



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

AUTHORS:

Shaakira Abrahams^{1,2}

Salerwe Mosebi¹

Muhammed Q. Fish^{1,2}

Maria A. Papathanasopoulos²

Raymond Hewer³

AFFILIATIONS:

¹Centre for Metal-Based Drug Discovery, Advanced Materials Division, Johannesburg, South Africa

²Department of Molecular Medicine and Haematology, University of the Witwatersrand Medical School, Johannesburg, South Africa

³Discipline of Biochemistry, University of KwaZulu-Natal, Pietermaritzburg, South Africa

CORRESPONDENCE TO:

Raymond Hewer

EMAIL:

hewerr@ukzn.ac.za

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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 cefixime trihydrate and epigallocatechin gallate found to reduce integrase strand transfer activity at IC_{50} values of $6.03 \pm 1.29 \mu M$ and $9.57 \pm 1.62 \mu M$, respectively. However, both cefixime trihydrate and epigallocatechin gallate retained their low micromolar inhibitory activity when tested against a raltegravir-resistant integrase double mutant (FCIC₅₀ values of 0.83 and 0.06, respectively), were 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 (μ HTS; 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 toxicology failure and physicochemical properties.¹ Similarly, a subset of compounds eliciting growing interest because of their subversive effects in drug discovery efforts are promiscuous inhibitors^{2,3} 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 similarity.

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 Sildenafil. 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 but 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 stimulators⁵ and coronavirus inhibitors⁶ 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 pINS.D.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, pINS.D, cloned into pET15B (Merck Millipore, Darmstadt, Germany). Cells were grown to logarithmic phase in Luria-Bertani medium and induced with 1 mM isopropyl-thiogalactoside. The recombinantly expressed protein was purified through affinity chromatography using a nickel (Ni)-affinity column and buffer exchanged into storage buffer (20 mM HEPES pH 7.2, 1 M NaCl, 4 mM EDTA, 2 mM dithiothreitol and 50% glycerol) using a PD-10 Sephadex column (GE Healthcare, Buckinghamshire, UK). The expression and purification of the HIV-1 IN was confirmed through SDS-PAGE and subsequent Western blot analysis. Similarly, recombinant IN which incorporated raltegravir-resistant mutations, IN_{Q148H/G140S}¹⁰ was prepared. Briefly, the pINS.D.His plasmid was used as a template for mutagenesis with the QuickChange Lightning Site Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Raltegravir-resistant mutations were inserted into the pINS.D.His backbone and included Q148H/G140S substitutions.

Radiolabelling of target DNA for scintillation proximity assays

Annealed oligonucleotides for target DNA (tDNA), T 56-S (AAAAGGAGGA-GAAGGAAAGGAGAGAGAGCGAATTAGCCCTTGGTC) and T 56-A (AAAAGGA GGAGAAGGAAAGGAGAGAGAGACCAAGGGCTAATTCG) 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 10x 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 ³H-tDNA 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). Percentage inhibition was determined for single-dose experiments while IC₅₀ values were determined as the compound concentration required to reduce HIV-1 recombinant IN ST activity by 50% and calculated using OriginPro 8.0 software (Origin Lab Corporation, Northampton, MA, USA). All inhibition values are the average of at least triplicate experiments.

HIV-1 integrase strand transfer enzyme-linked immunosorbent assay

The HIV-1 IN strand transfer inhibition enzyme-linked immunosorbent assay (ELISA) was adapted from previously described methods.^{9,11} Briefly, 0.15 μM double-stranded biotinylated dDNA (5'-biotin-ACCCTTTAGTCAGTGT GGAAAATCTCTAGCA-3' and 5'-ACTGCTAGAGATTTCCACACTGACTAA AAG-3') was added to the wells of streptavidin-coated 96-well microtitre plates (R&D Systems, Minneapolis, MN, USA). Following incubation at room temperature for 60 min and a stringent wash step, 1 μM purified recombinant HIV-1 subtype B IN (in the presence of MgCl₂) was assembled onto the pre-processed dDNA through incubation for 30 min at 22 °C. Following a wash step, the test compounds were titrated into individual wells at a final concentration of 100 μM for single-dose evaluation or concentrations ranging from 100 to 0.78 μM for dose-response experiments. The microtitre plates were incubated for 30 min at 37 °C, washed and the strand transfer reaction was initiated through the addition of 0.25 μM double-stranded FITC-labelled target DNA (5'-TGACCAAGGGCTAATTCACACT-FITC-3' and 5'-AGTGAATTAGCCCTTGGTCA-FITC-3') in HEPES buffer containing MgCl₂ and MnCl₂. After an incubation period of 60 min at 37 °C, the plates were washed as before and an alkaline phosphatase conjugated anti-FITC secondary antibody (Sigma-Aldrich, St Louis, MO, USA) was added. Finally, the plates were washed and substrate (BluePhos, KPL, Gaithersburg, MD, USA) was added to allow for detection at 620 nm using a microplate reader (xMark™, Bio-Rad, Hercules, CA, USA). Percentage inhibition was determined for single-dose experiments while IC₅₀ values were determined as the compound concentration required to reduce HIV-1 recombinant IN ST activity by 50% and calculated using OriginPro 8.0 software (Origin Lab Corporation). All inhibition values are the average of at least triplicate experiments.

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.¹²⁻¹⁴ The cytotoxicity assay was performed as per standard methods and as described previously.^{9,15} Briefly, MT-4 cells were plated in 96-well microtitre plates at 3.0 x 10⁵ 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 was spinoculated at 3000 x g for 90 min. After washing off unbound virus, cells were plated in 96-well microtitre plates at 100 μL/well and allowed to stabilise for 1 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. Cell-free supernatants were collected from each well, and p24 concentration was determined using the Vironostika HIV-1/2 p24 Antigen ELISA (bioMérieux, Marcy-l'Etoile, France) as per manufacturer's instructions. EC₅₀ values were determined as the concentration of the test compound required to reduce p24 concentration by 50% and were calculated using OriginPro 8.0 software (Origin Lab Corporation). The values obtained are averages of at least three separate experiments. Selectivity index (SI) values were calculated as the ratio of CC₅₀/EC₅₀.

Results and discussion

Inhibition of recombinant integrase strand transfer activity

To begin the screening process, all 727 small molecules within the NCC library were pooled in an orthogonal manner into 171 pools; each pool comprised a combination of 8 or 10 different compounds with each compound present in two pools. The pools were then screened through an automated SPA, in triplicate, with each compound evaluated at a final single-dose concentration of 10 μM. Initially, 24 pools were found to be affected by colour-quenching which was only resolved through the identification and removal of 12 coloured compounds. On completion of the screening process, four pools were found to reduce recombinant IN_{WT} ST activity by the pre-defined minimum cut-off of ≥50%, indicating the presence of two active compounds. Through cross-referencing, the two common compounds were identified as cefixime trihydrate (CEF) and epigallocatechin gallate (EGCG). CEF, a third-generation orally administered cephalosporin is a potent, broad-spectrum bactericidal. Like other cephalosporins, CEF possesses a dihydrothiazine ring fused to a beta-lactam ring and derives its therapeutic effect through inhibition of cell-wall synthesis by disruption of the transpeptidation process. Modulation of HIV-1 replication by CEF has not been previously described in the literature; however, cephalosporin oligonucleotides and monocyclic β-lactams have been reported as HIV-1 protease inhibitors. EGCG is a polyphenolic acid ester that has been proclaimed as an effective agent for an exceedingly broad range of diseases (including HIV-1 through several distinct mechanisms of action) despite its well-documented promiscuous nature.^{4,16} A search of the US NIH registry and results database of clinical studies revealed 92 clinical trials in varying stages of completion (from recruiting to completed) examining EGCG as treatment for 276 different clinical conditions.¹⁷

In our experience, the HIV-1 integrase ST SPA is a robust assay that yields a low number of HIT compounds per screen. The inclusion of detergent within the protocol design serves, perhaps inadvertently, to reduce the identification of false positive responses caused by promiscuous molecules. We therefore opted to continue investigating EGCG to verify a non-specific mechanism, and as such, both CEF and

EGCG were subjected to dose-response evaluation within the previously described SPA. Here we established IC₅₀ values of 6.03±1.29 μM and 9.57±1.62 μM for CEF and EGCG, respectively (Table 1) with steep slopes observed for both compounds (Hill slope > 1). In comparison, raltegravir, the first-in-class HIV-1 integrase drug marketed as Isentress®, inhibited recombinant IN_{WT} activity by 92±5% at a single-dose concentration of 10 μM and produced an IC₅₀ of 9.98±0.83 nM (Table 1) with a Hill slope of 1. Thereafter, the compounds were tested for inhibitory activity of the raltegravir-resistant IN_{Q148H/G140S} double-mutant.¹⁸ Replication capacity of this double mutant was reduced to 59% of IN_{WT} and, as anticipated,¹⁹ raltegravir was significantly less effective against this mutant (p<0.01) as reflected by a fold change in IC₅₀ (FCIC₅₀) value of 214 (Table 1). On the contrary, the two identified compounds retained their micromolar inhibitory activity against the raltegravir-resistant double mutant with FCIC₅₀ values of 0.83 and 0.06 calculated for CEF and EGCG, respectively (Table 1). As a further direct evaluation of activity, the compounds were tested for ST inhibition within an HIV-1 IN ST assay based on an ELISA platform. As a true, indisputable IN ST inhibitor, raltegravir efficiently inhibited IN_{WT} in this orthogonal assay to the same degree as that observed within the SPA-based assay (IC₅₀ = 10.25±0.75 nM) while dose-response evaluations were not warranted for CEF and EGCG as both proved ineffective inhibitors at a high single-dose concentration (<30% inhibition at 100 μM; Table 1). Up to this point, the behaviour of CEF and EGCG strongly supported a non-specific role for both molecules through a related mechanism of action that disrupted the SPA-based platform. This observation subsequently led us to the work of another group that speculated a non-specific mechanism for CEF within an SPA – in this case as an inhibitor of both NADH dehydrogenase (at an IC₅₀ ~ 8 μM) as well as MurG (at an IC₅₀ ~ 16 μM).²⁰

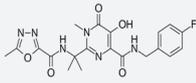
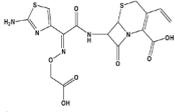
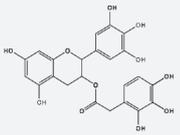
In-vitro evaluation

EGCG and CEF were both evaluated for toxicity within the MT-4 mammalian cell line. While EGCG yielded a CC₅₀ of 23 μM, CEF was not found to be toxic within the limits of the assay (≥200 μM). Thereafter, inhibition of HIV-1 replication by CEF and EGCG was evaluated in vitro in the MT-4 cell line following infection by HIV-1_{NL4-3}. For EGCG, an EC₅₀ of 24 μM was determined through dose-response studies (Table 1). The observed viral inhibition by EGCG closely correlated the toxicity profile of the compound in the same cell line and led to a negligible SI value (~1; Table 1). An SI value for CEF could not be determined as no observable viral inhibition was found in the cell-based HIV-1 inhibition assay up to the maximum tested compound concentration of 200 μM (Table 1). As a control, raltegravir was found to inhibit HIV-1 replication within this assay with an EC₅₀ of 16.82 nM with no observable toxicity up to the limit of the assay (CC₅₀ ≥ 200 μM; SI value ≥ 11 890.61).

Conclusion

Drug repurposing has proven successful in the past and offers a viable 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 unsusceptible 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 inhibition. 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.

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

Compound	HIV-1 integrase strand-transfer inhibition					MT-4 cell based assays		
	Scintillation proximity assay (SPA)			ELISA		CC ₅₀ ± SE (μM)	EC ₅₀ ± SE (μM)	SI
	IN _{WT} IC ₅₀ ± SE (μM)	IN _{G148H/G140S} IC ₅₀ ± SE (μM)	FCIC ₅₀	% Inhibition	IC ₅₀ ± SE (nM)			
Raltegravir 	9.98 ± 0.83 (x 10 ⁻³)	1.50 ± 0.50	214	96% (at 10 μM)	10.25 ± 0.75	>200	16.82 (x 10 ⁻³)	≥ 11 890.61
Cefixime trihydrate 	6.03 ± 1.29	5.01 ± 1.29	0.83	21% (at 100 μM)	ND	>200	>200	–
Epigallocatechin gallate 	9.57 ± 1.62	0.62 ± 1.50	0.06	29% (at 100 μM)	ND	23 ± 1	24 ± 3	~ 1

IN_{WT}, wild-type HIV-1 integrase

IC₅₀, 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

FCIC₅₀, fold-change in IC₅₀; calculated as the ratio of IN_{G148H/G140S}/IN_{WT}

CC₅₀, 50% cytotoxic concentration; calculated as the concentration of compound required to reduce cell viability by 50%

EC₅₀, 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 CC₅₀/EC₅₀

ND, not done

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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.

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