Protection of Mice From a *Chlamydia trachomatis* Vaginal Infection Using a Salicylidene Acylhydrazide, a Potential Microbicide

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The salicylidene acylhydrazide INP0341 inhibits growth of *Chlamydia* in HeLa cells, has negligible cell toxicity, and does not inhibit the growth of lactobacilli. The antichlamydial activity of INP0341 was retained when tested in vaginal and semen simulants. Vaginal tissue from INP0341-treated mice appeared similar to control sham-treated mice. To determine whether INP0341 can protect mice from a vaginal challenge, C3H/HeJ mice were either sham or INP0341 treated intravaginally pre- and postinoculation with $5 \times 10^2$ inclusion-forming units (IFUs) of *Chlamydia trachomatis* serovar D. Vaginal cultures taken over a month-long period showed a significant difference in the number of control mice that were culture positive versus the number in the INP0341-treated group, 100% (25/25) and 31% (8/26), respectively ($P < .05$). The quantity of IFUs shed and antibody titers to *Chlamydia* were significantly higher for the control group ($P < .05$). In summary, INP0341 is a promising compound to be considered for formulation as a vaginal microbicide.

*Chlamydia trachomatis* is a leading cause of sexually transmitted infections (STIs) and has been proposed as a main cause of infertility. *Chlamydia* infections and other STIs have been linked to increased acquisition and transmission of human immunodeficiency virus (HIV) [1–3]. Studies of populations with a high HIV incidence have found *Chlamydia* in as many as 17% of those infected with HIV [4]. Thus, reducing chlamydial infections may have the potential to reduce the transmission of HIV [5–7]. Although there are antibiotics that are effective against *Chlamydia*, infected individuals are often asymptomatic and do not seek treatment or do not have access to antibiotics. As with HIV, there is no effective human *Chlamydia* vaccine available, although much progress has been made in identifying key protective components of *Chlamydia* [8–11]. Therefore, in the absence of an effective vaccine for *Chlamydia*, alternative strategies are needed to control this STI agent. Among such approaches to STI control is the development of vaginal microbicides that provide short-term local protection [12, 13].

We recently investigated the ability of a group of salicylidene acylhydrazides to inhibit *C. trachomatis* [14]. We demonstrated that these compounds inhibit *Chlamydia* and *Neisseria gonorrhoeae* directly or indirectly through iron restriction. In contrast, when tested in vitro against hydrogen peroxide–producing lactobacilli, *Lactobacillus jensenii* and *Lactobacillus crispatus*, which are essential to the maintenance of normal vaginal flora, these compounds had little to no activity [15].

Previously we used N’-(5-chloro-2-hydroxy-3-methylbenzylidene)-2,4-dihydroxybenzhydrazide (INP0341) in a mouse model to see if it was able to attenuate a *Chlamydia* vaginal challenge up to day 4 after infection, a time when mice were receiving a daily vaginal dose of INP0341 [16]. We demonstrated a significant difference in *Chlamydia* levels...
between INP0341-treated and control mice. In this report, we have expanded our work with the mouse model using INP0341 as a vaginal microbicide, following mice for a month after infection, > 3 weeks after the last dose of microbicide was administered. Vaginal tissue exposed to multiple vaginal applications of the compound was examined histologically. In addition, we tested INP0341 in a simulated vaginal environment for up to 24 hours and over a pH range of 4.5–7.0 to determine the stability of the antichlamydial properties of the compound.

**MATERIALS AND METHODS**

**Organisms, Cell Lines, and INP0341**

We obtained *C. trachomatis* serovar D (UW-3) and HeLa 229 cells from the American Type Culture Collection. We raised HeLa cells and *Chlamydia* stocks as previously described [17]. We froze *Chlamydia* stocks at −80°C in 0.2 mol/L sucrose; 0.02 mol/L sodium phosphate, pH 7.2; 5 mmol/L glutamic acid (SPG).

INP0341 was synthesized from 5-chloro-2-hydroxy-3-methylbenzaldehyde and 2,4-dihydroxybenzhydrazide as described previously [18]. We recorded nuclear magnetic resonance (NMR) spectra in DMSO-d6 (residual DMSO-d5 δH = 2.50 ppm and DMSO-d6 δC = 39.52 ppm) using a Bruker spectrometer. We recorded mass spectra by detecting negative and positive molecular ions with an electrospray (ES) Waters Micromass ZG 2000 instrument using an XTerra mass spectrometry (MS) column (C18, 5 μmol/L, 4.6 × 50 mm) and a water-acetonitrile-formic acid eluent system. 1H NMR (recorded at 360 MHz): δH 12.09 (bs, 2H), 12.01 (s, 1H), 10.31 (s, 1H), 8.54 (s, 1H), 7.79 (d, 1H, J = 8.67 Hz), 7.42 (d, 1H, J = 1.87 Hz), 7.27 (d, 1H, J = 1.61 Hz), 6.39 (dd, 1H, J = 1.68, 8.77 Hz), 6.34 (d, 1H, J = 1.87 Hz), 2.20 (s, 3H) ppm. 13C NMR (recorded at 90 MHz): δC 165.01, 163.07, 162.03, 154.77, 148.40, 131.52, 130.05, 127.68, 127.24, 122.27, 118.65, 107.76, 105.89, 102.86, 15.33. MS (ES−) was calculated for C15H14ClN2O4 m/z 319.06 m/z (M − H)− and observed at 319.08 m/z. MS (ES−) was calculated for C15H14ClN2O4 m/z 321.06 m/z (M + H)+ and observed at 321.04 m/z. We dissolved INP0341 (Creative Antibiotics) in dimethyl sulfoxide (DMSO, Fisher Scientific) at a concentration of 25 mM and stored it at −20°C.

**Chlamydia Cultures**

HeLa 229 cells were grown to confluency in 1-dram glass shell vials overnight at 37°C in 5% carbon dioxide. Confluent monolayers were infected, incubated for 44–48 hours at 37°C in 5% carbon dioxide and stained as previously described using monoclonal antibodies (MAbs) to the *C. trachomatis* major outer membrane protein [17]. We viewed the resulting inclusion-forming units (IFUs) by light microscopy using an Olympus BH2 microscope.

**Stability of INP0341 in Vaginal-Fluid and Seminal-Fluid Simulants**

We prepared vaginal-fluid simulant (VFS) and seminal-fluid simulant as described by Owen and Katz [19, 20]. We made a series of VFS with a pH range of 4.5–7.0. The seminal-fluid simulant was adjusted to a pH of 7.7. We added INP0341 dissolved in DMSO to each simulant alone as well as to a 1:1 mixture of the simulants to achieve a final INP0341 concentration of 250 μmol/L. All mixtures were incubated at room temperature for 15 minutes. In addition, INP0341 was incubated in VFS for up to 24 hours. To test for compound activity, we added an aliquot of the INP0341–simulant mixture to newly infected *Chlamydia* cell cultures. The final concentration of INP0341 in the cell cultures was 25 μM. Cultures were incubated for 48 hours, fixed, stained with MAbs to *C. trachomatis*, and the IFUs were enumerated. Control cultures were processed in the same way as the compound-treated cultures minus INP0341. In experiments where the end point was infectious progeny, after incubation of the primary cultures, cultures were washed twice with SPG, sonicated in 0.5 mL SPG and this was used to infect HeLa cell monolayers as described previously [16]. On transfer and after 48 hours of incubation, cultures were fixed and stained and the IFUs and progeny were counted.

**Animal Model**

We used a mouse model to test the ability of INP0341 to attenuate a genital infection by *C. trachomatis* serovar D [11]. All experiments were approved by the University of California Irvine Institutional Animal Care and Use Committee. In each experiment, 7- to 8-week-old female C3H/HeJ (H-2k) mice (Jackson Laboratories) received 2 subcutaneous doses of 2.4 mg/ mouse of medroxyprogesterone acetate (SICOR Pharmaceuticals) on days 10 and 3 prior to a vaginal challenge with *C. trachomatis*. Mice were treated intravaginally with 0.05 mL of 1 mM INP0341 dissolved in 25% DMSO and diluted in SPG 2 days prior to challenge. 1 hour before challenge, at the time of infection, 4 hours and 8 hours after challenge, and daily for 5 days after the *Chlamydia* vaginal challenge. Control mice were sham treated, receiving SPG with DMSO without INP0341. All mice were infected on day 0 with 5 × 10⁵ IFU of *C. trachomatis* in 0.01 mL of Eagle minimal essential media (MEM, GIBCO). We collected vaginal swabs twice a week for a month after infection. Specimens were cultured, stained, and evaluated as previously described [11]. The experiments were repeated on 3 separate occasions with 5–11 mice per experimental group.

**Toxicity Testing on Vaginal Tissue**

Mice were given a single subcutaneous injection of medroxyprogesterone acetate, 1 mg (SICOR Pharmaceuticals) 3 days before being treated with 1 mM INP0341 in MEM containing 15% DMSO, or in the case of sham treated controls, the MEM-DMSO diluent. One pair of control mice was sacrificed immediately.
before all other mice received their first vaginal treatment and served as the normal controls. INP0341 treated and sham treated control pairs were sacrificed at 24-hour intervals following 1, 2 and 3 treatments that were administered at 24-hour intervals. On sacrifice, the vaginal tissue was harvested intact, cut into thirds as to retain the tubular structure, and immediately immersed in formalin and held until processed. Tissue was embedded in paraffin, sectioned and stained with hematoxylin-eosin.

**Antibody Titers**

Serum was collected at the start and on conclusion of the animal experiments. Antibody titers to elementary bodies (EBs) of *C. trachomatis* serovar D were established using an enzyme immunoassay [10]. *C. trachomatis* serovar D (UW3), EBs (10 μg/well in 100 μL of phosphate-buffered saline [PBS], pH 8.0) was used to coat 96-well microtiter plates of high protein-binding capacity (Corning) at 4°C overnight. The plates were washed with PBS–0.05% Tween 20, and blocked with 0.2 mL of the coated plates for 1 hour at 37°C 2-fold, from 1:100 to 1:204 800, in BLOTTO and incubated in goat antimouse immunoglobulin (IgG, IgA, and IgM; ICN Biomedical) was added, incubated for 1 hour at 37°C and washed as before. Signal was developed using a solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma) and hydrogen peroxide. Plates were read on a Multiskan RC reader (Thermo Electron) at 405 nm. Antibody titers were calculated by subtracting the background value obtained for normal mouse serum.

**Statistical Analysis**

Fisher exact test was used to determine differences between mouse groups as to the number of mice infected. The Mann–Whitney *U* test was performed to evaluate the number of IFUs recovered from the 2 groups of mice. In vitro data were analyzed by *t* test and Mann–Whitney *U* test using SigmaStat 3.5 software (SYSTAT Software). A *P* value < .05 was considered significant.

**RESULTS**

**Stability of INP0341 in Vaginal-Fluid Simulant and Seminal-Fluid Simulant**

Prior to testing the compound in vivo, we tested the stability of INP0341 in vitro using VFS over the pH range of 4.5–7.0, mimicking the range seen with human vaginal secretions [21]. Compared with controls, preincubation of INP0341 from 15 minutes up to 24 hours resulted in minimal effect to the antichlamydial activity of this compound as judged by infectious progeny (final concentration 25 μM) (Figures 1 and 2). Cultures to which INP0341 had been incubated in VFS at pH 6.5 and 7.0 compared with those incubated in simulant at pH 4.5 showed an increase in inclusion size that was easier to detect, yet considerably smaller than the control cultures without INP0341 (Figure 2). However, regardless of the pH of the VFS and the time of exposure up to 24 hours, the yield of infectious progeny in INP0341-treated cultures in comparison with those in control cultures, regardless of the simulant to which the compound was exposed was < 0.001% that of sham-treated controls (*P* < .001). Although more progeny were produced when the compound had been incubated with the SFS compared with the VFS, the resulting IFUs in the presence of the SFS was < 0.01% of control cultures. Error bars represent standard deviation (SD).

![Figure 1. Effect of vaginal-fluid and seminal-fluid simulants on the antichlamydial activity of INP0341. Vaginal-fluid simulant (VFS), seminal-fluid simulant (SFS), and a 1:1 mixture of the simulants were incubated with 250 μM of INP0341 for 15 minutes. Subsequently, the mixtures were added to HeLa cell monolayers along with *C. trachomatis* serovar D. After infected cultures were incubated for 48 hours, they were washed and transferred to fresh monolayers without simulant or INP0341. The resulting cultures that reflect *Chlamydia* progeny were then incubated for 48 hours, fixed and stained with a monoclonal antibody (MAb) to *C. trachomatis* and the resulting IFU were enumerated. There was a statistically significant reduction in inclusion-forming units (IFUs) in INP0341-treated cultures in comparison with those in control cultures, regardless of the simulant to which the compound was exposed (*P* < .001). Although more progeny were produced when the compound had been incubated with the SFS compared with the VFS, the resulting IFUs in the presence of the SFS was < 0.01% of control cultures. Error bars represent standard deviation (SD).](https://academic.oup.com/jid/article-abstract/204/9/1313/850061)
Compared with controls, cultures to which the simulant mixture containing INP0341 was added yielded a 4 log10 lower number of progeny compared with control cultures (P < .05) (Figure 1).

Effect of INP0341 on the Vaginal Tissue
We have previously reported that INP0341 has minimal toxicity when tested in vitro using HeLa cells [16]. To determine the effect of this compound on vaginal tissue, mice were inoculated vaginally with 0.05 mL of either 1 mmol/L of INP0341 in MEM containing 15% DMSO or the diluent alone. Mice were given a daily treatment and 2 pairs of control and INP0341 treated mice were sacrificed 24 hours after the first, second, and third treatments. There was little difference in the appearance of the vaginal mucosa throughout the entire vaginal canal in either the INP0341-treated or control mice (Figure 3). In all cases, the stratified squamous epithelium was intact and was of the same thickness in matching vaginal sections; the basal layer and submucosa were similar; no hemorrhagic lesions or blood vessel dilation was present; and the number of leukocytes, when present, was the same in both groups, with no obvious signs of inflammation in the INP0341-treated group. Therefore, INP0341 did not have any deleterious effects on the vaginal tissue even with multiple applications.

Ability of INP0341 to Protect Mice From a Chlamydia Vaginal Infection
In all, 25 sham-treated control and 26 INP0341-treated C3H mice were challenged with 5 × 10^2 IFU of C. trachomatis serovar D. Both groups were given multiple vaginal treatments up to 5 days after infection with either diluent alone (control group) or INP0341. We performed vaginal cultures twice a week over a 4-week period. For all time points, the number of control mice that were culture positive in comparison with the INP0341-treated mice was statistically significant (Figure 4). All 25 mice in the control group had at least 1 positive culture in the month following infection, whereas only 31% of the INP0341-treated mice (8/26) had ≥ 1 positive culture (P < .05).

The number of culture-positive control mice did not vary much throughout the first 2–3 weeks of infection but gradually declined in the last week (Figure 4). The number of culture-positive INP0341-treated mice increased the second week (days 11–17) and decreased slightly the third week. The last day INP0341 was administered was day 5, and one could speculate that this may be the reason that cultures performed on days 4 and 7 would have been lower than those taken on days 11–17, when the effect of the daily INP0341 treatment may have diminished.

The total number of IFUs recovered in the control group was statistically higher than that of the INP0341-treated group (P < .05) (Figure 5). In addition, with few exceptions, the number of IFUs recovered from vaginal cultures in individual mice was higher in the control group compared with that in the treated group. In both groups, the number of IFUs recovered gradually decreased throughout the month of observation.
Chlamydia Antibody Titers

We obtained sera from 20 control and 21 INP0341-treated mice 42 days after infection and evaluated the samples for the level of antibody to \textit{C. trachomatis} serovar D (Figure 6). The geometric mean titers for the control and treated groups were 16,890 and 913, respectively ($P < .001$). All INP0341 treated mice that were culture negative, with the exception of 1, had titers that ranged from undetectable ($< 100$) to 200. Only 1 mouse had a titer of 1600 despite negative vaginal cultures. There were 3 mice in the control group with titers below the detectable range ($< 100$). These 3 mice were the only culture-positive control mice that had a single positive vaginal culture, which in each case yielded $< 10$ IFU and was taken within the first 7 days after infection. Therefore, the early positive cultures may have represented inoculum rather than a true infection, especially in light of the lack of antibody response. With the exception of the single culture-negative, INP0341-treated mouse with a positive antibody titer (1600), the vaginal cultures mirrored the level of antibody response to \textit{C. trachomatis}.

DISCUSSION

The prevention of sexually transmitted infections (STI) in light of the explosive HIV epidemic, has taken on a new dimension. Many of the STIs, including \textit{C. trachomatis}, have been associated with an increase in transmission of HIV \cite{22,23}. It is estimated that over half of the genital infections caused by \textit{Chlamydia} are asymptomatic, therefore many infected individuals remain undiagnosed. Furthermore, in areas of the world where access to antibiotics is limited, many individuals remain untreated. The transmissibility of this agent and the sequela resulting from an...
infection, namely infertility and increased susceptibility to HIV, are reasons for developing alternative strategies to reduce infection and thus transmission of this pathogen. Although there has been much effort in developing an effective vaccine, we currently do not have a human vaccine for *C. trachomatis*. An alternative strategy is the development of vaginal microbicides, which would allow females to protect themselves from infection and thus prevent the transmission of *C. trachomatis* to their sexual partners and newborns [13].

We have previously described that INP0341, when tested in vitro, attenuates the infectivity of *C. trachomatis* serovar D, one of the most common genital strains, as well as the more aggressive lymphogranuloma venereum strain serovar L2 [14, 16]. This inhibitory compound class was first thought to act primarily by interrupting the type 3 secretion (T3S) pathway of this pathogen [24, 25]. We have also shown that the inhibition seen with this compound can be reversed specifically by the addition of ferrous or ferric ion [14]. Furthermore, the inhibitory activity of this compound toward *Chlamydia* can be reversed by the addition of holo-transferrin but not apo-transferrin. This finding led to the hypothesis that INP0341 works directly or indirectly by sequestering iron within the host cell, ultimately resulting in the inability of *Chlamydia* to develop within the host.

Iron restriction is a mechanism used by the host as a defense against pathogens that also need iron to survive [26]. Host proteins such as ferritin, transferrin, and lactoferrin have the ability to bind iron and thus sequester it from invading microorganisms. Pathogens have also developed a way to hijack iron from the host iron withholding defense system and depending on the organism these mechanisms vary. Compounds that interfere with this “tug of war” between host and pathogen in favor of the host have the potential to be a powerful weapon in the fight against infectious diseases. *N. gonorrhoeae* is a pathogen, like *C. trachomatis*, that needs iron to survive and as such has developed multiple mechanisms for iron acquisition from the host [27, 28]. The compound described in this report is also bactericidal against *N. gonorrhoeae*, thus it appears to have multiple STI targets [16].

In developing a vaginal microbicide, we must take into account many factors [29]. Among them is the effect of the microbicide on members of the normal vaginal flora. Lactobacilli

![Figure 5](https://academic.oup.com/jid/article-abstract/204/9/1313/850061)

**Figure 5.** *Chlamydia* inclusion-forming units (IFUs) obtained from vaginal cultures for control (○ or □, *n* = 25) and INP0341-treated (▲ or ■, *n* = 26) mice. Shown are the IFU counts for vaginal cultures obtained at the end of each week. The median number of IFUs is indicated by the horizontal line. Culture-negative mice (○ or △) and culture-positive (● or ▲) mice are shown in each group.

![Figure 6](https://academic.oup.com/jid/article-abstract/204/9/1313/850061)

**Figure 6.** Antibody titers to *Chlamydia* for sera obtained 42 days after vaginal inoculation of mice with *C. trachomatis* serovar D. Titers were measured by an enzyme immunoassay as described in Materials and Methods. All control mice (●, *n* = 20) were culture positive. INP0341-treated mice (*n* = 21) were both culture positive (▲) and culture negative (■). Horizontal lines represent the median titer in both control and INP0341-treated groups.
are critical in maintaining the homeostasis of the vaginal tract [15, 30]. In particular L. jensenii and L. crispatus, hydrogen peroxide–producing lactobacilli, have been shown to play an important role in maintaining the balance of bacterial and yeast flora in the vagina. Lactobacillus species do not depend on iron to survive; they instead rely on manganese and cobalt [31]. Therefore, it is not too surprising that when L. jensenii and L. crispatus were tested in vitro against INP0341, there was no effect on the growth of these 2 organisms, whereas the same concentrations proved microbicidal to both C. trachomatis and N. gonorrhoeae [16].

Microbicides also need to be effective across a broad pH range, that is, 4.2–7.5. Although the normal vaginal pH is ~4.5, it is increased during menstruation, intercourse, or as a result of STIs such as with Trichomonas [21]. As shown in this report, INP0341 showed a slight reduction in antichlamydial activity when tested in a VFS at a fairly high vaginal pH, 6.5 or 7.0; however, the compound-treated cultures were still significantly lower in infectious progeny than control cultures not incubated with INP0341. When tested with a seminal-fluid simulant at pH 7.7 or a 1:1 mixture of the simulants as to mimic coitus, there was a slight decrease in the antichlamydial activity of the compound. However, even here the INP0341-treated cultures were still inhibited, yielding < 0.1% infectious progeny compared with that of controls.

Although in vitro testing is crucial to the initial identification of promising microbicidal agents, in vivo animal models are essential in assessing different microbicidal candidates prior to and after formulation. In this report, we have shown that upon infection with C. trachomatis, INP0341 was able to significantly protect mice from a vaginal infection. Even after the “pressure” of the microbicide was removed, most mice remained culture negative throughout the observation period that extended 3 weeks after the last vaginal administration of the compound. Of the culture-positive INP0341 treated mice, the yield of IFUs from the vaginal cultures was significantly less than that from control mice. Therefore, even lowering the infectious burden may be important in reducing transmission of this pathogen.

We have shown that INP0341 is stable for retaining activity against C. trachomatis when tested in a simulated vaginal and coital environment; does not affect key members of the vaginal microbial flora, notably hydrogen peroxide–producing lactobacilli; has no deleterious effects on the integrity of the vaginal mucosa; has minimal in vitro cytotoxicity; does not produce an inflammatory response in the mouse vagina; and is able to protect mice from a Chlamydia vaginal infection. The next logical steps toward development of a microbicide for human use are formulation of the inhibitory agent into a vehicle with the desired pharmacokinetics, acceptance, and adherence [32]. Only with clinical trials will we know the ultimate effectiveness of the strategy of using iron restriction to fight STIs; if successful, this approach can play a significant role in controlling this most important human pathogen.

Notes

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References


