Published in final edited form as:

Antiviral Res. 2019 July ; 167: 110–116. doi:10.1016/j.antiviral.2019.04.010.

Structural and Pharmacological Evaluation of a Novel Non-nucleoside Reverse Transcriptase Inhibitor as a Promising Long Acting Nanoformulation for Treating HIV

Shalley N. Kudalkar^{a,b,1}, Irfan Ullah^{c,1}, Nicole Bertoletti^{a,b}, Hanna K. Mandl^d, José A. Cisneros^e, Jagadish Beloor^c, Albert H. Chan^{a,b}, Elias Quijano^d, W. Mark Saltzman^d, William L. Jorgensen^e, Priti Kumar^c, and Karen S. Anderson^{a,b,2}

^aDepartment of Pharmacology, Yale University School of Medicine, New Haven, CT 06520-8066

^bDepartment of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06520-8066

^cDepartment of Internal Medicine, Section of Infectious Diseases, Yale University School of Medicine, New Haven, CT 06520

^dDepartment of Biomedical Engineering, Yale University, New Haven, CT 06511

^eDepartment of Chemistry, Yale University, New Haven, CT 06520-8107

Abstract

Combination antiretroviral therapy (cART) has been proven effective in inhibiting human immunodeficiency virus type 1 (HIV-1) infection and has significantly improved the health outcomes in acquired immune deficiency syndrome (AIDS) patients. The therapeutic benefits of cART have been challenged because of the toxicity and emergence of drug-resistant HIV-1 strains along with lifelong patient compliance resulting in non-adherence. These issues also hinder the clinical benefits of non-nucleoside reverse transcriptase inhibitors (NNRTIs), which are one of the vital components of cART for the treatment of HIV-1 infection. In this study, using a computational and structural based drug design approach, we have discovered an effective HIV-1 NNRTI, compound I (Cmpd I) that is very potent in biochemical assays and which targets key residues in the allosteric binding pocket of wild-type (WT)-RT as revealed by structural studies. Furthermore, Cmpd I exhibited very potent antiviral activity in HIV-1 infected T cells, lacked cytotoxicity (therapeutic index >100,000), and no significant off-target effects were noted in pharmacological assays. To address the issue of non-adherence, we developed a long-acting nanoformulation of Cmpd I (Cmpd I-NP) using poly(lactide-coglycolide) (PLGA) particles. The

²Corresponding author: Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520-8066 karen.anderson@yale.edu (Karen S. Anderson).

¹S.N.K. and I.U. contributed equally to this work.

Author contributions: K.S.A., W.L.J., W.M.S., P.K., and S.N.K. designed research; S.N.K., I.U., N.B., J.B., H.K.M., E.Q., K.A.S., and J.A.C. performed research; S.N.K., N.B., H.K.M., and J.A.C. analyzed data; and S.N.K., N.B., H.K.M., K.S.A., W.L.J., W.M.S., and P.K. wrote the paper.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

pharmacokinetic studies of free and nanoformulated Cmpd I were carried out in BALB/c mice. Intraperitoneal administration of Cmpd I and Cmpd I-NP in BALB/c mice revealed prolonged serum residence time of 48 h and 30 days, respectively. The observed serum concentrations of Cmpd I in both cases were sufficient to provide >97% inhibition in HIV-1 infected T-cells. The significant antiviral activity along with favorable pharmacological and pharmacokinetic profile of Cmpd I, provide compelling and critical support for its further development as an anti-HIV therapeutic agent.

Graphical Abstract

Keywords

HIV-1; Non-nucleoside reverse transcriptase inhibitor; Long-acting nanoformulation; Pharmacokinetic studies; Mice Model; X-ray Crystallography

Introduction

Worldwide, antiretroviral therapy has markedly improved the morbidity and mortality associated with human immunodeficiency virus (HIV)-1 infection. Patients receiving combination antiretroviral therapy (cART) show significant reduction in hospitalizations and improved survival rates. One of the vital components of cART for the treatment of HIV-1 infection is non-nucleoside reverse transcriptase inhibitors (NNRTIs), which disrupt the normal functions of HIV-1 reverse transcriptase (RT) via binding to NNRTI binding pocket (NNIBP) close to the polymerase active site (Kohlstaedt, et al., 1992). In 1996, US Food and Drug Administration approved Nevirapine (NVP) as the first NNRTI for the treatment of HIV-1 infection, followed by delavirdine (1997), efavirenz (EFV) (1998), etravirine (ETV) (2008), and rilpivirine (RPV) (2011).

Although these NNRTIs are key components of effective cART regimens, their effectiveness is often outweighed by their poor pharmacokinetic properties, dose-limiting toxicities, and requirement of chronic treatment (Delaugerre, et al., 2001, Huang, et al., 2015, Rhee, et al., 2015). These properties increase the chances of developing drug resistant mutations and non-adherence in patients receiving cART. Additionally, other factors like cost of medication, lack of social support, high dosing, and wide fluctuations between peak and trough levels between dosing interval reduces adherence. For instance, use of RPV is restricted in patients with viral loads of <100,000 copies due to the dose-limiting cardiotoxicity caused by inhibition of hERG- ion channel (Bowes, et al., 2012). Additionally, EFV's low genetic barrier to resistance combined with various side effects in

patients makes it very hard to achieve required level of adherence (de Waal, et al., 2013, Riddler, et al., 2008).

In continuous efforts to develop next-generation NNRTIs with improved antidrug resistance, pharmacokinetics and toxicity profiles we have used computational and structure-based drug design to discover a class of inhibitors known as catechol diethers (Lee, et al., 2016, Lee, et al., 2014, Lee, et al., 2015, Lee, et al., 2013). One of the most potent inhibitors from this series is Cmpd I (Fig. 1). Cmpd I showed significant potency along with remarkable efficacy on clinically relevant drug resistant strains of HIV-1, and lack of cytotoxicity as described previously (Lee, et al., 2016, Lee, et al., 2014, Lee, et al., 2015, Lee, et al., 2013). In the current study, we further explored Cmpd I as a potential anti-HIV drug candidate by dissecting the molecular mechanism through biochemical and structural studies as well as investigating Cmpd I's in vitro and in vivo pharmacological profiles.

To reduce dosing frequency and to improve the quality of life of HIV/AIDS patients by tackling non-adherence, long-acting parenteral therapeutics containing cART are being developed and tested for their efficacy in animal models (Gao Y, 2018, Mandal S, 2018). Hence, Cmpd I was further developed into a long-acting nanoformulation (Cmpd I-NP) using a biodegradable polymer, poly(lactide-coglycolide) (PLGA), that has been by the FDA for drug delivery and other applications. The pharmacokinetic profiles of free Cmpd I and Cmpd I-NP were then investigated in BALB/c mice.

2. Materials and Methods

2.1. Chemicals and Synthesis of Compound I

Poly(D,L lactic-co-glycolic acid), 50:50 with inherent viscosity 0.55–0.75 dl/g, was purchased from DURECT Corporation (Birmingham, AL). Cmpd I was synthesized as detailed previously (Lee, et al., 2014).

2.2. Animals

Six- to eight-week-old female BALB/c mice from Jackson laboratories were used. All mice were housed in cages containing water, food and bedding. All procedures were approved by Yale University Animal Care and Use Committee.

2.3. Inhibition Assay with Pico Green and Solubility Measurements for Cmpd I

Inhibition assays were carried out with the PicoGreen Enzcheck Reverse Transcriptase Assay Kit (Life Technologies) as described previously (Gray, et al., 2015). The compound solubility was measured using a shake-flask procedure as described previously (Bollini, et al., 2013, Lee, et al., 2013, Lee, et al., 2013)

2.4. Measurement of the Inhibitory Activity of Cmpd I-NP in Infected TZM-bl Cells

The in vitro anti- HIV-1 activity from serum samples collected at various time intervals after intraperitoneal injection of free and nanoformulated Cmpd I was determined using a luciferase reporter gene assay as described previously (Kudalkar, et al., 2017).

2.4. In vitro Pharmacological Profiling

In vitro pharmacological profiling of Cmpd I and efavirenz was carried out by Eurofins Groups (Cerep and Eurofins Panlabs) in a panel of enzyme and radio-ligand assays with 34 targets as described previously (Kudalkar, et al., 2017).

2.5. Expression, purification and crystallization of the recombinant RT52A protein

Recombinant RT52A enzyme was expressed and purified as described previously (Das, et al., 2008, Frey, et al., 2012). Crystals of RT52A in complex with Cmpd I were prepared using similar methods as already described (Frey, et al., 2012, Kudalkar, et al., 2017)

2.6. Data collection, processing and structure determination and refinement

Crystals of sufficient quality were collected at APS on beam line 24-ID-E through NE-CAT. The data collection of protein-Cmpd I complex (PDB ID code: 6OE3) was achieved at a wavelength of 0.98Å and a temperature of 100 K on a silicon Pilatus HF-4M pixel detector. Data sets for the best diffracting crystals were indexed, processed and scaled with XDS (Kabsch, 2010). Phases were solved by molecular replacement with the program PHASER MR(McCoy, et al., 2007) from the CCP4 suite (Collaborative Computational Project, 1994) using the structure 4H4M as a search model. A subset corresponding to 5% of all reflections were omitted during refinement and used for the calculation of R_{free} . Model building was performed in Coot (Emsley and Cowtan, 2004, Emsley, et al., 2010) and refinement using PHENIX refine version 1.14-3260 (Adams, et al., 2010). Cmpd I SMILE codes were created with Molinspiration v2018.10, (<http://www.Molinspiration.com>) built with the Grade Web Server (Smart, 2011) and Cmpd I restraints were generated with eLBOW(Moriarty, et al., 2009). Cartesian simulated annealing, applying default parameters, was used as a first refinement step. Subsequently, refinement of XYZ coordinates, occupancies and individual B-factors were alternated with structural adaption until the model was readily built and gave the best possible explanation of the electron density, acceptable R-factors, geometry statistics, and Ramachandran statistics were achieved. Crystallography programs were compiled by SBGrid (Morin, et al., 2013).

2.7. Fabrication of Cmpd I loaded nanoparticles (Cmpd I-NPs)

PLGA nanoparticles were formulated by a single-emulsion solvent evaporation technique (Kudalkar, et al., 2018, Le Thi Mai, et al., 2009). For the 10 wt % Cmpd I-loaded formulation, 5.0 mg of compound I was dissolved in 0.250 ml of dimethylformamide, then added to 50 mg of 30-55 kDa PLGA in 1 ml methylene chloride to further co-dissolve overnight (for a concentration of 50 mg/ml).

To create a single-emulsion, the dissolved polymer solution was added drop wise to 1.25% polyvinyl alcohol under vortex followed by three sonications (10 seconds each) using a probe sonicator (38% amplitude). The final mixture was added to 25 ml of 0.3% polyvinyl alcohol, and stirred at room temperature for 3 h to evaporate the methylene chloride and DMF. The nanoparticles were then collected and transferred to a VWR 20K Ultra High Performance centrifugal filter unit and washed three times at 16,000 g at 4°C for 15 min with water. After the final wash, nanoparticles were resuspended in trehalose as a cryoprotectant at 100% (wt/wt) before lyophilization.

2.8. Characterization of Cmpd I loaded nanoparticles (Cmpd I-NPs)

NP size, polydispersity index (PDI) and zeta (ζ) potential were measured as described previously (Kudalkar, et al., 2018). Reverse-phase high-pressure liquid chromatography (HPLC) was used to measure drug loading (DL) as detailed previously (Kudalkar, et al., 2018), which is defined as the measured mass of Cmpd I per mass of PLGA-NP, and encapsulation efficiency (EE), which is defined as the ratio of the compounds loaded to the total drugs used for fabricating the nanoparticles (equations 1 and 2)

$$\text{Drug loading (DL) (\%)} = \frac{\text{mass of compound I in nanoparticles}}{\text{total mass of nanoparticles}} * 100$$

$$\text{Encapsulation efficiency (EE) (\%)} = \frac{\text{mass of drug in nanoparticles}}{\text{mass of polymer used in formulation}} * 100$$

2.9. Pharmacokinetic Studies in BALB/c mice

To determine dose-dependent serum drug concentrations, the in vivo pharmacokinetics of free Cmpd I and Cmpd I-NP were investigated on BALB/c mice obtained from Jackson Laboratory. BALB/c mice were randomly divided into two groups containing 3 mice each. One group of mice was injected intraperitoneally (i.p.) with a single 100 mg/kg dose of free Cmpd I suspended in sterile saline solution containing 10 % Tween 80. The second group was injected intraperitoneally with single 125 mg/kg Cmpd I-NP dose suspended in sterile saline solution. The blood samples were collected at predetermined time points from the ocular venous plexus by retro-orbital venipuncture and used for subsequent analysis as detailed previously.

3.0 Pharmacokinetic Analyses

The data were plotted as concentration as a function of time using PRISM 7.0 (GraphPad Software Inc, LaJolla, CA). The predicted area under the curve (AUC) of the concentration of drug in blood plasma against time ($\text{AUC}_{\text{predicted}}$) was calculated based on the linear trapezoid method (Bourget and Delouis, 1993). The maximum concentration (C_{max}) of a drug observed in serum after its administration and the maximum time (T_{max}) taken to reach C_{max} were also calculated from the concentration-time curve. The total body clearance (CL) value was obtained by the following equation (Ratain and Plunkett, 2003):

$$\text{CL} = \text{Dose} / \text{AUC}_{0 - \text{last}} \quad (1)$$

3. Results and Discussion

The molecular mechanism of inhibition of HIV-1 RT Cmpd I was investigated by the biochemical and structural studies. As illustrated in Table 1, very potent inhibition of RT ($\text{IC}_{50} = 4.8 \pm 1.1$ nM) was observed using a picogreen assay. The biochemical data were further supported by crystallographic studies via co-crystallization of Cmpd I with the RT protein. The crystal structure (PDB ID code 6OE3) had a resolution of 2.90 Å and exhibited a monoclinic symmetry in space group $C2$ with one heterodimer in the asymmetric unit. Table 2 shows the data collection, processing and refinement statistics of RT:Cmpd I

structure. The overall structure was similar to one of the previously described naphthyl catechol phenyl ether inhibitors (Kudalkar, et al., 2017). The Cmpd I was deeply buried in the open NNRTI binding pocket (NNBP) and made an extensive contacts with the residues in the non-nucleoside binding pocket (NNBP) (Fig.2A). The naphthyl moiety of Cmpd I was located in the hydrophobic region of NNBP and was stabilized by several van der Waals interactions with P95, L100, V108, Y188, W229, F227 and L234. Additionally, the naphthyl ring formed a π - π stacking interaction with Y188 and W229 residues. The nitrile group of the naphthyl ring resides in the tunnel region close to the polymerase active site. The central catechol ring is stabilized via π - π interaction with Y181. The uracil moiety is anchored in the groove region of the NNBP via π - π interaction with Y318 and its 2-carbonyl group is in H-bond distance with the amino group of the side chain of K102 (3.5 Å) and with the amide of the backbone of K103 and P236 (3.3 Å and 3.4 Å Fig. 2B).

The cellular anti-HIV activity of Cmpd I against WT RT is shown in Table 1 and is compared to FDA approved NNRTIs; rilpivirine and efavirenz (Fig. 1). Cmpd I showed single digit nanomolar EC₅₀ (1.1 nM) against WT RT in the cell based assays suggesting high potency (Lee, et al., 2014). The solubility of Cmpd I was 9.1 µg/ml, which was >100-fold greater than for rilpivirine and within 8-fold the value determined for efavirenz. The CLogP value of 3.59 of Cmpd I was in the normal range of 0-5 for oral drugs (Jorgensen, 2009) while for efavirenz, it was 4.6 and for rilpivirine it was above 5 (Bollini, et al., 2013, Jorgensen, 2009, Jorgensen and Duffy, 2002, Lee, et al., 2014, Lee, et al., 2013). The cytotoxic concentration (CC₅₀) for Cmpd I was >100 µM providing a therapeutic index of >100,000 whereas efavirenz and rilpivirine were cytotoxic to cells at a lower concentration range of 8-15 µM. The potent intrinsic inhibitory activity, better solubility profile, and low ClogP values of Cmpd I compared to rilpivirine (Janssen, et al., 2005, Sun, et al., 2012) and efavirenz (Frey, et al., 2014, Lee, et al., 2013) suggests that Cmpd I is a good drug candidate for further preclinical evaluation.

Additionally, Cmpd I was characterized along with efavirenz utilizing in vitro pharmacological profiling assays in order to identify any off-target effects. A panel of 34 targets including various ion channels, receptors, hormones and enzymes were used. The significant response from each assay is plotted as a heat map (Fig. 3). No adverse response was observed for Cmpd I to any of the targets tested except for an isoform cytochrome P450 2C19(CYP2C19) where a little over 50 % response was seen. This CYP2C subfamily is responsible for metabolizing ~20% of marketed drugs(Goldstein, 2001). Notably, Cmpd I did not hit any major targets like the hERG channel, blockade of which is responsible for major cardiac arrhythmias (Bowes, et al., 2012). Additionally, no adverse reaction was seen against CYP3A4, the major cytochrome P450 enzyme responsible for the metabolism of the majority of currently approved drugs (Nazir, et al., 2016, Wang, et al., 2016). In contrast, inhibition of binding of ligands to DHP and Na channels and 5HT2B receptors were observed with efavirenz suggesting that these might contribute to some of the side effects seen with efavirenz treatment.

The excellent in vitro pharmacological qualities of Cmpd I led us to develop a nanoformulation of Cmpd I (Cmpd I-NP) and explore the in vivo pharmacokinetics of both free and nanoformulated Cmpd I in BALB/c mice. Cmpd I-loaded PLGA based

nanoparticles (Cmpd I-NP) were made using emulsion-solvent evaporation technique as described in methods (Le Thi Mai, et al., 2009). Characterization of Cmpd I showed that it was encapsulated in PLGA with an average diameter of 320 ± 38 nm, polydispersity index (PDI) of 0.15 ± 0.1 , and an average negative zeta potential of -24 ± 0.9 mV. Representative scanning electron microscope (SEM) images illustrated that Cmpd I-NP exhibited spherical shapes (Fig. 4) and the size estimates from SEM were consistent with the size of Cmpd I-NP reported by DLS. The diameter of the Cmpd I-NP were in the range that has been previously reported to avoid clogging of the lymphatic ducts and fine capillaries (Bose T, 2014). Additionally, the slightly negative surface charge suggests that the nanoparticles will be less cytotoxic and will have prolonged circulation time in vivo by escaping uptake in cells and tissues (Jiang, et al., 2015, Zhang, et al., 2008). A theoretical drug loading of 10 wt% was input into the nanoparticle synthesis; the drug loading (DL%) in the formulated mixture was of 10 ± 0.7 wt% resulting in an encapsulation efficiency (EE) of $100 \pm 7\%$.

To determine the delivery and sustainability in vivo, free Cmpd I and Cmpd I-NP were carried injected into BALB/c mice, and the persistence of Cmpd I in the circulation was measured. The mice were divided into two groups of three mice each where one group received a single dose of Cmpd I (100 mg/kg) and the second group received 125 mg/kg dose of Cmpd I-NP, intraperitoneally. The blood samples were collected over a period of time until Cmpd I reached undetectable levels in both groups.

The serum concentration as a function of time after a single i.p. dose of Cmpd I showed that Cmpd I was rapidly absorbed, achieving a maximum concentration (C_{max}) of 7.8 ± 0.9 $\mu\text{g/ml}$ at 8 h; this concentration is 10,000-fold higher, than the therapeutic/effective concentrations (EC_{50}) (Fig.5A). The plasma concentration of Cmpd I remained near this level for ~36 h than decreased to undetectable levels by 72 h. A slightly lower C_{max} of 5.2 ± 1.6 $\mu\text{g/ml}$ was observed at 48 h for Cmpd I-NP (Fig.5B). However, Cmpd I was detected in serum for 30 days after a single i.p. dose of the nanoformulation. Importantly, the observed serum concentration of 1.7 $\mu\text{g/ml}$ for Cmpd I-NP at day 30 was substantially greater (>3000-fold) than the EC_{50} for the wild-type HIV-1 in the MT-2 assay (Fig. 5C) (Lee, et al., 2013).

Table 3 shows the PK parameters of Cmpd I and Cmpd I-NP in BALB/c mice. The AUC_{0-last} ($1004 \pm 60.45 \mu\text{g/ml/h}$) for Cmpd I injected as the nanoformulation was 2.3 times higher and the clearance rate (CL 2.0 ml/min/kg) was ~2 times lower than free injected Cmpd I (AUC_{0-last} $431.9 \pm 27.3 \mu\text{g/ml/h}$ and CL 3.9 ml/min/kg). In Fig.5D the serum levels for first 72 h are superimposed to highlight the differences in distribution and clearance over time between free Cmpd I and Cmpd I-NP. The larger AUC_{0-last} values and lower CL value for Cmpd I-NP could suggest that a higher volume of distribution can be achieved by nanoformulation of Cmpd I and its subjected to slower metabolism to maintain long-lasting serum levels. The longer residence time of Cmpd I in the serum when administered as Cmpd I-NP compared to the free drug can be attributed to a number of factors including slow release of Cmpd I from the polymer nanoparticle directly into body fluids and/or the cellular uptake of the particles followed by the slow release of the Cmpd I from intracellular breakdown of the polymer.

The serum samples collected at specified time were further analyzed for antiviral potency in an assay using TZM-bl indicator cell line infected with HIV. This cell line expresses a luciferase reporter driven by the HIV long terminal repeats and thus provides a linear measure for HIV infection (Kudalkar, et al., 2017). The assay was carried out as described in the methods section and > 97% inhibition was observed in serum samples collected as early as 2 hours post-administration of Cmpd I and Cmpd I-NP (Fig. 6). This level of inhibition was maintained till day 2 for free compound I and day 30 for Cmpd I-NP suggesting that the levels of Cmpd I in both groups (free or nanoformulated) were higher than the effective therapeutic concentrations and will be sufficient to control the HIV-infection in our future efficacy studies.

In conclusion, we have discovered a new HIV-1 NNRTI that exhibits significant antiviral activity and has favorable in vitro pharmacological characteristics. The data presented in this study further showed that the effective therapeutic concentrations of Cmpd I can be achieved in mice administered with free or nanoformulated Cmpd I. Additionally, all the mice appeared healthy with no signs of morbidity or mortality throughout the study. Furthermore, the nanoformulation of Cmpd I provided a slow and sustained release of compound I in BALB/c mice and the levels of Cmpd I were maintained well above the required therapeutic levels for a month even after a single dose. Given the favorable in vitro and in vivo profile of Cmpd I the next logical step is to measure the therapeutic potential of free and nanoformulated Cmpd I in an animal model of HIV infection.

Acknowledgements

This work was supported by grants from the National Institutes of Health [Grants AI044616, GM049551, AI141172, AI12443, AI122384, and EB000487]. Crystals screening was conducted with support from the Yale Macromolecular X-ray Core Facility [1S10OD018007-01]. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. This work is based upon research conducted at the Northeastern Collaborative Access Team beamlines, which are funded by the National Institute of General Medical Sciences from the National Institutes of Health (P30 GM124165). The Eiger 16M detector on 24-ID-E beam line is funded by a NIH-ORIP HEI grant (S10OD021527).

Abbreviations

HIV	Human immunodeficiency virus
HIV-RT	HIV reverse transcriptase
NNRTI	Non-nucleoside reverse transcriptase inhibitor
EC₅₀	Half maximal effective concentration of a compound
IC₅₀	Half maximal inhibitory concentration of a compound
HPLC	High performance liquid chromatography
PK	Pharmacokinetics
C_{max}	The maximum concentration of a drug observed in serum after its administration

T_{max}	The time taken to reach C _{max}
AUC	A plot of concentration of drug in blood plasma against time
CL	Clearance
NP	Nanoparticle
PKs	Pharmacokinetics

References

- Adams PD, et al., 2010 PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta crystallographica. Section D, Biological crystallography* 66, 213–221 10.1107/S0907444909052925. [PubMed: 20124702]
- Bollini M, et al., 2013 Optimization of diarylazines as anti-HIV agents with dramatically enhanced solubility. *Bioorganic & medicinal chemistry letters* 23, 5213–5216 10.1016/j.bmcl.2013.06.091. [PubMed: 23937980]
- Bose T, L. D, Mondal PP, Mandal S, 2014 Overview of nano-drugs characteristics for clinical application: the journey from the entry to the exit point. *J Nanoparticle Res* 16, 2527.
- Bourget P and Delouis JM, 1993 [Review of a technic for the estimation of area under the concentration curve in pharmacokinetic analysis]. *Therapie* 48, 1–5. [PubMed: 8356539]
- Bowes J, et al., 2012 Reducing safety-related drug attrition: the use of in vitro pharmacological profiling. *Nature reviews. Drug discovery* 11, 909–922 10.1038/nrd3845. [PubMed: 23197038]
- Collaborative Computational Project, N., 1994 The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr.* 1, 760–763
- Das K, et al., 2008 High-resolution structures of HIV-1 reverse transcriptase/TMC278 complexes: Strategic flexibility explains potency against resistance mutations. *Proc Natl Acad Sci U S A.*
- de Waal R, et al., 2013 Systematic review of antiretroviral-associated lipodystrophy: lipoatrophy, but not central fat gain, is an antiretroviral adverse drug reaction. *PloS one* 8, e63623 10.1371/journal.pone.0063623. [PubMed: 23723990]
- Delaugerre C, et al., 2001 Resistance profile and cross-resistance of HIV-1 among patients failing a non-nucleoside reverse transcriptase inhibitor-containing regimen. *Journal of medical virology* 65, 445–448. [PubMed: 11596076]
- Emsley P and Cowtan K, 2004 Coot: model-building tools for molecular graphics. *Acta crystallographica. Section D, Biological crystallography* 60, 2126–2132 10.1107/S0907444904019158. [PubMed: 15572765]
- Emsley P, et al., 2010 Features and development of Coot. *Acta crystallographica. Section D, Biological crystallography* 66, 486–501 10.1107/S0907444910007493. [PubMed: 20383002]
- Frey KM, et al., 2012 Crystal structures of HIV-1 reverse transcriptase with picomolar inhibitors reveal key interactions for drug design. *Journal of the American Chemical Society* 134, 19501–19503 10.1021/ja3092642. [PubMed: 23163887]
- Frey KM, et al., 2014 Structure-based evaluation of C5 derivatives in the catechol diether series targeting HIV-1 reverse transcriptase. *Chemical biology & drug design* 83, 541–549 10.1111/cbdd.12266. [PubMed: 24289305]
- Gao Y, K. J, Yu D, Ho RJY, 2018 Recent developments of nanotherapeutics for targeted and long-acting, combination HIV chemotherapy. *Eur J Pharm Biopharm.* 10.1016/j.ejpb.2018.04.014 10.1016/j.ejpb.2018.04.014.
- Goldstein JA, 2001 Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *British journal of clinical pharmacology* 52, 349–355. [PubMed: 11678778]
- Gray WT, et al., 2015 Potent Inhibitors Active against HIV Reverse Transcriptase with K101P, a Mutation Conferring Rilpivirine Resistance. *ACS Medicinal Chemistry Letters* 6, 1075–1079 10.1021/acsmedchemlett.5b00254. [PubMed: 26487915]
- <http://www.Molinspiration.com>.

- Huang B, et al., 2015 Fused heterocycles bearing bridgehead nitrogen as potent HIV-1 NNRTIs. Part 3: optimization of [1,2,4]triazolo[1,5-a]pyrimidine core via structure-based and physicochemical property-driven approaches. *European journal of medicinal chemistry* 92, 754–765 10.1016/j.ejmech.2015.01.042. [PubMed: 25626145]
- Janssen PA, et al., 2005 In search of a novel anti-HIV drug: multidisciplinary coordination in the discovery of 4-[[4-[[4-[(1E)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile (R278474, rilpivirine). *J Med Chem* 48, 1901–1909. [PubMed: 15771434]
- Jiang Y, et al., 2015 Nanoparticle-Based ARV Drug Combinations for Synergistic Inhibition of Cell-Free and Cell-Cell HIV Transmission. *Mol Pharm* 12, 4363–4374 10.1021/acs.molpharmaceut.5b00544. [PubMed: 26529558]
- Jorgensen WL, 2009 Efficient drug lead discovery and optimization. *Accounts of chemical research* 42, 724–733 10.1021/ar800236t. [PubMed: 19317443]
- Jorgensen WL and Duffy EM, 2002 Prediction of drug solubility from structure. *Advanced drug delivery reviews* 54, 355–366. [PubMed: 11922952]
- Kabsch W, 2010 Xds. *Acta crystallographica. Section D, Biological crystallography* 66, 125–132 10.1107/S0907444909047337. [PubMed: 20124692]
- Kleywegt GJZ, J. Y; Kjeldgaard M; Jones TA, 2001 *International Tables for Crystallography, Volume F. Crystallography of Biological Macromolecules.*
- Kohlstaedt LA, et al., 1992 Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 256, 1783–1790. [PubMed: 1377403]
- Kudalkar SN, et al., 2017 Structural and Preclinical Studies of Computationally Designed Non-Nucleoside Reverse Transcriptase Inhibitors for Treating HIV infection. *Mol Pharmacol* 91, 383–391 10.1124/mol.116.107755. [PubMed: 28167742]
- Kudalkar SN, et al., 2018 From in silico hit to long-acting late-stage preclinical candidate to combat HIV-1 infection. *Proc Natl Acad Sci U S A* 115, E802–E811 10.1073/pnas.1717932115. [PubMed: 29279368]
- Laskowski R. a. M., M. W; Moss DS; Thornton JM, 1993 PROCHECK: A Program to Check the Stereochemical Quality of Protein Structures. *J. Appl. Crystallogr.* 26 283–291.
- Le Thi Mai H, et al., 2009 Preparation of drug nanoparticles by emulsion evaporation method. *Journal of Physics: Conference Series* 187, 012047.
- Lee WG, et al., 2016 Design, Conformation, and Crystallography of 2-Naphthyl Phenyl Ethers as Potent Anti-HIV Agents. *ACS Med Chem Lett* 7, 1156–1160 10.1021/acsmedchemlett.6b00390. [PubMed: 27994756]
- Lee WG, et al., 2014 Picomolar Inhibitors of HIV-1 Reverse Transcriptase: Design and Crystallography of Naphthyl Phenyl Ethers. *ACS Med Chem Lett* 5, 1259–1262 10.1021/ml5003713. [PubMed: 25408842]
- Lee WG, et al., 2015 Discovery and crystallography of bicyclic arylaminoazines as potent inhibitors of HIV-1 reverse transcriptase. *Bioorganic & medicinal chemistry letters* 25, 4824–4827 10.1016/j.bmcl.2015.06.074. [PubMed: 26166629]
- Lee WG, et al., 2013 Picomolar Inhibitors of HIV Reverse Transcriptase Featuring Bicyclic Replacement of a Cyanovinylphenyl Group. *Journal of the American Chemical Society* 135, 16705–16713 10.1021/ja408917n. [PubMed: 24151856]
- Lee WG, et al., 2013 Picomolar inhibitors of HIV reverse transcriptase featuring bicyclic replacement of a cyanovinylphenyl group. *Journal of the American Chemical Society* 135, 16705–16713 10.1021/ja408917n. [PubMed: 24151856]
- Mandal S, K. G, Prathipati PK, Fan W, Li Q, Destache CJ, 2018 Long-acting parenteral combination antiretroviral loaded nano-drug delivery system to treat chronic HIV-1 infection: A humanized mouse model study. *Antiviral Res.* 156, 85–91 10.1016/j.antiviral.2018.06.005. [PubMed: 29885378]
- Matthews BW, 1968 Solvent content of protein crystals. *Journal of molecular biology* 33, 491–497. [PubMed: 5700707]
- McCoy AJ, et al., 2007 Phaser crystallographic software. *Journal of applied crystallography* 40, 658–674 10.1107/S0021889807021206. [PubMed: 19461840]

- Moriarty NW, et al., 2009 electronic Ligand Builder and Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta crystallographica. Section D, Biological crystallography* 65, 1074–1080 10.1107/S0907444909029436. [PubMed: 19770504]
- Morin A, et al., 2013 Collaboration gets the most out of software. *eLife* 2, e01456 10.7554/eLife.01456. [PubMed: 24040512]
- Nazir S, et al., 2016 Variation in pharmacokinetics of omeprazole and its metabolites by gender and CYP2C19 genotype in Pakistani male and female subjects. *Pakistan journal of pharmaceutical sciences* 29, 887–894. [PubMed: 27166533]
- Ratain MJ and Plunkett WK, 2003 Principles of Pharmacokinetics, in: Kufe DW PR, Weichselbaum RR, et al. (Ed.), *Holland-Frei Cancer Medicine*, 6th ed Hamilton (ON).
- Rhee S-Y, et al., 2015 Geographic and Temporal Trends in the Molecular Epidemiology and Genetic Mechanisms of Transmitted HIV-1 Drug Resistance: An Individual-Patient- and Sequence-Level Meta-Analysis. *PLOS Medicine* 12, e1001810 10.1371/journal.pmed.1001810. [PubMed: 25849352]
- Riddler SA, et al., 2008 Class-sparing regimens for initial treatment of HIV-1 infection. *The New England journal of medicine* 358, 2095–2106 10.1056/NEJMoa074609. [PubMed: 18480202]
- Smart OSW, T. O; Sharff A; Flensburg C; Keller P; Paciorek W; Vonnrhein C; Bricogne G , 2011 <http://www.globalphasing.com>
- Sun LQ, et al., 2012 Optimization of 2,4-diarylanilines as non-nucleoside HIV-1 reverse transcriptase inhibitors. *Bioorganic & medicinal chemistry letters* 22, 2376–2379 10.1016/j.bmcl.2012.02.055. [PubMed: 22406117]
- The PyMOL Molecular Graphics System, V. x. S, LLC.
- Wang Y, et al., 2016 Association Between CYP2C19 Loss-of-Function Allele Status and Efficacy of Clopidogrel for Risk Reduction Among Patients With Minor Stroke or Transient Ischemic Attack. *Jama* 316, 70–78 10.1001/jama.2016.8662. [PubMed: 27348249]
- Zhang Y, et al., 2008 Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells. *Biomedical microdevices* 10, 321–328 10.1007/s10544-007-9139-2. [PubMed: 18165903]

Highlights

- Biochemical and structural studies show a potent HIV-1 NNRTI (Compound I) bound in allosteric pocket.
- Pharmacological profiling of Compound I (Cmpd I) reveal no significant off-target effects.
- PKs show sustained levels of Cmpd I (free drug >48 h and long acting nanoparticle formulation, Cmpd I-NP, >30 d).
- Serum levels of Cmpd I inhibit HIV-1 infection in T cell-based assay with wide therapeutic index (>100,000).
- The favorable PKs of Cmpd I and Cmpd I-NP should be further explored in efficacy trials in animal models of HIV infection.

B.

C.

Figure 1:
Chemical structures of (A) efavirenz, (B) rilpivirine, and (C) Cmpd I

Figure 2. Close-up views of NNBP of the crystal structures of RT in complexes with NNRTIs: compound I (PDB code: 6OE3).

The protein is displayed in cyan. The carbon atoms of compound I are shown in magenta. Compound I is shown as stick model. (A) The protein is represented as ribbon model and the amino acids within a distance of 5 Å are shown as stick model. Fo-Fc polder electron density map is shown as gray mesh at a contour level of 4σ . (B) Close-up view on the binding mode of compound I. H-bond interactions are depicted as black dotted lines. Distances are given in Å. π - π stacking interactions are depicted as orange dotted line. All structural representations were prepared with PyMOL. (The PyMOL Molecular Graphics System)

Figure 3: In-vitro pharmacological profiling of efavirenz, and Cmpd I against targets for adverse drug reactions (ADRs).

Rows represent compounds tested and columns represent targets. Percentage inhibition at 10 μ M concentration of the compounds is color-coded. CYP, cytochrome P450, ADOR, Adenosine receptor, ADR, adrenergic receptor, DHP, dihydropyridine or calcium channel L-type, CNR1, cannabinoid receptor, DRD, Dopamine receptor, NMDAR, Glutamate receptor, HRH1, Histamine receptor, Imidazole I₂, Imidazole I₂ receptor, CHRM, Muscarinic, nAChR, nicotinic acetylcholine, OPRM, opiate μ , Phorbol ester receptor, KATP, potassium channel, hERG, the human Ether-à-go-go-Related Gene, EP4, prostanoid receptor, Rolipram, phosphodiesterase-4 inhibitor, 5HT2B, serotonin receptor 2B, Sigma σ 1 receptor, Na channel, sodium channel, NET, norepinephrine transporter

Figure 4: Scanning electron microscopy (SEM) of fabricated antiviral nanoparticles
The PLGA nanoparticles were loaded with Cmpd I as described in Methods.

Figure 5: Pharmacokinetics of Cmpd I and Cmpd I-NP in BALB/c mice.

The serum levels of Cmpd I after i.p. administration of 100 mg/kg of free Cmpd I (A) and 125 mg/kg of Cmpd I-NP (B). The blood samples were collected at different intervals and analyzed as described in Methods. Data points represent mean $\pm$ SD. (C) The log of serum concentrations for Cmpd I-NP were plotted against time and compared to EC₅₀ (★)- 1.1 nM. Note the >3 log difference between serum concentration and EC₅₀. (D) The serum levels of free Cmpd I and Cmpd I-NP are superimposed for the first 72h to show the difference in Cmpd I distribution.

Figure 6: HIV-1 inhibition by serum containing Cmpd I and Cmpd I-NP in TZM-bl cells
TZM-bl cells presented with serum containing Cmpd I or Cmpd I-NP collected at different intervals as described in Methods, were infected with HIV-1 (JRCSF strain). Virus infection was measured 2 days later by measuring luciferase activity in the cells. The percentage of the luciferase activity was normalized relative to the HIV-1 infected control TZM-bl cells that were not exposed to Cmpd I or Cmpd I-NP. The values are mean $\pm$ SD.

Table 1

HIV-RT inhibitory activity (IC₅₀ and EC₅₀ in nM), computed C log P, experimental aqueous solubility (in µg/ml) at pH 6.5, and cell cytotoxicity (in µM).

Data are the mean ± S.D. values from three different experiments involving triplicate measurements.

Compounds	IC ₅₀ WT (nM)	EC ₅₀ WT (nM)	ClogP	S (µg/ml)	CC ₅₀ (µM)
Cmpd I ^a	4.8 ± 1.1	1.1	3.59	9.1 ^a	>100
Rilpivirine	1.5 ± 0.4	0.67 ^b	5.75	0.02 ^c ; 0.24 ^d	8
Efavirenz	41 ± 2	2 ^e	4.67 ^b	68 ^b	15

^aLee et al. (2014).

^bKudalkar et al. (2017).

^cJanssen et al. (2005), pH 7.

^dSun et L. (2012), PH 7.4.

^eKing et al. (2002).

Table 2

Data collection and refinement statistics for RT (WT) in the complex with Cmpd I.

PDB ID code ^a	6OE3
(A) Data collection and processing	
Wavelength (Å)	0.9791
space group	C2
unit cell parameters a, b, c, (Å); α, β, γ, (°)	226.5, 69.9, 105.0; 90.0, 105.2, 90.0;
Matthews coefficient ^b (Å ³ /Da)	3.45
solvent content ^b (%)	64.4
(B) Diffraction data	
resolution range (Å)	50-2.90 (3.07-2.90)
unique reflections	34181 (5280)
R(I)sym (%)	9.0 (109.8)
Wilson B factor (Å ²)	18.2
completeness (%)	97.8 (94.7)
redundancy	3.1 (2.7)
<I/σ(I)>	11.5 (1.1)
CC(1/2)	99.6 (63.2)
(C) Refinement	
resolution range (Å)	43.17-2.90
reflections used in refinement (work/free)	32471/1709
final R value for all reflections (work/free) (%)	0.23/0.27
protein residues (chain A/B)	546/409
water molecules	8
RMSD from ideality: bond lengths (Å)	0.006
RMSD from ideality: bond angles (°)	0.821
Ramachandran plot ^c :	
residues in most favored regions (%)	92.8
residues in additionally allowed regions (%)	6.7
residues in generously allowed regions (%)	0.2
residues in disallowed regions (%)	0.2
Mean B factor protein (Å ²) ^c	91.5
Mean B factor ligand (Å ²) ^c	67.8
Mean B factor water molecules (Å ²) ^c	59.7

^aValues in parenthesis describe the highest resolution shell.^bCalculated with Matthews_coef program from CCP4 suite version 7.0.053.(Matthews, 1968)^cCalculated with PROCHECK. (Laskowski, 1993)

^d Mean B factors were calculated with MOLEMAN.(Kleywegt, 2001)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 3

Pharmacokinetic parameters of Cmpd I and Cmpd I-NP following i/p injection in BALB/c mice. Data are the mean $\pm$ S.D. values of triplicate measurement; n represents the number of mice

Pharmacokinetic Parameters	Cmpd I (n=3)	Cmpd I-NP (n=3)
Dose (mg/kg)	100	125
C _{max} (μ g/ ml)	7.8 $\pm$ 0.9	5.2 $\pm$ 1.6
T _{max} (h)	8	48
AUC _{0-last} (μ g h / ml)	431.9 $\pm$ 27.3	1004 $\pm$ 60.45
CL (ml/min/kg)	3.9	2.0