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

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### **Abstract**

**Introduction**—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. We determined the *in vitro* anti-HIV-1 activity, cytotoxicity and mechanism of action of several salicylidene acylhydrazides.

**Methods**—Inhibitory activity was assessed using TZMbl cells and primary peripheral blood mononuclear cells (PBMCs) as targets for HIV-1 infection. Anti-viral activity was measured against cell-free and cell-associated virus and in vaginal fluid and semen simulants. Since the anti-bacterial activity of salicylidene acylhydrazides is reversible by Fe<sup>2+</sup>, we determined whether Fe<sup>2+</sup> and other cations could reverse the anti-HIV-1 activity of the compounds. We also employed real-time PCR to determine the stage affected in the HIV-1 replication cycle.

**Results**—We identified four compounds with 50% HIV-1 inhibitory concentrations of 1 to 7 µM. *In vitro* toxicity varied but was generally limited. Activity was similar against three R5 clade B primary isolates and whether targets for virus replication were TZMbl cells or PBMCs. Compounds inhibited cell-free and cell-associated virus and were active in vaginal fluid and semen simulants. Fe<sup>2+</sup>, but not other cations, reversed the anti-HIV-1 effect. Finally, inhibitory effect of the compounds occurred at a post-integration step.

**Conclusions**—We identified salicylidene acylhydrazides with *in vitro* anti-HIV-1 activity in the µM 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.

### **Keywords**

Salicylidene acylhydrazides; HIV; microbicide; iron chelation

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## 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 of *Chlamydia* [2–4]. However, at least in the case 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 *C. trachomatis* and *Neisseria gonorrhoeae*, can be inhibited *in vitro* by  $\mu\text{M}$  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 anti-chlamydial, anti-gonococcal and iron-chelating activity, we sought to determine if the salicylidene acylhydrazides with activity against Chlamydia might also be effective in inhibiting HIV-1 infection. We conducted virus inhibition and toxicity assays using both primary CD4<sup>+</sup> 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  $\mu\text{M}$  range. These compounds inhibited diverse HIV-1 strains, and inhibition was reversible by iron repletion.

## 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 program was approved by the University of California, Irvine Institutional Review Board. Written informed consent was obtained from each participant.

### 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-naphthalenylmethylene)-4-chlorobenzhydrazide (INP0400) were the kind gift of Creative Antibiotics, Umeå, Sweden (Figure 1). For use in the assays described, compounds were dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ). The compounds were synthesized from the corresponding salicylic aldehydes and hydrazides according to published procedures [13]. The compounds were analyzed by nuclear magnetic resonance spectroscopy and mass spectrometry.

### Virus inhibition assays

Virus inhibition was measured using either TZMbl luciferase reporter cells or phytohemagglutinin (PHA)-activated primary peripheral blood mononuclear cells (PBMCs) as target cells. TZMbl cells, at  $5 \times 10^4$ /well, were plated into 96-well microtiter plates and incubated overnight until confluent. Compounds, diluted in DMSO, were next added to the

monolayers, followed immediately by polybrene (10  $\mu\text{g/ml}$ ) and HIV-1 (MOI = 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). 24-hours post-infection, monolayers were washed three times with medium to remove the compounds. Wells were then replenished with fresh medium (without compound), and the plates were incubated for an additional 24 hours. To determine virus yield, cell culture supernatant was removed from the wells, the monolayers were lysed with 40  $\mu\text{l}$  of a 1X solution of cell lysis buffer (Promega) for 10 minutes, and 25  $\mu\text{l}$  of the cell lysate was transferred to a white opaque microtiter plate for luminescence reading. A Biotek Synergy 2 multi-purpose plate reader with automatic injectors was used to inject 100  $\mu\text{l}$  of Luciferase 1000 assay substrate (Promega) in each well, and relative light units (RLUs) were read with a 1 second integration time one well at a time. Inhibition was calculated as follows:  $100 \times (\text{RLU}_{\text{DMSO control}} - \text{RLU}_{\text{test compound}}) / \text{RLU}_{\text{DMSO control}}$ . 50% inhibitory concentrations ( $\text{IC}_{50}$ ) were determined from the linear portion of the % inhibition-logarithmic concentration curve.

In some experiments, compounds were first mixed for 15 minutes 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 (vol/vol) 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 hours, washed, and replaced with fresh medium. Vaginal fluid and semen simulants were prepared by following the formulas proposed by Owen & 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<sub>US657</sub> for 3 days. After washing to remove cell-free virus, the infected CEM.NKr.CCR5 cells were added to TZMbl cells treated with compounds as above.

PBMCs were separated from whole blood of healthy volunteers via Ficoll-Paque (GE Healthcare, Piscataway, NJ). PBMCs were PHA stimulated for 2–3 days, washed three times with medium and plated into round bottom 96-well microtiter plates. The compounds and HIV-1<sub>US657</sub> were added as above. After washing 24 hours post-infection to remove salicylidene acylhydrazides and replenishing with fresh medium, plates were incubated for an additional 6 days. On day 7, HIV-1 was quantified in a p24 ELISA (ZeptoMetrix). Inhibition was calculated by dividing the concentration of p24 in wells with compound by the concentration of p24 in control (DMSO) wells.

### 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  $\mu\text{M}$  final concentrations of  $\text{FeSO}_4$ ,  $\text{MgCl}_2$ , or  $\text{CaCl}_2$  during the first 24-hour incubation period.

### Cytotoxicity assays

Compound cytotoxicity was measured by lactate dehydrogenase (LDH) release and by uptake of propidium iodide (PI). For LDH release, the Cytotox 96<sup>®</sup> non-radioactive cytotoxicity assay (Promega Corporation, Madison, WI) was used. TZMbl cells were seeded into 96-well microtiter plates at a concentration of  $5 \times 10^4$  cells per well. Cells were incubated at 37°C in 5%  $\text{CO}_2$  using RPMI media with 10% FBS, 4 mM of glutamate and 50  $\mu\text{g/ml}$  of gentamicin. Subsequently, concentrations of salicylidene acylhydrazides ranging from 0.78  $\mu\text{M}$  to 100  $\mu\text{M}$  diluted in DMSO were added along with 10  $\mu\text{g/ml}$  of polybrene to confluent monolayers. The final DMSO concentrations of controls were the same as those of the test compounds in all experiments. After 24-hours of incubation, media was collected to

assay LDH release, and cells were lysed for total LDH estimation. Media and cell lysates were diluted with PBS-1% FBS to obtain the linear range of LDH activity. Aliquots of 50  $\mu$ l of each sample were combined with the substrate and transferred to a flat bottom 96-well plate. Reactions were incubated for 30 minutes at room temperature in the dark following the addition of 50  $\mu$ l 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 Spectra MAX 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  $\times$  100).

To determine compound cytotoxicity by PI uptake, TZMbl cells were plated into 24-well tissue culture plates and incubated overnight until confluent. Concentrations of salicylidene acylhydrazides ranging from 3.125 to 100  $\mu$ M were added to the wells, incubated for 24 hours, washed, and replaced with fresh medium. Plates were then incubated for an additional 24 hours. Subsequently, cells were trypsinized and resuspended in 1x phosphate buffered saline (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 using CFlow Plus software. Toxicity due to the compounds was expressed relative to PI positive cells treated with DMSO.

#### Quantification of early reverse-transcription products

DNA was extracted (Dneasy Blood and Tissue kit, QIAgen, Valencia, CA) from trypsinized TZMbl cells that had been treated with compound and infected with HIV-1<sub>US657</sub> as above but in 24-well plates. The following primers and probe were used to quantify R-U5 DNA by real-time PCR: R-U5 sense M667 (5'-GGCTAACTAGGGAACCCACTG-3'); R-U5 antisense AA55 (5'-CTGCTAGAGATTTTCCACACTGAC-3'); and RU5 probe (5'-6-FAM-TGTGTGCCCGTCTGTTGTGTGACT-DDQI-3'). The human apolipoprotein B (HAPB) gene was quantified in the same reaction to adjust for experimental variability using the following primers and probe: HAPB forward primer (5'-TGAAGGTGGAGGACATTCCTCTA-3'); HAPB reverse primer (5'-CTGGAATTGCGATTTCTGGTAA-3'); and HAPB probe (5'-JOE-CGAGAATCACCCCTGCCAGACTTCCGT-TAMRA-3'). Real-time PCR was done with a RotoGene 3000 (Corbett Research, Sydney, Australia) using cycling parameters of 10 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C.

#### Quantification of integrated HIV-1 DNA

DNA was extracted from trypsinized TZMbl cells that had been treated with compound and infected with HIV-1<sub>US657</sub> as above. Alu-Gag (chromosomally integrated) DNA was amplified using the following primers: Alu forward primer (5'-GCCTCCCAAAGTGCTGGGATTACAG-3') and HIV Gag reverse primer (5'-GTTCTGCTATGTCACCTCC-3'). PCR cycling parameters were 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 15 seconds at 50°C, and 3.5 minutes at 72°C. Once completed, 2  $\mu$ L from this first PCR was used to amplify RU5 and HAPB in real time PCR as above. PCR standards consisted of DNA extracted from a known number of 8E5/LAV cells (AIDS Research and Reference Reagent Program, Germantown, MD from Dr. Thomas Folks), which contain one integrated copy of HIV-1 DNA per cell. Standards were nested and treated the same way as unknowns.

## Results

### Anti-HIV-1 activity of salicylidene acylhydrazides

We initially screened a total of 25 salicylidene acylhydrazide compounds for anti-HIV-1 activity at concentrations from 50 to 6.25  $\mu\text{M}$  using HIV-1<sub>US657</sub>, a primary, clade B R5 isolate. In these assays, target cells consisted of TZMbl cells, which allow for rapid quantification of infection. In addition, compound cytotoxicity was measured by LDH release and by microscopic examination of cells. Four compounds, INP0149, INP0161, INP0341 and INP0400 (structures illustrated in Figure 1), inhibited virus, had limited cytotoxicity, and were evaluated further (see below).

### Four active compounds inhibit HIV-1 on both the TZMbl cell line and on primary CD4+ lymphocytes

Serial dilutions of INP0149, INP0161, INP0341 and INP0400 were next tested for HIV-1 inhibitory activity on TZMbl cells. Compounds were added to the cells immediately prior to infection, and the compounds were washed off 24 hours later. Virus yield was quantified by luminescence (see Methods) 48 hours after infection. On TZMbl cells, all four compounds inhibited virus with 50% inhibitory concentrations between about 0.9 and 7  $\mu\text{M}$  (figure 2a; table 1). In these experiments, the TZMbl cells were confluent and were infected in the presence of polybrene. Similar results were obtained with infection of 50%-confluent cells or of confluent cells in the absence of polybrene (data not shown).

We also measured inhibition on primary, PHA-stimulated PBMCs, with virus yield determined seven days after infection by measuring p24 in supernatant fluid. In the absence of compound, virus yield ranged from 751 to 1015 pg/ml. Maximum inhibition on PBMCs was somewhat less than on the TZMbl cells, but the potency of the individual compounds relative to each other was similar on both cell types (with INP0149>0161>0341>0400 (figure 2b).

### Compounds inhibit diverse clade B strains of HIV-1

An effective topical microbicide must be active against a broad range of clinical HIV-1 strains. Using TZMbl cells as targets, we tested the four active compounds against two additional R5 clade B strains of HIV-1, HIV-1<sub>US005</sub> and HIV-1<sub>US712</sub> (figure 2c and 2d). These two additional strains were inhibited at concentrations similar to those that inhibited HIV-1<sub>US657</sub>.

### Salicylidene acylhydrazides inhibit virus replication initiated by HIV-1-infected cells

It is possible that both infected cells and cell-free virus initiate sexual transmission of HIV infection. To explore the impact of the compounds on infection by cell-associated virus, we incubated infected CEM.NKr-CCR5 cells (which had been washed to remove cell-free virus) with TZMbl target cells in the presence or absence of salicylidene acylhydrazides. The two compounds tested (INP0149 and INP0341) both resulted in inhibition of HIV-1<sub>US657</sub> (figure 2e). However, 50% inhibitory concentrations were somewhat higher than those seen with cell-free virus (compare with figure 2a), likely because virus yield on the TZMbl cells was about ten-times greater (based on RLUs) after exposure to infected cells (data not shown).

### Compounds are effective inhibitors of HIV-1 in vaginal fluid simulant, in semen simulant and in combined vaginal fluid-semen stimulants

Since a topical microbicide must retain activity on the vaginal mucosa, we used a simulant of vaginal fluid to evaluate the anti-viral activity and cytotoxicity of the salicylidene



acylhydrazides. The compounds were incubated with the simulant adjusted to a pH of 6.5, 5.5, or 4.5. Under these conditions, all four compounds continued to have similar activity against HIV-1<sub>US657</sub> (figure 3a) and demonstrated no increased toxicity (data not shown). Similarly, using either semen simulant (data not shown) or semen simulant in combination with vaginal fluid simulant (figure 3b) had little effect on the anti-HIV-1 activity of the compounds.

### Compounds have limited cytotoxicity

Cytotoxicity was measured by LDH release and by PI staining of TZMbl cells under the same conditions used to measure virus inhibition. By LDH release, no compound resulted in more than 20% cytotoxicity at concentrations up to 100  $\mu\text{M}$ . Furthermore, in the active HIV-1 inhibitory range (1 – 7  $\mu\text{M}$ ), cytotoxicity was <3% for all four compounds (data not shown). Based on PI staining, three of the four compounds (INP0161, INP0341 and INP0400) gave <25% cytotoxicity ( $\text{Cyt}_{25}$ ) at concentrations up to 25  $\mu\text{M}$ , whereas compound INP0149 resulted in about 25% cytotoxicity at a concentration of 12.5  $\mu\text{M}$  (table 1). At concentrations up to 100 $\mu\text{M}$ , cytotoxicity did not reach 50% for any of the four compounds when determined by PI staining. However, some of the compounds began to precipitate at concentrations above 50  $\mu\text{M}$ . We therefore calculated a therapeutic index (TI) based on 25% cytotoxicity concentrations, rather than the more standard 50% cytotoxicity concentration. Compounds INP0149, INP0161 and INP0341 had TIs ( $\text{Cyt}_{25}/\text{IC}_{50}$ ) of 10 and compound INP0400 had a TI of >3.4. It should be noted that PI staining was conducted at 48 hours following a wash at 24 hours. Unattached cells would have been washed away at that time, and PI staining might have therefore underestimated the number of cells staining with PI. To explore this possibility, we quantified the number of unattached TZMbl cells at 24 hours after the addition of compounds. Controls treated with DMSO alone demonstrated about 8.8% of unattached cells at 24 hours (compared to all cells). At concentrations of 25  $\mu\text{M}$  and 12.5 $\mu\text{M}$ , respectively, unattached cells with INP0149 were 14.7% and 9.4%, with INP0161 12.3% and 9%, with INP0341 7% and 6%, and with INP0400 15.7 and 15.7%. Thus, in some cases, there may have been a slight underestimation of cytotoxicity by the PI staining due to the unattached cells that would have not been accounted for. These results engender some concern about toxicity; however, the LDH results, as well as histological findings in mice [6], suggest that the toxicity of the compounds is limited.

### HIV-1 inhibitory activity is reversed by $\text{Fe}^{2+}$

The salicylidene acylhydrazides have been previously shown to limit iron availability [4], and iron depletion can result in inhibition of HIV-1 replication [7–11]. We therefore added  $\text{Fe}^{2+}$  ( $\text{FeSO}_4$  250  $\mu\text{M}$ ) to the medium prior to adding compounds and infecting TZMbl cells. At this concentration,  $\text{Fe}^{2+}$  was able to almost substantially reverse the inhibition of all four compounds (figure 4). Interestingly, reversal by  $\text{Fe}^{2+}$  was most modest for the least active compound (INP0400). The use of the cations  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  did not reverse inhibition of HIV-1 (data not shown), suggesting that chelation of  $\text{Fe}^{2+}$  is involved in the mechanism underlying the HIV-1 inhibitory activity of the salicylidene acylhydrazides.

### HIV-1 inhibition occurs at a post-entry, post-reverse transcription, post-integration step

To determine the stage of the HIV-1 replication cycle inhibited by the compounds, we measured R-U5 DNA after infection of TZMbl cells in the presence or absence of compound. R-U5 DNA is reverse-transcribed from the HIV-1 RNA genome very early after virus entry. Using real-time PCR, we demonstrated that compounds did not inhibit R-U5 DNA levels relative to the DMSO control, indicating that neither virus entry nor early reverse transcription were affected by the compounds (table 2).

We next measured integrated HIV-1 DNA using primers specific for both Alu (which occurs frequently in chromosomal DNA) and HIV-1. Again, we found similar amounts of integrated HIV-1 DNA in the presence or absence of the two salicylidene acylhydrazides tested (table 2). Thus, the compounds must be acting at steps in the virus replication cycle subsequent to integration.

## Discussion

We have demonstrated the anti-HIV-1 activity of four salicylidene acylhydrazides. These compounds inhibit diverse clade B HIV-1 strains in the low  $\mu\text{M}$  range, are active with primary CD4<sup>+</sup> lymphoblast target cells and maintain their activity in the presence of vaginal fluid and semen simulant. Moreover, we have shown that HIV-1 inhibitory activity is reversible when high concentrations of Fe<sup>2+</sup> are added to the medium. Finally, the compounds appear to inhibit infection at a post-integration stage.

An ideal vaginal microbicide would have inhibitory activity against a number of sexually transmitted pathogens. Notably, the four compounds we have tested inhibit three different R5 clinical isolates of HIV-1 and have similar inhibitory potency against *C. trachomatis* [5]. Moreover, compound INP0341 was also active against five of six *N. gonorrhoeae* strains with minimal bactericidal concentrations of 6.3  $\mu\text{M}$  [5]. In the case of *Chlamydia*, compound INP0341 has been shown to be active in a mouse model of vaginal infection [5]. While inhibiting a number of pathogens, the compounds tested do not have inhibitory activity (at concentrations up to 100 $\mu\text{M}$ ) against two of the most common hydrogen peroxide-producing members of the vaginal normal flora, *Lactobacillus jensenii* and *Lactobacillus crispatus* [5]. These two organisms are considered crucial to the maintenance of normal vaginal flora. Thus, the specificity of these compounds in targeting pathogens and sparing essential organisms make them well-suited for development as topical vaginal microbicides.

The four compounds had activity against HIV-1 in primary CD4<sup>+</sup> lymphoblasts obtained from healthy donors. Since CD4<sup>+</sup> lymphocytes are the major target cell for HIV-1 replication, compound activity could translate into *in vivo* efficacy. However, we have not measured the effect of the salicylidene acylhydrazides on other cells, such as macrophages and dendritic cells, which are likely very early targets following mucosal exposure. Moreover, although a vaginal fluid simulant at three different pH values, as well as semen simulant and a combination of semen and vaginal fluid simulants had little or no effect on activity, it remains to be determined if the compounds will inhibit HIV infection after exposure to intact or inflamed vaginal mucosa.

In the absence of details regarding the exact nature of the inoculum during natural sexual transmission of HIV, effective preventions should be active against incoming virus in both the cell-free and cell-associated forms. In this regard, the compounds tested were able to inhibit virus after exposure of target cells to either cell-free or cell-associated virus.

Our experiments clearly show that the addition of iron can reverse the inhibitory effect of the four compounds tested. Previous studies have also found that iron chelating agents, including the salicylidene acylhydrazide 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazine, inhibit HIV-1 [7–11]. Moreover, increased iron stores associated with a number of conditions, adversely affect HIV infection [16–18]. Our results are also consistent with those of others who have observed that iron depletion affects HIV replication at the level of transcription and that such an effect is related to perturbations in cell cycling [7, 10, 11]. We have previously observed that the inhibition of *C. trachomatis* and *N. gonorrhoeae* by salicylidene acylhydrazides is also reversible by iron [4, 5]. Finally, a group of

acylhydrazones has been shown to inhibit SIV, bind to HIV-1 capsid, and inhibit capsid assembly [19]. Whether the compounds we tested also target capsid assembly or solely act as iron chelators is unknown.

By likely acting on a post-integration step in virus replication, the compounds tested will allow the entry of virus and the integration of its genome. This implies that, in order to be effective, the compounds will need to be maintained at inhibitory concentrations for the duration of the life of infected cells. In this regard, we are in the process of testing the compounds in a humanized mouse model to gauge their *in vivo* efficacy. Moreover, the mechanism of inhibition will likely require the use of these compounds in combination with substances that inhibit early steps in the virus life cycle.

The compounds used in our study have been shown to slow cell progression, but as demonstrated by the LDH and PI results, there is minimal toxicity to the cells at the active concentrations. We note that the TI values are rather small, particularly for INP0400. However, because of compound precipitation at high concentrations, TI's were calculated using an unconventional 25% cytotoxicity value, rather than the usual 50% value. In addition, 1mM of INP0341 applied intravaginally to mice on three separate occasions over three days did not result in histological abnormalities [6]. In a previous study, INP0161, 0341 and 0400 also showed minimal toxicity to HeLa 299 cells when measured by mitochondrial dehydrogenase activity; however, in that study, INP0149 resulted in 9.4% and 81.1% cytotoxicity at 20 $\mu$ M and 50 $\mu$ M [4]. Additional *in vivo* studies will be required to further establish the safety profile of these compounds.

In summary, we have identified salicylidene acylhydrazides with *in vitro* anti-HIV-1 activity in the  $\mu$ M range. Although not as potent as some anti-retroviral agents, these compounds appear to have relatively low cytotoxicity *in vitro* and *in vivo* at well above inhibitory concentrations [6]. Finally, the activity of the compounds against other sexually transmitted pathogens makes them, likely in combination with other agents, potential candidates for formulation and use in a broad-spectrum topical microbicide.

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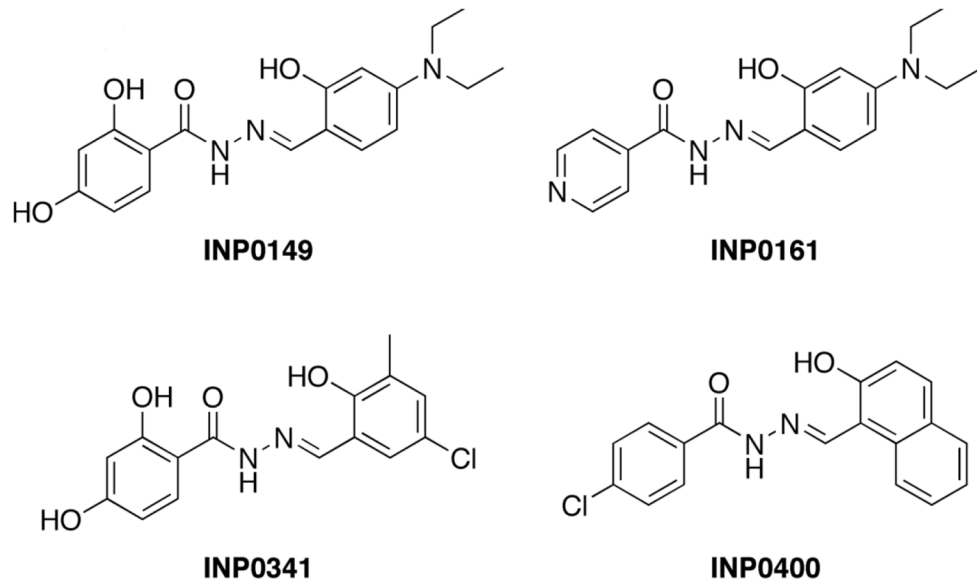
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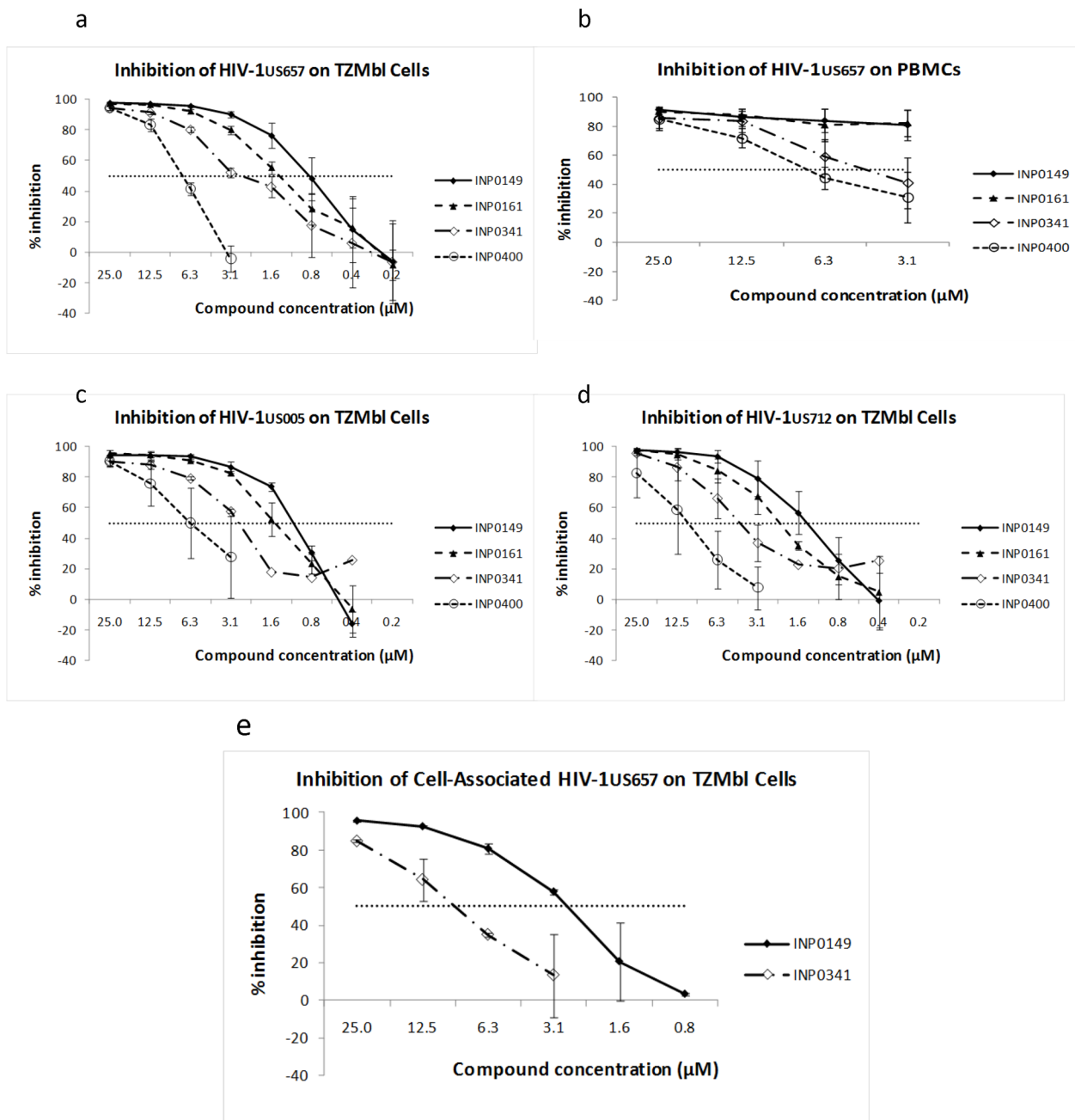
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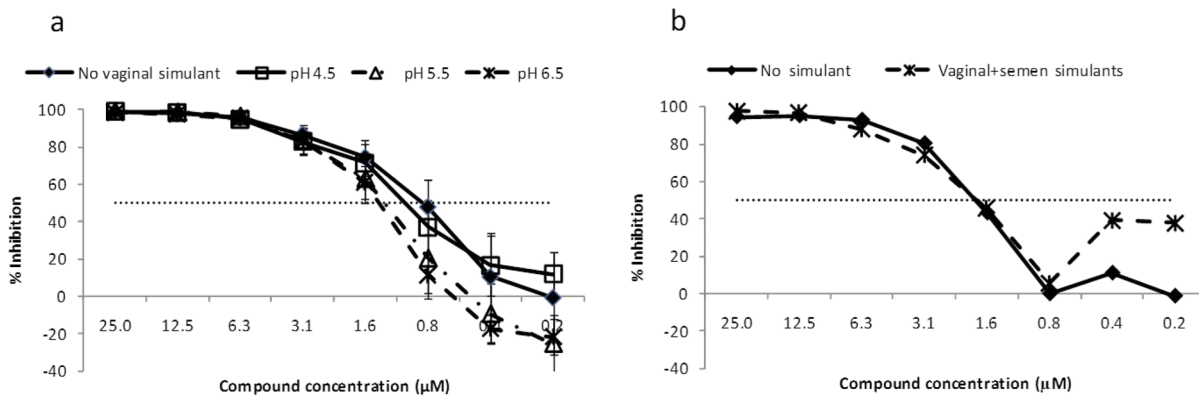
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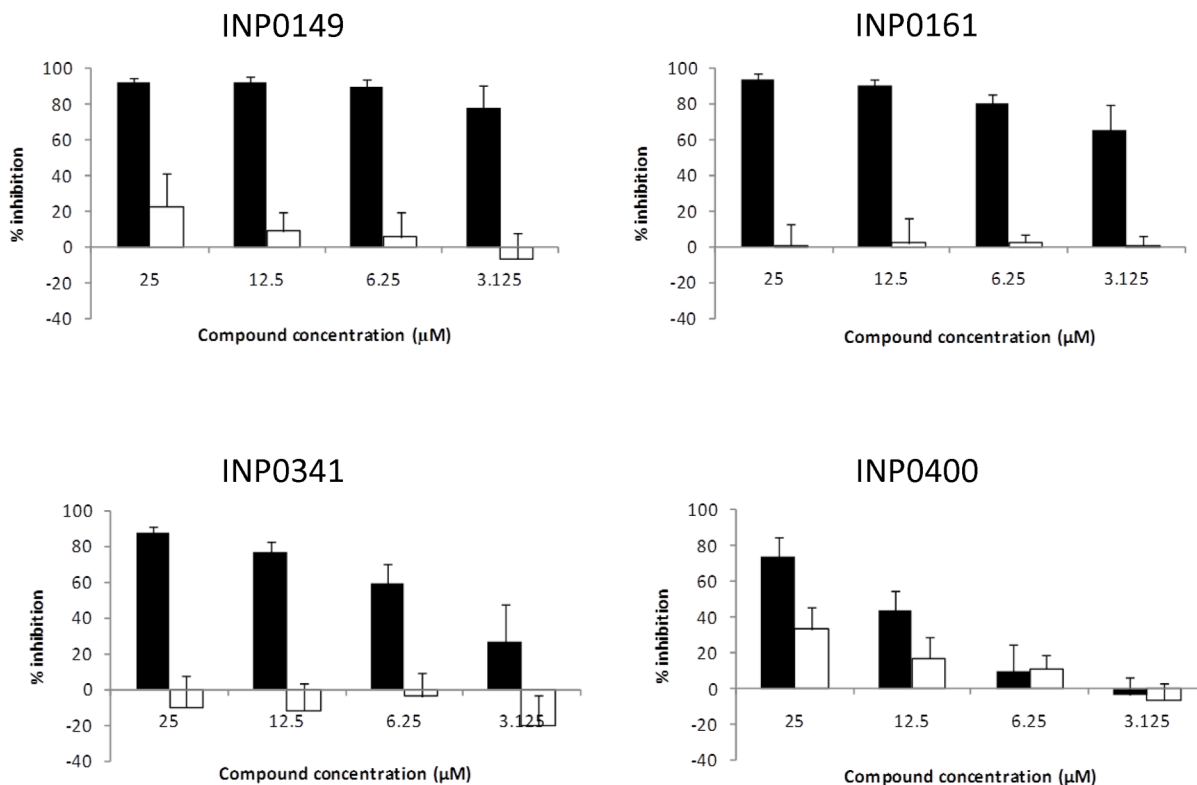
**Figure 1.**  
Structures of salicylidene acylhydrazides



**Figure 2.** HIV-1 is inhibited by salicylidene acylhydrazides using TZMbl indicator cells (a) or PHA-stimulated primary PBMCs (b) as targets for virus growth. A clinical clade B R5 strain (HIV-1<sub>US657</sub>) was used as cell-free virus with both target cells. Similar virus inhibition was observed with two additional clade B R5 strains, HIV-1<sub>US005</sub> (c) and HIV-1<sub>US712</sub> (d). The compounds also inhibit HIV-1 after exposure of TZMbl target cells to cell-associated virus (e), where CEM.NK<sub>r</sub>-CCR5 cells, infected for three days with HIV-1<sub>US657</sub> were incubated with TZMbl cells in the presence of compounds INP0149 or INP0341. Shown are means ± standard errors of assays repeated two or three times. Horizontal dotted line indicates 50% virus inhibition.



**Figure 3.** Vaginal fluid simulant at pH 4.5, 5.5 or 6.5 has little effect on inhibition of HIV-1 by the compounds (a). Inhibition of HIV-1<sub>US657</sub> by compound INP0149 is shown, and similar results were obtained with compounds INP0161, INP0341, and INP0400. Similarly, combined vaginal (pH 4.5) and semen (pH 7.7) simulants at a 1:1 vol/vol ratio (b) have little effect on compound activity. Horizontal dotted line indicates 50% virus inhibition.



**Figure 4.** Fe<sup>2+</sup> reverses the HIV-1 inhibitory activity of salicylidene acylhydrazides. Compounds were added in the absence (solid bars) or presence (open bars) of FeSO<sub>4</sub> (250 μM). Virus yield was determined using TZMbl cells. Data represent the mean and standard errors of 2–4 separate experiments.



**Table 1**Anti-HIV-1 activity of salicylidene acylhydrazides<sup>1</sup>.

<b>Compound</b>	<b>IC<sub>50</sub> (μM)</b>	<b>Cyt<sub>25</sub> (μM)</b>
INP0149	0.9	12.5
INP0161	1.3	>25
INP0341	2.3	>25
INP0400	7.3	>25

<sup>1</sup>Compounds were assayed against HIV-1US657, a primary R5 isolate, using TZMbl target cells. Percentage inhibition was calculated based on a DMSO control (no compound). IC<sub>50</sub> refers to concentration of compound resulting in 50% virus inhibition; Cyt<sub>25</sub> refers to concentration resulting in 25% cytotoxicity of TZMbl cells by propidium iodine staining.

**Table 2**

Effect of salicylidene acylhydrazides on an early HIV-1 reverse transcription product (R-U5 DNA) and on integrated viral DNA<sup>1</sup>.

<b>Compound</b>	<b>R-U5 DNA/HAPB</b>	<b>Integrated HIV DNA/HAPB</b>
Control (no compound)	17.3	9.8
INP0149	17.5	9.0
INP0161	30.6	ND
INP0341	39.2	7.7
INP0400	27.3	ND

<sup>1</sup> All compounds were tested at 25 $\mu$ M. Data are reported as DNA copies of HIV sequence per HAPB copy. HAPB serves as a “housekeeping” gene to adjust for the number of TZMbl cells from which DNA was extracted. ND = not done.