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(54) Title: NELFINAVIR FOR TREATMENT OF ADENOVIRAL INFECTION

(57) Abstract: The present invention relates to a compound for use in a method for treating or preventing an adenoviral infection, particular a human adenovirus infection or a murine adenovirus infection, wherein said compound is selected from Nelfinavir or a pharmaceutically acceptable formulation thereof, particularly Nelfinavir mesylate.

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Nelfinavir in treatment of adenoviral infection

The present invention relates to the use of Nelfinavir in treatment of adenoviral infection.

This International Application claims the right of priority of European Application EP18205033.6 filed 7 November 2018.

5 Description

Adenoviruses (AdVs) infect numerous species with high selectivity, including fish, birds, reptiles and human. Human Adenovirus (HAdV) was first isolated in 1953. HAdV belong to the family of *Mastadenoviruses*, comprising 103 genotypes that are grouped into seven species (<http://hadvwg.gmu.edu>). The species show heterogenous tissue tropism, and are associated with different diseases, such as respiratory disease (species B and C), conjunctivitis (species B and D), gastroenteritis (species A, F and G), acute haemorrhagic cystitis, meningoencephalitis and kidney disease, or fatality in immunocompromised patients, especially children. In immunocompetent individuals, ~50% of HAdV infections were found to be asymptomatic, while symptomatic infections were generally mild and resolved without sequelae. Certain cohorts e.g. US military appear to be more prone to HAdV transmission. Accordingly, anti-HAdV vaccinations have been available for US militaries in the past, but have been discontinued.

Targeted therapies or vaccinations against HAdV are not available to this date. HAdV vectors are the most widely used gene therapy and genetic vaccination vehicles in the clinic, due to their genetic stability, inability to incorporate into the host genome and ease of genetic and biochemical editing.

The need for treatment options of HAdV infection is emphasized by a recent outbreak of HAdV-B7 in the USA causing the death of 10 children in a Nursing and Rehabilitation centre in New Jersey. Recent studies on HAdV epidemiology have been fostered by advancement of molecular biology techniques and reveal prevalence and associated risk, especially among young children under 7.

AdVs have an icosahedral capsid of about 90 nm in diameter (not counting the protruding fiber proteins), and T= 25 triangulation number symmetry. The capsid contains a double-stranded DNA of 27-38 kbp, depending on the virus type. The primary receptor for species A, C, D, E and F types is the Coxsackievirus adenovirus receptor (CAR), a cellular membrane protein which functions in cell-cell adhesion. For types grouped into the B species, CD46 and desmoglein 2 act as primary receptors. It was shown that the majority of HAdV types with an arginine /glycine /aspartate (RGD) motif can utilize $\alpha\beta_5$ or $\alpha\beta_3$ integrins as a secondary and signalling receptor. HAdV deliver their genome to the cell nucleus, replicate their genome in

the nucleus, and assemble progeny in the nucleus. Limited proteolysis in the immature virions is mediated by the viral cysteine protease, which yields mature infectious particles with appropriate capsid stiffness and internal pressure. Virions exit the infected cells mostly upon disruption of the nucleus and lysis of the plasma membrane, involving the HAdV-C E3-11.6-kDa adenovirus death protein (ADP).

Based on the above-mentioned state of the art, the objective of the present invention is to provide means and methods for a treatment or vaccination against adenoviral infection. This objective is attained by the subject-matter of the independent claims of the present specification.

Summary of the invention

The invention in the broadest sense relates to the use of Nelfinavir or a derivative, salt or analogue thereof, in treatment or prevention of adenoviral infection.

Terms and definitions

For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any definition set forth below conflicts with any document incorporated herein by reference, the definition set forth shall control.

The terms “comprising,” “having,” “containing,” and “including,” and other similar forms, and grammatical equivalents thereof, as used herein, are intended to be equivalent in meaning and to be open ended in that an item or items following any one of these words is not meant to be an exhaustive listing of such item or items, or meant to be limited to only the listed item or items. For example, an article “comprising” components A, B, and C can consist of (i.e., contain only) components A, B, and C, or can contain not only components A, B, and C but also one or more other components. As such, it is intended and understood that “comprises” and similar forms thereof, and grammatical equivalents thereof, include disclosure of embodiments of “consisting essentially of” or “consisting of.”

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit, unless the context clearly dictate otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure.

Reference to “about” a value or parameter herein includes (and describes) variations that are directed to that value or parameter per se. For example, description referring to “about X” includes description of “X.”

As used herein, including in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., *Short Protocols in Molecular Biology* (1999) 4th Ed, John Wiley & Sons, Inc.) and chemical methods.

As used herein, the term *treating* or *treatment* of any disease or disorder (e.g. cancer) refers in one embodiment, to ameliorating the disease or disorder (e.g. slowing or arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment "treating" or "treatment" refers to alleviating or ameliorating at least one physical parameter including those which may not be discernible by the patient. In yet another embodiment, "treating" or "treatment" refers to modulating the disease or disorder, either physically, (e.g., stabilization of a discernible symptom), physiologically, (e.g., stabilization of a physical parameter), or both. Methods for assessing treatment and/or prevention of disease are generally known in the art, unless specifically described herein below.

Detailed description of the invention

Medical use of Nelfinavir and related pharmaceutical compounds

A first aspect of the invention relates to a compound for use in a method for treating or preventing an adenoviral infection or a medical condition caused by an adenoviral infection, particularly a disease or a clinical symptom, wherein the compound is selected from Nelfinavir (CAS Number 159989-64-7) or a pharmaceutically acceptable formulation thereof, i.e. the pharmaceutical formulation comprises Nelfinavir and one or more other ingredients, such as Nelfinavir mesylate (CAS number 159989-65-8).

In certain embodiments, the adenoviral infection is a human adenovirus infection or a murine adenovirus infection.

In certain embodiments, the adenoviral infection is an infection by HAdV-B7.

In certain embodiments, the medical condition is selected from a respiratory disease, conjunctivitis, gastroenteritis, acute haemorrhagic cystitis, meningoencephalitis and kidney disease. In certain embodiments, the compound as specified herein is administered for use

in prevention of death associated with adenoviral infection, particularly with an infection by HAdV-B7.

In certain embodiments, the compound is administered to reach a plasma concentration of $\geq 1,25 \mu\text{mol/l}$. In certain embodiments, the compound is administered to reach a plasma concentration of $\geq 3 \mu\text{mol/l}$. In certain embodiments, the compound is administered to reach a plasma concentration of $\geq 10 \mu\text{mol/l}$.

In certain embodiments, the compound is administered within 72 h of infection. In certain embodiments, the compound is administered within 48 h of infection. In certain embodiments, the compound is administered within 24 h of infection.

In certain embodiments, the compound is administered within 72 h of detection of infection. In certain embodiments, the compound is administered within 48 h of detection of infection. In certain embodiments, the compound is administered within 24 h of detection of infection.

In certain embodiments, the compound is formulated for oral application.

Pharmaceutical Composition and Administration

A second aspect of the invention relates to a dosage form for use in a method for treating or preventing an adenoviral infection or a medical condition caused by an adenoviral infection, wherein said dosage form comprises a compound is selected from Nelfinavir (CAS Number 159989-64-7) and a pharmaceutically acceptable formulation thereof, i.e. the pharmaceutical formulation comprises Nelfinavir and one or more other ingredients, or a Nelfinavir salt, such as Nelfinavir mesylate (CAS number 159989-65-8).

In certain embodiments, the dosage form is formulated for oral application.

Alternatively, this aspect can be formulated as relating to a pharmaceutical composition comprising a compound for use according to the present invention, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier. In further embodiments, the composition comprises at least two pharmaceutically acceptable carriers, such as those described herein.

In certain embodiments of the invention, the compound for use according to the present invention is typically formulated into pharmaceutical dosage forms to provide an easily controllable dosage of the drug and to give the patient an elegant and easily handleable product.

The pharmaceutical composition can be formulated for oral administration, parenteral administration, or rectal administration. In addition, the pharmaceutical compositions of the present invention can be made up in a solid form (including without limitation capsules,

tablets, pills, granules, powders or suppositories), or in a liquid form (including without limitation solutions, suspensions or emulsions).

The dosage regimen for the compounds of the present invention will vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent and its mode and route of administration; the species, age, sex, health, medical condition, and weight of the recipient; the nature and extent of the symptoms; the kind of concurrent treatment; the frequency of treatment; the route of administration, the renal and hepatic function of the patient, and the effect desired. In certain embodiments, the compounds of the invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three, or four times daily.

In certain embodiments, the pharmaceutical composition or combination of the present invention can be in unit dosage of about 1-1,000 mg of active ingredient(s) for a subject of about 50 - 70 kg. The therapeutically effective dosage of a compound, the pharmaceutical composition, or the combinations thereof, is dependent on the species of the subject, the body weight, age and individual condition, the disorder or disease or the severity thereof being treated. A physician, clinician or veterinarian of ordinary skill can readily determine the effective amount of each of the active ingredients necessary to prevent, treat or inhibit the progress of the disorder or disease.

The pharmaceutical compositions of the present invention can be subjected to conventional pharmaceutical operations such as sterilization and/or can contain conventional inert diluents, lubricating agents, or buffering agents, as well as adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers and buffers, etc. They may be produced by standard processes, for instance by conventional mixing, granulating, dissolving or lyophilizing processes. Many such procedures and methods for preparing pharmaceutical compositions are known in the art, see for example L. Lachman et al. The Theory and Practice of Industrial Pharmacy, 4th Ed, 2013 (ISBN 8123922892).

Dosage forms may be for enteral administration, such as nasal, buccal, rectal, transdermal or oral administration, or as an inhalation form or suppository. Alternatively, parenteral administration may be used, such as subcutaneous, intravenous, intrahepatic or intramuscular injection forms. Optionally, a pharmaceutically acceptable carrier and/or excipient may be present.

Methods of Manufacture of a Pharmaceutical Composition

A third aspect of the invention relates to a method of manufacture of a medicament for treating or preventing an adenoviral infection or a medical condition caused by an adenoviral infection comprising the use of a compound selected from Nelfinavir (CAS Number 159989-

64-7) or a pharmaceutically acceptable formulation thereof, i.e. the pharmaceutical formulation comprises Nelfinavir and one or more other ingredients, or a Nelfinavir salt, such as Nelfinavir mesylate (CAS number 159989-65-8). In certain embodiments, the medicament is manufactured for treatment or prevention of a human adenovirus infection or a murine
5 adenovirus infection. In certain embodiments, the medical condition for which the medicament is administered is selected from a respiratory disease, conjunctivitis, gastroenteritis, acute haemorrhagic cystitis, meningoencephalitis and kidney disease. In certain embodiments, the medicament is administered for prevention of death associated with adenoviral infection, particularly with an infection by HAdV-B7.

10 *Methods of Treatment*

A fourth aspect of the invention relates to a method for treating a patient having been diagnosed with adenoviral infection, or who is suspected of suffering from an adenoviral infection, or preventing an adenoviral infection or a medical condition caused by an adenoviral infection in a patient at risk of adenoviral infection. A method of treatment or
15 prevention according to this aspect of the invention comprises administering to said patient a compound selected from Nelfinavir (CAS Number 159989-64-7) and a pharmaceutically acceptable formulation thereof, i.e. the pharmaceutical formulation comprises Nelfinavir and one or more other ingredients, or a Nelfinavir salt, such as Nelfinavir mesylate (CAS number 159989-65-8), in a therapeutically effective concentration to a patient in need thereof.

20 In certain embodiments, the method of treatment according to the invention is applied in cases of a human adenovirus infection or a murine adenovirus infection. In certain embodiments, the medical condition in which the method is applied is selected from a respiratory disease, conjunctivitis, gastroenteritis, acute haemorrhagic cystitis, meningoencephalitis and kidney disease, or fatality.

25 In certain embodiments, the compound is administered to reach a plasma concentration of $\geq 1,25 \mu\text{mol/l}$. In certain embodiments, the compound is administered to reach a plasma concentration of $\geq 3 \mu\text{mol/l}$. In certain embodiments, the compound is administered to reach a plasma concentration of $\geq 10 \mu\text{mol/l}$.

In certain embodiments, the compound is administered within 72 h of infection. In certain
30 embodiments, the compound is administered within 48 h of infection. In certain embodiments, the compound is administered within 24 h of infection.

In certain embodiments, the compound is administered within 72 h of detection of infection. In certain embodiments, the compound is administered within 48 h of detection of infection. In certain embodiments, the compound is administered within 24 h of detection of infection.

35 In certain embodiments, the compound is administered orally.

The skilled person is aware that any specifically mentioned drug may be present as a pharmaceutically acceptable salt of said drug. Pharmaceutically acceptable salts comprise the ionized drug and an oppositely charged counterion. Non-limiting examples of pharmaceutically acceptable anionic salt forms include acetate, benzoate, besylate, bitartrate, bromide, carbonate, chloride, citrate, edetate, edisylate, embonate, estolate, fumarate, gluceptate, gluconate, hydrobromide, hydrochloride, iodide, lactate, lactobionate, malate, maleate, mandelate, mesylate, methyl bromide, methyl sulfate, mucate, napsylate, nitrate, pamoate, phosphate, diphosphate, salicylate, disalicylate, stearate, succinate, sulfate, tartrate, tosylate, triethiodide and valerate. Non-limiting examples of pharmaceutically acceptable cationic salt forms include aluminium, benzathine, calcium, ethylene diamine, lysine, magnesium, meglumine, potassium, procaine, sodium, tromethamine and zinc.

Wherever alternatives for single separable features such as, for example, an isotype protein or ligand type or medical indication are laid out herein as “embodiments”, it is to be understood that such alternatives may be combined freely to form discrete embodiments of the invention disclosed herein. Thus, any of the alternative embodiments for a ligand type may be combined with any of the alternative embodiments of medical indication mentioned herein.

The invention is further illustrated by the following examples and figures, from which further embodiments and advantages can be drawn. These examples are meant to illustrate the invention but not to limit its scope.

Brief description of the figures

Fig. 1 Image-based screening the Prestwick Chemical Library identifies Nelfinavir as safe and potent inhibitor of HAdV-C infection. **A)** Schematic overview of the AntiVir screening pipeline. PCL compounds and DFT positive control in DMSO as well as DMSO alone as negative control were pre-spotted onto 384well imaging plates by Echo acoustic liquid handling at 10 nl corresponding to a final concentration of 1.25 μ M in 80 μ l assay volume/well and stored at -20°C. Compound-blinded plates were thawed and 40,000 A549 cells/wells seeded. The following day, the cells were inoculated with HAdV-C2-dE3B-CMV-GFP at 31 pfu/well. Allowing for multiple viral replication rounds, the cells were PFA-fixed at 72 h pi and the nuclei stained using Hoechst. The infection phenotype was imaged using an epifluorescence HT microscope and score using Plaques2.0. The data of the four technical replicates was further processed in R. **B)** Exemplary epifluorescence microscopy 384well images stitched to a screening plate overview of consistent of 16 replicates of negative (two most left columns) and positive control (two most right columns) and 352 blinded PCL compounds (centre 20 columns). Hoechst-stained nuclei are shown in blue, viral GFP

in green. **C)** Representative 384well epifluorescence microscopy images of the DMSO negative control (most left), the DFT positive control (most right) and the top hit Nelfinavir mesylate (centre). Hoechst-stained nuclei are shown in blue, viral GFP in green. Scale bar is 5 mm. **D)** Structure of Nelfinavir mesylate. **E)** Cell index (CI)-based TC_{50} for long-term incubation of Nelfinavir shows stable toxicity over time. TC_{50} s and hereon based mean TC_{50} over time of 25.66 μ M were derived from impedance measurements indicating the cytopathic effects of A549 treated with 0.10 to 100 μ M Nelfinavir. Impedance was recorded every 15 min using an xCELLigence system. The time on the x-axis indicates hours after cell seeding. Vertical line shows the time of drug addition. **F)** Nelfinavir displays a broad therapeutic window against HAdV-C2-dE3B-CMV-GFP infection of A549 cells between the concentration causing 50% inhibition of HAdV spread in treated, infected wells (IC_{50} = 0.37 μ M) and the concentration causing 50% toxicity in treated, non-infected wells (TC_{50} = 10.01 μ M) measured as plaque numbers and number of nuclei, respectively. The ratio TC_{50} / IC_{50} = 27.05 is referred to as therapeutic index (TI_{50}). Image-based progression of infection was quantified using Plaque2.0 at 82 h pi . Plaque numbers per well are depicted as red circles, numbers of infected nuclei as green circles. Numbers of nuclei in Nelfinavir-treated, non-infected wells are shown in blue; treated, infected wells shown in orange. Data points represent one of four biological replicates. IC_{50} s are derived from non-linear curve fitting. For detailed information and statistics, see Table 3.

Fig. 2 **Nelfinavir has no effect on early or late steps of the HAdV-C replication cycle.**

A) Neither the expression level of transgenic GFP expressed under the CMV promoter (shown in green) nor that of the late structural HAdV protein hexon (shown in red) are affected by Nelfinavir late pre-lysis stages of HAdV-C2-dE3B-CMV-GFP infection in A549 cells. Data points represent for each of the four biological replicates mean median nuclear intensities per well normalized to the mean median nuclear intensities of the DMSO-treated wells. Epifluorescence microscopy images were segmented and analysed using CellProfiler. **B)** Representative TEM images of late stage HAdV-C2-dE3B-infected A549 cells at 41 h pi unveil viral capsid assembly (red arrowy) inside the nucleus (black arrows pointing at the nuclear envelop from the nuclear lumen) in both DMSO-treated and Nelfinavir-treated cells. Scale bar indicates 2 μ m. **C)** Negative staining EM images of double CsCl gradient-purified HAdV-C5 progeny from Nelfinavir-treated ($HAdV-C5^{+Nelfinavir}$) and untreated A549 cells confirm fully assembled, full progeny capsids. Scale bar indicates 200 nm. **D)** $HAdV-C5^{+Nelfinavir}$ harbours fully HAdV protease-processed proteins VI and VII detected by identical band size in Western Blot indicating unaffected HAdV protease activity.

HAdV-C2-ts1 is a temperature-sensitive HAdV-C2 mutant with impaired HAdV protease resulting in full length unprocessed precursor proteins pVI and pVII. **E)** HAdV-C5^{+Nelfinavir} shows no defects in binding to naïve A549 cells. Virus was bound to A549 cells for 1 h at 4°C, followed by PFA-fixation, nuclei staining by Hoechst (shown in blue), immunofluorescent staining of virus capsids (in green) and cytosolic staining by SuccEst (in red). Images represent max projections of confocal z stacks. Grey squares indicate position of right-handed zoom-outs. Scale bars indicate 20 µm. **F)** Protein-concentration normalized titration of HAdV-C5^{+Nelfinavir} demonstrates unaffected infectivity compared to virus purified from non-treated cells quantified as elicited number of infected A549 cells / well at 44 h pi based on α-hexon immunofluorescent staining. Infected nuclei were segmented and classified in CellProfiler. Total numbers of nuclei / well are shown in blue. Bars represent means of four technical replicates, error bars indicate standard deviation. Note that x-axis is discrete.

Fig. 3 Nelfinavir is a post-exposure inhibitor of HAdV-C egress. A) A549 cells at 3 days post infection (dpi) inoculated with 1:10 diluted cell lysates or supernatants harvested from Nelfinavir-treated HAdV-C2-dE3B-infected A549 cells at indicated time points reveal delayed viral progeny release to the supernatant. Nuclei signal shown in blue, viral GFP in green. **B)** Productivity per 12-well of HAdV-C2-dE3B-infected A549 cells treated with Nelfinavir (shown in orange) or DMSO control (in blue) at indicated time post infection determined by titration of whole well (cell and supernatant) lysate on naïve A549 cells. Line indicates mean slope for whole well lysate volume to infected nuclei linear regression of three biological triplicates, dotted line indicates SE. **C)** Plaques and nuclei per well at 72 h pi of HAdV-C2-dE3B-infected A549 cells treated with 1.25 µM Nelfinavir during different periods post infection. Plaques were segmented using Plaque2.0, single nuclei numbers in CellProfiler. Nuclei are shown in blue, plaques in infected, non-treated wells shown in green and Nelfinavir-treated wells in orange. Data points represent one of 8 biological replicas, long vertical lines indicate means and error bars give standard deviations. **D)** Nelfinavir's ability to inhibit HAdV-C2-dE3B-CMV-GFP spread in A549 at least up to 96 h pi is limited by the amount of virus inoculum as pfu/well. Total number of nuclei shown in blue, number of dead, PI-positive cells shown in red and number of infected, GFP-positive cells shown in green are normalized by the respective mean number in infected, non-treated wells. Note that the number of infected cells at 43 h pi is not affected by the Nelfinavir treatment. Data points represent means of 4 biological replicas, dotted lines indicate standard deviation. **E)** Treatment of HAdV-C2-dE3B-CMV-GFP infection in A549 cells with 1.25 µM Nelfinavir delays plaque formation and causes slowly

growing plaques of rounder morphology. Viral GFP expression level shown as 16-color LUT. Scale bar is 1 mm. **F)** 1.25 μ M Nelfinavir significantly delays the increase in HAdV-C2-dE3B-infected A549 cells by delaying plaque formation. Plaques were segmented using Plaque2.0, single nuclei numbers in CellProfiler. Number of infected cells and plaques per well of DMSO-treated, infected wells are shown in green, those of Nelfinavir-treated, infected well in orange. Data points represent means of 24 biological replicas including the example well micrographs shown in **D**, error bars indicate standard deviation. **G)** The delayed HAdV-C2-dE3B-CMV-GFP plaques under 1.25 μ M Nelfinavir are significantly rounder analysed by Kolmogorov-Smirnov test of plaque roundness detected by Plaque 2.0. Data points indicate centre well plaque regions harbouring a single peak region from 24 biological replicates including the example well micrographs shown in **D**. Only regions consisting of at least 5 infected cells ($\geq 1,500 \mu\text{m}^2$) were considered as plaque. Plaque morphologies in control wells could not be quantified later than 3 dpi, since no single peak plaque regions were detected. DMSO-treated plaques at 3 dpi vs. 1.25 μ M Nelfinavir-treated plaques at 5 dpi: approximate p value < 0.0001 (****), Kolmogorov-Smirnov D = 0.5755. DMSO-treated plaques at 3 dpi vs. 1.25 μ M Nelfinavir-treated plaques at 6 dpi: approximate p value < 0.0001 (****), Kolmogorov-Smirnov D = 0.7171.

Fig. 4: Nelfinavir interferes with ADP translocation and inhibits HAdV-C lysis in an ADP-dependent manner. A) Representative high-magnification confocal images of HAdV-C2-dE3B-infected A549 cells fixed at 44 h pi comparing the effect of Nelfinavir on ADP localization. Nuclei, shown in blue in the merged most right panel, were stained with Hoechst. Viral GFP is displayed in green. ADP, depicted in red, was immunofluorescently detected using a rabbit α -HAdV-C2-ADP₈₇₋₁₀₁ antibody. Whole cells, shown in grey scale, were stained using SuccEst. Images are maximal projection of 30 z planes with 0.5 μ m z step, scale bar indicates 10 μ m. **B)** Plaque formation in ADP-depleted HAdV-C2-dE3B-CMV-GFP in A549 is delayed by 1 day but does not affect plaque shapes at the indicated time points. Cells were infected with 34 and 41 pfu/well HAdV-C2-dE3B-CMV-GFP and -dADP, respectively. Nuclei signal shown in blue, viral GFP in green, scale bar is 1 mm. **C)** Depletion of ADP from HAdV-C2-dE3B-CMV-GFP renders Nelfinavir ineffective in A549 ($\text{IC}_{50} = 5.82$ compared to 0.22 μ M). Image-based progression of infection was quantified using Plaque2.0 at 72 h pi for HAdV-C2-dE3B-CMV-GFP and 96 h pi for the ADP-deleted mutant. Plaque numbers per well are normalized to the mean DMSO control and depicted as full green triangles for HAdV-C2-dE3B-CMV-GFP and empty red triangles for the ADP-deleted mutant. Nuclei numbers of non-infected, treated wells are normalized to the mean DMSO control and depicted as full blue circles for 72 h

incubation and empty blue circles for 96 h incubation. Data points represent means of 4 biological replicates, error bars indicate standard deviation. Epifluorescence microscopy images were analysed using Plaque 2.0 for plaque numbers, nuclei were segmented in CellProfiler. IC_{50} s are derived from non-linear curve fitting. For detailed information and statistics, see Table 3. **D) and E)** Cell index profiles from impedance measurements of infected A549 indicate cytopathic effects. Nelfinavir inhibits cytotoxicity in DMSO-treated HAdV-C2-dE3B-infection (**D**, green profile), but not the ADP-depleted mutant (**E**, red profile). Impedance was recorded every 15 min using an xCELLigence system. Each point represents the average value from two replicates with standard deviations. The time on the x-axis indicates hours after cell seeding. Vertical lines show the time of infection and drug addition. Profiles of non-infected cells are shown in blue. The concentrations of Nelfinavir with which the infected cells were incubated with are represented by different shades of orange. **F)** The delay of cytotoxicity is calculated as time between each treatment's highest mean cell index and its half maximum, depicted in green for infection with HAdV-C2-dE3b and red for -dADP infection. For HAdV-C2-dE3B-infected A549 treated with 25 μ M Nelfinavir, the measurement was aborted due to overgrowth-causing cytotoxicity before the maximal cell index was reached. Thus, no delay of cytotoxicity could be calculated, as indicated by the striped line. Treatment with 100 μ M Nelfinavir is toxic. **G)** Therapeutic index (TI_{50}) calculated as Nelfinavir concentration causing 50% toxicity (TC_{50}) divided by the concentration leading to 50% reduction in numbers of plaques per well (IC_{50}) for infection with HAdV-C2-dE3B-CMV-GFP and -dADP in different cancer and primary cell lines. For detailed information and statistics, see Table 3.

Fig. 5: Nelfinavir inhibits cell-free and reveals delayed cell-to-cell transmission of HAdV-C. **A)** Systematic overview on pathogen transmission routes. Lytic cell-free spread releases progeny following lysis of the host cell, while non-lytic egress is characterized by persistence of the first-round infected host cell. In both cases, convection in the media leads to asymmetric, long-distance, scattered comet-shaped plaques, which can be inhibited by the addition of neutralizing antibodies. In contrast, direct cell-to-cell pathogen spread from persisting first round infected host cells causes symmetric, small-growing, dense round plaques, which cannot be inhibited by neutralizing antibodies. Non-infected cells are shown in grey with blue nuclei. First round infected cells are shown in dark green, red nuclei indicates lysis at the end of the pathogen replication cycle. Subsequently second round infected cells are shown in light green. Grey arrow represents direction of convective flow. Axes indicate side or top-down view. **B)** Example epifluorescence micrographs of plaque morphologies caused by lytic cell-free transmission (HAdV-C2-dE3B), mixed cell-free and cell-to-

cell transmission (IAV H1N1) and direct cell-to cell transmission (HSV-1). Nuclei are shown in green, viral transgenic GFP in green. Scale bar is 5 mm. **C)** Impairment of cell-free transmission of HAdV-C2-dE3B-CMV-GFP progeny by addition of 1:10 HAdV-C2/5-neutralizing serum to the media reveals delayed round plaques in A549 cells. Nuclei are shown in blue, viral GFP in green.

Fig. 6: **A HAdV genotype's susceptibility to Nelfinavir correlates with its plaque shape.**

A) Therapeutic index (TI_{50}) calculated as Nelfinavir concentration causing 50% toxicity (TC_{50}) divided by the concentration leading to 50% reduction in numbers of plaques per well (IC_{50}) for different HAdV, mouse adenoviruses (MAdV) and herpes simplex virus 1 (HSV-1) in different cancer and primary cell lines. For detailed information and statistics, see Table 3. **B)** Representative microscopic and macroscopic plaques morphologies of Nelfinavir-sensitive and -insensitive HAdV genotypes. Black/white images show plaques based on epifluorescence microscopy of hexon immunostaining or GFP expression in a 96well of A549 cells infected with the indicated HAdV genotype, scale bar is 1 mm. Purple/white images show plaques based on infection-caused cytotoxicity creating lesions visualized by crystal violet staining in A549 monolayers in a 12well of A549 cells infected with the indicated HAdV genotype, scale bar is 5 mm.

Fig. 7: **Morphological characterization of Nelfinavir-induced delayed round plaque phenotype.**

A) End-point epifluorescence microscopy of HAdV-C2-dE3B-CMV-GFP infection in A549 cells revealing increased convective flow by plate tilting does not affect the spatio-temporal spread pattern under Nelfinavir. Nelfinavir delays formation and number of plaques in a concentration-dependent manner. Early plaques show comet-shaped morphologies as highlighted by the red squares 1 and 2, while late plaques under Nelfinavir appear round as exemplified by red square 3. GFP intensity is shown as 16-color LUT. Arrow points uphill, the direction of convective flow and comet plaque development. Scale bar is 1 mm. **B)** 3 dimensional topological views of plaque morphologies assessed as viral GFP expression levels along the z-axis. Red numbers correspond to regions of interest (ROIs) 1 to 3 indicated by red squares in **A**. x- and y-axis are oriented as labelled in **A**. GFP intensity is shown as rainbow colour LUT, indicating spatial origin of plaque formation at peak GFP intensity in purple. **C)** Quantification of delay in plaque formation by Nelfinavir over time. Epifluorescence microscopic images were analysed using Plaque2.0. Data points represent means of 12 replicas from two experiments including the example well micrographs shown in **A**, error bars indicate STDs. DMSO-treated infected wells are shown in green, infected wells treated with 1.25 to 10 μ M Nelfinavir are shown in shades of orange. **D)** Morphological analysis of plaque roundness vs. size over the

course of Nelfinavir treatment at 1.25 and 3 μ M compared to the DMSO-treated control wells over the course of infection indicated by shades of orange. Epifluorescence microscopic images were analysed using Plaque2.0. Data points indicate centre well plaque regions harbouring a single peak region from 12 wells from two experiments including the example well micrographs shown in **A**. Plaque morphologies in control wells could not be quantified later than 3 dpi, since no single peak plaque regions were detected. Only regions consisting of at least 5 infected cells ($\geq 1,500 \mu\text{m}^2$ indicated by the dotted vertical lines) were considered as plaque.

Examples

Example 1: Design of the phenotypic screening

Conventional screenings for anti-viral drugs have been limited to inhibitors of early infection events, or viral replication. The inventors recently developed automated high-throughput image analysis framework Plaque2.0 allows to screen for compounds targeting any stage of the full virus life cycle - from virus entry and replication to protein expression, progeny formation, virion egress and spread. The assay detects viral gene products by immunohistochemistry, RNA *in situ* hybridization or fluorescent transgene expression. Virus transmission from a single infected host cell leads to infection of neighbouring cells, which is detectable as dense foci of infected cells, termed plaque. Plaque2.0 readily analyzes screening-scale high-throughput images, extracts both single cell-level features such as infection status or object-level parameters including number of plaques per well, plaque area and shape, infection density and viral gene product intensity. Plaque2.0 is used to thoroughly quantify multi-round viral infections.

Targeting the viral host is a promising anti-viral strategy, since viruses inherently provide few targets for attack, and viral proteins are difficult targets for broad and long-lasting inhibitors.

In order to identify chemicals with novel anti-viral activity, the inventors have performed a phenotypic image-based screening of an established library of 1,280 small molecules.

The Prestwick Chemical Library (PCL) comprises off-patent chemicals of high chemical and pharmacological diversity, most of them approved by the FDA, EMA and other agencies. For an outline of the experimental and analysis pipeline, see Figure 1A. All compounds were blinded and tested in temporally separated biological quadruplicates. A549 cells (adenocarcinomic human alveolar basal epithelium, ATCC) were incubated with compound (1.25 μ M in DMSO), solvent control (0.0125% DMSO) or positive control compound (1.25 μ M 3'-Deoxy-3'-fluorothymidine, DFT) overnight prior to infection with HAdV-C2-dE3B-CMV-GFP at 36 plaque forming units (pfu)/well. In this replicating reporter virus, the E3B region is exchanged for a GFP transgene under the constitutively active CMV promoter. DFT is a 3'-

deoxy-3'-substituted thymidine nucleoside analogue, which prevents HAdV replication in infected cells by DNA chain termination. 72 hours post infection (h pi), cells were fixed, the nuclei stained with Hoechst and subsequently imaged on a high-throughput microscope (IXM-C, Molecular Devices, LLC, San Jose, USA). Figure 1B shows example wells from a 384-well plate overview. Number of nuclei and number of infected nuclei; number, area and shape of plaques and GFP intensity were quantified using Plaque2.0.

The calculated Z' scores shown in Table 1 underline the high statistical power of the HAdV AntiVir screen, for three out of the four infection quantifiers number of infected nuclei, infection index (infected nuclei / total nuclei per well), Z' scores are greater than 0.5, the remaining quantifier integrated GFP intensity still indicates good statistical power. All infection quantifier read-outs were further normalized relative to the DMSO solvent control. 128 PCL compounds that showed toxicity in absence of infection (Presto Blue assay) and 76 PCL compounds in presence of HAdV (nuclei count exceeding solvent control mean - 2 standard deviations, STDs) were excluded. Compounds exceeding solvent control parameter means +/- 3 STDs for the four infection quantifiers number of infected nuclei, infection index (infected nuclei / total nuclei per well), number of plaques or integrated GFP intensity were selected as hits. Two hits showed significant reduction of number of infected nuclei and infection index, four hits led to significantly reduced integrated GFP intensity. None of the PCL compounds fulfilled the 3 STDs filter for plaque number reduction. Additionally, all non-toxic compounds were ranked according to their scores for the four infection quantifiers. Of all 1,280 small molecules contained in the PCL, Nelfinavir was identified as the top hit and chosen for in-depth follow-up (Table 1). The example wells shown in Figure 1C indicate that Nelfinavir inhibits a viral life cycle stage post entry, nuclear import and early translation of viral DNA as the transgenic GFP is successfully expressed in single cells, but no viral spread observable by plaque formation is detected.

Example 2: Nelfinavir is a clinical compound inhibiting the HIV protease and a promising anti-cancer therapeutic

Nelfinavir (structure depicted in Figure 1D, (3S,4aS,8aS)-N-tert-butyl-2-[(2R,3R)-2-hydroxy-3-[(3-hydroxy-2-methylbenzoyl)amino]-4-phenylsulfanylbutyl]-3,4,4a,5,6,7,8,8a-octahydro-1H-isoquinoline-3-carboxamide, Mono-methane Sulfonate, Viracept, AG1343, CAS number 159989-65-8) was first introduced as a competitive inhibitor of human immunodeficiency virus (HIV) aspartyl protease. The small molecule (MW = 663.889 g/mol) inhibits the HIV-1 protease with an K_i of 2 nM by interacting with the S1 and S2 subsites.

Nelfinavir received FDA approval in 1997 and is to date used in combination with reverse transcriptase inhibitors and/or other protease inhibitors to treat HIV-infected patients, exhibiting an ED_{50} of 14 nM. While initially administered orally at 750 mg three times a day, a

twice daily dose of 1,250 mg was found to be equally effective, to which the standard treatment plan was adjusted accordingly in 1999. Treatment of HIV-infected individuals has been found effective and save in combination with numerous other diseases and conditions including pregnancy. Side-effects of Nelfinavir-based combination therapy include diarrhoea, metabolic disturbances (hypercholesterolaemia, hyperglycaemia and lipodystrophy) and liver toxicity. Moreover, Nelfinavir is associated with a number of clinically significant drug interactions and coadministration of some drugs (e.g. astemizole, cisapride, triazolam) is contraindicated.

Nelfinavir plasma levels have been found to be highly variable between patients. Concentrations ranged from 0.14 to 11.74 mg/L (0.21 to 17.7 μ M) and from 0.36 to 10.57 mg/L (0.54 to 15.9 μ M) under TID and BID regimens, respectively. Nelfinavir is bound to plasma proteins, and metabolized via 3A4 and 2C19 to an active metabolite (M8), which competitively inhibits 3A4 activity, and induces cytochrome P450 enzymes at chronic dosage. The M8 metabolite undergoes metabolism via 3A4. Nelfinavir was also significantly enriched in blood monocytes, and was only moderately exported by P-glycoprotein-mediated efflux. It is unclear if monocyte adsorption accounted for the observed fast clearing of CX from the plasma of mice (54 ug/ml at 30 min to 3 ug/ml 4 h post injection), or if other mechanisms were involved, such as accumulation in the liver. Measurements of plasma levels likely underestimated the effective intracellular concentrations of CX.

Nelfinavir has been tested *in vivo* in mice, rats, dogs, monkeys, marmoset and human, but not hamsters. Nelfinavir was administered both orally as well as *intra venously* in 5% dextrose except for marmots where propylene glycol:water (50:50) was used. Nelfinavir demonstrated significant oral bioavailability across a range of species including dogs (47%), marmosets (17%), cynomolgus monkeys (26%) and human (20-80%, increased when taken with food). Notably, the twice daily *ip* administration of 50 mg/kg Nelfinavir instead of once 100 mg/kg in mice was not well tolerated and lead to significant reduction of body weight. An overview on the pharmacokinetics in different mammals is given in Table 2.

Nelfinavir demonstrates promising anti-cancer activity. It inhibits Akt phosphorylation, signal transducer and activation of transcription factor 3 (STAT3) signalling, cyclin-dependent kinase 2 (CDK2) function, heat shock protein 90 (HSP90) function, and general kinase activity. Notably, Nelfinavir also downregulates and blocks androgen receptor signalling in hormone-sensitive prostate cancer cells. Nelfinavir is currently under heavy investigation in 135 clinical trials including single and combinational treatment of various cancers, HIV and HIV co-infection with other pathogens. NIH's NCI currently supports 5 clinical trials using Nelfinavir alone and in combination with other therapies, which are at phase I to II. Most relevant is AMC-098 "Nelfinavir Mesylate in treating patients with Kaposi Sarcoma: This pilot

phase II trial studies how well Nelfinavir mesylate works in treating patients with kaposi sarcoma. Nelfinavir mesylate may stop the growth of tumour cells by blocking some of the enzymes needed for cell growth.” Patients are receiving standard dose Nelfinavir po BID *po* high dose *po* BID.

5 Example 3: Nelfinavir is a potent inhibitor of HAdV-C infection spread

The *in vitro* TC₅₀ of Nelfinavir was determined over 4 days of continuous treatment of A549 cells based on impedance, where Nelfinavir shows low toxicity (TC₅₀ = 25.66 µM, Figure 1E). Previous studies have shown an anti-proliferative effect of Nelfinavir already at 5 µM for A549 cells *in vitro*.

10 The inventors further determined the efficacy to inhibit the spread of HAdV in A549 monolayer cell culture of Nelfinavir by 96well plaque assay as summarized in Figure 1F. Following overnight adhesion, the cells were inoculated with HAdVC2-dE3B-CMV-GFP at 55 plaque forming units per well as well as a titration of Nelfinavir. The cells were fixed after 3 days, and number of nuclei and number of infected nuclei assessed using CellProfiler and
15 plaques detected using Plaque2.0. By determining Nelfinavir's therapeutic index 50 (TI₅₀ = 27.05) as ratio between the concentration causing 50% inhibition of HAdV spread (IC₅₀ = 0.37 µM) and the concentration causing 50% toxicity (TC₅₀ = 10.01 µM), the inventors highlight its safety and efficacy. IC₅₀ and TC₅₀ were measured as plaque numbers and number of nuclei, respectively.

20 Example 4: Nelfinavir does not inhibit the HAdV life cycle including maturation of progeny virions

The viral life cycle of HAdV infection is a multi-step process. It is comprised of virus binding and entry, endosomal escape and nuclear HAdV genome delivery, replication, assembly and maturation in the nucleus. Finally, progeny virus is released to the extracellular space by host
25 cell lysis. To identify which step of the viral life cycle is affected by Nelfinavir, the inventors first tested its effect on HAdV replication assessed by quantification of viral protein expression levels at a late stage HAdV-C2-dE3B-CMV-GFP of A549 cells (46 hours post infection, h pi) incubated at 0 to 6.25 µM Nelfinavir. Neither the number of infected nuclei nor the expression level of HAdV GFP (viral transgene under CMV promoter) or that of HAdV
30 hexon (late structural capsid protein) are affected by Nelfinavir (Figure 2A), even at non-toxic concentrations causing total spread inhibition of HAdV-C2-dE3B-CMV-GFP (≥2.5 µM, compare Figure 1F). The inventors further examined Nelfinavir's impact on the subsequent capsid assembly. Transmission electron microscopy (TEM) of HAdV-C2-dE3B-CMV-GFP - infected, Nelfinavir-treated A549 cells depicted in Figure 2B revealed viral progeny
35 assembled regardless of the presence of the drug. The inventors further double CsCl gradient-purified full HAdV-C5 capsids from Nelfinavir-treated A549 cells (HAdV-C5^{Nelfinavir})

and performed negative staining electron microscopy (EM). The inventors found HAdV-C5^{Nelfinavir} capsids to be fully assembled, yet slightly deformed, darker stained and more clustered as shown in Figure 2C. Nevertheless, HAdV-C5^{Nelfinavir} capsids harboured unaffected cleavage of the HAdV precursors of pVI and pVII to their mature forms VI and VII (see Figure 2D), indicating uninhibited HAdV protease. The inventors further tested if HAdV-C5^{Nelfinavir} were infectious and found no defects on binding to naïve cells (Figure 2E) or subsequent viral gene expression (Figure 2F). In summary, the inventors found early and late stages of the first HAdV-C replication cycle including the infectivity of the intracellularly produced viral progeny unaffected by Nelfinavir.

Example 5: Nelfinavir is a post-exposure inhibitor of HAdV-C egress

To confirm that Nelfinavir acts at release of viral progeny, the inventors determined the number of infectious particles present intracellularly compared to that released to the supernatant at different time points (Figure 3A). Cells were infected with HAdV-C2-dE3B, washed and detached to remove unbound input virus and reseeded in the presence of different concentrations of Nelfinavir. At the indicated timepoints, supernatant and cell lysates were harvested separately and titrated on naïve cells for 3 days to allow for plaque formation. While the infected cell lysates harvested at 44 hours post infection (h pi) show comparable virus concentrations, the supernatants are free of viral progeny confirming infected host cells have not lysed yet. At 72 h pi however, only the supernatant of the non-treated infected cells harbours infectious viruses and the respective cell lysate gives rise to more plaques compared to the Nelfinavir-treated samples. This observation indicates that Nelfinavir impaired progeny release. Even at 120 h pi (5 days post infection, dpi), hardly any viral progeny is detectable in the supernatant of infected cells treated with 3 µM Nelfinavir. In comparison, both cell lysate and supernatant from the non-treated controls harbour so much virus, that all cells infected with either have lysed completely.

These observations were confirmed by quantification of the total infectious HAdV-C2-dE3B-CMV-GFP progeny in the A549 cells and the supernatant depicted in Figure 3B in a separate experiment in triplicates. Following the protocol described above, viral progeny in the cell monolayer and supernatant was harvested at the indicated time post infection by 3 freeze/thaw cycles and titrated on naïve A549 cells. The number of GFP-positive, infected cells at 44 h pi was determined and the yield per 12well extrapolated. Already a single treatment with 1.25 µM Nelfinavir delays the propagation of infection by at least 1, by 4 days for 10 µM. The total yield of infectious units 7 dpi was reduced by 3-logs emphasizing Nelfinavir's efficacy to inhibit HAdV-C-dE3B's virulence. In agreement with these observations, the inventors determined that Nelfinavir's inhibition of plaque formation is sustained at >50% even if Nelfinavir is administered to infected cells at 40 h pi (Figure 3C). In a wash-in/ wash-out

assay, HAdV-C2-dE3B-infected A549 cells were incubated with Nelfinavir over various stages of the infection cycle, namely early (0-20 h pi), late (20-40 h pi) and very late stage (≥ 40 h pi). Taken together, these findings indicate that Nelfinavir impairs a post-maturation step in the late stage of the HAdV-C2 life cycle necessary for progeny egress from the host cell.

The inventors next quantified the potency and longevity of Nelfinavir's inhibitory effect against HAdV-C2 infection. By assessing the number of infected nuclei and plaques at 4 dpi after inoculation with increasing HAdV-C2-dE3B-CMV-GFP pfu/well treated with 3 μ M Nelfinavir, the inventors show that spread is completely abolished up to 100 pfu/well (Figure 3D), underlining Nelfinavir's potency. When the inventors intended to quantify the longevity of Nelfinavir's egress inhibition (Figure 3E), the inventors noticed that HAdV-C2-dE3B-CMV-GFP in fact forms plaques under 1.25 μ M Nelfinavir delayed by 2 days (Figures 3F and G). However, they show a strikingly different, significantly rounder phenotype as quantified in Figure 3E and G. This phenotype was further confirmed in uphill assays (Figure 7A-D), where tilting of the plate over the course of the experiment increases convection. Moreover, the inventors found no correlation between the size of the plaques and their roundness (Figure 7D).

Example 6: Nelfinavir interferes with ADP protein levels and trafficking

Since the late AdV protein ADP was found to mediate efficient host cell lysis, the inventors suspected that Nelfinavir acts on ADP. It is highly expressed during the late stage of HAdV-C infection and requires multi-step post-translational modification at the ER and Golgi and proteolytic cleavage in order to finally assert its function in the nuclearenvelope. The inventors performed immunofluorescence staining of late stage HAdV-C2-dE3B-infected A549 cells (44 h pi, representative images shown in Figure 4A). Under non-perturbed conditions, ADP accumulates in cytoplasmic vesicular foci and the nuclear envelop. According to ADP's trafficking kinetics, the inventors interpret these foci as the trans-Golgi network (TGN) and COPI and COPII vesicles trafficking to and from the nucleus, respectively. Note that ADP expression levels were heterogeneous, but correlated with GFP expression levels irrespective of treatment with Nelfinavir. Moreover, the infection-induced nuclear remodelling was not affected by Nelfinavir. All strongly GFP-positive cells harboured strongly condensed chromatin and Hoechst-negative compartments indicating assembled progeny virus clusters as well as indentations of the nuclear envelop around these replication centres. The inventors conclude that Nelfinavir interferes with post-translational modification and / or proteolytic cleavage of ADP and thereby impairs ADP-mediated host cell lysis.

Example 7: Nelfinavir's anti-AdV efficacy is ADP-dependent

In order to conform that Nelfinavir's mode of action depends on ADP, the inventors have created an ADP-depleted HAdV-C2-dE3B-CMV-GFP mutant (HAdV-C2-dE3B-dADP) and characterized its spread kinetics in A549 cells. As depicted in Figure 4B, the onset of plaque formation in HAdV-C2-dE3B-dADP is delayed by one day compared to HAdV-C2-dE3B. This observation agrees with previous kinetic studies of ADP deleted HAdV-C mutants, if not as severe. HAdV-C2-dE3B-dADP plaques are comet-shaped, albeit their comet-heads appear bigger and denser. The inventors further assessed its sensitivity to Nelfinavir (Figure 4C) and found that depletion of ADP renders HAdV-C2-dE3B-CMV-GFP completely insensitive to spread inhibition by Nelfinavir ($TI_{50} = 2.11$ compared to 66.80 for HAdV-C2-dE3B, see Table 3 for details). Nelfinavir's ineffectiveness to inhibit HAdV-C2-dE3B-dADP-induced cell death was also detected via impedance-based measurement of cell viability. While the cell index profiles of HAdV-C2-dE3B-infected cells converge to the non-infected cells with increasing Nelfinavir concentrations (Figure 4D), the profiles of the Nelfinavir-treated HAdV-C2-dE3B-dADP-infected cells are nearly congruent with the non-treated infected control regardless of the Nelfinavir concentration (Figure 4E). Nelfinavir's lacking effect on the HAdV-C2-dE3B-dADP-induced cytopathic effect is further emphasized when calculating its delay of cytotoxicity (Figure 4F). Nelfinavir's ADP-dependence was confirmed in additional cell lines HeLa and HBEC as summarized in Figure 4G, for details see Table 3.

Example 8: Nelfinavir inhibits cell-free, but not direct cell-to-cell transmission of HAdV

The pathways by which intracellularly replicating pathogens such as viruses are transmitted between host cells can be grouped into two mechanistically distinct modes. These lead to characteristic plaque phenotypes visualized in unlimited convection *in vitro* plaque assays as sketched in Figure 5A. Both lytic, as described for HAdV-C, and non-lytic cell-free transmission gives rise to comet-shaped plaques whose density of infected cells decreases with increasing distance from the lysed first round infected cell *in vitro*. This spatial pattern is caused by convective passive mass flow in the cell culture medium. In contrast, direct cell-to-cell transmission as employed by HSV-1 leads to round plaques with a high density of infected cells unaffected by convection in the medium. For representative microscopy images, see Figure 5B. The inventors took advantage of HAdV-C-neutralizing serum to characterize the spatio-temporal transmission pattern of HAdV-C2-dE3B-CMV-GFP when second round infection via cell-free progeny is impaired by the addition of neutralizing antibodies (Figure 5A). As shown in Figure 5C, the inventors found that the onset of plaque-development is delayed by a day when cell-free virus is neutralized, while increasing viscosity by the addition of glycerol had no effect. Moreover, delayed plaques under neutralizing serum are round. In summary, the spatio-temporal transmission pattern of

HAdV-C2-dE3B-CMV-GFP under cell-free progeny-neutralizing conditions resembles that under Nelfinavir.

Example 9: Nelfinavir is a broad HAdV inhibitor

The inventors assessed Nelfinavir's inhibition breadth for various HAdV genotypes from species A, B, C and D in the cancer cell lines A549 (human adenocarcinomic alveolar basal epithelial cells) and HeLa (human epithelial cervix carcinoma cells) as well as the immortalized primary cell lines HCE (normal human corneal epithelial cells) and HBEC3-KT (normal human bronchial epithelial cells), HSV-1 in A549 and Mouse adenovirus (MAdV) 1 and 3 in CMT93 (mouse rectum carcinoma cells) as summarized in Figure 6A, for details see Table 3. The assay could not be established for HAdV-E4 and MAdV-2. The inventors first determined the optimal amount of inoculum and duration of infection for each virus - cell line combination in the 96well format to balance statistical significance and automated plaque segmentation. Based hereon, the inventors determined Nelfinavir's therapeutic index (TI_{50}) as ratio between toxicity (TC_{50}) and spread inhibition (IC_{50}) measured as number of nuclei and plaque numbers, respectively, infection for each virus - cell line combination. The inventors found a highly heterogenous susceptibility to Nelfinavir across HAdV species, which was consistent in the tested cell lines. While all tested HAdV-C genotypes as well as HAdV-B14 show high TI_{50} s (>10) ranging from 12.22 (HAdV-C1) to 71.09 (HAdV-C2), members of HAdV species A, D and most of the HAdV-B genotypes show intermediate (2 - 10) to no susceptibility (<2) to Nelfinavir, notably HAdV-B7 and B11 with $TI_{50}<0$. MAdV-1 and 3 also showed no to intermediate susceptibility, respectively. Nelfinavir has been shown to impair HSV egress by inhibition of envelop protein glycosylation. The inventors show that HSV-1 plaque formation is inhibited at a TI_{50} comparable to HAdV-C.

Morphological examinations of the microscopic 96-well images across the tested viruses under non-treated conditions (Figure 6B, black and white images) revealed that a virus' susceptibility to Nelfinavir correlates with their plaque shapes. Viruses with low TI_{50} showed a high fraction of round plaques already without the addition of Nelfinavir, while those HAdV genotypes with high TI_{50} s show exclusively comet-shaped plaques. This observation was confirmed macroscopically by crystal violet-stained non-treated, non-agar-overlaid infections in 12-well plates (Figure 6B, purple images).

Table 1. Scores from the HAdV AntiVir screening. All PCL compounds were tested in biological, temporally separated quadruplicates. 32 positive and negative controls (DFT and DMSO respectively) were included on every 384-well screening plate (521 in total). (ns = not significant)

	Number of nuclei	Number of infected	Infection index	Number of plaques	Integrated GFP
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		nuclei			intensity
Z 'score	-0.41	0.51	0.51	0.53	0.31
Nelfinavir	ns	0.91	0.88	1.00	0.98

Table 2: Pharmacokinetic parameters for Nelfinavir in selected mammals. BID (bi-daily dosage), F (Bioavailability), ip (intraperitoneal injection), iv (intravenous injection), pi (post injection).

Species	Dose	Route	C _{max} [ug/ml]	T _{max} [h]	T _{1/2-el} [h]	AUC _{0-∞} [μg/ml]	AUC ₀₋₁₂ [μg/(ml*h)]	F [%]
Mouse	50 mg/kg	ip	23.21 *)					
Mouse	100 mg/kg	ip	54.33 *)					
Rat	25 mg/kg	iv	12.73	0.06	1.26	7.01		
Rat	50 mg/kg	oral	1.34	2.82		6.09		43
Dog	15 mg/kg	iv	10.36	0.08	1.05	10.00		
Dog	30 mg/kg	oral	1.20	2.50	9.72	9.74		47
Monkey	12.5 mg/kg	iv	7.85	0.08	1.43	8.90		
Monkey	25 mg/kg	oral	0.93	2.50	2.29	4.07		25.5
Marmoset	12.5 mg/kg	iv	4.56	0.08	0.66	6.33		
Marmoset	25 mg/kg	oral	0.61	0.75	2.04	1.97		17
Human	800 mg	oral	3.13	3.50	2.28	7.56		
Human	1250 mg +)	oral					29.3	
Human	1250 mg +)	oral	4.3	4.00	4.20		30.5	

5 *) (30 min pi); +) BID; #)

Table 3: Statistical information on Nelfinavir's TI₅₀ against various viruses in different human cell lines. Indicated cell lines were infected with the indicated pfu/well virus and fixed at the indicated time pots infection. F: Fixation (dpi); BR: number of biological replicates; RO: readout; PN: Plaque number quantified based on viral GFP expression or HAdV hexon immunofluorescence staining as indicated, IC₅₀ (inhibitory concentration 50%, Nelfinavir concentration leading to 50% reduction in plaque number/ well), TC₅₀ (toxic concentration 50%, Nelfinavir concentration causing 50% reduction in nuclei number/ well) and TI₅₀ (TC₅₀/IC₅₀ ratio) are means of the indicated number of replicates. SE gives standard error. All IC₅₀, TC₅₀, SE values in μmol/L. Plaque numbers were quantified using Plaque2.0, nuclei numbers were quantified based on Hoechst signal using CellProfiler. Non-linear regression was performed in GraphPad.

Table 3

Genotype	F	BR	RO	PN (DMSO)	IC50	SE IC50	TC50	SE TC50	TI50 (TC50/IC50)	SE TI50
HAdV-A31	6	4	Hexon	13.00	14.13	8.97	19.08	7.39	1.35	1.38
HAdV-B3-pIX-FS2A-GFP	3	4	Hexon	6.50	10.22	10.91	15.29	4.97	1.50	2.08
HAdV-B7	4	4	Hexon	17.50	16.92	14.08	12.61	4.23	0.75	0.87
HAdV-B11	3	4	Hexon	10.80	28.89	41.04	15.29	4.97	0.53	0.92
HAdV-B14 clinical	3	4	Hexon	10.50	0.40	0.15	15.29	4.97	38.23	27.07
HAdV-B14 reference	3	3	Hexon	8.33	0.84	0.43	12.00	3.45	14.22	11.40
HAdV-B16	4	4	Hexon	8.25	12.27	9.16	20.35	7.78	1.66	1.87
HAdV-B21	4	4	Hexon	5.25	16.04	15.56	20.35	7.78	1.27	1.72
HAdV-B34	4	4	Hexon	12.75	3.18	1.18	20.35	7.78	6.40	4.81
HAdV-B35-pIX-FS2A-GFP	4	4	Hexon	12.25	2.49	0.76	15.29	4.97	6.13	3.87
HAdV-C1	4	4	Hexon	8.00	1.94	0.47	23.75	9.91	12.22	8.09
HAdV-C2	4	4	Hexon	5.75	0.24	0.09	17.26	1.61	71.09	33.69
HAdV-C2-dE3B_CMV-GFP	4	4	GFP	34.25	0.22	0.05	14.89	7.34	66.80	47.02
HAdV-C2-dE3B-dADP_CMV-GFP	4	3	GFP	41.33	5.82	1.65	12.29	2.06	2.11	0.95
HAdV-C5	3	4	VI	6.25	0.57	0.23	11.91	2.88	21.01	13.75
HAdV-C6	3	4	Hexon	7.75	0.60	0.20	21.19	6.55	35.14	22.45
HAdV-D8	5	4	Hexon	8.50	9.09	6.47	21.43	8.88	2.36	2.65
HAdV-D30	5	4	Hexon	9.25	4.20	1.78	13.36	1.13	3.18	1.61
HAdV-D37	5	4	Hexon	9.00	16.66	16.38	21.43	8.88	1.29	1.80
HSV-1-CMV-GFP	1	4	GFP	96.50	2.87	0.74	49.50	9.55	17.25	7.78
HAdV-C2-dE3B_CMV-GFP	4	4	GFP	12.00	0.49	0.20	29.85	4.15	61.03	33.52
HAdV-C2-dE3B-dADP_CMV-GFP	5	4	GFP	37.80	4.68	0.91	41.69	3.54	8.91	2.50

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CMT-93	MAV-1-pIX-FS2A-GFP	4	4	GFP	17.75	20.72	22.72	21.29	10.62	1.03	1.64
	MAV-3-pIX-FS2A-GFP	4	4	GFP	13.50	8.70	6.19	26.07	6.53	3.00	2.88
	HAdV-A31	8	4	Hexon	20.25	18.06	19.45	19.59	13.50	1.08	1.92
HFC	HAdV-C2-dE3B_CMV-GFP	5	4	GFP	54.75	0.55	0.10	29.73	15.31	54.04	37.20
	HAdV-B3-pIX-FS2A-GFP	8	4	GFP	18.25	6.41	1.21	12.74	1.10	1.99	0.55
	HAdV-B35-pIX-FS2A-GFP	6	4	GFP	25.50	6.99	1.50	13.33	1.26	1.91	0.59
HFC	HAdV-C2-dE3B_CMV-GFP	4	4	GFP	23.25	0.53	0.10	28.97	2.78	55.18	15.51
	HAdV-C2-dE3B-dADP_CMV-GFP	4	4	GFP	27.50	2.72	0.58	12.40	0.77	4.56	1.25

Claims

1. A compound for use in a method for treating or preventing an adenoviral infection, particular a human adenovirus infection or a murine adenovirus infection, wherein said compound is selected from Nelfinavir and a pharmaceutically acceptable Nelfinavir formulation or a Nelfinavir salt.
2. The compound for use in a method for treating or preventing an adenoviral infection according to claim 1, wherein said compound is Nelfinavir mesylate.
3. The compound for use in a method for treating or preventing an adenoviral infection according to claim 1 or 2, wherein said compound is administered to reach a plasma concentration of $\geq 1,25 \mu\text{mol/l}$, particularly of $\geq 3 \mu\text{mol/l}$, more particularly of $\geq 10 \mu\text{mol/l}$.
4. The compound for use in a method for treating or preventing an adenoviral infection according to any one of the preceding claims, wherein said compound is administered within 72 h of infection, particularly within 48 h of infection, more particularly within 24 h of infection.
5. The compound for use in a method for treating or preventing an adenoviral infection according to any one of the preceding claims, wherein said compound is administered within 72 h of detection of infection, particularly within 48 h of detection of infection, more particularly within 24 h of detection of infection.
6. The compound for use in a method for treating or preventing an adenoviral infection according to any one of the preceding claims, wherein said compound is formulated for oral application.
7. A dosage form for use in a method for treating or preventing an adenoviral infection, wherein said dosage form comprises a compound selected from Nelfinavir and a pharmaceutically acceptable Nelfinavir formulation or a Nelfinavir salt, particularly wherein said compound is Nelfinavir mesylate.
8. The dosage form for use in a method for treating or preventing an adenoviral infection according to claim 7, wherein said dosage form is formulated for oral application.
9. A method of manufacture of a medicament for treating or preventing an adenoviral infection, particularly a human adenovirus infection or a murine adenovirus infection, comprising the use of a compound selected from Nelfinavir and a pharmaceutically acceptable Nelfinavir formulation or a Nelfinavir salt, particularly wherein said compound is Nelfinavir mesylate.

10. A method for treating or preventing an adenoviral infection, particularly a human adenovirus infection or a murine adenovirus infection, comprising administering a compound selected from Nelfinavir and a pharmaceutically acceptable Nelfinavir formulation or a Nelfinavir salt in a therapeutically effective concentration to a patient in need thereof.
11. The method for treating or preventing an adenoviral infection according to claim 10, wherein said compound is Nelfinavir mesylate.
12. The method for treating or preventing an adenoviral infection according to claim 10, wherein said compound is administered to reach a plasma concentration of $\geq 1,25 \mu\text{mol/l}$, particularly of $\geq 3 \mu\text{mol/l}$, more particularly of $\geq 10 \mu\text{mol/l}$.
13. The method for treating or preventing an adenoviral infection according to claim 10, wherein said compound is administered within 72 h of infection, particularly within 48 h of infection, more particularly within 24 h of infection.
14. The method for treating or preventing an adenoviral infection according to claim 10, wherein said compound is administered orally.

Fig. 1

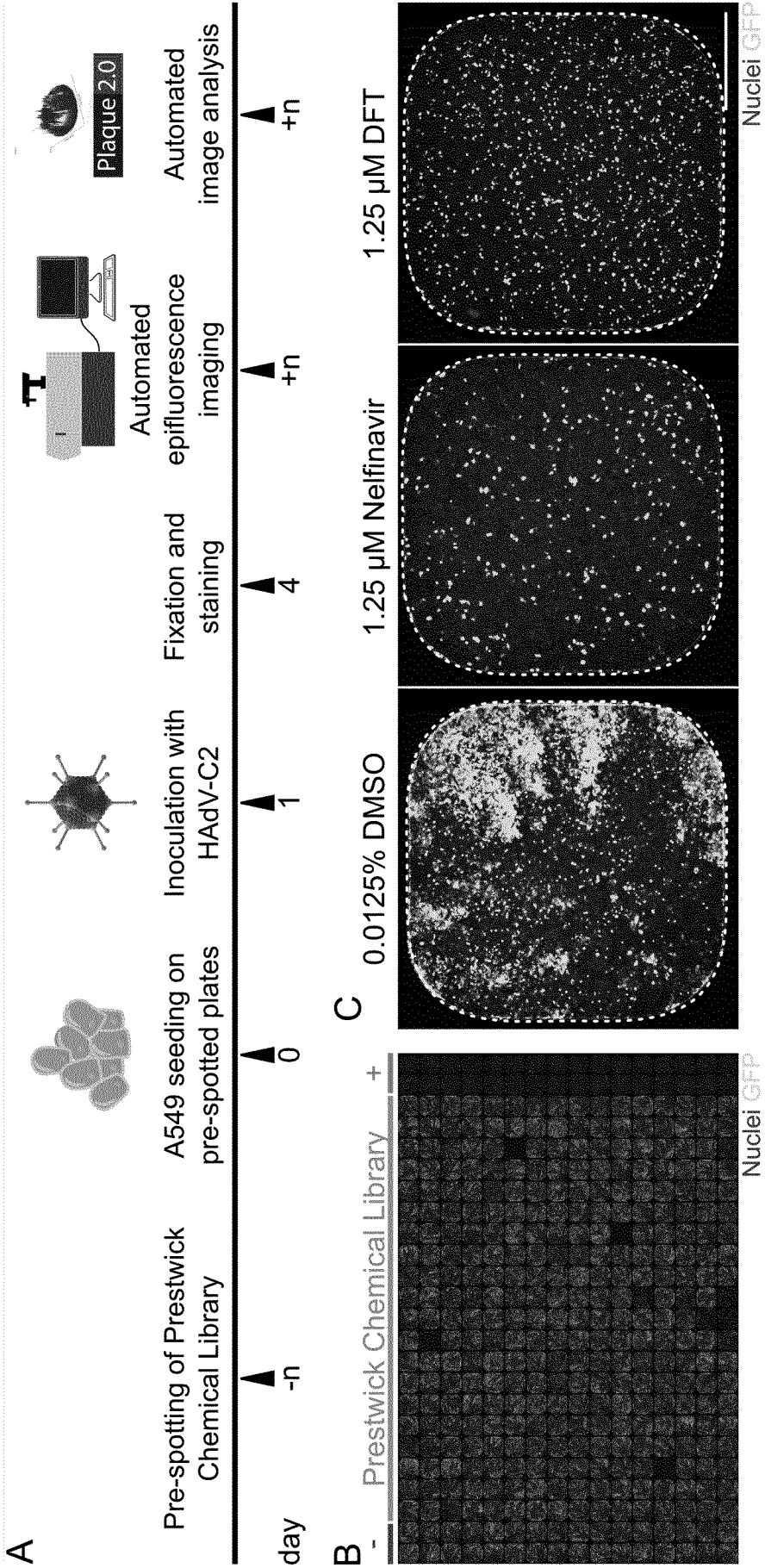
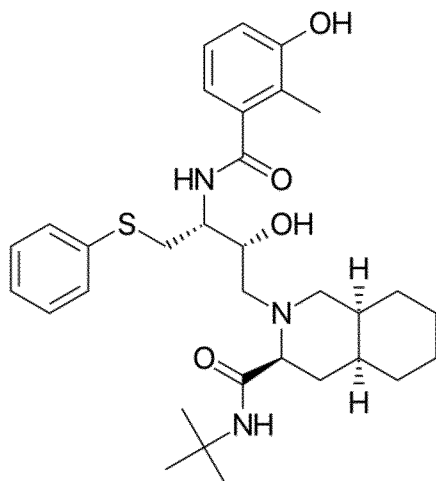
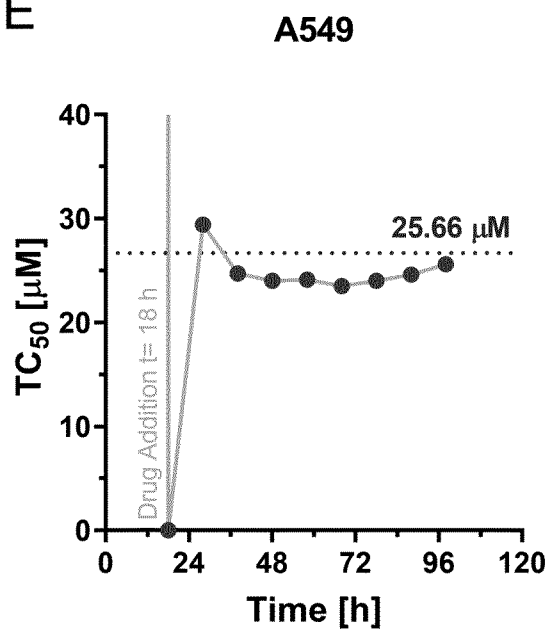


Fig. 1 (continued)

D



E



F

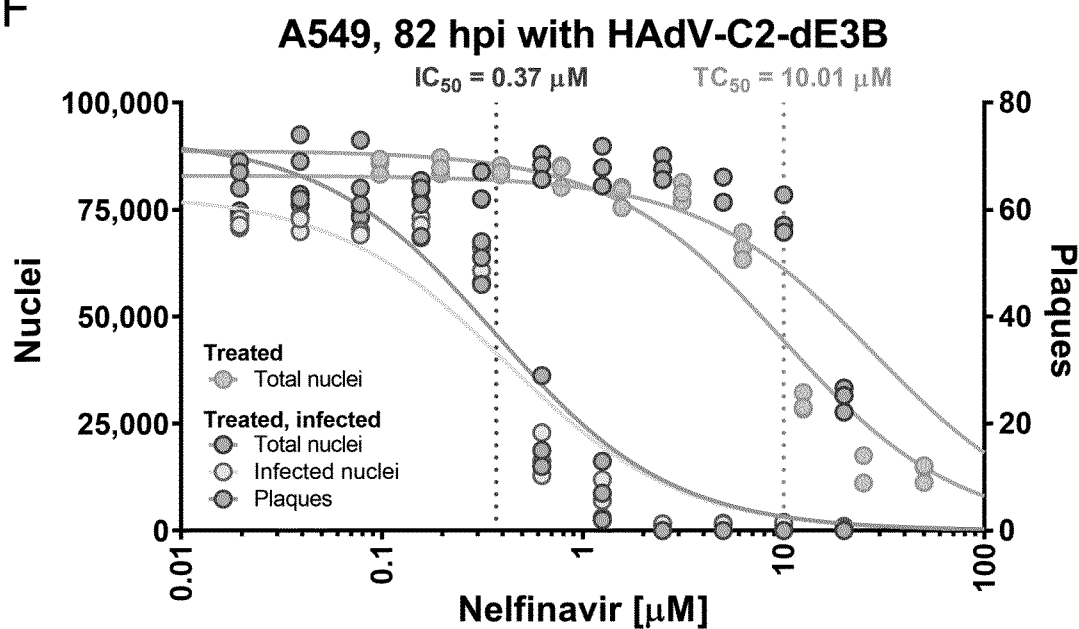


Fig. 2

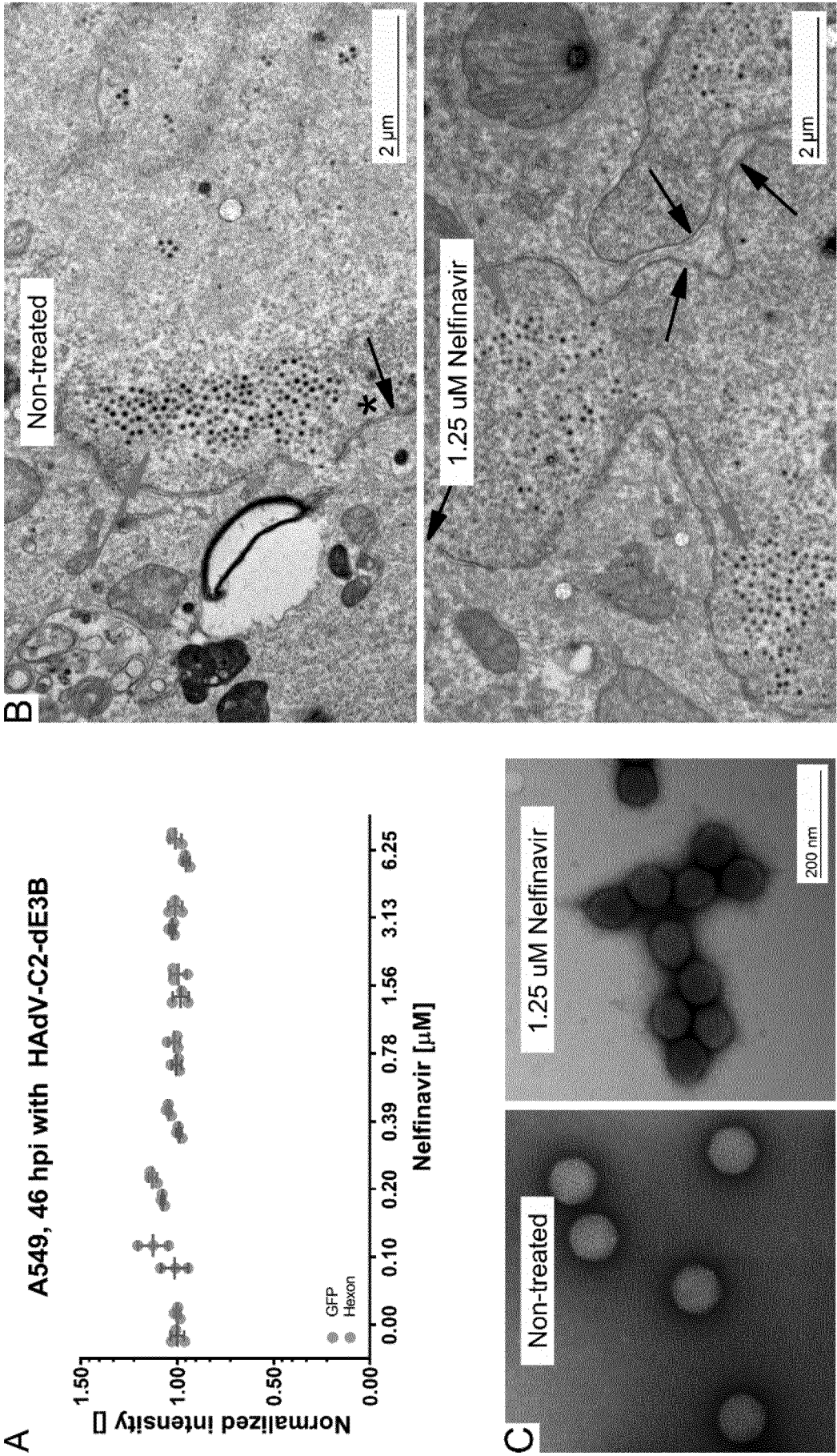


Fig. 2 (continued)

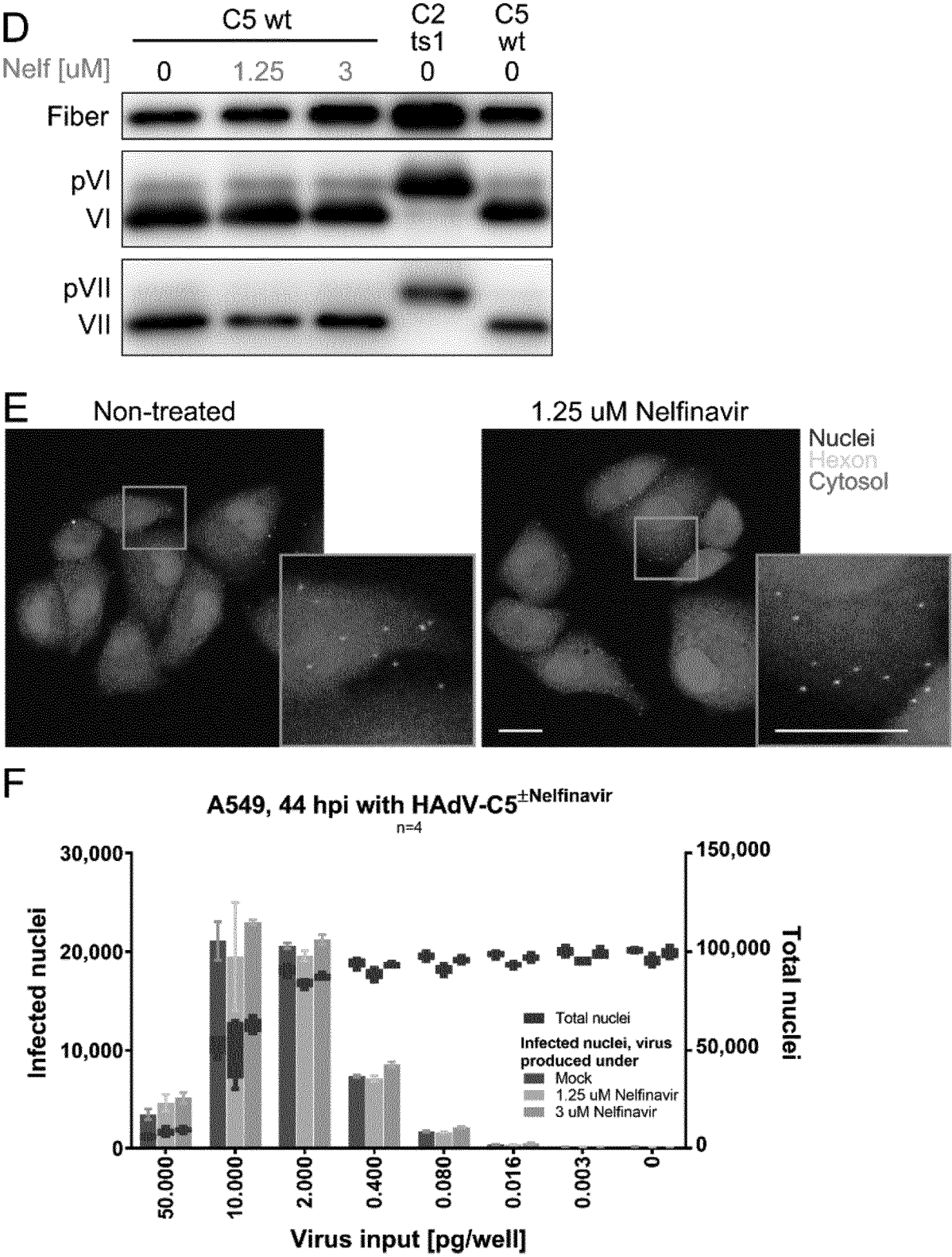


Fig. 3

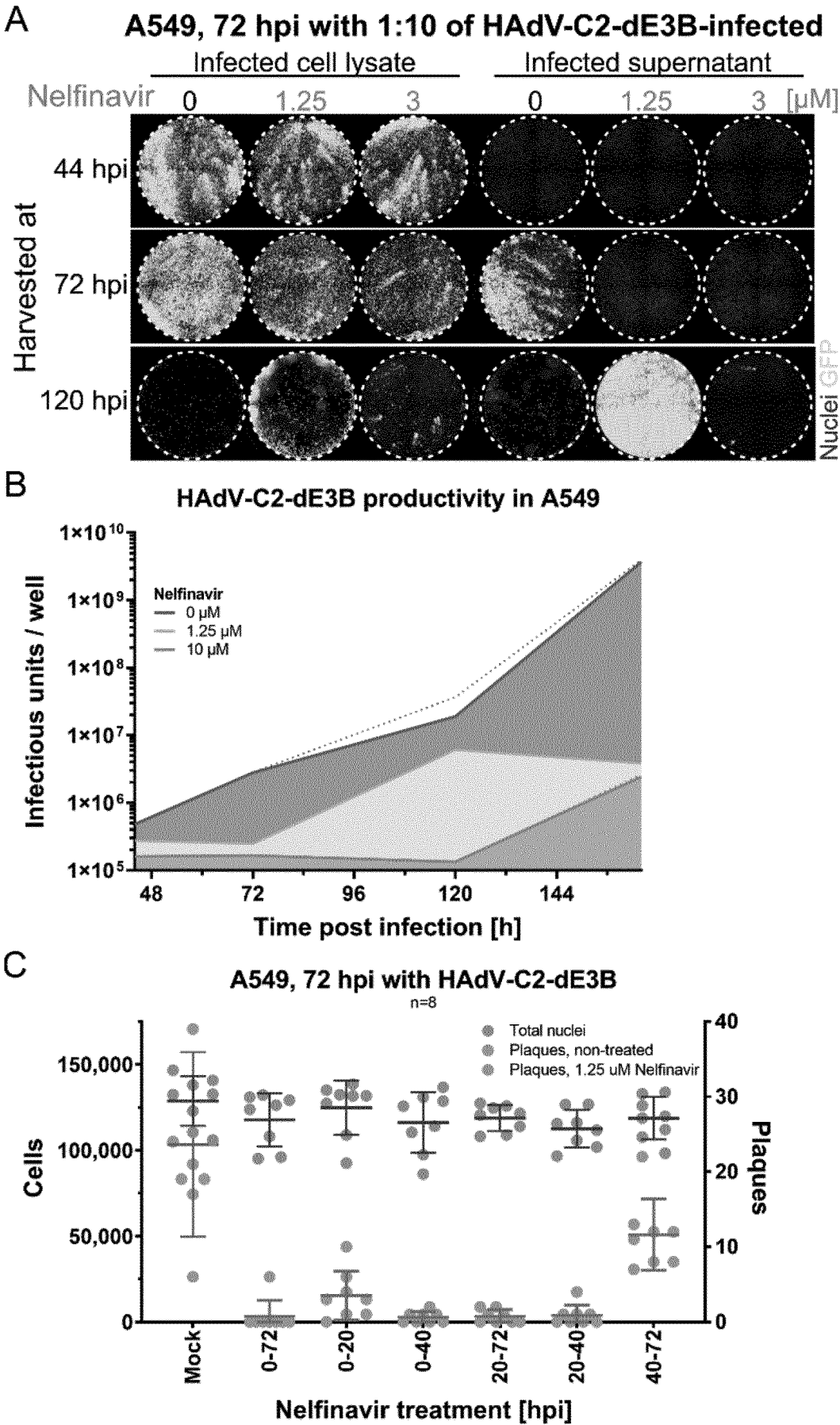


Fig. 3 (continued)

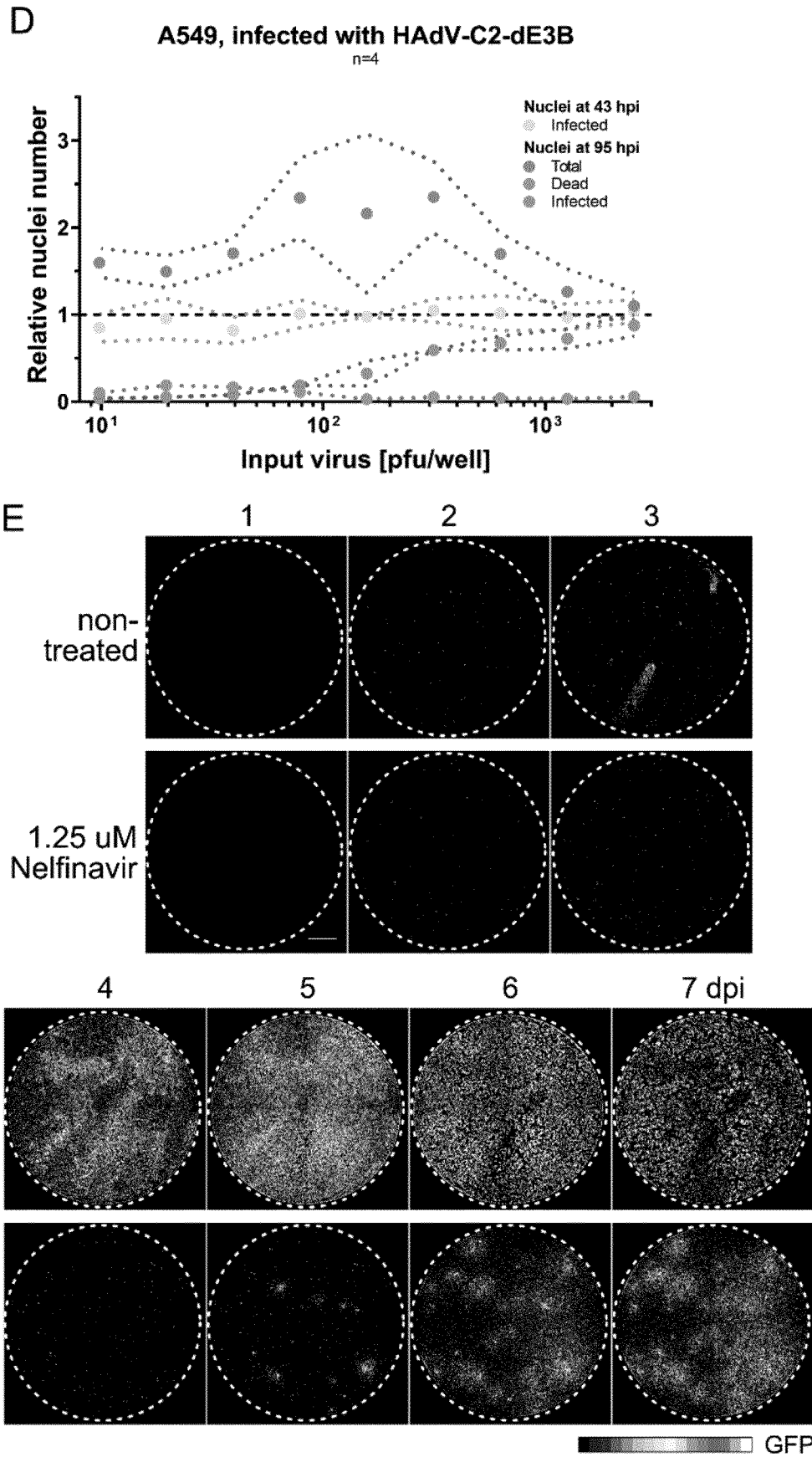


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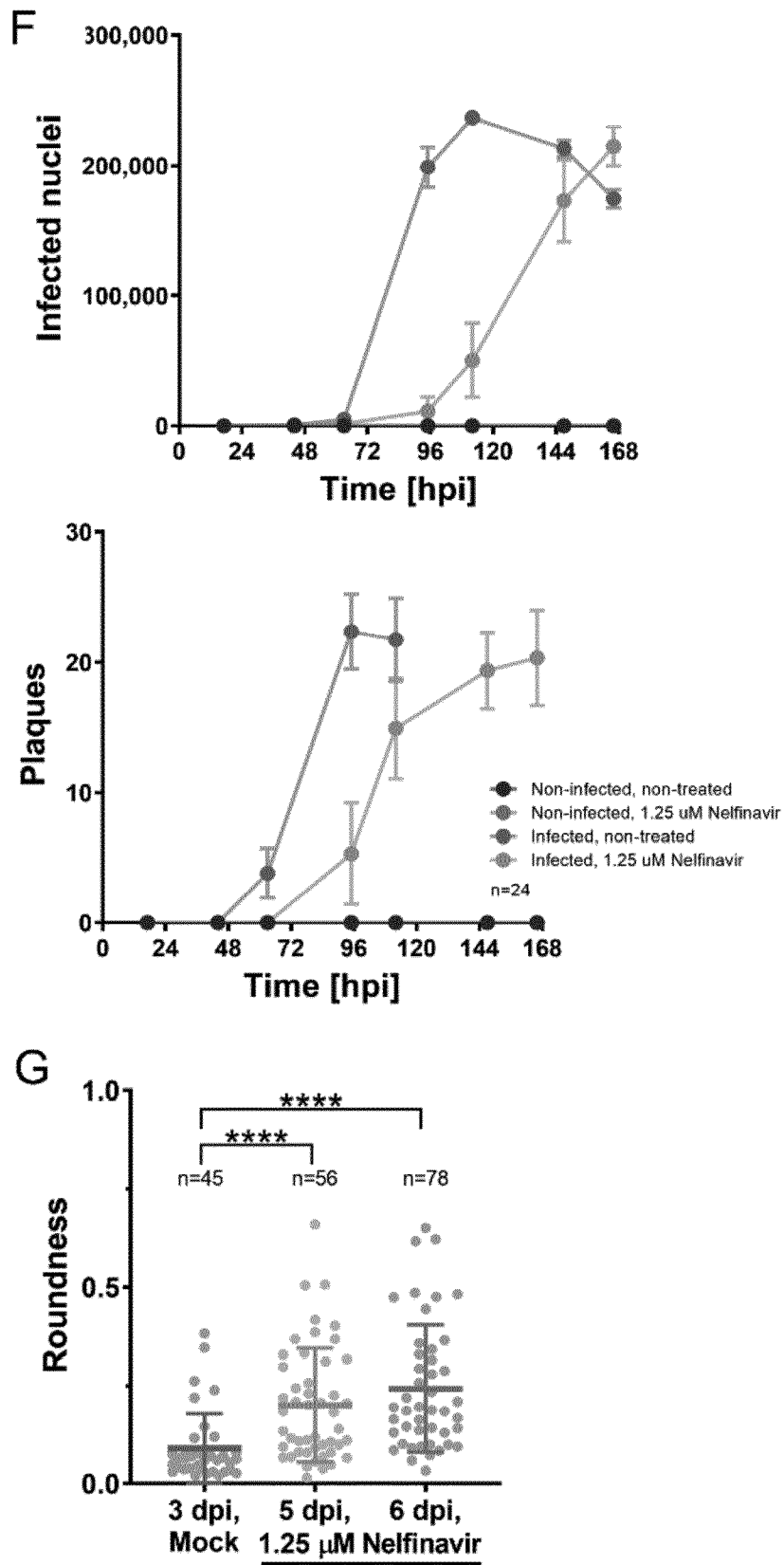


Fig. 4

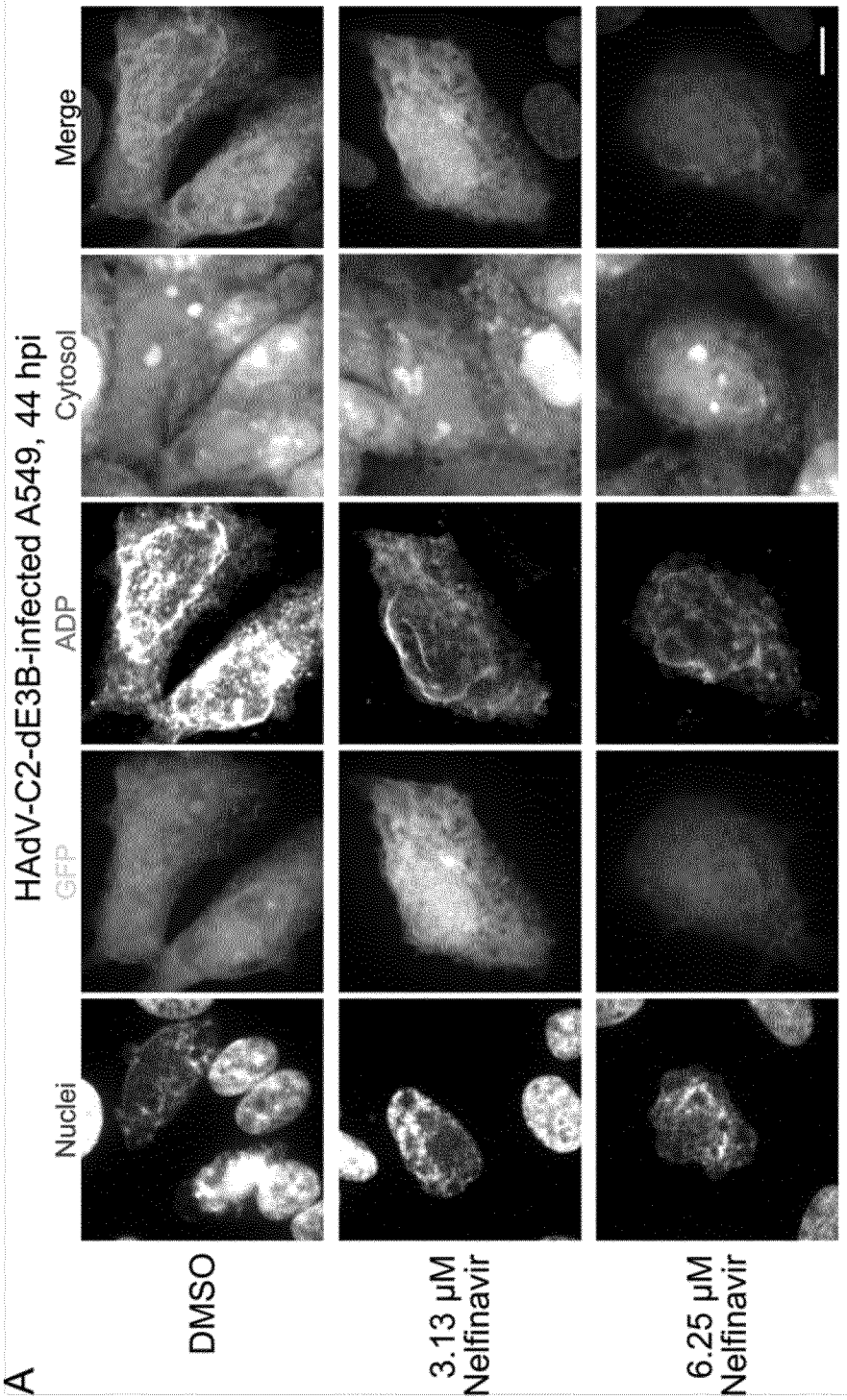


Fig. 4 (continued)

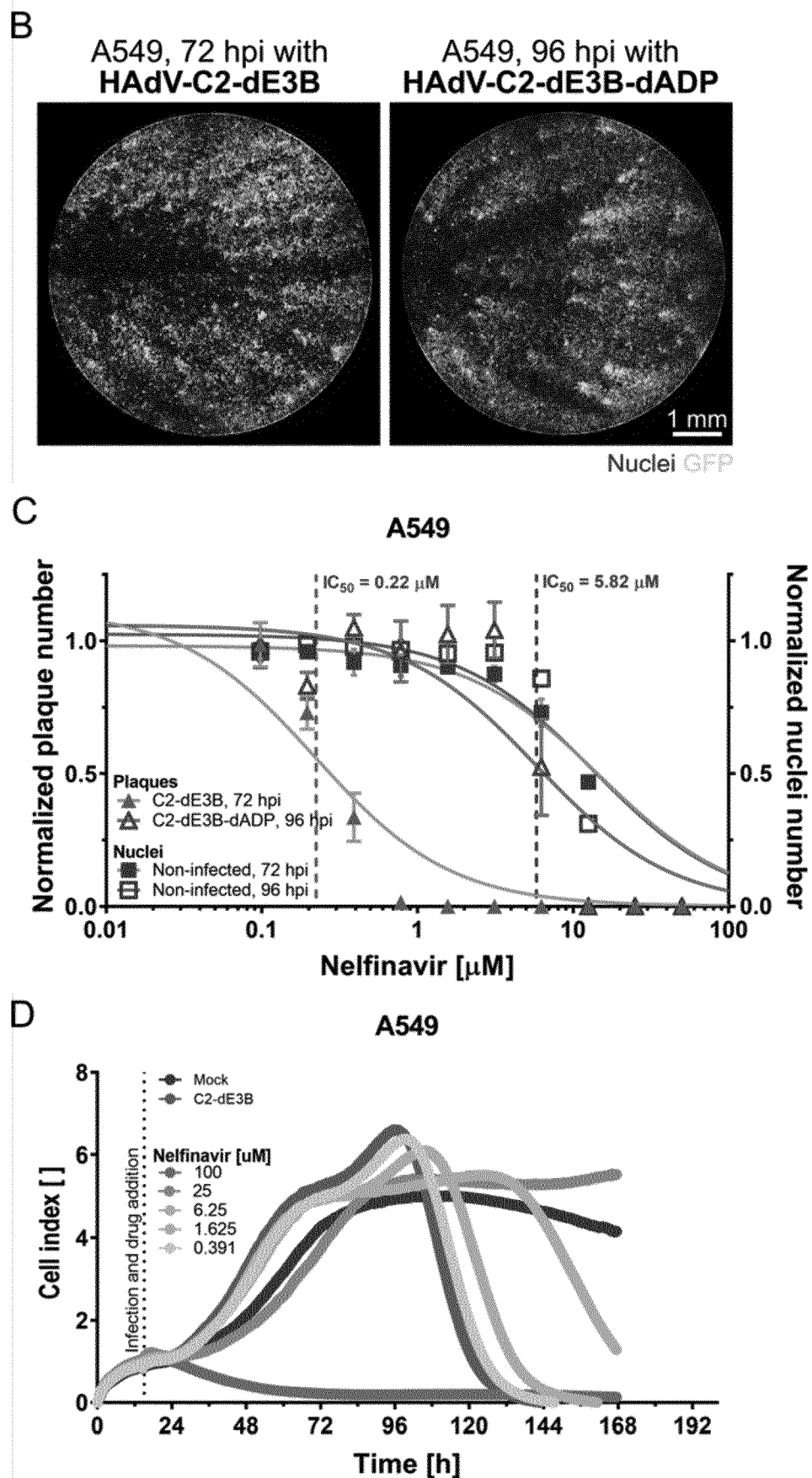


Fig. 4 (continued)

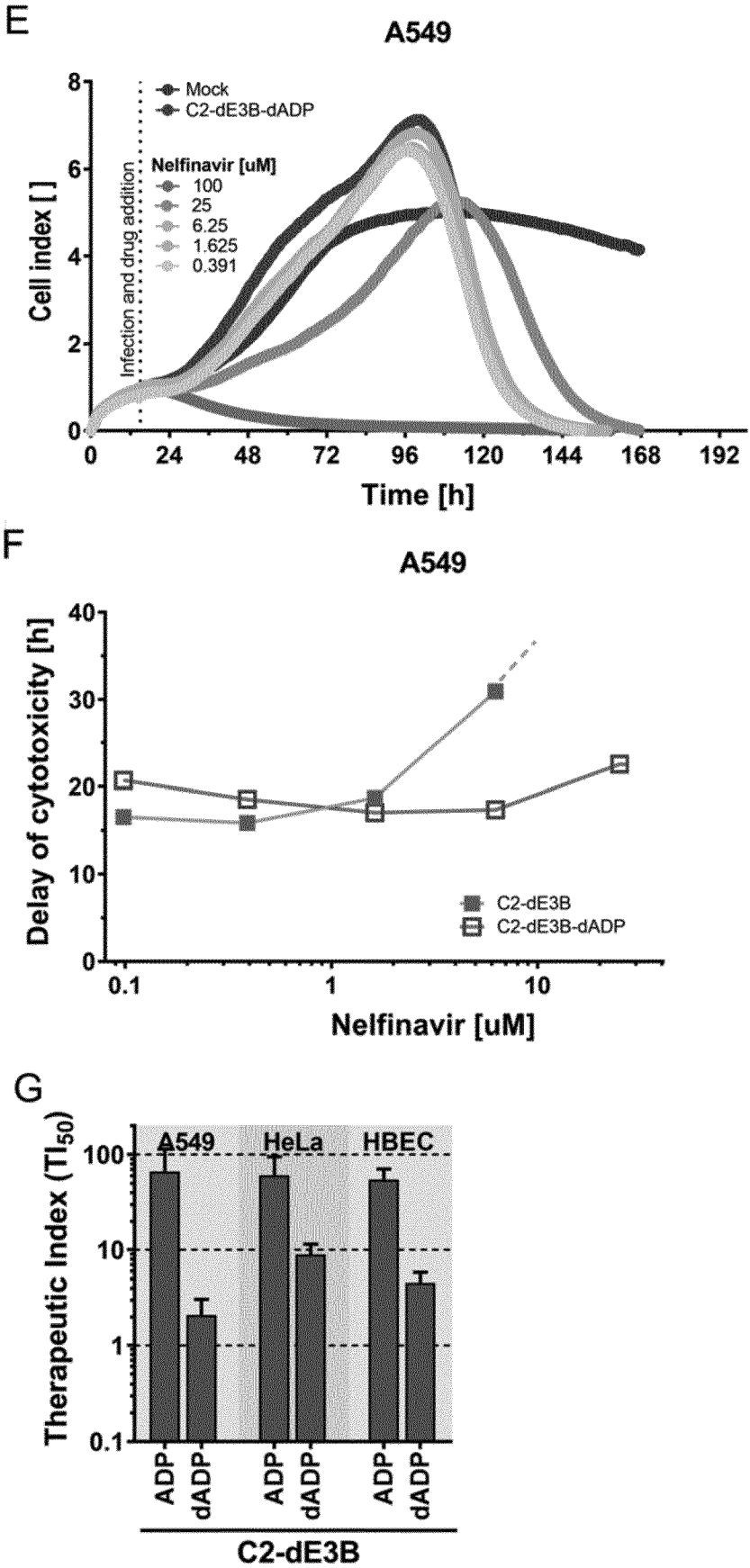


Fig. 5

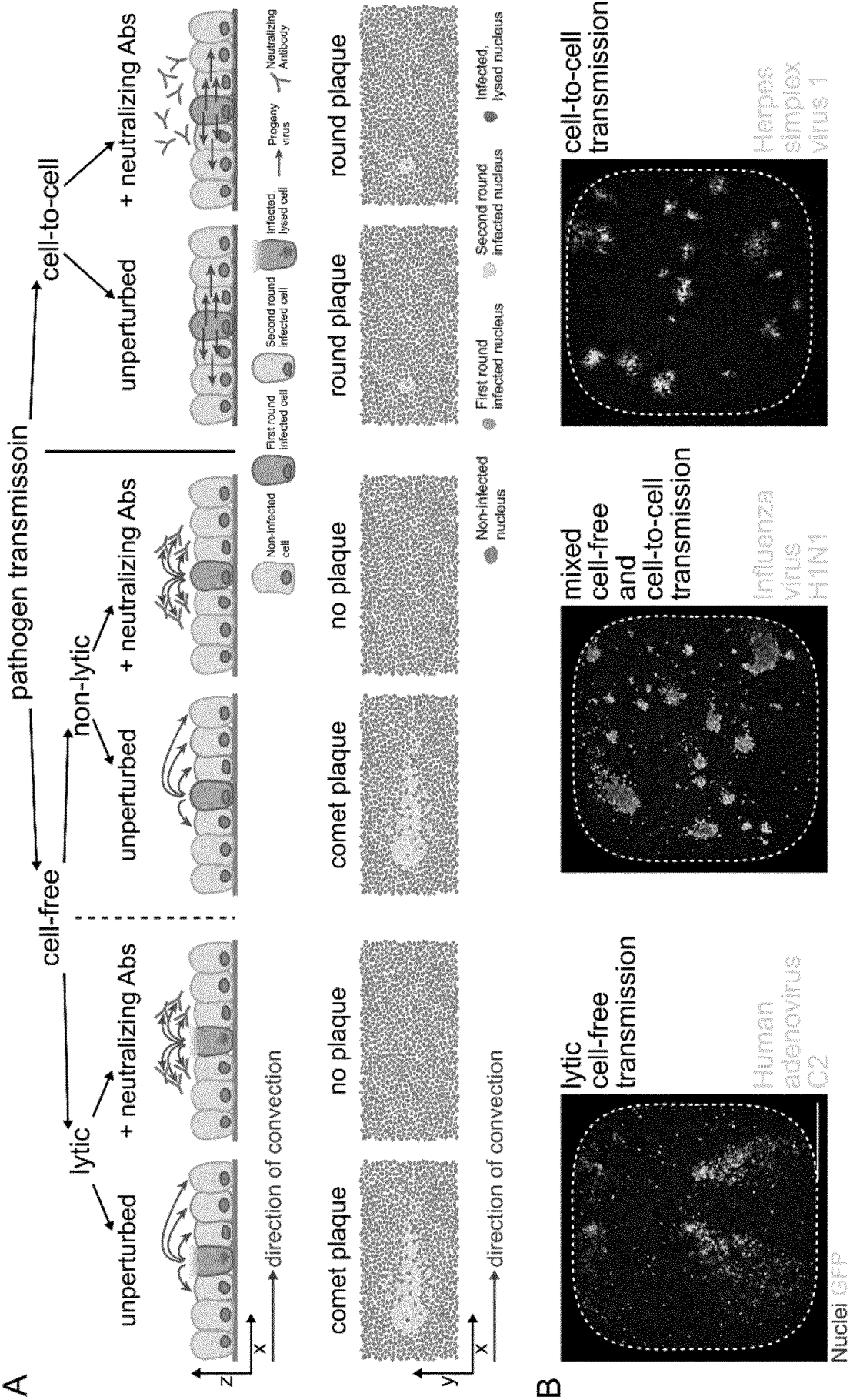


Fig. 5 (continued)

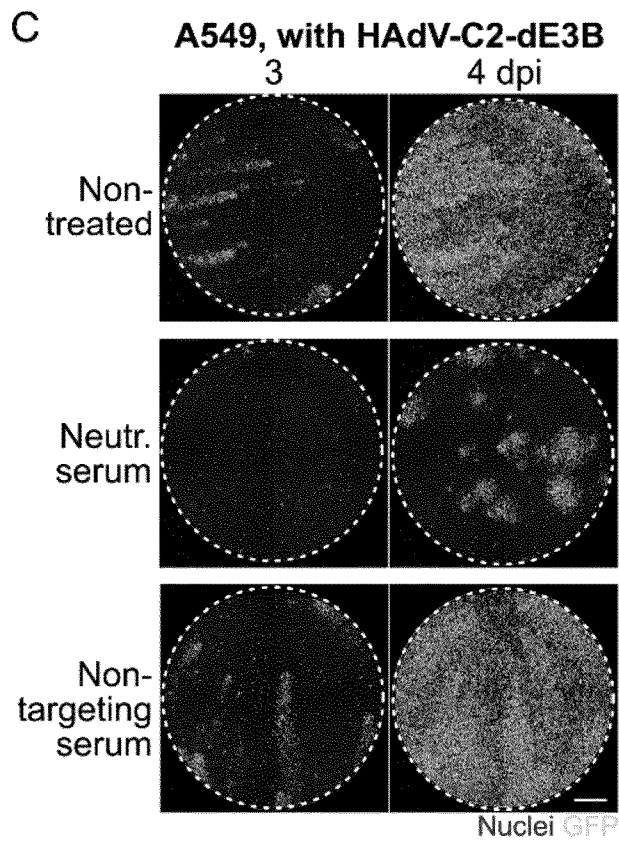


Fig. 6

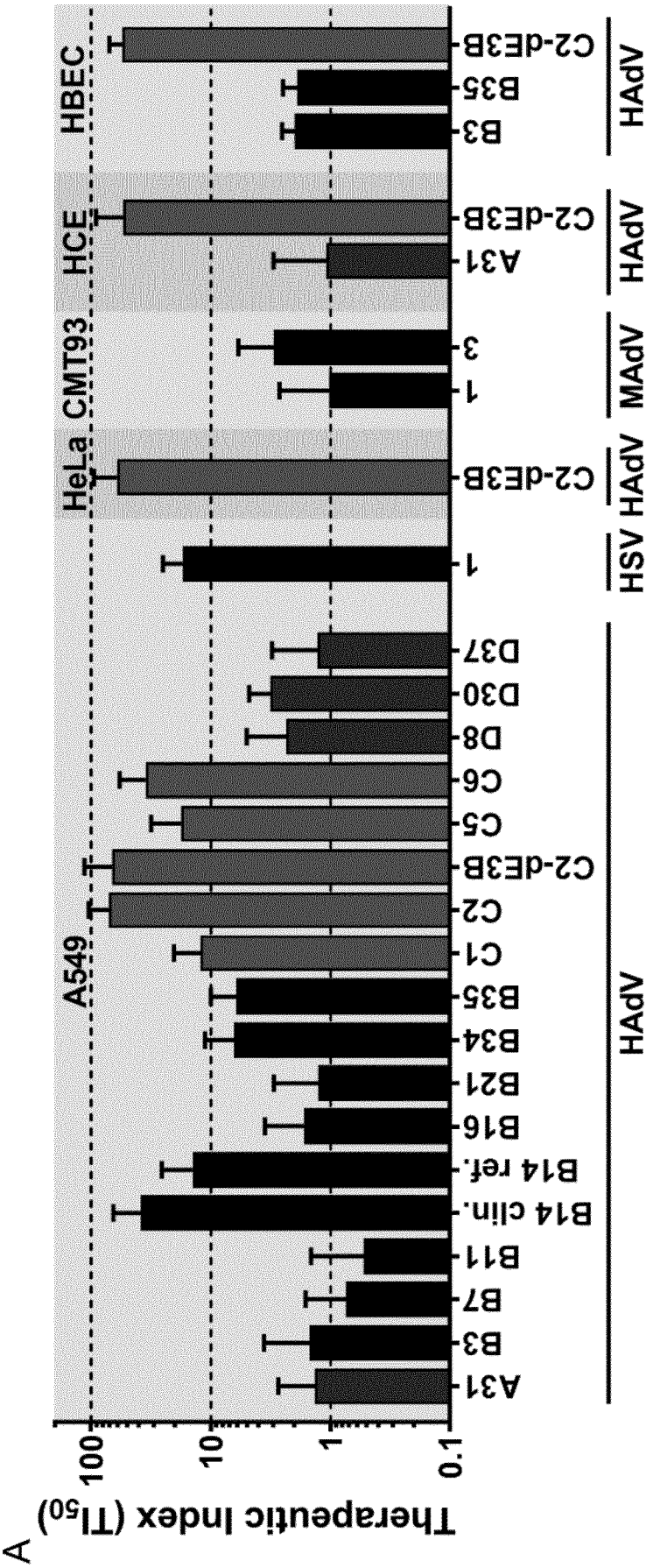


Fig. 6 (continued)

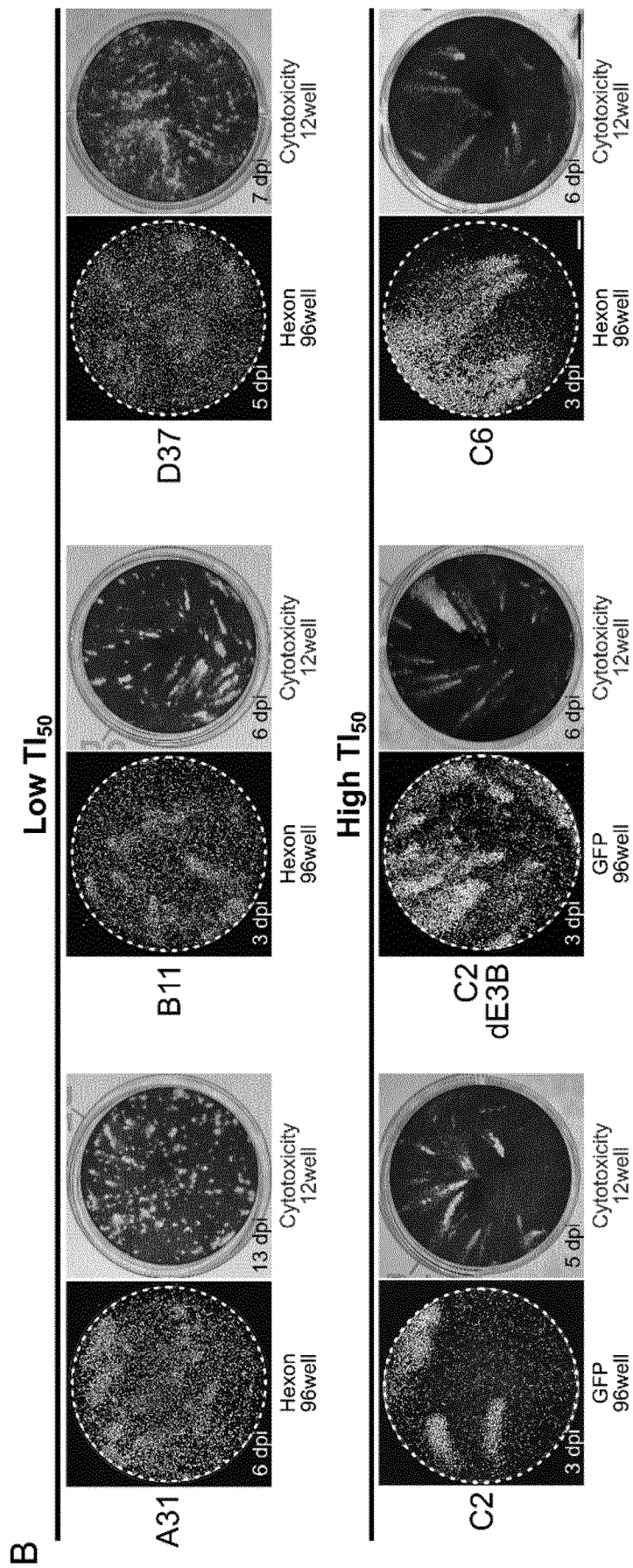


Fig. 7

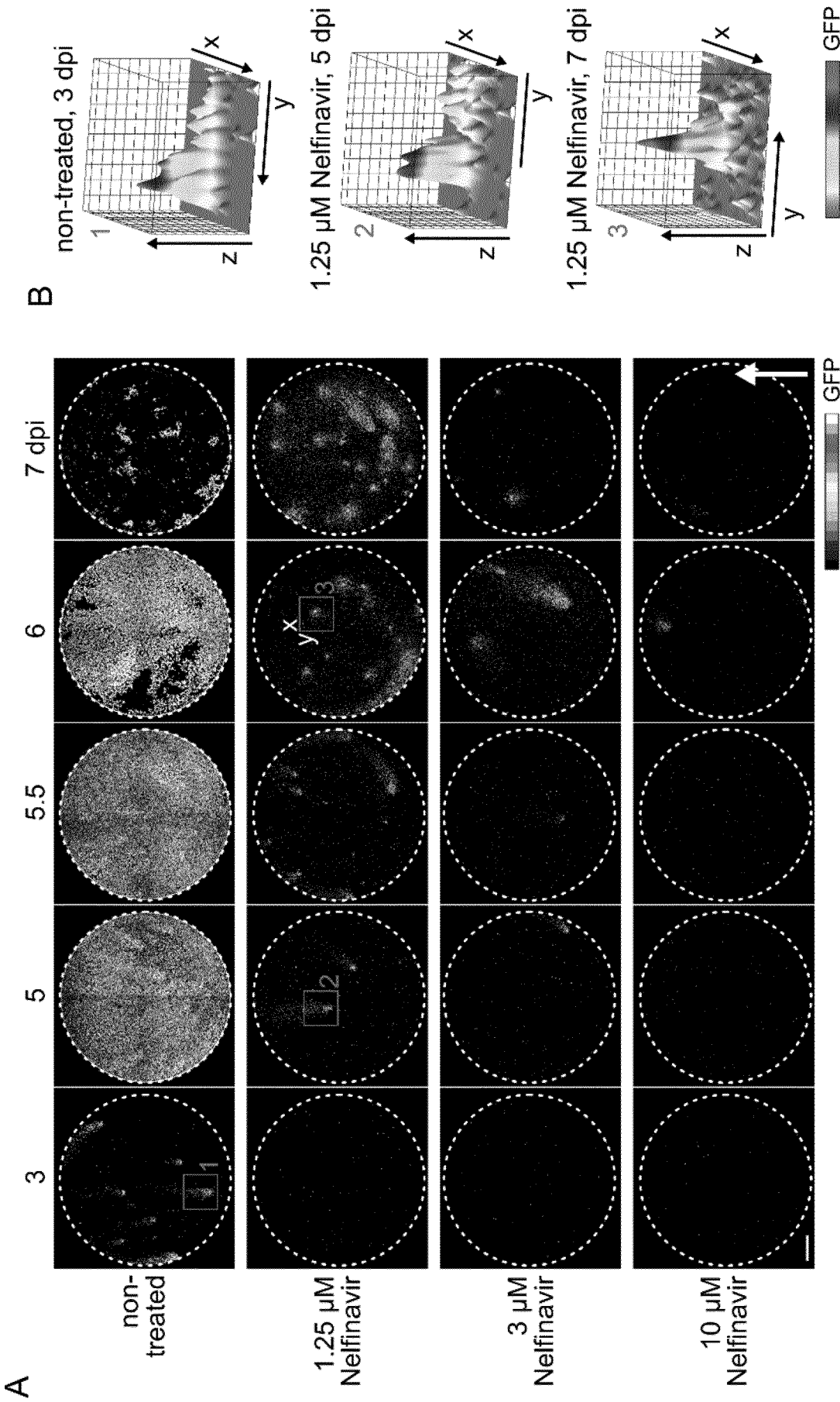
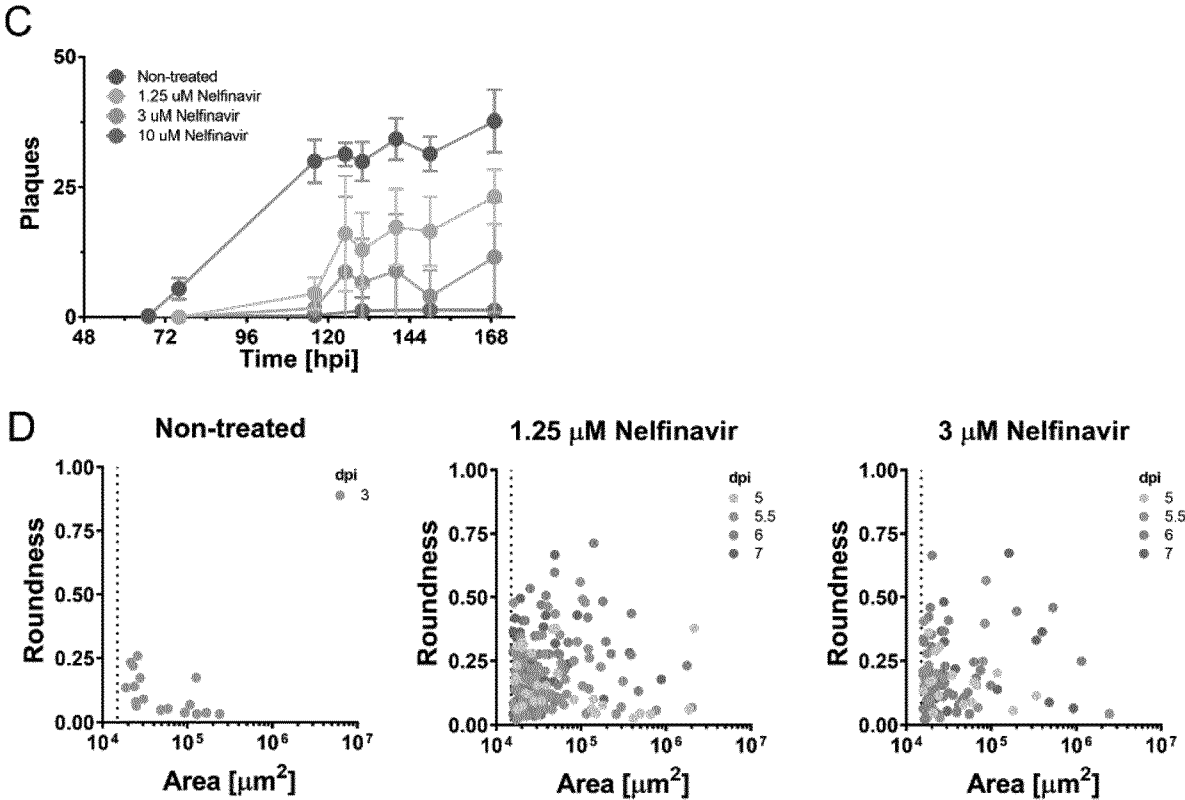


Fig. 7 (continued)



International application No
PCT/EP2019/080446

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/080446

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SOREN GANTT ET AL: "The HIV Protease Inhibitor Nelfinavir Inhibits Kaposi's Sarcoma-Associated Herpesvirus Replication In Vitro", ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 55, no. 6, June 2011 (2011-06), pages 2696-2703, XP055666466, US ISSN: 0066-4804, DOI: 10.1128/AAC.01295-10 abstract page 2698, column 2, paragraph 4 -----	1-14
A	N. N. KALU ET AL: "Nelfinavir Inhibits Maturation and Export of Herpes Simplex Virus 1", JOURNAL OF VIROLOGY., vol. 88, no. 10, 15 May 2014 (2014-05-15), pages 5455-5461, XP055666460, US ISSN: 0022-538X, DOI: 10.1128/JVI.03790-13 abstract -----	1-14
A	PRASHANT HIWARKAR ET AL: "Management of adenovirus infection in patients after haematopoietic stem cell transplantation: State-of-the-art and real-life current approach : A position statement on behalf of the Infectious Diseases Working Party of the European Society of Blood and Marrow Transplantation", REVIEWS IN MEDICAL VIROLOGY, vol. 28, no. 3, 6 April 2018 (2018-04-06), page e1980, XP055666472, GB ISSN: 1052-9276, DOI: 10.1002/rmv.1980 the whole document -----	1-14