage-polyadenylation site (*e.g.*, Figure 1 of WO 02/067861 and WO 02/068627). Typically, an almost invariant AAUAAA (SEQ ID

NO: 14) hexamer lies 20 to 50 nucleotides upstream of a more variable element rich in U or GU residues. Cleavage between these two elements is usually on the 3' side of an A residue and, in vitro, is mediated by a large, multicomponent protein complex. The choice of a suitable polyadenylation signal sequence will consider the strength of the polyadenylation signal sequence, as completion of polyadenylation process correlates with poly(A) site strength (Chao et al., Molecular and Cellular Biology, 1999, 19:5588-5600). For example, the strong SV40 late poly(A) site is committed to cleavage more rapidly than the weaker SV40 early poly(A) site. The person skilled in the art will consider to choose a stronger polyadenylation signal sequence if a more substantive reduction of nonspecific transcription is required in a particular vector construct. In principle, any polyadenylation signal sequence may be useful for the purposes of the present invention. However, in preferred embodiments of this invention the termination signal sequence is either the SV40 late polyadenylation signal sequence or the SV40 early polyadenylation signal sequence. In one embodiment of the invention, the termination signal sequence is isolated from its genetic source and inserted into the viral vector at a suitable position upstream of an E2F or TERT promoter.

The term "expression" refers to the transcription and/or translation of an endogenous gene, transgene or coding region in a cell. In the case of an antisense construct, expression may refer to the transcription of the antisense DNA only.

The term "up-regulated" as used herein means that a greater quantity of the RNA for a specific gene can be detected in the target cell as compared to another cell. For example, if a tumor cell that produces more telomerase RNA as compared to a non-tumor cell, the tumor cell has up-regulated expression of telomerase. Expression is considered up regulated when the quantity of specific RNA in a target cell (e.g.

tumor cell) is at least 3-fold greater than in another cell (non-tumor cell). In another embodiment, the quantity of specific RNA is at least 5-fold greater. In another embodiment, the quantity of specific RNA is at least 10-fold greater. One skilled in the art knows how to measure RNA levels for a specific RNA sequence (e.g. Northern Assay).

As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. Jackson R J, Howell M T, Kaminski A (1990) Trends Biochem Sci 15(12):477-83) and Jackson R J and Kaminski, A. (1995) RNA 1(10):985-1000). The present invention encompasses the use of any IRES element, which is able to promote direct internal ribosome entry to the initiation codon of a cistron. "Under translational control of an IRES" as used herein means that translation is associated with the IRES and proceeds in a cap-independent manner. Examples of "IRES" known in the art include, but are not limited to IRES obtainable from picornavirus (Jackson et al., 1990, Trends Biochem Sci 15(12):477-483); and IRES obtainable from viral or cellular mRNA sources, such as for example, immunoglobulin heavy-chain binding protein (BiP), the vascular endothelial growth factor (VEGF) (Huez et al. (1998) Mol. Cell. Biol. 18(11):6178-6190), the fibroblast growth factor 2, and insulin-like growth factor, the translational initiation factor eIF4G, yeast transcription factors TFIID and HAP4. IRES have also been reported in different viruses such as cardiovirus, rhinovirus, aphthovirus, HCV, Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV). As used herein, "IRES" encompasses functional variations of IRES sequences as long as the variation is able to promote direct internal ribosome entry to the initiation codon of a cistron. In preferred embodiments, the

IRES is mammalian. In other embodiments, the IRES is viral or protozoan. In one illustrative embodiment disclosed herein, the IRES is obtainable from encephelomyocarditis virus (ECMV) (commercially available from Novogen, Duke et al. (1992) J. Virol 66(3):1602-1609). In another illustrative embodiment disclosed
5 herein, the IRES is from VEGF. Examples of IRES sequences are described in U.S. patent 6,692,736.

A “self-processing cleavage site” or “self-processing cleavage sequence” as referred to herein is a nucleotide or amino acid sequence, wherein upon translation, rapid intramolecular (cis) cleavage of a polypeptide comprising the self-processing
10 cleavage site occurs to result in expression of discrete mature protein or polypeptide products. Such a “self-processing cleavage site”, may also be referred to as a post-translational or co-translational processing cleavage site, e.g., a 2A site, sequence or domain. A 2A site, sequence or domain demonstrates a translational effect by modifying the activity of the ribosome to promote hydrolysis of an ester linkage,
15 thereby releasing the polypeptide from the translational complex in a manner that allows the synthesis of a discrete downstream translation product to proceed (Donnelly, 2001). Alternatively, a 2A site, sequence or domain demonstrates “auto-proteolysis” or “cleavage” by cleaving its own C-terminus in cis to produce primary cleavage products (Furler; Palmenberg, Ann. Rev. Microbiol. 44:603-623 (1990)).

20 The term “E2F promoter” as used herein refers to a native E2F promoter and functional fragments, mutations and derivatives thereof. The E2F promoter does not have to be the full-length or wild type promoter. One skilled in the art knows how to derive fragments from an E2F promoter and test them for the desired selectivity. An E2F promoter fragment of the present invention has promoter activity selective for

tumor cells, i.e. drives tumor selective expression of an operatively linked coding sequence.

The term “tumor selective promoter activity” as used herein means that the promoter activity of a promoter fragment of the present invention in tumor cells is
5 higher than in non-tumor cell types.

The term “telomerase promoter” or “TERT promoter” as used herein refers to a native TERT promoter and functional fragments, mutations and derivatives thereof. The TERT promoter does not have to be the full-length or wild type promoter. One skilled in the art knows how to derive fragments from a TERT promoter and test them
10 for the desired selectivity. A TERT promoter fragment of the present invention has promoter activity selective for tumor cells, i.e. drives tumor selective expression of an operatively linked coding sequence. In one embodiment, the TERT promoter of the invention is a mammalian TERT promoter. In another embodiment, the mammalian TERT promoter is a human TERT promoter.

15 In one embodiment, an E2F promoter according to the present invention has a full-length complement that hybridizes to the nucleotide sequence shown in SEQ ID NO:1 under stringent conditions. In another embodiment, the TERT promoter according to the present invention has a full-length complement that hybridizes to the nucleotide sequence shown in SEQ ID NO:2 under stringent conditions. The phrase
20 “hybridizing to” refers to the binding, duplexing, or hybridizing of a molecule to a particular nucleotide sequence under stringent conditions when that nucleotide sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. “Bind(s) substantially” refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by

reducing the stringency of the hybridization media to achieve the desired detection of the target nucleotide sequence.

“Stringent hybridization conditions” and “stringent wash conditions” in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes part 1 chapter 2 “Overview of principles of hybridization and the strategy of nucleic acid probe assays” Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C to 20°C (preferably 5°C) lower than the thermal melting point (T_m) for the specific nucleotide sequence at a defined ionic strength and pH. Typically, under highly stringent conditions a probe will hybridize to its target subsequence, but to no other unrelated sequences.

The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids that have more than 100 complementary nucleotides on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of high stringency wash conditions is 0.15M NaCl at 72°C for about 15 minutes. An example of high stringent wash conditions is a 0.2xSSC wash at 65°C for 15 minutes (see, Sambrook, *infra*, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*,

more than 100 nucleotides, is 1xSSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4-6xSSC at 40°C for 15 minutes. For short probes (*e.g.*, about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.0M Na ion, typically about
5 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

10 The term “homologous” as used herein with reference to a nucleic acid molecule refers to a nucleotide sequence naturally associated with a host virus or cell.

The terms “identical” or percent “identity” in the context of two or more nucleotide or protein sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that
15 are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described herein, *e.g.* the Smith-Waterman algorithm, or by visual inspection.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm,
20 test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), by the BLAST algorithm, Altschul *et al.*, *J. Mol. Biol.* 215: 403-410 (1990), with software that is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), or by visual inspection (see generally, Ausubel *et al.*, *infra*). For purposes of the present invention, optimal alignment of sequences for comparison is most preferably conducted by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981).

In the context of the present invention, the term "isolated" refers to a nucleic acid molecule, polypeptide, virus, or cell that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid molecule or polypeptide may exist in a purified form or may exist in a non-native environment such as, for example, a recombinant host cell. An isolated virus or cell may exist in a purified form, such as in a cell culture, or may exist in a non-native environment such as, for example, a recombinant or xenogeneic organism.

A "normal cell status" or "normal physiological state" is the status of a cell which exists in normal physiological conditions and which is non-dividing or divides in a regulated manner, *i.e.*, a cell in a normal physiological state.

An aberrant cell status is defined in relation to a cell of the same type, which is in a non-dividing/regulated dividing state and under normal physiological conditions.

As used herein, the terms “cancer”, “cancer cells”, “neoplastic cells”, “neoplasia”, “tumor”, and “tumor cells” (used interchangeably) refer to cells that exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype or aberrant cell status characterized by a significant loss of control of cell proliferation. Neoplastic cells can be malignant or benign. It follows that cancer cells are considered to have an aberrant cell status.

The term “consists essentially of” or “consisting essentially of” as used herein with reference to a particular nucleotide sequence means that the particular sequence may have additional residues on either the 5’ or 3’ end or both, wherein the additional residues do not materially affect the basic and novel characteristics of the recited sequence.

The terms “candidate bioactive agent,” “drug candidate”, “compound” or grammatical equivalents as used herein describes any molecule, *e.g.*, protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc., to be tested for bioactive agents that are capable of directly or indirectly altering the cancer phenotype or the expression of a cancer sequence, including both nucleic acid sequences and protein sequences.

ADENOVIRAL VECTORS OF THE INVENTION

The present invention provides novel replication-competent adenoviral vectors, examples of which are described in US20010053352, WO 96/17053 and WO 99/25860. In particular, oncolytic adenoviral vectors are disclosed in which expression of an adenoviral gene, which is essential for replication, is controlled by a regulatory region that is selectively transactivated in cancer cells. Thus the adenoviral vectors of the invention are considered to the “pan cancer” vectors. In accordance with the

present invention, the pan cancer vectors comprise a cancer selective regulatory region, such as an E2F or TERT promoter described in further detail herein.

The adenoviral vectors of the invention are made by standard techniques known to those skilled in the art. The adenoviral vectors of the invention are transferred into packaging cells in order to generate viral particles by techniques known to those skilled in the art. Packaging cells typically complement any functions deleted from the wild-type adenoviral genome. The production of such particles requires that the vector be replicated and that those proteins necessary for assembling an infectious virus be produced. The packaging cells are cultured under conditions that permit the production of the desired viral vector particle. Viral particles are recovered by standard techniques. The preferred packaging cells are those that have been designed to limit homologous recombination that could lead to wild-type adenoviral particles. Examples of cells that may be used to produce adenoviral particles include the human embryonic kidney cell line 293 (Graham *et al.*, *J. Gen. Virol.* 36:59-72 (1977)), the human embryonic retinoblast cell line PER.C6 (U.S. Patent Nos. 5,994,128 and 6,033,908; Fallaux *et al.*, *Hum. Gene Ther.* 9: 1909-1917 (1998)), and the human cervical tumor-derived cell line HeLa-S3 (U.S. patent application 60/463,143; ATCC #CCL-2.2).

The present invention contemplates the use of all adenoviral serotypes to construct the oncolytic adenoviral vectors and adenoviral particles according to the present invention. For example, the adenoviral nucleic acid backbone is derived from adenovirus serotype 2(Ad2), 5 (Ad5) or 35 (Ad35), although other serotype adenoviral vectors can be employed. Adenoviral stocks that can be employed according to the invention include any adenovirus serotype. A large number of adenovirus serotypes are currently available from American Type Culture Collection (ATCC, Manassas,

VA), and the invention includes any other serotype of adenovirus available from any source including those serotypes listed in Table 1. The adenoviruses that can be employed according to the invention may be of human or non-human origin. For instance, an adenovirus can be of subgroup A (*e.g.*, serotypes 12, 18, 31), subgroup B (5 *e.g.*, serotypes 3, 7, 11, 14, 16, 21, 34, 35), subgroup C (*e.g.*, serotypes 1, 2, 5, 6), subgroup D (*e.g.*, serotypes 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47), subgroup E (serotype 4), subgroup F (serotype 40, 41), or any other adenoviral serotype.

10 TABLE 1

Examples Of Human And Animal Adenoviruses Including The American Type Culture Collection Catalog # For A Representative Virus Of The Respective Classification

Adenovirus Type	ATCC #
Adenovirus Type 21	ATCC VR-1099
SA18 (Simian adenovirus 18)	ATCC VR-943
SA17 (Simian adenovirus 17)	ATCC VR-942
Adenovirus Type 47	ATCC VR-1309
Adenovirus Type 44	ATCC VR-1306
Avian adenovirus Type 4	ATCC VR-829
Avian adenovirus Type 5	ATCC VR-830
Avian adenovirus Type 7	ATCC VR-832
Avian adenovirus Type 8	ATCC VR-833
Avian adenovirus Type 9	ATCC VR-834
Avian adenovirus Type 10	ATCC VR-835
Avian adenovirus Type 2	ATCC VR-827
Adenovirus Type 45	ATCC VR-1307
Adenovirus Type 38	ATCC VR-988
Adenovirus Type 46	ATCC VR-1308
Simian adenovirus	ATCC VR-541
SA7 (Simian adenovirus 16)	ATCC VR-941
Frog adenovirus (FAV-1)	ATCC VR-896
Adenovirus type 48 (candidate)	ATCC VR-1406
Adenovirus Type 42	ATCC VR-1304
Adenovirus Type 49 (candidate)	ATCC VR-1407

Adenovirus Type 43	ATCC VR-1305
Avian adenovirus Type 6	ATCC VR-831
Avian adenovirus Type 3	
Bovine adenovirus Type 3	ATCC VR-639
Bovine adenovirus Type 6	ATCC VR-642
Canine adenovirus	ATCC VR-800
Bovine adenovirus Type 5	ATCC VR-641
Adenovirus Type 36	ATCC VR-913
Ovine adenovirus type 5	ATCC VR-1343
Adenovirus Type 29	ATCC VR-272
Swine adenovirus	ATCC VR-359
Bovine adenovirus Type 4	ATCC VR-640
Bovine adenovirus Type 8	ATCC VR-769
Bovine adenovirus Type 7	ATCC VR-768
Adeno-associated virus Type2 (AAV-2H)	ATCC VR-680
Adenovirus Type 4	ATCC VR-4
Adeno-associated virus Type3 (AAV-3H)	ATCC VR-681
Peromyscus adenovirus	ATCC VR-528
Adenovirus Type 15	ATCC VR-661
Adenovirus Type 20	ATCC VR-662
Chimpanzee adenovirus	ATCC VR-593
Adenovirus Type 31	ATCC VR-357
Adenovirus Type 25	ATCC VR-223
Chimpanzee adenovirus	ATCC VR-592
Chimpanzee adenovirus	ATCC VR-591
Adenovirus Type 26	ATCC VR-224
Adenovirus Type 19	ATCC VR-254
Adenovirus Type 23	ATCC VR-258
Adenovirus Type 28	ATCC VR-226
Adenovirus Type 6	ATCC VR-6
Adenovirus Type 2 Antiserum:	ATCC VR-1079
Adenovirus Type 6	ATCC VR-1083
Ovine adenovirus Type 6	ATCC VR-1340
Adenovirus Type 3	ATCC VR-847
Adenovirus Type 7	ATCC VR-7
Adenovirus Type 39	ATCC VR-932
Adenovirus Type 3	ATCC VR-3
Bovine adenovirus Type 1	ATCC VR-313
Adenovirus Type 14	ATCC VR-15
Adenovirus Type 1	ATCC VR-1078
Adenovirus Type 21	ATCC VR-256
Adenovirus Type 18	ATCC VR-1095
Baboon adenovirus	ATCC VR-275
Adenovirus Type 10	ATCC VR-11
Adenovirus Type 33	ATCC VR-626
Adenovirus Type 34	ATCC VR-716
Adenovirus Type 15	ATCC VR-16

Adenovirus Type 22	ATCC VR-257
Adenovirus Type 24	ATCC VR-259
Adenovirus Type 17	ATCC VR-1094
Adenovirus Type 4	ATCC VR-1081
Adenovirus Type 16	ATCC VR-17
Adenovirus Type 17	ATCC VR-18
Adenovirus Type 16	ATCC VR-1093
Bovine adenovirus Type 2	ATCC VR-314
SV-30	ATCC VR-203
Adenovirus Type 32	ATCC VR-625
Adenovirus Type 20	ATCC VR-255
Adenovirus Type 13	ATCC VR-14
Adenovirus Type 14	ATCC VR-1091
Adenovirus Type 18	ATCC VR-19
SV-39	ATCC VR-353
Adenovirus Type 11	ATCC VR-849
Duck adenovirus (Egg drop syndrome)	ATCC VR-921
Adenovirus Type 1	ATCC VR-1
Chimpanzee adenovirus	ATCC VR-594
Adenovirus Type 15	ATCC VR-1092
Adenovirus Type 13	ATCC VR-1090
Adenovirus Type 8	ATCC VR-1368
SV-31	ATCC VR-204
Adenovirus Type 9	ATCC VR-1086
Mouse adenovirus	ATCC VR-550
Adenovirus Type 9	ATCC VR-10
Adenovirus Type 41	ATCC VR-930
C1	ATCC VR-20
Adenovirus Type 40	ATCC VR-931
Adenovirus Type 37	ATCC VR-929
Marble spleen disease virus	
Adenovirus Type 35	ATCC VR-718
SV-32 (M3)	ATCC VR-205
Adenovirus Type 28	ATCC VR-1106
Adenovirus Type 10	ATCC VR-1087
Adenovirus Type 20	ATCC VR-1097
Adenovirus Type 21	ATCC VR-1098
Adenovirus Type 25	ATCC VR-1103
Adenovirus Type 26	ATCC VR-1104
Adenovirus Type 31	ATCC VR-1109
Adenovirus Type 19	ATCC VR-1096
SV-36	ATCC VR-208
SV-38	ATCC VR-355
SV-25 (M8)	ATCC VR-201
SV-15 (M4)	ATCC VR-197
Adenovirus Type 22	ATCC VR-1100
SV-23 (M2)	ATCC VR-200

Adenovirus Type 11	ATCC VR-12
Adenovirus Type 24	ATCC VR-1102
Avian adenovirus Type 1	
SV-11 (M5)	ATCC VR-196
Adenovirus Type 5	ATCC VR-5
Adenovirus Type 23	ATCC VR-1101
SV-27 (M9)	ATCC VR-202
Avian adenovirus Type 2 (GAL)	ATCC VR-280
SV-1 (M1)	ATCC VR-195
SV-17 (M6)	ATCC VR-198
Adenovirus Type 29	ATCC VR-1107
Adenovirus Type 2	ATCC VR-846
SV-34	ATCC VR-207
SV-20 (M7)	ATCC VR-199
SV-37	ATCC VR-209
SV-33 (M10)	ATCC VR-206
Avian adeno-associated virus	ATCC VR-865
Adeno-associated (satellite) virus Type 4	ATCC VR-646
Adenovirus Type 30	ATCC VR-273
Adeno-associated (satellite) virus Type 1	ATCCVR-645
Infectious canine hepatitis (Rubarth's disease)	
Adenovirus Type 27	ATCC VR-1105
Adenovirus Type 12	ATCC VR-863
Adeno-associated virus Type 2	
Adenovirus Type 7a	ATCC VR-848

The recombinant adenoviral vectors of this invention are useful as therapeutics for prevention and/or treatment of cancer. The vectors of the invention preferentially replicate in and effect selective cytolysis of tumor cells. In one embodiment, the

5 vectors of the invention, with an E2F promoter operatively linked to a gene essential to replication, preferentially kill Rb-pathway defective tumor cells as compared to cells that are non-defective in the Rb-pathway. In another embodiment, the vectors of the invention, with a TERT promoter operatively linked to a gene essential to

10 replication, preferentially kill tumor cells with up-regulated expression of telomerase as compared to non-tumor cells. In another embodiment, vectors of the invention with both an E2F promoter operatively linked to a gene essential to replication and a TERT

promoter operatively linked to a gene essential to replication, preferentially kill tumor cells with both a defect in the Rb-pathway and with up-regulated expression of telomerase. Without wishing to be limited by theoretical considerations, in one embodiment the specific regulation of viral replication by an E2F or TERT promoter
5 is shielded from read-through transcription by an upstream termination signal sequence. In some embodiments, the recombinant viral vectors of the invention further comprise a selective promoter linked to the E4 gene. In other embodiments, the recombinant viral vectors of the invention comprise a 19k deletion in the adenoviral E1b gene. In further embodiments, the recombinant viral vectors of the invention
10 comprise an IRES or self-processing cleavage site (e.g., a 2A) sequence. In still further embodiments the recombinant viral vectors of the invention comprise a heterologous coding sequence or transgene, operatively linked to a native or heterologous promoter. In yet another embodiment, the adenoviral vectors of the invention comprise a targeting ligand. Thus, the combination and the sequential positioning of the genetic
15 elements employed in the vectors of the invention provide for and enhance the vector's selectivity, while at the same time minimizing toxicity and side effects in an animal.

In one embodiment, the recombinant viral vector of the invention comprises a termination signal sequence (as described e.g., in patent publication US20030104624). Insertion of the polyadenylation signal sequences may reduce replication of the
20 oncolytic adenoviral vector in nontarget cells and therefore toxicity. A termination signal sequence may also be placed before (5') any promoter in the vector. In one embodiment, the terminal signal sequence is placed 5' to the E2F promoter, which is operatively linked to the E1a or E1b coding sequences. In another embodiment, the terminal signal sequence is placed 5' to the TERT promoter, which is operatively
25 linked to the E1a or E1b coding sequences.

In an alternative embodiment, the invention further comprises a mutation or deletion in the E1b gene. In one embodiment, the mutation or deletion in the E1b gene is such that the E1b-19kD protein becomes non-functional. This modification of the E1b region may be combined with vectors where all or a part of the E3 region is present. In one embodiment, there is a deletion of E1b (provided as SEQ ID NO:4 or SEQ ID NO:6). In one embodiment the first 261 nucleotides of the E1b open reading frame are deleted. In one embodiment, the nucleotide sequence in SEQ ID NO: 12 is deleted from the vector. In one embodiment, the E1b deletion is the same as the E1b deletion in the viruses CG5757 or OV945.

10 The oncolytic adenoviral vectors of the invention may optionally comprise a heterologous coding sequence or transgene. For example, the heterologous coding sequence may encode an immunostimulatory protein including, but not limited to, cytokines (GM-CSF, IL1, IL2, IL4, IL5, IFN α , IFN γ , TNF α , IL12, IL18, and flt3), proteins that stimulate interactions with immune cells (B7, CD28, MHC class I, MHC class II, TAPs), tumor-associated antigens (immunogenic sequences from MART-1, gp100(pmel-17), tyrosinase, tyrosinase-related protein 1, tyrosinase-related protein 2, melanocyte-stimulating hormone receptor, MAGE1, MAGE2, MAGE3, MAGE12, BAGE, GAGE, NY-ESO-1, β -catenin, MUM-1, CDK-4, caspase 8, KIA 0205, HLA-A2R1701, α -fetoprotein, telomerase catalytic protein, G-250, MUC-1, carcinoembryonic protein, p53, Her2/neu, triosephosphate isomerase, CDC-27, LDLR-FUT, telomerase reverse transcriptase, and PSMA), antibodies that block inhibitory signals (CTLA4 blockade), chemokines (MIP1 α , MIP3 α , CCR7 ligand, and calreticulin), and other proteins.

In another embodiment, the heterologous coding sequence codes for an anti-angiogenic protein. Anti-angiogenic proteins include, but are not limited to, METH-1, METH -2, TrpRS fragments, proliferin-related protein, prolactin fragment, PEDF, vasostatin, various fragments of extracellular matrix proteins and growth factor/cytokine inhibitors. Various fragments of extracellular matrix proteins include, but are not limited to, angiostatin, endostatin, kininostatin, fibrinogen-E fragment, thrombospondin, tumstatin, canstatin, and restin.

In another embodiment, the heterologous coding sequence codes for a growth factor/cytokine inhibitor. Growth factor/cytokine inhibitors include, but are not limited to, VEGF/VEGFR antagonist, sFlt-1, sFlk, sNRP1, angiopoietin/tie antagonist, sTie-2, chemokines (IP-10, PF-4, Gro-beta, FGF/FGFR antagonist (sFGFR), Ephrin/Eph antagonist (sEphB4 and sephrinB2), inhibitors of factors including IFN-gamma (Mig), IFN α , PDGF, TGF β and IGF-1; and the like.

In another embodiment, the heterologous coding sequence codes for a suicide gene. A "suicide gene" encodes for a protein which itself can lead to cell death, as with expression of diphtheria toxin A, or the expression of the protein can render cells selectively sensitive to certain drugs, e.g., expression of the Herpes simplex thymidine kinase gene (HSV-TK) renders cells sensitive to antiviral compounds, such as acyclovir, gancyclovir and FIAU (1-(2-deoxy-2-fluoro-.beta.-D-arabinofuranosil)-5-io- douracil). Other suicide genes include, but are not limited to, genes that encode for carboxypeptidase G2 (CPG2), carboxylesterase (CA), cytosine deaminase (CD), cytochrome P450 (cyt-450), deoxycytidine kinase (dCK), nitroreductase (NR), purine nucleoside phosphorylase (PNP), thymidine phosphorylase (TP), varicella zoster virus thymidine kinase (VZV-TK), and xanthine-guanine phosphoribosyl transferase (XGPRT). The heterologous gene can exert its effect at the level of RNA, for instance,

by encoding an antisense message or ribozyme, a protein that affects splicing or 3' processing (e.g., polyadenylation), or a protein that affects the level of expression of another gene within the cell, e.g. by mediating an altered rate of mRNA accumulation, an alteration of mRNA transport, and/or a change in post-transcriptional regulation.

- 5 The addition of a heterologous gene to the virus could result in a virus with an additional antitumor mechanism of action.

In another embodiment, the adenoviral vectors of the invention further comprise a targeting ligand included in a capsid protein of the particle. In one embodiment, the capsid protein is a fiber protein and the ligand is in the HI loop of the
10 fiber protein. In another embodiment, the capsid protein is a fiber protein or pIX. In yet another embodiment, the targeting ligand is in the HI loop of the fiber protein. In a further embodiment, the ligand is added to the carboxyl end of the adenovirus fiber protein. In an additional embodiment, the virus is targeted by replacing the fiber knob with a fiber knob from another adenovirus serotype. For examples of targeted
15 adenoviruses, see for example, WO 00/67576, WO 99/39734, US6,683,170, US6,555,368, US5,922,315, US5,543,328 and US5,846,782,

The adenoviral vector particle may also include other mutations to the fiber protein. Examples of these mutations include, but are not limited to those described in US application no. 10/403,337, WO 98/07877, WO 01/92299, and US Patent Nos.
20 5,962,311, 6,153,435, 6,455,314 and Wu et al. (J Virol. 2003 Jul 1;77(13):7225-7235). These include, but are not limited to mutations that decrease binding of the viral vector particle to a particular cell type or more than one cell type, enhance the binding of the viral vector particle to a particular cell type or more than one cell type and/or reduce the immune response to the adenoviral vector particle in an animal. In addition, the
25 adenoviral vectors of the present invention may also contain mutations to other viral

capsid proteins. Examples of these mutations include, but are not limited to those described in US Patent Nos. 5,731,190, 6,127,525, and 5,922,315. Other mutated adenoviruses are described in U.S. Patent Nos. 6,057,155, 5,543,328 and 5,756,086.

Accordingly, in another aspect there is provided a method of selective
5 cytolysis, i.e., killing a neoplastic cell in a cell population that comprises contacting an effective amount of the viral vectors and/or viral particles of the invention with said cell population under conditions where the viral vectors and/or particles can infect the neoplastic cells in the cell population, selectively replicate in and kill the neoplastic cells. The cell population may be in vivo, in vitro or in an ex vivo setting.

10 The invention further comprises adenoviral vector particles, in which a targeting ligand is included in a capsid protein of the particle. In a further embodiment, the capsid protein is a fiber protein and the ligand is in the HI loop of the fiber protein.

The adenoviral vectors of the invention are made by standard techniques
15 known to those skilled in the art. The vectors are transferred into packaging cells by techniques known to those skilled in the art. Packaging cells provide complementing functions to the adenovirus genomes that are to be packaged into the adenovirus particle. The production of such particles requires that the vector be replicated and that those proteins necessary for assembling an infectious virus be produced. The
20 packaging cells are cultured under conditions that permit the production of the desired viral vector particle. The particles are recovered by standard techniques. Examples of packaging cells include, but are not limited to, packaging cells that have been designed to limit homologous recombination that could lead to wild-type adenoviral particles, for example, cells disclosed in U.S. Patent Nos. 5,994,128 (Fallaux, et al.) and
25 6,033,908 (Bout, et al). Also, viral vector particles of the invention may be, for

example, produced in PerC6 or Hela-S3 cells (e.g. see US patent application 60/463,143).

E2F PROMOTERS

5 Without being bound by theory, the selectivity of E2F-responsive promoters (hereinafter sometimes referred to as E2F promoters) is reported to be based on the derepression of the E2F promoter/transactivator in Rb-pathway defective tumor cells. In quiescent cells, E2F binds to the tumor suppressor protein pRB in ternary complexes. In its complexed form, E2F functions to repress transcriptional activity
10 from promoters with E2F binding sites, including the E2F-1 promoter itself (Zwicker J, and Muller R., Prog. Cell Cycle Res 1995; 1:91-99). The E2F-1 promoter is transcriptionally inactive in resting cells. In normal cycling cells, pRB-E2F complexes are dissociated in a regulated fashion, allowing for controlled derepression of E2F and subsequent cell cycling (Dyson, N., Genes and Development 1998; 12:2245-2262).

15 In the majority of tumor types, the Rb cell cycle regulatory pathway is disrupted, suggesting that Rb-pathway deregulation is obligatory for tumorigenesis (Strauss M, Lukass J and Bartek J., Nat Med 1995; 12:1245-1246). One consequence of these mutations is the disruption of E2F-pRB binding and an increase in free E2F in tumor cells. Rb itself is mutated in some tumor types, and in other tumor types factors
20 upstream of Rb are deregulated (Weinberg, RA. Cell 1995; 81:323-330). One effect of these Rb-pathway changes in tumors is the loss of pRB binding to E2F, and an apparent increase in free E2F in tumor cells. The abundance of free E2F in turn results in high-level expression of E2F responsive genes in tumor cells, including the E2F-1 gene. Accordingly, the term "Rb-pathway defective cells" may be functionally defined
25 as cells which display an abundance of "free" E2F, as measured by gel mobility shift

assay or by chromatin immunoprecipitation (Takahashi Y et al., Genes Dev. 2000 Apr 1;14(7):804-16). The E2F-1 promoter has been shown to up-regulate the expression of marker genes in an adenovirus vector in a rodent tumor model but not normal proliferating cells in vivo (Parr MJ et al., Nature Med 1997; Oct;3(10):1145-1149).

5 An E2F-responsive promoter has at least one E2F binding site. In one embodiment, the E2F-responsive promoter is a mammalian E2F promoter. In another embodiment, it is a human E2F promoter. For example, the E2F promoter may be the human E2F-1 promoter. Further, the human E2F-1 promoter may be, for example, a E2F-1 promoter having the sequence as described in SEQ ID NO:1. A number of
10 examples of E2F promoters are known in the art (e.g. Parr et al. Nature Medicine 1997;3(10) 1145-1149, WO 02/067861, US20010053352 and WO 98/13508). E2F responsive promoters typically share common features such as Sp I and/or ATT7 sites in proximity to their E2F site(s), which are frequently located near the transcription start site, and lack of a recognizable TATA box. E2F-responsive promoters include
15 E2F promoters such as the E2F-1 promoter, dihydrofolate reductase (DHFR) promoter, DNA polymerase A (DPA) promoter, c-myc promoter and the B-myc promoter. The E2F-1 promoter contains four E2F sites that act as transcriptional repressor elements in serum-starved cells. In one embodiment, an E2F-responsive promoter has at least two E2F sites. In another embodiment, an E2F promoter is
20 operatively linked to the adenovirus E1a region. In a further embodiment, an E2F promoter is operatively linked to the adenovirus E1b region. In yet a further embodiment, an E2F promoter is operatively linked to the adenovirus E4 region.

 In one embodiment of the invention, the recombinant viral vectors of the present invention selectively replicate in and lyse Rb-pathway defective cells. In one
25 embodiment, the E2F promoter of the invention is a mammalian E2F promoter. In

another embodiment, the mammalian E2F promoter is a human E2F promoter, for example a human E2F promoter which comprises or consists essentially of SEQ ID NO:1. Embodiments of the invention include adenoviral vectors comprising an E2F promoter wherein the E2F promoter comprises a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence shown in SEQ ID NO:1; (b) a fragment of the nucleotide sequence shown in SEQ ID NO: 1, wherein the fragment has tumor selective promoter activity; (c) a nucleotide sequence having at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% or more % identity over its entire length to the nucleotide sequence shown in SEQ ID NO: 1, wherein the nucleotide sequence has tumor selective promoter activity; and (d) a nucleotide sequence having a full-length complement that hybridizes under stringent conditions to the nucleotide sequence shown in SEQ ID NO:1, wherein the nucleotide sequence has tumor selective promoter activity. In another embodiment of the invention, the E2F promoter comprises nucleotides 7 to 270 of SEQ ID NO:1. In another embodiment of the invention, the E2F promoter comprises nucleotides 7 to 270 of SEQ ID NO:1, wherein nucleotide 75 of SEQ ID NO:1 is a T instead of a C.

In other embodiments, a E2F promoter according to the present invention has at least 80, 85, 87, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% or more sequence identity to the nucleotide sequence shown in SEQ ID NO:1, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. In one embodiment, the given % sequence identity exists over a region of the sequences that is at least about 50 nucleotides in length. In another embodiment, the given % sequence identity exists over a region of at least about 100 nucleotides in length. In another embodiment, the given % sequence identity exists over a region of at least about 200 nucleotides in

length. In another embodiment, the given % sequence identity exists over the entire length of the sequence.

The E2F-responsive promoter does not have to be the full-length or wild type promoter, but should have a tumor-selectivity of at least 3-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, at least 100-fold or even at least 300-fold. Tumor-selectivity can be determined by a number of assays using known techniques, such as the techniques employed in WO 02/067861, Example 4, for example RT-PCR or a comparison of replication in selected cell types. The tumor-selectivity of the adenoviral vectors can also be quantified by E1A RNA levels, as further described in WO 02/067861, Example 4, and the E1A RNA levels obtained in H460 (ATCC, Cat. # HTB-177) cells can be compared to those in PERC (Clonetics Cat. #CC2555) cells in order to determine tumor-selectivity for the purposes of this invention. The relevant conditions of the experiment may vary, but typically follow those described in WO 02/067861.

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TELOMERASE (TERT) PROMOTERS

Without being bound by theory, the understanding of selective TERT expression in cancer is based on the current knowledge that TERT is the rate-limiting catalytic subunit of telomerase, a multicomponent ribonucleoprotein enzyme that has also been shown to be active in ~ 85 % of human cancers but not normal somatic cells (Kilian A et al. Hum Mol Genet. 1997 Nov;6(12):2011-9; Kim NW et al. Science. 1994 Dec 23;266(5193):2011-5; Shay JW et al. European Journal of Cancer 1997; 5, 787-791; Stewart SA et al. Semin Cancer Biol. 2000 Dec;10(6):399-406). Cancer cells appear to require immortalization for tumorigenesis and telomerase activity is almost always necessary for immortalization (Kim NW et al. Science. 1994 Dec

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23;266(5193):2011-5; Kiyono T et al. Nature 1998;396:84). Thus, the majority of tumor cells have a disregulated telomerase pathway. Such tumor cells are specifically targeted by viruses of the invention utilizing a TERT promoter operatively linked to a gene and/or coding region essential for replication (e.g. E1a, E1b or E4).

5 The term TERT promoter as used herein refers to a full-length TERT promoter and functional fragments, mutations and derivatives thereof. The TERT promoter does not have to be a full-length or wild type promoter. One skilled in the art knows how to derive fragments from a TERT promoter and test them for the desired specificity. In one embodiment, a TERT promoter of the invention is a mammalian
10 TERT promoter. In a further embodiment the mammalian TERT promoter, is a human TERT promoter (hTERT). In one embodiment of the invention, the TERT promoter comprises or consists essentially of SEQ ID NO:2, which is a 239 bp fragment of the hTERT promoter. In another embodiment of the invention, the TERT promoter comprises or consists essentially of SEQ ID NO:3, which is a 245 bp
15 fragment of the hTERT promoter. In one embodiment, a TERT promoter is operatively linked to the adenovirus E1a region. In another embodiment, the TERT promoter is operatively linked to the adenovirus E1b region. In yet a further embodiment, the TERT promoter is operatively linked to the adenovirus E4 region.

Embodiments of the invention include adenoviral vectors comprising a TERT
20 promoter wherein the TERT promoter comprises a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence shown in SEQ ID NO:2; (b) a fragment of the sequence shown in SEQ ID NO:2, wherein the fragment has tumor selective promoter activity; (c) a nucleotide sequence having at least 90% identity over its entire length to the sequence shown in SEQ ID NO:2, wherein the nucleotide
25 sequence has tumor selective promoter activity; and (f) a nucleotide sequence having a

full-length complement that hybridizes under stringent conditions to the sequence shown in SEQ ID NO:2, wherein the nucleotide sequence has tumor selective promoter activity. Other examples of TERT promoters are known to those skilled in the art (e.g. WO 98/14593).

5 In other embodiments, an TERT promoter according to the present invention has at least In other embodiments, a E2F promoter according to the present invention has at least 80, 85, 87, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% or more sequence identity to the sequence shown in SEQ ID NO:2 or SEQ ID NO:3, when compared and aligned for maximum correspondence, as measured using one of the following
10 sequence comparison algorithms or by visual inspection. In one embodiment, the given % sequence identity exists over a region of the sequences that is at least about 50 nucleotides in length. In another embodiment, the given % sequence identity exists over a region of at least about 100 nucleotides. In another embodiment, the given % sequence identity exists over a region of at least about 200 nucleotides. In another
15 embodiment, the given % sequence identity exists over the entire length of the sequence.

COMPOSITIONS AND METHODS FOR PRACTICING THE INVENTION

 In a further aspect of the invention, a pharmaceutical composition comprising
20 the recombinant viral vectors and/or particles of the invention and a pharmaceutically acceptable carrier is provided. Such compositions, which can comprise an effective amount of adenoviral vector of the invention in a pharmaceutically acceptable carrier, are suitable for local or systemic administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral
25 solutions or suspensions, oil in water or water in oil emulsions and the like.

Formulations for parenteral and non-parenteral drug delivery are known in the art. Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors and particles of the invention. Acceptable pharmaceutical carriers are, for example, saline solution, protamine sulfate (Elkins-Sinn, Inc., Cherry Hill, N.J.),
5 water, aqueous buffers, such as phosphate buffers and Tris buffers, or Polybrene (Sigma Chemical, St. Louis MO) and phosphate-buffered saline and sucrose. The selection of a suitable pharmaceutical carrier is deemed to be apparent to those skilled in the art from the teachings contained herein. These solutions are sterile and generally free of particulate matter other than the desired adenoviral virions. The compositions
10 may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. Excipients that enhance infection of cells by adenovirus and/or anaesthetics, e.g., lidocaine, may be included.

15 The viral vectors are administered to a host in an amount that is effective to inhibit, prevent, or destroy the growth of the tumor cells through replication of the viral vectors in the tumor cells. Such administration may be by systemic administration as herein described, or by direct injection of the vectors into a tumor. In one approach, the vectors are administered systemically in an amount of at least 5×10^9 viral particles per kilogram body weight and in general, such an amount does not
20 exceed 1×10^{13} viral particles per kilogram body weight. In another approach, the vectors are administered intratumorally in an amount of at least 2×10^{10} viral particles and in general such an amount does not exceed 1×10^{13} viral particles per kilogram body weight. In yet another approach, the vectors are instilled into the bladder of the
25 subject. In such cases, the transduction may be pre-treated with a transduction

enhancer such as described in USSN 10/327869. The exact dosage to be administered is dependent upon a variety of factors including the age, weight, and sex of the patient, and the size and severity of the tumor being treated. The vectors may be administered one or more times. Single or multiple administrations of the compositions can be
5 carried out with dose levels and pattern being selected by the treating physician. If necessary, the immune response may be diminished by employing a variety of immunosuppressants, or removal or preexisting antibodies, so as to permit repetitive administration and/or enhance replication by reducing the immune response to the viruses. Administration of the adenoviral vectors of the present invention may be
10 combined with other antineoplastic protocols, numerous examples of which are known in the art. Such antineoplastic protocols will vary dependent upon the type of cancer under treatment.

Delivery can be achieved in a variety of ways, employing liposomes, direct injection, systemic injection, catheters, topical applications, inhalation, etc.

15 It follow that the invention provides a method of treating a subject having a neoplastic condition, comprising administering a therapeutically effective amount of an adenoviral vector of the invention to the subject, typically a patient with cancer. While the mechanism is not part of the invention, the viral vectors described herein are believed to distribute selective to tumor cells and essentially throughout a tumor mass
20 due to the capacity for selective replication in the tumor tissue.

All neoplastic conditions are potentially amenable to treatment with the methods of the invention. Tumor types include, but are not limited to hematopoietic, pancreatic, neurologic, hepatic, gastrointestinal tract, endocrine, biliary tract, sinopulmonary, head and neck, soft tissue sarcoma and carcinoma, dermatologic,
25 reproductive tract, respiratory, and the like. In one embodiment, the tumors for

treatment are those with a high mitotic index relative to normal tissue. In another embodiment, the tumors are solid tumors.

In one embodiment the subject is a human patient. For human patients, if a heterologous coding sequence is included in the vector, the heterologous coding sequence may be of human origin although genes of closely related species that exhibit high homology and biologically identical or equivalent function in humans may be used if the product of the heterologous coding sequence does not produce/cause an adverse immune reaction in the recipient. In one embodiment, the heterologous coding sequence codes for a therapeutic protein or therapeutic RNA. A therapeutic active amount of a nucleic acid sequence or a therapeutic gene is an amount effective at dosages and for a period of time necessary to achieve the desired result. This amount may vary according to various factors including but not limited to sex, age, weight of a subject, and the like.

The invention also provides for screening candidate drugs to identify agents useful for modulating the expression of E2F and/or TERT, and hence useful for treating cancer. Appropriate host cells are those in which the regulatory region of E2F and/or TERT is capable of functioning. In one embodiment, the regulatory region of E2F and/or TERT is used to make a variety of expression vectors to express a marker that can then be used in screening assays. In one embodiment, the marker is E1a, E1b, viral replication or combinations thereof, all of which can be measured using techniques well known to those skilled in the art. The expression vectors may be either self-replicating extrachromosomal vectors or vectors that integrate into a host genome. Generally, these expression vectors include a transcriptional and translational regulatory nucleic acid sequence of E2F and/or TERT operatively linked to a nucleic acid encoding a marker. The marker may be any protein that can be readily detected.

It may be detected on the basis of light emission, such as luciferase or FITC, color, such as β -galactosidase, enzyme activity, such as alkaline phosphatase or antibody reaction, such as a protein for which an antibody exists. In addition, the marker system may be a viral vector or particle of the present invention.

5 In one embodiment, a viral vector of the invention is used to assess the anti-cancer efficacy of a candidate therapeutic agent. According to this embodiment, an effective amount of the viral vectors is contacted with a cell population under conditions where the viral vectors can infect the neoplastic cells in the cell population, selectively replicate in, and kill the neoplastic cells. The LD₅₀ of the viral vector in
10 the presence and absence of the candidate agent is compared to identify the candidate agents that modulate the expression of the E2F and/or TERT gene or enhance the efficacy of the viral vector in terms of selective cytolysis of cancer cells. If the level of expression is different as compared to similar viral vector controls lacking the E2F and/or TERT promoter, the candidate agent is capable of modulating the expression of
15 E2F and/or TERT and is a candidate for treating cancers and for further development of active agents on the basis of the candidate agent so identified.

 In a second embodiment, the candidate agent is added to host cells containing the expression vector and the level of expression of the marker is compared with a control. If the level of expression is different, the candidate agent is capable of
20 modulating the expression of E2F or TERT and is a candidate for treating cancers involving these genes and for further development of active agents on the basis of the candidate agent so identified.

 Active agents so identified may also be used in combination treatments, for example with oncolytic adenoviruses of the invention.

25 In preferred embodiments, the bioactive agents modulate the expression

profiles, or expression profile nucleic acids or proteins provided herein. In a particularly preferred embodiment, the candidate agent suppresses a cancer phenotype, for example relative to a normal tissue phenotype.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, e.g. small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Preferred small molecules are less than 2000, or less than 1500 or less than 1000 or less than 500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, e.g. at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, *e.g.*, Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992, *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Anand, 1992, *Techniques for the Analysis of Complex Genomes*, Academic Press, New York; Guthrie and Fink, 1991, *Guide to Yeast Genetics and Molecular Biology*, Academic Press, New York; Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Jakoby and Pastan, 1979; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Hogan *et al.*, *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press,

Cold Spring Harbor, N.Y., 1986).

EXAMPLES

The present invention is described by reference to the following examples,
5 which are offered by way of illustration and are not intended to limit the invention in
any manner. Standard techniques well known in the art or the techniques specifically
described below are utilized.

Example 1: Construction of the OV945 and Sequencing Verification

Based on an E2F-1 promoter sequence (GenBank S74230), primers 1405.77.1
10 (5'-ataccggtggtaccatccggacaaagcctgcgcg; SEQ ID NO:8) and 1405.77.2
(5'-agaccggtcgagggctcgatcccgcctccg; SEQ ID NO:9) were designed and a 270 nt E2F-1
promoter (SEQ ID NO:1) was cloned from human genomic DNA by PCR. The AgeI
(or PinAI) sites from the primers were used to replace the human uroplakin II
promoter in CP1131 (US Patent 6,692,736, Zhang et al., Cancer Res. 2002 Jul
15 1;62(13):3743-50) with the E2F-1 promoter, resulting in plasmid CP1493. CP1493
has an E2F-1 promoter operatively linked to E1a, an IRES operatively linked to the
E1b region and most of the E1B 19-KDa region deleted. The CP1493 plasmid was
sequenced and confirmed as correct. CP1493 was cotransfected into 293 cells with
pBHGE3 (from Microbix Biosystems, Inc. Toronto, Ontario, Canada and described in
20 US Patent 6,140,087) to yield recombinant virus OV945. The structure of OV945 was
confirmed by PCR amplification, followed by diagnostic enzymatic digestion for the
corresponding specific regions. Further sequencing of the OV945 E1 region also
confirmed the genomic structure (SEQ ID NO: 6).

Example 2: Construction of CG5757 and sequencing verification

The hTERT promoter was PCR cloned from plasmid pGRN316 (from Geron, Menlo Park, California; also described in Günes et al., Cancer Research 60, 2116-2121, 2000) with primers 1244.39.1 (SEQ ID NO:10; 5 aagtcgaccggtaccgtggcgaggaggactggggac) and 1244.39.2 (SEQ ID NO:11; aagtcgaccggtgcgggggtggccggggccaggg). The SalI cloning sites in the primers were used to replace the IRES fragment in CP1493 with the hTERT promoter. The derived plasmid, CP1509, has the E1a and E1B 55K genes under transcriptional control of the E2F and hTERT promoters, respectively. CP1509 was cotransfected into 293 cells 10 with pBHGE3 (from Microbix Biosystems, Inc. Toronto, Ontario, Canada; described in US Patent 6,140,087) to yield recombinant virus. The nucleotide sequence of the left end of the CG5757 virus is provided (SEQ ID NO: 4).

Example 3: Construction of OV947 and genomic structure verification

OV947 is structurally similar to CG5757 but lacks the E1B 19k deletion. The E2F-1 15 and hTERT promoters were derived from CP1509, a 0.27Kb AgeI fragment and a 0.24Kb SalI fragment, respectively. These promoters were cloned into CP686 (US Patent 6,692,736; Li et al., Cancer Res. 2001 Sep 1;61(17):6428-36) 5' to the E1A and E1B genes, respectively, to yield CP1498. The 0.27Kb AgeI fragment (E2F-1 promoter) was cloned in place of the AFP promoter and the 0.24Kb SalI fragment 20 (hTERT promoter) was cloned in place of the IRES in CP686. The plasmid CP1498 was used to cotransfect with pBHGE3 (from Microbix Biosystems, Inc. Toronto, Ontario, Canada; US Patent 6,140,087) into 293 cells to yield OV947. The structure of OV947 (Figure 1) was confirmed following PCR amplification and diagnostic enzymatic digestion for the corresponding specific regions.

Example 4: Construction of OV1025 and genomic structure verification

The OV1025 virus has both E1A and E1B under transcriptional control of the hTERT promoter. In contrast to OV945 and CG5757, in OV1025, the E1B 19K region is not deleted. The AgeI sites in PCR primers 1244.39.1 (SEQ ID NO:10) and 1244.39.2

- 5 (SEQ ID NO:11) were used to clone the hTERT promoter in place of the AFP promoter in CP686 (US Patent 6,692,736, Li et al., Cancer Res. 2001 Sep 1;61(17):6428-36). The derived DNA construct CP1429 was cotransfected with pBHGE3 into 293 cells resulting in generation of the recombinant virus OV1025. The structure of OV1025 (Figure 1) was confirmed by PCR amplification, followed by
- 10 diagnostic enzymatic digestion of the corresponding specific regions.

Example 5: Cell Lines

Table 2 provides a listing of exemplary cell lines used to characterize the replication of the pan cancer viruses of the invention.

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Table 2. Cell lines for evaluation of viruses.

Cell Name	Cell Origin	ATTC	Notes
Hep3B	hepatocellular carcinoma	HB-8064	
A549	Lung carcinoma	CCL-185	
LoVo	colorectal adenocarcinoma	CCL-229	
SW480	colorectal adenocarcinoma	CCL-228	
LNCaP	Prostate cancer	CRL-1740	
Panc-1	Pancreatic cancer	CRL-1469	
HeLa	Cervical epithelial adenocarcinoma	CCL-2	
253J BV	Human bladder transitional cell carcinoma		Dr. Dr. Colin Dinney's Lab in MD Anderson Cancer Center
WI-38/VA-13	SV40 transformed human lung fibroblast	CCL-75.1	
HBL-100	Normal breast cell	HTB-124	ATCC discontinued
WI-38	Human normal lung fibroblast cell	CCL-75	
IMR-90	Normal lung fibroblast cell	CCL-186	

ARPE-19	Normal retinal pigmented epithelial cell	CRL-2302	
BSM	Bladder smooth muscle primary cell		Human primary cell from Cambrex, East Rutherford, New Jersey
Lung FB	Primary lung fibroblast cell		Human primary cell from Cambrex, East Rutherford, New Jersey
HMEC	Primary mammary epithelial cell		Human primary cell from Cambrex, East Rutherford, New Jersey
HMVEC-L	Primary lung microvascular endothelial cell		Human primary cell from Cambrex, East Rutherford, New Jersey
HRE	Primary renal epithelial cell		Human primary cell from Cambrex, East Rutherford, New Jersey
SAEC	Primary small airway epithelial cell		Human primary cell from Cambrex, East Rutherford, New Jersey
PrEC	Primary prostate epithelial cell		Human primary cell from Cambrex, East Rutherford, New Jersey

Example 6: Assay For Detecting Selective Expression Of E1

Transcriptional control of the E1 gene is typically evaluated by Western blot.

A sample virus is compared to wild type Adenovirus type 5 (designated OV802) for

- 5 E1A and E1B expression 24 hours following infection (Multiplicity of infection (MOI) of 10 pfu/cell). In a typical study, about 25 ug of cell lysate from each sample is subjected to immunostaining by mouse monoclonal antibodies against E1A or E1B and visualized by ECL.

Example 7: Tumor Selective Cytotoxicity Assays

Cytopathic Effect & Crystal Violet Assay: In a typical study cancer cells (Hep3B, LoVo, A549, 253J B-V) and normal cells (HRE, HMVEC-L and WI-38 cells) are tested in a crystal violet assay for cell killing. Briefly, the cells are plated in 6-well plates. One day later the cells are infected with CG5757 or OV802 at various MOIs of 10, 1, 0.1, 0.01, 0.001 and 0 PFUs per cell. At a selected time point(s) after the infection, cells are observed microscopically for CPE. Then cells are fixed with 10% Formalin and stained with 1% Crystal Violet using standard procedures and the amount of staining is observed.

MTT Assay: In a typical study, cells are plated on 96-well plates at 10,000 cells/well one day prior viral infection. Cells are infected at different MOIs (pfu/cell) for selected time points and viral cytotoxicity is determined by percentage of control in the MTT assay as previous described (Li et al., 2001). The cytotoxicity data is analyzed for sigmoidal dose response curve fit using GraphPad software and the LD50 determined. Comparing the relative LD50 between tumor and normal cells, the cytotoxic specificity of an oncolytic vector is represented by the selectivity index (SILD). The formula for SILD is $((LD_{50} \text{ of Ad5 on tumor}) / (LD_{50} \text{ of oncolytic virus on tumor})) / ((LD_{50} \text{ of Ad5 on normal}) / (LD_{50} \text{ of oncolytic virus on normal}))$. Relative LD50 (OV802/oncolytic vector) values of 1 indicate the vector has the identical cell killing as wild type OV802. SILD values above "1" indicate tumor cell selectivity.

Ex Vivo Primary Tumor Culture Assay: In a typical study, samples of primary human tumors or normal tissue were collected from patients who had undergone surgical resection for colorectal cancer or pancreatic cancer. Tissue samples were quickly placed in an ice-cold solution appropriate culture medium, dissected on ice and homogeneous tissue slices were selected. Cube-sized pieces of each tissue sample

were prepared, rinsed and placed in medium suitable for viral infection. A known amount of wild type adenovirus or recombinant viral vector was added. After two hours, the culture medium was replaced with fresh medium supplemented with insulin and hydrocortisone. The tissue samples were placed on Millicell membrane culture inserts (0.45 μ M pore size; Millipore, Billerica, MA) placed inside each well of a 6-well plate and incubated under appropriate conditions. The tissue samples were examined by immunohistochemical staining using an adenoviral-specific antibody at 24 hours post-infection using standard procedures or harvested at day 5 post-infection for LD₅₀ determination of progeny viruses after a specified number of freeze-thaw cycles. The SILD was then calculated from normal tissue and tumor tissue as described above. SILD values of greater than "1" indicate tumor cell selectivity.

Ex: Vivo Primary Tissue Culture Assay

Example 8: A Viral Production and Growth Kinetics Assay For Oncolytic Adenoviruses

In a typical study, cells are plated on 6-well plates at 5E5 cells/well one day prior virus infection. Oncolytic virus and OV802 are infected at MOI of 2 (pfu/cell) for a selected amount of time, for example 72 hours. Cell lysates are harvested and plaque titered on 293 cells. For a growth kinetics study, infected cells are harvested and titrated at corresponding time points.

Example 9: In vivo antitumor efficacy

A549 xenograft model: In a typical study, six-to-eight weeks old athymic BALB/C nu/nu mice are used. Generally, 5×10^6 A549 cells in 0.1ml are mixed with 0.1ml of Matrigel and injected into each animal subcutaneously. Nude mice bearing subcutaneous A549 tumors are injected intratumorally at a dosage of about 4×10^8 viral particles/mm³. Virus may be injected on more than one day depending upon the

regimen. Tumor volume and body weight are monitored and statistically analyzed using Prism GraphPad.

253JB-V xenograft model: In a typical study, six-to-eight weeks old athymic BALB/C nu/nu mice are used. About 2E6 253J B-V cells in 0.1ml are mixed with
5 0.1ml of Matrigel and injected to each animal subcutaneously. Nude mice bearing subcutaneous 253J B-V tumors are injected intratumorally at a dosage of about 4×10^8 viral particles/mm³ for four consecutive days beginning at day 20. Tumor volume and body weight are monitored and statistically analyzed using Prism GraphPad.

Example 10: CG5757 - Selective Expression Of E1

10 The following example demonstrates that the specific expression of the E1A and E1B genes in CG5757 results in viral replication and cell killing.

Using the procedure described in Example 6, the normal lung fibroblast cell WI-38 (ATCC #CCL-75) (Rb+) was compared to the isogenic WI-38/VA-13 cell (ATCC #CCL-75.1). WI-38/VA-13 was transformed by SV40 and therefore has a
15 defective Rb-pathway. During infection, wild type OV802 showed no selectivity for relative E1 expression in WI-38/VA-13 cells. In comparison, CG5757 only expressed detectable E1 in Rb defective VA13 cells. This selective E1 expression indicates that CG5757 is under the transcriptional control of the E2F-1 promoter. The control of the E1B 55K gene in CG5757 was also demonstrated to be cancer-specific in that it is
20 only detected in infected cancer cells but not normal HRE cells. Therefore, the observed expression of both E1A and E1B genes following infection with CG5757 is tumor-specific and the viral replication is selective for cancer cells that are Rb-defective and telomerase positive. To demonstrate the tumor selectivity of CG5757, a broad panel of cancer cell lines (Hep3B, LoVo, A549, 253J B-V, Panc-1, and Hela)
25 and normal cells (HRE and WI-38) were analyzed for E1A expression after viral

infection. CG5757 induced E1A expression was only detected in the cancer cells. CG5757 clearly demonstrated tumor-selective expression of both the E1A and E1B genes, the products of which play an essential role in activating other adenoviral genes.

5 Example 11: CG5757 In Vitro Tumor Selective Cytotoxicity

The cytopathic effect induced following infection in different cells with wild type adenovirus OV802 and the tumor selective CG5757 virus was compared by both microscopy and crystal violet staining. OV802 showed no selectivity for killing of cancer versus normal cells, whereas infection with CG5757 resulted in cytolysis
10 (killing) specific to cancer cells. CG5757 showed 1,000 to 10,000 fold less infectivity relative to OV802 in normal cells.

An in vitro MTT assay was also used to quantitatively compare the cytotoxicity of CG5757 and wild type Ad5 (OV802) on different cells. In the tested cancer cells, CG5757 performed similarly to wild type Ad5 in a dose response study
15 (Figure 3). At a selected MOI of, for example, 1 pfu/cell (Figure 3 panels C and D), CG5757 exhibited the same killing kinetics as wild type Ad5 in Hep3B cells. Furthermore, the cytotoxicity data were analyzed for Sigmoidal dose response curve fit using GraphPad software and the LD50 determined. The comparison of the LD50 value between wild type adenovirus, OV802, and the oncolytic vector, CG5757,
20 indicated the strength of viral cytotoxicity and can be used to normalize the transduction efficiency of different cells. Table 3 lists the SILD of CG5757, that is the LD50 of CG5757 relative to Ad5 on primary cells (HRE, PrEC, HMEC, HMVEC-L, BSM, Lung FB, SAEC, WI-38 and tumor cells (Hep3B, A549, LoVo, SW480, LNCap, Panc-1, Hela and 253J B-V) using the SILD formula. SILD values above "1"
25 indicate tumor cell selectivity.

Table 3. Selectivity index of CG5757

	HRE	PrEC	HMEC	HMVEC-L	BSM	Lung FB	SAEC	WI-38
Hep3B	1676.74	6957.82	85.50	154.79	75.76	2.27	62.99	75.76
A549	84.14	349.13	4.29	7.77	3.80	0.11	3.16	3.80
LoVo	340.18	1411.60	17.35	31.40	15.37	0.46	12.78	15.37
SW480	571.53	2371.64	29.14	52.76	25.83	0.77	21.47	25.82
LNCaP	115.65	479.88	5.90	10.68	5.23	0.16	4.34	5.23
Panc-1	118.55	491.93	6.05	10.94	5.36	0.16	4.45	5.36
Hela	1731.10	7183.40	88.28	159.81	78.22	2.34	65.04	78.22
253J B-V	278.22	1154.52	14.19	25.68	12.57	0.38	10.45	12.57

- Based on the selectivity index derived from the cytotoxicity assay using a variety of different cells, 57 of 64 comparisons of the relative cytotoxicity of CG5757 and wild type Ad5 between tumor and normal cell lines yielded a selectivity index greater than 1, demonstrating strong tumor selectivity.

Tissue-specific viral expression and production of CG5757 in clinically derived tissue samples were determined using the *ex vivo* primary tumor culture model of Example 7.

- Following the procedure in Example 7, tissue samples isolated from primary human tumors or normal tissue and quickly placed in an ice-cold solution of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) and 100 μ g/ml penicillin and streptomycin. Tissue samples were dissected on ice, homogeneous tissue slices were selected and 1 mm³ cubes of each tissue sample were prepared, rinsed and placed in 6-well plates in IMDM supplemented with 5% FCS. An aliquot of 1 x 10⁹ pfu of CG5757 or Ad5 (also known as OV802) was added to the

appropriate tissue-containing well. After two hours, the culture medium was replaced with fresh IMDM supplemented with 5% FCS, 10 μ M insulin and 1 μ M hydrocortisone. The tissue samples were placed on Millicell membrane culture inserts (0.45 μ M pore size; Millipore, Billerica, MA) placed inside each well of a 6-well plate and incubated at 37°C in a 5% CO₂ atmosphere. At day 5 post-infection, the medium and infected tissue were collected and subjected to three freeze-thaw cycles. The lysates were titrated on 293 cells and the TCID₅₀ titers were calculated for each sample.

The results demonstrate that an average more than 10-fold more CG5757 was detected in tumor colon tissues than in adjacent normal tissues. For instance, the primary tumor isolated from one patient had about 10,000-time higher infectious CG5757 present in tumor tissue than the normal tissue. In contrast, there was no significant difference in virus production between tumor or normal tissues following wild-type Ad5 infection. To evaluate tumor selectivity of virus production, the TCID₅₀ of CG5757 was compared to that of Ad5 within the same pair of tissues to control for differences in infectivity and viral production to generate the Selectivity Index, as calculated above. The Selectivity Index obtained from each of the various primary tumors ranged from about 4 to greater than 3000, with the majority of samples having a Selectivity Index of greater than 75.

Tissue-specific viral expression was also demonstrated by immunohistochemical staining. Following the procedure in Example 7, a portion of the primary tumor samples infected with CG5757 or wild type Ad5 was analyzed 24 hours post-infection for expression of Ad E1A using an anti-E1A antibody. The results show that E1A expression was detectable only in colon tumor tissues but not in the normal tissues such as colon, pancreas and spleen. These results demonstrate that

the E2F-1 promoter is active in and selective for primary colon tumors. Preferential viral production of CG5757 in tumor tissue was observed in tissues samples collected from multiple patients.

These results from the ex vivo experiments using clinical primary tissues
 5 provide further evidence of the high tumor selectivity of CG5757.

Results Ex Vivo Model

Example 12: OV945 and OV947 In Vitro Tumor Selective Cytotoxicity

10 Using an MTT assay, the in vitro tumor selective cytotoxicity of OV945 and OV947 was evaluated on Hep3B and BSM cells. The results are shown in Figure 8. The Cytopathic Potency Index is the relative LD50 by ratio of LD50 of OV802 over LD50 of the test viral vector on a selected cell (Johnson et al., 2002, Cancer Cell, 1:325-337). As listed in Table 4, both of the test viruses had essentially the same
 15 potency on the tumor cell line Hep3B as OV802. On the other hand, the sample viruses were both about 333-fold less potent on the normal cell line, BSM.

Table 4. Cytopathic Potency Index

	OV802	OV945	OV947
Hep3B cells	1	1	1
BSM cells	1	0.003	0.003

Example 13: In Vitro Tumor Selective Cytotoxicity for CG5757, OV945 and
 20 OV947

The Crystal Violet Assay was also used to evaluate CV945 and CV947. OV802 was used as a control. Tumor cells, Hep3B, A549 and LoVo, were infected at

an MOI of 1 pfu/cell. Normal cells, Lung FB, IMR90 and WI-38, were infected at an MOI of 10 pfu/cell. All infections were performed in triplicate. Six days after infection with CV945, CV947 or OV802, for each of the tumor cell lines, most if not all of the cells displayed CPE. In contrast, the normal cells infected CV945 and CV947 displayed little, if any, CPE, but normal cells infected with OV802 displayed complete CPE. Cells were then stained with crystal violet. The tumor cells infected with any of three viruses displayed very little staining. Whereas, the normal cells displayed staining over the majority of the surface except for those infected with CV802, which displayed very little staining.

The in vitro cytotoxicity MTT assay was used to compare oncolytic vectors to the wild type OV802. Table 5 summarizes the LD50 values following MTT assay in different cells.

Table 5. Comparison of LD50 (pfu/cell) on different cells

	Hep3B	A549	LoVo	SW480	LNCaP	Panc-1	HRE	PrEC
OV802	0.0003	0.0008	0.0124	0.0146	0.0018	0.0030	0.0006	0.0184
CG5757	0.0011	0.0577	0.2275	0.1601	0.0948	0.1590	3.6946	477.0356
OV945	0.0017	0.0135	0.0412	1.0753	0.8655	0.0806	0.9781	0.5058
OV947	0.0311	0.1923	0.4903	1.1968	0.7299	1.3509	1.3710	16.9500

Example 14: The tumor selective production of CG5757

Using the procedure described in Example 8, in vitro selective replication of CG5757 was compared to wild type virus by burst size (viral production) and growth kinetics assay in a panel of tumor and normal cells (Figures 4 and 5). In the burst size assay, all cells were infected at an MOI of 2 pfu/cell for 72 hrs and harvested for plaque titration on 293 cells (Figure 4). For the growth kinetic study, infected cells

were harvested and titrated at corresponding time points (Figure 5). CG5757 showed selective replication in the tumor cells at a level comparable to wild type OV802. On the normal cells, the burst size was about 1,000 to 100,000 fold lower than that of wild type OV802. Further viral growth curves on Hep3B and HRE cells (Figure 5) also indicate that CG5757 replicates similarly to wild type OV802 in the cancer cells, but not in the normal cells.

The same analysis was performed for the viruses OV945 and OV947. On the normal cells, the burst size was about 1,000 to 100,000 fold lower for OV945, OV947 and CG5757 than that of wild type OV802 (Figure 6).

Example 15: The tumor selective production of OV945 and OV947

Using the procedure described in Example 8, in vitro selective replication of OV945 and OV947 was compared to wild type virus by burst size (viral production) on various cells, including HBL 100 cells. See Figure 7.

Example 16: CG5757 and OV945 - *In vivo* antitumor efficacy

In vivo antitumor efficacy of CG5757 and OV945 was determined using the procedures described in Example 9. CG5757 was tested in an A549 xenograft model. CG5757 was injected on days 20, 21, 22, and 23 after tumor implantation. Figure 13 shows that the treatment group (n=10) demonstrated significant anti-tumor efficacy relative to the control group (n=10) ($p < 0.01$ by Dunnett's method of ANOVA with PBS/glycerol as the control group). With a total viral dose as high as 1.6×10^{13} viral particles per kilogram of body weight, there was no body weight change as compared to the control group.

The A549 model was also used to demonstrate the antitumor efficacy of OV945. After intratumoral administration of OV945, the treatment group demonstrated a statistically significant inhibitory effect on the tumor growth as

compared to the control group (Figure 2). The body weight also had no significant change indicating the toxicity after intratumoral injection is minimized.

Similar studies were done using nude mice bearing subcutaneous 253J B-V tumors. CG5757 or OV945 was administered by intratumoral injection of 4×10^8 vp/mm³ at various dosing regimens.

For CG5757, three different regimens were carried out and tumors were injected on the following days after tumor implantation: Regimen 1: 20, 21, 22, 23, 24; Regimen 2: 20, 24, 27, 30; Regimen 3: 20, 27, 34, 41. All of the CG5757 treatment groups in different regimens had significant tumor regression compared to the controls ($p < 0.01$ by Dunnette's method of ANOVA with PBS/glycerol as the control group). See Figure 10. Four weeks after treatment, the average volume of 253J B-V tumors in animals treated with five consecutive daily intratumoral injections of CG5757 (4×10^8 particles/mm³ of tumor) decreased to 72% of baseline while the control group had an increase to 944% of baseline. Furthermore, about 50% of animals in each treatment group had complete regression of the 253J B-V tumor xenografts, suggesting strong antitumor efficacy of CG5757.

For OV945 tested in the 253J B-V tumor model, the results are shown in Figure 11.

Example 17: CG5757: *In vivo* toxicity assay

The *in vivo* toxicity profile of CG5757 was compared to wild-type Ad5 and the replication defective Addl312 in immune-deficient SCID mice following intravenous administration. SCID mice were injected with a single intravenous dose of 8.5×10^{11} vp/kg of CG5757, wild-type Ad5, replication defective Addl312, or PBS supplemented with 10% glycerol (vehicle control) and animals were closely monitored. No morbidity was seen in the vehicle, Addl312 or CG5757 treatment

groups.

Individual body weights were collected over a 28-day period and compared among the different treatment groups. Relative to study day 1, all treatment groups had no body weight gain between day 3 and day 8. Ad5 treated animals lost weight steadily between day 1 and day 5, at which time they became moribund and were terminated. In contrast, the CG5757 treatment group gained body weight after the initial loss between day 3 to 8, and the difference in mean body weight change for this group was not significant compared to the vehicle- or Addl312 treated groups.

In addition, selected clinical chemistry parameters were monitored on study day 2, 4, 14 and 28. During the course of the study, observed levels of creatine kinase (CK) were not different between any treatment group at any time point, indicating that there was no overt toxicity in skeletal muscle, cardiac muscle, brain, or kidney (Figure 4B and data not shown). Furthermore, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was evident on day 2 and showed no difference between any treatment group; however, significantly elevated AST and ALT levels ($p < 0.05$) were seen in mice treated with wt Ad5 (OV802) on day 4 compared to control or Addl312 treatment groups. The CG5757 treatment group had an elevation of both ALT and AST on study day 4, however, there was no significant difference relative to vehicle or Addl312 treated mice. By day 14, all serum parameters returned to normal. Taken together, these observations demonstrate that CG5757 is less toxic than wild-type Ad5 and, therefore, may have potential as a therapeutic agent.

Example 18: *In vitro* combination of CG5757 and chemotherapeutic agents

Potential synergism between existing chemotherapeutic agents and the replication competent vectors of the present invention was investigated. Co-

application of CG5757 with a variety of known chemotherapeutic agents was evaluated using an *in vitro* cell assay. A variety of known chemotherapeutic agents were tested in combination with CG5757 for their *in vitro* cytotoxic effect on Hep3B and LNCaP cells. The concentration of each chemotherapeutic agent was optimized to a particular concentration for each cell type that did not generate extensive cytotoxic effect from the agent alone. Under such conditions, particular chemotherapeutic agents exhibited a non-additive effect in combination with CG5757. Of the agents tested, doxorubicin and Taxol showed potential synergistic cytotoxicity with CG5757. Doxorubicin at 5 ng/ml did not generate cytotoxicity on Hep3B cells, whereas CG5757 at an MOI of 0.005 (pfu/cell) destroyed only 30% of the cells by day 7; however, co-administration of doxorubicin and CG5757 was cytotoxic to about 85% of the cells 7 days after treatment. A similar result was observed when Taxol (1ng/ml) and CG5757 (moi 0.1) were co-administered to LNCaP cells.

These results demonstrate that the recombinant viral vectors of the present invention can provide a synergistic benefit to existing chemotherapeutic compositions and approaches.

BRIEF DESCRIPTION OF THE SEQUENCES

The following is a description of the sequences relied upon the description provided herein.

5 SEQ ID NO:1 is a 270 bp fragment containing sequences from the human E2F promoter.

SEQ ID NO:2 is a 239 bp fragment containing sequences from the human telomerase (*TERT*) promoter.

10 SEQ ID NO:3 is a 245 bp fragment containing sequences from the human *TERT* promoter.

SEQ ID NO:4 is a 2751 bp fragment containing sequences from the adenoviral vector CG5757.

SEQ ID NO:5 is a 4022 bp fragment containing the predicted sequences for the adenoviral vector OV947.

15 SEQ ID NO:6 is a 3207 bp fragment containing sequences from the adenoviral vector OV945.

SEQ ID NO:7 is a 4304 bp fragment containing the predicted sequences for the adenoviral vector OV1025.

SEQ ID NO:8 and 9 are primer sequences for amplifying an E2F-1 promoter.

20 SEQ ID NO:10 and 11 are primer sequences for amplifying a TERT promoter.

SEQ ID NO:12: is a portion of E1b that is deleted in various adenoviral vectors of the invention including CG5757 and OV947.

SEQ ID NO: 13: is a polyadenylation consensus DNA sequence.

SEQ ID NO: 14: is a polyadenylation consensus RNA sequence.

WHAT IS CLAIMED IS:

1. 1. A recombinant viral vector comprising an adenoviral nucleic acid backbone,
wherein said nucleic acid backbone comprises in sequential order: a left ITR,
5 an adenoviral packaging signal, an E2F responsive promoter operatively linked
to an E1a coding region, a transcriptional element operatively linked to an E1b
coding region and a right ITR wherein said transcriptional element is selected
from the group consisting of a TERT promoter and an IRES.
- 10 2. The recombinant viral vector of Claim 1, wherein the E2F responsive promoter
is the human E2F-1 promoter.
3. The recombinant viral vector of Claim 1, wherein the human E2F-1 promoter
comprises SEQ ID NO:1 .
- 15 4. The recombinant viral vector of Claim 1, wherein the transcriptional element
operatively linked to an E1b coding region is an IRES.
5. The recombinant viral vector of Claim 1, wherein the transcriptional element
20 operatively linked to an E1b coding region is a TERT promoter.
6. The recombinant viral vector of Claim 5, wherein the TERT promoter is a
human TERT promoter.

7. The recombinant viral vector of Claim 5, wherein the TERT promoter comprises SEQ ID NO:2 or SEQ ID NO:3.
8. The recombinant viral vector of Claim 1, wherein at least one of the adenoviral elements selected from the group consisting of the left ITR, the adenoviral packaging signal, the E1a coding region, the E1b coding region, and the right ITR are derived from adenovirus serotype 5 (Ad5).
9. The recombinant viral vector of Claim 1, further comprising a mutation or deletion in an E3 coding region.
10. The recombinant viral vector of Claim 1, wherein at least one of the E3 coding regions have been deleted from said backbone.
11. The recombinant viral vector of Claim 1, wherein the E3 region in said backbone codes for at least one of the native E3 proteins.
12. The recombinant vector of Claim 11, wherein said E3 coding region is selected from the group consisting of E3-6.7, KDa, gp19KDa, 11.6KDa (ADP), 10.4 KDa (RID α), 14.5 KDa (RID β), and E3-14.7Kda.
13. The recombinant viral vector of Claim 1, wherein the E3 region in said backbone codes for all of the native E3 proteins.

14. The recombinant viral vector of Claim 1, further comprising a mutation or deletion in the E1b gene.
15. The recombinant viral vector of Claim 14, wherein said mutation or deletion results in the loss of the active 19kD protein expressed by the wild-type E1b gene.
16. The recombinant viral vector of Claim 15, wherein said deletion in the E1b gene comprises SEQ ID NO:12.
17. The recombinant viral vector of Claim 1, comprising SEQ ID NO: 4 or SEQ ID NO:5.
18. The recombinant viral vector of Claim 1, comprising SEQ ID NO:6.
19. The recombinant viral vector of Claim 1, comprising SEQ ID NO:7.
20. The recombinant viral vector of Claim 1, further comprising a transgene.
21. A pharmaceutical composition comprising the adenoviral vector of Claim 17 and a pharmaceutically acceptable carrier.
22. A pharmaceutical composition comprising the adenoviral vector of Claim 18 and a pharmaceutically acceptable carrier.

23. A pharmaceutical composition comprising the adenoviral vector of Claim 19
and a pharmaceutically acceptable carrier.
24. A method of treating a host organism having a neoplastic condition,
5 comprising administering a therapeutically effective amount of the composition
of Claim 17 to said host organism.
25. A method of treating a host organism having a neoplastic condition,
comprising administering a therapeutically effective amount of the composition
10 of Claim 18 to said host organism.
26. A method of treating a host organism having a neoplastic condition,
comprising administering a therapeutically effective amount of the composition
15 of Claim 19 to said host organism.
27. The method of treatment of Claim 24, wherein the neoplastic condition is lung,
breast, prostate, or colon cancer.
28. The method of treatment of Claim 25, wherein the neoplastic condition is lung,
20 breast, prostate, or colon cancer.
29. The method of treatment of Claim 26, wherein the neoplastic condition is lung,
breast, prostate, or colon cancer.

30. The method of treatment of Claim 27, wherein administration is by intratumoral injection.

5 31. The method of treatment of Claim 28, wherein administration is by intratumoral injection.

32. The method of treatment of Claim 29, wherein administration is by intratumoral injection.

10 33. A method of selective cytolysis of a cancer cell, comprising contacting a cell population with an effective amount of an adenoviral vector comprising an adenoviral nucleic acid backbone, wherein said nucleic acid backbone comprises in sequential order: a left ITR, an adenoviral packaging signal, an E2F responsive promoter operatively linked to an E1a coding region, a
15 transcriptional element operatively linked to an E1b coding region and a right ITR wherein said transcriptional element is selected from the group consisting of a TERT promoter an E2F responsive promoter and an IRES, under conditions where the adenoviral vector infects the cells of the cell population resulting in selectively cytolysis of cancer cells within said cell population.

20

34. The method of Claim 33, wherein the E2F responsive promoter operatively linked to an E1a coding region is the human E2F-1 promoter.

25 35. The method of Claim 34 wherein the human E2F-1 promoter comprises SEQ ID NO:1 .

36. The method of Claim 33, wherein the transcriptional element operatively linked to an E1b coding region is an IRES.
- 5 37. The method of Claim 33, wherein the transcriptional element operatively linked to an E1b coding region is a TERT promoter.
38. The method of Claim 37, wherein the TERT promoter is a human TERT promoter.
- 10 39. The method of Claim 38, wherein the TERT promoter comprises SEQ ID NO:2 or SEQ ID NO:3.
40. The method of Claim 33, wherein the transcriptional element operatively linked to an E1b coding region is the E2F responsive promoter.
- 15 41. The method of Claim 40, wherein the E2F responsive promoter is a human E2F-1 promoter.
42. The method of Claim 41 wherein the human E2F-1 promoter comprises SEQ ID NO:1 .
- 20 43. The method of Claim 33, further comprising a mutation or deletion in an E3 coding region.

44. The method of Claim 36, wherein at least one of the E3 coding regions have been deleted from said backbone.
45. The method of Claim 36, wherein the E3 region in said backbone codes for at least one native E3 protein.
46. The recombinant viral vector of Claim 36, further comprising a mutation or deletion in the E1b gene.
47. The method of Claim 33, wherein said mutation or deletion results in the loss of the active 19kD protein expressed by the wild-type E1b gene.
48. The method of Claim 33, wherein said deletion in the E1b gene comprises SEQ ID NO: 12
49. The method of Claim 33, wherein said adenoviral vector comprises SEQ ID NO:4 or SEQ ID NO:5.
50. The method of Claim 33, wherein said adenoviral vector comprises SEQ ID NO:6.
51. The method of Claim 33, wherein said adenoviral vector comprises SEQ ID NO:7.

Figure 1

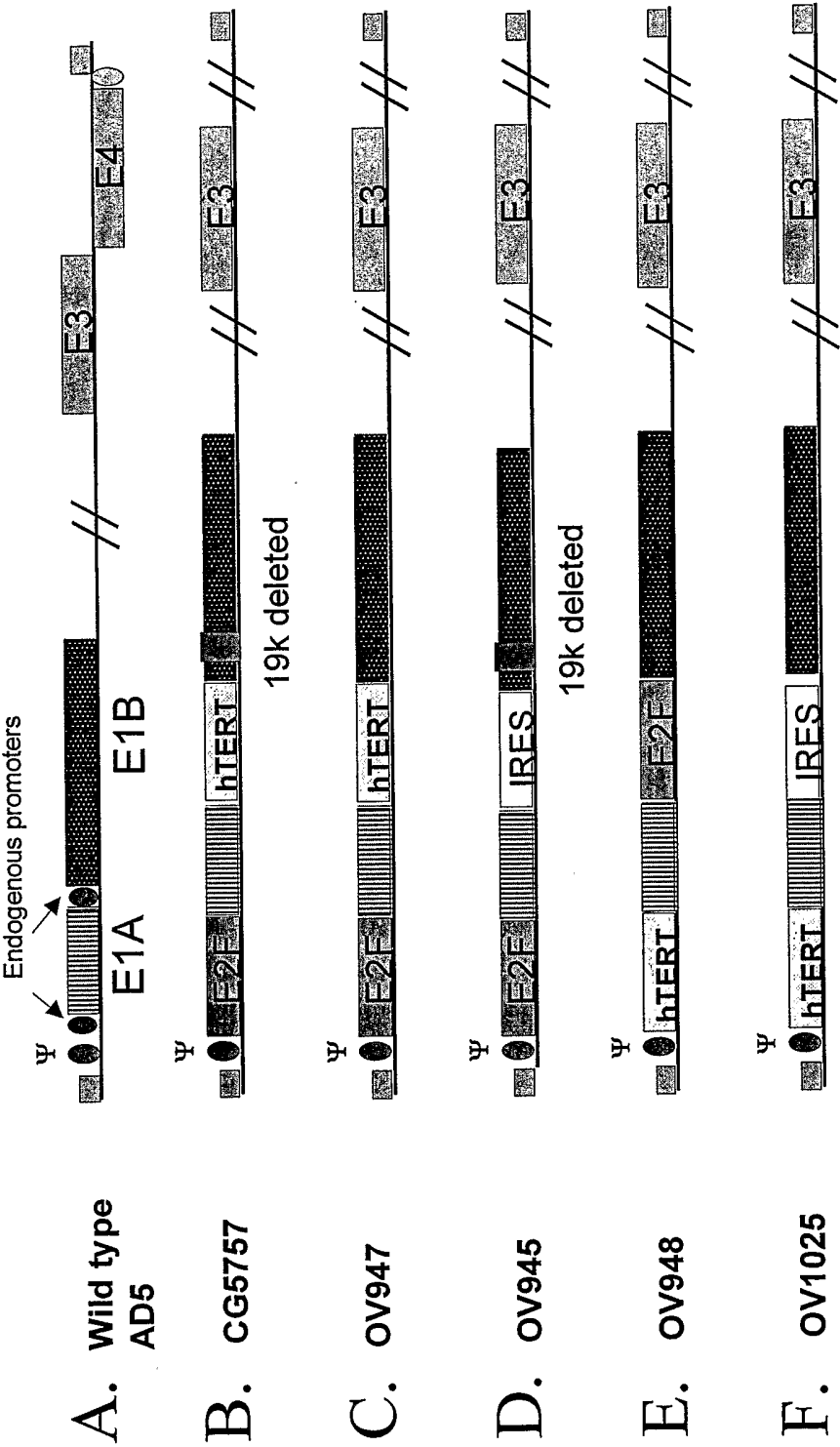


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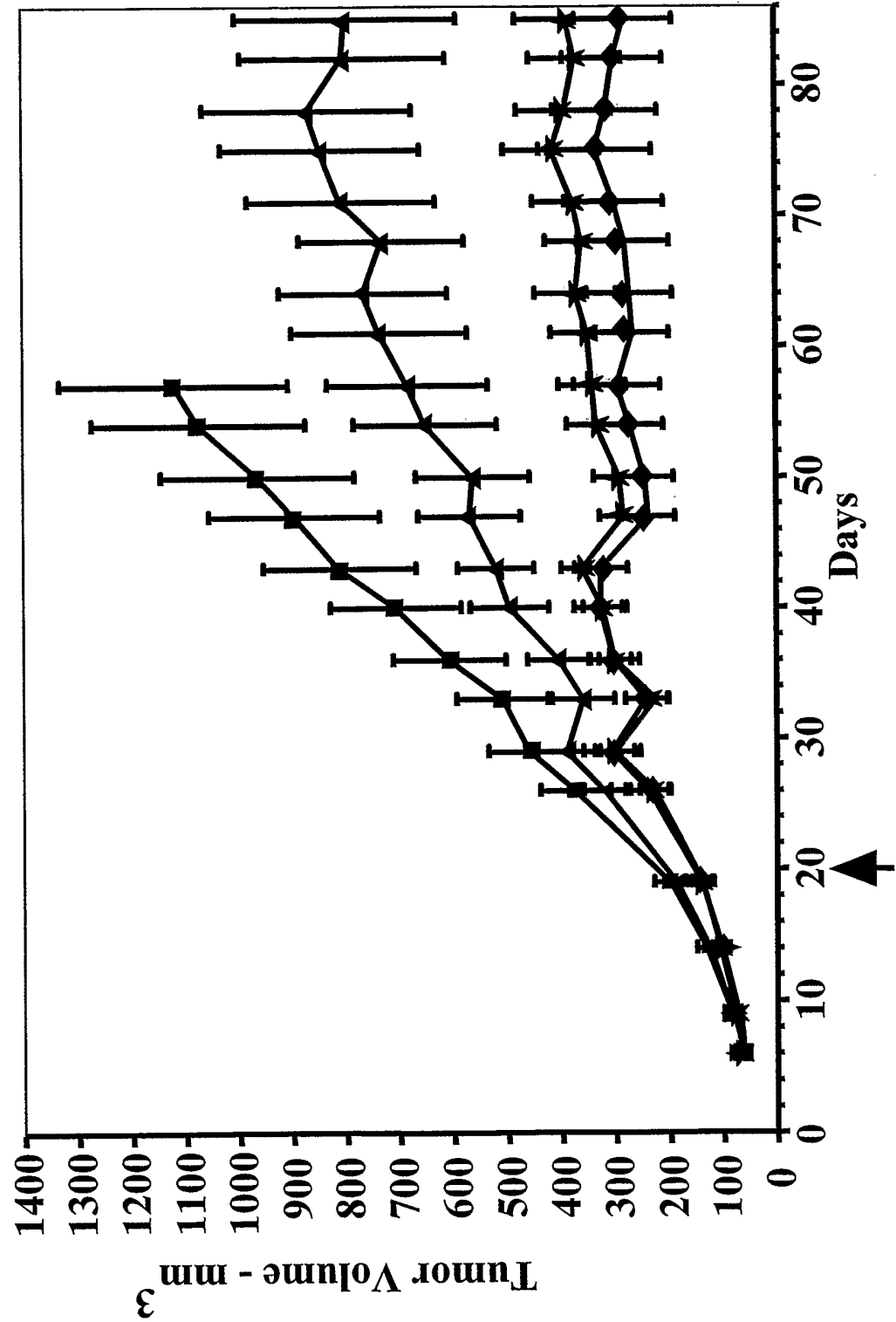


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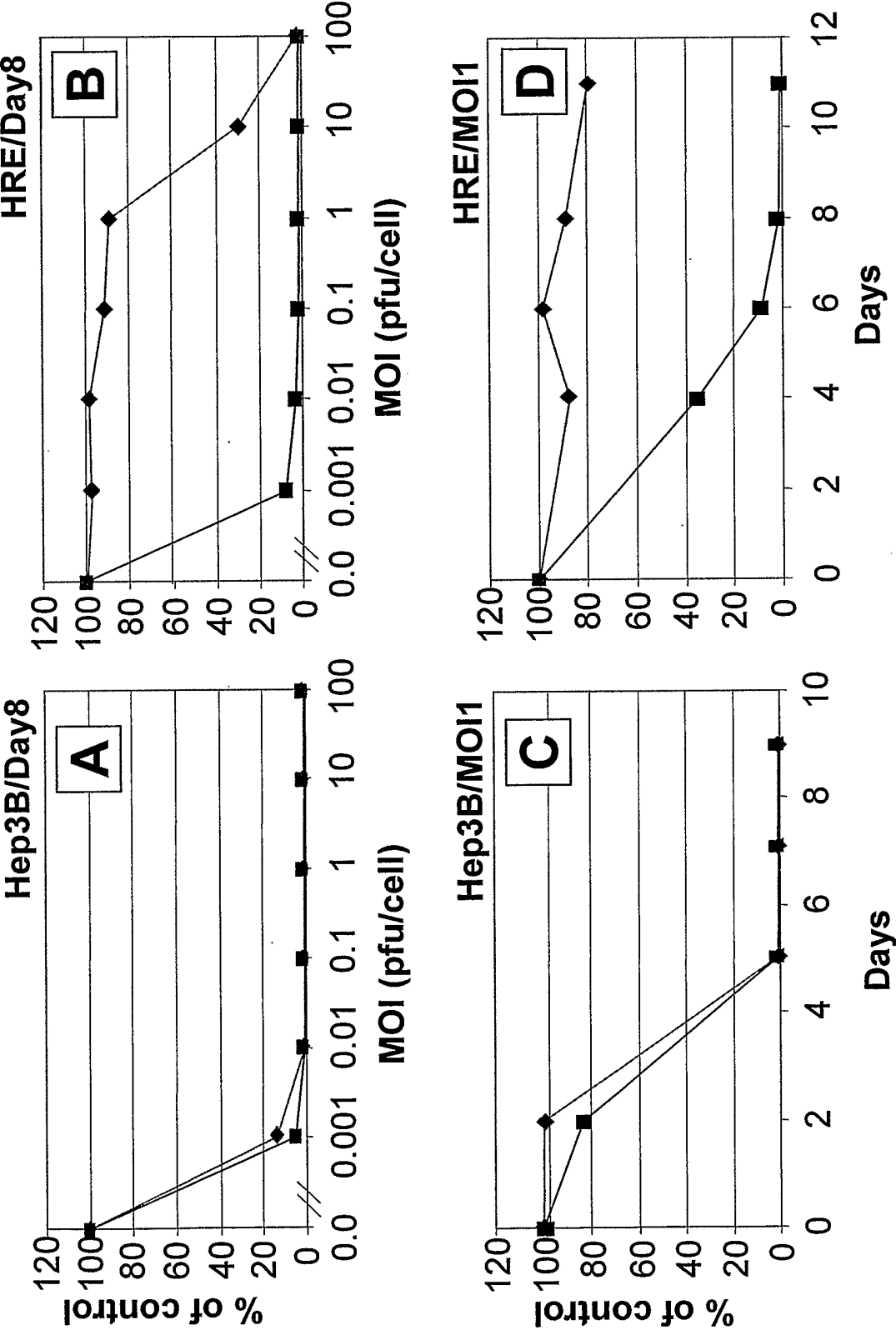


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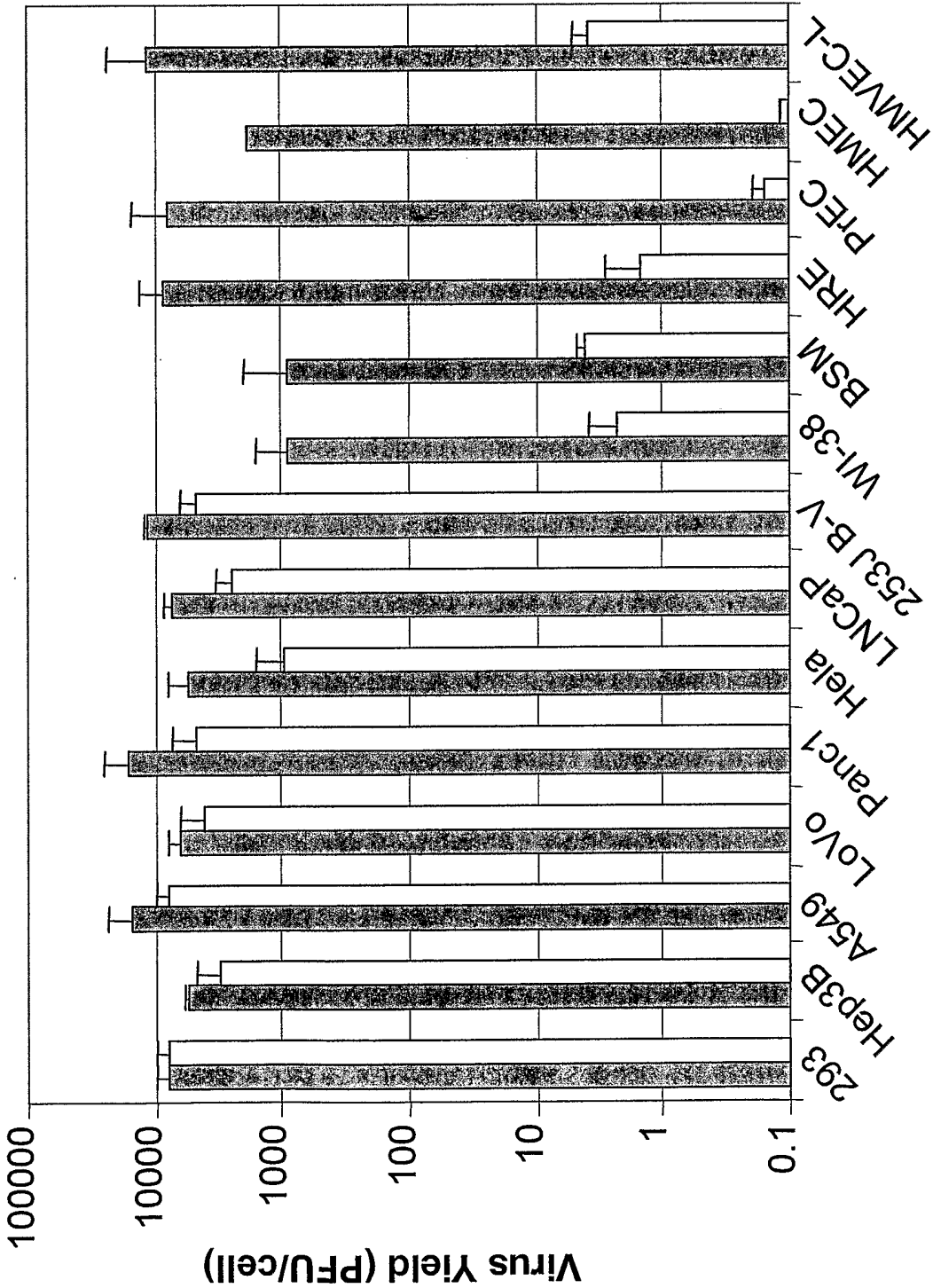


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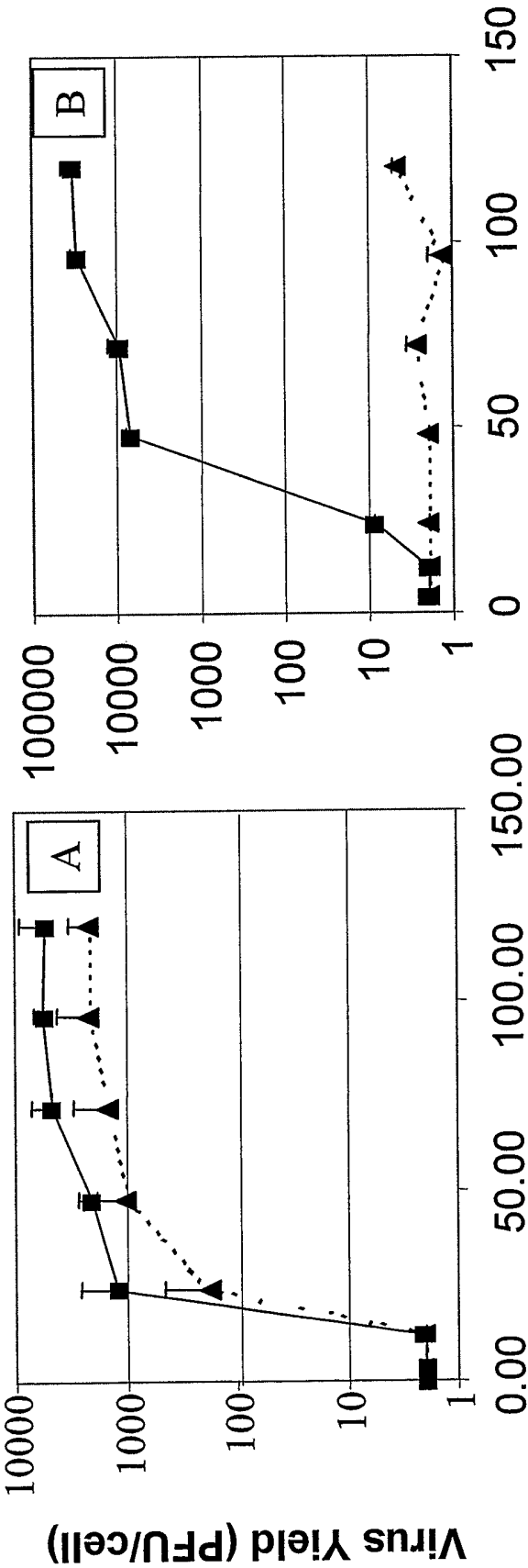


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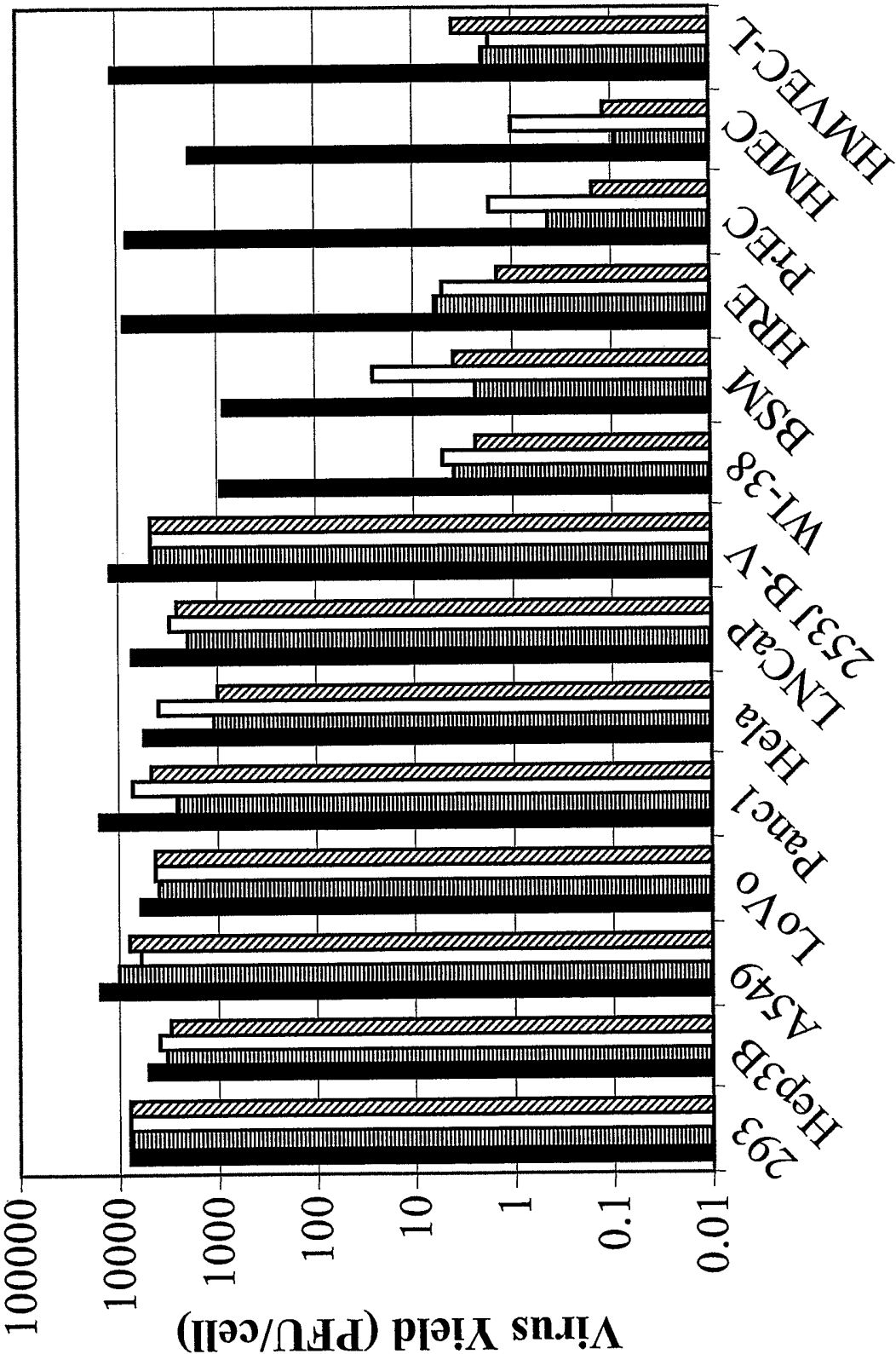


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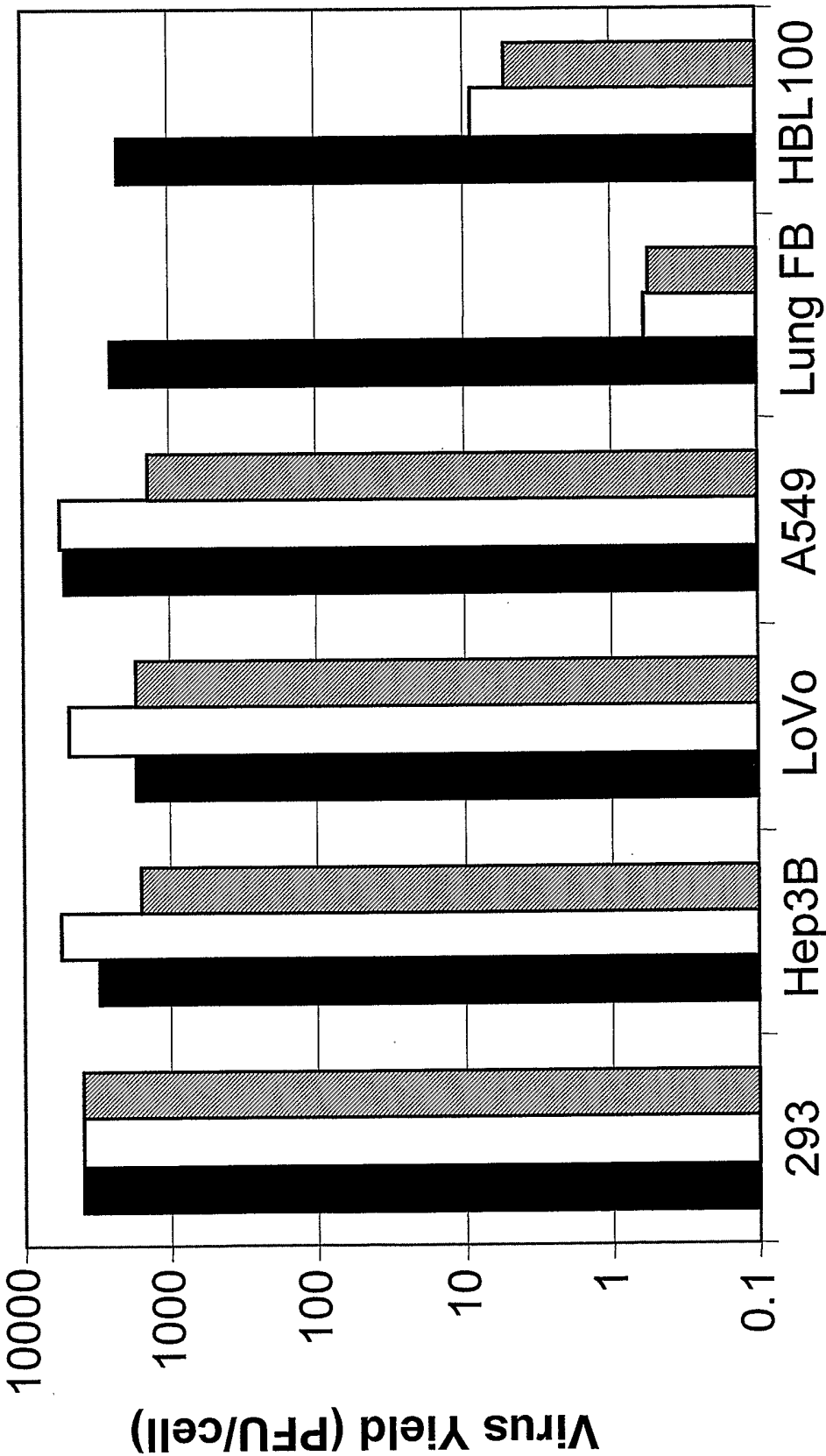


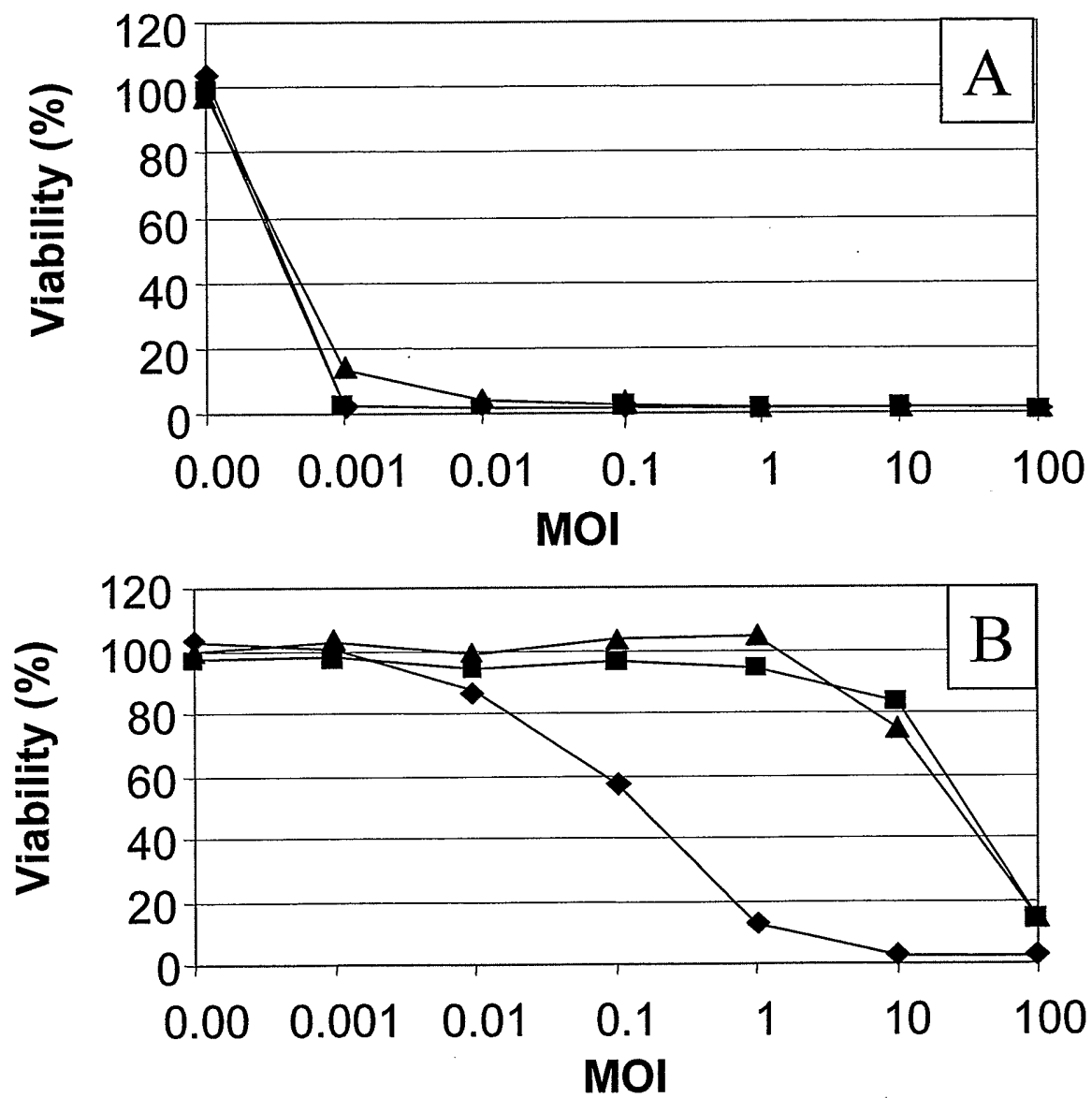
Figure 8

Figure 9

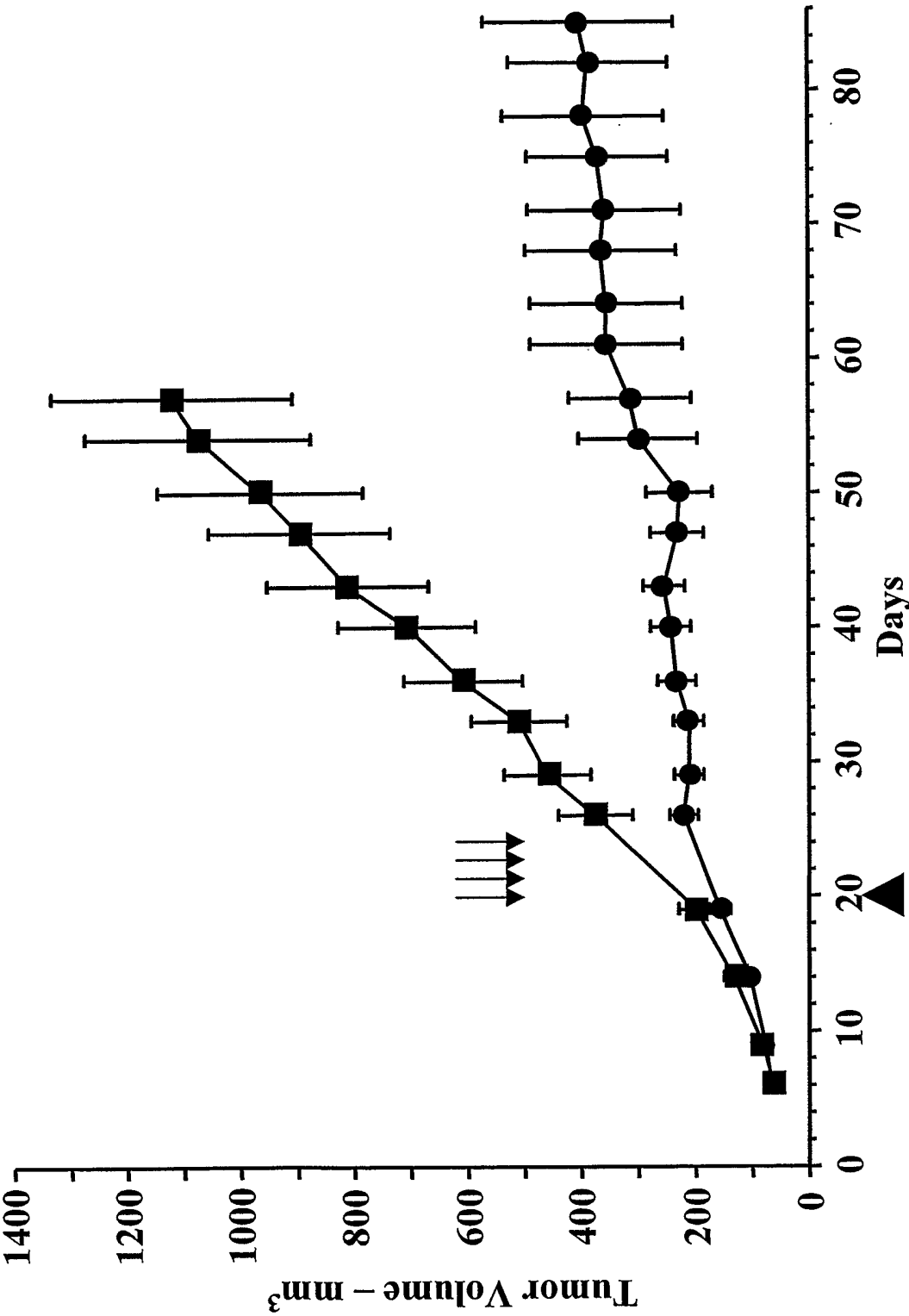


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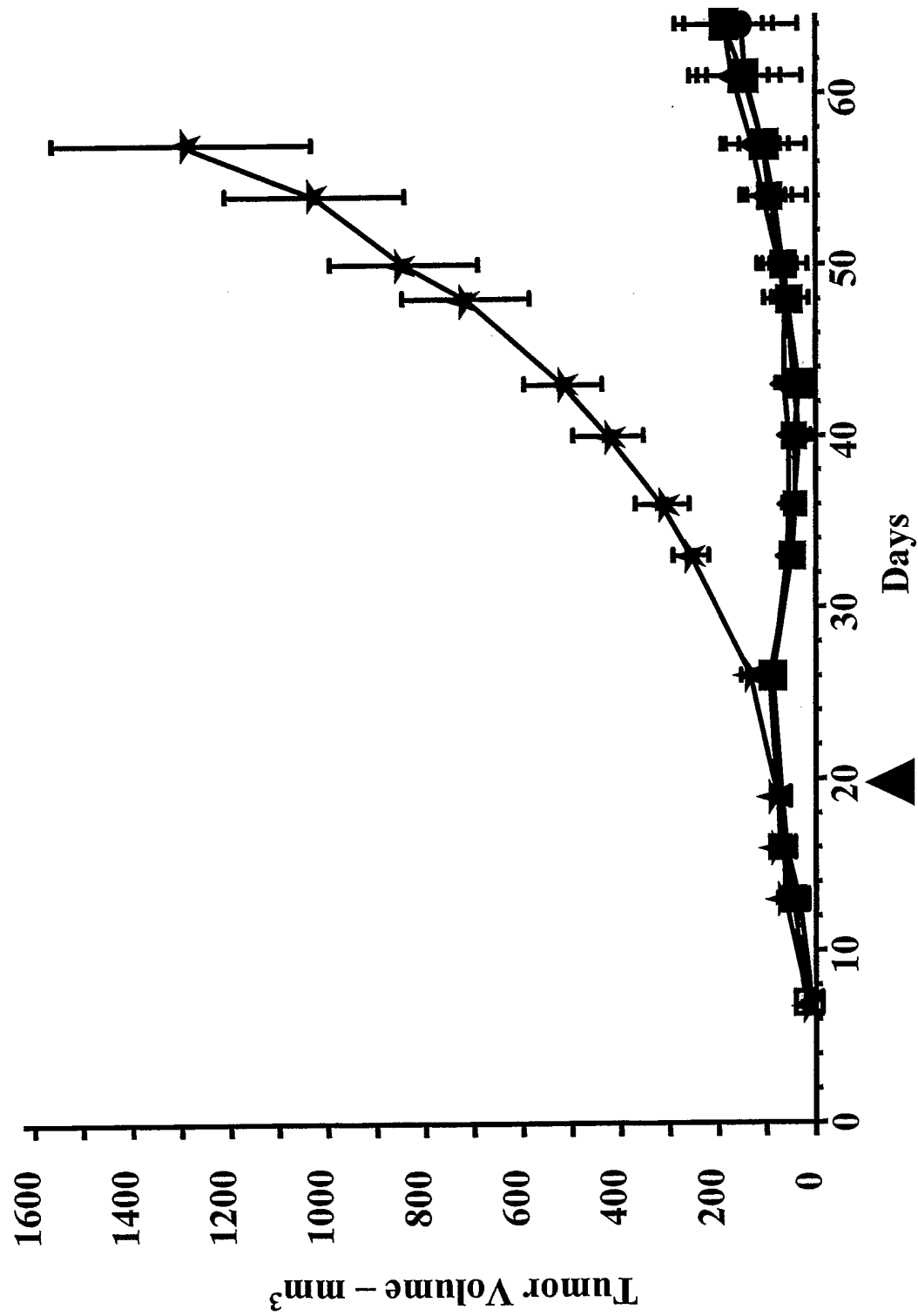
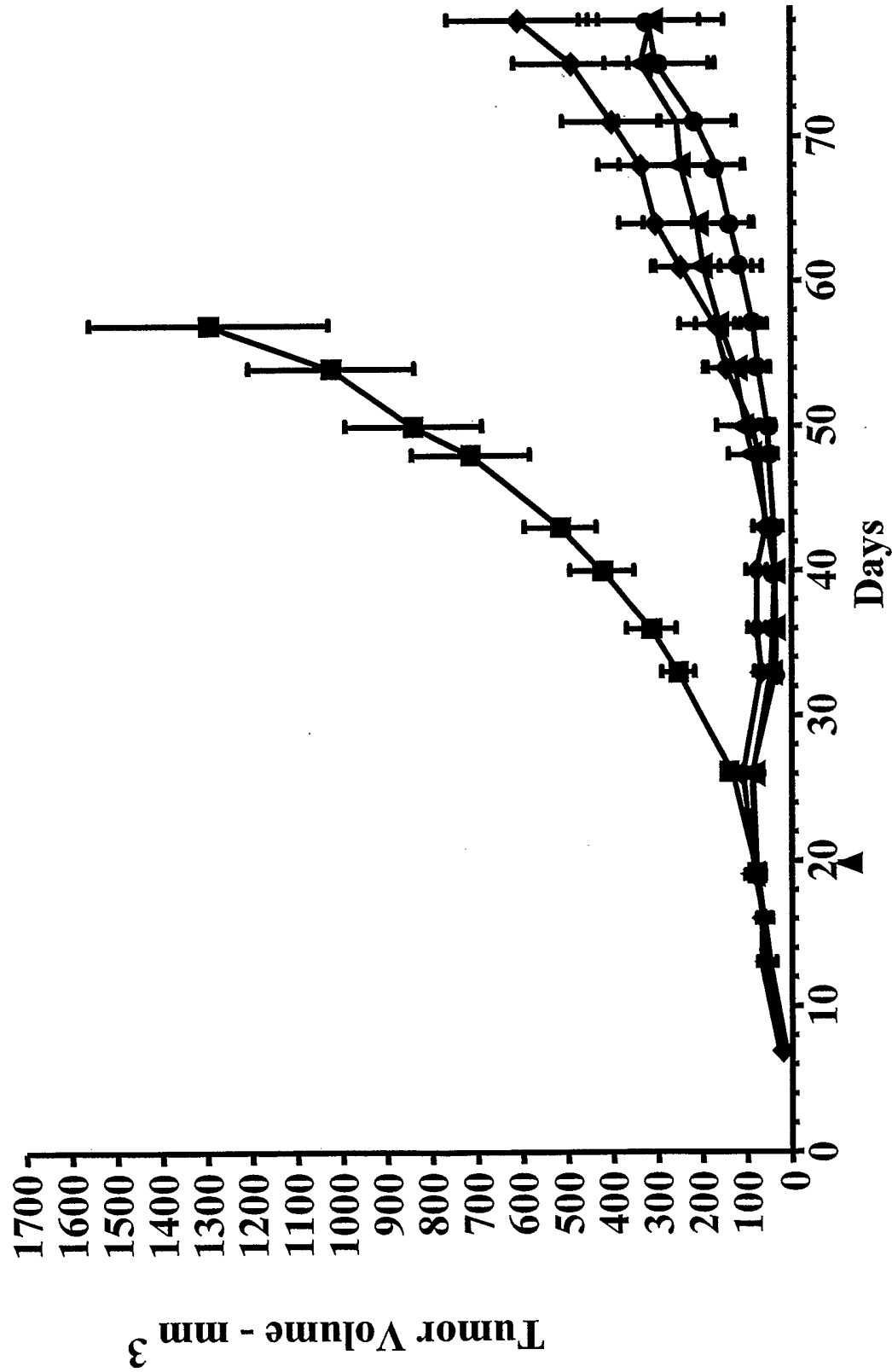


Figure 11



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