International Journal of Antimicrobial Agents 40 (2012) 354–360

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

In vitro anti-HIV-1 activity of salicylidene acylhydrazide compounds

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ARTICLE INFO

Article history:
Received 30 March 2012
Accepted 24 May 2012

Keywords:
Salicylidene acylhydrazides
HIV
Microbicide
Iron chelation

ABSTRACT

Salicylidene acylhydrazide compounds have been shown to inhibit bacterial pathogens, including *Chlamydia* and *Neisseria gonorrhoeae*. If such compounds could also target HIV-1, their potential use as topical microbicides to prevent sexually transmitted infections would be considerable. In this study, the in vitro anti-HIV-1 activity, cytotoxicity and mechanism of action of several salicylidene acylhydrazides were determined. Inhibitory activity was assessed using TZM-bl cells and primary peripheral blood mononuclear cells (PBMCs) as targets for HIV-1 infection. Antiviral activity was measured against cell-free and cell-associated virus and in vaginal fluid and semen simulants. Since the antibacterial activity of salicylidene acylhydrazides is reversible by Fe^{2+}, the ability of Fe^{2+} and other cations to reverse the anti-HIV-1 activity of the compounds was determined. Real-time PCR was also employed to determine the stage affected in the HIV-1 replication cycle. Four compounds with 50% inhibitory concentrations against HIV-1 of 1–7 μM were identified. In vitro toxicity varied but was generally limited. Activity was similar against three R5 clade B primary isolates and whether the target for virus replication was TZM-bl cells or PBMCs. Compounds inhibited cell-free and cell-associated virus and were active in vaginal fluid and semen simulants. Fe^{2+}, but not other cations, reversed the anti-HIV-1 effect. Finally, the inhibitory effect of the compounds occurred at a post-integration step. In conclusion, salicylidene acylhydrazides were identified with in vitro anti-HIV-1 activity in the micromolar range. The activity of these compounds against other sexually transmitted pathogens makes them potential candidates to formulate for use as a broad-spectrum topical genital microbicide.

1. Introduction

A clinical trial of topical tenofovir, applied as a gel, has demonstrated a reduction in sexually acquired HIV infections among women [1]. The modest reduction associated with tenofovir gel establishes the utility of topical microbicides and underscores the need to develop more potent compounds. Compounds capable of simultaneously inhibiting both HIV and other sexually transmitted pathogens would constitute an ideal prevention strategy.

Previous studies have suggested that salicylidene acylhydrazides can inhibit the type III secretion apparatus of *Yersinia pseudotuberculosis* and *Chlamydia* [2–4]. However, at least with of *Chlamydia* spp., the salicylidene acylhydrazides exert an antibacterial effect either directly or indirectly by limiting iron availability to host cells [4]. We have recently shown that sexually transmitted pathogens, including *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, can be inhibited in vitro by micromolar concentrations of selected salicylidene acylhydrazides [5]. In the case of *Chlamydia*, these compounds were further shown to protect mice from a vaginal challenge [6].

Iron-chelating compounds, including a salicylidene acylhydrazide, have been previously shown to inhibit HIV infection [7–11]. It is thought that limiting iron uptake by cells susceptible to HIV slows cell cycle progression and reduces integrated viral gene expression, although other mechanisms have also been proposed [7,8,12]. Given their antichlamydial, antigonococcal and iron-chelating activity, we sought to determine whether the salicylidene acylhydrazides with activity against *Chlamydia* might also be effective in inhibiting HIV-1 infection. Virus inhibition and toxicity assays were conducted using both primary CD4+ lymphocytes and a cell line that allows rapid detection of successful infection. Four compounds were identified with limited cytotoxicity and with antiviral activity in the low micromolar range. These compounds inhibited diverse HIV-1 strains and inhibition was reversible by iron repletion.

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http://dx.doi.org/10.1016/j.ijantimicag.2012.05.023

2. Materials and methods

Research on this project involved the use of blood samples taken from human subjects enrolled in the University of California, Irvine Normal Blood Donors Program. This programme was approved by the University of California, Irvine Institutional Review Board. Written informed consent was obtained from each participant.

2.1. Compounds

The salicylidene acylhydrazides *N′*-(4-diethylamino-2-hydroxybenzylidene)-2,4-dihydroxybenzhydrazide (INP0149), *N′*-(4-diethylamino-2-hydroxybenzylidene)-isonicotinic acid hydrazide (INP0161), *N′*-(5-chloro-2-hydroxy-3-methylbenzylidene)-2,4-dihydroxybenzhydrazide (INP0341) and *N′*-(2-hydroxy-1-naphtalenylmethylene)-4-chlorobenzhydrazide (INP0400) (Fig. 1) were a kind gift from Creative Antibiotics (Umeå, Sweden). For use in the assays described, compounds were dissolved in dimethyl sulphoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ). Compounds were synthesised from the corresponding salicylic aldehydes and hydrazides according to published procedures [13]. Compounds were analysed by nuclear magnetic resonance spectroscopy and mass spectrometry.

2.2. Virus inhibition assays

Virus inhibition was measured using either TZM-bl luciferase reporter cells or phytohaemagglutinin (PHA)-activated primary peripheral blood mononuclear cells (PBMCs) as target cells. TZM-bl cells (5×10^4/well) were plated into 96-well microtitre plates and were incubated overnight until confluent. Compounds diluted in DMSO were next added to the monolayers, followed immediately by polybrene (10 μg/mL) and HIV-1 (multiplicity of infection = 0.02). Note that the use of polybrene increased the yield of virus as expected but had little or no impact on the magnitude of inhibition due to test compounds (data not shown). At 24 h post infection, monolayers were washed three times with medium to remove the compounds. Wells were then repleted with fresh medium (without compound) and the plates were incubated for an additional 24 h. To determine virus yield, cell culture supernatant was removed from the wells, the monolayers were lysed with 40 μL of a 1× solution of cell lysis buffer (Promega, Madison, WI) for 10 min, and 25 μL of the cell lysate was transferred to a white opaque microtitre plate for luminescence reading. A Synergy 2 Multipurpose Plate Reader (BioTek, Winooski, VT) with automatic injectors was used to inject 100 μL of Luciferase 1000 Assay Substrate (Promega) in each well, and relative light units (RLUs) were read with a 1 s integration time one well at a time. Inhibition was calculated as follows: $100 \times (RLU_{DMSO\ control} - RLU_{test\ compound})/RLU_{DMSO\ control}$. The 50% inhibitory concentration (IC_{50}) was determined from the linear portion of the % inhibition–logarithmic concentration curve.

In some experiments, compounds were first mixed for 15 min with a vaginal fluid simulant at pH 4.5, 5.5 or 6.5, a semen simulant at pH 7.7 or a 1:1 (v/v) combination of vaginal fluid simulant (pH 4.5) and semen simulant (pH 7.7) prior to incubating with cells. In these experiments, compound in vaginal fluid or semen simulant was left on the target cells for 24 h, washed and then replaced with fresh medium. Vaginal fluid and semen simulants were prepared by following the formulas proposed by Owen and Katz [14,15].

To evaluate the impact of salicylidene acylhydrazides on limiting infection initiated by infected cells, as opposed to cell-free virus, CEM.NKR-CCR5 cells were exposed to HIV-1_{US657} for 3 days. After washing to remove cell-free virus, infected CEM.NKR-CCR5 cells were added to TZM-bl cells treated with compounds as above.

PBMCs were separated from whole blood of healthy volunteers via Ficoll-Paque (GE Healthcare, Piscataway, NJ). PBMCs were stimulated with PHA for 2–3 days, washed three times with medium and plated into round bottom 96-well microtitre plates. The compounds and HIV-1_{US657} were added as above. After washing at 24 h post infection to remove salicylidene acylhydrazides and repleting with fresh medium, plates were incubated for an additional 6 days. On Day 7, HIV-1 was quantified in a p24 enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix, Buffalo, NY). Inhibition was calculated by dividing the concentration of p24 in wells with compound by the concentration of p24 in control (DMSO) wells.

2.3. Iron and other cations

Experiments with added iron, magnesium or calcium were set up similarly to the virus inhibition assays with the addition of 250 μM final concentrations of $FeSO_4$, $MgCl_2$ or $CaCl_2$ during the first 24-h incubation period.

2.4. Cytotoxicity assays

Compound cytotoxicity was measured by lactate dehydrogenase (LDH) release and by uptake of propidium iodide (PI). For LDH release, the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used. TZM-bl cells were seeded into 96-well microtitre plates at a concentration of 5×10^4 cells/well. Cells were incubated at 37 °C in 5% CO_2 using RPMI media with 10% foetal bovine serum (FBS), 4 mM of glutamate and 50 μg/mL gentamicin. Subsequently, concentrations of salicylidene acylhydrazides ranging from 0.78 μM to 100 μM diluted in DMSO were added along with 10 μg/mL polybrene to confluent monolayers. The final DMSO concentrations of controls were the same as those of the test compounds in all experiments. After 24 h of incubation, medium was collected to assay LDH release, and cells were lysed for total LDH estimation. Media and cell lysates were diluted with phosphate-buffered saline (PBS)/1% FBS to obtain the linear range of LDH activity. Aliquots of 50 μL of each sample were combined with the substrate and transferred to a flat-bottom 96-well plate. Reactions were incubated for 30 min at room temperature in the dark following the addition of 50 μL of 1 M acetic acid. Each sample was assayed in triplicate. All assays were performed on three separate occasions. Reactions were read at 492 nm using a SpectraMax 340 Plate Reader (Molecular Devices, Sunnyvale, CA). DMSO-treated samples were used as a background control, and salicylidene acylhydrazide-treated cells were used to calculate the percentage of LDH leakage [(LDH activity in medium/total LDH activity) × 100].

To determine compound cytotoxicity by PI uptake, TZM-bl cells were plated into 24-well tissue culture plates and were incubated overnight until confluent. Concentrations of salicylidene acylhydrazides ranging from 3.125 μM to 100 μM were added to the wells, incubated for 24 h, washed and replaced with fresh medium. Plates were then incubated for an additional 24 h. Subsequently, cells were trypsinised and re-suspended in 1× PBS. Cytotoxicity was determined by adding PI and determining the percentage of cells containing PI from the FL3 channel of an Accuri C6 flow cytometer (Accuri Instruments Inc., Ann Arbor, MI) using CFlow Plus software. Toxicity due to the compounds was expressed relative to PI-positive cells treated with DMSO.

2.5. Quantification of early reverse transcription products

DNA was extracted (DNeasy Blood and Tissue Kit; QIAGEN, Valencia, CA) from trypsinised TZM-bl cells that had been treated with compound and infected with HIV-1_{US657} as above but in 24-well plates. The following primers and