Method for producing recombinant adeno-associated viral particles

The current invention is in the field of gene therapy with adeno-associated viral particles. In more detail, herein is reported a method for the improved production of recombinant adeno-associated viral particles comprising a therapeutic payload.

Background of the Invention

Gene therapy is opening unprecedented opportunities for novel therapeutic approaches. Based on the concept of rescuing function mutations by co-expressing the correct gene, to allow biological functions to be restored, it requires the use of specific delivery to ensure the proper delivery of therapeutic genes to the intended site of action. In this context, recombinant adeno-associated virus particles (rAAVp) are most widely used for therapeutic gene delivery.

Bioprocessing of viruses is complex and requires systematic and coordinated steps both in upstream processing as well as downstream processing. However, the use of traditional cultivation processes for the production of therapeutic virus particles does not support effective commercial manufacturing strategies. This is even more pronounced by the large size of therapeutic viral particles as compared to therapeutic biomolecules, such as, e.g., antibodies. Additionally, viral particles are much more complex.

The manufacturing process of therapeutic recombinant adeno-associated virus particles requires the insertion of the therapeutic transgene into the recombinant AAV capsid shell (full viral articles, i.e. recombinant viral particles comprising an encapsidated nucleic acid).

However, a certain percentage of the recombinantly produced viral particles that does not contain the desired transgene might also be produced, as well as partly filled recombinant viral particles.

Additionally, in view of the high doses required for this therapeutic approach, high titer production processes are required.

WO 99/11764 reported methods and compositions for producing high titer, substantially purified preparations of recombinant adeno-associated virus (AAV) that can be used as vectors for gene delivery.

EP 4 239 063 reported Adeno-associated virus producer cell lines in which DNA fragmentation caused by programmed cell death is inhibited, uses of cell lines in which DNA fragmentation caused by programmed cell death is inhibited for the production of AAV, related methods of producing AAV, and methods of producing AAV, comprising the step of exposing the cells in which AAV is produced to an inhibitor of DNA fragmentation caused by programmed cell death during the AAV production phase.

Summary of the Invention

The current invention is based, at least in part, on the finding that the productivity of mammalian cells producing a recombinant adeno-associated viral particle (rAAVp) can be increased when the cultivation is carried out in the presence of certain production enhancers, i.e. the titer of the produced rAAVps is increased compared to a cultivation in the absence of said enhancers.

The current invention is based, at least in part, on the finding that the fraction of full rAAVps obtained from a cultivation of mammalian cells producing said rAAVp can be increased when the cultivation is carried out in the presence of certain production enhancers, i.e. the fraction of full rAAVps is increased compared to a cultivation in the absence of said enhancers.

The invention is based, at least in part, on the finding that capsid titer, genomic titer and full-to-empty ratio of rAAVps produced with a mammalian cell is influenced by the addition of production enhancers. It has been found that by the addition of production enhancers the viral genomic titer and the full-to-empty ratio can be increased.

The invention is based, at least in part, on the finding that not all compounds are useful as production enhancers as it has been found that for the majority of compounds the resulting increased full-to-empty ratio resulted concomitantly not in higher capsid titers and vice versa.

The invention is based, at least in part, on the finding that production enhancers used in DDR (DNA damage response signaling) need to induce DNA damage response without actually damaging the replicating viral vector DNA, as it would otherwise negatively impact viral genome integrity. Thus, compounds such as, e.g., cisplatin

or DNA integrating substances, have been found to be not useful in a method according to the current invention.

The invention is based, at least in part, on the finding that production enhancers used in to enhance dNTP synthesis by co-factors or other building blocks (such as folinic acid) or upregulating dNTP synthesis or feeder pathways, for example by DDR signaling.

The invention is based, at least in part, on the finding to delay cell death by addition of anti-apoptotic compounds, such as inhibitors of caspases.

It has to be pointed out that some compounds may have effects in more than one group, i.e., e.g., BN82002 is a CD25 phosphatase inhibitor and thereby has an effect on cell cycle as well as κB kinase via NF-κB or AICAR is an AMPK activator as well as inducing oxidative phosphorylation. Therefore, come compounds a mentioned with respect to more than one effect associated with the current invention and therefore serve as example of such an individual as well as combined effect.

The current invention comprises at least the following embodiments:

1. A method for producing a recombinant adeno-associated viral particle (rAAVp) preparation comprising the steps of

   - cultivating a mammalian cell comprising

     (a) a gene encoding a non-adeno-associated-virus gene, which is operably linked to two AAV inverted terminal repeats (ITRs) (i.e. the non-adeno-associated virus gene is interspaced between the two AAV ITRs),

     (b) a gene encoding the AAV Cap protein VP1 and/or VP3,

     (c) a gene encoding the AAV Rep protein Rep78 or Rep68 and/or a gene encoding the AAV Rep protein Rep52 or Rep40,

     (d) a gene encoding the adenoviral helper function E4orf6, and

     (e) a gene encoding the adenoviral helper function E2A,

   - recovering the rAAVp from the cell and/or the cultivation medium,

- optionally purifying the rAAVp,

and thereby producing the rAAVp preparation,

whereby the cultivating of the mammalian cell is in the presence of at least one compound selected from one of the following groups of compounds

(i) the caspase inhibitor Q-VD-OPh and IDN6556,

(ii) DNA damage response activation inducing substances, including ATM/ATR inducing substances,

(iii) antiviral response inhibitors,

(iv) AMPK activators,

(v) nutrient starvation induction, hypoxia, toxins,

(vi) oxidative phosphorylation agents,

(vii) inhibitors of κB kinase,

(viii) induction genotoxic and oxidative stress,

(ix) dNTP synthesis enhancers, including co-factors and building blocks,

(x) cell cycle modulators.

2. A method for producing a recombinant adeno-associated viral particle (rAAVp) preparation comprising the steps of

- cultivating a mammalian cell comprising

(a) a gene encoding a non-adeno-associated-virus gene, which is operably linked to two AAV inverted terminal repeats (ITRs) (i.e. the non-adeno-associated virus gene is interspaced between the two AAV ITRs),

(b) a gene encoding the AAV Cap protein VP1 and/or VP3,

(c) a gene encoding the AAV Rep protein Rep78 or Rep68 and/or a gene encoding the AAV Rep protein Rep52 or Rep40,

(d) a gene encoding the adenoviral helper function E4orf6, and

(e) a gene encoding the adenoviral helper function E2A,

- recovering the rAAVp from the cell and/or the cultivation medium,

- optionally purifying the rAAVp,

and thereby producing the rAAVp preparation,

whereby the cultivating of the mammalian cell is in the presence of at least two compounds each selected from one of the following groups of compounds

(i) the caspase inhibitor Q-VD-OPh and IDN6556,

(ii) DNA damage response activation inducing substances, including ATM/ATR inducing substances,

(iii) antiviral response inhibitors,

(iv) AMPK activators,

(v) nutrient starvation induction, hypoxia, toxins,

(vi) oxidative phosphorylation agents,

(vii) inhibitors of κB kinase

(viii) induction genotoxic and oxidative stress,

(ix) dNTP synthesis enhancers,

(x) cell cycle modulators.

3. The method according to embodiment 1, wherein the cultivating of the mammalian cell is in the presence of one compound selected from one of the groups of compounds.

4. The method according to any one of embodiments 1 to 3, wherein the group of DNA damage response activation inducing substances comprises MK-1775, AZD7762 and Resveratrol.

5. The method according to any one of embodiments 1 to 4, wherein the group of antiviral response inhibitors comprises BAY 11-7082, BN82002 and C176.

6. The method according to any one of embodiments 1 to 5, wherein the group of AMPK activators comprises AICAR.

7. The method according to any one of embodiments 1 to 6, wherein the group of oxidative phosphorylation agents comprises AICAR.

8. The method according to any one of embodiments 1 to 7, wherein the group of inhibitors of κB kinase comprises BAY 11-7082 and BN82002.

9. The method according to any one of embodiments 1 to 8, wherein the group of compounds inducing genotoxic and oxidative stress comprises resveratrol.

10. The method according to any one of embodiments 1 to 9, wherein the group of dNTP synthesis enhancers comprises folinic acid.

11. The method according to any one of embodiments 1 to 10, wherein the group of cell cycle modulators comprises BN82002.

13. The method according to any one of embodiments 1 to 11,

whereby the cultivating of the mammalian cell is in the presence of at least one compound selected from the following compounds

(i) Q-VD-OPh,

(ii) MK-1775,

(iii) AZD7762,

(iv) AICAR,

(v) BAY 11-7082,

(vi) folinic acid,

(vii) resveratrol,

(viii) C176,

(ix) BN82002,

(x) Y-27632,

(xi) IDN-6556.

14. The method according to any one of embodiments 1 to 13, wherein the cultivating is at least in the presence of IDN-6556 at a starting concentration of about 10-50 µM or in the presence of Q-VD-OPh at a starting concentration of about 20-50 µM.

15. The method according to any one of embodiments 1 to 14, wherein the cultivating is at least in the presence of MK-1775 at a starting concentration of about 0.05-0.3 µM or/and in the presence of AZD7762 at a starting concentration of about 0.01-0.1 µM.

16. The method according to any one of embodiments 1 to 15, wherein the cultivating is at least in the presence of AICAR at a starting concentration of about 500-1000 µM.

17. The method according to any one of embodiments 1 to 16, wherein the cultivating is at least in the presence of BAY 11-7082 at a starting concentration of about 2.5 µM.

18. The method according to any one of embodiments 1 to 17, wherein the cultivating is at least in the presence of folinic acid at a starting concentration of about 5 µM.

19. The method according to any one of embodiments 1 to 18, wherein the cultivating is at least in the presence of resveratrol at a starting concentration of about 10-50 µM.

20. The method according to any one of embodiments 1 to 19, wherein the cultivating is at least in the presence of C176 at a starting concentration of about 0.5-2 µM.

21. The method according to any one of embodiments 1 to 20, wherein the cultivating is at least in the presence of BN82002 at a starting concentration of about 50 µM.

22. The method according to any one of embodiments 1 to 21, wherein the cultivating is at least in the presence of Y-27632 at a starting concentration of about 30 µM.

23. The method according to any one of embodiments 1 to 22, whereby the cultivating of the mammalian cell is in the presence of at least one compound selected from the following compounds

(i) Q-VD-OPh at a starting concentration of about 20-50 µM,

(ii) MK-1775 at a starting concentration of about 0.05-0.3 µM,

(iii) AZD7762 at a starting concentration of about 0.01-0.1 µM,

(iv) AICAR at a starting concentration of about 500-1000 µM,

(v) BAY 11-7082 at a starting concentration of about 2.5 µM,

(vi) folinic acid at a starting concentration of about 5 µM,

(vii) resveratrol at a starting concentration of about 10-50 µM,

(viii) C176 at a starting concentration of about 0.5-2 µM,

(ix) BN82002 at a starting concentration of about 50 µM,

(x) Y-27632 at a starting concentration of about 30 µM, and

(xi) IDN6556 at a starting concentration of about 10-50 µM.

24. The method according to any one of embodiments 1 to 22, whereby the cultivating of the mammalian cell is in the presence of

(i) Q-VD-OPh at a starting concentration of about 20-50 µM, or/and

(ii) MK-1775 at a starting concentration of about 0.05-0.3 µM, or/and

(iii) AZD7762 at a starting concentration of about 0.01-0.1 µM, or/and

(iv) AICAR at a starting concentration of about 500-1000 µM, or/and

(v) BAY 11-7082 at a starting concentration of about 2.5 µM, or/and

(vi) folinic acid at a starting concentration of about 5 µM, or/and

(vii) resveratrol at a starting concentration of about 10-50 µM, or/and

(viii) C176 at a starting concentration of about 0.5-2 µM, or/and

(ix) BN82002 at a starting concentration of about 50 µM, or/and

(x) Y-27632 at a starting concentration of about 30 µM, or/and

(xi) IDN6556 at a starting concentration of about 10-50 µM.

25. The method according to any one of embodiments 1 to 24, wherein the cultivating is at least in the presence of IDN6556 at a starting concentration of about 20 µM.

26. The method according to any one of embodiments 1 to 25, wherein the cell comprises at least one gene encoding the AAV Cap protein VP1, VP2 and VP3.

27. The method according to any one of embodiments 1 to 26, wherein each gene of (b) to (d) is in an expression cassette.

28. The method according to any one of embodiments 1 to 27, wherein the culturing of the mammalian cell is under conditions suitable for producing the rAAVp.

29. The method according to any one of the embodiments 1 to 28, wherein

- one or both of the genes encoding the adenoviral helper functions is/are gene under control of a derepressible promoter, or/and
- the gene encoding the AAV Cap protein or/and the gene encoding the AAV Rep protein is/are under control of a derepressible promoter, and
- the cell further comprises a nucleic acid molecule encoding a repressor element of the first and the second derepressible promoters.

30. The method according to any one of embodiments 1 to 28, wherein

- one or both of the genes encoding the adenoviral helper functions is/are gene under control of an inducible promoter, or/and
- the gene encoding the AAV Cap protein or/and the gene encoding the AAV Rep protein is/are under control of an inducible promoter.

31. The method according to embodiment 30, wherein the inducible promoter is a doxycycline-inducible promoter.

32. The method according to any one of embodiments 30 to 31, wherein the cultivating step comprises a first phase and a second phase, whereby the cell is cultivated in the first phase in the absence of the substance and at the start of the second phase the inducible promoter is induced and the compound is added.

33. The method according to embodiment 32, wherein the first phase is 1, 2 or 3 days.

34. The method according to any one of embodiments 30 to 31, wherein the cultivating step comprises a first phase, a second phase and a third phase, whereby the cell is cultivated in the first and the second phase in the absence of the substance, at the start of the second phase the inducible promoter is induced and at the start of the third phase the compound is added.

35. The method according to embodiment 34, wherein the first phase is 1, 2 or 3 days and the second phase is 1 or 2 days.

36. The method according to any one of embodiments 1 to 35, wherein the cultivating is for a total of 7 to 10 days.

37. The method according to any one of embodiments 1 to 36, wherein the cultivating is at a temperature of about 36°C to 42 °C.

38. The method according to any one of embodiments 1 to 37, wherein the cultivating is in a first temperature phase at a temperature of about 36 to 38 °C and in a second temperature phase at a temperature of about 38°C to about 42°C.

39. The method according to any one of embodiments 32 to 38, wherein the first phase and the first temperature phase are the same phase and the second or the second and third phase and the second temperature phase are the same phases.

40. The method according to any one of embodiments 32 to 38, wherein the first and the second phase and the first temperature phase are the same phases and the third phase and the second temperature phase are the same phase.

41. The method according to any one of embodiments 1 to 40, wherein the mammalian cell is a HEK or CHO cell.

42. The method according to any one of embodiments 1 to 41, wherein the cultivating is at a pH value in the range of and including pH 7.4 to pH 7.6.

43. The method according to any one of embodiments 1 to 42, wherein the rAAVp is a therapeutic rAAVp.

44. The method according to any one of embodiments 1 to 43, wherein the rAAVp is for transfer of a nucleic acid that is transcribed into a polypeptide with therapeutic effect into target cells.

45. The method according to any one of embodiments 1 to 44, wherein the rAAVp is for transfer of a nucleic acid that has therapeutic effect into target cells.

46. The method according to any one of embodiments 1 to 45, wherein the rAAVp preparation comprises recombinant adeno-associated viral particles (rAAVps) comprising at least one coding nucleic acid sequence interspaced between two adeno-associated viral inverted terminal repeats.

47. The method according to any one of embodiments 1 to 46, wherein the rAAV in the rAAVp is derived from a wild-type AAV particle selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV 2i8, AAV rh.74, AAV rh.10 and AAV 7m8, as well as variants thereof.

48. The method according to any one of embodiments 1 to 47, wherein the rAAV is of the serotype AAV2 or a variant thereof.

49. The method according to any one of embodiments 1 to 48, wherein the rAAVp comprises one or two ITR sequences of a wild-type AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or AAV12.

50. The method according to any one of embodiments 1 to 49, wherein the cultivating encompasses the inoculation of the bioreactor and the harvest of the rAAVp.

51. The method according to any one of embodiments 1 to 50, wherein the cultivating starts with the inoculation of the bioreactor.

52. The method according to any one of embodiments 1 to 51, wherein one or more or all of the expression cassettes for the non-adeno-associated viral gene, which is interspaced between two AAV ITRs, for the adeno-associated virus rep gene, for the adeno-associated virus cap gene, for the adeno-associated virus E2A gene, for the adeno-associated virus E4orf6 and for the adeno-associated virus VA RNA gene are introduced into the mammalian cell after the inoculation of the bioreactor.

53. The method according to any one of embodiments 1 to 52, wherein one or more or all of the expression cassettes for the non-adeno-associated viral gene, which is interspaced between two AAV ITRs, for the adeno-associated virus rep gene, for the adeno-associated virus cap gene, for the adeno-associated virus E2A gene, for the adeno-associated virus E4orf6 and optionally for the

adeno-associated virus VA RNA gene are introduced into the mammalian cell or the HEK cell after the inoculation of the bioreactor, whereby up to three plasmids are co-transfected into the mammalian cell, whereby one of the plasmids comprises the expression cassette for the non-adeno-associated viral gene, which is interspaced between two AAV ITRs, one of the plasmids comprises the expression cassettes for the rep and cap genes and one of the plasmids comprises the expression cassettes for the adenoviral E2A, E4orf6 and VA RNA genes.

54. The method according to any one of embodiments 1 to 53, wherein expression of one or more or all of the non-adeno-associated viral gene, which is interspaced between two AAV ITRs, the adeno-associated virus rep gene, the adeno-associated virus cap gene, the adeno-associated virus E2A gene, the adeno-associated virus E4orf6 and optionally the adeno-associated virus VA RNA gene is induced after the inoculation of the bioreactor.

55. The method according to any one of embodiments 53 to 54, wherein the introduction is about 16 to 32 hours after the inoculation of the bioreactor.

56. The method according to embodiment 55, wherein the induction is about 16 to 32 hours after the inoculation of the bioreactor.

57. The method according to any one of embodiments 52 to 56, wherein the introduction or induction is about 24 hours after the inoculation of the bioreactor.

58. The method according to any one of embodiments 32 to 57, wherein the introduction is at the end of the first phase.

59. The method according to any one of embodiments 1 to 58, wherein the method further comprises after the cultivating step the step of isolating the rAAVp from the cells and/or the cultivation medium and optionally purifying the rAAVp.

60. The method according to embodiment 59, wherein the purifying is by one or more column chromatography steps and/or a CsCl or iodixanol gradient centrifugation step.

61. The method according to any one of embodiments 59 or 60, wherein the first chromatography step is an affinity chromatography step.

62. The method according to any one of embodiments 59 to 61, wherein the purifying is by a sequence of chromatography steps wherein the first is an affinity chromatography, followed by an anion exchange chromatography or a cation exchange chromatography, and an optional size exclusion chromatography.

63. A pharmaceutical composition comprising the rAAVp preparation obtained with a method according to any one of embodiments 1 to 62.

64. A pharmaceutical composition comprising the rAAVp preparation obtained with a method according to any one of embodiments 1 to 62 and a pharmaceutically acceptable excipient.

65. Use of the method according to any one of embodiments 1 to 62 for increasing the yield of a recombinantly produced rAAVp.

66. Use of the method according to any one of embodiments 1 to 62 for increasing the percentage of full particles in a rAAVp.

67. Use of at least one of the compounds

(i) the caspase inhibitor Q-VD-OPh and IDN6556,

(ii) DNA damage response activation inducing substances, including ATM/ATR inducing substances,

(iii) antiviral response inhibitors,

(iv) AMPK activators,

(v) nutrient starvation induction, hypoxia, toxins,

(vi) oxidative phosphorylation agents,

(vii) inhibitors of κB kinase,

(viii) induction genotoxic and oxidative stress,

(ix) dNTP synthesis enhancers,

(x) cell cycle modulators

for increasing the yield of a recombinantly produced rAAVp.

68. Use of at least one of the compounds

(i) the caspase inhibitor Q-VD-OPh and IDN6556,

(ii) DNA damage response activation inducing substances, including ATM/ATR inducing substances,

(iii) antiviral response inhibitors,

(iv) AMPK activators,

(v) nutrient starvation induction, hypoxia, toxins,

(vi) oxidative phosphorylation agents,

(vii) inhibitors of κB kinase,

(viii) induction genotoxic and oxidative stress,

(ix) dNTP synthesis enhancers,

(x) cell cycle modulators.

69. The use according to any one of embodiments 67 to 68,

whereby the at least one compound is selected from the following compounds

(i) Q-VD-OPh,

(ii) MK-1775,

(iii) AZD7762,

(iv) AICAR,

(v) BAY 11-7082,

(vi) folinic acid,

(vii) resveratrol,

(viii) C176,

(ix) BN82002,

(x) Y-27632,

(xi) IDN-6556.

70. The use according to any one of embodiments 67 to 68,

whereby the at least one compound is selected from the following compounds

(i) Q-VD-OPh at a starting concentration of about 20-50 µM, or/and

(ii) MK-1775 at a starting concentration of about 0.05-0.3 µM, or/and

(iii) AZD7762 at a starting concentration of about 0.01-0.1 µM, or/and

(iv) AICAR at a starting concentration of about 500-1000 µM, or/and

(v) BAY 11-7082 at a starting concentration of about 2.5 µM, or/and

(vi) folinic acid at a starting concentration of about 5 µM, or/and

(vii) resveratrol at a starting concentration of about 10-50 µM, or/and

(viii) C176 at a starting concentration of about 0.5-2 µM, or/and

(ix) BN82002 at a starting concentration of about 50 µM, or/and

(x) Y-27632 at a starting concentration of about 30 µM, or/and

(xi) IDN6556 at a starting concentration of about 10-50 µM.

71. The use to embodiment 70, wherein the compound is IDN6556 at a starting concentration of about 20 µM.

In addition to the various embodiments depicted and claimed, the disclosed subject matter is also directed to other embodiments having other combinations of the features disclosed and claimed herein. As such, the particular features presented herein can be combined with each other in other manners within the scope of the disclosed subject matter such that the disclosed subject matter includes any suitable combination of the features disclosed herein. The foregoing description of specific embodiments of the disclosed subject matter has been presented for purposes of

illustration and description. It is not intended to be exhaustive or to limit the disclosed subject matter to those embodiments disclosed.

Description of the Figures

Figure 1 Best results with respect to viral genomic titer and showing also the corresponding capsid titer and full-to-empty ratio.

Detailed Description of the Invention

The current invention is directed to a method for producing a recombinant adeno-associated viral particle (rAAVp) preparation comprising the steps of

- cultivating a mammalian cell comprising

(a) a gene encoding a non-adeno-associated-virus gene, which is operably linked to two AAV inverted terminal repeats (ITRs) (i.e. the non-adeno-associated virus gene is interspaced between the two AAV ITRs),

(b) a gene encoding the AAV Cap protein VP1 and/or VP3,

(c) a gene encoding the AAV Rep protein Rep78 or Rep68 and/or a gene encoding the AAV Rep protein Rep52 or Rep40,

(d) a gene encoding the adenoviral helper function E4orf6, and

(e) a gene encoding the adenoviral helper function E2A,

- recovering the rAAVp from the cell and/or the cultivation medium,

- optionally purifying the rAAVp,

and thereby producing the rAAVp preparation,

whereby the cultivating of the mammalian cell is in the presence of at least one compound selected from one of the following groups of compounds

(i) the caspase inhibitor Q-VD-OPh and IDN6556,

(ii) DNA damage response activation inducing substances, including ATM/ATR inducing substances,

(iii) antiviral response inhibitors,

(iv) AMPK activators,

(v) nutrient starvation induction, hypoxia, toxins,

(vi) oxidative phosphorylation agents,

(vii) inhibitors of κB kinase,

(viii) induction genotoxic and oxidative stress,

(ix) dNTP synthesis enhancers,

(x) cell cycle modulators.

DEFINITIONS

Unless otherwise defined herein, scientific and technical terms used in connection with the current invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

Useful methods and techniques for carrying out the current invention are described in e.g. Ausubel, F.M. (ed.), Current Protocols in Molecular Biology, Volumes I to III (1997); Glover, N.D., and Hames, B.D., ed., DNA Cloning: A Practical Approach, Volumes I and II (1985), Oxford University Press; Freshney, R.I. (ed.), Animal Cell Culture – a practical approach, IRL Press Limited (1986); Watson, J.D., et al., Recombinant DNA, Second Edition, CHSL Press (1992); Winnacker, E.L., From Genes to Clones; N.Y., VCH Publishers (1987); Celis, J., ed., Cell Biology, Second Edition, Academic Press (1998); Freshney, R.I., Culture of Animal Cells: A Manual of Basic Technique, second edition, Alan R. Liss, Inc., N.Y. (1987). The content of which is incorporated herein by reference,

The use of recombinant DNA technology enables the generation of derivatives of a nucleic acid. Such derivatives can, for example, be modified in individual or several nucleotide positions by substitution, alteration, exchange, deletion or insertion. The modification or derivatization can, for example, be carried out by means of site directed mutagenesis. Such modifications can easily be carried out by a person skilled in the art (see e.g. Sambrook, J., et al., Molecular Cloning: A laboratory manual (1999) Cold Spring Harbor Laboratory Press, New York, USA; Hames,

B.D., and Higgins, S.G., Nucleic acid hybridization – a practical approach (1985) IRL Press, Oxford, England).

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and equivalents thereof known to those skilled in the art, and so forth. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably.

The term “about” denotes a range of +/- 20 % of the following numerical value. In certain embodiments, the term about denotes a range of +/- 10 % of the thereafter following numerical value. In certain embodiments, the term about denotes a range of +/- 5 % of the thereafter following numerical value.

The terms “comprise(s)”, “include(s)”, “having”, “has”, “can”, “contain(s)” and variants thereof, as used herein, are intended to be open-ended transitional phrases, terms or words that do not preclude the possibility of additional acts or structures. The term “comprising” also encompasses the term “consisting of”. The present disclosure also contemplates other embodiments “comprising”, “consisting of” and “consisting essentially of” the embodiments or elements presented herein, whether explicitly set forth or not.

The terms “empty recombinant AAV particle” and “empty rAAVp”, which can be used interchangeably, denote a protein shell composed of adeno-associated capsid polypeptides without a therein encapsidated/packaged functional nucleic acid (rAAVp = recombinant adeno-associated virus particle). That is, an empty rAAVp either may be free of encapsidated nucleic acid or comprises a nucleic acid or part thereof that is not transcribed at all or not transcribed into a functional transcript. Accordingly, an empty rAAVp does not function to transfer a nucleic acid that encodes a functional protein or is transcribed into a functional transcript of interest into a target cell. In certain embodiments of all aspects and embodiments, the functional protein or the functional transcript of interest has a therapeutic effect.

The terms “full recombinant AAV particle” or “full rAAVp”, which can be used interchangeably, denote a non-covalent complex formed of a protein shell composed of adeno-associated capsid polypeptides and a therein encapsidated/packaged functional nucleic acid sequence. That is, a full rAAVp comprises a nucleic acid that

is transcribed into a functional transcript. Accordingly, the full rAAVp functions to transfer a nucleic acid that encodes a protein or is transcribed into a transcript of interest into a target cell. In certain embodiments, a functional nucleic acid comprises at least one coding nucleic acid sequence interspaced between two adeno-associated viral inverted terminal repeats (ITRs).

The term “full to empty ratio” denotes the mathematical ratio of the number of full recombinant AAV particles (full rAAVp) to the total number of recombinant AAV particles (sum of full rAAVp and empty rAAVp) in a sample or in a recombinant AAV particle preparation. As the number of full rAAVp can be at most the same as the total number of rAAVp, the ratio can be at most 1. Generally, the ratio is less than 1 and is expressed as a percentage. The number of full rAAVp can be determined by determining the number of nucleic acid sequences interspaced between two AAV ITRs in the sample or preparation. This can be done by PCR, especially digital droplet PCR (ddPCR) or quantitative PCR (qPCR). The total number of rAAVp can be determined by determining the number of protein shells formed of adeno-associated capsid polypeptides in the sample or preparation. This can be done by ELISA, especially by a capsid polypeptide specific ELISA.

The terms “recombinant AAV vector" or “rAAVv” or “transgene”, which can be used interchangeably herein, denote a nucleic acid derived from a wild-type genome of an adeno-associated virus, wherein except for the ITR (adeno-associated virus Inverted Terminal Repeat) sequences all endogenous AAV nucleic acids are replaced by one or more exogenous nucleic acid(s). For example, such an exogenous nucleic acid can be a nucleic acid transcribed into a transcript of interest or that encodes a therapeutic protein or a therapeutic nucleic acid. Typically, for a rAAVv one or both ITR sequences of the wild-type AAV genome are retained. Thus, a rAAVv can be distinguished from a wild-type AAV vector, since all or at least a part of the viral genome has been replaced with a non-native (i.e. exogenous) nucleic acid with respect to the virus. Incorporation of a non-native nucleic acid therefore defines the AAV vector as a "recombinant" vector. It has to be pointed out that the serotype of the ITRs in the rAAVv does not need to be the same as the serotype of the adeno-associated capsid polypeptides forming the shell of the rAAVp comprising said rAAVv.

In principle, any non-AAV nucleic acid can be packaged into a shell composed of adeno-associated capsid polypeptides resulting in a rAAVp, e.g. for subsequent infection (transduction) of a cell, ex vivo, in vitro or in vivo.

As used herein, the term "serotype" is used to classify different wild-type and recombinant AAV particles based on the amino acid sequence of the polypeptides forming the protein shell (capsid) of the respective AAV particle. Originally, serologic distinctiveness was determined based on the lack of cross-reactivity between antibodies to one AAV particle as compared to another AAV particle. Such cross-reactivity differences are usually due to differences in capsid polypeptide sequences and the respective antigenic determinants (e.g., due to VP1, VP2, and/or VP3 sequence differences of AAV serotypes). Despite the possibility that AAV variants including capsid variants may not be serologically distinct from a reference or wild-type AAV or other AAV serotype, they differ by at least one amino acid residue compared to the reference or wild-type or other AAV serotype.

Under the traditional definition, a serotype means that the virus of interest has been tested against serum specific for all existing and characterized serotypes for neutralizing activity and no antibodies have been found that neutralize the virus of interest. As more naturally occurring virus isolates are discovered and/or capsid mutants generated, there may or may not be serological differences with any of the currently existing serotypes. Thus, in cases where the new AAV particle has no serological difference, this new AAV particle would be a subgroup or variant of the corresponding wild-type serotype. In many cases, serology testing for neutralizing activity has yet to be performed on mutant viruses with capsid sequence modifications to determine if they are of another serotype according to the traditional definition of serotype.

The term “vector" denotes the portion of a larger nucleic acid, e.g. of a recombinant plasmid, that is ultimately packaged or encapsulated or encapsidated either directly or in form of a single strand or in form of RNA into a protein shell composed of adeno-associated virus capsid polypeptides to form a rAAVp. In cases where recombinant plasmids are used to construct or manufacture rAAVps, the viral particle does not include the portion of the "plasmid" that does not correspond to the vector part of the recombinant plasmid. For example, in case of a rAAVp the recombinant vector comprises that part of the recombinant plasmid that is interspaced between two AAV ITRs. The non-vector portion of the recombinant plasmid is referred to as the "plasmid backbone". The plasmid backbone is important for cloning and amplification of the plasmid, a process that is needed for propagation and recombinant virus production, but is not itself packaged or encapsulated or encapsidated into the recombinant AAV particle. Thus, a “vector" refers to the

nucleic acid that is packaged or encapsulated or encapsidated by a protein shell composed of adeno-associated virus capsid polypeptides, i.e. in a rAAVp.

GENERAL METHODS FOR PRODUCING rAAVp

WO 1999/11764 reported methods for generating high titer helper-free preparations of recombinant AAV vectors. Not further defined AAV producer cells grown in suspension in bioreactors were infected with Adenovirus Type 5 (Ad5) at a multiplicity of infection (MOI) of 10 in low serum media at 1.5 L scale at different pH values. At a culture pH of 7.2, 4.7 E+12 total particles were obtained, at a culture pH of 7.4 1.95 E+13 total particles were obtained, at a culture pH of 7.6 1.84 E+13 total particles were obtained and at a culture pH of 8.0 1.63 E+13 total particles were obtained. The cultivation was performed in a 1.5 L bioreactor and, thus, the cultivation volume can be calculated (75 % of the nominal value) to have been about 1.125 L. Therefore the total particle number correspond to 4.2 E+09 vp/mL (pH 7.2), 1.7 E+10 vp/mL (pH 7.4), 1.6 E+10 vp/mL (pH 7.6) and 1.5 E+10 vp/mL (pH 8), respectively.

WO 2000/14205 reported the production of AAV particles in a non-defined cell type denoted as JL-14 cells by co-infection with adenoviral helper virus, whereby at a pH value of 7.4 the highest number of AAV particles (sum of intracellular and secreted AAV particles), at a pH value of 8 AAV particles with the highest infectivity and at a pH of 7.6 the highest ratio of number of AAV particles to infectivity was obtained. The cultivation was performed in a volume of 1.5 L medium and, thus, the total particle number correspond to 3.0 E+09 vp/mL (pH 7.2), 1.3 E+10 vp/mL (pH 7.4), 1.2 E+10 vp/mL (pH 7.6), 3.3 E+09 vp/mL (pH 7.8) and 1.1 E+10 vp/mL (pH 8), respectively. Based on the provided infectivity data it can be assumed that the full/empty ratio of the thereby produced rAAV particles is below 1 %.

Piras, B.A., et al. (Mol. Ther. Meth. Clin. Dev. 3 (2016) 16015) compared distribution of AAV8 in cell culture media and lysates on days 3, 5, 6 and 7 post-transfection and found increasing viral production through day 6, with the proportion of viral particles in the media increasing from 76% at day 3 to 94% by day 7. Larger-scale productions showed that the ratio of full-to-empty AAV particles is similar in media and lysate, and that AAV harvested on day 6 post-transfection provides equivalent function in mice compared to AAV harvested on day 3.

Piras et al. employed adherent HEK293T/17 cells cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum supplemented with 2 mmol/l

GlutaMAX (Life Technologies, Grand Island, NY). AAV was produced by two-plasmid transfection using PEIpro(TM) (Polyplus-transfection SA, Illkirch, France) 1 day after seeding cells at a density of 7.26×1 E+04 cells/cm^2.

Powers, A.D., et al. (Hum. Gene Ther. Meth. 27 (2016) 112-121) reported the development and optimization of AAV hFIX particle production by transient transfection in an iCELLis(R) fixed-bed bioreactor. A yield to as high as 9 E+14 viral particles per square meter of fixed bed were obtained. On day 3 after inoculation with HEK293T/17 cells, the vessel was transfected with plasmid scAAV-LP1-hFIXco-helpv3 and plasmid CR21+LTAAV help 2-8 at a plasmid mass ratio of 3:1, respectively, using polyethylenimine (PEIpro(TM) Transfection Reagent Cat #115-375; Polyplus) in IMDM (Lonza) or DMEM supplemented with 10% FBS and 6 mM GlutaMAX(TM). The PEI and DNA solutions were combined at a 2:1 ratio.

Poulain, A., et al. (J. Biotechnol. 255 (2017) 16-27) reported rapid protein production from stable CHO cell pools using plasmid vector and the cumate gene-switch. Cells were transfected using linear polyethylenimine (PEIpro(TM)) from Polyplus-Transfection (Illkirch, France). On the day of transfection, cells were suspended at a density of 2 × 1 E+06 cells/mL in CD DG44 medium (Life Technologies Inc., Burlington, ON, Canada), supplemented with 4 mM glutamine and 0,1% Kolliphor® P 188. The cell suspension was distributed in 6-well plates (1.8 mL/well). The DNA:PEIpro(TM) complexes were prepared at a ratio of 1:5 (w:w), with a total of 2 µg DNA per well to transfect in 100 µL of complete culture medium.

WO 2017/096039 reported scalable methods for producing recombinant AAV vectors in serum-free suspension cell culture systems suitable for clinical use. Production of rAAV vectors was performed in bioreactors with HEK293F cells using triple transfection at a cell density of 1 E+06 cells/mL (1.000.000 cells / mL) with a plasmid ratio of 1:1:1 and a PEI-based transfection reagent (PEI/DNA weight ratio of 2:1 with ½ of PEI as free PEI) at a temperature of 37 °C and a pH value of 7.2.

Nyamay'antu, A., et al. (Cell Gen. Ther. Ins. 4 (2018) 71-79) reported that PEI is widely used due to its affordability and high DNA delivery efficiency, in both adherent and suspension cells grown in serum-free medium. PEIpro(TM) is suited for small- to large-scale production of various viruses, notably AAV particles. In stirred-tank bioreactors using HEK293 or HEK293T cells titers in the range of 0.8-1.5 E+09-E+10 vg/mL can be obtained.

Koo, T., et al. (Nat. Commun. 9 (2018) 1855) reported that CRISPR-LbCpf1 prevents choroidal neovascularization in a mouse model of age-related macular degeneration. To produce AAV vectors, they were pseudotyped in AAV9 capsids. HEK293T cells (ATCC, CRL-3216) were transfected with pAAV-ITR-LbCpf1-crRNA, pAAV2/9 encoding for AAV2rep and AAV9cap, and helper plasmid. HEK293T cells were cultured in DMEM with 2% FBS. Recombinant pseudotyped AAV vector stocks were generated using PEI co-precipitation with PEIpro(TM) (Polyplus-transfection) and triple-transfection with plasmids at a molar ratio of 1:1:1 in HEK293T cells. After 72 h of incubation, cells were lysed and particles were purified by iodixanol step-gradient ultracentrifugation.

The Rep proteins from AAV2 are commonly and nearly exclusively used in the production of rAAVps derived from the serotypes AAV1 to AAV13 (Daya, S., and Berns, K.I., Clin. Microbiol. Rev. 21 (2008) 583-593; Zincarelli, C., et al., Mol. Ther. 16 (2008) 1073-1080).

WO 2019/094253 reported means and methods for preparing viral vectors and uses thereof. Adherent HEK293 cells were cultivated in bioreactors at a pH value of 7.23 and triple transfected (plasmid ratio 1:1:1) with PEI/DNA at a PEI-plasmid ratio of about 1:1 by weight.

Collaud, F. et al. (Mol. Ther. Meth. Clin. Dev. 12 (2019) 157-174) reported titers for recombinant AAV8 particles of 6.0 ± 1.89 E+04 vg/cell and 1.77 ± 1.37 E+04 vg/cell for (single stranded) and (self-complementary) AAV, respectively, for adherent HEK293 cells. A fully scalable method based on triple transfection of HEK293 cells cultured in suspension was also reported. Triple transfection of HEK293 cells was performed with polyethylenimine (PEIpro(TM), Polyplus) directly in 10 L bioreactors. AAV vectors were recovered from both supernatant and cells by mild detergent lysis followed by AVB Sepharose affinity column purification. Purified vectors were then concentrated and tested for quality and potency. No information about the pH value and obtained titers are provided.

Nyamay'antu, A., et al. (Cell Gen. Ther. Ins. 6 (2020) 655-661) reported that the efficiency of the delivery process is essential to obtain a high number of producing cells. Of the existing transfection methods, the use of PEI-based transfection reagent is predominant in gene therapy as it combines affordability and compatibility for transfection of adherent and suspension cells. In comparison to the gold standard PEIpro(TM) used for viral vector manufacturing, FectoVIR(TM)-AAV has been

found to improve significantly recombinant AAV2 particle production yield of both viral genome production and packaging efficiency in suspension cells of an rAAV2-GFP of up to 10-fold compared to PEIMax(TM) and up to 2-fold compared to PEIpro(TM), respectively, when each transfection reagent is used under the recommended conditions. In more detail, suspension HEK293T cells were transfected using the respective transfection reagent under the recommended conditions. rAAV2-GFP were harvested 72 hours post transfection. The obtained titer with FectoVIR(TM) is in the range of 1 E+04 to 4.5 E+04 vg/cell depending on the used volume of complexation (1%-10%) corresponding to 1 E+12 vg/mL. The respective functional titers are about 2-8 E+08 TU/mL. The results are almost independent of the employed cultivation medium.

In a blog article entitled "Optimization of AAV production for high-yielding and scalable GMP processes with Catalent" (www.polyplus-transfection.com) different transfection reagent to DNA ratios were tested with the two serotypes AAV9 (1:1 and 2:1) and AAV2 (3:1.5 and 5:2.5). The AAV2 vector yield was not affected as notably, with a 4-5-fold increase in the vector genome titer and a 3-6-fold increase in the viral particle titer with FectoVIR(TM)-AAV as compared to PEIpro(TM). These results show that improvement in yield may vary with the AAV serotype. In a further study comparing additional AAV2 and AAV5 vectors (different from the previous AAV2 and AAV5 vectors) and using a DoE approach to optimization, experiments were conducted varying transfection reagent to DNA ratios (3:2, 3:1.5) and plasmid DNA molar ratios (1:1:1, 2:1:2, 1:2:1) were performed. A 3-5-fold increase for AAV2 and a 1.1-1.6-fold increase for AAV5 in the vector genome titer with FectoVIR(TM)-AAV compared to PEIpro(TM) was observed. The viral particle titer increased 3.5-4.5-fold for AAV2 and 2.5-3.75-fold for AAV5. Reagent-to-DNA ratios of 2:1 and 1.5:1 and plasmid ratios of 1:1:1 to 2:1:2 to 1:2:1 were used. The obtained titer with FectoVIR(TM) was in the range of 4 E+11 to 1 E+12 vg/mL.

Rossi, A. and Peigné, C-M. (Cell Culture Dish Article May 17, 2021) outlined that, typically, AAV production titers are around 1 E+11 to 1 E+12 in vg/mL and 1 E+08 to 1 E+09 TU/mL.

Wosnitzka, K., et al. (Cell Gen. Ther. Ins. 7 (2021) 1-7) reported that analysis of physical titers revealed a 3-fold increase in both viral particles (VP) and viral genome (VG) per ml of cell culture when using FectoVIR(TM)-AAV transfection reagent compared to PEIpro(TM).

Porte, M., et al. (poster entitled "Next-Generation Transfection Reagent for Large Scale AAV Manufacturing", Polyplus, Illkirch, France) reported the transfection of suspension-HEK293T cells with the optimal conditions for the other PEI- based reagent (1.5 µg/million cells, ratio DNA : PEI of 1 µg : 4 µL) and FectoVIR(TM)-AAV (1 µg/million cells, ratio DNA : reagent of 1 µg : 1 µL) following the recommended protocol for each reagent. A titer of about 5 E+11 vg/mL versus 1.5 E+11 vg/mL using FectoVIR(TM) and PEI-based transfection reagent, respectively, with a packaging efficiency of 20 % vs. about 13.5 %, respectively, was obtained.

Nakamura et al. (Eur. J. Haematol. 73 (2004) 285-294) reported about the development of packaging cell lines for generation of adeno-associated virus vectors by lentiviral gene transfer of trans-complementary components. It is outlined that adeno-associated virus (AAV) vector systems have several useful advantages with regard to in vitro and in vivo gene transfer. However, their usages have been limited by cumbersome and labor-intensive vector production in the traditional method. To overcome limitations in AAV production, Nakamura et al. explored the possibility of generating AAV packaging cell line, 293T R/C.VA.E2A.E4. cells, by using lentivirus-mediated transduction of Rep/Cap gene of AAV-2, VA RNA, E2A, and E4 genes of Ad5 into 293T cells. In packaging cell lines, it is important that supply of the AAV vector can be stably performed for long time. They showed that the 293T R/C.VA.E2A.E4. cells have stably maintained the transduced components after more than 10 passages and yielded high-titer AAV vectors, and the titer of AAV vectors did not decline even if culture of the packaging cells was continued for long time. The Rep/Cap and E4 gene products caused no remarkable cytotoxicity. The 293T R/C.VA.E2A.E4. cells might be able to tolerate the Rep/Cap and E4 gene products, or have less copy numbers of the Rep/Cap and E4 genes than the traditional method. Moreover, they showed that the AAV vectors derived from 293T R/C.VA.E2A.E4. cells infected the primary human CD34+ hematopoietic progenitor cells with high efficiency (50–70%). In the 293T R/C.VA.E2A.E4. cells, the AAV vectors can be generated by the transfection of one AAV vector plasmid, and large-scale AAV production can be easily achieved. It is important that cumbersome, variable, and costly transfection is avoided.

WO 2018/192983 reported an adeno-associated virus (AAV) producer cell comprising nucleic acid sequences encoding rep/cap gene; helper virus genes; and the DNA genome of the AAV vector particle, wherein said nucleic acid sequences are all integrated together at a single locus within the AAV producer cell genome. Reported are also nucleic acid vectors comprising a non-mammalian origin of

replication and the ability to hold at least 25 kilo bases (kb) of DNA, characterized in that said nucleic acid vector comprises nucleic acid sequences encoding: rep/cap gene, and helper virus genes as well as uses and methods using said nucleic acid vector in order to produce stable AAV packaging and producer cell lines.

WO 2018/194438 reported a cell line for producing a non-replicating adenovirus, and a preparation method therefor and, more specifically, to: a cell line for producing a replication-deficient adenovirus by expressing any one or more selected from an E1 protein, and an E1A protein or an E1B protein of an adenovirus; and a method for preparing the same. In addition, it is reported the use of the cell line, for expressing any one or more selected from an E1 protein, and an E1A protein or an E1B protein of an adenovirus.

WO 2020/078953 reported adeno-associated virus (AAV) vector producer cell comprising nucleic acid sequences encoding AAV rep and cap genes, helper virus genes, and a DNA genome of the AAV vector; the AAV rep gene comprising an intron, the intron comprising a transcription termination sequence with a first recombination site located upstream and a second recombination site located downstream of the transcription termination sequence; and the nucleic acid sequences all integrated together at a single locus within the AAV vector producer cell genome.

WO 2020/132059 reported a mammalian cell line for producing adeno-associated virus (AAV), suitably including nucleic acids encoding helper genes and AAV genes, under the control of derepressible promoters. The disclosure also relates to isolated nucleic acid molecules that encode such genes, as well as methods of using the mammalian cells for producing AAVs. Especially is reported a mammalian cell for producing an adeno-associated virus (AAV), comprising (a) a nucleic acid molecule encoding a viral helper gene under control of a first derepressible promoter; (b) a nucleic acid molecule encoding an AAV gene under control of a second derepressible promoter; and (c) a nucleic acid molecule encoding a repressor element of the first and the second derepressible promoters.

EP 3 822 346 reported the use of an engineered mammalian packaging cell line for producing recombinant virus particles, wherein the cell line is engineered to lack cell surface expression of heparan sulfate. Further disclosed are a method for producing recombinant virus particles and a recombinant virus particle obtainable by the

method. Further disclosed is a mammalian packaging cell line deposited under number DSM ACC3355 or DSM ACC3356.

WO 2022/112218 reported methods for the production of Adeno-associated vims (AAV), comprising steps of providing a stable AAV producer cell line in which at least some or all genes encoding the components necessary for the production of AAV are stably integrated into the cell genome, and culturing said cells in perfusion culture during the AAV production step (i.e., during the N step), wherein said perfusion culture encompasses continuous replacement of spent media with fresh media, and wherein said continuous replacement of spent media with fresh media continues after the induction of AAV production. In the cell at least (a) a gene encoding the AAV Rep protein Rep78 or Rep68, (b) a gene encoding the AAV Rep protein Rep52 or Rep40; (c) the genes encoding the adenoviral helper functions E4orf6 and E2A stably integrated into the host cell genome. Further at least the following genes are stably integrated into the host cell genome (a) the genes encoding the AAV Cap proteins VP1, VP2, VP3; (b) a gene encoding the AAV Rep protein Rep78 or Rep68; (c) a gene encoding the AAV Rep protein Rep52 or Rep40; (d) the genes encoding the adenoviral helper functions E4orf6 E2A; (e) the gene of interest flanked by AAV ITRs.

WO 2022/173944 reported methods for producing an adeno-associated virus (AAV) in an E1 complementary producer cell. Especially is reported a method of producing an adeno-associated virus (AAV) in an E1 complementary producer cell, comprising (a) transfecting the E1 complementary producer cell with one or more vectors comprising (1) an E1A adenovirus helper gene; (2) an adenovirus helper gene selected from E2A, E4, or both; (3) a viral-associated, non-coding RNA (VA RNA); and (4) an AA V gene selected from Rep, Cap, or both; (b) culturing the transfected E1 complementary producer cell under conditions suitable for producing the AAV; and (c) purifying the AAV from the cultured E1 complementary producer cell, thereby obtaining the AAV.

WO 2022/192261 reported compositions and methods for producing and characterizing stable viral vector producer cell lines that enable industrial scale production of viral vectors. Novel viral vector genome constructs, in which the constructs can be precisely mapped and viral vector genome constructs precisely quantified, are also disclosed for efficient production and characterization of viral vectors in mammalian cells.

WO 2023/077078 reported recombinant adeno-associated virus (rAAV) packaging and/or producer cell lines which have been engineered to reduce expression and/or activity of one or more genes and/or proteins to increase rAAV titers. Especially is reported a recombinant adeno-associated virus (rAAV) packaging and/or producer cell line comprising cells in which the expression of a gene selected from the group consisting of RIG-1 (DDX58), IFIT3, MDA5 (IFIH1), CGAS (cGAS), CHUK (IKK-a), DDX41, DHX58 (LGP2), IFI6, IKBKB (IKK-α), IRF3, IRF7, MAVS, MYD88, NFKB1, NFKB2, TBK1, TRIP, and TRIM25, and any combination thereof is reduced compared to that in control parental cells.

WO 2023/102549 reported systems for increasing AAV particle production. These systems comprise producer cell lines adapted for the production of AAV particles, as well as methods of producing AAV particles using said producer cell lines. Also provided are AAV particles produced by said production systems, producer cell lines and methods. Especially it is reported a genetically engineered producer cell line in which the expression of at least one of TMED10, MON2, TMED2, HS2ST1, C3orfS8, SPPL3, SURF4, LSMS, ARFl, and PI4KB is reduced as compared to a control cell line, and/or in which the expression of at least one of B4GALT7, B3GAT3, OAF, EXT2, COMMD3, SLC3SD1, B3GALT6, SDCl, NDSTl, RACl, CSK, GLCE, PDCL, FAM20B, TM4SFS, DGAT2, POMTl, YYl, and DPF2 is increased as compared to a control cell line.

WO 2023/114897 reported methods for the production of recombinant adeno-associated virus (rAAV) particles. These methods are particularly useful for the large-scale production of AAV particles. Especially it is reported A method for producing recombinant AAV (rAAV) particles, comprising (a) introducing into a mammalian cell a first polynucleotide comprising an rAAV genome, to generate an AA V producer cell; (b) culturing the AA V producer cell in a first culture medium at a first temperature for a first period of time; (c) culturing the AAV producer cell in a second culture medium at a second temperature for a second period of time, wherein the second temperature is about 38°C to about 42°C, such that rAAV particles are produced by the AAV producer cell, wherein the rAAV particles comprise an rAAV genome comprising a transgene, and an AAV capsid comprising an AAV capsid protein.

WO 2023/166026 reported cell lines in which DNA fragmentation is inhibited, uses of cell lines in which DNA fragmentation is inhibited for the production of adeno-associated virus (AAV), related methods of producing AAV, and methods of

producing AAV, comprising the step of exposing the cells in which AAV is produced to an inhibitor of DNA fragmentation during the AAV production phase.

WO 2023/171698 reported a producer cell for the production of an adeno-associated virus wherein cell damage is avoided or suppressed at the establishment of a cell line, a method for producing the producer cell, and a method for producing an AAV using the producer cell. In the producer cell a Cap gene under the control of a foreign promoter and a Rep gene under the control of a foreign promoter is integrated in the chromosome, that is free from a VA-RNA gene and/or an E4 gene, and that is a mammalian cell.

The content of all documents outlined in this section are expressly incorporated by reference herein.

RECOMBINANT CELL

Generally, for efficient as well as large-scale production of a rAAVp a cell expressing and, if possible, also secreting said rAAVp is used. Such a cell is termed "recombinant producer cell" or short "producer cell".

For the generation of a recombinant producer cell a suitable mammalian cell is transfected with the nucleic acids required for producing said rAAVp, including the required AAV helper functions.

Generally, for expression of a coding sequence, i.e. of an open reading frame, additional regulatory elements, such as a promoter and a polyadenylation signal (sequence), are necessary. Thus, for functional transcription an open reading frame has to be and is operably linked to said additional regulatory elements. This can be achieved by combining these parts into a so-called expression cassette. The minimal regulatory elements required for an expression cassette to be functional in a mammalian cell are a promoter functional in said mammalian cell, which is located upstream, i.e. 5', to the open reading frame, and a polyadenylation signal (sequence) functional in said mammalian cell, which is located downstream, i.e. 3', to the open reading frame. Additionally a terminator sequence may be present 3' to the polyadenylation signal (sequence). For expression, the promoter, the open reading frame/coding region and the polyadenylation signal sequence have to be arranged in an operably linked form.

Likewise, a nucleic acid that is transcribed into a non-protein coding RNA is called "RNA gene". Also for expression of an RNA gene, additional regulatory elements, such as a promoter and a transcription termination signal or polyadenylation signal (sequence), are necessary. The nature and localization of such elements depends on the RNA polymerase that is intended to drive the expression of the RNA gene. Thus, an RNA gene is normally also integrated into an expression cassette.

In case of an rAAVp, which is composed of different (monomeric) capsid polypeptides and a therein encapsidated single stranded DNA molecule and which in addition requires other viral helper functions for production and encapsidation, a multitude of expression cassettes differing in the contained open reading frames/coding sequences are required. In this case, at least an expression cassette for each of the transgene, for the polypeptides forming the capsid of the rAAVp, for the required viral helper functions are required. Thus, individual expression cassettes at least for each of the helper functions E1A, E1B, E2A, E4orf6, the rep and cap genes are required. HEK293 cells express the E1A and E1B helper functions constitutively.

ADENO-ASSOCIATED VIRUS (AAV)

For a general review of AAVs and of the adenovirus or herpes helper functions see, Berns and Bohensky, Advances in Virus Research, Academic Press., 32 (1987) 243-306. The genome of AAV is described in Srivastava et al., J. Virol., 45 (1983) 555-564. In US 4,797,368 design considerations for constructing recombinant AAV vectors are described (see also WO 93/24641). Additional references describing AAV vectors are West et al., Virol. 160 (1987) 38-47; Kotin, Hum. Gene Ther. 5 (1994) 793-801; and Muzyczka J. Clin. Invest. 94 (1994) 1351. Construction of recombinant AAV vectors is described in US 5,173,414; Lebkowski et al., Mol. Cell. Biol. 8 (1988) 3988-3996; Tratschin et al., Mol. Cell. Biol. 5 (1985) 3251-3260; Tratschin et al., Mol. Cell. Biol., 4 (1994) 2072-2081; Hermonat and Muzyczka Proc. Natl. Acad. Sci. USA 81 (1984) 6466-6470; Samulski et al. J. Virol. 63 (1989) 3822-3828.

An AAV is a replication-deficient parvovirus. It can replicate only in cells, in which certain viral functions are provided by a co-infecting helper virus, such as adenoviruses, herpesviruses and, in some cases, poxviruses such as vaccinia. Nevertheless, an AAV can replicate in virtually any cell line of human, simian or rodent origin provided that the appropriate helper viral functions are present.

Without helper viral genes being present, an AAV establishes latency in its host cell. Its genome integrates into a specific site in chromosome 19 [(Chr) 19 (q13.4)], which is termed the adeno-associated virus integration site 1 (AAVS1). For specific serotypes, such as AAV2 other integration sites have been found, such as, e.g., on chromosome 5 [(Chr) 5 (p13.3)], termed AAVS2, and on chromosome 3 [(Chr) 3 (p24.3)], termed AAVS3.

AAVs are categorized into different serotypes. These have been allocated based on parameters, such as hemagglutination, tumorigenicity and DNA sequence homology. Up to now, more than 12 different serotypes and more than a hundred sequences corresponding to different clades of AAV have been identified.

The capsid protein type and symmetry determines the tissue tropism of the respective AAV. For example, AAV2, AAV4 and AAV5 are specific to retina, AAV2, AAV5, AAV8, AAV9 and AAV-rh.10 are specific for brain, AAV1, AAV2, AAV6, AAV8 and AAV9 are specific for cardiac tissue, AAV1, AAV2, AAV5, AAV6, AAV7, AAV8, AAV9 and AAV10 are specific for liver, AAV1, AAV2, AAV5 and AAV9 are specific for lung.

Pseudotyping denotes a process comprising the cross packaging of the AAV genome between various serotypes, i.e. the genome is packaged with differently originating capsid proteins.

The wild-type AAV genome has a size of about 4.7 kb. The AAV genome further comprises two overlapping genes named rep and cap, which comprise multiple open reading frames (see, e.g., Srivastava et al., J. Viral., 45 (1983) 555-564; Hermonat et al., J. Viral. 51 (1984) 329-339; Tratschin et al., J. Virol., 51 (1984) 611-619). The Rep protein encoding open reading frame provides for four proteins of different size, which are termed Rep78, Rep68, Rep52 and Rep40. These are involved in replication, rescue and integration of the AAV. The Cap protein encoding open reading frame provides four proteins, which are termed VP1, VP2, VP3, and AAP. VP1, VP2 and VP3 are part of the proteinaceous capsid of the AAV particles. The combined rep and cap open reading frames are flanked at their 5'- and 3'-ends by so-called inverted terminal repeats (ITRs). For replication, an AAV requires in addition to the Rep and Cap proteins the products of the genes E1A, E1B, E4orf6, E2A and VA of an adenovirus or corresponding factors of another helper virus.

In the case of an AAV of the serotype 2 (AAV2), for example, the ITRs each have a length of 145 nucleotides and flank a coding sequence region of about 4470

nucleotides. Of the ITR's 145 nucleotides 125 nucleotides have a palindromic sequence and can form a T-shaped hairpin structure. This structure has the function of a primer during viral replication. The remaining 20, non-paired, nucleotides are denoted as D-sequence.

The wild-type AAV genome harbors three transcription promoters P5, P19, and P40 (Laughlin et al., Proc. Natl. Acad. Sci. USA 76 (1979) 5567-5571) for the expression of the rep and cap genes.

The ITR sequences have to be present in cis to the coding region. The ITRs provide a functional origin of replication (ori), signals required for integration into the target cell's genome, and efficient excision and rescue from host cell chromosomes or recombinant plasmids. The ITRs further comprise origin of replication like-elements, such as a Rep-protein binding site (RBS) and a terminal resolution site (TRS). It has been found that the ITRs themselves can have the function of a transcription promoter (Flotte et al., J. Biol. Chem. 268 (1993) 3781-3790; Flotte et al., Proc. Natl. Acad. Sci. USA 93 (1993) 10163-10167).

For replication and encapsidation, respectively, of the viral single-stranded DNA genome an in trans organization of the rep and cap gene products is required.

The rep gene locus comprises two internal promoters, termed P5 and P19. It comprises open reading frames for four proteins. Promoter P5 is operably linked to a nucleic acid sequence providing for non-spliced 4.2 kb mRNA encoding the Rep protein Rep78 (chromatin nickase to arrest cell cycle), and a spliced 3.9 kb mRNA encoding the Rep protein Rep68 (site-specific endonuclease). Promoter P19 is operably linked to a nucleic acid sequence providing for a non-spliced mRNA encoding the Rep protein Rep52 and a spliced 3.3 kb mRNA encoding the Rep protein Rep40 (DNA helicases for accumulation and packaging).

The two larger Rep proteins, Rep78 and Rep68, are essential for AAV duplex DNA replication, whereas the smaller Rep proteins, Rep52 and Rep40, seem to be essential for progeny and single-strand DNA accumulation (Chejanovsky & Carter, Virology 173 (1989) 120-128).

The larger Rep proteins, Rep68 and Rep78, can specifically bind to the hairpin conformation of the AAV ITR. They exhibit defined enzyme activities, which are required for resolving replication at the AAV termini. Expression of Rep78 or Rep68

could be sufficient for infectious particle formation (Holscher, C., et al. J. Virol. 68 (1994) 7169-7177 and 69 (1995) 6880-6885).

It is deemed that all Rep proteins, primarily Rep78 and Rep68, exhibit regulatory activities, such as induction and suppression of AAV genes as well as inhibitory effects on cell growth (Tratschin et al., Mol. Cell. Biol. 6 (1986) 2884-2894; Labow et al., Mol. Cell. Biol., 7 (1987) 1320-1325; Khleif et al., Virology, 181 (1991) 738-741).

Recombinant overexpression of Rep78 results in phenotype with reduced cell growth due to the induction of DNA damage. Thereby the host cell is arrested in the S phase, whereby latent infection by the virus is facilitated (Berthet, C., et al., Proc. Natl. Acad. Sci. USA 102 (2005) 13634-13639).

Tratschin et al. reported that the P5 promoter is negatively auto-regulated by Rep78 or Rep68 (Tratschin et al., Mol. Cell. Biol. 6 (1986) 2884-2894). Due to the toxic effects of expression of the Rep protein, only very low expression has been reported for certain cell lines after stable integration of AAV (see, e.g., Mendelson et al., Virol. 166 (1988) 154-165).

The cap gene locus comprises one promoter, termed P40. Promoter P40 is operably linked to a nucleic acid sequence providing for 2.6 kb mRNA, which, by alternative splicing and use of alternative start codons, encodes the Cap proteins VP1 (87 kDa, non-spliced mRNA transcript), VP2 (72 kDa, from the spliced mRNA transcript), and VP3 (61 kDa, from alternative start codon). VP1 to VP3 constitute the building blocks of the viral capsid. The capsid has the function to bind to a cell surface receptor and allow for intracellular trafficking of the virus. VP3 accounts for about 90 % of total viral particle protein. Nevertheless, all three proteins are essential for effective capsid production.

It has been reported that inactivation of all three capsid proteins VP1 to VP3 prevents accumulation of single-strand progeny AAV DNA. Mutations in the VP1 amino-terminus ("Lip-negative" or "Inf-negative") still allows for assembly of single-stranded DNA into viral particles whereby the infectious titer is greatly reduced.

The AAP open reading frame is encoding the assembly activating protein (AAP). It has a size of about 22 kDa and transports the native VP proteins into the nucleolar region for capsid assembly. This open reading frame is located upstream of the VP3 protein encoding sequence.

In individual AAV particles, only one single-stranded DNA molecule is contained. This may be either the "plus" or "minus" strand. AAV particles containing a DNA molecule are infectious. Inside the infected cell, the parental infecting single stranded DNA is converted into a double stranded DNA, which is subsequently amplified. The amplification results in a large pool of double stranded DNA molecules from which single strands are displaced and packaged into capsids.

Adeno-associated viral (AAV) vectors can transduce dividing cells as well as resting cells. It can be assumed that a transgene introduced using an AAV vector into a target cell will be expressed for a long period. One drawback of using an AAV vector is the limitation of the size of the transgene that can be introduced into cells.

Parvovirus particles, including AAV serotypes and variants thereof, provide a means for ex vivo, in vitro and in vivo delivery of nucleic acid, which encode proteins, into cells such that the infected cells express the encoded protein. AAVs are viruses useful as gene therapy vectors as they can penetrate cells and introduce nucleic acid/genetic material so that the nucleic acid/genetic material may be stably maintained in the infected cells. Because AAV are not associated with pathogenic disease in humans, AAVs are able to deliver heterologous polynucleotide sequences (e.g., therapeutic proteins and agents) to human patients without causing substantial AAV-related pathogenesis or disease.

AAV particles used as vehicles for effective gene delivery possess a number of desirable features for such applications, including tropism for dividing and non-dividing cells. Early clinical experience with these vectors also demonstrated no sustained toxicity and immune responses were minimal or undetectable. AAV are known to infect a wide variety of cell types in vivo and in vitro by receptor-mediated endocytosis or by transcytosis. These vector systems have been tested in humans targeting retinal epithelium, liver, skeletal muscle, airways, brain, joints and hematopoietic stem cells.

Recombinant AAV particles do not typically include viral genes associated with pathogenesis. Such particles typically comprise a genome, wherein one or more of the wild-type AAV genes have been deleted in whole or in part, for example, rep and/or cap genes, but retain at least one functional flanking ITR sequence, as necessary for the rescue, replication, and packaging of the recombinant vector into an rAAV. Thus, an AAV vector includes sequences required in cis for replication and packaging (i.e. functional ITR sequences).

Recombinant AAV particles, as well as methods and uses thereof, can be based on any wild-type AAV genome or serotype or combination thereof. As a non-limiting example, a rAAV can be based upon any wild-type AAV genome, i.e. comprise the respective ITR sequences, such as AAV1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, 2i8, rh.74, rh.10 or 7m8 for example. Such particles can be based on the same strain or serotype (or subgroup or variant), or be different from each other. As a non-limiting example, a rAAV based upon one wild-type genome can be identical or different to one or more of the capsid proteins that package the vector. In addition, a recombinant AAV vector can be based upon an AAV (e.g., AAV2) wild-type serotype genome distinct from one or more of the AAV capsid proteins that package the vector. For example, the AAV vector can be based upon AAV2, whereas at least one of the three capsid proteins could be an AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV-rh.74, AAV-rh.10 or AAV-7m8 or a variant thereof, for example. AAV variants include variants and chimeras of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV-rh.74, AAV-rh.10 and AAV-7m8 capsids.

In certain embodiments of all aspects and embodiments of the invention, the rAAVp is derived from a wild-type AAV particle selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV-rh.74, AAV-rh.10 and AAV-7m8, as well as variants (e.g., capsid variants, such as amino acid insertions, additions, substitutions and deletions) thereof, for example, as set forth in WO 2013/158879, WO 2015/013313 and US 2013/0059732 (disclosing LK01, LK02, LK03, etc.).

In certain embodiments of all aspects and embodiments of the invention, the rAAVp comprises a capsid polypeptides with an amino acid sequence having 70 % or more sequence identity to an wild-type AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV-2i8, AAV-rh.10, AAV-rh.74, or AAV-7m8 capsid sequence.

In certain embodiments of all aspects and embodiments of the invention, the rAAVp comprises one or two ITR sequence having 70 % or more sequence identity to a wild-type AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or AAV12 ITR sequence.

Recombinant AAV particles can be incorporated into pharmaceutical compositions. Such pharmaceutical compositions are useful for, among other things, administration and delivery to a subject in vivo or ex vivo. In certain embodiments, the pharmaceutical composition contains a pharmaceutically acceptable carrier or excipient. Such excipients include any pharmaceutical agent that does not itself induce an immune response harmful to the individual receiving the composition, and which may be administered without undue toxicity.

Protocols for the generation of adenoviral vectors have been described in US 5,998,205; US 6,228,646; US 6,093,699; US 6,100,242; WO 94/17810 and WO 94/23744, which are incorporated herein by reference in their entirety.

RECOMBINANT ADENO-ASSOCIATED VIRAL PARTICLES (rAAVps)

Different methods are known in the art for generating recombinant AAV particles. For example, transfection with an AAV vector comprising plasmid and a plasmid comprising AAV helper sequences (rep and cap) in conjunction with co-infection with one AAV helper virus (e.g., adenovirus, herpesvirus, or vaccinia virus) or transfection with a recombinant AAV vector comprising plasmid, an AAV helper plasmid (comprising rep and cap), and an helper function plasmid. Non-limiting methods for generating rAAV are described, for example, in US 6,001,650, US 6,004,797, WO 2017/096039, and WO 2018/226887. Following rAAV production (i.e. particle generation in cell culture systems), rAAV can be obtained from the host cells and/or cell culture supernatant and purified.

For the generation of recombinant AAV particles, expression of the Rep and Cap proteins, the helper proteins E1A, E1B, E2A and E4orf6 as well as optionally the adenoviral VA RNA in a single mammalian cell is required. The helper proteins E1A, E1B, E2A and E4orf6 can be expressed using any promoter as shown by Matsushita et al. (Gene Ther. 5 (1998) 938-945), especially the CMV IE promoter. Thus, any promoter can be operably linked to said genes for functional expression.

Generally, to produce rAAV, different, complementing plasmids are co-transfected into a host cell. One of the plasmids comprises the transgene sandwiched between the two cis acting AAV ITRs. The missing AAV elements required for replication and subsequent packaging of progeny recombinant genomes, i.e. the open reading frames for the Rep and Cap proteins, are contained in trans on a second plasmid. The overexpression of the Rep proteins results in inhibitory effects on cell growth (Li, J., et al., J. Virol. 71 (1997) 5236-5243). Additionally, a third plasmid comprising the

genes of a helper virus, i.e. E1, E4orf6, E2A and VA from adenovirus, is required for rAAV production.

To reduce the number of required plasmids, rep, cap and the adenovirus helper genes may be combined on a single plasmid.

Alternatively, the host cell may already stably express the E1 gene products. Such a cell is a HEK293 cell. The human embryonic kidney clone denoted as 293 was generated back in 1977 by integrating adenoviral DNA into human embryonic kidney cells (HEK cells) (Graham, F.L., et al., J. Gen. Virol. 36 (1977) 59-74). The HEK293 cell line comprises base pair 1 to 4344 of the adenovirus serotype 5 genome. This encompasses the E1A and E1B genes as well as the adenoviral packaging signals (Louis, N., et al., Virology 233 (1997) 423-429).

When using HEK293 cells the missing E2A, E4orf6 and VA genes can be introduced either by co-infection with an adenovirus or by co-transfection with an E2A-, E4orf6- and VA-expressing plasmid (see, e.g., Samulski, R.J., et al., J. Virol. 63 (1989) 3822-3828; Allen, J.M., et al., J. Virol. 71 (1997) 6816-6822; Tamayose, K., et al., Hum. Gene Ther. 7 (1996) 507-513; Flotte, T.R., et al., Gene Ther. 2 (1995) 29-37; Conway, J.E., et al., J. Virol. 71 (1997) 8780-8789; Chiorini, J.A., et al., Hum. Gene Ther. 6 (1995) 1531-1541; Ferrari, F.K., et al., J. Virol. 70 (1996) 3227-3234; Salvetti, A., et al., Hum. Gene Ther. 9 (1998) 695-706; Xiao, X., et al., J. Virol. 72 (1998) 2224-2232; Grimm, D., et al., Hum. Gene Ther. 9 (1998) 2745-2760; Zhang, X., et al., Hum. Gene Ther. 10 (1999) 2527-2537). Alternatively, adenovirus/AAV or herpes simplex virus/AAV hybrid vectors can be used (see, e.g., Conway, J.E., et al., J. Virol. 71 (1997) 8780-8789; Johnston, K.M., et al., Hum. Gene Ther. 8 (1997) 359-370; Thrasher, A.J., et al., Gene Ther. 2 (1995) 481-485; Fisher, J.K., et al., Hum. Gene Ther. 7 (1996) 2079-2087; Johnston, K.M., et al., Hum. Gene Ther. 8 (1997) 359-370).

In order to limit the transgene activity to specific tissues, i.e. to limit the site of action, the transgene can be operably linked to an inducible or tissue specific promoter (see, e.g., Yang, Y., et al. Hum. Gene. Ther. 6 (1995) 1203-1213).

The coding sequences of E1A and E1B (open reading frames) can be derived from a human adenovirus, such as, e.g., in particular of human adenovirus serotype 2 or serotype 5. An exemplary sequence of human Ad5 (adenovirus serotype 5) is found in GenBank entries X02996, AC_000008 and that of an exemplary human Ad2 in GenBank entry AC_000007. Nucleotides 505 to 3522 comprise the nucleic acid

sequences encoding E1A and E1B of human adenovirus serotype 5. Plasmid pSTK146 as reported in EP 1 230 354, as well as plasmids pGS119 and pGS122 as reported in WO 2007/056994, can also be used as a source for the E1A and E1B open reading frames.

E1A is the first viral helper gene that is expressed after adenoviral DNA enters the cell nucleus. The E1A gene encodes the 12S and 13S proteins, which are based on the same E1A mRNA by alternative splicing. Expression of the 12S and 13S proteins results in the activation of the other viral functions E1B, E2, E3 and E4. Additionally, expression of the 12S and 13S proteins force the cell into the S phase of the cell cycle. If only the E1A-derived proteins are expressed, the cell will die (apoptosis).

E1B is the second viral helper gene that is expressed. It is activated by the E1A-derived proteins 12S and 13S. The E1B gene derived mRNA can be spliced in two different ways resulting in a first 55 kDa transcript and a second 19 kDa transcript. The E1B 55 kDa protein is involved in the modulation of the cell cycle, the prevention of the transport of cellular mRNA in the late phase of the infection, and the prevention of E1A-induced apoptosis. The E1B 19 kDa protein is involved in the prevention of E1A-induced apoptosis of cells.

The E2 gene encodes different proteins. The E2A transcript codes for the single strand-binding protein (SSBP), which is essential for AAV replication

In addition, the E4 gene encodes several proteins. The E4 gene derived 34 kDa protein (E4orf6) prevents the accumulation of cellular mRNAs in the cytoplasm together with the E1B 55 kDa protein, but also promotes the transport of viral RNAs from the cell nucleus into the cytoplasm.

The viral associated RNA (VA RNA) is a non-coding RNA of adenovirus (Ad), regulating translation. The adenoviral genome comprises two independent copies: VAI (VA RNAI) and VAII (VA RNAII). Both are transcribed by RNA polymerase III (see, e.g., Machitani, M., et al., J. Contr. Rel. 154 (2011) 285-289) from a type 2 polymerases III promoter. For recombinant AAV particle production, the adenoviral VA RNA gene can be driven by any promoter.

The structure, function, and evolution of adenovirus-associated RNA using a phylogenetic approach was investigated by Ma, Y. and Mathews, M.B. (J. Virol. 70 (1996) 5083-5099). They provided alignments as well as consensus VA RNA

sequences based on 47 known human adenovirus serotypes. Said disclosure is herewith incorporated by reference in its entirety into the current application.

VA RNAs, VAI and VAII, are consisting of 157-160 nucleotides (nt).

Depending on the serotype, adenoviruses contain one or two VA RNA genes. VA RNAI is believed to play the dominant pro-viral role, while VA RNAII can partially compensate for the absence of VA RNAI (Vachon, V.K. and Conn, G.L., Virus Res. 212 (2016) 39-52).

The VA RNAs are not essential, but play an important role in efficient viral growth by overcoming cellular antiviral machinery. That is, although VA RNAs are not essential for viral growth, VA RNA-deleted adenovirus cannot grow during the initial step of vector generation, where only a few copies of the viral genome are present per cell, possibly because viral genes other than VA RNAs that block the cellular antiviral machinery may not be sufficiently expressed (see Maekawa, A., et al. Nature Sci. Rep. 3 (2013) 1136).

Maekawa, A., et al. (Nature Sci. Rep. 3 (2013) 1136) reported efficient production of adenovirus vector lacking genes of virus-associated RNAs that disturb cellular RNAi machinery, wherein HEK293 cells that constitutively and highly express flippase recombinase were infected to obtain VA RNA-deleted adenovirus by FLP recombinase-mediated excision of the VA RNA locus.

The human adenovirus 2 VA RNAI corresponds to nucleotides 10586-10810 of GenBank entry AC_000007 sequence. The human adenovirus 5 VA RNAI corresponds to nucleotides 10579-10820 of GenBank entry AC_000008 sequence.

GENERAL DESCRIPTION OF RECOMBINANT AAV PARTICLE PRODUCTION

After entry into the host cell nucleus, AAV can follow either one of two distinct and interchangeable pathways of its life cycle: the lytic or the lysogenic. The former develops in cells infected with a helper virus such as Ad or herpes simplex virus (HSV) whereas the latter is established in host cells in the absence of a helper virus.

When a latently infected cell is super-infected with a helper virus, the AAV gene expression program is activated leading to the AAV Rep-mediated rescue (i.e., excision) of the provirus DNA from the host cell chromosome followed by replication and packaging of the viral genome. Finally, upon helper virus-induced

cell lysis, the newly assembled virions (particles) are released. Thus, the lytic phase of the AAV life cycle is induced.

Therefore, in the presence of Ad helper functions, the rAAV vector is subjected to the wild-type AAV lytic processes by being rescued from the plasmid backbone, replicated and packaged into preformed AAV capsids as single-stranded molecules (Gonçalves, M.A.F.V., Virol. J., 2 (2005) 43).

Generation of a recombinant AAV particle involves replacing a majority of the AAV's wild-type genome with a desired transgene and providing the viral genes that are essential for virus packaging in-trans on a separate plasmid. Once all components are transfected together into a packaging cell line, recombinant AAV particles are assembled using the cell's cellular machineries. The process of viral assembly and encapsulation takes roughly two days, after which the cells are lysed to release the rAAV for further purification and concentration (https://old.abmgood.com/marketing/knowledge_base/Adeno_Associated_Virus_Production_and_Modification_of_AAV.php).

AAV is not released very efficiently from the cells, although major differences have been observed between serotypes (see, e.g., Strobel, B., et al., Lamla T. Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. Hum. Gene Ther. Methods 26 (2015) 147-157). When harvesting the culture, a cell disruption method is usually applied to recover the vectors entrapped in the cells.

Historically, manufacturing of rAAVps was performed by double transfection of a plasmid containing the rep and the cap ORFs and a plasmid with the gene of interest flanked by ITRs. Then, a helper virus, typically Adenovirus, was co-infected (see, e.g., Aponte-Ubillus, J.J., et al., Appl. Microbiol. Biotechnol. 102 (2018) 1045-1054; Muzyczka, N., Curr. Top. Microbiol. Immunol. 158 (1992) 97-129). In this setting, the separation of the helper virus from the final product was difficult, but a critical element to avoid induction of inflammatory responses after injection into patients (see, e.g., Schnell, M.A., et al., Mol. Ther. 3 (2001) 708-722.). Therefore, production of rAAVps nowadays moved towards an adenovirus-free approach by utilizing triple transfection (see, e.g., Large, E.E., et al., Viruses 13 (2021) 1336). To this end, three components are needed: one plasmid encoding the genes for Rep and Cap without the ITRs, a second plasmid with the transgene of interest flanked by ITRs, and a helper plasmid to provide the helper genes of the helper virus (see, e.g., Aponte-

Ubillus, J.J., et al., Appl. Microbiol. Biotechnol. 102 (2018) 1045-1054; Farris, K.D. and Pintel, D.J., Hum. Gene Ther. 19 (2008) 1421-1427; Grimm, D., et al., Hum. Gene Ther. 9 (1998) 2745-2760; Ferrari, F.K., et al., Nat. Med. 3 (1997) 1295-1297). For example, the Adenovirus helper bears the minimal required adenoviral genes E2A, E4 and VA. It is important to note, that the Human Embryonic Kidney cells 293 (HEK293) constitutively express the adenoviral genes E1A/B, which are also required for production of rAAVps. Therefore, HEK293 cells are classic producer cells for rAAVps and for manufacturing. Other cell types require a supplementation of E1A/B.

Carter et al. have shown that the entire rep and cap open reading frames in the wild-type AAV genome can be deleted and replaced with a transgene (Carter, B. J., in "Handbook of Parvoviruses", ed. by P. Tijssen, CRC Press, pp. 155-168 (1990)). Further, it has been reported that the ITRs have to be maintained to retain the function of replication, rescue, packaging, and integration of the transgene into the genome of the target cell.

When cells comprising the respective viral helper genes are transduced by an AAV vector, or, vice versa, when cells comprising an integrated AAV provirus are transduced by a suitable helper virus, then the AAV provirus is activated and enters a lytic infection cycle again (Clark, K.R., et al., Hum. Gene Ther. 6 (1995) 1329-1341; Samulski, R.J., Curr. Opin. Genet. Dev. 3 (1993) 74-80).

Producer cells contain the rep and cap gene sequences, as well as the transgene cassette flanked by ITR sequences on one or more plasmids that are retained, e.g., via drug selection. Production of rAAV in these cell lines generally occurs after their infection with the required helper functions. Therefore, cells are infected either with replication-competent adenovirus (usually wild type Ad5) or a plasmid comprising the respective helper genes to supply helper virus proteins and initiate rAAV production. A packaging cell line differs from a producer cell line as it only contains the rep and cap genes.

More generally, cells transfected or transduced with DNA for the recombinant production of AAV particles can be referred to as a "recombinant cell". Such a cell can be any mammalian cell that has been used as recipient of a nucleic acid (plasmid) encoding packaging proteins, such as AAV packaging proteins, a nucleic acid (plasmid) encoding helper proteins, and a nucleic acid (plasmid) that encodes a protein or is transcribed into a transcript of interest, i.e. a transgene placed between

two AAV ITRs. The term includes the progeny of the original cell, which has been transduced or transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total nucleic acid complement as the original parent, due to natural, accidental, or deliberate mutation.

Numerous cell growth media appropriate for sustaining cell viability or providing cell growth and/or proliferation are commercially available. Examples of such medium include serum free eukaryotic growth mediums, such as medium for sustaining viability or providing for the growth of mammalian (e.g., human) cells. Non-limiting examples include Ham's F12 or F12K medium (Sigma-Aldrich), FreeStyle (FS) F17 medium (Thermo-Fisher Scientific), MEM, DMEM, RPMI-1640 (Thermo-Fisher Scientific) and mixtures thereof. Such media can be supplemented with vitamins and/or trace minerals and/or salts and/or amino acids, such as essential amino acids for mammalian (e.g., human) cells.

For producing rAAV, three plasmids are co-transfected into a mammalian cell. The transgene plasmid encodes the expression cassette, which is cloned between the AAV ITRs, whereas rep and cap genes are provided in trans by co-transfecting a second, packaging plasmid (rep/cap plasmid) to ensure AAV replication and packaging. The third plasmid, also referred to as helper plasmid, contains the minimal helper virus factors, commonly adenoviral E2A, E4orf6 and VA genes, but lacking AAV ITRs.

Diverse methods for the DNA transfer into mammalian cells have been reported in the art. These are all useful in the methods according to the current invention. In certain embodiments of all aspects and embodiments, electroporation, nucleofection, or microinjection for nucleic acid transfer/transfection is used. In certain embodiments of all aspects and embodiments, an inorganic substance (such as, e.g., calcium phosphate/DNA co-precipitation), a cationic polymer (such as, e.g., polyethylenimine, DEAE-dextran), or a cationic lipid (lipofection) is used for nucleic acid transfer/transfection is used. Calcium phosphate and polyethylenimine are the most commonly used reagents for transfection for nucleic acid transfer in larger scales (see, e.g., Baldi et al., Biotechnol. Lett. 29 (2007) 677-684), whereof polyethylenimine is preferred.

The growth in serum-free suspension culture and improvement of efficiency and reproducibility of transfection conditions using PEI as a transfection reagent permits

ready scale-up the AAV production using shake-flasks, wave, or stirred-tank bioreactors.

The composition may comprise further plasmids or/and cells. Such plasmids and cells may be in contact with free PEI.

In addition to PEI, valproic acid (VPA) can be used to improve transfection efficiency. VPA, a branched short-chain fatty acid and inhibits histone deacetylase activity. Due to this reason, it is commonly added to mammalian cell culture as an enhancer of recombinant protein production.

Encoded AAV packaging proteins include, in certain embodiments of all aspects and embodiments, AAV rep and/or AAV cap. Such AAV packaging proteins include, in certain embodiments of all aspects and embodiments, AAV rep and/or AAV cap proteins of any AAV serotype.

Encoded helper proteins include, in certain embodiments of all aspects and embodiments, adenovirus E1A and E1B, adenovirus E2 and/or E4, VA RNA, and/or non-AAV helper proteins.

The cultivation can be performed using the generally used conditions for the cultivation of eukaryotic cells of about 37 °C, 95 % humidity and 8 vol.-% CO2. The cultivation can be performed in serum containing or serum free medium, in adherent culture or in suspension culture. The suspension cultivation can be performed in any fermentation vessel, such as, e.g., in stirred tank reactors, wave reactors, rocking bioreactors, shaker vessels or spinner vessels or so called roller bottles. Transfection can be performed in high throughput format and screening, respectively, e.g. in a 96 or 384 well format.

Methods according to the current invention can include AAV particles of any serotype, or a variant thereof. In certain embodiments of all aspects and embodiments, a recombinant AAV particle comprises any of AAV serotypes 1-12, an AAV VP1, VP2 and/or VP3 capsid protein, or a modified or variant AAV VP1, VP2 and/or VP3 capsid protein, or wild-type AAV VP1, VP2 and/or VP3 capsid protein. In certain embodiments of all aspects and embodiments, an AAV particle comprises an AAV serotype or an AAV pseudotype, where the AAV pseudotype comprises an AAV capsid serotype different from an ITR serotype.

Expression control elements include constitutive or regulatable control elements, such as a tissue-specific expression control element or promoter.

ITRs can be any of AAV2 or AAV6 or AAV8 or AAV9 serotypes, or a combination thereof. AAV particles can include any VP1, VP2 and/or VP3 capsid protein having 75 % or more sequence identity to any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV10, AAV11, AAV12, AAV 2i8, AAV rh.10, AAV rh.74 or AAV 7m8 VP1, VP2 and/or VP3 capsid proteins, or comprises a modified or variant VP1, VP2 and/or VP3 capsid protein selected from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAV-2i8, AAV-rh.10, AAV-rh.74 and AAV-7m8 AAV serotypes.

Following production of recombinant viral (e.g., AAV) particles, if desired, the viral (e.g., rAAV) particles can be purified and/or isolated from host cells using a variety of conventional methods. Such methods include column chromatography, CsCl gradients, iodixanol gradient and the like.

For example, a plurality of column purification steps such as purification over an anion exchange column, an affinity column and/or a cation exchange column can be used. (See, e.g., WO 02/12455 and US 2003/0207439). Alternatively, or in addition, an iodixanol or CsCl gradient steps can be used (see, e.g., US 2012/0135515; and US 2013/0072548). Further, if the use of infectious virus is employed to express the packaging and/or helper proteins, residual virus can be inactivated, using various methods. For example, adenovirus can be inactivated by heating to temperatures of approximately 60 °C for, e.g., 20 minutes or more. This treatment effectively inactivates the helper virus since AAV is heat stable while the helper adenovirus is heat labile.

An objective in the rAAV production and purification systems is to implement strategies to minimize/control the generation of production related impurities such as proteins, nucleic acids, and vector-related impurities, including wild-type/pseudo wild-type AAV species (wtAAV) and AAV-encapsulated residual DNA impurities.

Considering that the rAAV represents only a minor fraction of the biomass, rAAV need to be purified to a level of purity, which can be used as a clinical human gene therapy product (see, e.g., Smith P.H., et al., Mo. Therapy 7 (2003) 8348; Chadeuf G., et al, Mo. Therapy 12 (2005) 744; report from the CHMP gene therapy expert group meeting, European Medicines Agency EMEA/CHMP 2005, 183989/2004).

In certain embodiments of all aspects and embodiments of the method according to the current invention, as an initial step, typically the cultivated cells that produce the rAAVps are harvested, optionally in combination with harvesting cell culture supernatant (medium) in which the cells (suspension or adherent) producing recombinant AAV particles have been cultured. The harvested cells and optionally cell culture supernatant may be used as is, as appropriate, lysed or concentrated. Further, if infection is employed to express helper functions, residual helper virus can be inactivated. For example, adenovirus can be inactivated by heating to temperatures of approximately 60 °C for, e.g., 20 minutes or more, which inactivates only the helper virus since AAV is heat stable while the helper adenovirus is heat labile.

The cells in the harvested cultivation broth can be lysed using methods now in the art, such as, e.g., detergent lysis or freeze-thaw cycles, to release the rAAV particles. Concurrently during cell lysis or subsequently after cell lysis, a nuclease, such as, e.g., benzonase, is added to degrade contaminating DNA. Typically, the resulting lysate is clarified to remove cell debris, e.g. by filtering or centrifuging, to render a clarified cell lysate. In a particular example, the lysate is filtered with a micron diameter pore size filter (such as a 0.1-10.0 μm pore size filter, for example, a 0.45 μm and/or pore size 0.2 μm filter), to produce a clarified lysate.

The lysate (optionally clarified) contains recombinant AAV particles (comprising full as well as empty rAAVps) and production/process related impurities, such as soluble cellular components from the host cells that can include, inter alia, cellular proteins, lipids, and/or nucleic acids, and cell culture medium components. The optionally clarified lysate is then subjected to purification steps to purify the rAAV (comprising rAAV vectors) from impurities using chromatography. The clarified lysate may be diluted or concentrated with an appropriate buffer prior to the first chromatography step.

After cell lysis, optional clarifying, and optional dilution or concentration, a plurality of subsequent and sequential chromatography steps can be used to purify the rAAV.

The first chromatography step is preferably an affinity chromatography step using an AAV affinity chromatography ligand.

If the first chromatography step is affinity chromatography the second chromatography step can be anion exchange chromatography. Thus, in certain embodiments of all aspects and embodiments, rAAV purification is via affinity

chromatography, followed by purification via anion exchange chromatography or/and cation exchange chromatography or/and size exclusion chromatography, in any order or sequence or combination.

The removal of empty capsids from full ones, for example, during downstream processing is based on their different isoelectric points (pI) in anion exchange chromatography. The average calculated pI across all serotypes is 5.9 for full capsids and 6.3 for empty capsids (Venkatakrishnan, B., et al., J. Virol. 87 (2013) 4974-4984).

Cation exchange chromatography functions to separate the AAV from cellular and other components present in the clarified lysate and/or column eluate from an affinity or size exclusion chromatography. Examples of strong cation exchange resins capable of binding rAAV over a wide pH range include, without limitation, any sulfonic acid based resin as indicated by the presence of the sulfonate functional group, including aryl and alkyl substituted sulfonates, such as sulfopropyl or sulfoethyl resins. Representative matrices include but are not limited to POROS HS, POROS HS 50, POROS XS, POROS SP, and POROS S (strong cation exchangers available from Thermo Fisher Scientific, Inc., Waltham, MA, USA). Additional examples include Capto S, Capto S ImpAct, Capto S ImpRes (strong cation exchangers available from GE Healthcare, Marlborough, MA, USA), and commercial DOWEX®, AMBERLITE®, and AMBERLYST® families of resins available from Aldrich Chemical Company (Milliwaukee, WI, USA). Weak cation exchange resins include, without limitation, any carboxylic acid based resin. Exemplary cation exchange resins include carboxymethyl (CM), phospho (based on the phosphate functional group), methyl sulfonate (S) and sulfopropyl (SP) resins.

Anion exchange chromatography functions to separate rAAV from proteins, cellular and other components present in the clarified lysate and/or column eluate from an affinity or cation exchange or size exclusion chromatography. Anion exchange chromatography can also be used to reduce and thereby control the amount of empty rAAV in the eluate. For example, the anion exchange column having full and empty rAAV bound thereto can be washed with a solution comprising NaCl at a modest concentration (e.g., about 100-125 mM, such as 110-115 mM) and a portion of the empty rAAV can be eluted in the flow through without substantial elution of the full rAAV. Subsequently, full rAAV bound to the anion exchange column can be eluted using a solution comprising NaCl at a higher concentration (e.g., about 130-300 mM NaCl), thereby producing a column eluate with reduced or depleted amounts of

empty rAAVps and proportionally increased amounts of full rAAV comprising an rAAV vector.

Exemplary anion exchange resins include, without limitation, those based on polyamine resins and other resins. Examples of strong anion exchange resins include those based generally on the quaternized nitrogen atom including, without limitation, quaternary ammonium salt resins such as trialkylbenzyl ammonium resins. Suitable exchange chromatography materials include, without limitation, MACRO PREP Q (strong anion-exchanger available from BioRad, Hercules, CA, USA); UNOSPHERE Q (strong anion-exchanger available from BioRad, Hercules, CA, USA); POROS 50HQ (strong anion-exchanger available from Applied Biosystems, Foster City, CA, USA); POROS XQ (strong anion-exchanger available from Applied Biosystems, Foster City, CA, USA); POROS SOD (weak anion-exchanger available from Applied Biosystems, Foster City, CA, USA); POROS 50PI (weak anion-exchanger available from Applied Biosystems, Foster City, CA, USA); Capto Q, Capto XQ, Capto Q ImpRes, and SOURCE 30Q (strong anion-exchanger available from GE healthcare, Marlborough, MA, USA); DEAE SEPHAROSE (weak anion-exchanger available from Amersham Biosciences, Piscataway, NJ, USA); Q SEPHAROSE (strong anion-exchanger available from Amersham Biosciences, Piscataway, NJ, USA). Additional exemplary anion exchange resins include aminoethyl (AE), diethylaminoethyl (DEAE), diethylaminopropyl (DEPE) and quaternary amino ethyl (QAE).

A commercial manufacturing process to purify recombinant AAV particles intended as a product to treat human disease should achieve the following objectives: 1) consistent particle purity, potency and safety; 2) manufacturing process scalability; and 3) acceptable cost of manufacturing.

Exemplary processes for recombinant AAV particle purification are reported in WO 2019/006390.

Methods to determine infectious titer of rAAV particles containing a transgene are known in the art (see, e.g., Zhen et al., Hum. Gene Ther. 15 (2004) 709). Methods for assaying for empty rAAV and full rAAV with packaged transgenes are known (see, e.g., Grimm et al., Gene Therapy 6 (1999) 1322-1330; Sommer et al., Malec. Ther. 7 (2003) 122-128).

To determine the presence or amount of degraded/denatured capsid, purified rAAV can be subjected to SDS-polyacrylamide gel electrophoresis, consisting of any gel

capable of separating the three capsid proteins, for example, a gradient gel, then running the gel until sample is separated, and blotting the gel onto nylon or nitrocellulose membranes. Anti-AAV capsid antibodies are then used as primary antibodies that bind to denatured capsid proteins (see, e.g., Wobus et al., J. Viral. 74 (2000) 9281-9293). A secondary antibody that binds to the primary antibody contains a means for detecting the primary antibody. Binding between the primary and secondary antibodies is detected semi-quantitatively to determine the amount of capsids. Another method would be analytical HPLC with a SEC column or analytical ultracentrifuge.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The invention is based, at least in part, on the finding that capsid titer, genomic titer or/and full-to-empty ratio of rAAVps produced with a mammalian cell is influenced by the addition of production enhancers. It has been found that by the addition of production enhancers the viral genomic titer or/and the full-to-empty ratio can be increased.

In more detail, it has been found that the effect is substance class or even compound dependent. That is, it has been found that in certain substance classes not all compounds have an effect but only certain members thereof.

The invention is based, at least in part, on the finding that not all compounds are useful as production enhancers.

The invention is based, at least in part, on the finding that production enhancers used in DDR (DNA damage response signaling) need to induce DNA damage response without actually damaging the replicating viral vector DNA as it would otherwise negatively impact viral genome integrity. Thus, compounds such as, e.g., cisplatin or DNA integrating substances, have been found to be not useful in a method according to the current invention.

The invention is based, at least in part, on the finding that suitable production enhancers should enhance dNTP synthesis by co-factors or other building blocks (such as folinic acid) or upregulating dNTP synthesis or feeder pathways, for example by DDR signaling.

The invention is based, at least in part, on the finding to delay cell death by addition of anti-apoptotic compounds, such as inhibitors of caspases, is beneficial.

The current invention is exemplified in the following with an exemplary stable recombinant AAV producer HEK cell line with the respective elements being doxycycline inducible. This is presented as a mere exemplification of the general teaching of the current invention and shall not be construed as a limitation. Any other stable or transient AAV produced cell line can be used in the method according to the current invention.

The substances selected based on their potential usefulness as AAV production enhancer are listed in Table 1. The identified concentrations, addition time point and solvent are also listed.

Table 1: Compounds.
dai = directly after induction (= day 3 of the cultivation); 2dai = 2 days after induction (= day 5 of the cultivation); empty concentration = condition not tested

substance-no	substance description	concentration 1	concentration 2	concentration 3	concentration 4	addition timing	pathway	solubility	Supplier, Catalog Number	CAS Number
#01	CGP74514A	0.5 µM	5 µM			dai	S-phase induction/ prolongation	DMSO (10 mg/ mL)	Merck (Sigma) 217696	190654-01-4
#02	Etoposide	0.1 µM	1 µM	10 µM		dai	S-phase induction/ prolongation	DMSO (25 mg/ mL)	Merck (Sigma) 341205	33419-42-0
#03	Topotecan	10 µM	100 µM	500 µM		dai	S-phase induction/ prolongation	DMSO (10 mg/ mL)	Merck (Sigma) T2705	123948-87-8

substance-no	substance description	concentration 1	concentration 2	concentration 3	concentration 4	addition timing	pathway	solubility	Supplier, Catalog Number	CAS Number
#04	Purvalanol A	10 µM	50 µM			dai	S-phase induction/ prolongation	DMSO (100 mg/ mL)	Merck (Sigma) 540500	212844-53-6
#05	CD437	1.5 µM	5 µM	15 µM	30 µM	dai	polymerase alpha inhibitor	EtOH (7.97 mg/mL); DMSO (39.85 mg/	Merck (Sigma) C5865	125316-60-1
#06	Suramin	8 µM	24 µM	30 µM		dai	polymerase alpha inhibitor	H2O (10 mg/ mL)	Merck (Sigma) 574625	129-46-4
#07	Z-VAD-FMK	20 µM	50 µM			dai	caspase inhibitor	DMSO (20 mg/ mL)	Fisher Scientific 15826258	187389-52-2
#08	Q-VD-OPh	20 µM	50 µM			dai	caspase inhibitor	solution in DMSO	Merck (Sigma) 551476	1135695-98-5
#09	IDN-6556 (Emricasan)	10 µM	20 µM	50 µM		dai	caspase inhibitor	DMSO (2 mg/ mL)	Merck (Sigma) SML2227	254750-02-2
#10	Z-DEVD-FMK	10 µM				dai	caspase inhibitor	DMSO (20 mg/ mL)	Merck (Sigma) 264155	210344-95-9
#11	Pifithrin-α	10 µM	30 µM			dai	Apoptosis inhibition	DMSO (73 mg/ mL)	Merck (Sigma) 506132	63208-82-2

substance-no	substance description	concentration 1	concentration 2	concentration 3	concentration 4	addition timing	pathway	solubility	Supplier, Catalog Number	CAS Number
#12	Pifithrin-μ	10 μM	30 μM			dai	Apoptosis inhibition	EtOH (36 mg/mL); DMSO (36 mg/mL)	Merck (Sigma) P0122	64984-31-2
#13	Y-27632 (ROCK) inhibitor	10 μM	30 μM			dai	Apoptosis inhibition	solution in H2O	Merck (Sigma) 688001	146986-50-7
#14	BAY 11-7082	2.5 μM	8 μM			dai	antiviral response inhibitor	solution in DMSO	Merck (Sigma) 196871	19542-67-7
#15	C-176	0.5 μM	2 μM			dai	antiviral response inhibitor	EtOH (2 mg/mL); DMSO (72 mg/mL)	Merck (Sigma) SML2559	314054-00-7
#16	ML323	5 μM	50 μM			dai	DDR	EtOH (38 mg/mL); DMSO (76 mg/mL)	Merck (Sigma) SML1177	1572414-83-5
#17	Mirin	5 μM	50 μM			dai	DDR	DMSO (44 mg/mL)	Merck (Sigma) 475954	299953-00-7
#18	GSK2830371	1 μM	50 μM			dai	DDR	DMSO (92 mg/mL)	Merck (Sigma) SML1048	1404456-53-6
#19	BN82002	5 μM	50 μM			dai	DDR + antiviral response	EtOH (10 mg/mL); DMSO (10 mg/mL)	Merck (Sigma) 217691	396073-89-5

substance-no	substance description	concentration 1	concentration 2	concentration 3	concentration 4	addition timing	pathway	solubility	Supplier, Catalog Number	CAS Number
#20	Resveratrol	10 µM	50 µM			dai	DDR, cell cycle	DMSO (50 mg/mL)	Merck (Sigma) 554325	501-36-0
#21	AZD7762	0.01 µM	0.1 µM			dai	DDR	DMSO (15 mg/mL)	Merck (Sigma) SML0350	1246094-78-9
#22	MK-1775 (Adavosertib)	0.05 µM	0.3 µM			dai	DDR	DMSO (100 mg/mL)	Fisher Scientific NC1097015	955365-80-7
#23	Coenzyme Q10	5 µM				dai	Survival, oxidative phosphorylation	EtOH (0.3 mg/mL)	Merck (Supelco) V-060	303-98-0
#24	AICAR	500 µM	1000 µM			dai	Survival, oxidative phosphorylation	DMSO (2 mg/mL)	Thermo J64720.MF	2627-69-2
#25	Leucovorin (folinic acid)	5 µM	50 µM			dai	dNTP synthesis	DMSO (20 mg/mL)	Thermo 230312500	6035-45-6
#26	Ac-DEVD-CHO	1 µM	50 µM	100 µM		dai	caspase inhibitor	H2O (1 mg/mL)	Merck (Sigma) A0835	169332-60-9
#27	Hydroxy-chloroquine	5 µM	15 µM			2dai	endocytosis/ trafficking	PBS (pH 7.2) (5 mg/mL); H2O (87 mg/mL)	VWR (Thermo) ACRO-263010250	747-36-4

substance-no	#28	#29	#30	#31	#32	#33	#34
substance description	Bafilomycin A1	Chloroquine	Brefeldin A	Retro-2	Monensin	MMG-11	Celastrol
concentration 1	0.2 µM	5 µM	0.1 µM	10 µM	0.25 µM	5 µM	1 µM
concentration 2	1 µM	15 µM	3 µM	30 µM	2.5 µM	10 µM	3 µM
concentration 3							
concentration 4							
addition timing	2dai	2dai	2dai	2dai	2dai	dai	dai
pathway	endocytosis/ trafficking	endocytosis/ trafficking	endocytosis/ trafficking	endocytosis/ trafficking	endocytosis/ trafficking	antiviral inhibitor	antiviral inhibitor
solubility	DMSO (100 mg/ mL)	H2O (50 g/L)	DMSO (56 mg/ mL	DMSO (10 mg/ mL)	solution in MeOH	DMSO (2 mg/ mL)	DMSO (>10 mg/ mL)
Supplier, Catalog Number	VWR (Thermo) ACRO-328120001	Merck (Sigma) C6628	Thermo: 00-4506-51 Merck (Sigma): B5936	Merck (Sigma) SML1085	Thermo 00-4505-51	Merck (Sigma) SML3577	Merck (Sigma) C0869
CAS Number	88899-55-2	50-63-5	20350-15-6	1429192-00-6	67-56-1	313254-94-3	34157-83-0

substance-no	substance description	concentration 1	concentration 2	concentration 3	concentration 4	addition timing	pathway	solubility	Supplier, Catalog Number	CAS Number
#35	Caffeic acid	0.25 µM	2.5 µM			dai	AdV replication enhancer	EtOH (50 mg/mL)	Merck (Sigma) 205546	331-39-5
#36	Telenzepine dihydrochloride	0.25 µM	2.5 µM			dai	AdV replication enhancer	H2O (100 mM)	Merck (Sigma) T122	147416-96-4
#37	Altretamine	0.25 µM	2.5 µM			dai	AdV replication enhancer	EtOH (33 mg/mL); DMSO (15 mg/ml)	Thermo 549835	0645-05-06
#38	Maprotiline hydrochloride	0.25 µM	2.5 µM			dai	AdV replication enhancer	solution in MeOH	Merck (Sigma) M-920	10347-81-6
#39	Nifurtimox	0.25 µM	2.5 µM			dai	AdV replication enhancer	DMSO (57 mg/mL)	Merck (Sigma) N3415	23256-30-6

The results obtained in parallel ambr15 cultivations according to Example 1 with the compounds of Table 1 are listed in the following Table 2.

Table 2: Cultivation results; empty = no addition; dai = directly after induction (= day 3 of the cultivation); 2dai = 2 days after induction (= day 5 of the cultivation).

condition – first set	Addition timing	Capsid Titer (ELISA)	Genomic Titer (dPCR)	Percentage full capsids
Reference	no addition	4.4249E+12	1.34E+11	3.0%
Reference	no addition	4.1096E+12	1.87E+11	4.6%

condition – first set	Addition timing	Capsid Titer (ELISA)	Genomic Titer (dPCR)	Percentage full capsids
Reference	no addition	6.0805E+12	1.66E+11	2.7%
Reference	no addition	3.9477E+12	7.29E+10	1.8%
Reference	no addition	3.5946E+12	1.32E+11	3.7%
DMSO 3%	dai	3.7927E+12	3.18E+10	0.8%
MeOH 1.7%	dai	5.5364E+12	7.6E+10	1.4%
Etoposide 0.1 µM	dai	6.9191E+12	9.09E+10	1.3%
Etoposide 1 µM	dai	4.2946E+12	4.79E+10	1.1%
Etoposide 10 µM	dai	4.123E+12	3.95E+10	1.0%
Topotecan 10 µM	dai	1.5025E+12	9.73E+10	0.6%
Topotecan 100 µM	dai	8.3261E+11	2.47E+10	3.0%
Topotecan 500 µM	dai	8.4241E+11	7.52E+10	0.9%
CD437 1.5 µM	dai	3.2608E+12	1.06E+11	3.3%
CD437 15 µM	dai	3.0068E+12	4.81E+10	1.6%
Suramin 8 µM	dai	3.8333E+12	1.08E+11	2.8%
Suramin 24 µM	dai	3.6678E+12	1.29E+11	3.5%
Q-VD-OPh 20 µM	dai	4.6876E+12	2.03E+11	4.3%
Q-VD-OPh 50 µM	dai	5.4748E+12	2.08E+11	3.8%
IDN6556 10 µM	dai	5.5896E+12	1.02E+11	1.8%
IDN6556 50 µM	dai	5.2209E+12	1.48E+11	2.8%
Z-DEVD-FMK 10 µM	dai	4.4221E+12	1.72E+11	3.9%
Y-27632 30 µM	dai	4.037E+12	1.91E+11	4.7%
Pifithrin-α 10 µM	dai	6.4202E+12	1.41E+11	2.2%
Pifithrin-α 30 µM	dai	6.8427E+12	4.59E+10	0.7%
Pifithrin-µ 10 µM	dai	3.2822E+12	5.48E+10	1.7%
Pifithrin-µ 30 µM	dai	6.2661E+12	1.05E+11	1.7%
Y-27632 10 µM	dai	3.3749E+12	1.29E+11	3.8%
Y-27632 30 µM + BAY 11-7082 2.5 µM	dai	4.2796E+12	1.83E+11	4.3%
BAY 11-7082 2.5 µM	dai	5.8512E+12	2.96E+11	5.1%
C-176 0.5 µM	dai	4.9155E+12	1.87E+11	3.8%
C-176 2 µM	dai	4.3801E+12	2.13E+11	4.9%
ML323 5 µM	dai	6.7691E+12	1.75E+11	2.6%
ML323 50 µM	dai	3.2898E+11	1.55e+10	4.7%
Mirin 5 µM	dai	4.9676E+12	1.88E+11	3.8%
Mirin 50 µM	dai	5.1992E+12	1.69E+11	3.3%
GSK2830371 1 µM	dai	5.662E+12	1.07E+11	1.9%

condition – first set	Addition timing	Capsid Titer (ELISA)	Genomic Titer (dPCR)	Percentage full capsids
GSK2830371 50 µM	dai	6.8884E+12	1.65E+11	2.4%
Resveratrol 10 µM	dai	5.5344E+12	1.93E+11	3.5%
Resveratrol 50 µM	dai	5.7442E+12	2.53E+11	4.4%
AZD7762 0.01 µM	dai	5.8099E+12	1.52E+11	2.6%
AZD7762 0.1 µM	dai	5.3298E+12	2.56E+11	4.8%
MK-1775 0.05 µM	dai	4.8404E+12	1.92E+11	4.0%
MK-1775 0.3 µM	dai	4.3979E+12	2.23E+11	5.1%
Coenzyme Q10 5 µM	dai	5.4673E+12	1.74E+11	3.2%
AICAR 500 µM	dai	4.1705E+12	1.49E+11	3.6%
AICAR 1000 µM	dai	4.6269E+12	2.01E+11	4.3%
Leucovorin 5 µM	dai	4.2593E+12	2.39E+11	
Leucovorin 50 µM	dai	4.2874E+12	6.04E+10	1.4%
Ac-DEVD-CHO 1 µM	dai	4.1541E+12	7.25E+10	1.7%
Ac-DEVD-CHO 50 µM	dai	3.3098E+12	1.04E+11	3.1%
Ac-DEVD-CHO 100 µM	dai	3.3022E+12	7.66E+10	2.3%
MMG-11 5 µM	dai	5.7442E+12	8.54E+10	1.5%
MMG-11 10 µM	dai	6.7493E+12	8.83E+10	1.3%
Celastrol 1 µM	dai	5.0129E+12	1.24E+11	2.5%
Celastrol 3 µM	dai	6.587E+12	1.12E+11	1.7%
Caffeic acid 0.25 µM	dai	4.2217E+12	7.27E+10	1.7%
Caffeic acid 2.5 µM	dai	2.9063E+12	1.02E+11	3.5%
Telenzepine dihydrochloride 0.25 µM	dai	4.3105E+12	4.14E+10	1.0%
Telenzepine dihydrochloride 2.5 µM	dai	4.4805E+12	7.27E+10	1.6%
Altretamine 0.25 µM	dai	4.7535E+12	8.65E+10	1.8%
Altretamine 2.5 µM	dai	5.8428E+12	6.54E+10	1.1%
Maprotiline hydrochloride 0.25 µM	dai	4.178E+12	9.41E+10	2.3%

condition – first set	Addition timing	Capsid Titer (ELISA)	Genomic Titer (dPCR)	Percentage full capsids
Maprotiline hydrochloride 2.5 µM	dai	4.7168E+12	1.33E+11	2.8%
Nifurtimox 0.25 µM	dai	4.4964E+12	8.09E+10	
Nifurtimox 2.5 µM	dai	5.4548E+12	4.99E+10	0.9%
Chloroquine 5 µM	dai	4.0142E+12	1.18E+11	2.9%
Chloroquine 15 µM	dai	3.6492E+12	5.16E+10	1.4%
Pifrithin µ 30µM + Etoposide 1 µM	dai	7.0062E+12	1.29E+11	1.8%
Hydroxychloro-quine 5 µM	2dai	3.4625E+12	9.69E+10	2.8%
Hydroxychloro-quine 15 µM	2dai	3.5187E+12	1.53E+11	4.3%
Bafilomycin A1 0.2 µM	2dai	4.16E+12	1.07E+11	2.6%
Bafilomycin A1 1 µM	2dai	4.5097E+12	9.65E+10	2.1%
Chloroquine 5 µM	2dai	4.1847E+12	9.86E+10	2.4%
Chloroquine 15 µM	2dai	4.2351E+12	8.95E+10	2.1%
Brefeldin A 0.1 µM	2dai	3.9421E+12	8.63E+10	2.2%
Brefeldin A 3 µM	2dai	3.9975E+12	1.04E+11	2.6%
Retro-2 10 µM	2dai	4.1775E+12	7.47E+10	1.8%
Retro-2 30 µM	2dai	4.3553E+12	8.48E+10	1.9%
Monensin 0.25 µM	2dai	4.3734E+12	9.38E+10	2.1%
Monensin 2.5 µM	2dai	4.2807E+12	1.06E+11	2.5%

condition – second set	Addition timing	Capsid Titer (ELISA)	Genomic Titer (dPCR)	Percentage full capsids
Reference	no addition	4.6475E+12	2.78E+11	6.0%
Reference	no addition	4.8103E+12	2.66E+11	5.5%
Reference	no addition	4.6477E+12	2.95E+11	6.3%
Reference	no addition	4.8793E+12	2.64E+11	5.4%
Z-VAD-FMK 20 µM	dai	5.1136E+12	2.31E+11	4.5%
Z-VAD-FMK 50 µM	dai	5.7025E+12	2.68E+11	4.7%

condition – second set	Addition timing	Capsid Titer (ELISA)	Genomic Titer (dPCR)	Percentage full capsids
CGP74514A 0.5 µM	dai	5.5989E+12	2.51E+11	4.5%
CGP74514A 5 µM	dai	5.2707E+12	2.12E+11	4.0%
Purvalanol A 10 µM	dai	6.2072E+12	1.03E+11	1.7%
Purvalanol A 50 µM	dai	8.4814E+11		0.0%
BN82002 5 µM	dai	5.7253E+12	2.39E+11	4.2%
BN82002 50 µM	dai	6.0768E+12	3.99E+11	6.6%
Topotecan 100 µM	dai	6.6468E+11	3.50E+10	0.5%
IDN6556 5 µM	dai	5.6944E+12	2.59E+11	4.5%
CD437 30 µM	dai	2.8063E+12	9.69E+10	0.3%
Suramin 30 µM	dai	4.8332E+12	2.58E+11	5.3%
CD437 1.5 µM	dai	3.9982E+12	2.07E+11	5.2%
DMSO 1.5%	dai	7.8835E+12	2.37E+11	3.0%
MK-1775 0.05 µM	dai	5.8076E+12	2.82E+11	4.9%
IDN6556 10 µM	dai	6.4945E+12	2.43E+11	3.7%
MMG-11 5 µM	dai	5.7351E+12	2.44E+11	4.3%
IDN6556 40 µM	dai	5.5021E+12	2.79E+11	5.1%
BAY 11-7082 8 µM	dai	2.0639E+11	2.43E+10	0.1%
Q-VD-OPh 50 µM	dai	6.234E+12	3.49E+11	5.6%
Caffeic acid 0.25 µM	dai	5.208E+12	2.63E+11	5.0%
Ac-DEVD-CHO 1 µM	dai	4.7401E+12	2.77E+11	5.8%
Celastrol 3 µM	dai	7.1033E+12	2.49E+11	3.5%
DMSO 3%	dai	6.055E+12	3.49E+10	0.6%

condition – second set	Addition timing	Capsid Titer (ELISA)	Genomic Titer (dPCR)	Percentage full capsids
EtOH 0.5%	dai	5.6386E+12	2.72E+11	4.8%
Telenzepine dihydrochloride 2.5 µM	dai	4.613E+12	2.75E+11	6.0%
Etoposide 0.1 µM	dai	6.8326E+12	2.1E+11	3.1%
CD437 5 µM	dai	3.5852E+12	9.16E+10	2.6%
CD437 15 µM	dai	4.3874E+12	3.96E+10	0.9%
IDN6556 20 µM	dai	5.2548E+12	3.51E+11	6.7%
Q-VD-OPh 20 µM	dai	5.2497E+12	3.13E+11	6.0%
Leucovorin 50 µM	dai	5.1136E+12	2.43E+11	4.8%
Suramin 24 µM	dai	4.596E+12	3.06E+11	6.7%

As can be seen from the data higher viral capsid titer did not correlate with higher viral genomic titer. Especially the addition of Etoposide 0.1 µM, MMG-11 10µM are unexpectedly negatively impacting the vg yield, but not capsid titers.

The compounds with the best viral genomic titers are listed in the following Table 3. The titers are above the upper limit of the standard deviation of the reference process, i.e. the identical process except for the addition of the substance to be tested.

Table 3: Highest genomic titer.

substance description	**concentration**	**Vg titer increase compared with reference average**	**% full increase compared with reference average**	**Pathway**
BAY11-7082	2.5µM	>+100%	>+100%	Antiviral Response
AZD7762	0.1 µM > 0.01 µM	>+50%	>+50%	DDR (ATM/ATR induction)

substance description	concentration	Vg titer increase compared with reference average	% full increase compared with reference average	Pathway
resveratrol	50 µM > 10 µM	>+80%	>+80%	DDR (ATM/ATR induction)
leucovorin (folinic acid)	5 µM > 50 µM	>+50%	>+50%	dNTP synthesis
MK-1775	0.3 µM > 0.05 µM	>+80%	>+80%	DDR (ATM/ATR induction)
C176	2 µM > 0.5 µM	>+30%	>+30%	Antiviral Response inhibitor
BN82002	50 µM	>+50%	>+50%	cell cycle + anti-inflamm.
Q-VD-OPh	50 µM	>+50%	>+50%	Caspase (pan)
BAY11-7082	> 20µM	>+50%	>+50%	antiviral response inhibitor
AZD7762	0.1 µM > 0.01 µM	>+50%	>+50%	DDR (ATM/ATR induction)
AICAR	1000 µM > 500 µM	>+30%	>+30%	Oxidative phosphorylation
Y-27632	30 µM	>+30%	>+30%	Apoptosis inhibitor
IDN-6556	20 µM	+30%	+15%	Caspase

In Figure 1 the best results with respect to viral genomic titer (= above upper limit standard deviation reference processes) are marked by striping and the results are sorted by descending full-to-empty ratio.

It has been unexpectedly found that the tested substance from the following classes show no suitable effect:

- Polymerase alpha inhibitors;
- Endocytosis inhibitors;
- Topoisomerase inhibitors;

- Coenzyme Q10 (essential component of the oxidative phosphorylation pathway).

Thus, it has been found that the following compounds show a beneficial effect when added at least at the induction of recombinant AAV particle expression in a stable AAV producing cell line:

Caspase inhibitor:	IDN-6556 @ 10-50 µM Q-VD-OPh @ 20-50 µM
DDR / ATM/ATR induction:	especially MK-1775 @ 0.05-0.3 µM and AZD7762 @ 0.01-0.1 µM Resveratrol @10-50 µM
Antiviral response inhibitors:	especially BAY 11-7082 @ 2.5 µM C176 @ 0.5-2 µM BN82002 @ 50 µM
Oxidative phosphorylation agents:	especially AICAR @ 500-1000 µM
Phosphorylation /AMPK activator:	especially AICAR @ 500-1000 µM
Inhibitor of κB kinase (IKK):	especially BAY 11-7082 @ 2.5 µM
dNTP (thymidylate) synthesis co-factor:	especially Leucovorin® @ 5 µM
Cell cycle modulators:	especially Resveratrol @ 10-50 µM BN82002 @ 50 µM

The invention is based, at least in part, on the finding that apoptosis inhibition by caspase inhibition with the caspase inhibitor Q-VD-OPh (pan caspase inhibitor) is beneficial for rAAVp production.

The invention is based, at least on part, on the finding that inhibition of antiviral response is beneficial with respect to viral genome titer.

The invention is based, at least on part, on the finding that increase of oxidative phosphorylation is beneficial for rAAVp production.

The invention is based, at least on part, on the finding that an increase in dNTP synthesis is beneficial for rAAVp production.

It can be seen that the compounds ML323, chloroquine and Topotecan, are not suitable.

It can be seen that the tested adenoviral replication enhancing substances have no enhancing effect on rAAVp production.

It can be seen that the tested endocytosis inhibition substances have no enhancing effect on rAAVp production.

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The examples and the figure are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

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Examples

Example 1

Cultivation of HEK293 cells and production of recombinant AAV preparations

Generally, the cultivation methods have been adapted from standard protocols (see, e.g., Lindl, T., "Zell- und Gewebekultur: Einführung in die Grundlagen sowie ausgewählte Methoden und Anwendungen", Spektrum Akademischer Verlag GmbH, Heidelberg/Berlin, 2002) and operating instructions of the respective supplier.

Pre-cultivation

HEK cells were thawed and propagated in shake flasks at 37 °C, 85 % humidity, a pCO2 of 5% and a shaking frequency of 120 rpm for two to three weeks in cultivation medium. Cells were split every three to four days and expanded in medium to the volume required for inoculation of the production cultivation.

Production cultivation

For producing recombinant AAV particles, the respective pre-cultivated HEK293 cells were cultured in the respective reactor in a batch or fed-batch process under the indicated conditions.

rAAVp preparation: 1) with particles comprising capsid variant derived from AAV2 serotype and therapeutic transgene; 2) with particles comprising an AAV2 wild type capsid and green fluorescent protein (GFP) transgene:

reactor: Ambr15
cell line: HEK293 cell line stably transfected with all elements required for rAAVp production; doxycycline inducible cell line
cultivation medium: Gibco™ Protein Expression Medium (PEM; Thermo Fisher Scientific; Cat-No.: 10621864) + 4 mM GlutaMAX™ (Thermo Fisher Scientific; Cat-No.: 35050061)
feed: HEK FS (Sartorius; Cat-No.: 871-0001)

temperature: about 37 °C
pH: about 7.2
inoculation VCD: ~ 30 E+05 cells/mL
induction: day 3; doxycycline
duration: 7 days

Example 2

Lysis

In case lysis was included in the process, it has been performed as follows: To release the AAV particles into the cell culture broth, 5 % (v/v) of lysis buffer (10 % Triton CG 110, 40 mM $MgCl_2$) was added to the culture broth. Additionally, 100 U/ml Benzonase™ nuclease (Merck). The cell culture broth was then incubated for about one hour at 37 °C with stirring, without aeration and pH control. After the respective incubation, 5 M NaCl solution was added and the lysate was sterile filtered.

Example 3

AAV particle purification

For the affinity chromatography step a column comprising 10.5 mL AAVX resin from Thermo Fisher was used on an Äkta Avant 25 chromatography system. The system was run at a flow rate of about 300 cm/h. After equilibration with buffer A (1x PBS, pH 7.4, 0.001% Pluronic F-68) 200 mL of the lysed culture broth was applied to the column followed by 2 wash steps with equilibration buffer and 0.5 M NaCl, pH 6.0, respectively. AAV particles were eluted with 0.1 M sodium citrate solution, pH 2.4. The pH of the eluate was adjusted to pH 7.5 by addition of 2 M Tris, pH 10.

step	**buffer**	**column volumes [CV]**
equilibration	1xPBS, pH 7.4	3
load	lysate	
wash I	1xPBS, pH 7.4	4
wash II	0.5 M sodium chloride pH 6.0	4

wash III	1xPBS, pH 7.4	4
elution	0.1 M sodium citrate pH 2.4	3

Example 4

Analytical methods

Enzyme-linked immunosorbent assay (ELISA) for total titer determination

For AAV capsid titer determination the kit from PROGEN (Cat. no PRAAV8) was used according to the manufacturer's instructions.

In short, this assay is a sandwich ELISA using as capture antibody a recombinant AAV capsid specific antibody and a biotin-labeled detection antibody.

The wells of the pre-coated multi-titer plate (MTP) were incubated overnight with 100 µL standard, sample or control, respectively, at 4 °C. The next day the wells were washed three times with ASSB buffer (1x) as provided in the kit. Thereafter 100 µL per well of a solution comprising the biotinylated detection antibody (diluted according to the manufacturer's instructions) were added and incubated for two hours at room temperature with shaking. Afterwards, the wells were washed three times with ASSB buffer (1x) as provided in the kit. In the next step 100 µl of a solution comprising horseradish peroxidase conjugated to streptavidin was added to each well and incubated for 30 min. At room temperature with shaking. Afterwards, the wells were washed three times with ASSB buffer (1x) as provided in the kit. For color reaction 100 µL of a solution comprising ABTS prepared according to the manufacturer's instructions was added to each well and incubated with shaking. The color intensity was determined suing an MTP-ELISA-Reader Versa Max (Molecular Devices) at 405 nm with a reference wavelength of 490 nm until the difference in the extinction between blank and the standard with the highest concentration reaches about 1.5.

Each sample, standard and control was measured in duplicate.

The amount of capsids (capsids/mL) was calculated based on a standard curve determined by a 4-parameter fitting, e.g. according to the WiemerRodbard algorithm, using the average values of the standards.

Digital droplet polymerase chain reaction (ddPCR) for genomic titer determination

Reagents for enzymatic sample treatment:

1) DNase I buffer (NEB): 100 mM Tris-HCl, pH 7.6, 25 mM $MgSO_4$, 5 mM $CaCl_2$

2) DNase I (NEB): 0.2 U/µL

3) Proteinase K (NEB; approx. 20 mg/mL = 800 U/mL): 16 U / mL

4) proteinase K buffer (BioRad): 400 mM Tris-HCl, 20 mM EDTA, 2000 mM NaCl, 1 % SDS, pH 8

5) sodium dodecyl sulfate (SDS) solution: 10 % (w/v)

Enzymatic sample treatment:

- mix 30 µL H2O, 5 µL DNase I buffer, 5 µL DNase I, 10 µL sample
- incubate at 37 °C for 30 min.
- heat to 75 °C for 15 min. to obtain an incubated DNase I-Mix
- short cool down and centrifugation
- mix 42 µL H2O + 2 µL proteinase K + 5 µL proteinase K buffer + 1 µL 10 % SDS solution and add the incubated DNase I-Mix
- incubate for 60 min. at 50 °C
- heat to 95 °C for 15 min.
- cooling to 4 °C

ddPCR:

For viral genome titration, a duplexing ddPCR assay was performed. Primer and probes were designed against the used CMV promoter and against the polyA/3'UTR sequence. The PCR mastermix was prepared according to the following Table (droplet digital PCR guide - Bio-Rad).

components	volume per well [µL]	final concentration
Supermix (2x)	11	1x
20 µM CMV primer fwd.	0.99	900 nM
20 µM CMV primer rev	0.99	900 nM
20 µM CMV probe	0.275	250 nM
template	5.5	-
water	0.99	-
total	22	

The prepared mastermix was pipetted into a 96 well plate with 16.5 µL per well. Then, dilution series of the pretreated samples were conducted: 10 µL of samples were transferred with LoRentention Tips into 90 µL water in LoBind Tubes and thoroughly mixed. Thereafter, 5.5 µL of the samples were added to the mastermix solution in the 96 well plate in several dilution steps. The plate was sealed at 180 °C, vortexed at 2,200 rpm for 1 min. and centrifuged at 1,000 rpm for another 1 min. With an automatic droplet generator device, which takes 20 µL PCR mix out of each well, up 20,000 droplets per well were produced and transferred into another 96 well plate. After sealing the droplet plate at 180 °C, a PCR run was carried out. The respective conditions are shown in the following Table.

number of cycles	denaturation	annealing	final elongation	end
1	94 °C, 10 min.			
39	94 °C, 30 sec.	58 °C, 1 min.		

1			98 °C, 10 min.	constant at 12 °C

In a droplet reader, the fluorescence signal was measured for each droplet. The QuantaSoft software processed the reader data and calculated copy numbers per 20 µL well for the target sequences. Initial sample titers can be determined with following equation:

$$copy\ number\left[\frac{copies}{mL}\right] = \frac{output\left[\frac{copies}{20\mu L\ well}\right]}{5\left[\frac{\mu L\ sample}{20\mu L\ well}\right]} \cdot dilution\ factor \cdot\ 1000\left[\frac{\mu L}{mL}\right]$$

Patent Claims

1. A method for producing a recombinant adeno-associated viral particle (rAAVp) preparation comprising the steps of

- cultivating a mammalian cell comprising

(a) a gene encoding a non-adeno-associated-virus gene, which is operably linked to two AAV inverted terminal repeats (ITRs) (i.e. the non-adeno-associated virus gene is interspaced between the two AAV ITRs),

(b) a gene encoding the AAV Cap protein VP1 and/or VP3,

(c) a gene encoding the AAV Rep protein Rep78 or Rep68 and/or a gene encoding the AAV Rep protein Rep52 or Rep40,

(d) a gene encoding the adenoviral helper function E4orf6, and

(e) a gene encoding the adenoviral helper function E2A,

- recovering the rAAVp from the cell and/or the cultivation medium,

- optionally purifying the rAAVp,

and thereby producing the rAAVp preparation,

whereby the cultivating of the mammalian cell is in the presence of at least one compound selected from the following compounds,

(i) Q-VD-OPh,

(ii) MK-1775,

(iii) AZD7762,

(iv) AICAR,

(v) BAY 11-7082,

(vi) folinic acid,

(vii) resveratrol,

(viii) C176,

(ix) BN82002,

(x) Y-27632,

(xi) IDN-6556.

2. The method according to claim 1, wherein the cultivating is at least in the presence of Q-VD-OPh at a starting concentration of about 20-50 µM.

3. The method according to any one of claims 1 to 2, wherein the cultivating is at least in the presence of MK-1775 at a starting concentration of about 0.05-0.3 µM or/and in the presence of AZD7762 at a starting concentration of about 0.01-0.1 µM.

4. The method according to any one of claims 1 to 3, wherein the cultivating is at least in the presence of AICAR at a starting concentration of about 500-1000 µM.

5. The method according to any one of claims 1 to 4, wherein the cultivating is at least in the presence of BAY 11-7082 at a starting concentration of about 2.5 µM.

6. The method according to any one of claims 1 to 5, wherein the cultivating is at least in the presence of folinic acid at a starting concentration of about 5 µM.

7. The method according to any one of claims 1 to 6, wherein the cultivating is at least in the presence of resveratrol at a starting concentration of about 10-50 µM.

8. The method according to any one of claims 1 to 7, wherein the cultivating is in the presence of IDN-6556 at a starting concentration of about 10-50 µM or in the presence of Q-VD-OPh at a starting concentration of about 20-50 µM.

9. The method according to any one of claims 1 to 8, wherein

- one or both of the genes encoding the adenoviral helper functions is/are gene under control of an inducible promoter, or/and
- the gene encoding the AAV Cap protein or/and the gene encoding the AAV Rep protein is/are under control of an inducible promoter.

10. The method according to claim 9, wherein the inducible promoter is a doxycycline-inducible promoter.

11. The method according to any one of claim 9 to 10, wherein the cultivating step comprises a first phase and a second phase, whereby the cell is cultivated in the first phase in the absence of the substance and at the start of the second phase the inducible promoter is induced and the compound is added.

12. The method according to claim 11, wherein the first phase is 1, 2 or 3 days.

13. The method according to any one of claims 9 to 10, wherein the cultivating step comprises a first phase, a second phase and a third phase, whereby the cell is cultivated in the first and the second phase in the absence of the substance, at the start of the second phase the inducible promoter is induced and at the start of the third phase the compound is added.

14. The method according to claim 13, wherein the first phase is 1, 2 or 3 days and the second phase is 1 or 2 days.