# CHEMICAL-BIOLOGY APPROACH TO DELINEATE THE MECHANISM OF ACTION OF HIV-1 LATENCY REVERSING AGENTS

by

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## ABSTRACT

Prostratin exhibits potent HIV-1 latency reversing activity, yet its molecular mechanism of action has not been defined in detail. Here, we used a novel chemical biology approach which revealed that the protein kinase C-mitogen activated protein kinase kinase (PKC-Mel) pathway is essential for prostratin-induced reactivation of latent HIV-1 provirus. We identified 7 different Mek inhibitors using our approach that constantly impaired prostratin-induced reactivation. We also identified PKC inhibitors, Raf kinase inhibitors, and extracellular signal-regulated kinases (Erk) inhibitors that blocked prostratin-induced reversal of HIV-1 latency. Consistent with these observations, the PKC-Ras-Mek-Erk pathway plays an essential role in the mechanism by which prostratin reactivates latent HIV-1. In addition, we identified several kinase inhibitors that activated HIV-1 latent genes in a significant level individually, including CUDC-101, XMD8-92, PD173074, Quizartinib, and CUDC-907. Therefore, this chemical biology approach provides new methods of revealing the molecular mechanism of LRAs into the HIV-1 eradication research.

The work represented by this thesis holds public health significances lying mainly in enriching the knowledge of HIV-1 latency reversing agents, contributing to the translational study, and the overall advancement of HIV eradication in public health.

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# PREFACE

I would first like to thank my thesis advisor Dr. Nicolas Sluis-Cremer. The door to Prof. Sluis-Cremer's office was always open whenever I had a question about my research or study. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my M.S. study.

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# 1.0 INTRODUCTION

Since HIV-1 (human immunodeficiency virus type 1) was discovered 30 years ago as the causative agent of the acquired immunodeficiency syndrome (AIDS), the viral infection is always one of the major global health issues [1]. More than 75 million people worldwide have been infected with HIV-1, and there are approximately 36.7 million of people living with the infection [2]. Without the intervention treatments, the viral replication causes the loss of $CD4^+$ T cells and a wide range of immunological dysfunction, increasing the risks of tumorigenesis and other accidental infections such as Kaposi's sarcoma-associated herpesvirus (KSHV) infection. The immunodeficiency caused by HIV-1 infection will lead to the further syndromes such as cardiovascular disease, bone diseases, and hepatic dysfunction. Antiretroviral drugs have been applied to the clinical treatment and intervention, which dramatically decreased the AIDS-related mortality rate since 2000. The continuous therapy can suppress viral replication and enable immune recovery, which also contributes to the epidemiological intervention. However, scientists are still seeking for a cure of HIV-1 to address this public health issue.

## 1.1 HIV-1 LATENCY

Viruses have evolved a variety of mechanisms to evade the host immune system. One of the mechanisms adapted by HIV-1 is the establishment of the latency in the infected cells. HIV-1

provirus can be expressed immediately in acute infections, followed by the viral replication cycle and the release of progeny viruses. Alternatively, depending on the status of the host cells, the provirus can become dormant. Once the latently infected cells encounter antigens or other activating stimuli, latency may be reversed, and the cells may begin to produce viruses[3].

The latency occurs when the provirus enters a non-productive, albeit reversible, state of replication. The HIV-1 proviral DNA integrates into cellular chromosomal DNA, existing in a permanent post-integration latent state. HIV-1 establishes latent infection mainly in resting memory $CD4^+$ T cells with a long-life span[4]. The level of the viral gene transcription is very low, and little or no viral proteins are synthesized, which protects the infected cells from detection by the host immune system. There are several factors which contribute to the maintenance of latency, including the absence of nuclear forms of key host transcription factors (e.g. NFκB and NFAT) in resting $CD4^+$ T cells, the absence of viral transactivator (e.g. Tat) [3], the presence of transcriptional suppressors such as CTIP2 (COUPTF Interacting Protein 2)[5], and the cellular microRNAs (miRNAs) which inhibits histone acetylation [6]. The regulated viral transcription in resting cells can be re-activated following exposure of appropriate stimuli, which was shown by numerous studies [7-9].

## 1.2 TREATMENT OF HIV-1 INFECTION

### 1.2.1 Antiretroviral therapy

Currently, the most common way to treat HIV infection is through the use of a combination of targeted antiviral drugs, typically referred to as antiretroviral therapy (ART). ART is not a cure,

but it can control the viral load to reduce the AIDS-related death rate and transmission among a population. The approved antiretroviral drugs were classified into six categories, including nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, CCR5 antagonists, and HIV integrase inhibitors (Figure 1). The antiretroviral drugs are usually used in combinations of three or more drugs from more than one class as called “combination therapy” (cART) to prevent drug resistance.

### 1.2.2 HIV eradication

Two important features of HIV are its vast genetic diversity and its ability to rapidly evolve, which affect pathogenesis, prevention, diagnosis, and clinical treatment[10]. The vast diversity also implies the potential for rapid selection of drug resistance mutations during ART. In addition, new findings suggest that cells infected prior to initiating therapy can proliferate to very high numbers both before and during the ART[11], and a rebound in viremia can occur in patients after long-term cART[12]. Moreover, the pharmacokinetics properties of the drugs used to treat HIV-1 requires the delivery of high doses to maintain the half-life of ARTs, which brings inconvenience to both clinicians and patients[13]. These studies highlight the need to find eradication methods.

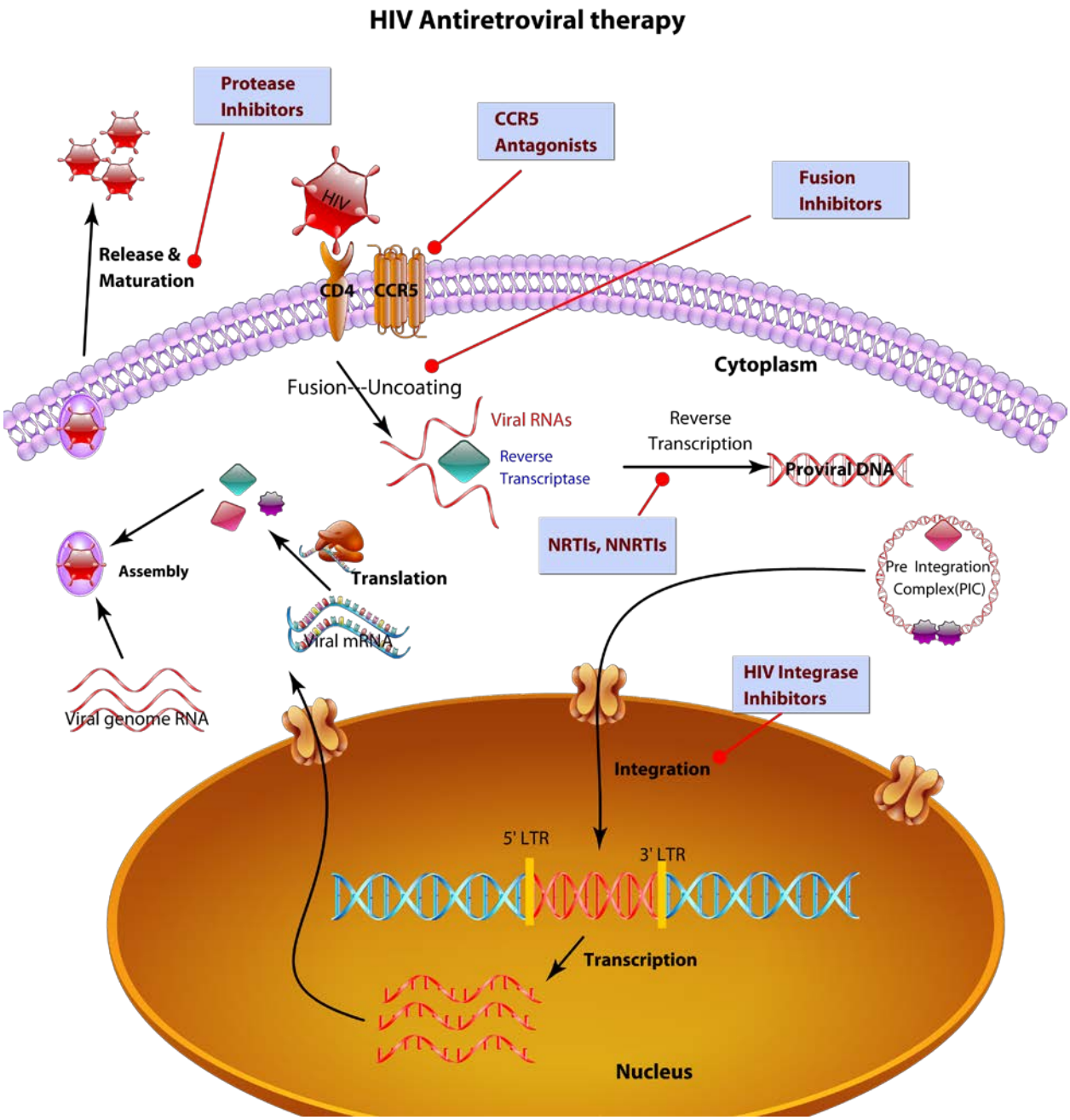



**Figure 1. The mechanism of antiretroviral therapy.**

The six drug classes include more than 25 HIV medicines that were approved by FDA to treat HIV infection. Nucleoside Reverse Transcriptase Inhibitors (NRTIs) are competitive analogues of deoxynucleotides that are necessary for the viral DNA chain synthesis. As the result, incorporation of NRTIs leads to the interruption of DNA synthesis due to the lack of 3'-hydroxyl group on the deoxyribose moiety. Non- Nucleoside Reverse Transcriptase Inhibitors (non-NRTIs) block reverse transcriptase by binding directly to the enzyme. Fusion inhibitors block the HIV envelope from merging with the host $CD4^+$ T cell membrane. Protease inhibitors (PIs) block the viral protease activity that is crucial for the maturation of progeny viruses to become contagious. CCR5 antagonists block the HIV coreceptor to prevent HIV binding and fusion, and ultimately entry into cells. HIV integrase inhibitors block integration which is a vital step in retroviral replication.

## 1.3 BARRIERS TO HIV-1 ERADICATION

### 1.3.1 Cellular HIV-1 Reservoirs

During the HIV-1 infection, the activated $CD4^+$ T cells are responsible for producing more progeny viruses, while the resting $CD4^+$ T cells produce less or no viruses but are able to persist after ART[14]. There are two main populations of resting $CD4^+$ T cells: naïve and memory $CD4^+$ T cells, and infection occurs in both populations[15]. Memory T cells are the main reservoirs due to their long-life span and the regulation of cell division. The naïve $CD4^+$ T cells also contribute to the latency reservoirs due to their ability to ability to proliferate and differentiate into any of the memory cell subsets[16].

Due to the invulnerability of the resting $CD4^+$ T cells reservoirs to antiretroviral drugs, cell-associated proviral DNA and RNA are still detectable in peripheral blood mononuclear cells (PBMC) by PCR-based assays, even after several years of cART [17], and therapy interruption leads to the re-emergence of viremia [18]. Moreover, one study showed that in individuals where cART might provide a period of control to the re-emergence of viremia after the interruption of therapy, the HIV-1 DNA predicted the time of plasma viruses rebound [19]. This suggests that the size of the reservoir indicates the period of time when viral rebound is controlled by cART after interruption of the treatment.

### 1.3.2 The Shock and Kill Strategy

A broadly proposed approach to reduce HIV-1 reservoir size involves reversing latency in patients on cART, as this would theoretically expose such cells to the killing by immune-

mediated clearance, viral cytopathic effects, or other therapeutic strategies such as therapeutic vaccines (Figure 2). The early attempts of latency reversal were performed by using IL-2 and anti-CD3 monoclonal antibody (OKT3) combination [8, 20]. However, OKT3/IL-2 therapy resulted in global T cell activation and proliferation, which was toxic, and anti-OKT3 antibodies were rapidly induced[21]. Further research to find latency reversing agents (LRAs) that work without inducing T cell activation activity have led to the identification of individual compounds capable of reactivating HIV-1 latency. The identified LRAs can be categorized into the following groups based on their pharmacological targets: (1) histone deacetylase inhibitors (HDACi) such as Vorinostat; (2) cytokines and chemokines (e.g. IL-7); (3) DNA methyltransferase inhibitors (DNMTi); (4) protein C kinase (PKC) agonists such as Prostratin; (5) p-TEFb activators; (6) histone methyltransferase inhibitors (HMTi); and (7) unclassified agents such as disulfiram.

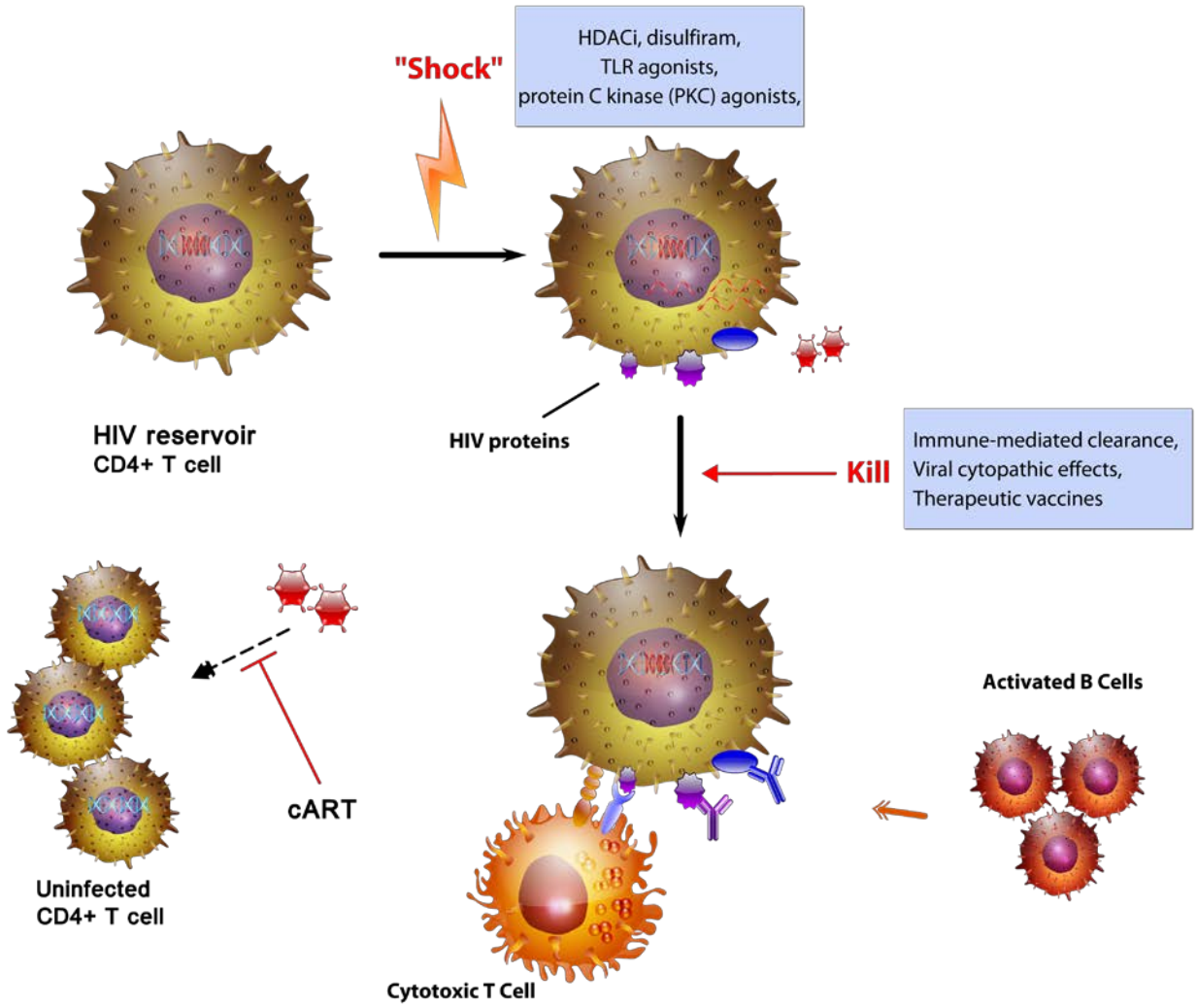



**Figure 2 "Shock and Kill" strategy.**

The "shock and kill" strategy includes three steps: 1) LRAs are applied to the HIV reservoirs to activate the expression of latent genes. 2) Latent gene expression results in the production of viral proteins and progenies, which make them vulnerable to immune targeting and other treatment methods. 3) The combinatorial use of cART prevents the uninfected cells from new HIV infection.

## 1.4 THE DEVELOPMENT OF LATENCY REVERSING INTERVENTION

### 1.4.1 Clinical Experiences with LRAs

The clinical studies described that most of the identified LRAs were able to increase the HIV RNA production and/or the level of viremia when the single drug was applied alone in vivo, but the size of latent reservoirs was barely affected. Vorinostat, an HDACi approved by FDA for the treatment of cutaneous T-cell lymphoma[22], did increase the HIV-1 transcription in a significant level for two weeks, but this change remained at the same level when Vorinostat was given for 8 weeks[23]. Disulfiram, known as the acetaldehyde dehydrogenase inhibitor, was not able to reduce latent reservoirs at a significant level, despite the treatment increased the residual viremia in patients [24]. Moreover, the single use of LRAs that do not cause T cell activation is ineffective for the elimination of latency reservoirs[25]. On the other hand, studies focusing on a combinatorial use of LRAs suggested that the combination of proviral activators from different classes may have important implications for reducing the size of reservoirs[26]. Based on those observations, a combination of LRAs from two independent classes might be required for reservoir clearance. However, the combined effects of multiple LRAs involve with a complex mechanism which is not fully understood.

### 1.4.2 PKC-θ agonist Prostratin functions as a LRA candidate

Then PKC-θ was first isolated in the 1990s by Altman and his group[27] as a member of novel isoform (nPKCs) in $Ca^{2+}$-independent PKC family. PKC-θ activates multiple transcriptional factors such NFκB and Activating protein-1 (AP-1), which are critical for the expression of

many T-cell activation-related genes such as IL-2[28]. Therefore, PKC-θ is regarded as one of important factors in the reactivation of HIV latent genes.

Prostratin [13-O-acetyl-12-deoxyphorbol] is a phorbol ester that was first isolated from Strathmore weed *Pimelea prostrate* in 1976[29]. It showed the ability to prevent HIV-1 infections by downregulating the HIV-1 cellular receptors CD4 and CXCR4 without any significant side effects[30]. Moreover, it was proved that prostratin can activate latent HIV viral reservoirs *in vitro* by stimulating IκB kinase (IKK)-dependent phosphorylation (through the stimulation of novel and atypical translocation protein PKC-θ) which leads to the translocation of NF-κB and NF-κB-dependent activation of HIV-1 LTR, while showing no effect to NFAT and AP-1[31]. Furthermore, unlike other phorbols which hyperactivate PKC and trigger cell proliferation leading to carcinogenesis, prostratin induces T-cell activation by upregulating surface marker CD25 and CD69 while inhibiting mitogen triggered T cell proliferation simultaneously[32].

## 2.0 STATEMENT OF THE PROJECT

Previous studies have revealed that prostratin binds to PKC-θ and stimulates its kinase activity. Due to its unique pharmaceutical characteristics contributing to both HIV prevention and eradication, it is necessary to completely understand the mechanism in prostratin-induced latency reversal. Therefore, we designed a novel chemical biology approach to identify the cellular pathways that are involved with the maintenance and reversal of HIV latency, as well as the mechanism upon prostratin-induced latency reversal. Moreover, there is a possibility that this verified approach can be applied to delineate the mechanism of latency reversal induced by other drugs and identify the new potential LRAs.

# 3.0 SPECIFIC AIMS

Aim 1: Establish a validated chemical biology approach that identifies potential LRAs by inhibiting crucial pathways involved with the maintenance of HIV latency.

We orchestrated High-throughput screening by using different kinase inhibitor (From Kinase Inhibitor L-1200) individually to challenge HIV-1 latency model 24ST1NLESG cell line. Then we selected reagents with latency reversing ability according to the relative activity of *seap* (*seap* functions as an indicator of HIV late gene expression in the model). The selected reagents will be used in dose-response study to determine $IC_{50}$ for the further studies

Aim 2: Validated the approach by using prostratin to delineate the mechanism of prostratin-induced reactivation.

We used prostratin as the reference to verify the chemical biology approach on its ability to delineate the molecular events during the prostratin-induced latency reversal. By comparing the differences of inhibitor-treated cells with or without the presence of prostratin, we demonstrated that some inhibited pathways were essential in prostratin-induced reactivation, which was in consistency of previous studies. In the further step, we used dose-response assay and cell viability assay to eliminate the system error that affected internal validity.

# 4.0 MATERIALS AND METHODS

## 4.1 CELL LINES AND MEDIA

24ST1NLESG cell line (obtained from Joseph P. Dougherty's Lab) was cultured in RPMI 1640 L-glutamine (+) supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin. Cells were cultured at a concentration of $2 \times 10^6$ cells/ml for all experiments. This human $CD4^+$ T-cell clones were derived from the SupT1 cell inoculated with $NLE^-S$-G vector. This pNL4-3-based lentiviral vector contains all necessary cis-acting elements for HIV replication, and harbors both secreted alkaline phosphatase (*seap*) in the *env* position and enhanced green fluorescence protein (*egfp*) in the *nef* position (Figure 3). Moreover, the SEAP/GFP construct has a 2.5-kb deletion in *pol* and a 1.0-kb deletion in *env* to render the vectors replication-incompetent. Additionally, the *vpu* start codon in both constructs is mutated for robust marker gene expression.[33]. The seap gene encodes a truncated form of placental alkaline phosphatase, which catalyzes the alkaline phosphatase substrate CSPD [*Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate*] to generate chemiluminescent signals.

Human SupT1 cell line (obtained from Dr. Zandrea Ambrose's Lab) was cultured in RPMI 1640 L-glutamine (+) supplemented with 10% fetal bovine serum and 5%

penicillin/streptomycin. Cells were cultured at a concentration of 2 × $10^6$ cells/ml for all experiments.

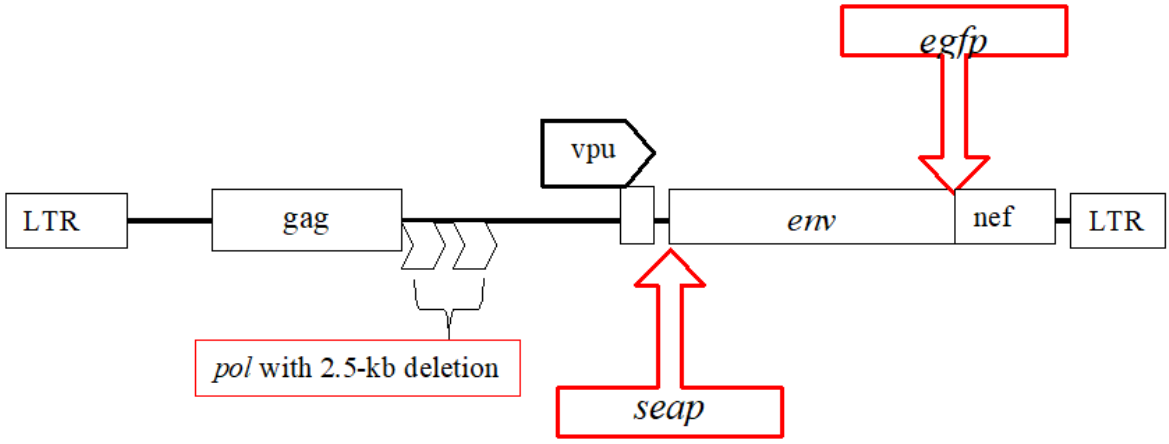



**Figure 3 The schematic diagram of HIV genes in NLE-S-G vector**

The vector contains two parts of deletion on *pol* and *env* gene for the safety purpose. It also contains *egfp* reporter gene in nef start codon, which serves as an early gene marker used in single cell analysis by flowcytometry.

## 4.2 LATENCY-REVERSING AGENTS

Prostratin was purchased from Sigma-Aldrich with a white powder form, which was further rehydrated in DMSO to a concentration of 50 mM, and diluted in DMSO to a working stock solution of 100 μM. Recombinant Human TNF-α was purchased from R&D System Inc with powder form, which was further reconstituted at 100 μg/mL in sterile PBS, and diluted to a working stock solution of 50 ng/mL. Romidepsin were purchased from Selleckchem and rehydrated in DMSO into a concentration of 5mM. The concentration of the working stock is 500nM. All stocks and working solutions were stored at −20°C and used avoiding repeated freezing–thawing cycles.

## 4.3 LATENCY-REVERSING AGENT TREATMENT CONDITIONS.

### 4.3.1 High-throughput screening

24ST1NLESG cells were seeded into F-bottom 96 well cell culture plates at a concentration of 5 × $10^5$ cells/ml for all wells. Each well contains 100 µl of cell culture. Cells were stimulated with kinase inhibitors or latency-reversing agents at the following concentrations for all single and combination treatments unless otherwise indicated: 20 µM kinase inhibitor as screening concentration, 1 µM prostratin, 50 ng/ml TNF-α, or media alone plus DMSO. The final DMSO percentage was 1% (v/v) for all single and 2% (v/v) for all combinatorial treatments. Concentrations and incubation time were chosen based on the previous time course and dose-response studies *in vitro* with 24ST1NLESG cells as well as.

### 4.3.2 Drug dose-responses assays

To determine drug $IC_{50}$, 24ST1NLESG cells were seeded in F-bottom 96 well cell culture plates at a density of 50,000 cells per well in a volume of 98 µl. 1 µl of prostratin was added into each well with a final concentration of 1µM. 1 µl of selected inhibitors (diluted to working stocks with following concentrations: 2 mM, 500 µM, 125 µM, 31.25 µM, 7.8 µM, and 1.95 µM) were added into each well in combination with prostratin. A DMSO control and a prostratin control were used on each plate. The final DMSO percentage was 2% (v/v) for all the treatments. All results were normalized to the average DMSO control for individual wells. $IC_{50}$ values were calculated by plotting luminescence counts per second (LCPS) against the drug concentration and fitting a

nonlinear regression curve using GraphPad Prism vision 6 and SigmaPlot version 11.0 for Windows.

## 4.4 MEASUREMENT OF SEAP CHEMILUMINESCENCE

After treatment, the cell cultures were heated at 65 °C for 30 minutes to inactivate endogenous alkaline phosphatase, while the SEAP was stable under this condition. The cell SEAP gene expression was assayed using alkaline phosphatase substrate CSPD with Sapphire-II™ Enhancer (purchased from ThermoFisher). The CSPD and cell culture were mixed in a white opaque 96-well plate. Each well contains 50 μl CSPD and 10 μl cell culture (heat inactivated). Then the plate was set in 1450 MicroBeta TriLux machine to incubate 15 minutes and read the chemiluminescence.

## 4.5 CELL VIABILITY ASSAY

The CellTiter-Glo® Luminescent Cell Viability Assay (Purchased from Promega Corporation) was used to estimate the biological viability of the SupT1 cells. This assay determines the number of viable cells in culture based on quantitation of ATP, which indicates the presence of metabolically active cells. SupT1 cells was placed in the opaque 96-well plate at a density of 50,000 cells per well in a volume of 98 μl. The cells were incubated with/without reagents that was used in dose-response study for 48 h at 37 °C. The 100 μl assay reagent was added into each

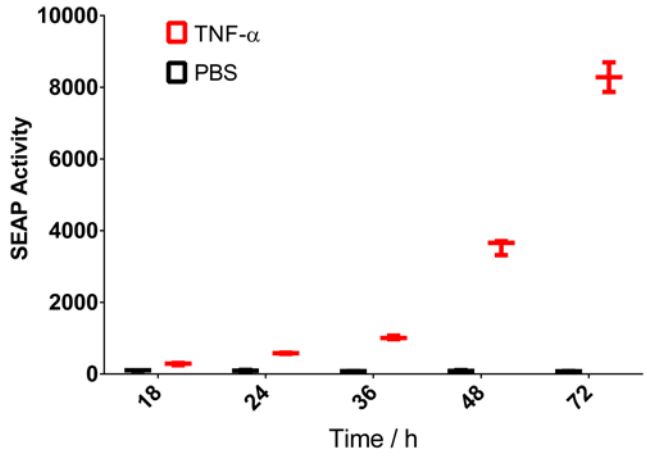
well. After 10 minutes of incubation, the 96-well plate was analyzed using an luminescence reader 1450 MicroBeta TriLux machine.

# 5.0 RESULTS

## 5.1 24ST24ST1NLESG CELLS SERVES AS HIV-1 LATENCY MODEL

For the assay to be reliable in an HTS in a small-well format, the HIV-1 latency model 24ST24ST1NLESG cells were stimulated with TNF-α to test the latent gene expression. The results showed that *seap*, the indicator of late gene expression, were detected from the cell culture, and the level of expression was increasing following the time courses (Figure 4A). This verified the reliability of the model as previously described [33]. Then we designed a dose-response study to determine the best concentration of prostratin to use in this model. The result showed that prostratin induced the peak level of seap expression at the concentration of 1 μM (Figure 4B). Based on these studies, we designed a time course study to determine the optimal incubation time for prostratin-induced latency reversal. After 48 hours of incubation, the level of seap expression in cells treated with prostratin was more than 3 times higher than that in cells treated with DMSO (Figure 4C).

A

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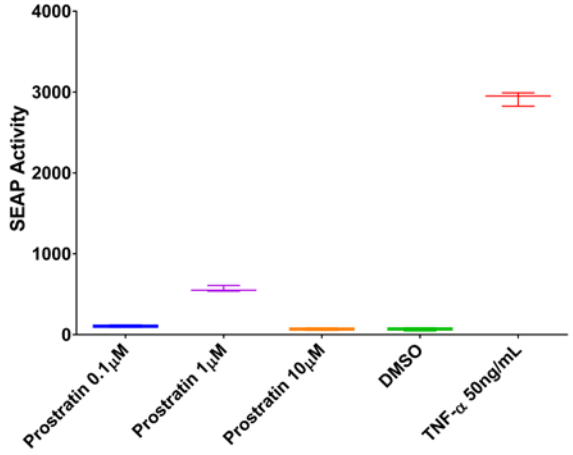


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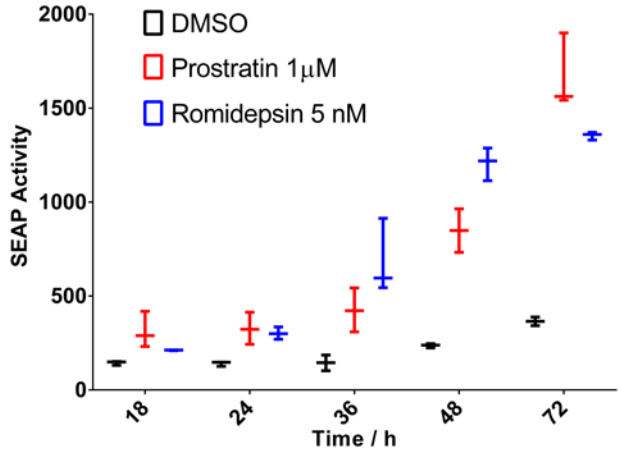


**Figure 4. Time course and dose-response studies.**

3A. 24ST24ST1NLESG cells ($10^6$/ml) were exposed to TNF-α (50ng/ml). The expression of seap gene was measured at different time points. The negative vehicle control was sterile PBS containing 0.1%FBS (due to the reason that TNF-α was added 0.1% FBS to maintain stability). 3B. 24ST24ST1NLESG cells ($10^6$/ml) were exposed to TNF-α (50ng/ml), different concentration of prostratin, and DMSO. *Seap* expression was measured after 48 hours of incubation. 3C. 24ST24ST1NLESG cells (106/ml) were exposed to 1μM prostratin, 5 nM Romidepsin, and DMSO. Romidepsin and DMSO served as positive and negative controls. Seap expression was measured after different incubation time periods. Results shown are the means and standard deviations of three representative experiments.

## 5.2 SCREENING AGENTS THAT CAN REACTIVATE HIV-1 LATENCY

To identify the cellular pathways that are involved with latency reversal, we conducted a high-throughput screening using Kinase Inhibitor Library L-1200 (418 inhibitors) targeting kinases such as receptor tyrosine kinases (RTKs), phosphoinositide 3-kinase (PI3K), Aurora Kinase, Cyclin-dependent kinases (CDK), extracellular signal–regulated kinase (Erk), and Mitogen-activated protein kinase kinase (MEK). DMSO and prostratin were used as the negative and positive control.

For each well that was treated with single kinase inhibitor alone, the LCPS was normalized with LCPS in the DMSO-treated group. We selected the reagents with $\bar{L}$ (the average of normalized LCPS values) larger than 3 as the potential drugs with latency reversing ability. The screening was repeated 6 times under same conditions. The results showed that most of the inhibitors reversed latent genes by targeting HDAC, JAK/STAT pathway, and different types of growth factor receptors (Table 1). CUDC-101 and XMD8-92 showed the reproducible results of reversing HIV-1 latent genes in every screening.

**Table 1. Pathways that are involved with HIV-1 latency reversal in 24ST24ST1NLESG cells model.**

| Frequency | Target | Drug | $\bar{L}$ | SD |
|---|---|---|---|---|
| **6/6** | EGFR,HER2,HDAC | CUDC-101 | 5.4427 | 1.7907 |
| | ERK, Brd4 | XMD8-92 | 3.8233 | 0.2696 |
| **5/6** | VEGFR,FGFR | PD173074 | 6.3228 | 1.5877 |
| | FLT3 | Quizartinib | 4.9727 | 1.6423 |
| | PI3K,HDAC | CUDC-907 | 5.0932 | 2.3393 |
| **4/6** | Bcr-Abl | Nilotinib | 5.4289 | 2.4202 |
| | PDGFR,c-Kit | Masitinib | 6.2045 | 3.0210 |
| | CDK | Palbociclib-HCl | 94.7300 | 34.4822 |
| | PI3K | TGX-221 | 5.0351 | 1.3368 |
| | PDGFR | Imatinib | 12.2218 | 7.6163 |
| | DNA-PK,PI3K | NU7441 | 4.1203 | 1.0548 |
| | JAK | CEP-33779 | 13.3691 | 4.5881 |
| | JAK | Filgotinib | 3.4344 | 0.5282 |
| **3/6** | ATM/ATR | KU-55933 | 3.1717 | 0.0976 |
| | PLK | Ro3280 | 7.4889 | 5.6895 |
| | AMPK | HTH-01-015 | 7.1006 | 1.4822 |

## 5.3 IDENTIFIED CELLULAR PATHWAYS AFFECTING PROSTRATIN-INDUCED LATENCY REVERSAL BY USING HTS

To identify the potential cellular pathways which are involved with prostratin-induced latency reversal, we used the same L-1200 library screening in combination with prostratin. DMSO and prostratin alone were used as negative and positive controls.

For each well, an R-value was given to function as the parameter. R value for the screening was calculated based on the analysis of SEAP expression from one single treatment (one kinase inhibitor alone) and one combinatorial treatment (same kinase inhibitor plus prostratin) using the equation:

$$R = \frac{L_{KI+P} \div L_P}{L_{KI} \div L_{DMSO}}$$

with L representing the luminescence counts per second (LCPS), while KI and P represent treatment with kinase inhibitor and/or prostratin. We used $R \leq 0.36$ as the cut-off value to select reagents that inhibit the prostratin-induced latency reversing. To eliminate false positive results due to cytotoxicity, the drugs were excluded if the denominator ( $L_{KI} \div L_{DMSO}$ ) of R value was less than 0.4. The screening was repeated three times under same conditions.

The screening results showed that Palbociclib, Nilotinib (Bcr-Abl inhibitor), HTH-01-015 (AMPK inhibitor), and 7 Mek inhibitors are reproducible among all three repeats (Table 2). This result implied that Mek inhibitors negatively affect prostratin-induced latency reversal. The Palbociclib and Nilotinib were showed in the former screening with the ability to reverse latency in a significant level (Table 1). However, the combinatorial use of them with prostratin showed the opposite effect reproducibly.

**Table 2. Summary of reagents that inhibit prostratin-induced latency reversing.**

| Frequency | Target | Drug | $\bar{R}$ | SD |
|---|---|---|---|---|
| 3/3 | MEK | Selumetinib | 0.1327 | 0.0713 |
| | MEK | U0126-EtOH | 0.2031 | 0.1409 |
| | MEK | PD0325901 | 0.1620 | 0.0935 |
| | MEK | Refametinib | 0.1664 | 0.0942 |
| | MEK | Trametinib | 0.1776 | 0.1072 |
| | MEK | TAK-733 | 0.2006 | 0.1122 |
| | MEK | MEK162 | 0.0945 | 0.0451 |
| | CDK | Palbociclib-HCl | 0.1287 | 0.0772 |
| | Bcr-Abl | Nilotinib | 0.2289 | 0.0531 |
| | AMPK | HTH-01-015 | 0.2203 | 0.0649 |
| 2/3 | MEK | Pimasertib | 0.1588 | 0.1466 |
| | MEK | PD318088 | 0.1457 | 0.1373 |
| | MEK | AZD8330 | 0.0493 | 0.0109 |
| | Raf | GDC-0879 | 0.2472 | 0.1157 |
| | Raf | Dabrafenib | 0.2731 | 0.0291 |
| | ERK | Ulixertinib | 0.1926 | 0.1490 |
| | PKC | Sotrastaurin | 0.1404 | 0.0254 |
| | JAK | LY2784544 | 0.3071 | 0.0031 |
| | JAK | CEP-33779 | 0.0902 | 0.0643 |
| | JAK | Filgotinib | 0.1446 | 0.0543 |
| | PI3K | TGX-221, | 0.1690 | 0.0577 |
| | PI3K, HDAC | CUDC-907 | 0.1839 | 0.0332 |
| | Aurora Kinase | AMG-900 | 0.2264 | 0.1462 |
| | EGFR,HER2,HDAC | CUDC-101 | 0.1161 | 0.0251 |
| | FLT3 | Quizartinib | 0.3496 | 0.0019 |
| | JAK,EGFR | AG-490 | 0.158 | 0.0990 |
| | LRRK2 | GNE-7915 | 0.1815 | 0.2195 |
| | PAK | FRAX597 | 0.2213 | 0.0224 |
| | PDGFR,c-Kit | Masitinib | 0.2238 | 0.0884 |
| | PLK | Ro3280 | 0.1623 | 0.1241 |
| | Syk | Piceatannol | 0.3096 | 0.0549 |

## 5.4 PROSTRATIN-INDUCED REACTIVATION IS SINSITIVE TO MEK INHIBITORS

To verify the screening results, 24ST1NLESG cells were cultured in 96 well plates at a density of 50,000 per well. Prostratin was used at concentrations previously shown to be effective in reversing latency in the model system. The kinase inhibitors were applied at the beginning concentration of 20 μM. The concentration was diluted to one-fourth with DMSO, and the concentration of DMSO remains same among all the wells. In the Mek inhibitors dose-response tests, all of the Mek inhibitors exhibited a dose-response, showing increased inhibition at increased doses. (Figure 5 A). However, U0126-EtOH showed a lower level of inhibition activity to prostratin-induced reactivation even at a high concentration (Figure 5 A). On the contrary, CDK inhibitor Palbociclib-HCl and AMPK inhibitor HTH-01-015 didn't show an obvious dose-response curve to the inhibition effect (Figure 5 E).

## 5.5 PROSTRATIN INDUCED LATENCY REVERSAL THROUGH PKC-MEK-ERK PATHWAY

To determine the pathway which prostratin induced in the latency reactivation, we further did dose-response study using inhibitors that target different kinases specifically in PKC-Mek pathway including PKC, Raf, and Erk. The inhibitors were applied at the beginning concentration of 20 μM and then diluted to one-fourth with DMSO as same as that was in Mek inhibitors dose-responses study. The results showed dose-response curves on both PKC and Erk

inhibitors (Figure 5B and C). The inhibitory activity of Raf inhibitors dropped slowly following the increasing doses (Figure 5D).

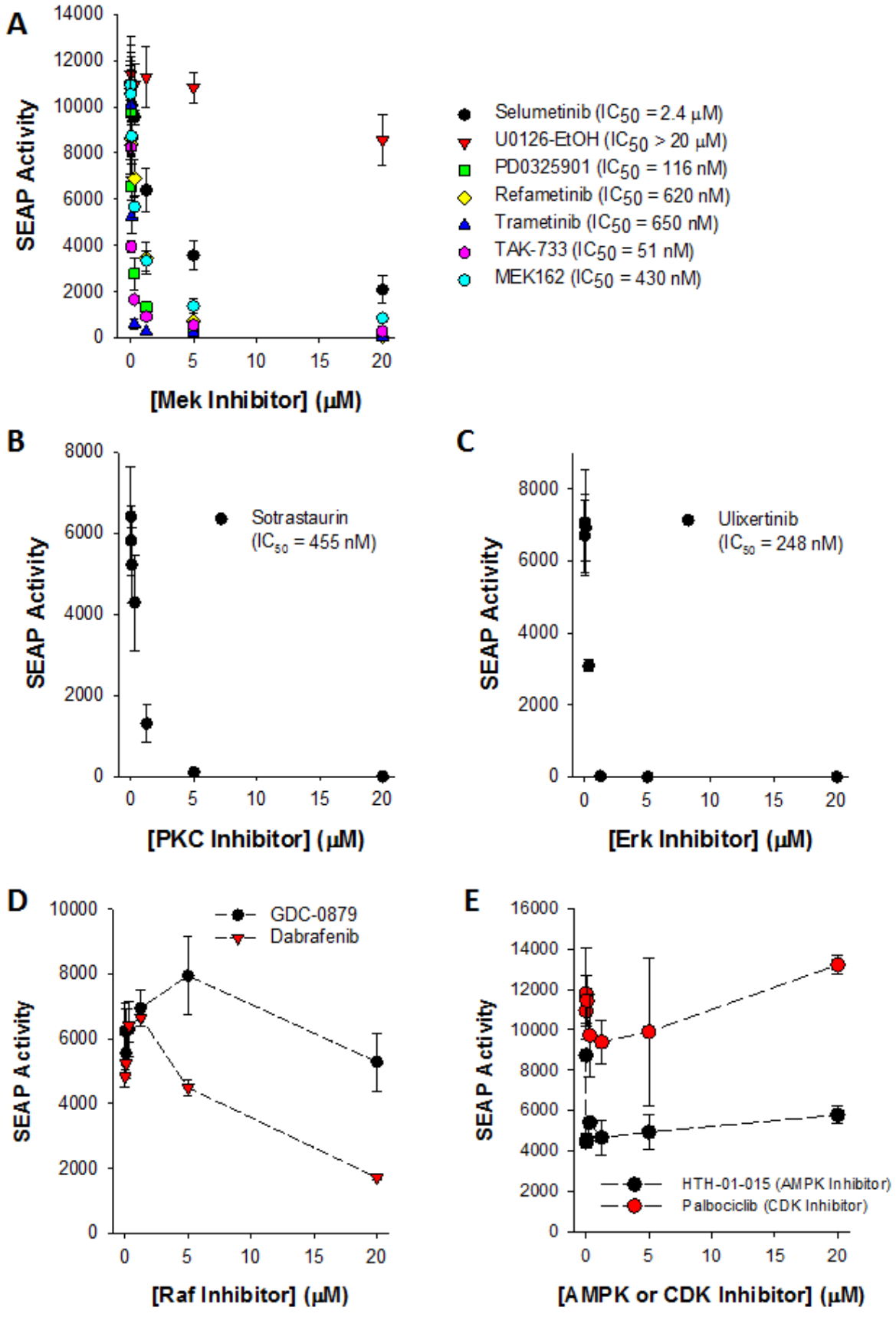
A
SEAP Activity
[Mek Inhibitor] (μM)
Selumetinib (IC50 = 2.4 μM)
U0126-EtOH (IC50 > 20 μM)
PD0325901 (IC50 = 116 nM)
Refametinib (IC50 = 620 nM)
Trametinib (IC50 = 650 nM)
TAK-733 (IC50 = 51 nM)
MEK162 (IC50 = 430 nM)
B
SEAP Activity
Sotrastaurin
(IC50 = 455 nM)
[PKC Inhibitor] (μM)
C
SEAP Activity
Ulixertinib
(IC50 = 248 nM)
[Erk Inhibitor] (μM)
D
SEAP Activity
GDC-0879
Dabrafenib
[Raf Inhibitor] (μM)
E
SEAP Activity
HTH-01-015 (AMPK Inhibitor)
Palbociclib (CDK Inhibitor)
[AMPK or CDK Inhibitor] (μM)

**Figure 5. Drug dose-responses study and $IC_{50}$ determination.**

4A and 4E. The cells were treated with different Mek inhibitors, HTH-01-015, or Palbociclib at the diluted concentrations (20 µM, 5 µM, 1.25 µM, 312.5 nM, 78.13 nM, and 19.53 nM) individually, combined with 1 µM prostratin for 48 hours of incubation. The data was normalized with cells treated with 2% DMSO (v/v). 4B, 4C, and 4D. Cells were treated with Sotrastaurin, Ulixertinib, or selected Raf inhibitors at the different concentrations (20 µM, 5 µM, 1.25 µM, 312.5 nM, 78.13 nM, and 19.53 nM) individually, combined with 1 µM prostratin for 48 hours of incubation. Results shown are the means and standard deviations of three representative experiments. The $IC_{50}$ was calculated with SigmaPlot.

## 5.6 CELL VIABILITY ASSAY

As the screening and dose-response assay showed earlier, Prostratin-induced reactivation was inhibited by several reagents including 7 Mek inhibitors (Selumetinib, U0126-EtOH, PD0325901, Refametinib, Trametinib, TAK-733, and MEK162), two Raf inhibitors (GDC-0879 and Dabrafenib), Erk inhibitor (Ulixertinib), and PKC inhibitor (Sotrastaurin). Our next step was to determine if the reduction of prostratin-induced reactivation was caused by cytotoxicity. For this purpose, we carried out the cell viability assay with or without the challenge of kinase inhibitors, and in both cases, we measure the relative viability using PBS-treated cells as the reference. We observed that the stimulation with Mek inhibitors, especially Trametinib, significantly reduced the viability at the concentration of 20 µM compared with PBS-treated cells (Figure 6 A). The Ulixertinib and Sotrastaurin led to the decrease of viability around 50% with the high-dose treatment (Figure 6 B and C). However, GDC-0879 and Dabrafenib were able to maintain the viability over 90% above even at high-dose treatment (Figure 6 D). Moreover, we also tested Palbociclib-HCl and HTH-01-015 to verify the latency reversing ability showed in the Screening (Table 1). The results showed that the viability remained steady following the increasing doses, but dramatically reduced at the high-dose treatment (20 µM) (Figure 6 E).

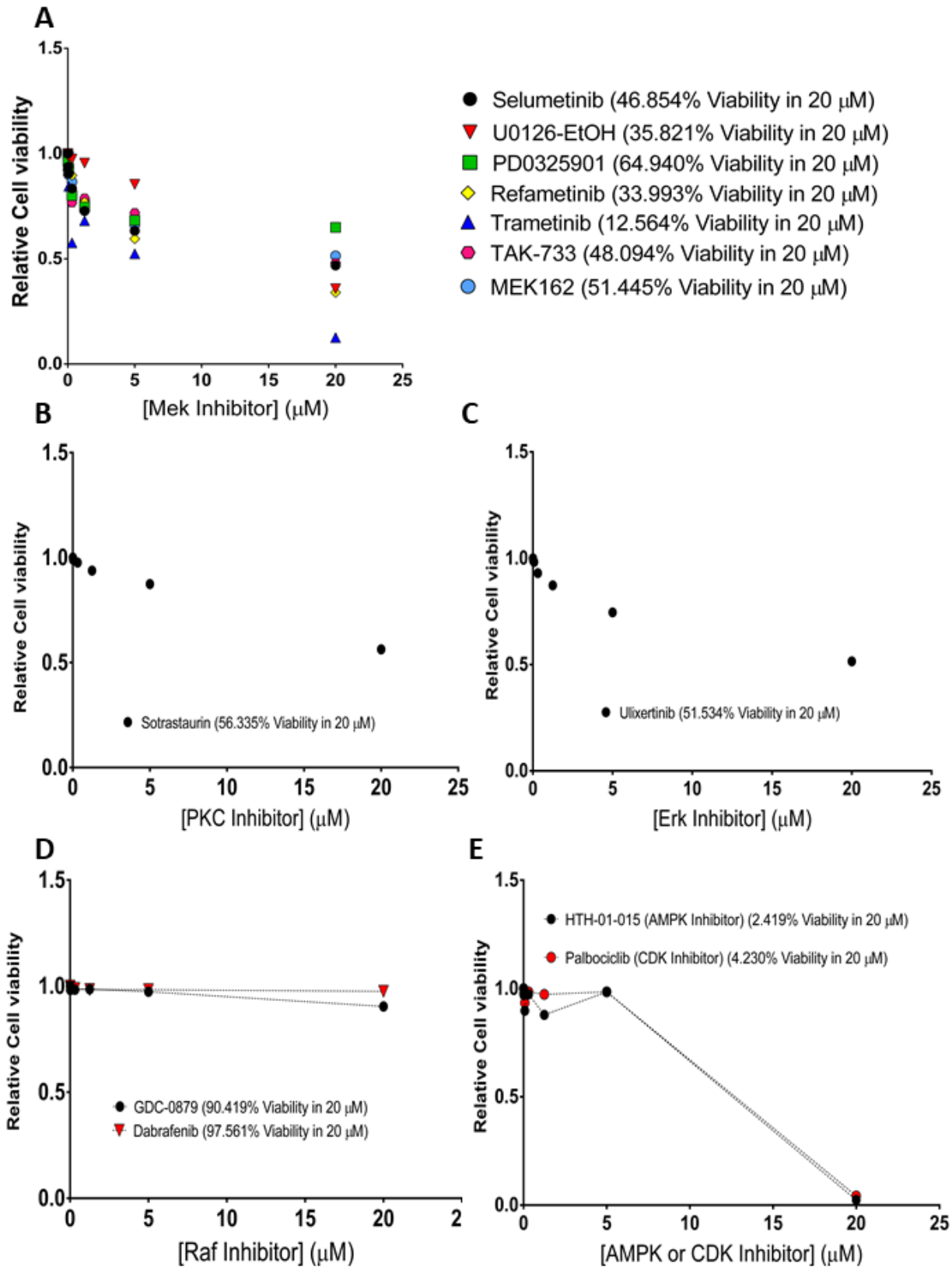
A
Relative Cell viability
[Mek Inhibitor] (μM)
Selumetinib (46.854% Viability in 20 μM)
U0126-EtOH (35.821% Viability in 20 μM)
PD0325901 (64.940% Viability in 20 μM)
Refametinib (33.993% Viability in 20 μM)
Trametinib (12.564% Viability in 20 μM)
TAK-733 (48.094% Viability in 20 μM)
MEK162 (51.445% Viability in 20 μM)
B
Relative Cell viability
Sotrastaurin (56.335% Viability in 20 μM)
[PKC Inhibitor] (μM)
C
Relative Cell viability
Ulixertinib (51.534% Viability in 20 μM)
[Erk Inhibitor] (μM)
D
Relative Cell viability
GDC-0879 (90.419% Viability in 20 μM)
Dabrafenib (97.561% Viability in 20 μM)
[Raf Inhibitor] (μM)
E
Relative Cell viability
HTH-01-015 (AMPK Inhibitor) (2.419% Viability in 20 μM)
Palbociclib (CDK Inhibitor) (4.230% Viability in 20 μM)
[AMPK or CDK Inhibitor] (μM)

**Figure 6. Cell viability assay using PKC, Raf, Mek, Erk, AMPK, and CDK inhibitors**

5A. Cell viability test for prostratin-treated SupT1 cells under the stimulation of Mek inhibitors. 5B and 5C. Cells were grown in the presence of prostratin and PKC or Erk inhibitor for 48 hours and were used to measure the final cell viability. Responses were normalized using PBS-treated cells as reference. The relative viability at the highest dose (dose of 20 µM, used in previous screenings) was showed in the figures.

# 6.0 DISCUSSION

## 6.1 CELL LINE MODEL

In this study, 24ST24ST1NLESG cell line was applied to mimic the HIV-1 infected resting $CD4^+$ T-cells in patients. The cell clones were derived from human SupT1 cells, latently infected with an HIV-$1_{NL4-3}$-based reporter construct SEAP/GFP. As the report, genes carried on this vector can be induced by TNF-α[34]. This cell model was also used by Dougherty's group in the study of SAHA for treating T-cell lymphoma research[35] and Cochrane's group in Digoxin-suppressed HIV-1 replication study[36]. Our study also suggests the consistency and reliability of the replication-competent HIV-1 model in drug-induced reactivation. Moreover, our study shows that Prostratin induces a higher level of latency reversal compared with Romidepsin in the cell line model, which was in consistency with the study from Siliciano's group who used resting $CD4^+$ T-cells from infected individuals on suppressive ART[37]. This suggests that 24ST24ST1NLESG cell line is capable of representing latently infected resting $CD4^+$ T-cells in HIV-infected patients.

The cell line was derived from SupT1 cells which are T lymphoblast cells expressing CD4 and CD45RO on the surface (showed on CD45RO Antibody (UCH-L1) from Santa Cruz Biotechnology). This indicates that 24ST1NLESG cells are similar to resting memory $CD4^+$ T-cells. It is established that Resting memory $CD4^+$ T-cells represent main reservoirs, however,

additional reservoirs could further complicate eradication efforts. Evidence for additional reservoirs was showed from a detailed analysis of residual viremia. The study from Toronto Mucosal Immunology Group and the University of Toronto suggests some latently infected reservoirs (especially $T_{H1}$ and $T_{H17}$ reservoirs) were established under the regulation of immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor-beta (TGF-β) which inhibit $CD4^+$ T-cells activation[38]. In addition, macrophages are regarded as potential reservoirs due to their higher resistance to viral cytopathic effects than activated $CD4^+$ T-cells[39]. Furthermore, the high expression of P-glycoprotein transporter in macrophages has been reported to limit the availability of ART drugs[40]. Therefore, macrophages could serve as reservoirs if they can persist in an infected state during the years of ART. To increase the internal validity of this study, the other cell models should be applied to this chemical biology approach such as Bosque/Planelles' model using naïve $CD4^+$ T-cells that were activated by anti-CD3 and anti-CD28 *in vitro* and infected with an NL4-3-based virus. Moreover, PBMCs isolated from HIV-1-infected patients under the ART should be used in the further study to verify the results.

## 6.2 THE IDENTIFIED LRAS BY SCREENING

The chemical biology approach in this study identified several kinases that are involved with the reversal of HIV latency, including Erk, HDAC, EGFR/FGFR, PI3K, and JAK. Five agents were identified to reverse HIV-1 latency reproducibly, including CUDC-101, XMD8-92, PD173074, Quizartinib, and CUDC-907. Inhibition of HDAC was showed in the earlier study that induced the latency reversing both in vitro and in vivo[41]. CUDC-101 and CUDC-907 inhibit HDAC[42, 43] which may reverse the latency as same as other HDACi LRAs. XMD8-92 is an

Erk 5 and Brd4 specific inhibitor, which was used to regulate TCR/CD3 expression in Xavier Rovira-Clavé's study[44]. Erk 5 is the last MAP kinase family member which involves in MEK5-ERK5 pathway linked to different cellular processes, playing a crucial role in cell proliferation in normal and cancer cells[45]. However, recent studies show that the anticancer activity of Erk 5 should be carefully interpreted. It has been reported that several kinase inhibitors can strongly inhibit the bromodomain-containing protein-4 (Brd4), a general transcription co-activator[46]. XMD8-92 shows similar potency in which its antitumor effects could be compromised by the Brd4-inhibiting activity[47]. Compared what we found in this study, the XMD8-92 shows the ability to activate latent gene rather than inhibits latent gene activation by deactivating Erk and Brd4. This suggests that the further study of XMD8-92 using PBMCs isolated from HIV-infected patients is required. Quizartinib is used on a clinical trial for the treatment of Acute myeloid leukemia (AML)[48]. It has been reported that the presence of FMS-like tyrosine kinase-3 (FLT3) was highly associated with the signal transducers and activators of transcription 5 (STAT5)[49]. Therefore, one explanation is that Quizartinib suppressed FLT3 activity leading to the inhibition of JAK/STAT pathway, which is in consistency with other JAK inhibitors (such as CEP-33779 and Filgotinib) inducing latency reactivation (Table 1). Above all, our next step should focus on the verification of the theory by using FLT3-siRNA or Brd4-siRNA and the application of those reagents in PBMCs. Per se, future studies of these speculative matters may bring new concepts to what is known so far.

This approach is based on 24ST24ST1NLESG cell line, which introduces biological variance due to the variability in individual cell responding to the pharmaceutical stimuli. Moreover, the screening was processed using a multichannel pipette, which brings the random error to this system. It is showed that the standard deviation of Palbociclib-HCl is 34.4822 (Table

1), which is infeasible to be fixed by increasing the internal validity. To increase the external validity by minimizing the random error caused by multichannel pipette, a large number of replicants should be used in this study. However, this may bring another obstacle into the procedure, i.e. a large number of experimental replications requires a high-volume workload operated by individuals.

## 6.3 VARIFICATION OF THE BIOLOGY APPROACH USING PROSTRATIN

Three agents, Palbociclib-HCl, Nilotinib, and HTH-01-015 showed activity to reverse the latency alone three or more times among 6 parallel repeat experiments. Among these three agents, Palbociclib and HTH-01-015 were reported only for their ability to suppress tumor cell proliferation[50, 51], while their ability to reactivate HIV-1 latency remains unknown. However, when those agents were used in combination with prostratin, the reversal of latency was significantly inhibited. The cell viability assay result suggests that the combinatorial use of prostratin with Palbociclib or Nilotinib leads to the significant reduction of cell viability (Figure 6 E). One explanation of the screening results (showed in Table 2) is that the combination is highly cytotoxic to the cell line, which affected the numerator and lowered the R value. Further studies are required to elucidate the mechanism of the cytotoxicity from the combination of those drugs.

This approach also revealed that several kinases are critical in prostratin-induced latency reversal, including Mek, Erk, Raf, and PKC. The following dose-response assays suggest that prostratin may activate the signaling cascade through PKC-Raf-Mek-Erk to induce the expression of proviral latent genes. Xiaoyu Yang’s study showed similar results that Erk

activated latent HIV-1 infection by stimulating a cooperative interaction of AP-1 and NF-κB[52]. Egbert Flory's study showed that Raf agonists activated NF-κB and HIV-1 replication in T cells[53]. In summary, these earlier studies suggest that activation of Raf/Mek/Erk pathway upregulates HIV replication and infectivity, and PKC and Erk activation induce the reactivation of HIV-1 latency. Based on the results from this study, we conclude that Mek is activated in prostratin-induced HIV-1 latency reversal, which stimulates NF-κB-dependent transcription activation through PKC/Raf/Mek/Erk signaling cascade. Additional assays such as phosphor-specific Western Blot analysis and RNAi will be required in future studies to verify the signaling cascade and the functions of kinases.

# 7.0 PUBLIC HEALTH SIGNIFICANCE

The proposed research is relevant to the public health because the HIV-1 infection is still one of the major global costs measured by both patient suffering and economic burden. Despite effective treatment, HIV-infected individuals have a higher risk of heart diseases, kidney dysfunction, and neurological disease. In 2015, an estimated 36.7 million people were living with HIV, including 1.8 million children[54]. Among these people, 18.2 million people were accessing ART. However, the prevalence of HIV infection is higher among developing countries and poverty-stricken areas such as Angola, Kenya, Zimbabwe, Indonesia, and Brazil[55]. Among all the infected individuals, males accounted for 81% of all diagnoses of HIV infection, which indicates that HIV transmission remains common in many vulnerable populations, including men who have sex with men and injection drug users. As of December 2015, 46% of all adults and 49% of all children living with HIV were accessing to ART[54]. On the other hand, clinical studies focusing on HIV eradication showed that candidate LRAs including HDACi (Vorinostat, panobinostat, romidepsin), disulfiram, and JQ1 (bromo and extra terminal bromodomain inhibitor) only activated HIV-1 latency in a small subtype of resting $CD4^+$ T cells[25]. Moreover, some combinatorial uses of LRAs from different classes showed an outstanding effect on latency reactivation ex vivo[37], yet the mechanism of this remains unclear. Therefore, by using this chemical biology approach, the action on LRA-induced latency reversal will be

delineated to provide a better understanding of the mechanism. Thus, it contributes to the overall advancement of HIV eradication in public health.

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