Screening of the NIH Clinical Collection for inhibitors of HIV-1 integrase activity

Drug repurposing offers a validated approach to reduce drug attrition within the drug discovery and development pipeline through the application of known drugs and drug candidates to treat new indications. Full exploitation of this strategy necessitates the screening of a vast number of molecules against an extensive number of diseases of high burden or unmet need and the subsequent dissemination of the findings. In order to contribute to endeavours within this field, we screened the 727 compounds comprising the US National Institutes of Health (NIH) Clinical Collection through an HIV-1 (human immunodeficiency virus type 1) integrase strand transfer inhibition assay on an automated scintillation proximity assay platform. Only two compounds were identified within the initial screen, with ceftixime trihydrate and epigallocatechin gallate found to reduce integrase strand transfer activity at IC_{50} values of 6.03±1.29 µM and 9.57±1.62 µM, respectively. However, both ceftixime trihydrate and epigallocatechin gallate retained their low micromolar inhibitory activity when tested against a raltegravir-resistant integrase double mutant (FCIC_{50} values of 0.83 and 0.06, respectively), ineffective in an orthogonal strand transfer ELISA (<30% inhibition at 100 µM) and produced negligible selectivity index values (<1) in vitro. While no useful inhibitors of HIV-1 integrase strand transfer activity were found within the NIH Clinical Collection, the identification of two assay-disrupting molecules demonstrates the importance of consideration of non-specific inhibitors in drug repurposing screens.

Significance:

- This study is the first to screen the US NIH Clinical Collection for potential HIV-1 integrase inhibitors.
- The pervasive nature of promiscuous inhibitors is emphasised.

Introduction

Early-stage drug discovery fulfils a critical role within the broader drug discovery process and the entire drug discovery and development pipeline. Early-stage drug discovery is typically – but not always – undertaken following target identification and validation, and involves the screening of compounds with the intent purpose of identifying compounds with promising activity (HIT compounds) that can then be developed further (into LEAD compounds) within the drug discovery phase. Early-stage drug discovery activities can range from the evaluation of a limited set of compounds, typically selected through rational drug design methodologies, to the assessment of large compound libraries through high throughput screening (HTS, defined as the screening of >10 000 compounds per day) and even ultra-HTS (µHTS, defined as the screening of >100 000 compounds per day) operations. Owing to the sheer number of compounds screened, early-stage drug discovery ostensibly carries the highest failure rate and, accordingly, the highest risk of all activities within the pipeline. However, the true bottleneck to success in the broader drug discovery and development process lies less with the quantity of compounds identified as HITS during screening and more with the quality of these compounds and their suitability as drug candidates. Specifically, the highest cause for compound attrition in the pipeline, by far, is attributed to non-clinical toxicity which accounts for the termination of >40% of all compounds from the drug discovery and development pipeline.1 To mitigate the potential significant financial loss resulting from compound failures, in particular the high cost of late-stage failures, most pharmaceutical organisations adopt the ‘fail early, fail cheap’ paradigm. To support this approach, researchers aim to recognise ADMET-related issues through an ever-increasing number of tests undertaken at progressively earlier stages of the pipeline. Equally, findings from these tests have been retrospectively accumulated to delineate physicochemical properties (i.e. LogP, LogD, molecular weight, aromatic rings, rotatable bonds, polar surface area, etc.) that influence drug-likeness and then subsequently collated into ‘rules of thumb’ (such as the Lipinski rule of five, the rule of three and many other variations and extensions) and property prediction software programs. Application of these predictive models has allowed for the identification and judicious removal of non-favourable compounds either following screening or directly from the physical compound library prior to screening. While immeasurably useful, these tools have not proven infallible as evinced through a recent study of 812 failed compounds (oral development candidates from four different major pharmaceutical companies) that could draw no correlation between non-clinical toxicity failure and physicochemical properties.1 Similarly, a subset of compounds eliciting growing interest because of their subversive effects in drug discovery efforts are promiscuous inhibitors2,3 or pan-assay interference compounds (PAINS)4. These compounds yield convincing false-positive results in biological assays and significant efforts have been undertaken to identify them and ultimately remove them from screening libraries.4 Broadly categorised and inclusive of several classes of compounds with varying mechanisms of action (i.e. aggregate-inducing compounds, redox-cyclers, covalent modifiers, metal complexes), these compounds do not readily lend themselves to predictive algorithms. Nonetheless, databases of existing PAINS highlight common structures (i.e. flavonoids, quinones, rhodamines) and some can also be searched for similarly.

Of other approaches aimed at minimising compound attrition, the concept of drug repurposing (or drug repositioning) has drawn significant interest. Herein, the underlying principle is the evaluation of clinically approved drugs or
late-stage clinical trial failures (all off-patent or generics) as disease-modifying agents in therapeutic areas other than the one for which they were designed or proved effective. The main appeal of this approach is the decreased risk of failure arising from safety issues while the extensive prior development allows for a quicker transitioning through the pipeline (up to 60%) with reduced costs (up to 40%). If successful, the drug can be granted patent protection on grounds of a new application or new formulation. The classic example of drug repositioning is Viagra™—the blockbuster erectile dysfunction drug from Pfizer which first served as an angina medication under the name Silidenafil. Numerous other examples exist, including azidothymidine (the cancer turned anti-HIV drug), ropinirole (a dual Parkinson’s and restless legs syndrome treatment) and Rogaine® (a hair-loss drug repurposed from a blood pressure drug from a failed ulcer candidate), to mention a few. The scientific merit of the concept has driven growth in its popularity as is clearly evident through interest from major pharmaceutical companies, the growth in focused start-up companies, the rise in related literature and a recently launched journal (Drug Repurposing, Rescue and Repositioning) with dedicated content.

Applying the drug repurposing approach to the field of HIV drug discovery has been previously described. While the treatment options for HIV-1 are formidable – both in the number of antiretroviral agents approved and the efficacy of combination therapy – the absence of an effective therapeutic vaccine or cure and the persistent threat of antiretroviral drug resistance has substantiated the continued exploration for novel inhibitors. In this study, we sought to identify an existing drug with activity against HIV-1 integrase (IN) – a virally encoded enzyme that catalyses the integration of viral DNA into the host chromosome. For this purpose we screened the US National Institutes of Health (NIH) Clinical Collection (NCC), which is a 727 small-molecule library of FDA-approved and late-stage candidates that has been previously explored for proteasome stimulators3 and coronavirus inhibitors4 but not, to the best of our knowledge, for HIV-1 IN inhibition. The NCC library was screened by means of an automated process through an HIV-1 IN strand-transfer (ST) inhibition scintillation proximity assay (SPA) in order to identify novel catalytic IN inhibitors.

**Methods**

**Expression and purification of recombinant HIV-1 integrase**

The reagent pINSD.His (Cat. #2957) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr Robert Craigie.7,8 Recombinant His-tagged HIV-1 IN was expressed and purified as previously described.9 Briefly, wild-type HIV-1 IN was overexpressed in E. coli BL21 (DE3) bacterial cells using the NL4-3 histidine (HIS) tagged HIV-1 IN coding sequence, pINSD, cloned into pET15B (Merck, Craigie. AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr Robert Richman.10 The reagent MT-4 (Cat. #120) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr Douglas Richman.11,12

**Radiolabelling of target DNA for scintillation proximity assays**

Annealed oligonucleotides for target DNA (tDNA), T 5′-S (AAAGGGAGGA-GAAGGGAAAGGAAGAAGCAGATTACCCGGTGGT) and T 5′-A (AAAGGA-GGAGAGAGAGAGAGAGAGGAGAGAGAGAGAGAGGAGAGAAGACCTTACATTTCG) oligonucleotides (Inqaba Biotech, Pretoria, South Africa) were radiolabelled with 3H-dCTP and 3H-dTTP (AEC Amersham, Johannesburg, South Africa) by filling in the 5′-overhangs according to the Fermentas Klenow fragment DNA labelling kit instructions (ThermoFisher Scientific, Waltham, MA, USA). Unincorporated nucleotides were removed from the radiolabelled tDNA using the QIAquick nucleotide removal kit (Qiagen, Hilden, Germany).

**HIV-1 integrase strand transfer scintillation proximity assay**

The SPA was carried out as previously described10 and adapted to an automated platform on a Hamilton Starlet robotic system (Hamilton, Bonaduz, Switzerland). Briefly, a 1x reaction buffer was prepared containing 200 mM HEPES (pH 7.5), 300 mM NaCl, 50 mM dithiothreitol and 0.5% Igepal (nonidet-P40). Polyvinyltoluene streptavidin-coated scintillation beads (GE Healthcare Sciences, Marlborough, MA, USA) were reconstituted in 1x reaction buffer at a final concentration of 10 mg/mL. Biotinylated donor DNA (dDNA) was added at a final concentration of 500 nM and rocked at room temperature for 1 h. The bead suspension was washed twice with 1x reaction buffer and centrifuged at 1000 x g for 5 min. The pellet was resuspended at 2 mg/mL in 2x reaction buffer to which recombinant IN (wild-type or Q148H/G140S mutant) was added at a final concentration of 1 µM and rocked at room temperature for 30 min. The final SPA reactions comprised, per well: 1 mg/mL SPA bead-dDNA-IN complex with 8 to 10 test compounds at 10 µM each for single-dose experiments or concentrations ranging from 100 to 0.78 µM for dose-response experiments (substituted with DMSO buffer solution for blank control). This reaction mixture was incubated at 22 °C for 30 min whilst shaking gently. The reactions were initiated by adding 500 nM H-dDNA to each well at a final concentration of 50 nM and incubated at 37 °C shaking for 90 min before the enzymatic reaction was stopped using 62 mM EDTA. The reaction product formation was measured using the Top Count Scintillation Counter NXT (Perkin Elmer, Waltham, MA, USA).

**Cytotoxicity assays**

The reagent MT-4 (Cat. #120) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr Douglas Richman.11,12 The cytotoxicity assay was performed as per standard methods and as described previously.9,13 Briefly, MT-4 cells were plated in 96-well microtitre plates at 3.0 x 10³ cells/mL and allowed to stabilise
for 2 h at 37 °C and 5% CO₂. Thereafter, test compounds were added to the plate through twofold serial dilution to allow for eight final compound concentrations ranging from 200 to 1.56 µM in a total volume of 200 µL/well. The cells and compounds were then incubated for 96 h at 37 °C and 5% CO₂. To each well, 20 µL CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA) was added. The plates were incubated for 4 h and absorbance was read at 490 nm on a multiplate reader (xMark, Bio-Rad). CC₅₀ values were determined as the concentration of the test compound required to reduce the cell viability by 50% and were calculated using OriginPro 8.0 software (Origin Lab Corporation). The values obtained are averages of at least three separate experiments.

**Antiviral activity**

To determine antiviral activity, 50 µL HIV-1 NL4-3 virus was added to 3.0x10⁵ MT-4 cells/mL at a multiplicity of infection of 0.1 and the mixture of cell-well synthesis by disruption of the transpeptidation process. Modulation of HIV-1 replication by CEF has not been previously documented promiscuous nature.

In our experience, the HIV-1 integrase ST SPA is a robust assay that will be elucidated through future studies to facilitate further screening strategy for the discovery and development of therapeutic agents. In an endeavour to contribute to efforts in this field, we screened the NCC library to identify new inhibitors of HIV-1 integrase strand transfer activity. While no true inhibitors of HIV-1 IN ST activity were discovered, the identification of two non-specific inhibitors through our screen demonstrated that drug repurposing is not insusceptible to the presence of assay disruptors. In particular, the identification of EGCG demonstrates the invasiveness of even the most well-documented PAINS into chemical screening libraries. Furthermore, and perhaps more interestingly, the confirmation of the clinically relevant antibiotic CEF as an SPA disruptor demonstrates necessity to interrogate the action of well-characterised molecules within specific assay platforms and also supports the mandatory use of secondary or orthogonal assays to confirm the primary results. The findings from this study suggest that both EGCG and CEF disrupt the SPA through a similar non-aggregating mechanism that will be elucidated through future studies to facilitate further screening projects based on this assay platform.
Acknowledgements

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Authors’ contributions

R.H. was responsible for the conceptualisation, methodology, data analysis, validation, critically reviewing the writing, writing revisions, student supervision, project leadership, project management and funding acquisition. S.M. was responsible for the methodology, data analysis, validation, data curation, writing revisions, student supervision and project leadership. S.A. was responsible for the methodology, data collection, data analysis, sample analysis, validation, data curation and writing the initial draft. M.Q.F. was responsible for the methodology, data collection and data analysis. M.A.P. was responsible for the methodology, data analysis, validation, writing revisions and student supervision.

References


### Table 1: Comparison of cefixime trihydrate, epigallocatechin gallate and raltegravir as inhibitors of HIV-1 integrase strand transfer activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>HIV-1 integrase strand-transfer inhibition</th>
<th>MT-4 cell based assays</th>
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<tbody>
<tr>
<td></td>
<td>Scintillation proximity assay (SPA)</td>
<td>ELISA</td>
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<tr>
<td></td>
<td>IN$<em>{WT}$ IC$</em>{50}$ ± SE (µM)</td>
<td>IN$<em>{Q148H/G140S}$ IC$</em>{50}$ ± SE (µM)</td>
</tr>
<tr>
<td>Raltegravir</td>
<td>9.98 ± 0.83 (x 10$^{-3}$)</td>
<td>1.50 ± 0.50</td>
</tr>
<tr>
<td>Cefixime trihydrate</td>
<td>6.03 ± 1.29</td>
<td>5.01 ± 1.29</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>9.57 ± 1.62</td>
<td>0.62 ± 1.50</td>
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IN$_{WT}$, wild-type HIV-1 integrase

IC$_{50}$, 50% inhibitory concentration; calculated as the concentration of compound required to reduce the HIV-1 integrase strand transfer activity by 50%

SE, Standard error; standard error of the mean for at least three separate experiments

FCIC$_{50}$, fold-change in IC$_{50}$; calculated as the ratio of IN$_{Q148H/G140S}$/IN$_{WT}$

CC$_{50}$, 50% cytotoxic concentration; calculated as the concentration of compound required to reduce cell viability by 50%

EC$_{50}$, 50% effective concentration; defined as the concentration of compound required to reduce HIV-1 replication by 50%

SI, selectivity index; calculated as the ratio of CC$_{50}$/EC$_{50}$

ND, not done


