PKC Agonists as Small Molecule Inducing Agents for Enhancing Lentiviral Vector Production

Charles Moore-Kelly, Thomas M. Evans, Jordan Wright, Dan C. Farley, Ian T. Matthews, David Pulido-Gomez, Olotu Ogonah, Kyri Mitrophanous, Nicholas Clarkson, R. André Raposo

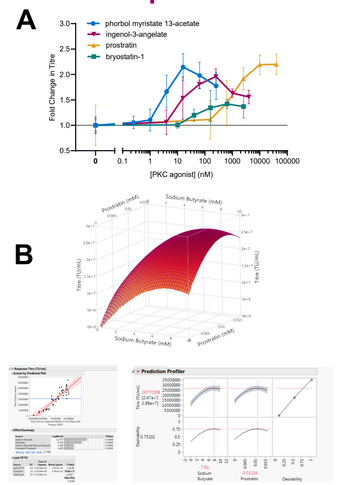
Overview

Achieving high upstream lentivector (LV) titres is fundamental to the development and manufacture of commercially viable gene therapy products. However, product titres can vary considerably with different therapeutic transgene sequences despite being produced in the same cell system, so implementing new technologies that are able to recover low titres or improve high titres further can be invaluable.

Induction is a key stage of the upstream LV production process that typically involves increasing the expression of LV genes in production cells with the histone deacetylase inhibitor (HDACi), sodium butyrate. A series of recent screening experiments revealed to us that titres of LV products can be further increased by using an additional class of molecules known as PKC agonists alongside the induction step. Here, we describe how, through optimisation of dosing concentration, LV titres can be enhanced 2- to 9-fold with the PKC agonists prostratin and ingenol 3-angelate in a product-specific manner. Importantly, LV produced using PKC agonists have comparable or superior particle-to-infectivity ratios, and residual PKC agonists are removed from the vector product following downstream processing. Furthermore, we demonstrate that PKC agonists act synergistically with an in-house technology based on LV RNA-targeted U1 snRNA to achieve log-fold-increases in titre without detriment to product quality attributes.

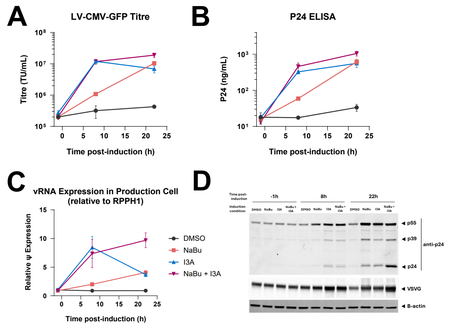
Having established the benefits of introducing PKC agonists to our platform process and in our packaging/producer cell lines, we provide insight into the mechanisms by which these agonists are acting on production cells and now intend to transfer this technology to GMP manufacturing for commercial products.

1. PKC agonists combine with HDAC inhibitors to improve lentiviral vector titres



The PKC agonists phorbol myristate 13-acetate (PMA), ingenol 3-angelate (I3A), prostratin and bryostatin-1 have been reported to invoke HIV-1 latency reversal in CD4⁺ cells in combination with HDAC inhibitors (Reuse et al., 2009; Brogdon et al., 2016). These four PKC agonists were tested in the production of an internal benchmark LV carrying a GFP reporter transgene (LV-CMV-GFP) in suspension adapted HEK293T cells (**A**). The phorbol esters, PMA and prostratin, and diterpene-class molecule, I3A, were shown to induce a 2-fold increases in LV-CMV-GFP titre when combined with sodium butyrate. The optimal concentration of HDAC inhibitor and PKC agonist were determined by full-factorial DOE at 24-well plate scale (**B**).

2. Induction with I3A rapidly induces release of LV particles

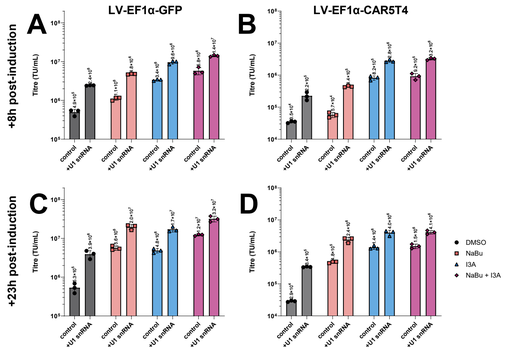


The kinetics of vector production were assessed at two time points post-induction (8h and 22h). Induction with I3A resulted in a rapid release of infectious viral particles from production cells (**A** & **B**). Examination of viral RNA (**C**) and structural proteins, Gag and VSVG (**D**), shows that particle release coincides with a rapid increase in transcription of viral RNA and a modest increase in expression of viral proteins in production cells.

3. Combined expression of modified U1 snRNA and PKC agonists improve vector titres considerably

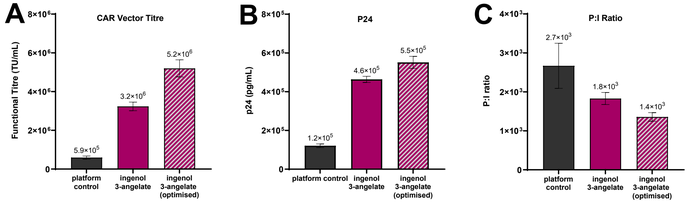
Modified U1 snRNA targets the 5' UTR to suppress aberrant splicing of genomic RNA and increase the availability of full-length transcripts of genomes that contain internal EF1α promoters. Transient co-expression of modified U1 snRNA improved titres of EF1α-GFP vector and an in-house chimeric antigen receptor (CAR5T4) vector compared to the platform control (sodium butyrate induction) by 3.6-fold and 5-fold, respectively (**C** & **D**).

The rate of vector production was increased by I3A for both vector products, with distinctly higher titres measured 8h after induction (**A** & **B**). Interestingly, I3A induction alone was sufficient to increase LV-CAR5T4 titres 3-fold compared to sodium butyrate induction alone with no significant improvement when combined with sodium butyrate, whereas the synergistic interaction was more apparent for GFP reporter virus. The combined use of modified U1 snRNA and I3A improved CAR5T4 vector titres 5.7 and 8.5-fold compared to platform conditions (**C** & **D**).



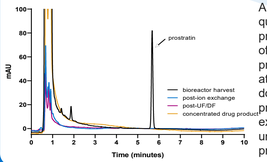
4. Optimisation of PKC induction protocol increased CAR vector titres at bioreactor scale

2024, Baltimore US



Optimisation of the induction protocol resulted in a further 60% increase in CAR vector titre at Ambr®250 scale and 9-fold improvement over platform conditions (**A**). Furthermore, the optimised protocol resulted in 50% lower P:I ratio compared to platform conditions (**B** & **C**).

5. PKC agonist is cleared during downstream processing

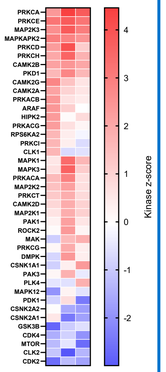


An RP-HPLC assay was developed to quantify the concentration of prostratin in process samples and assess the clearance of the PKC agonist from the final drug product. Process samples were analysed at different stages of the upstream and downstream process, demonstrating that prostratin was removed following ion exchange chromatography and was undetectable in the final concentrated drug product.

6. Kinase substrate enrichment analysis

Phosphopeptide mass spectrometry was performed to identify kinases activated by PKC agonists and their downstream substrate. Kinase substrate enrichment analysis (KSEA) showed strong enrichment in phosphorylated substrate associated with several PKC isotypes, indicating strong constitutive activity of the calcium-dependent isoform, PKCα, and calcium-independent isoforms, PKCε and PKCδ from 10 mins to 6h post-induction (Katti et al., 2022). Activity of MAPK pathways were also detected, with a positive enrichment of ERK1 (MAPK3), ERK2 (MAPK1), as well as activity of known downstream kinases of ERK signalling, including p70 ribosomal S6 kinase (RPS6KB1).

KSEA showed strong downregulation of the activity of cyclin dependent kinases 2 and 4 (CDK2/4) and glycogen synthase kinase 3 beta (GSK3B) immediately following treatment with prostratin. At 6 hours post-induction, treated samples demonstrated a lower enrichment in substrate associated with mammalian target of rapamycin (MTOR) and 3-phosphoinositide-dependent kinases (PDK1).



References

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Contact – Oxford Biomedica • partnering@oxb.com

Oxford Biomedica (UK) Limited, Windrush Court, Transport Way, Oxford, OX4 6LT, UK

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