Enhancing Titers of Therapeutic Lentiviral Vectors using PKC Agonists

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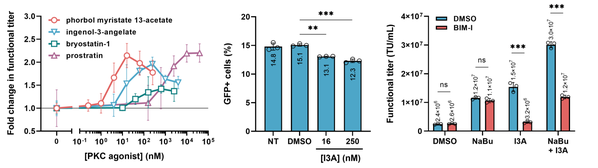
Introduction

Lentiviral vector (LV)-based therapies employ the molecular machinery of HIV-1 to stably integrate therapeutic genes in patient cells for long-term disease correction. However, suboptimal expression of LV components in HEK293T-based production systems can limit titers and hinder clinical product development. Here, we identify protein kinase C (PKC) agonists as robust enhancers of LV production. PKC activation resulted in rapid transcription of LV genomic RNA and accelerated vector particle release in a manner that complemented the use of the histone deacetylase (HDAC) inhibitor sodium butyrate. Stimulation strongly upregulated AP-1 transcription factor subunits independently of NF-κB pathway activation. Application of PKC agonists in LV production resulted in a ~3-fold improvement in the titer of a chimeric antigen receptor (CAR)-LV. Furthermore, a ~9-fold increase in titer was achieved when this induction method was combined with co-expression of an LV RNA-targeted U1 snRNA enhancer. Importantly, LV produced using PKC agonists had comparable particle-to-infectivity ratios and preserved T cell transduction efficiency. These findings suggest that incorporating PKC agonists into commercial LV manufacturing could considerably reduce the cost per patient dose of new LV-based gene therapies.

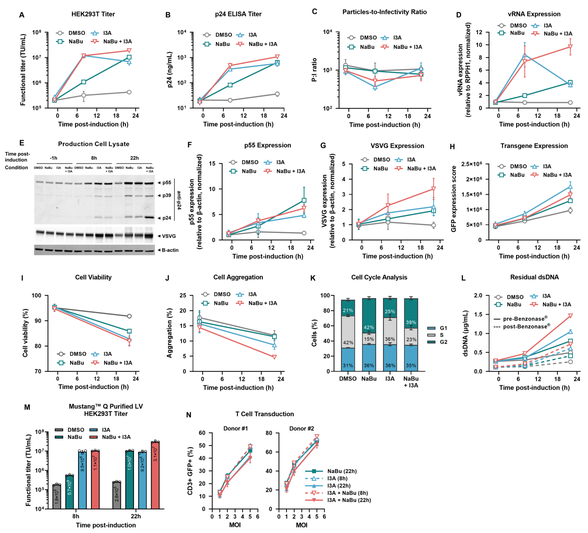
1. PKC agonists increase LV titers in HEK293T-derived production cells.



The PKC agonists phorbol myristate 13-acetate (PMA), ingenol-3-angelate (I3A), prostratin, and bryostatin-1 were evaluated for their impact on the output titer of a 3rd generation LV containing a GFP reporter transgene (LV-CMV-GFP) (1A). PKC agonist or DMSO was added to transfected HEK293T production cells immediately following the addition of sodium butyrate (NaBu), an HDAC inhibitor that is commonly used in LV manufacture. PMA, I3A and prostratin increased LV titer by ~2-fold compared to the control condition.

PKC agonist spiked into GFP vector reference control at transduction indicated that residual PKC agonist in LV harvest material was not inflating functional titer values reported by the transduction assay (1B). Pre-treatment of production cells with the PKC-inhibitor bisindolylmaleimide I (BIM-I) specifically abolished PKC-induced LV production, highlighting the distinct mechanisms mediated by the two drug classes (1C).

2. I3A induces rapid transcription of vector RNA and release of LV particles.

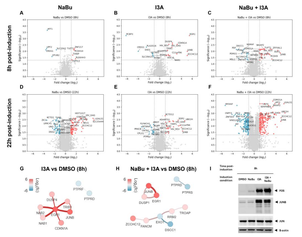


Treatment of HEK293T-based production cells with I3A resulted in a rapid increase in LV titer and particle release (2A & 2B). Combined induction with I3A and NaBu resulted in the highest LV titer at harvest. The use of PKC agonists only resulted in a modest improvement (decrease) in particle-to-infectivity ratio at the early harvest timepoint (8h post-induction) (2C). PKC activation resulted in a rapid increase in the rate of viral RNA (vRNA) transcription (2D). This coincided with increases in the expression of LV structural proteins (Gag and VSVG) (2E, 2F & 2G) and transgene protein (GFP) (2H) with respect to the vehicle control.

The use of I3A alone and in combination with NaBu was well tolerated by production cells (2I & 2J). NaBu treatment triggered cell cycle checkpoints, whereas treatment with I3A alone resulted in a similar cell-phase distribution to the control (2K). Residual dsDNA (a monitored process impurity) was equivalent across all conditions at the 8h harvest time-point. Combined I3A and NaBu treatment resulted in the highest dsDNA levels at final harvest (2L).

LV was purified using anion exchange chromatography and re-titered on HEK293T cells (2M). The transduction efficiency of CD3+ T cells with purified vector produced under each tested induction condition was equivalent at each corresponding multiplicity of infection (MOI) (2N). These results demonstrate that LV infectivity was not compromised by the use of PKC agonist or earlier harvest time points.

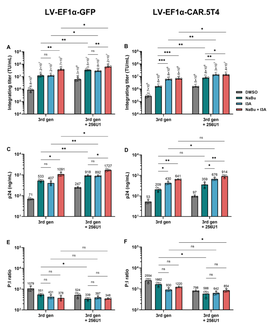
3. PKC treatment upregulates cellular immediate early genes during LV production.



Changes in the suspension-adapted HEK293T production cell proteome effected by NaBu, I3A and their combination were assessed by peptide mass spectrometry (3A-3F). At 22 hours post-induction, 131 and 39 differentially expressed proteins (|log2(FC)| > 1, log10(FDR) > 1.3) were identified in cells treated with NaBu or I3A, respectively, indicating the comparatively broad influence of HDAC inhibition on the production cell proteome (3D & 3E). Combined NaBu and I3A dosing resulted in substantial dysregulation of protein expression, with 342 host cell proteins identified as being differentially expressed at the final timepoint (3F).

PKC stimulation by I3A resulted in a rapid and prominent expression of cellular immediate early genes within 8 hours of treatment, including a sustained upregulation of AP-1 transcription factor subunit, JUNB, and early growth response factor, EGR1. Graphing of differentially expressed proteins by known functional and physical associations highlighted the strength of the JUNB/EGR1 axis in the immediate cellular response to PKC activation for both treatment groups (3G & 3H). Activation of AP-1 subunits FOS and JUNB was confirmed by Western blot (3I).

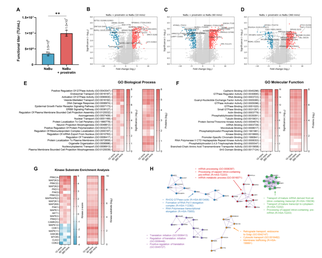
4. PKC activation induces the production of therapeutic LV-CAR and synergizes with a modified U1 snRNA-based LV enhancer to increase titers.



The individual and combined effects of NaBu and I3A were assessed in the small-scale production of a therapeutic LV (4A-F). The transgene for a therapeutic CAR targeting the tumor associated antigen 5T4 (CAR.5T4) with an internal EF1α promoter was selected as an example of a product that is known to yield comparatively low titers under standard conditions. LV with a GFP transgene (and otherwise identical cassette sequences) was produced in parallel to directly compare the influence of induction conditions across the different transgenes. In addition, vector was produced with and without co-expression of a modified U1 snRNA LV enhancer "256U1", which binds to a region within the packaging signal to stabilise vRNA.[1,2]

Interestingly, we observed that the relative fold change induced by I3A compared to NaBu differed between the two vector products. I3A treatment alone was effective at increasing LV-EF1α-CAR.5T4 titers by >3-fold vs NaBu (4B). Co-expression of 256U1 increased LV titers across all induction conditions for both transgenes (4A & 4B). The particle-to-infectivity ratio of vector product was not compromised by the use of PKC agonists (4E & 4F).

5. Identification of kinases activated by PKC agonists and their downstream substrates.



Phosphopeptide mass spectrometry was used to identify the kinases activated by PKC agonists and their downstream substrates in HEK293T-derived production cells. Induction with NaBu and prostratin resulted in a ~3-fold increase in LV-EF1α-CAR.5T4 functional titer compared to NaBu alone (5A). A total of 422, 645, and 411 differentially phosphorylated peptides (|log2(FC)| > 3 and log10(FDR) > 1.3) were detected at 10, 60 and 360 mins following treatment with PKC agonist, respectively (5B-D). These kinase substrate were associated with "Positive Regulation of GTPase Activity", "Endosomal Transport, "Cadherin Binding" and "GTPase Regulator Activity" (5E-F). Kinase substrate enrichment analysis identified strong activity of PKCα, δ and ε isozymes, alongside pronounced activity of mitogen activated protein kinase (MAPK) pathway (5G). Molecular complex prediction analysis identified clusters of kinase substrate associated with "mRNA processing", "RNA polymerase II elongation" and "mRNA transport" (5H).

6. Conclusion

PKC agonists are a class of inducing agents that complement the use of sodium butyrate to enhance the manufacture of therapeutic HIV-1-based LV. PKC agonists increase the rate of transcription and vector particle release in a manner that coincides with strong MAPK and AP-1 activation. Furthermore, their combined application with 256U1 enhances titers of therapeutic CAR-LV by ~9-fold compared to standard conditions.[1,2] Taken together, these results emphasize the productivity improvements that can be achieved by maximizing vRNA availability in LV production.

7. References

1. Farley, D., and Wright, J. (2021). Oxford Biomedica (UK) Limited. Enhancing production of lentiviral vectors. Patent Application #WO/2021/014157.

2. Wright, J., Alberts, B.M., Hood, A.J., Nogueira, C., Miskolczi, Z., Vieira, C.R., Chipchase, D., Lamont, C.M., Goodyear, O., Moyce, L.J., et al. (2024). Improved Production and Quality of Lentiviral Vectors By Major-Splice-Donor Mutation and Co-Expression of a Novel U1 Snrna-Based Enhancer. Preprint at Social Science Research Network, https://doi.org/10.2139/ssrn.4929949.