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Preclinical evaluation of a mercaptobenzamide and its prodrug for NCp7-targeted inhibition of human immunodeficiency virus

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ABSTRACT

Although the effective use of highly active antiretroviral therapy results in the suppression of virus production in infected individuals, it does not eliminate the infection and low level virus production in cells harboring virus in sanctuary sites. Thus, the continued search for new antiretroviral agents with unique and different mechanisms of HIV inhibition remains critical, and compounds that can reduce the level of virus production from cells already infected with HIV, as opposed to preventing *de novo* infection, would be of great benefit. A mercaptobenzamide (MDH-1-38) and its prodrug (NS1040) are being developed as potential therapeutic compounds targeting the zinc finger of HIV nucleocapsid. In the presence of esterase enzymes, NS1040 is designed to be converted to MDH-1-38 which has antiviral activity. While we presume that NS1040 is rapidly converted to MDH-1-38 in all experiments, the two compounds were tested side-by-side to determine whether the presence of a prodrug affects the antiviral activity or mechanism of action. The two compounds were evaluated against a panel of HIV-1 clinical isolates in human PBMCs and monocyte-macrophages and yielded EC_{50} values ranging from 0.7 to 13 μM with no toxicity up to 100 μM. MDH-1-38 and NS1040 remained equally active in human PBMCs in the presence of added serum proteins as well as against HIV-1 isolates resistant to reverse transcriptase, integrase or protease inhibitors. Cell-based and biochemical mechanism of antiviral action assays demonstrated MDH-1-38 and NS1040 were virucidal at concentrations of 15 and 50 μM, respectively. Cell to cell transmission of HIV in multiple passages was significantly reduced in CEM-SS and human PBMCs by reducing progeny virus infectivity at compound concentrations greater than 2 μM. The combination of either MDH-1-38 or NS1040 with other FDA-approved HIV drugs yielded additive to synergistic antiviral interactions with no evidence of antiviral antagonism or synergistic toxicity. Serial dose escalation was used in attempts to select for HIV strains resistant to MDH-1-38 and NS1040. Virus at several passages failed to replicate in cells treated at increased compound concentrations, which is consistent with the proposed mechanism of action of the virus inactivating compounds. Through 14 passages, resistance to the compounds has not been achieved. Most HIV inhibitors with mechanism of antiviral action targeting a viral protein would have selected for a drug resistant virus within 14 passages. These studies indicate that these NCp7-targeted compounds represent new potent anti-HIV drug candidates which could be effectively used in combination with all approved anti-HIV drugs.

1. Introduction

Fixed-dose combination antiretroviral therapy has reduced AIDS-associated morbidity and mortality in patients infected with HIV-1 due to excellent efficacy and tolerability. Despite the development of highly effective antiretroviral agents (ARVs), therapy

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does not eradicate HIV infections and life-long treatment is necessary (Berg, 1986). In the course of long-term treatment, emergence of drug-resistant HIV which reduces the effectiveness of antiretroviral therapy can threaten virologic suppression of HIV. Developing novel therapeutics with a high genetic barrier to HIV resistance and low toxicity thus remains a necessity. The development of a specific virucidal compound with the ability to interfere with multiple stages of HIV replication has the potential to add significantly to the ability to treat HIV infection.

The nucleocapsid protein 7 (NCp7) of HIV is an unexploited target to disrupt HIV with small molecules (Mori et al., 2015). Established HIV targets for therapeutic applications are enzymes (namely HIV protease, integrase, or reverse transcriptase) or viral entry/fusion inhibitors. HIV-1 NCp7 is a small, 55 amino acid protein that contains two highly conserved zinc fingers derived from a Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa4-Cys (CCHC) motif. The main role of NCp7 is to bind to viral RNA and protect it from degradation (Covey, 1986; Guo et al., 2002; Ramboarina et al., 1999; Tanchou et al., 1998). The alteration of any one of the zinc-chelating amino acids or surrounding amino acids results in the production of non-infectious virus. NCp7 functions at multiple stages of HIV replication, including: reverse transcription, integration, Tat-promoted transcription, auto-catalysis of Gag-Pol precursors to form functional protease, promotion of Gag assembly and interaction with viral genomic RNA, and protection of double-stranded HIV genomic RNA from degradation (Buckman et al., 2003; Guo et al., 2000; Hargittai et al., 2004; Turpin et al., 1996; Zybarth and Carter, 1995; Berkowitz et al., 1993; Dawson and Yu, 1998; Shubsda et al., 2002; Lapadat-Tapolsky et al., 1993). The mechanistic activities of NCp7 suggest a potentially high genetic barrier to the selection of resistance and suggest that any resistant strains that may emerge might suffer significant loss of fitness. *In vitro* evaluations have confirmed that removal of zinc from the zinc fingers results in a loss of viral infectivity, impaired reverse transcription, and defective Gag-precursor maturation.

Since the discovery of inhibitors which attack the zinc finger, a variety of classes of inhibitors have been described (Rice et al., 1993; Ryser et al., 1994; Turpin et al., 1999; Sancineto et al., 2015; Kim et al., 2015), many of which are described in a recent review (Mori et al., 2015). Some NCp7 inhibitors act by covalent modification of the target, although these molecules typically lead to undesirable toxicity. The recent report using diselenobisbenzamides to inhibit HIV replication via covalent modification of NCp7 is a promising new development in this area (Sancineto et al., 2015). Another strategy to inhibit NCp7 is to screen for non-covalent inhibitors. There is recent success in this area that has identified compounds based on a rhodanine core structure (Kim et al., 2015). The entire collection of NCp7 inhibitors, however, all have EC$_{50}$ values for antiviral activity against HIV in the low micromolar range. Compared to most drugs used to treat HIV infection, the antiviral activity of NCp7 inhibitors is significantly weaker. Therefore, alternative strategies to target NCp7 should be considered.

We have previously identified a specific class of benzamide thioesters (N-substituted S-acyl-2-mercaptobenzamides (SAMTs, such as 247 in Fig. 1) that selectively inactivate NCp7 and exhibit minimal *in vitro* cytotoxicity (Jenkins et al., 2005; Loo et al., 1996; Schito et al., 2003; Srivastava et al., 2004; Huang et al., 1998; Rice et al., 1995, 1996, 1997a, 1997b). SAMTs also inhibit HIV replication in both acutely and chronically HIV-infected cells. SAMTs specifically transfer an acetyl group to a cysteine of NCp7, which is then transferred intramolecularly to a neighboring lysine side chain. After NCp7 is acetylated by the SAMT, the protein loses zinc coordination, unfolds, and can no longer function to support HIV replication. We also discovered that after acetyl transfer to NCp7, the conjugate thiol of the SAMT can be reacetylated via a pathway involving acetyl CoA. This mechanism potentially allows SAMTs to inactivate multiple copies of NCp7 via a catalytic mechanism shown in Fig. 1.

However, the thioester bond in SAMTs is highly susceptible to hydrolysis, making them difficult to formulate as a therapeutic product. Additional studies on the mechanism of SAMTs determined that the acetyl group did not need to be part of the molecule at the time of delivery to an infected cell. Instead, treatment with the conjugate thiol of the SAMT (see conjugate thiol of 247, Fig. 1) was sufficient to achieve antiviral activity due to the intracellular activation of the conjugate thiol with acetyl CoA (Miller Jenkins et al., 2010). While the thiol is relatively stable in solid form, in solution it can slowly oxidize to form a disulfide. Therefore, we wanted to investigate whether a thiol and its corresponding prodrug (which would avoid oxidation in solution) may provide an easier drug formulation solution to designing SAMT-based therapy to treat HIV infection.

In this manuscript, we demonstrate that the S-acetyl portion of SAMT can be removed from formulation while still retaining all beneficial antiviral activity. In this regard, MDH-1-38 and its prodrug NS1040 were evaluated to investigate their potential as NCp7-targeted antiviral agents, including antiviral efficacy, cellular toxicity and mechanism of antiviral activity. In the presence of esterase enzymes, NS1040 is rapidly converted to MDH-1-38 (Fig. 2). Preclinical toxicity and pharmacokinetic properties for NS1040 were also evaluated in rats.

2. Materials and methods

2.1. Chemicals, synthesis, and characterization of molecules

Unless otherwise stated, all reactions were carried out under an atmosphere of nitrogen. Solvents were purified in a J. C. Meyer Glass Contour Solvent System. Reagents were purchased from Sigma Aldrich (St. Louis, MO) and used as received. β-alanine amide hydrochloride was purchased from Pharmaron (Beijing, China) and used as received. NMR spectra were obtained at room temperature on a Bruker Ultrashield 400 Plus. Signals are reported as δ ppm (multiplicity, number of protons); s = singlet, d = doublet, t = triplet, q = quartet, sext = sextet, m = multiplet, dd = doublet of doublets, td = triplet of doublets, b = broad. Mass spectra were recorded on a Waters LCT Premier and reported as $[M+Na]^+$.

2.1.1. 2,2'-disulfanediylbis(N-(3-amino-3-oxopropyl)benzamide) (1)

Thiosalicylic acid (1.0 g, 6.5 mmol, 1.0 eq) was combined with β-alanine amide hydrochloride (0.85 g, 6.8 mmol, 1.05 eq) and HCTU (2.8 g, 6.8 mmol, 1.05 eq) in 5 mL DMF at room temperature. The mixture was stirred for 5 min and then Hünig's base was added drop wise (4 mL). After stirring overnight, the majority of the solvent was removed under vacuum, and the resulting residue poured over ~50 mL of crushed ice/water. The mixture was left open to air and stirred vigorously overnight. The resulting precipitate was isolated by filtration. The sticky filter cake was washed repeatedly with 50 mL portions of water, isolated with a spatula and dried in vacuo to give 1.05 g (73% yield) of a white solid of sufficient purity to be used in the next step. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67–8.64 (t, 1H), 7.63–7.61 (m, 2H), 7.47–7.42 (td, 1H), 7.39 (bs, 1H), 7.30–7.26 (td, 1H), 6.86 (bs, 1H), 3.48–3.42 (q, 2H), 2.40–2.37 (t, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.5, 166.9, 136.7, 133.8, 131.1, 127.9, 125.9, 125.7, 36.0, 34.9; HRMS (ESI) m/z calcd. for $C_{20}H_{22}N_4O_4S_2$ $[M+Na]^+$: 469.0980, found 469.0972.

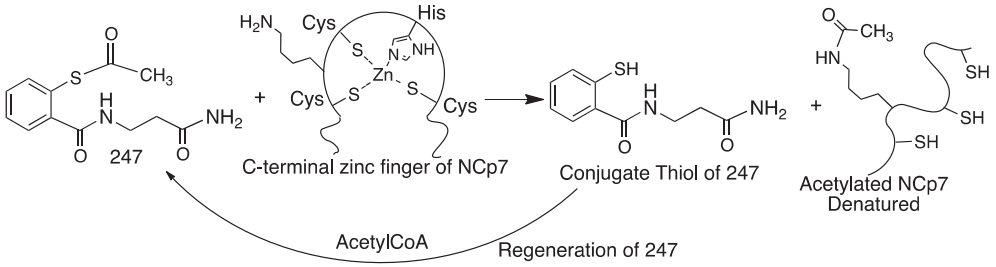


Fig. 1. SAMT inactivation of NCp7.

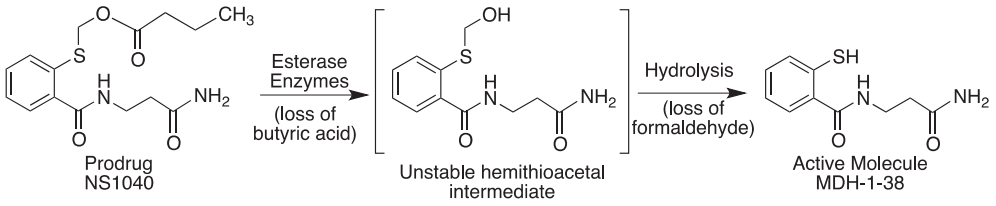


Fig. 2. Cleavage of NS1040 to MDH-1-38 by cellular esterases.

2.1.2. N-(3-amino-3-oxopropyl)-2-mercaptobenzamide (MDH-1-38)

A round bottom flask was charged with **1** (0.5 g, 1.12 mmol, 1.0 eq) and TCEP hydrochloride (0.481 g, 1.68 mmol, 1.5 eq). Then, 9:1 DMF/water was added (3 mL) followed by Hünig's base (0.39 mL, 2.24 mmol, 2.0 eq) and the mixture was stirred overnight at room temperature. Roughly half of the solvent was removed in vacuo, and the residue treated with 10 mL of water. The mixture was placed in refrigerator overnight to form a white precipitate. The resulting solids were isolated by filtration. The sticky filter cake was washed repeatedly with 25 mL portions of water, isolated with a spatula and vacuum dried to give 0.395 g of an off-white solid, 79% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45–8.42 (t, 1H), 7.48–7.46 (dd, 1H), 7.41–7.39 (dd, 1H), 7.36 (bs, 1H), 7.30–7.26 (td, 1H), 7.18–7.14 (td, 1H), 6.84 (bs, 1H), 5.36 (bs, 1H), 3.42–3.37 (m, 2H), 2.36–2.33 (t, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.4, 167.6, 133.4, 132.9, 130.3, 130.2, 128.3, 124.5, 35.9, 34.8; HRMS (ESI) m/z calcd. for $C_{10}H_{12}N_2O_2S$ $[M+Na]^+$: 247.0517, found 247.0520.

2.1.3. ((2-((3-amino-3-oxopropyl)carbamoyl)phenyl)thio)methyl butyrate (NS1040)

To a round bottom flask was added thiosalicylic acid (0.250 g, 1.62 mmol, 1.00 eq), HBTU (0.645 g, 1.70 mmol, 1.05 eq) and β-alanine amide hydrochloride (0.212 g, 1.70 mmol, 1.05 eq). DMF (4 mL) was added, followed by Hünig's base (0.988 mL, 5.67 mmol, 3.5 eq). The mixture was stirred at RT for 12 h. Then, chloromethyl butyrate (0.203 mL, 1.62 mmol, 1.0 eq) was injected, and the reaction stirred for 4 h at RT. The reaction was quenched with water (5 mL) and extracted 4× with 5 mL of CH_2Cl_2. The combined organics were washed with saturated $NaHCO_3$ and brine before being dried over Na_2SO_4. The organics were evaporated, and the residue was purified by flash chromatography over silica gel in MeOH/DCM to yield 0.270 g (52% yield) of a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56–7.53 (dd, 1H), 7.45–7.43 (dd, 1H), 7.38–7.34 (td, 1H), 7.27–7.23 (td, 1H), 7.02–6.99 (bt, 1H), 6.21, (bs, 1H), 5.78 (bs, 1H), 5.36 (s, 2H), 3.68–3.63 (m, 2H), 2.56–2.53 (m, 2H), 2.32–2.29 (t, 2H), 1.67–1.57 (sext, 2H), 0.93–0.89 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) HRMS (ESI) m/z calculated for $C_{15}H_{20}N_2O_4S$ $[M+Na]^+$: 347.1041, found 347.1042.

2.2. Compounds, cell lines and viruses

Mercaptobenzamide (MDH-1-38) and its prodrug NS1040 were provided by NIDDK/NIH. Control compounds were either purchased from Sigma Aldrich (St. Louis, MO) or obtained from NIAID AIDS Research and Reference Reagent Program (Germantown, MD). Established cell lines and HIV strains were obtained from NIAID AIDS Research and Reference Reagent Program (Rockville, MD).

2.3. PBMC and monocyte macrophage assays

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll hypaque gradient centrifugation from whole blood (Biological Specialty Corporation; Colmar, PA) and activated with PHA. Monocytes were further purified by adherence to plastic and washing to remove lymphocytes. Following a seven day acute infection with the clinical wild type and drug resistant HIV isolates in the presence of compound, supernatant reverse transcriptase (RT) activity or p24 expression by ELISA was measured to quantify virus replication (Buckheit et al., 2007, 2008). Cell viability was measured in parallel using XTT dye reduction.

2.4. Mechanism of anti-HIV-1 action assays

2.4.1. Attachment inhibition assay

Test compound was added to TZM-bl-FcRI cells that had been plated in a 96-well flat-bottomed plate 24 h prior to assay initiation (Buckheit et al., 2007, 2008). A pre-determined titer of virus was added 15 min following the cell pretreatment with test compound at 37°C/5% CO_2 and incubated for an additional 2 h. At the end of the incubation, residual virus and compound were removed by washing, fresh media was added and the culture was incubated for an additional 48 h β–galactosidase expression was determined by chemiluminescence for the efficacy plate (Tropix Gal-screen™, Bedford Mass.).

2.4.2. Fusion inhibition assay

Compound was incubated with HeLa-CD4-LTR-β-Gal cells in a 96-well flat-bottom plate for 1 h at 37°C/5% CO2 before being co-cultured with HL2/3 cells for 48 h (Buckheit et al., 2007, 2008). Following the incubation, the cells were lysed and evaluated for β

-galactosidase expression using Gal-Screen.

2.4.3. HIV-1 reverse transcription inhibition

The biochemical RT inhibition assay utilized recombinant, purified HIV-1 RT enzyme (Buckheit et al., 2007, 2008). RT enzyme was assayed in a 100 μL reaction mixture containing 25 mM Tris-HCl, pH 8.0, 75 mM KCl, 8 mM $MgCl_2$, 2 mM DTT, 10 μM dGTP, 0.01U rC:dG template primer (Amersham Pharmacia Biotech; Piscataway, NJ), 10 μCi [^{32}P]-α-dGTP (800 Ci/mmol), and the test compound. The enzyme reactions are allowed to proceed for 30 min at 37 °C before termination of the reaction by addition of 10% TCA and 100 μg of heat-denatured, sonicated salmon sperm DNA was added to aid DNA precipitation and recovery. TCA-precipitated DNA was harvested onto glass-fiber filters, washed twice with ice-cold 10% TCA and subjected to liquid scintillation counting. Incorporated radioactivity in the labeled DNA samples was quantified using a Wallac MicroBeta scintillation counter.

2.4.4. HIV-1 integrase inhibition

The HIV-1 Integrase Assay Kit (Bioproducts MD; Middletown, MD) was used to examine the integrase inhibition activity (Buckheit et al., 2007, 2008). The plate was coated with double-stranded HIV-1 LTR U5 donor substrate (DS) oligonucleotide containing an end-labeled biotin. Full-length recombinant HIV-1 integrase protein was added, followed by test articles, then a different double-stranded target substrate (TS) oligo containing 3'-end modifications. The products of the reaction were detected colorimetrically using an HRP-labeled antibody directed against the TS 3'-end modification.

2.4.5. HIV-1 protease inhibition

The SensoLyte 520 HIV-1 Protease Assay Kit (Anaspec Inc.; San Jose, CA) and recombinant HIV-1 protease were used to examine the protease inhibition activity (Buckheit et al., 2007, 2008). The cleavage of the p17/p24 junction by HIV-1 protease and HIV-1 protease activity in the presence of test compound was monitored in a fluorescent plate reader using excitation and emission wavelengths of 490nm/520 nm.

2.4.6. Virucidal assay

Compound was incubated with undiluted HIV_{IIIB} for 20 min at 37 °C (Buckheit et al., 2007, 2008). Following incubation, the tubes were centrifuged at 13,800 rpm at 4 °C for 90 min then washed five times with tissue culture medium. The virus pellet was resuspended in RPMI medium to a predetermined titer for the untreated virus and incubated with CEM-SS cells for 6 day at 37 °C/5%CO_2 to quantify the effect of compound treatment on virus infectivity. Cell viability was measured by XTT tetrazolium dye.

2.4.7. Cell-to-cell virus transmission assay

HIV-infected fresh human PBMCs were treated with six concentrations of test compound for three days. The cultures are then passaged in the presence of the fixed concentration of compound by replacing 80% of the culture volume with fresh compound-containing medium and uninfected cells every 3 days for three passages (Buckheit et al., 2007, 2008). Cell-free supernatant samples are collected on the day of sub-culturing to define the level of virus replication which is occurring in the presence of each fixed concentration of test compound by RT and extracellular p24 antigen measurement. $TCID_{50}$ values of each passage will be determined by endpoint dilution.

2.5. Combination drug interaction evaluation

The efficacy and toxicity of the compounds alone and in combination were analyzed in microtiter plate based PBMC assays as described above using an in-house software program, which calculates % CPE reduction or % reduction in virus replication, % cell viability, EC_{25}, EC_{50} and EC_{95}, TC_{25}, TC_{50} and TC_{95} and selectivity indices and displays the results graphically (Hartman et al., 2011). Combination antiviral effects (efficacy and toxicity) are analyzed statistically by the Prichard and Shipman MacSynergy II methodology. The MacSynery II analysis includes calculation of the dose response of the two compounds when used alone followed by calculation of the expected additive level of antiviral inhibition when the compounds are used together based on the individual dose response curves. The expected level of activity at each of the concentration data points was then compared to the experimentally determined antiviral activity in the assay. The expected activity was subtracted from the realized activity, yielding a negative, zero or positive value. These values were plotted, yielding a three-dimensional representation of the data. Zero values indicating that the expected and realized activity were the same yields a flat surface which is indicative of an additive interaction of the two compounds. Positive values indicate that the realized activity was greater than the expected activity and thus a potentially synergistic interaction of the compounds was observed in the assay. The accumulation of positive points on the synergy plot yields a three-dimensional surface projecting above the plane of additivity and the volume of the surface above the plane can be calculated and expressed (in units of $\mu M^2\%$ for MacSynergy II). In contrast, negative values indicate that the realized activity was less than the expected activity, yielding a surface projecting below the plane of additivity indicative of an antagonistic interaction of the compounds. Our extensive experience with the evaluation of combination assay data has resulted in the following interpretations of the synergy volumes derived from MacSynergy II data: >100 $\mu M^2\%$ is defined as a highly synergistic interaction; 51–99 $\mu M^2\%$ is defined as synergistic; 0–50 $\mu M^2\%$ and −50 to 0 $\mu M^2\%$ is defined as additive; -99 to −51 $\mu M^2\%$ is defined as antagonistic; and <-100 $\mu M^2\%$ is defined as highly antagonistic.

2.6. Drug-resistant virus selection

The serial dose escalation method for resistance selection was utilized in CEM-SS cells with the laboratory-derived $HIV\text{-}1_{IIIB}$ virus strain (Buckheit et al., 2013). This method was performed by culturing virus in the presence of suboptimal concentrations of MDH-1-38 and NS1040 with small increases in drug concentration made at each passage to gradually increase the selective pressure on the virus replicating in the cells. Virus production was quantified by RT assay to determine peak day of virus passage. The titer of virus harvested at the peak day of virus production and its sensitivity to the selecting and control compounds was defined at each passage.

2.7. In vivo maximum tolerated dose evaluation

The maximum tolerated dose (MTD) of NS1040 in Sprague-Dawley rats following a dose escalation design vial oral administration. Five groups of 8 animals (4 each female and male) were dosed once vial oral administration at a dose level of 750, 1000, 1500 or 2000 mg/kg/dose using 5% DMSO/70% PEG400 in water or at 2000 mg/kg/dose using 2% CMC-Na in water. Parameters evaluated include cage side and detailed clinical observations, body weight, and food consumption. The general procedures for animal care and housing were conducted in accordance with the National Research Council for the Care and Use of Laboratory Animals and the Animal Welfare Standards (Code of federal regulations, 2012). All procedures and protocols used in the described *in vivo* studies

were reviewed and approved by the Institutional Animal Care and Use Committee and were performed in an AAALAC accredited facility.

2.8. Repeat dose toxicity study in rats

A fourteen-day repeat dose toxicity evaluation of daily, oral NS1040 administration was performed in thirty-two Sprague-Dawley rats. The reversibility, progression and potential delayed effects of NS1040 were determined following a fourteen-day recovery period. Four groups of 8 animals (4 main study animals sacrificed on Day 15 and 4 recovery animals sacrificed on Day 29) were orally dosed on a daily basis with NS1040 at a dose level of 500, 1000 or 2000 mg/kg/dose in parallel with a control article. Blood samples for clinical pathology analyses were collected from the animals prior to terminal sacrifice. All main study animals were euthanized and necropsied on Day 15 and the recovery animals were euthanized and necropsied on Day 29. Parameters evaluated included cageside and detailed clinical observations, body weight, food consumption, clinical pathology (clinical chemistry, hematology, coagulation and urinalysis), absolute and relative organ weights, gross pathology and microscopic pathology.

3. Results

3.1. Synthesis and plasma stability

The syntheses of MDH-1-38 and NS1040 are shown in Scheme 1. Both molecules are made starting from thiosalicylic acid. For MDH-1-38, an amide coupling between the hydrochloride salt of β-alanine amide and thiosalicylic acid is facilitated using the reagent HCTU. Once this reaction is complete, a standard work up yields the disfulfide 1 in 73% yield. This disulfide is reduced using TCEP to yield MDH-1-38. The synthesis of the prodrug NS1040 is performed in two-steps, in a single flask, without the isolation of any intermediates. After performing the same amide bond reaction between thiosalicylic acid and β-alanine amide, chloromethyl butyrate is added. After work up of the reaction, NS1040 is isolated in 52% yield (Scheme 1). To ensure that NS1040 could be easily converted to MDH-1-38, routine metabolite identification studies were performed *in vitro* using human plasma (Supporting Information). After incubation for 1 and 48 h in human plasma, MDH1-38 was clearly identified along with a minor metabolite (M3). Additional evidence for conversion of NS1040 to MDH-1-38 was obtained by studying *in vivo* pharmacokinetics in rats (see section 3.10).

3.2. Range of anti-HIV activity in fresh human PBMC assays

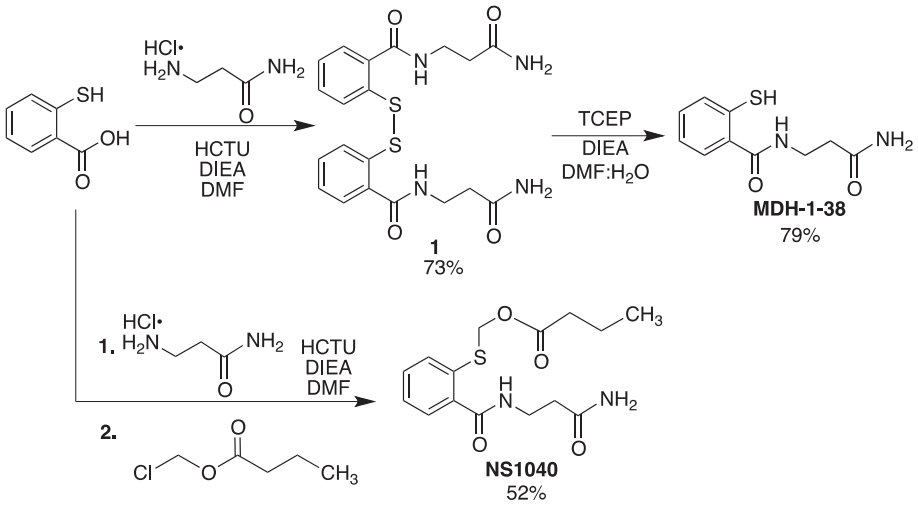
MDH-1-38 and its prodrug NS1040 were evaluated in parallel with AZT against a panel of low passage human clinical HIV-1 subtype viruses representative of group M subtypes A through G and group O in fresh human PBMCs to determine range of antiviral activity (Table 1). MDH-1-38 was active against all of the low passage clinical HIV-1 strains evaluated with EC_{50} values ranging from 1.44 ± 0.18 to 10.7 ± 2.38 μM. Similarly, NS1040 was active against the same low passage clinical HIV-1 strains with EC_{50} values ranging from 0.87 ± 0.88 to 12.3 ± 8.18 μM. Presumably NS1040 is rapidly converted to MDH-1-38 by esterases present in the fetal bovine serum in all assays. MDH-1-38 and NS1040 were cytotoxic in fresh human PBMCs at concentrations greater than 231 and 216 μM, respectively. Similarly, MDH-1-38 and its prodrug NS1040 were evaluated for co-receptor specificity against a panel of clinical HIV-1 subtype B viruses utilizing either CXCR4-only, CCR5-only or dual co-receptors (CXCR4/CCR5) in fresh human PBMCs. MDH-1-38 and NS1040 were similarly active against all of the clinical subtype B HIV-1 strains regardless of co-receptor usage.

3.3. Range of anti-HIV activity in fresh human monocyte-macrophage assays

MDH-1-38 and its prodrug NS1040 were evaluated in parallel with AZT against six CCR5 -tropic HIV strains in fresh human monocytes macrophages (Table 2). MDH-1-38 and NS1040 yielded EC_{50} values ranging from 2.5 to 19 μM and 0.6–20 μM, respectively, against the panel of CCR5-tropic HIV strains in fresh human monocytes macrophages. MDH-1-38 and NS1040 were cytotoxic in fresh human monocyte/macrophages at concentrations greater than 2000 and 1500 μM, respectively.

3.4. Serum protein binding

MDH-1-38 and its prodrug NS1040 were evaluated antiviral activity assays against HIV-1_{IIIB} in CEM-SS cells and against clinical HIV-1 subtype B virus 92US723 in fresh human PBMCs in the absence or presence of 50% human serum, 2.0 mg/mL alpha 1-acid



Scheme 1. Synthesis of MDH-1-38 and its prodrug NS1040.

Table 1
Antiviral activity against a range of geographically diverse HIV-1 subtypes in fresh human PBMCs.

HIV-1 subtype	Virus strain (coreceptor usage)	Mean EC_{50} ± SD				
		AZT (nM)	MDH-1-38 (μM)	TI	NS1040 (μM)	TI
A	92RW009 (CCR5)	4.03 ± 2.06	4.53 ± 0.52	51.0	2.87 ± 3.06	75.3
	92RW020 (CCR5)	1.43 ± 0.93	1.44 ± 0.18	161	0.87 ± 0.88	248
	92UG029 (CXCR4)	8.88 ± 0.81	6.82 ± 2.13	33.9	6.59 ± 8.50	32.8
B	92BR003 (CCR5)	6.89 ± 2.02	5.30 ± 2.67	43.6	3.67 ± 2.38	58.9
	92BR014 (CCR5/CXCR4)	2.49 ± 0.22	4.15 ± 0.54	55.7	3.41 ± 3.26	63.3
	93US073 (CCR5)	3.09 ± 1.28	3.45 ± 0.88	67.0	4.24 ± 1.08	50.9
C	92BR025 (CCR5)	5.16 ± 3.61	4.76 ± 1.15	48.5	2.73 ± 1.56	79.1
	93MW959 (CCR5)	4.36 ± 2.47	4.64 ± 0.24	49.8	3.08 ± 1.22	70.1
	98IN017 (CXCR4)	6.29 ± 0.11	4.42 ± 0.37	52.3	2.62 ± 1.12	82.4
D	92UG001 (CCR5/CXCR4)	6.51 ± 1.27	5.39 ± 0.74	42.9	3.14 ± 2.18	68.8
	92UG024 (CXCR4)	6.48 ± 6.39	2.13 ± 0.04	109	1.32 ± 0.83	164
	92UG035 (CCR5)	1.58 ± 0.90	3.93 ± 0.28	58.8	4.28 ± 1.03	50.5
E	93TH073 (CCR5/CXCR4)	10.2 ± 6.36	2.84 ± 2.50	81.3	1.85 ± 1.24	117
	CMU02 (CCR5)	4.80 ± 3.18	3.28 ± 1.47	70.4	2.30 ± 1.21	93.9
	93TH051 (CCR5)	7.09 ± 5.25	5.25 ± 2.78	44.0	5.91 ± 2.39	36.5
F	93BR019 (CXCR4)	5.80 ± 3.95	4.01 ± 1.11	57.6	3.52 ± 2.62	61.4
	93BR029 (CCR5)	7.07 ± 3.95	3.38 ± 0.85	68.3	3.45 ± 1.90	62.6
	93BR020 (CCR5/CXCR4)	7.64 ± 1.56	3.46 ± 1.85	66.8	2.75 ± 1.07	78.5
G	JV1083 (CCR5)	7.33 ± 1.10	4.63 ± 0.60	49.9	3.45 ± 1.90	62.6
	G3 (CCR5)	4.27 ± 5.00	3.94 ± 0.40	58.6	5.39 ± 4.09	40.1
	RU570 (CCR5)	26.9 ± 13.6	1.98 ± 0.10	117	3.13 ± 3.41	69.0
O	BCF01 (CCR5)	2.37 ± 0.08	3.24 ± 4.48	71.3	7.04 ± 1.74	30.7
	BCF02 (CCR5)	6.59 ± 1.26	10.7 ± 2.38	21.6	9.98 ± 8.37	21.6
	BCF03 (CCR5)	6.21 ± 0.74	9.63 +/−4.77)	24.0	12.3 ± 8.18	17.6

In each individual antiviral assay, efficacy and toxicity values are derived from a minimum of three replicate wells. Each antiviral assay was performed at least twice to determine mean EC_{50} values and standard deviation. Therapeutic index (TI) calculated from CC_{50} value of 231 μM for MDH-1-38 and 216 μM for NS1040 in fresh human PBMCs.

Table 2
Antiviral activity against CCR5-tropic HIV-1 in fresh human monocytes/macrophages.

HIV-1 subtype B virus isolate (CCR5)	Mean EC_{50}				
	AZT (nM)	MDH-1-38 (μM)	TI	NS1040 (μM)	TI
BaL	0.8	18.6	108	18.7	80.2
92BR017	0.7	1.04	1923	0.76	1974
92BR021	0.6	5.32	376	5.92	253
92TH026	0.6	11.0	182	0.66	2273
91US005	<0.3	2.50	800	0.60	2500
92US660	0.7	4.97	402	1.59	943
ADA	2.0	15.7	127	20.1	74.6

Therapeutic index (TI) calculated from CC_{50} value of 2000 μM for MDH-1-38 and 1500 μM for NS1040 in fresh human monocyte/macrophages.

glycoprotein (AAGP), or 10 mg/mL human serum albumin (HSA) to determine protein binding in cell culture (data not shown). MDH-1-38 and its prodrug NS1040 performed comparably in cell culture medium only and in the presence of AAGP with EC_{50} values ranging from 0.76 to 1.44 μM. Both compounds yielded an approximate 4.5-fold decrease in potency in the presence of 50% human AB serum. MDH-1-38 also yielded a 4.5-fold decrease in potency in the presence of HSA. NS1040 did not exhibit a significant decrease in potency with HSA. In PBMCs, MDH-1-38 and its prodrug NS1040 performed comparably in cell culture medium only and in the presence of the three serum protein additives with no significant change in antiviral activity.

3.5. Antiviral evaluation against drug-resistant HIV isolates

MDH-1-38 and its prodrug NS1040 were evaluated in parallel with a positive and negative control compound in antiviral activity assays against drug-resistant and wild type HIV-1 in MT-4 cells (Table 3). MDH-1-38 and its prodrug NS1040 performed comparably against the panel of drug-resistant HIV isolates with EC_{50} values ranging from 0.82 ± 0.37 μM to 10.3 ± 5.34 μM, similar to activity against wild type HIV. MDH-1-38 and its prodrug NS1040 were also evaluated against low passage human clinical HIV-1 viruses resistant to known antiretroviral agents in fresh human PBMCs (Table 4). The compounds performed comparably against the panel of drug resistant HIV-1 isolates with exhibited EC_{50} values ranging from 0.98 ± 0.64 μM to 10.0 ± 4.78 μM, similar to activity seen against wild type HIV isolates. Both compounds were found to be non-toxic to MT-4 cells in all assays up to a high test concentration of 100 μM.

3.6. Mechanism of anti-HIV activity evaluations (Table 5)

3.6.1. HIV entry and fusion inhibition

MDH-1-38 and prodrug NS1040 were evaluated for the ability to inhibit HIV attachment to HeLa-CD4-LTR-β-gal cells expressing cell surface CD4 and for inhibition of HL2/3 cells expressing HIV-1 gp120 and gp41 fusing with HeLa-CD4-LTR-β-gal cells. Chicago sky blue and enfuvirtide were evaluated as positive control compounds and yielded EC_{50} values of 0.64 ± 0.05 μg/mL and 0.08 ± 0.09 μM, respectively, in the attachment inhibition assay and in the fusion inhibition assy. MDH-1-38 yielded an EC_{50} value of 44.4 ± 7.21 μM in the attachment inhibition assay and 83.3 ± 26.4 μM in the fusion inhibition assay. NS1040 was inactive

Table 3
Antiviral activity against drug-resistant HIV-1 strains in MT-4.

Laboratory virus strain	Genotype	Mean EC_{50} (μM) ± SD		
		Positive control (nM)	MDH-1-38	NS1040
Enfuviritide-resistant	Env V38A/N126K	AZT 1.5 ± 0.5	7.09 ± 0.03	5.48 ± 0.01
Indinavir-resistant, Ritonavir-resistant	PR V82F/I84V	AZT 2.0 ± 0	4.10 ± 0.47	3.52 ± 1.70
Indinavir-resistant, Ritonavir-resistant	PR V82A	AZT 3.5 ± 0.5	10.3 ± 5.34	6.73 ± 1.06
Indinavir-resistant, Ritonavir-resistant	PR I84V	AZT 2.5 ± 1.5	2.54 ± 0.93	3.33 ± 0.44
Saquinavir-resistant	PR G48V/L90M	AZT 3.5 ± 2.5	1.0 ± 0.34	1.10 ± 0.32
Atazanavir-resistant	PR I50L	AZT 1.5 ± 0.5	0.82 ± 0.37	1.27 ± 0.19
NNRTI-resistant	RT K103N	AZT 0.9 ± 0.1	3.97 ± 1.31	5.02 ± 0.14
NNRTI-resistant	RT L100I	AZT 0.65 ± 0.5	1.22 ± 0.01	1.61 ± 0.41
NNRTI-resistant	RT Y181C	AZT 1.0 ± 0	3.99 ± 1.59	4.52 ± 1.07
NNRTI-resistant	RT V106A	AZT 2.0 ± 1.0	2.74 ± 2.17	4.07 ± 0.78
Lamivudine-resistant	RT M184V	AZT 0.55 ± 0.05	2.70 ± 1.52	3.50 ± 1.1
NNRTI-resistant	RT V108I	AZT 1.5 ± 0.5	3.84 ± 1.60	4.70 ± 0.17
AZT-resistant	RT 4xAZT (D67N/K70R/T215Y/K219Q)	NVP 35 ± 15	3.31 ± 2.20	4.24 ± 0.91
AZT- and NNRTI-resistant	RT 4xAZT/Y181C	AZT 7.0 ± 4.2	4.87 ± 0.22	5.24 ± 0.18
AZT- and NNRTI-resistant	RT 4xAZT/L100I	AZT 4.0 ± 0.71	3.59 ± 1.25	3.54 ± 2.02
Didanosine-resistant	RT L74V	AZT 2.0 ± 0	3.93 ± 2.33	4.59 ± 0.61
NNRTI-resistant	RT V179D	AZT 1.0 ± 0.3	3.57 ± 1.81	5.01 ± 0.31
NNRTI-resistant	RT Y188C	AZT 1.5 ± 0.5	3.37 ± 2.15	4.72 ± 0.55
Tenofovir-resistant	RT K65R	RTV 0.06 ± 0.02	4.29 ± 0.64	5.21 ± 0.08
NNRTI-resistant	RT K103N/Y181C	AZT 2.0 ± 0	8.03 ± 1.45	5.19 ± 0.06
Raltegravir-resistant	IN Q148H/G140S	AZT 2.0 ± 0	3.39 ± 2.15	4.16 ± 0.26

Table 4
Antiviral activity against drug-resistant HIV-1 in PBMCs.

Drug resistant clinical HIV isolate	PBMC/HIV-1 EC_{50} ± SD		
	Raltegravir (μM)	MDH-1-38 (μM)	NS1040 (μM)
Wild Type 92US727	10.5 ± 12.7	3.32 ± 1.95	5.58 ± 0.12
NNRTI-resistant N119	3.54 ± 0.89	2.58 ± 2.52	5.90 ± 1.31
NNRTI-resistant A17R	1.99 ± 0.04	11.4 ± 8.47	10.0 ± 4.78
Multi-drug resistant MDR769	2.37 ± 1.51	3.50 ± 1.45	4.77 ± 0.95
Multi-drug resistant AD. MDR	2.98 ± 0.54	10.6 ± 6.95	7.21 ± 4.38
PI-resistant 1002-60	2.97 ± 0.23	2.04 ± 1.03	3.66 ± 0.28
PI-resistant 144-44	3.87 ± 3.25	9.78 ± 8.23	8.38 ± 5.40
PI-resistant 1026-60	1.77 ± 0.85	2.67 ± 0.40	3.29 ± 0.57
PI-resistant 1064-52	0.86 ± 0.49	3.06 ± 0.28	2.62 ± 1.87
PI-resistant 1022-48	1.32 ± 0.17	0.98 ± 0.64	3.45 ± 2.26

up to 100 μM in both the attachment and fusion inhibition assays.

3.6.2. HIV reverse transcriptase inhibition

MDH-1-38 and NS1040 were evaluated for inhibition of wild type HIV-1 RT in a biochemical RT inhibition assay using a rC:dG template primer system. MDH-1-38 and NS1040 did not inhibit HIV RT activity when evaluated at concentrations up to 100 μM. Delavirdine was evaluated as a positive control compound and yielded an EC_{50} value of 0.53 ± 0.06 μM.

3.6.3. HIV integration inhibition

MDH-1-38 and prodrug NS1040 were evaluated for the ability to inhibit HIV integrase from cleaving the terminal two bases from the exposed 3'-end of the HIV-1 LTR DS and then catalyzing a strand-transfer reaction to integrate the double stranded viral DNA into the target substrate. MDH-1-38 and NS1040 did not inhibit HIV integrase activity when evaluated at concentrations up to 100 μM. The positive control compound, raltegravir, yielded an EC_{50} value of 0.13 ± 0.01 μM.

3.6.4. HIV protease inhibition

MDH-1-38 and prodrug NS1040 were evaluated for the ability to inhibit HIV protease cleavage. MDH-1-38 and NS1040 did not inhibit HIV protease activity when evaluated at concentrations up to 100 μM. Saquinavir evaluated as a positive control compound yielded an EC_{50} value of 0.02 ± 0 μM.

3.6.5. HIV virucidal evaluation

MDH-1-38 and prodrug NS1040 were evaluated for HIV_{IIIB} virucidal activity (Fig. 3). MDH-1-38 and NS1040 reduced the infectivity of HIV virions treated for 20 min and washed prior to infection of target CEM-SS cells at concentrations above 15 and 50 μM, respectively. The positive control compound, zinc finger inhibitor aldrithiol-2, yielded an EC_{50} value of 2.26 ± 1.85 μM.

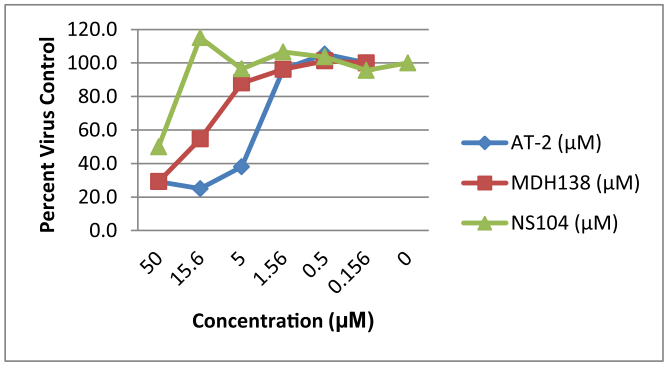


Fig. 3. Virucidal evaluation of MDH-1-38 and NS1040.

3.6.6. Inhibition of HIV transmission

The effect on virus infectivity from HIV-infected CEM-SS cells and HIV-infected fresh human PBMCs was evaluated for MDH-1-38 and NS1040 for three passages to measure HIV transmission. Cell to cell transmission of HIV_{IIIB} for three consecutive passages was significantly reduced by treatment of infected CEM-SS cells with 2 μM of MDH-1-38 as measured by quantification of extracellular virus (by RT assay and p24 ELISA) and virus infectivity ($TCID_{50}$/mL) (Table 6). The prodrug NS1040 was approximately 3-fold less effective than MDH-1-38 at reducing cell to cell HIV transmission, which we believe falls within the range of error for this particular assay. In fresh human PBMCs, HIV transmission using clade C strain 92US727 was similarly reduced by 3.25 μM of MDH-1-38 and 5 μM of NS1040 following 2 and 3 passages as measured by virus quantification using RT assay and p24 ELISA, as well as virus infectivity measured by $TCID_{50}$/mL (Table 7). Saquinavir was evaluated in parallel as a positive control compound in the both the CEM-SS and PBMC assays. Significant reduction in virus replication and subsequent infectivity of CEM-SS cells or PBMCs from virus treated with Saquinavir was demonstrated.

3.7. Combination therapy evaluations

MDH-1-38 and its prodrug NS1040 were evaluated in combination with twenty-three known anti-HIV agents for the inhibition of cytopathic effect in CEM-SS cells using the IIIB strain of HIV-1 (data not shown). The percent of virus replication inhibition above expected at each concentration for each two-drug combination was calculated at the 95%, 99% and 99.9% confidence interval.

Importantly, the combination assays demonstrated that within the concentration ranges employed in the combination antiviral assays, no evidence of either antagonism or synergistic toxicity was observed with any of the compound combinations, suggesting that the test compounds could be safely used with all approved HIV-1 inhibitors.

MDH-1-38 and NS1040 yielded slightly synergistic interactions of cytoprotection with the fusion inhibitor enfuviritide (mean synergy volume of 80.8 ± 32.9 and 115 ± 39.5 $\mu M^2\%$, respectively). In combination with reverse transcriptase inhibitors, MDH-1-38 and NS1040 yielded additive to synergistic interactions. MDH-1-38 combinations with delavirdine, efavirenz, nevirapine, zidovudine, tenofovir or stavudine (mean synergy volumes ranging from 74.4 ± 19.7 to 111 ± 51 $\mu M^2\%$), and NS1040 in combination with lamivudine or stavudine (mean synergy volume 180 ± 128 and 110 ± 68 $\mu M^2\%$, respectively) yielded synergistic interactions of cytoprotection. MDH-1-38 in combination with the integrase inhibitor dolutegravir demonstrated a synergistic interaction (mean synergy volume 95.7 ± 44.3 $\mu M^2\%$). MDH-1-38 or NS1040 in combination with protease inhibitors resulted in additive to highly synergistic interactions. MDH-1-38 or NS1040 in combination with amprenavir (mean synergy volume 101 ± 61 and 111 ± 27.8 $\mu M^2\%$, respectively), lopinavir (mean synergy volume 108 ± 64.5 and 212 ± 62.2 $\mu M^2\%$, respectively), ritonavir (mean synergy volume 282 ± 111 and 143 ± 33.5 $\mu M^2\%$, respectively), and nelfinavir (mean synergy volume 498 ± 288 and 282 ± 61.5 $\mu M^2\%$, respectively) resulted in synergistic to highly synergistic interactions of cytoprotection.

3.8. Selection of HIV-1_{IIIB} virus resistant to MDH-1-38 and NS1040

$HIV\text{-}1_{IIIB}$ virus was propagated in CEM-SS cells in the presence of increasing concentrations of MDH-1-38 and prodrug NS1040. The peak day of virus replication as determined by reverse transcriptase activity was used to evaluate compound susceptibility at each passage. The fold-increase in EC_{50} values were then compared to wild type virus susceptibility results. For MDH-1-38 and NS1040 it was necessary to escalate the dose in slight increments of increasing concentration following the first virus passage and larger volumes of compound-treated virus was needed for the consecutive acute infections as compared to wild type virus. Virus growth in the presence of MDH-1-38 and NS1040 was significantly impaired compared to wild type HIV, which made virus passaging difficult in the presence of increased concentrations. Attempts to grow virus in the presence of a fixed high concentration of compound for several passages were also made in an effort to select for drug-resistant virus. Fourteen passages of HIV in the presence of MDH-1-38 or NS1040 did not result in resistance.

3.9. In vitro and in vivo toxicity

Before examining toxicity *in* vivo, a few standard *in vitro* toxicity tests were performed. In tests for inhibition of cytochrome P450 enzymes, neither NS1040 nor MDH-1-38 had significant inhibitory activity. A standard hERG test for cardiotoxicity was also negative. To determine the toxicity *in vivo,* the toxicity of NS1040 in Sprague-Dawley rats was examined to determine the maximum tolerated dose (MTD) from a single oral administration and examine repeat dose toxicity from once daily oral administration for 14 consecutive days. NS1040 was administered by oral gavage as a suspension in 2% carboxymethyl cellulose (CMC-Na) in water. The suspensions of NS1040 were all vortexed and sonicated to obtain homogeneous solutions prior to administration. For the MTD studies, doses of 750, 1000, and 1500 mg/kg were delivered at a dose volume of 10 mL/kg. Doses of 2000 mg/kg were delivered at a dose volume of 20 mL/kg to account for increases in viscosity. Higher doses were not possible due to ethical limits on the quantity of test article that can be reasonably administered for evaluation of toxicity. After each dose, there was a four-day observation period to examine cageside and clinical observations, body weights, and food consumption. Remarkably, NS1040 did not exhibit any toxicity at any dose in the MTD study. In the repeat dose toxicity study, NS1040 was administered in the same formulation at doses of 500, 1000, and 2000 mg/kg for 14 consecutive days. Animals were separated into a main group that was sacrificed at day 15 of the study, and a recovery group that was sacrificed on day 29. During the study, cageside and clinical observations, body weights, and food consumption were all monitored. Clinical pathology was also examined after animals

Table 5
Mechanism of antiviral action.

Anti-HIV assay	Control EC_{50} ± SD	MDH-1-38 EC_{50} (μM) ± SD	NS1040 EC_{50} (μM) ± SD
Attachment inhibition	CSB 0.64 ± 0.05	44.4 ± 7.21	>100 ± 0
Fusion inhibition	T20 0.08 ± 0.09	83.3 ± 26.4	>100 ± 0
Reverse transcriptase inhibition	DLV 0.53 ± 0.06	>100 ± 0	>100 ± 0
Integrase inhibition	RAL 0.13 ± 0.01	>100 ± 0	>100 ± 0
Protease inhibition	SQV 0.02 ± 0	>100 ± 0	>100 ± 0
Virucidal activity	AD2 2.26 ± 1.85	19.7 ± 6.58	52.9 ± 4.10

Table 6
HIV transmission inhibition in CEM-SS cells.

Compound	Passage 1 (N = 3)			Passage 2 (N = 3)			Passage 3 (N = 3)		
	RT (cpm)	p24 (pg/mL)	$TCID_{50}$/mL	RT (cpm)	p24 (pg/mL)	$TCID_{50}$/mL	RT (cpm)	p24 (pg/mL)	$TCID_{50}$/mL
Saquinavir (20 nM)	25	794	0	107	1589	0	68	1085	0
NS1040 (2 μM)	27	725	0	1063	85,803	1.76×10^8	7036	1,570,937	1.31×10^4
MDH-1-38 (2 μM)	22	619	0	185	28,255	2.46×10^5	2820	394,996	2.41×10^4
Virus control	407	7740	7.78×10^4	6069	3,451,929	1.06×10^8	10,071	1,085,100	2.37×10^5

Table 7
HIV transmission inhibition in PBMCs.

Compound	Passage 1 (N = 3)			Passage 2 (N = 3)			Passage 3 (N = 3)		
	RT (cpm)	p24 (pg/mL)	$TCID_{50}$/mL	RT (cpm)	p24 (pg/mL)	$TCID_{50}$/mL	RT (cpm)	p24 (pg/mL)	$TCID_{50}$/mL
Saquinavir (12.5 nM)	281	971	3.08×10^3	1394	3505	8.30×10^3	7015	61,275	1.09×10^5
NS1040 (5 μM)	222	766	4.18×10^2	595	1731	6.86×10^3	9728	61,967	6.52×10^4
MDH-1-38 (3.25 μM)	216	816	3.75×10^2	730	2642	4.90×10^3	11,080	55,429	2.17×10^4
Virus Control	280	932	7.81×10^3	4773	26,482	1.14×10^5	14,090	136,435	8.50×10^5

were sacrificed. Across all parameters, there was no toxicity observed with NS1040. At the higher doses, cloudy urine samples were noted for some of the rats. However, this observation did not correlate with any abnormal kidney-related clinical chemistry or other histopathological changes in the urinary system. Other minor observations are documented, but none are considered to be significant toxicological findings directly related to NS1040.

3.10. Pharmacokinetics in rats

Pharmacokinetics (PK) profiles of NS1040 and its active metabolite MDH-1-38 were determined in male Sprague-Dawley rats (n = 3) after single intravenous (IV) and oral gavage (PO) administration. NS1040 was administrated by 50 mg/kg IV bolus injection of 12.5 mg/mL solution in 5% DMSO and 50% PEG300 in saline, and 1000 mg/kg PO of 10 mg/mL NS1040 in 2% carboxymethyl cellulose (CMC) suspension. Blood samples were collected at predetermined times over a 24-h period.

To prevent conversion of NS1040 after sample collection, 150 μL of blood was aliquoted in tubes containing 15 μL of sodium fluoride (1.0 M) and 7.5 μL of citric acid (1.0 M). Blood samples were kept on wet ice for less than 10 min until centrifugation at 4 °C to harvest plasma. Plasma samples were stored at −80 °C until analysis. The plasma samples were analyzed by an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method.

After IV and PO administration of NS1040, NS1040 was rapidly converted to MDH-1-38 by esterases in blood and tissues. The concentration of NS1040 was below the lower limit of quantitation (0.10 ng/mL) 1 h post IV dosing and 0.75 h post PO dosing. The area-under-the-plasma concentration–time curve ($AUC_{0-\infty}$) was 1.7 ± 0.9 ng h/mL and 34 ± 20 ng h/mL for IV and PO, respectively. The $AUC_{0-\infty}$ of MDH-1-38 was 3080 ± 763 ng h/mL and 39,400 ± 4770 ng h/mL after IV and PO administration of NS1040, respectively (Table 8). The PK profiles of NS1040 and MDH-1-38 after 50 mg/kg IV and 1000 mg/kg PO administration of NS1040 was depicted in Fig. 4.

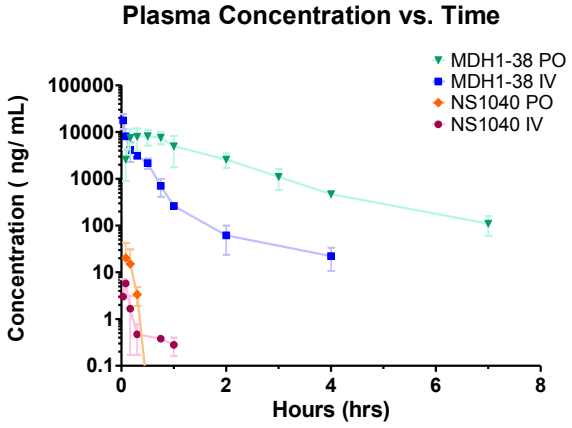


Fig. 4. Pharmacokinetics profiles of NS1040 and its active metabolite MDH-1-38 in Sprague-Dawley rats after single intravenous (IV) and oral gavage (PO) administration.

Table 8
Pharmacokinetic parameters of NS1040 and MDH-1-38 in rats after 50 mg/kg IV and 1000 mg/kg PO administration of NS1040.

PK parameter	50 mg/kg NS1040 IV		1000 mg/kg NS1040 PO	
	NS1040	MDH-1-38	NS1040	MDH-1-38
$AUC_{0-\infty}$ (ng h/mL)	1.7 ± 0.9	3080 ± 763	34 ± 20	39,400 ± 4770
$t_{1/2}$ (h)	0.4 ± 0.2	0.5 ± 0.3	0.5 ± 0.1	4.0 ± 2.4
T_{max} (h)	0.033[a]	0.033	1.1 ± 1.7	0.8 ± 0.7
C_{max} (ng/mL)	3.0 ± 4.1[b]	14,100 ± 6860	38.2 ± 50.0	5240 ± 2810
F %				64

[a] First sampling time point after IV administration.
[b] Concentration obtained at the first sampling time point after IV administration.

3.11. Tests for activity against other viruses

The molecule MDH-1-38 was submitted to the Division of Microbiology and Infectious Diseases (DMID) antiviral screening program within the National Institutes of Allergy and Infectious Diseases (NIAID). The molecule failed to show any activity against influenza A virus type H1N1, respiratory syncytial virus, and Venezuelan equine encephalitis virus. MDH-1-38 had weak activity (EC_{50} values ~20 μM) against rift valley fever virus, SARS corona virus, tacaribe virus, and ebola virus.

4. Discussion

MDH-1-38 and its prodrug NS1040 are being developed as potential therapeutic compounds targeting the zinc finger of HIV-1 nucleocapsid. The compounds were similarly and broadly active against a geographically diverse panel of clinical HIV-1 subtypes

and inhibited CXCR4-tropic, CCR5-tropic and dual-tropic virus strains with equal efficacy. MDH-1-38 and its prodrug were equally active against monocyte-tropic strains of HIV-1 in fresh human monocytes. These data demonstrate that MDH-1-38 and its prodrug NS1040 would be expected to be active against HIV-1 strains found throughout the world as a therapeutic product. The lack of cross resistance to HIV strains conferring resistance to reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors, as well as multidrug resistant isolates, indicates a distinct mechanism of antiviral action for MDH-1-38 and its prodrug when compared with approved HIV drugs. Following cell-based and biochemical inhibition assays to evaluate mechanism of antiviral action, it was concluded that MDH-1-38 and its prodrug act as a virucidal agent and reduces transmission of infectious virus over multiple rounds of virus replication. This reduction in virus infectivity proved a challenge when passaging virus in the presence of MDH-1-38 and NS1040 in attempts to select for drug resistant HIV. Virus culture in the presence of increased concentrations over fourteen passages in the presence of MDH-1-38 or NS1040 did not result in resistance, which is unique compared to most HIV inhibitors that reduce virus replication by targeting a viral protein. Combination assays performed with representatives of the various classes of HIV inhibitors, MDH-1-38 and the prodrug NS1040 resulted in additive to synergistic interactions with all of the approved inhibitors, with the greatest levels of synergy observed with compounds which inhibited protease (ritonavir, lopinavir and nelfinavir). Most importantly, there was no significant combination antagonism or synergistic toxicity for any of the combination therapy assays at the concentrations tested. The evaluation for *in vivo* toxicity in rats showed that NS1040 is non-toxic, even at doses of 2000 mg/kg repeated for 14 consecutive days. A detailed pharmacokinetic analysis showed that NS1040 rapidly breaks down to MDH-1-38 in blood. Subsequent studies are underway to characterize the metabolites of MDH-1-38.

Our studies indicate that these NCp7-targeted compounds represent new potent anti-HIV drug candidates which could be safely and effectively used in combination with all approved anti-HIV drugs.

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