Increased recombinant AAV production by HEK293 cells using small molecule chemical additives

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Abstract

Recombinant adeno-associated virus (rAAV) has established itself as a highly efficacious gene delivery vector with a well characterised safety profile allowing broad clinical application. Recent successes in rAAV-mediated gene therapy clinical trials will continue to drive demand for improved rAAV production processes to reduce costs. Here we demonstrate that small molecule bioactive chemical additives can significantly increase recombinant AAV vector production by HEK cells up to 3-fold. Nocodazole (an anti-mitotic agent) and M344 (a selective histone deacetylase inhibitor) were identified as positive regulators of rAAV8 genome titre in a microplate screening assay. Addition of nocodazole to triple-transfected HEK293 suspension cells producing rAAV arrested cells in G2/M phase, increased average cell volume, and reduced viable cell density relative to untreated rAAV producing cells at harvest.. Final crude genome vector titre from nocodazole treated cultures was >2-fold higher compared to non-treated cultures.. Further investigation showed nocodazole addition to cultures to be time critical.Genome titre improvement was found to be scalable and serotype independent across two distinct rAAV serotypes, rAAV8 and rAAV9. Furthermore, a combination of M344 and nocodazole produced a positive additive effect on rAAV8 genome titre, resulting in a 3-fold increase in genome titre compared to untreated cells.

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Abstract

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Nocodazole (an anti-mitotic agent) and M344 (a selective histone deacetylase inhibitor) were identified as positive regulators of rAAV8 genome titre in a microplate screening assay. Addition of nocodazole to triple-transfected HEK293 suspension cells producing rAAV arrested cells in G2/M phase, increased average cell volume, and reduced viable cell density relative to untreated rAAV producing cells at harvest.. Final crude genome vector titre from nocodazole treated cultures was >2-fold higher compared to non-treated cultures.. Further investigation showed nocodazole addition to cultures to be time critical.Genome titre improvement was found to be scalable and serotype independent across two distinct rAAV serotypes, rAAV8 and rAAV9. Furthermore, a combination of M344 and nocodazole produced a positive additive effect on rAAV8 genome titre, resulting in a 3-fold increase in genome titre compared to untreated cells.

Introduction

Recent regulatory approvals for recombinant adeno-associated virus (rAAV) mediated gene therapy products (Luxturna® in 2017 and Zolgensma® in 2019) - together with a significant increase in the number of rAAV gene therapies in clinical trials[1],[2] has highlighted the need for improved production process technology with respect to titre, product quality and cost. Indeed, the high cost of manufacturing rAAV gene therapies to support the typically high therapeutic dosages used (e.g. 6 x 10^{13} – 2 x 10^{14} vector genomes/kg[3]) has limited the economic viability of AAV gene therapies despite evidence of promising clinical efficacy. Therefore there is an urgent need for robust, intensified process technology to support production of Good Manufacturing Practice (GMP) quality material. As nearly all current rAAV production processes rely on co-transfection of three plasmids encoding the necessary AAV and helper genes into HEK293 cell hosts for transient production of rAAV within 3-5 days[4], efficient use of plasmid DNA (itself produced using a costly GMP-approved process) is also of paramount importance.

rAAV is a complex macromolecular product requiring a diverse network of cellular processes and molecular interactions to enable coordinated cellular synthesis - host-cell proteins, transiently expressed viral helper, capsid and replicase genes as well as the single stranded therapeutic viral DNA payload itself [5]–[7]. Small molecule enhancers of recombinant protein production have demonstrated efficacy in a wide variety of mammalian cell lines[3,10,11] and the addition of chemicals as diverse as sodium chloride, sodium butyrate, and soy peptones have been shown in previous studies to improve rAAV production yields[11]–[13]. Targeting of discrete pathways involved in the replication, packaging, and trafficking of viral particles by bioactive small molecule cell culture additives therefore offers a simple and cost-effective way of increasing viral titre and reducing overall production costs.

Here we show that microplate plate-based screening of chemical additives can be used to rapidly identify positive effectors of rAAV synthesis in HEK cells and that significant improvements in viral genome titre can be obtained by subsequent optimisation of dosing regimen. Furthermore, we also demonstrate that a combination of two functionally distinct small molecule enhancers may act additively, resulting in a 3-fold increase in viral vector titre (both increased genome titre and increased capsid titre) to substantially improve the efficient use of transfected plasmid DNA.

Materials and Methods

Cell culture

Proprietary suspension adapted Human Embryonic Kidney 293 (HEK293) cells were provided by REGENXBIO (Rockville, MD). Cells were cultured in a serum free medium supplemented with L-glutamine (Thermo Scientific, Waltham, MA). Cells were maintained in an orbital shaking incubator (Infors, Bottmingen, Switzerland) at 30 mL culture volumes in 125 mL Erlenmeyer flasks (Corning) at 37°C, 5% CO_2 and 85% humidity, with agitation at 140 rpm. Smaller scale cultures were grown in shallow-well 24-well plates (0.7 mL culture volume/well, 240 rpm shaking) (Corning, Acton, MA) using the Deutz system[14]. Routine cell density and cell viability measurements were performed on a ViCell automated cell counter (Beckman-Coulter, Brea, CA). Measurements of density and viability and determination of mean cell volume during rAAV production runs were performed using the Norma HT system (Iprasense) utilising 3 µl total cell culture and 20 µm slide chambers. Mean cell volume (V) was calculated as $V = \frac{4}{3}\pi r^3$ where r = measured cell diameter/2.

rAAV vector production in suspension adapted HEK293 cells

Proprietary ITR, Helper and Rep/Cap plasmid vectors used for rAAV8 and rAAV9 production were supplied by REGENXBIO using GFP as a reporter/transgene. Plasmids were transfected using polyethylenimine (PEI) as a gene delivery vehicle at optimised PEI:DNA and DNA:cell ratios. For rAAV production, plasmids and PEI were separately diluted in a serum free medium before being combined and incubated at RT. After incubation, the DNA:PEI polyplex transfection mixture was added to cells. For analysis, 450 µL of total cell culture was added to 50 µL 10x cell lysis buffer containing 1x cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche) and incubated for 1 h at 37 °C with gentle agitation. Samples were briefly centrifuged at 12,000 RPM to remove cell debris and the resulting supernatant used to determine viral genome and capsid titre.

Measurement of rAAV genome titre

Genome titre was quantified by digital droplet PCR (ddPCR). Aliquots of post-lysis supernatant (5 µL) from total cell culture were treated with DNAse I (Roche, Basel, Switzerland) to remove residual plasmid DNA. DNAse I-treated samples were diluted 1000- or 10,000-fold in PCR dilution buffer (GeneAmp PCR Buffer I (Thermo Scientific), 0.02% UltraPure Salmon Sperm DNA Solution (Invitrogen, Waltham, MA), 0.1% Pluronic F-68 non-ionic surfactant). Droplet formation and subsequent post-PCR droplet analysis was performed using the QX200 system (Bio-Rad, Hercules, CA), with absolute quantification of AAV genome copies/µl determined using the Quantasoft analysis software (Bio-Rad). Genome detection was achieved using primers and a FAM-labelled probe targeting the PolyA sequence of the pAAV-CAG-GFP plasmid. Capsid titre quantification was performed using the AAV8 titration ELISA (Progen, Heidelberg, Germany) from total cell lysis supernatant diluted in 1x ASSB assay buffer (Progen).

Chemical additive screening in microplates

Chemical additives for screening were diluted in either sterile water or dimethyl sulfoxide (DMSO, 100% v/v) where appropriate. Low, medium and high concentrations for each chemical were based on available literature describing their observed effects on recombinant protein expression*in vitro* (listed in Table S1). Cells were initially grown to a density of 4 x 10^6 viable cells/mL in Erlenmeyer flasks and mid-exponential phase cells were transfected with AAV-encoding plasmids prior to transferring to 24-shallow-well microplates (Corning) at 2.8x10^6 cells per well, 20 min post-transfection. Chemical additives were added to cells 24 hours post-transfection (HPT). Cell cultures were harvested at 72 HPT for viral genome titre analysis by ddPCR.

HEK cell DNA content analysis by flow cytometry

Cells (1 x 10^6) were harvested 72 HPT and fixed in 70% (v/v) EtOH at 4°C for 30 min, with gentle vortexing to prevent clumping. EtOH was removed and the cells washed twice in 1x PBS. Fixed cells were treated with 100 µl RNAse A (100 µg/mL in PBS; Qiagen) for 5 min at RT and the DNA stained with the subsequent addition of 400 µl propidium iodide (PI) (50 µg/mL in PBS; Thermo Scientific) at room temperature for a minimum of 30 min. PI-stained cells were analysed by flow cytometry using an LSRII instrument (BD Biosciences). Gated single cell populations were detected based on PI signal and the resulting histograms analysed using FlowJo software to determine the relative distribution of cells within the cell cycle (G1/S/G2-M) phases.

Immunocytochemistry

rAAV producing cells were harvested 24 hours after nocodazole addition. 1 x 10^6 cells were spun down at 250xg for 5 min, culture media was removed, and cells resuspended in 1 mL PBS. Resuspended cells were incubated with no agitation for 30 min at RT and allowed to adhere by gravity sedimentation to lysine-coated glass coverslips in 24-well plates. After 30 min, PBS and unadhered cells were removed by gentle aspiration. Adhered cells were fixed by 10min incubation at RT with 0.5 mL 4% (v/v) paraformaldehyde in PBS. Fixed cells were washed with PBS before permeabilization with 0.5 mL 0.5% (v/v) Triton-X100 for 10 min. Permeabilised cells were blocked by 10% normal goat serum (Life Technologies, Carlsbad, CA; #016201) in PBS for 30 min. Cells were then incubated with anti-Fibrillarin antibody (Abcam, Cambridge,

UK; ab5821, 0.1 µg/mL in blocking buffer) for 1 h at RT before incubation with goat anti-Rabbit Alexa-594 conjugated secondary antibody (Abcam; ab150080, 1:1000 dilution in blocking buffer) for 45 minutes at room temperature. Coverslips were transferred to a glass slide and mounted with Fluoroshield mounting media containing DAPI (Sigma-Aldrich; F6507) for visualisation of nuclei. Slides were imaged using a Nikon Eclipse Ti fluorescent microscope and images adjusted for brightness and contrast using ImageJ software.

Results

Initial screening of chemical additives to increase AAV genome titre

To identify novel small molecule enhancers of rAAV production, a panel of 15 small molecule culture additives was chosen based on either their reported propensity to increase recombinant protein expression in mammalian cell systems, or their reported positive effects on rAAV transduction efficiency. Due to the diverse mechanisms involved in AAV vector production, the panel featured chemicals that exert their influence on recombinant protein expression via a broad range of functional mechanisms. Specifically, we tested chemicals reported to be "chemical chaperones" (TUDCA, TMAO, betaine)[15]–[17], cell cycle modulators (nocodazole, BI-2536)[18],[19], caspase inhibitors (z-VAD-fmk)[20], histone deacetylase inhibitors (NaBu, VPA, M344, apicidin)[10],[21]–[23], insulin-mimetics (lithium chloride and zinc sulphate)[24], and proteasome inhibitors (ONX0912, MLN9708, MG132)[25]–[27].

To enable multi-parallel screening of small molecule additives, we employed pre-optimized small-scale cultures (700 µL culture volume) in 24-well microplates that exhibited a similar rAAV production profile to shake flask cultures (data not shown). Triple transfection was performed using PEI in a serum-free medium, and chemical additives were introduced to rAAV8-producing suspension HEK293 cells at 24 HPT. Each additive was used at three different concentrations (Low, medium, high – values based on previous literature, see Table S1). Total cell culture was harvested at 72 HPT, cells were chemically lysed and the rAAV8 genome titre in the crude supernatant was measured by ddPCR. Crude genome titre in cells containing chemical additives was compared to that of untreated rAAV8 producing cells to determine changes to titre mediated by the small molecules. Several chemicals displayed a clear concentration-dependent impact on genome titre, both positively (z-VAD-fmk) and negatively (LiCl, ONX0912) (Fig. 1A). Several compounds with similar mechanistic properties showed a marked reduction in rAAV titre at the tested concentrations – most notably inhibitors of proteasomal function (ONX0912, MLN9708, MG132) and the reported chemical chaperones (TUDCA, TMAO, betaine). Notably, the screening showed that high relative dose nocodazole (4 µM) added to culture 24 HPT resulted in a 1.34-fold increase in rAAV8 titer compared to untreated control cells ($p = 0.62$, $n = 2$), while M344 appeared to be effective at low dose (2.5 µM) with a 1.36-fold increase in rAAV8 titer ($p = 0.64$, $n = 2$). Of note, nocodazole treatment increased mean cell volume at all three concentrations, an attribute that appears to correlate positively with measured genome titre ($r^2 = 0.79$, $p < 0.0001$) (Fig. 1B) and has been shown in previous reports to be a major cellular determinant of recombinant protein productivity in Chinese hamster ovary (CHO) cells [28]–[30]. Nocodazole treated cells were found to be up to 27% larger than control cells. Nevertheless, this phenotypic variation was not observed with M344, in which the increase in titer was not accompanied by an increase in cell volume. It may therefore be inferred that these molecules modulate rAAV expression via distinct mechanisms independent of each other and that increased control of rAAV production may be achieved by the application of specific combinations of small molecule effectors.

Early addition of nocodazole enhances rAAV8 production in HEK293 cells

A recent study showed that *cap* gene expression, translation and assembly of Cap proteins into empty AAV particles is a kinetically rapid process[31]. ˜80% of capsids are assembled within the first 24 HPT whereas Rep mediated replication of rAAV genome from the *cis* -ITR plasmid is slower, peaking after capsid protein levels have plateaued due to the inhibitory action of Rep binding to the packaging plasmid and inhibiting transcription or by translational repression of *cap* mRNA. As capsid secretion from the nucleus into the cytoplasm is independent of genome loading, pre-assembled empty capsids can be secreted from the site of rAAV genome loading, thus depleting the pool of empty capsids available to newly replicated rAAV genome

[31],[32]. Therefore, we hypothesized that the effect of a given chemical effector may be positive (or further enhanced), neutral, or negative with respect to the timing of its deployment,

To further investigate the impact of nocodazole in enhancing rAAV production, we deployed high dose nocodazole (4 μM) between 0 – 48 HPT (Figure 2). Addition of 4 μM nocodazole immediately (0 HPT) and 4 HPT resulted in a considerable reduction in VCD at 72 HPT of 74% and 64% respectively, compared to rAAV8 producing cells without nocodazole (Ctrl) (Fig. 2A). Also apparent at early addition timepoints was a slight reduction in cell viability ($p > 0.05$; Fig. 2B) and a significantly higher mean cell volume compared to cultures not treated with nocodazole (up to 23%; Fig. 2C), consistent with previous work by Tait et al., (2004) who observed increased cell size and decreased viability in nocodazole treated CHO cells producing a recombinant monoclonal antibody. Most strikingly, addition of nocodazole at 4 HPT resulted in a 2.2-fold increase in rAAV8 genome titre at harvest compared to control cells (Fig. 2D). Addition at the earlier timepoint of 0 HPT was sub-optimal, with a 10% reduction in rAAV8 titer compared to the titer of cells treated at 4 HPT (Figure 2D). We therefore conclude that early addition (˜4 HPT) of nocodazole to rAAV producing cells is an effective positive mediator of rAAV production in a small-scale, transient expression, suspension HEK cell system.

Nocodazole improves rAAV genome titre in two different serotypes

To investigate the effect of nocodazole addition in a system more representative of large scale rAAV production, culture volume was scaled up from the initial microplate culture volume of 0.7 mL to 30 mL culture volume in shake flasks. To rule out a serotype-dependent effect of nocodazole addition, plasmids for both rAAV8 and rAAV9 serotypes were separately transfected in HEK293 cells and cultured with and without the addition of 4 μM nocodazole. rAAV genome titre was measured from 24 – 72 HPT. All measured parameters of nocodazole induced cell cycle arrest (reduced VCD, decreased viability, and increased mean cell volume) and increased rAAV genome titre were consistent with those seen at smaller culture volumes (Fig. 3A & B). Comparable to microplate-based cultures, mean viral genome titre was increased by up to 2.5-fold in nocodazole-treated cultures compared to untreated cultures (Fig. 3C). Importantly there were no observed significant differences in the above-described measurements between cultures producing rAAV8 or rAAV9, suggesting a broad applicability for nocodazole addition in rAAV manufacturing.

rAAV producing cultures treated with nocodazole have an increased proportion of cells in G2/M phase

Nocodazole is an anti-mitotic agent, used as both a chemotherapeutic and as a common agent of cell cycle synchronisation[18],[33]–[35]. Nocodazole exerts its effect by reversibly inhibiting the polymerisation of β-tubulin, destabilising microtubules and preventing the formation of mitotic spindles, thus arresting cells within the G2/M phase of the cell cycle. Cells treated with nocodazole typically enter mitosis but are unable to progress through cytokinesis, leading to either apoptosis in cells remaining in arrested mitosis for an extended period of time, or subsequent "mitotic slippage" into G0/G1 phase followed by apoptosis[35]–[38]. In addition to its use as a cell synchronisation agent, nocodazole has previously been shown to increase transient recombinant protein expression in a mammalian cell system[39].

Flow cytometry was carried out to determine the cell cycle status of rAAV8-producing cultures treated with nocodazole. Untreated rAAV8 producing cells were found to be almost entirely in either G1 or S phase at 72 HPT (Fig. 4A). Cells treated with 4 μM nocodazole at the point of transfection (0 HPT) resulted in a very significant proportion (38%) arresting in G2/M phase (Fig. 3B). The ratio of cells in G2/M : G1 phase was found to decrease the later nocodazole was added (Fig. 4C). Of note is the observation that a significant number (26%) of cells remain arrested in G2/M phase at 72 HPT when nocodazole is added as late as 24 HPT, yet the positive effect on rAAV8 genome titre is substantially lessened compared to earlier treatment. This would suggest a critical temporal component to the addition of nocodazole, such that later additions to cell culture are sub-optimal in terms of producing high rAAV titres, likely due to the rapid assembly process of cap proteins that largely occurred within 24 HPT[31].

The nucleolus is a dynamic compartment within the nucleus which undergoes extensive remodelling during

cell cycle progression, particularly during mitosis[40]. Previous immunocytochemical examination of nucleolar localisation throughout normal cell cycle progression shows a distinctive dispersal of nucleolar protein staining throughout the cytoplasm during prometaphase and metaphase – concomitant with the breakdown of the nuclear membrane during mitosis [41]–[43]. Nucleolar proteins in cells in interphase and prophase are typically found located within the nucleus itself. Of note, the nucleolus is considered to be a likely site for AAV capsid assembly and Rep-mediated loading of AAV genome into capsids [44]–[47], as well as being linked more generally to viral replication in other human viruses[48].

Cells from rAAV8 producing cultures - both with and without nocodazole addition 4 HPT - were harvested and fixed at 24 HPT. Cells were stained with a nucleolar marker (fibrillarin - a protein component of the nucleolus associated with ribosomal RNA processing[42]), and DAPI (a nuclear DNA stain). Widefield fluorescent microscopy was used to visualise the phenotypic changes induced by nocodazole addition. Untreated cells displayed highly localised or punctate staining of fibrillarin within the area of nuclear DNA staining (Fig. 4D), indicative of cells in interphase or prophase. In contrast, cells treated with nocodazole exhibited a proportion displaying a disorganised nuclear phenotype - with fibrillarin distributed broadly throughout the cytoplasm, and with condensed nuclear DNA (Fig. 4E) –indicative of cells either progressing through mitosis or arrested within G2/M phase.

Small molecule additives can be combined to further enhance rAAV8 production

In order to evaluate whether specific combinations of effectors could act synergistically to further increase rAAV production, we utilized nocodazole in combination with either z-VAD-fmk or M344 (see Figure 1). M344 is a synthetic analogue of the anti-fungal drug Trichostatin A, an inhibitor of Class I and IIB histone deacetylases, and recently used as an enhancer of recombinant protein expression in mammalian cell culture[10]. Z-VAD-fmk is a pan-caspase inhibitor[20]. Each chemical was added to rAAV8 producing HEK293 cultures together with nocodazole at 4 HPT in 24-well microplates. The inclusion of z-VAD-fmk in the initial screening experiment was predicated on its anti-caspase activity, as we hypothesised caspase mediated apoptosis - linked to rAAV production and expression of AAV2 Rep proteins - would negatively affect final crude titre[49],[50]. Additionally, caspase-mediated apoptosis induced by nocodazole-mediated cell cycle dysregulation would further increase cell death within the production cultures. Increased cell viability and VCD in cultures treated with nocodazole/z-VAD-fmk relative to untreated, nocodazole treated or nocodazole/M344 treated cells suggests that apoptosis was reduced (Fig. 5A & B) but with an unexpected reduction in crude genome titre (Fig. 5D). The observed reduction may be a consequence of off-target induction of autophagy via inactivation of n -glycanase 1 (NGLY1) that has been shown to occur in HEK293 cells treated with z-VAD-fmk[51]. While the role of autophagy in rAAV production is undetermined, inhibition of autophagy has been shown to increase recombinant protein expression in CHO cells[52],[53] and unintentional upregulation of autophagy may result in a reduction in viral component proteins necessary for production of high viral titres. Interestingly, the highest mean cell volume, which appeared to correlate positively with genome titre, was measured in cells treated with both nocodazole and z-VAD-fmk (Fig. 5C). This may be a result of reduced apoptotic cell death allowing cell size to increase but with the caveat that any benefit gained from this from a production perspective is attenuated by the potential negative off-target effects of z-VAD-fmk.

We observed that the combination of 2.5 μM M344 and 4 μM nocodazole produced an additive effect, increasing crude rAAV genome titre 2.6-fold compared to untreated cultures, an improvement on nocodazole alone (2-fold increase compared to untreated) (Fig 5D). This additive effect was also observed in 30 mL shake flask cultures, whereby measurements of VCD and viability closely replicated those observed in the screening assay (Fig. 6A), together with an improvement in genome titre with nocodazole/M344 from a 2.6-fold increase in the screening assay to a 3-fold increase compared to untreated cells in larger volume cultures (Fig. 6B). AAV8 intact capsid-specific ELISA analysis of total capsid titre showed a significant increase in intact capsids after addition of nocodazole (4.3-fold increase) and nocodazole/M344 (11.3-fold increase) compared to untreated controls (data not shown). We anticipate that rAAV vector development could complement the process engineering strategy to further maximize rAAV titer (e.g. high intact capsid levels are likely to benefit from hybrid Rep with improved genome packaging efficiency)[54] while combinatorial

empirical modeling will enable systematic determination of optimal chemical dosage and timing.

Taken together, these data show that nocodazole, either alone or in combination with select small molecules, can reproducibly boost both genome and total viral particle titre in a transient suspension HEK293 rAAV production system and that it is both scalable and applicable to production of rAAVs derived from two phylogenetically distinct and clinically translatable pseudotyped capsids.

Discussion

Improving the yield of intact, genome-containing rAAV particles during viral vector production is a critical step to reducing overall production costs. Here we describe a simple and robust method by which viral vector titre, a fundamental critical quality attribute of viral vector production, may be quickly improved in an established and previously optimised suspension culture system. Small molecule enhancers of recombinant protein expression have been extensively used across a wide range of mammalian cell systems to improve transient production performance[8]–[10],[22],[39],[55],[56]. The ease of use and low cost of small molecule enhancers (particularly for chemicals with pronounced biological activity at low dosages) makes them an attractive solution to improving rAAV yield.

Investigation of the cellular mechanisms underpinning increased viral genome titre point to the arrest of rAAV producing cells in mitosis soon after PEI-mediated transient transfection of rAAV producing plasmids. Prior studies have shown a strong preference for wildtype AAV replication within the G2/M phase of the cell cycle[57],[58]. Nocodazole has been shown to significantly improve transient transfection efficiency in CHO cells[39] which may stem from increased nuclear permissibility of transfection complexes due to the breakdown of the nuclear membrane during mitosis. A reduction in cell proliferation caused by nocodazole addition may also benefit viral production by reducing plasmid copy number dilution and maintaining mRNA transcript levels [9]. A recent study utilising a CRISPR-mediated genome wide screening strategy identified two target genes (ITPRIP and SKA2) that when modulated in cells increased rAAV genome titre and improved full/empty capsid ratios, with both target genes (strongly, in the case of SKA2) associated with cell cycle modulation [59]. Further to this, a proteomic study of HEK cells during AAV5 production highlighted a number of proteins involved in cell cycle and proliferation as being strongly downregulated during production [60]. The molecular effects of cell cycle arrest within the G2/M phase on rAAV production are unknown, but there is a potential correlation between the loss of essential nucleolar functions and the nucleolar localization of viral proteins, while the volume increase could possibly minimize crowding effects due to accumulation of viral proteins[61]. This apparent link between the cell cycle and AAV production, and the abundance of cell cycle modulating molecules, necessitates further investigation into the use of cell cycle modulators for both improving rAAV production yields and ultimately improving our understanding of the underlying biological processes governing rAAV production.

The apparent important temporal aspect of cell cycle regulation within the production process may also provide avenues for non-chemical interventions to improve vector yield as this relationship becomes better understood. Whilst we have not investigated the mechanism behind M344-mediated titre enhancement, HDAC6 (of which M344 is a selective inhibitor) has been shown to bind to, and regulate clearance of, ubiquitinated proteins via induction of the heat-shock cellular response[62]. AAV capsid proteins are a known target of ubiquitination post-viral entry[63], and the ubiquitin-proteasome pathway (UPP) has been suggested to play an active role in AAV capsid monomer degradation[64], therefore we posit a link between M344-mediated UPP dysregulation and increased crude viral titre. Due to the robustness of the results between small-scale plate-based cultures and larger scale shake flasks, we believe that the screening process shown here could be further scaled down and automated to increase throughput, due to the availability of instrumentation that can rapidly and accurately dispense very small volumes of drugs into culture. As minimal volumes are required for ddPCR analysis of genome titre, identification of novel enhancers of rAAV could be rapidly incorporated into existing rAAV production platforms with minimal changes to existing protocols.

In summary, we show that the use of readily available small molecule enhancers can significantly improve

rAAV production yield. We show that small molecule enhancers of rAAV production are amenable to optimisation in an existing suspension HEK293 cell system and that positive hits from initial small-scale screening of enhancer molecules can translate to larger scale production platforms. We also show that increased titre resulting from nocodazole treatment is consistent across two different serotypes, suggesting broad applicability in rAAV manufacturing.

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Conflicts of interest

The authors declare no conflicts of interest.

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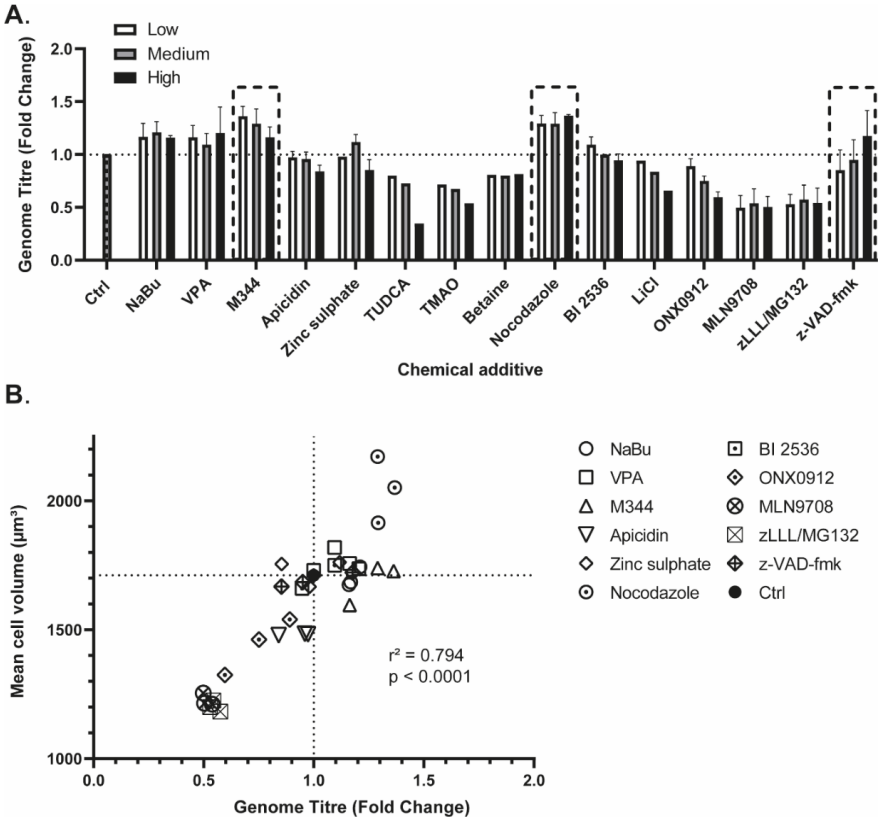

Figure 1 – Initial screen of small molecule culture additives identifies nocodazole, M344 and z-VAD-fmk as novel putative enhancers of rAAV genome titre. Cells were analysed 72 hours post transfection (HPT) for rAAV8 genome titre, shown here as the fold change relative to rAAV8 producing cells containing no small molecule enhancers (Ctrl). Nocodazole, M344, and z-VAD-fmk are highlighted (dashed boxes) (**A**). Mean cell volume vs genome titre fold change is shown in (**B**), dashed lines indicate control values. R^2 and p-value calculated from Pearson correlation coefficient of mean cell volume vs genome titre. Data shown in **A** are mean ± SEM for two independent experiments carried out in technical duplicate, with the exception of TUDCA, TMAO, Betaine and LiCl which are mean only data from one independent experiment carried out in technical duplicate (data from these experiments is omitted in **B**). NaBu - Sodium butyrate; VPA – Valproic acid; TUDCA - Tauroursodeoxycholic acid; TMAO - Trimethylamine N-oxide; LiCl – Lithium chloride

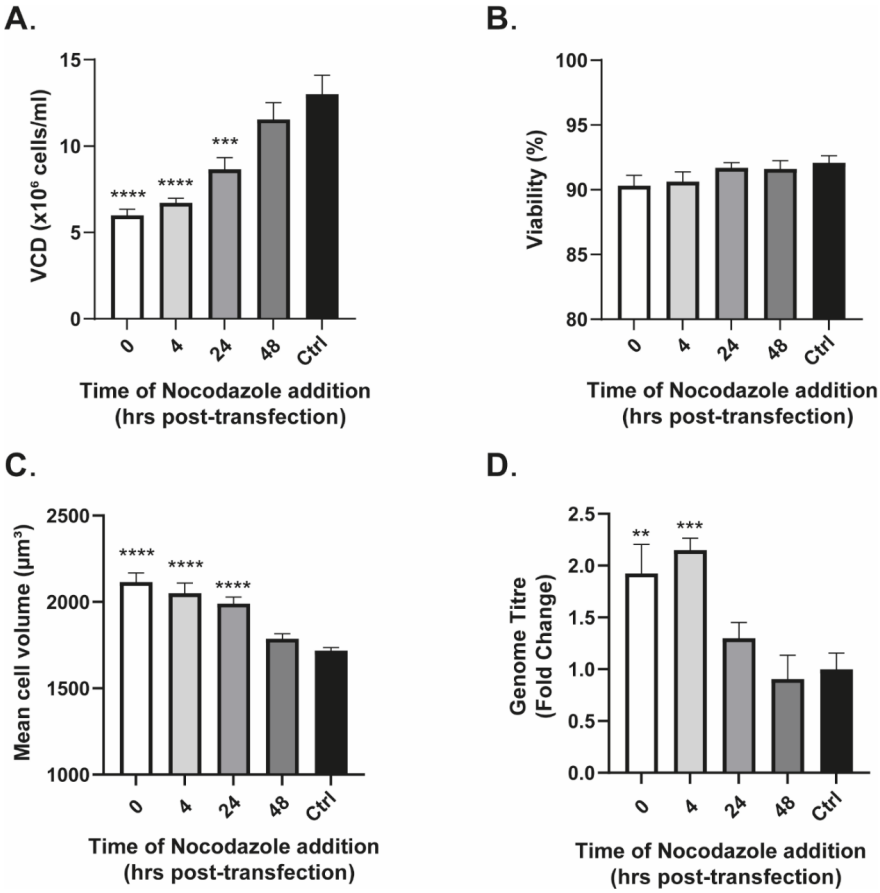

Figure 2 – Addition of nocodazole to rAAV8 producing cells to increase genome titre is time-dependent. rAAV8 producing cells were cultured in 24-well microplates and 4 µM nocodazole was added at 0-, 4-, 24-, or 48-HPT or left untreated (Ctrl). At 72 HPT cells were harvested and viable cell density (VCD) (**A**), viability (**B**) and mean cell volume (**C**) was measured. Final crude genome titre expressed as a fold change relative to Ctrl is shown in (**D**). Data shown for VCD, viability and mean cell diameter are the mean ± S.E.M. n > 3 independent biological replicates. Data shown for genome titre fold change are the mean ± S.E.M. n [?] 3 independent biological replicates. Data were analysed by one-way ANOVA followed by post-hoc Holm-Šídák multiple comparisons test with respect to Ctrl. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

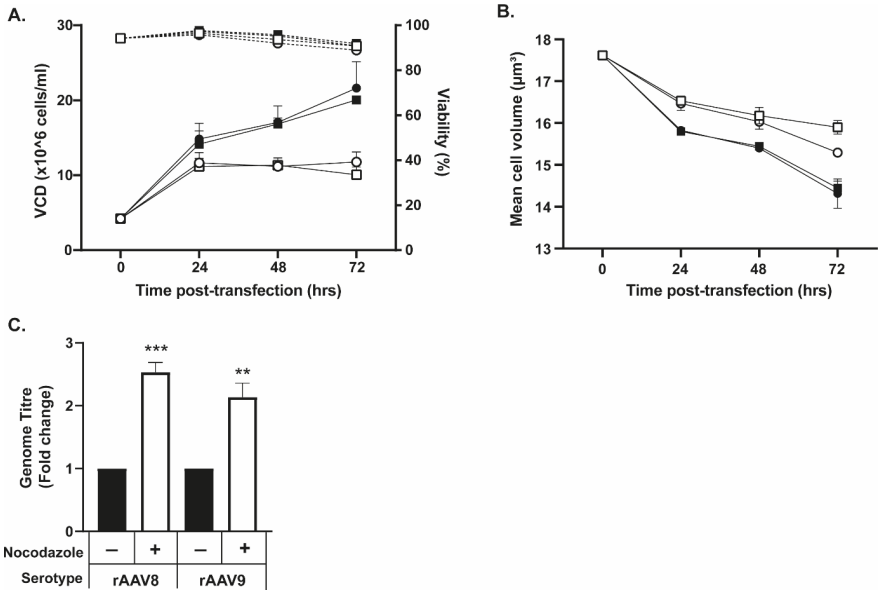

Figure 3 – Increased genome titre in nocodazole treated cells is maintained in larger scale cultures and across two separate serotypes. 30 mL shake flask cultures were transfected with rAAV8 or rAAV9 producing plasmids and cultured for 72 HPT in the presence (rAAV8: ; rAAV9:) or absence (rAAV8: ; rAAV9:) of 4 µM nocodazole added 4 hours post-transfection. Cultures were measured daily for viable cell density (VCD) (**A** – solid lines), viability (**A** – dashed lines) and mean cell volume (**B**). Fold change analysis of genome titre for both serotypes in the presence (+) or absence (–) of 4 µM nocodazole at 72 HPT is shown in (**C**). Data shown are the mean ± S.E.M. n = 3 independent biological replicates. Fold change between nocodazole treated and untreated cultures was analysed for each serotype separately by Student's unpaired two-tailed t-test with respect to untreated cultures. ** $p < 0.01$, *** $p < 0.001$.

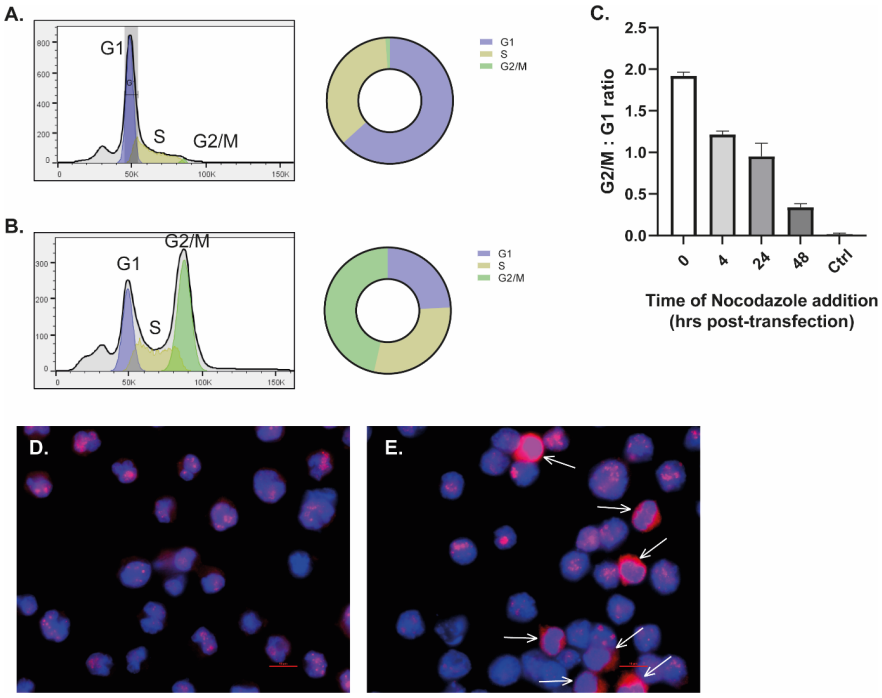

Figure 4 – rAAV8 producing cells treated with nocodazole arrest in G2/M phase. Flow cytometry histograms triggered against propidium iodide signal for cells show the relative abundance of cells in either G1 (blue), S (orange) or G2/M (green) phases of the cell cycle in the absence (**A**) or presence (**B**) of 4 μM nocodazole addition at 0 HPT. Ratio of cells in G2/M phase to G1 phase at 72 HPT with 4 μM nocodazole added at 0, 4, 24, 48 HPT or untreated (Ctrl) (**C**). Representative fluorescent composite images of rAAV-producing cells fixed 24 HPT show staining of the fibrillar component of the nucleolus with anti-fibrillarin (red) and nuclear DNA with DAPI (blue). Cells displaying disorganised nucleolar morphology are indicated by white arrows in non-treated cells (**D**) and cells treated 4 hours post-transfection with 4 μM nocodazole (**E**). Images taken using 100 x oil objective. Scale bar 10 μm. Data shown in (**C**) are mean ± SEM for three biological replicates.

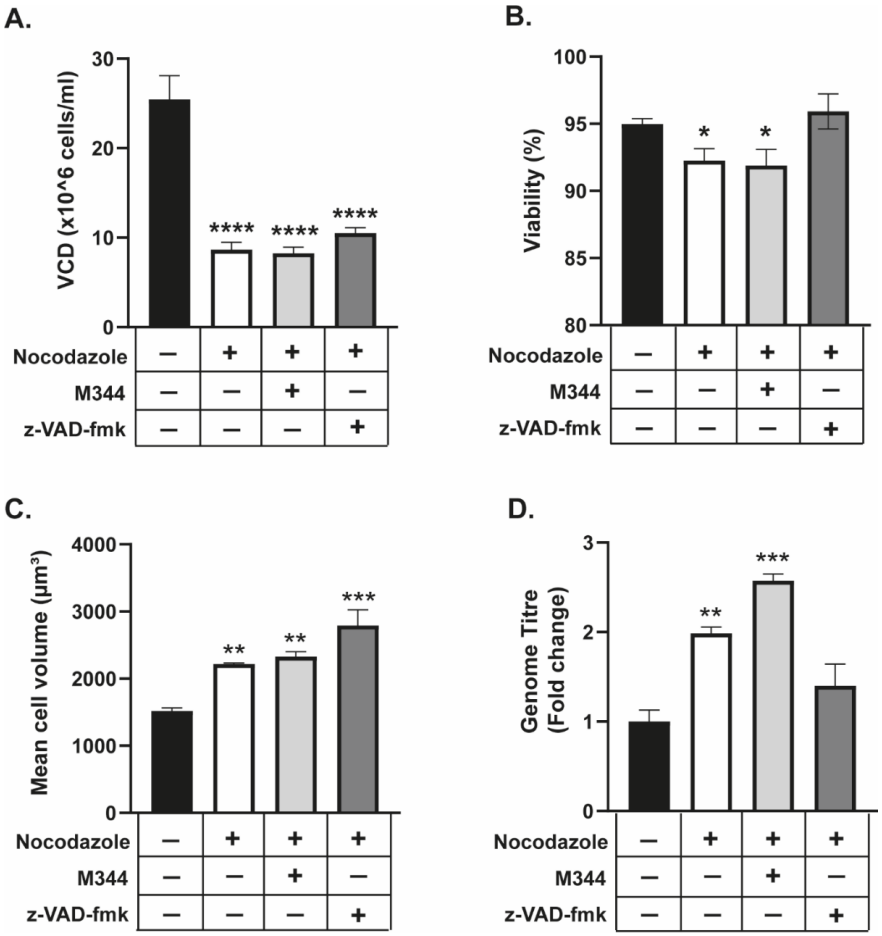

Figure 5 –Combinatorial use of small molecule enhancers has an additive effect on genome titre. rAAV8 producing cells were treated 4 HPT with small molecule enhancers of genome titre or left untreated. Cells were harvested 72 HPT and measurements taken of viable cell density (VCD) (**A**), viability (**B**), mean cell volume (**C**), and rAAV8 genome titre as a fold change with respect to untreated cells (-/-/-) (**D**). Data shown are the mean ± S.E.M. n = 3 independent biological replicates. Data were analysed by one-way ANOVA followed by post-hoc Holm-Šídák multiple comparisons test with respect to untreated cells (-/-/-). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

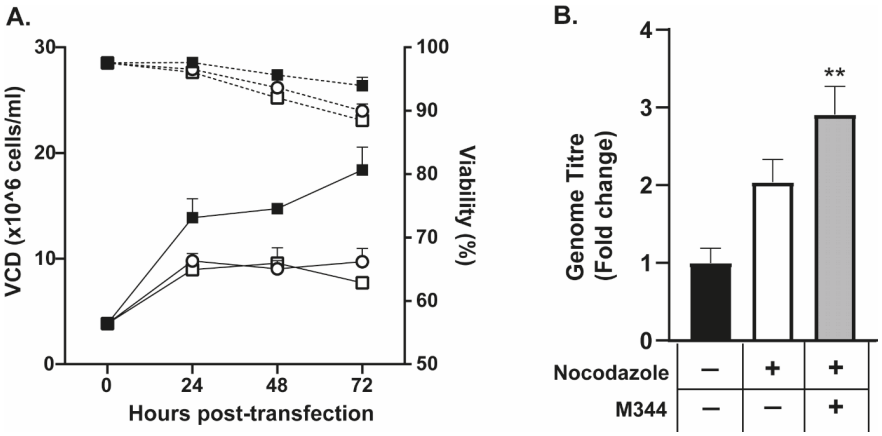

Figure 6 – Genome titre improvements are retained in larger scale cultures. 30mL shake flask cultures were transfected with rAAV8 producing plasmids with no chemical additives (), with the addition of 4μM nocodazole at 4 hours post-transfection (), or with the addition of 4 μM nocodazole and 2.5 μM M344 at 4 hours post-transfection (). Cultures were measured daily for VCD (**A** – solid lines) and viability (**A** – dashed lines). Genome titre was measured 72 hours post-transfection and is shown as fold change with respect to untreated cultures (**B**). Data shown are the mean ± S.E.M. n = 3 independent biological replicates. Data were analysed by one-way ANOVA followed by post-hoc Holm-Šídák multiple comparisons test with respect to untreated cells (-/-). * $p < 0.05$, ** $p < 0.01$