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Candidate vaginal microbicides with activity against *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

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Abstract

Vaginal microbicides with activity towards organisms that cause sexually transmitted infections have been proposed as a strategy to reduce transmission. Small-molecule inhibitors of *Chlamydia trachomatis* serovar D belonging to the class of salicylidene acylhydrazides (INPs) have been shown to work through a mechanism that involves iron restriction. Expanding on this work, ten INPs were tested against a lymphogranuloma venereum strain of *C. trachomatis* serovar L2, *Neisseria gonorrhoeae*, and hydrogen peroxide-producing *Lactobacillus crispatus* and *Lactobacillus jensenii*. Seven INPs had minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations of <50 µM towards *C. trachomatis* L2. Three INPs had an MIC <12.5 µM against *N. gonorrhoeae*. Inhibition by was reversed by iron, holo-transferrin and holo-lactoferrin but not by the iron-poor forms of these compounds. The compounds exhibited no bactericidal activity toward *Lactobacillus*. The INPs were not cytotoxic to HeLa 229 cells. When INP 0341 was tested in a mouse model of a *Chlamydia* vaginal infection there was a significant reduction in the number of mice shedding *C. trachomatis* up to 4 days after infection ($P < 0.01$). In summary, select INPs are promising vaginal microbicide candidates as they inhibit the growth of two common sexually transmitted organisms in vitro, are active in a mouse model against *C. trachomatis*, are not cytotoxic and do not inhibit organisms that compose the normal vaginal flora.

Keywords

Vaginal microbicide; Sexually transmitted infections; *Chlamydia trachomatis*; LGV; *Neisseria gonorrhoeae*

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Competing interests

None declared.

Ethical approval

Not required.

1. Introduction

Prevention of sexually transmitted infections (STIs) in the era of the human immunodeficiency virus (HIV) epidemic has taken on new importance [1–6]. As such, multiple strategies in addition to antibiotics and antivirals are needed to address this problem since the context for transmission of STIs varies greatly depending on geographical, economic and social situations. Progress in vaccine development for most STIs is still at the bench level; currently, the only STI vaccine available is for high-risk types of human papillomavirus. Meanwhile, pathogens such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* not only have the potential to cause significant morbidity on their own but have been linked to the increased transmission of HIV [1,2].

One strategy to reduce the transmission of STIs is vaginal microbicides [7,8]. In part this approach has been proposed based on the overall acceptance rate of contraceptive vaginal spermicides. Several agents have been proposed for incorporation into a vaginal gel and some are currently under development [8–12]. We have recently described a salicylidene acylhydrazide, INP 0341, that when tested in vitro attenuates the infectivity of a common genital strain, serovar D, of the intracellular pathogen *C. trachomatis* [13].

In this study, we have expanded our investigation of salicylidene acylhydrazides (INPs) against two STIs, a lymphogranuloma venereum (LGV) strain of *C. trachomatis* (serovar L2) and *N. gonorrhoeae*, as well as against bacterial organisms that are members of the normal human vaginal flora. In addition, INP 0341 was tested in a mouse model to determine whether this compound could attenuate a *Chlamydia* genital infection. A compound able to selectively attenuate both *C. trachomatis* and *N. gonorrhoeae* while not affecting the normal bacterial flora or vaginal mucosa would be an excellent candidate to formulate and test as a vaginal microbicide.

2. Materials and methods

2.1. Organisms and cell lines

Chlamydia trachomatis serovar L2 (434), *C. trachomatis* serovar D (UW-3), *N. gonorrhoeae* (ATCC 49226 and ATCC 43069), *Lactobacillus jensenii* strain 6G (ATCC 25258), *Lactobacillus crispatus* (ATCC 33197), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Neisseria mucosa* (ATCC 19695), *Neisseria lactamica* (ATCC 23970), *Neisseria flava* (ATCC 13120) and HeLa 229 cells were obtained from the American Type Culture Collection (Manassas, VA). In addition, a clinical isolate of *Neisseria subflava* was included in the testing. *Neisseria gonorrhoeae* strains FA 1090, FA 7163, FA 7034 and FA 6916 were obtained from the laboratory of Dr Fred Sparling (University of North Carolina, Chapel Hill, NC) [14,15].

HeLa cells and *Chlamydia* stocks were raised as previously described [16]. *Neisseria* spp. were maintained on chocolate agar (BD Becton Dickinson, Franklin Lakes, NJ), *E. coli* and *E. faecalis* on 5% sheep blood agar (BD Becton Dickinson) and *Lactobacillus* spp. on Difco™ Lactobacilli MRS agar (BD Becton Dickinson). All plates were incubated at 37 °C in 5% CO₂ for 48 h. Organisms were subcultured twice prior to being used in an assay.

2.2. Chemicals and salicylidene acylhydrazides (INPs)

INPs (Creative Antibiotics, Umeå, Sweden) were dissolved in dimethyl sulphoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ) at a concentration of 25 mM and stored at –20 °C. Ferrous ammonium sulphate (FeSO₄) (Sigma-Aldrich, St Louis, MO) was diluted in distilled water and filter-sterilised (0.22 µM) prior to use. Stock solutions (500 nM) of human holo-transferrin and human apo-transferrin (Sigma-Aldrich) were dissolved in sterile water, and human

lactoferrin saturated with iron (Sigma-Aldrich) and human apo-lactoferrin (Sigma) were dissolved in sterile phosphate-buffered saline and stored at -20°C .

2.3. Chlamydia cultures

HeLa 229 cells were grown to confluency in 1-dram glass shell vials overnight at 37°C in 5% CO_2 . Confluent monolayers were infected and stained as previously described [16]. The resulting inclusion-forming units (IFU) were viewed using an Olympus BH2 microscope (Olympus, Center Valley, PA), enumerated and compared with untreated controls.

2.4. Cytotoxicity assay

A lactate dehydrogenase (LDH) assay was used to determine the cytotoxic effects of INPs on HeLa cells. The LDH assay was performed with INP-treated and untreated HeLa cells using the Cytotox-96 kit (Promega, Madison, WI) according to the manufacturer's instructions. The percentage of LDH leakage was calculated using the formula $(\text{LDH activity in medium}/\text{total LDH} \times 100)$ [17]. Each sample was assayed in triplicate.

2.5. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs)

Serial dilutions of INPs (1.5–100 μM) in DMSO were added to brain–heart infusion (BHI) broth (BD Becton Dickinson) with and without supplementation with 2% IsoVitaleX (BD Becton Dickinson) in 96-well microtitre plates. A standardised suspension of bacteria from an overnight culture was added to achieve 1×10^5 colony-forming units (CFU)/mL. Plates were incubated at 37°C in 5% CO_2 for 24 h and subsequently the MIC was defined as the lowest concentration of the compound that prevented macroscopic growth.

To determine the MBC_{99} (the lowest concentration of INP at which the bacterial count was reduced to <99% of the inoculum), aliquots were cultured from wells with no visible growth and the highest INP concentration with visible growth. *Neisseria* spp. were subcultured on chocolate agar, *Lactobacillus* spp. on MRS agar, and *E. faecalis* and *E. coli* were subcultured on 5% sheep blood agar. All plates were incubated at 37°C in 5% CO_2 for 24–48 h.

The MIC for *C. trachomatis* L2 was determined by infecting HeLa cell monolayers at a multiplicity of infection of 0.2 IFU with two-fold dilutions of INPs (0.75–50 μM). Following incubation for 36 h, one set of cultures was fixed and stained to determine the MIC and the transitional MIC (MIC_{TP}) and one set was sonicated and passed to fresh monolayers to determine the MBC. The MIC_{TP} was the concentration of the compound at which abnormal inclusion size was observed for >90% of the inclusions, the MIC was the next highest concentration tested, and the MBC was the lowest concentration of the compound where <99% IFU were observed after passage to HeLa cells compared with control cultures [18].

2.6. Iron reversal assays

Neisseria gonorrhoeae grown in BHI broth with IsoVitaleX was incubated with the INP with and without free iron (Fe^{2+}), iron-rich human holo-transferrin or human holo-lactoferrin and their corresponding iron-poor (apo) forms. These compounds were also added to INP-treated *C. trachomatis* L2 cultures before centrifugation onto HeLa cell monolayers. Following incubation, cultures were fixed, stained and inclusions were counted as previously described [16]. *Neisseria* cultures were incubated for 24 h before subculture to chocolate agar. The average number of CFU generated by the INP-treated *N. gonorrhoeae* cultures with and without an added iron source was compared with cultures incubated in media alone. All assays were performed in triplicate.

2.7. Genomic DNA isolation and purification

Total genomic *Chlamydia* DNA was isolated from cell cultures at time 0, immediately after centrifugation (early) and 36 h (late) post infection. The Promega Wizard® Genomic DNA Purification Kit (Promega) was used to extract genomic DNA according to the manufacturer's instructions. DNA samples were precipitated, washed, reconstituted in 10 mM HEPES (pH 8.0), stored at -20 °C and/or used in real-time quantitative polymerase chain reaction (qPCR) assay. An ultraviolet spectrophotometer was used to determine the DNA concentration in each sample.

2.8. Real-time quantitative PCR

Plasmid control constructs were made from *C. trachomatis* L2 *ompA*, which was amplified using the primers 5'-ATAAGAATGCGGCCGCAATGAAAAA CTCTTGAAA-3' and 5'-GACTAGTTTAGAAGCGGAATTGTGCATTTACGTGA-3'. The PCR product and the pTRG vector (Stratagene, La Jolla, CA) were treated with *NotI* and *SpeI* enzymes (New England Biolabs, Beverly, MA) prior to ligation and transformation of *E. coli* DH5α (New England Biolabs). The resulting construct (pTRG-*ompA*) was propagated in *E. coli* and was subsequently purified using the Qiagen Miniprep Kit (QIAGEN, Valencia, CA).

A qPCR assay employing the AB Biosystems SYBR Green (PE Applied Biosystems, Warrington, UK) reaction mix and method was used to determine the amount of chlamydial DNA in INP-treated and untreated cultures. A 174-bp segment was amplified from the pTRG-*ompA* template. A 7-point standard curve was generated by amplifying known amounts of pTRG-*ompA* templates. Input DNA for all samples was normalised by DNA concentration (ng/μL). Thermal cycling reaction conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 54 °C for 30 s and 72 °C for 30 s. To determine relative numbers of *Chlamydia* genomic copies in each sample, threshold cycle (Ct) values between the standard and the test samples were compared [19]. Assays were performed in triplicate.

2.9. Animal model

A mouse model, as previously described, was used to test the ability of INP 0341 to attenuate a genital infection by *C. trachomatis* serovar D [20]. Seven-to-eight-week-old female C3H/HeJ (H-2^k) mice (Jackson Laboratories, Sacramento, CA) received two subcutaneous doses of medroxyprogesterone acetate (SICOR Pharmaceuticals, Inc., Irvine, CA) on Days 10 and 3 before a vaginal challenge with 1×10^3 IFU of *C. trachomatis* in 0.01 mL of 0.2 M sucrose/0.02 M sodium phosphate (pH 7.2) (SPG). Mice were treated intravaginally 2 days prior to challenge, 1 h before, with the challenge dose of *Chlamydia*, 4 h and 8 h after challenge, and daily up to 5 days after vaginal challenge with 0.02 mL of 1 mM INP 0341 dissolved in 20% DMSO and diluted in SPG. Control mice were treated the same but received SPG with DMSO without INP 0341. Vaginal swabs were collected 4 days after infection and were cultured, stained and evaluated as previously described [20]. The experiment was repeated three times with 5 to 15 mice per experimental group.

2.10. Statistical analysis

Fisher's exact test was used to determine differences between mouse groups regarding the number of mice infected. Mann-Whitney *U*-test was performed to evaluate the number of IFU recovered from two groups of mice. In vitro data were analysed by Student's *t*-test and Mann-Whitney *U*-test using SigmaStat 3.5 software (SYSTAT Software, Inc., Richmond, CA). A *P*-value of <0.05 was considered significant.

3. Results

3.1. Salicylidene acylhydrazide (INP) inhibition of *Chlamydia trachomatis* L2

The effect of ten INPs at a concentration of 25 μ M against *C. trachomatis* L2 could be grouped by light microscopy into those that gave maximum, moderate or no appreciable inhibition (Fig. 1). INPs 0341, 0149, 0161, 0029 and 0007 were the most inhibitory, yielding either no inclusions or inclusions that were difficult to see by light microscopy owing to their abnormally small size. Cultures incubated in the presence of INPs 0400 and 0269 resulted in *Chlamydia* inclusions with great heterogeneity in size and appearance. INPs 0328, 0143 and 0406 did not appear to inhibit *Chlamydia* growth compared with control cultures.

To quantitate the inhibitory effect of the compounds, copies of *C. trachomatis* L2 *ompA* in 36 h cultures were measured by qPCR. The yield of *Chlamydia* DNA corresponded to the appearance of the L2 inclusions (Fig. 1). INPs giving maximal inhibition yielded from 0.6% to 1.7% ($\log_{10}$ 3.25 to 3.67 vs. 5.45) of *Chlamydia* DNA compared with control cultures ($P < 0.05$). INPs 0400 and 0269 with moderate inhibition yielded *Chlamydia* DNA that was 4.3% to 5.1% ($\log_{10}$ 4.07 to 4.15 vs. 5.45) compared with control cultures ($P < 0.05$). INPs 0328, 0143 and 0406 yielded *Chlamydia* DNA amounts similar to control cultures ($P > 0.05$).

The MIC_{TP}, MIC and MBC for the INPs correlated with light microscopy and qPCR performed with 25 μ M INP (Fig. 1). Corroborating the qPCR data, the two compounds with the lowest MIC/MBC were INP 0341 and INP 0149, whilst INPs 0143, 0328 and 0406 exhibited no inhibitory activity.

3.2. Salicylidene acylhydrazide (INP) effect on *Neisseria gonorrhoeae* and normal vaginal flora

The MICs/MBCs of the INPs were established for *N. gonorrhoeae*, commensal *Neisseria* spp., hydrogen peroxide-producing *L. jensenii* and *L. crispatus*, *E. faecalis* and *E. coli*. No inhibition of growth of *N. gonorrhoeae* was seen with INPs 0328, 0400 and 0406. In contrast, INPs 0007, 0029 and 0341, which also exhibited good activity against *C. trachomatis*, gave the lowest MIC for *N. gonorrhoeae* at 12.5 μ M. With few exceptions, the MBC and MIC values were the same, suggesting a bactericidal effect of the active compounds towards *N. gonorrhoeae*. In contrast, the commensal *Neisseria* strains tested were less affected by the compounds, with MICs one- to two-fold higher than *N. gonorrhoeae*.

None of the INPs had bactericidal activity up to 100 μ M against either *L. jensenii* or *L. crispatus*, two of the most common hydrogen peroxide-producing members of the vaginal normal flora. *Escherichia coli* was not inhibited by any of the compounds tested. *Enterococcus faecalis* was inhibited by INP 0007, 0341 and 0400, with MICs ranging from 12.5 μ M to 50 μ M. With these three compounds, the MIC and MBC values were the same or within one dilution, suggesting a bactericidal effect against *E. faecalis*.

3.3. Reversal of salicylidene acylhydrazide (INP) inhibition

HeLa cells infected with *C. trachomatis* L2 were incubated for 36 h with and without INP 0341 (25 μ M) along with the iron-rich holo-form of lactoferrin and transferrin and their corresponding iron-poor apo-forms as well as iron supplied as Fe²⁺. Control cultures without INP 0341 but with added Fe²⁺, holo- and apo-lactoferrin or holo- and apo-transferrin appeared similar to control cultures, therefore none of the added iron sources alone had an effect on the number or appearance of *Chlamydia* inclusions. Normal inclusions were not seen in cultures incubated with 25 μ M INP 0341 compared with the control. However, when holo-transferrin, holo-lactoferrin or Fe²⁺ was added, the inhibitory effect of INP 0341 was abrogated. In contrast,

there was no reversal of the inhibitory effect when cultures containing INP 0341 were incubated with apo-transferrin or apo-lactoferrin.

To investigate whether iron-binding compounds were able to reverse the inhibitory effect of INP 0341 on *N. gonorrhoeae*, in addition to the two ATCC strains that contained the full complement of lactoferrin and transferrin genes, transferrin and lactoferrin mutants were obtained from Dr Fred Sparling. One of these strains (FA 1090) is a human isolate that was found to lack lactoferrin receptor protein expression but expressed the transferrin receptor proteins [15]. The other three strains have been constructed in vitro to either possess the full complement of lactoferrin and transferrin genes [FA 7163 (lactoferrin+/transferrin+)] or to lack one or both of the lactoferrin and/or transferrin genes [FA 6916 (lactoferrin-/transferrin-) and FA 7034 (lactoferrin+/transferrin-)] [14]. Unlike the ATCC strains, the laboratory-constructed strains did not grow well in BHI broth alone therefore the MICs/MBCs of all strains were compared using BHI broth supplemented with 2% IsoVitalEx (Table 1). There was no significant difference in the MIC/MBC whether or not the strains possessed genes for the lactoferrin or transferrin receptor proteins. The general pattern of activity of the ten INP compounds was the same for all *N. gonorrhoeae* strains, with INPs 0007, 0029 and 0341 being the most inhibitory, similar to that seen with *C. trachomatis*.

The same iron-carrier proteins used to test for reversal of inhibition of *C. trachomatis* by INP 0341 were used in a bactericidal assay with *N. gonorrhoeae* (Fig. 2). Here, 1×10^5 CFU/mL in BHI broth supplemented with IsoVitalEx was incubated overnight with and without INP 0341 (6.25 μ M). With the three strains of *N. gonorrhoeae* that had the full complement of lactoferrin and transferrin genes (ATCC 49226, ATCC 43069 and FA 7163), free iron supplied as FeSO₄, holo-lactoferrin and holo-transferrin, but not apo-lactoferrin and apo-transferrin, were able to reverse the inhibition by INP 0341. In contrast, these iron sources were not able to reverse the inhibition in laboratory-constructed strains lacking one or more of the lactoferrin and transferrin receptor genes.

3.4. Cytotoxicity

LDH measurement of INP-treated cells incubated for up to 48 h showed no significant membrane damage as determined by the lack of leakage of LDH into the supernatant media ($P > 0.05$) (data not shown).

3.5. Mouse model

Mice were treated intravaginally with 1 mM INP 0341 prior to, at the time of and after infection with 1×10^3 IFU of *C. trachomatis* serovar D. Control mice were treated the same as the INP-treated group minus INP 0341. There was a significant difference 4 days after infection between the two groups in terms of the number of mice infected as judged by a vaginal culture ($P = 0.013$). Of the control mice, 17/20 (85%) were culture-positive, in contrast to the INP-treated mice where 12/25 (48%) were culture-positive 4 days after infection. There was also a significant difference in the number of IFU recovered 4 days after infection (Fig. 3) ($P < 0.01$).

4. Discussion

STIs remain a worldwide problem especially in light of their association with increased HIV acquisition [1–6,8]. Vaginal microbicides have been proposed as an approach to reduce the spread of STIs [7–9], an attractive concept since it would afford women the ability to protect themselves [21]. A limited number of formulations of vaginal microbicides have been investigated in clinical trials, but in general results have been disappointing [22,23]. Some of this is due to the fact that they were shown to damage the vaginal epithelium resulting in inflammation and ulceration that ultimately led to an increase in STIs. The work presented here

lays the foundation for the development of a vaginal microbicide based on local iron withholding as a strategy to selectively attenuate pathogenic bacterial growth. The ability of pathogenic organisms to acquire iron is essential to their survival [24]. However, the human host keeps iron tightly regulated and bound to limit the free supply available. We have explored the concept of introducing iron-sequestering compounds into this ‘tug-a-war’ between host and pathogen in order to favour the host and thus limit the infectivity of the pathogen.

The INPs used in this report were originally described to interfere with the type three secretion system (T3S) of several virulent Gram-negative bacilli including *Yersinia* and *Chlamydia* [13,25–27]. In an attempt to dissect the mechanism for this apparent effect on the *Chlamydia* T3S, we explored their role as a chelating agent. Using different metal ions to reverse the inhibition of the genital pathogen *C. trachomatis* serovar D, we found this effect to be specifically reversed by iron [13]. Any effect these compounds had on host cell proliferation was also reversed by iron, leading us to hypothesise that these compounds were able to indirectly limit *Chlamydia* growth by restricting available iron within the host. *Chlamydia*, like most intracellular pathogens, is dependent upon host cell iron supplies [28,29].

With the ultimate goal of developing a vaginal microbicide, we wanted to expand our findings to determine whether other genital bacterial pathogens would be inhibited by these compounds. LGV serovars of *C. trachomatis* are more aggressive and invasive than the other genital serovars of *C. trachomatis*. LGV strains are prevalent in underdeveloped nations, many of which also have a high prevalence of HIV. However, over the last decade there have been several reports of increasing numbers of LGV from countries in Western Europe and North America [30,31]. The results obtained with the INPs for *C. trachomatis* serovar L2 mirrored those that we had previously reported for serovar D. We also wanted to determine whether lactoferrin could compete with INPs since it is in high concentration in the vaginal tract and a key protein in maintaining iron homeostasis. Apo-lactoferrin did not interfere or compete with the inhibitory properties of the INP; only when it was saturated with iron in its holo form did it reverse the INP effect.

Neisseria gonorrhoeae was also inhibited by the INPs and in general those compounds that were effective against *C. trachomatis* also had activity toward *N. gonorrhoeae*. This pathogen also requires iron for growth and has multiple mechanisms for acquiring iron, including high-affinity receptors for both transferrin and lactoferrin [14,32,33]. We attempted to determine whether natural and laboratory strains lacking lactoferrin or transferrin receptors were inhibited by the INPs and, if so, could the inhibition be reversed by the iron-rich form of either lactoferrin or transferrin. Our results showed that INP inhibition could be reversed by an iron source with strains possessing the full complement of lactoferrin and transferrin receptor genes, but was not reversed with strains lacking one or both set of genes coding for these receptors. The reason for this is not clear, but resolving this may add to our understanding of the complex iron acquisition systems in *N. gonorrhoeae* that have both unique features but also share some common pathways within the bacterial membrane. Results with *N. gonorrhoeae* corroborated those obtained with *Chlamydia*, suggesting that iron withholding is one, if not the main, mechanism by which the INPs attenuate the growth of these STI agents.

When developing a vaginal microbicide, candidate compounds should have minimal activity toward organisms that comprise the normal vaginal flora [34,35]. The hydrogen peroxide-producing lactobacilli have been shown to be part of the innate vaginal defence, being essential in maintaining homeostasis of the vaginal tract [36]. Since lactobacilli are somewhat unique in that they do not require iron for growth but instead rely on manganese, we reasoned that INPs should not have appreciable activity against these organisms [37]. To test this hypothesis, both *L. crispatus* and *L. jensenii*, two of the lactobacilli most frequently recovered from the normal human vaginal tract [34,35], were employed. None of the compounds tested had

bactericidal activity towards *L. crispatus* and *L. jensenii*. Therefore, whilst the compounds were able to kill the genital pathogens, they had minimal to no effect on organisms considered essential for the maintenance of pH and bacterial balance in the vagina.

In the vaginal milieu many factors may compromise the activity of the INPs. Among them are the vaginal secretions, pH, lactoferrin and blood cells. In addition, a microbicide needs to be carefully formulated for ease of delivery and retention of activity. To begin to address the in vivo activity of the INP, a genital mouse model of a *Chlamydia* infection was employed. Here it was possible to demonstrate that the group of mice that were INP-treated had a significantly lower rate of infection compared with the control untreated group. These experiments demonstrate the proof of principle that INP 0341 can attenuate *C. trachomatis* infection in vivo.

In conclusion, the in vitro results presented here demonstrate that select INPs, in particular 0029 and 0341, inhibited both *C. trachomatis* and *N. gonorrhoeae*, had minimal to no effect towards organisms considered to be part of the normal vaginal flora, were not cytotoxic to HeLa cells and were active in vivo. These findings suggest that they are good candidates to consider further for formulation and continued testing as a vaginal microbicide.

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Fig. 1.

Quantitation of *Chlamydia trachomatis* L2 DNA by quantitative polymerase chain reaction (qPCR). *Chlamydia*-infected HeLa cell cultures in the presence of 25 μM of the indicated salicylidene acylhydrazide (INP), as shown on the x-axis, were harvested 36 h after infection and the DNA was isolated. Primers for *ompA* were used to quantitate *Chlamydia* DNA. The horizontal dashed lines represent the *Chlamydia* DNA copies from control cultures without INPs at time 0 (input or *Chlamydia* inoculum) and at 36 h after infection. Error bars represent $\pm$ standard deviation. The INPs could be grouped by the level of inhibition into those giving: (A) maximum inhibition, <2% of *Chlamydia* DNA compared with control cultures with no INP present at 36 h; (B) moderate inhibition, ca. 5% of control; and (C) mild inhibition, 60–80% of control. Below the graph is a representative picture of the inclusion appearance at 36 h for infected cultures incubated in the presence of an INP from each of the three groupings: (A) INP 0341, (B) INP 0400 and (C) INP 0406, representing maximum, moderate and no significant inhibition, respectively. The transitional minimal inhibitory concentration (MIC_{TP}), minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values in μM are shown for the ten INPs tested. The values paralleled those seen with qPCR and light microscopy in that the INPs could be grouped into three categories.

Fig. 2.

Reversal of the inhibitory effect of the salicylidene acylhydrazide INP 0341 on *Neisseria gonorrhoeae*. Microtitre plates were inoculated with a standardised suspension of an overnight culture of *N. gonorrhoeae* with a final concentration of 1×10^5 colony-forming units (CFU)/mL. Cultures were incubated without or with 6.25 μ M INP 0341. In addition, cultures were incubated with and without each of the various iron sources as shown: 250 μ M Fe²⁺ and 10 nM each of holo-lactoferrin (Holo-Lfn), apo-lactoferrin (Apo-Lfn), holo-transferrin (Holo-Tfn) and apo-transferrin (Apo-Tfn). Cultures were incubated for 24 h at 37 °C in 5% CO₂ at which time aliquots were diluted in phosphate-buffered saline and plated onto chocolate agar. Subculture plates were incubated for 48 h at 37 °C in 5% CO₂ and the resulting CFU counted.

Fig. 3. Resulting inclusion-forming units (IFU)/mouse were compared from vaginal cultures taken 4 days after a *Chlamydia* genital infection from salicylidene acylhydrazide INP 0341-treated ($\circ$) and untreated control ($\blacklozenge$) mice. Median IFU in the INP-treated group was 0 and in the control group was 1476 IFU as shown by the horizontal lines. There was a statistical difference between the two groups in terms of the number of *Chlamydia* recovered on Day 4 ($P < 0.01$).

Minimal inhibitory concentrations (MICs)^a and minimal bactericidal concentrations (MBCs) of various salicylidene acylhydrazides (INPs) against *Neisseria gonorrhoeae*

Table 1

Organism	Lf/Tf ^b	MIC/MBC (μM)									
		INP 0007	INP 0029	INP 0143	INP 0149	INP 0161	INP 0269	INP 0328	INP 0341	INP 0400	INP 0406
<i>Neisseria gonorrhoea</i> e ATCC 49226	+/+	12.5/12.5	6.3/6.3	100/100	50/50	100/100	50/50	100/>100	6.3/6.3	>100/>100	>100/>100
<i>N. gonorrhoea</i> e ATCC 43069	+/+	12.5/25	12.5/12.5	100/>100	50/50	>100/>100	50/50	100/>100	12.5/12.5	>100/>100	>100/>100
<i>N. gonorrhoea</i> e FA 1090	-/+	3.1/6.3	3.1/6.3	50/50	50/50	100/100	12.5/12.5	100/100	6.3/6.3	50/100	>100/>100
<i>N. gonorrhoea</i> e FA 7163	+/+	3.1/3.1	3.1/6.3	25/25	50/50	100/100	12.5/25	50/100	6.3/6.3	25/50	>100/>100
<i>N. gonorrhoea</i> e FA 7034	+/-	6.3/6.3	6.3/6.3	25/25	50/50	100/100	12.5/12.5	100/100	6.3/6.3	50/50	>100/>100
<i>N. gonorrhoea</i> e FA 6916	-/-	12.5/12.5	12.5/12.5	50/50	50/50	100/100	25/25	100/100	6.3/6.3	100/100	>100/>100

^aMIC determination was performed in brain–heart infusion broth supplemented with 2% Iso VitaleX.

^bLf + denotes possession of genes for expression of the lactoferrin receptor proteins LbpA and LbpB; and Tf + denotes a strain with complete genes for expression of the transferrin receptor proteins TbpA and TbpB. – refers to strains missing these functional genes.