

## ORIGINAL ARTICLE

# Pre-clinical pharmacokinetics and anti-chlamydial activity of salicylidene acylhydrazide inhibitors of bacterial type III secretion

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Salicylidene acylhydrazides belong to a class of compounds shown to inhibit bacterial type III secretion (T3S) in pathogenic Gram-negative bacteria. This class of compounds also inhibits growth and replication of *Chlamydiae*, strict intracellular bacteria that possess a T3S system. In this study a library of 58 salicylidene acylhydrazides was screened to identify inhibitors of *Chlamydia* growth. Compounds inhibiting growth of both *Chlamydia trachomatis* and *Chlamydomphila pneumoniae* were tested for cell toxicity and seven compounds were selected for preliminary pharmacokinetic analysis in mice using cassette dosing. Two compounds, ME0177 and ME0192, were further investigated by individual pharmacokinetic analysis. Compound ME0177 had a relatively high peak plasma concentration ( $C_{max}$ ) and area under curve and therefore may be considered for systemic treatment of *Chlamydia* infections. The other compound, ME0192, had poor pharmacokinetic properties but the highest anti-chlamydial activity *in vitro* and therefore was tested for topical treatment in a mouse vaginal infection model. ME0192 administered vaginally significantly reduced the infectious burden of *C. trachomatis* and the number of infected mice.

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## INTRODUCTION

Inhibitors of bacterial type III secretion (T3S) are promising compounds for the treatment of infections caused by Gram-negative pathogens.<sup>1,2</sup> By inhibiting this system, delivery of bacterial virulence factors into the host cells is blocked. Salicylidene acylhydrazides inhibit T3S in *Yersinia*, *Salmonella*, *Shigella* and enterohemorrhagic *Escherichia coli*.<sup>3–8</sup> In the strict intracellular pathogens, *Chlamydia trachomatis* and *Chlamydomphila pneumoniae*, this compound class not only inhibits T3S but also arrests growth and replication.<sup>9–12</sup> *C. trachomatis* is the most common sexually transmitted pathogen worldwide and a major cause of infertility.<sup>13</sup> Genital *Chlamydia* infections also increase the susceptibility to other sexual transmitted agents such as HIV.<sup>14</sup> Vaginal microbicides may be considered for prevention and control of local *Chlamydia* infections, but established infections may ascend to the ovarian tubes and therefore require systemic treatment. In order to identify lead compounds for anti-chlamydial drug development, we screened a library of 58 salicylidene

acylhydrazides<sup>15</sup> for their ability to inhibit *Chlamydia* growth. Subsequently, preliminary pharmacokinetic (PK) analysis was performed in mice using seven of the most promising compounds. The *in vivo* efficacy was evaluated for the most potent compound.

## MATERIALS AND METHODS

### Chemicals

For *in vitro* testing, the 58 salicylidene acylhydrazides<sup>15</sup> and the previously evaluated T3S inhibitor ME0053 (INP0403)<sup>4,5,7,8,12</sup> were dissolved in dimethyl sulfoxide (DMSO) and stored at room temperature as 10 mM stock solutions. Other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless indicated otherwise.

### Organisms and cell lines

*Mycoplasma*-free HeLa 229 cells (CCL-2.1; ATCC, Manassas, VA, USA) and HEp-2 cells (CCL-23; ATCC) were propagated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, 20 mM HEPES (pH 8.0), 8  $\mu\text{g ml}^{-1}$  gentamicin, 1  $\mu\text{g ml}^{-1}$  amphotericin B

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(Invitrogen, Carlsbad, CA, USA) and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> (v/v). *C. trachomatis* serovar L2 (VR-902B; ATCC) was cultured in HeLa 229 cells and purified as previously described.<sup>16</sup> *C. trachomatis* serovar D strain UW-3 (VR-885; ATCC) was propagated in HeLa 229 and *C. pneumoniae* strain T45<sup>17</sup> was raised in HEp-2 cells.<sup>17</sup> *Chlamydiae* were stored in 0.2 M sucrose, 0.02 M sodium phosphate (pH 7.4) and 5 mM glutamic acid (SPG) at -80 °C until use. *Lactobacillus jensenii* (25258; ATCC) and *Lactobacillus crispatus* strain 6G (33197; ATCC) were maintained on Difco Lactobacilli MRS agar (BD, Becton Dickinson, Franklin Lakes, NJ, USA) and *E. coli* (25922; ATCC) on 5% sheep blood agar (BD) at 37 °C in 5% CO<sub>2</sub>.

#### Determination of MIC for *C. trachomatis* and *C. pneumoniae*

HeLa cells were seeded in 96-well flat-bottom cell culture plates (1 × 10<sup>4</sup> cells per well) in RPMI 1640 medium supplemented as above. After overnight incubation, cells were infected with *C. trachomatis* serovar L2 diluted in Hank's balanced salt solution at a multiplicity of infection of 0.3. After 1 h incubation at 37 °C, the Hank's balanced salt solution was removed and replaced with RPMI containing two-fold dilutions of test compounds (1.6–50 μM) or solvent alone (1% DMSO, final concentration). *C. pneumoniae* strain T45 was added to HeLa cells at multiplicity of infection 0.3 in cell culture medium and centrifuged at 900 × g for 1 h at 35 °C. Thereafter, the medium was removed and replaced with test compounds or solvent diluted in cell culture medium containing 1 μg ml<sup>-1</sup> cycloheximide. Cell cultures were fixed at 35 and 48 h for *C. trachomatis* and *C. pneumoniae*, respectively, immunostained (Pathfinder; Bio-Rad, Hercules, CA, USA) and examined at ×200 magnification with fluorescent microscopy. Tests were performed in duplicate and repeated three times to determine the lowest concentration where complete inhibition of intracellular growth was observed.

#### Cell viability assay

HeLa 229 cells (1 × 10<sup>4</sup> cells per well) cultured in 96-well microtiter plates were incubated for 28 h at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> (v/v) with compounds (50 and 25 μM) or solvent alone (0.5% DMSO, final concentration) diluted in Dulbecco's Modified Eagle Medium (Invitrogen) without indicator and supplemented with 0.5% fetal calf serum, 2 mM L-glutamine and 8 μg ml<sup>-1</sup> gentamicin. Cell viability was evaluated using resazurin (40 μM final concentration) that is reduced to fluorescent resorufin in living cells.<sup>18</sup> Fluorescence was measured using a Tecan Safire microplate reader (Tecan, Männedorf, Switzerland) with excitation at 535 nm and emission at 595 nm. Resazurin was added 4 h prior to measurement and cell-free wells with medium and compound were used to exclude chemical reduction of resazurin by the compounds. In addition, cell viability was scored microscopically relative to the DMSO control. All experiments were performed in triplicate and repeated at least three times. Low toxicity was defined as ≥65% cell viability at a compound concentration of 50 μM.

#### Confocal imaging of ME0192 in cell culture

The localization of the weakly fluorescent ME0192 in *Chlamydia*-infected cells was investigated by confocal microscopy (Nikon Eclipse CI plus; Nikon Instruments Inc., Melville, NY, USA). HeLa cells were cultivated on coverslips and infected with *C. trachomatis* serovar L2 at a multiplicity of infection of 0.3 as described above. Cultures were maintained for 48 h and in the last 8 h, the growth medium was supplemented with 100 μM ME0192 or solvent (1% DMSO, final concentration). After methanol fixation, nucleic acids were stained with 0.05 μM SYTOX Orange (Molecular probes, Eugene, OR, USA). Immunostaining of *Chlamydia* (Pathfinder; Bio-Rad) and DAPI nucleic acid staining was also performed on separate coverslips handled in parallel.

#### Pharmacological formulation

Compounds with the lowest MIC (3–12.5 μM) for *C. pneumoniae* and low toxicity to HeLa cells (Table 1) were chosen for administration in mice together with the previously studied ME0053.<sup>4,5,7,8,12</sup> The compounds were solubilized by pH adjustment or a co-solvent approach. ME0164, 0177, 0184 and 0264 had good aqueous solubility at an alkaline pH and were dissolved in 1 M NaOH to obtain stock solutions of 100–230 mM. Compounds ME0053,

0190 and 0192 were only soluble in DMSO at higher concentrations and 30–250 mM stock solutions were prepared. The stock solutions were diluted with aqueous solutions and the pH was adjusted with 1 M HCl. Stability of the compounds in the formulations was confirmed by LC-MS analysis (negative ionization) using a Waters Micromass ZQ 2000 instrument equipped with an electrospray ionization source (Waters, Milford, MA, USA) using an XTerra MS C<sub>18</sub> 5 μM 4.6 × 50-mm column and an H<sub>2</sub>O/acetonitrile/formic acid eluent system. To rapidly evaluate several compounds, a cassette dosing strategy was adopted. Two equivalent mixtures containing four compounds were prepared, 250 μM of each compound in phosphate-buffered saline with 1.7% DMSO, 5 mM NaOH and 39% propylene glycol, pH 9. ME0053 was included in both mixtures as a reference. Thereafter, high-dose formulations of ME0177 and ME0192 were prepared. A stock solution of ME0177 in 1 M NaOH was diluted in water to 15.3 mM and adjusted to pH 9. ME0192 was diluted from 250 mM DMSO stock to 8 mM in water with 67 mM NaOH, final DMSO concentration 6.4% and pH 9.

#### Systemic administration in mice

Ten-week-old female BALB/c mice (Taconic, Ry, Denmark) were injected intraperitoneally with 200 μl of either cassette 1 (containing 250 μM each of ME0053, 0164, 0177 and 0190) or cassette 2 (containing 250 μM each of ME0053, 0184, 0192 and 0264). Each mixture was administered to two mice and plasma was retrieved at 10, 30, 45, 60, 90 and 240 min after injection. The 25-μl blood samples were collected from the tail veins, mixed with 1 μl 100 mM EDTA and centrifuged at 1000 × g for 10 min.

The high-dose formulations of ME0177 (15.3 mM) and ME0192 (8 mM) were injected intraperitoneally (200 μl) to seven mice per compound and whole blood was collected over 5 h for ME0177 and over 12 h for ME0192. Five mice were sampled at each time point. Intravenous injections of ME0177 and ME0192 (50–100 μl) were given to two mice per compound and blood samples were obtained over the 2-h period after administration. The 25-μl blood samples were mixed with 75 μl water supplemented with Heparin 40 μg ml<sup>-1</sup>. Plasma and whole blood was also collected from mice that did not receive compounds. All the samples were stored at -20 °C until analysis. Mice were treated with food and water *ad libitum* according to the regulations and ethical permission from Umeå Ethical Committee on Animal Research, Sweden.

#### LC-MS/MS analysis of compound concentrations in blood

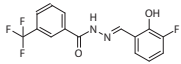
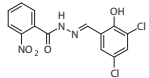
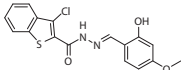
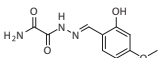
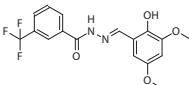
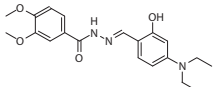
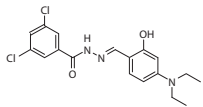
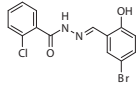
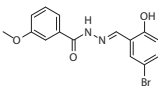
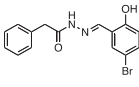
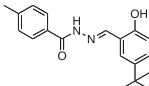
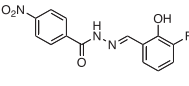
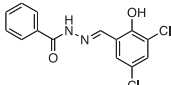
Concentrations were analyzed using a liquid chromatography tandem mass spectrometry system consisting of an AB Sciex Triple Quad 5500 mass spectrometer (AB Sciex, Foster City, CA, USA), two LC-20AD XR pumps plus a CBM-20A system controller (Shimadzu, Kyoto, Japan) and a CTC HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The analytical separation column (Aquasil 5 μm, 30 × 1 mm<sup>2</sup> (Dentsply, Milford, DE, USA) was coupled with pre-column (Aquasil 5 μm, 10 × 1 mm<sup>2</sup>). A gradient elution of mobile phase A (water/0.5% HOAc) and B (MeCN/HOAc/water 95/0.5/4.5) was run with a total run time of 4 min and flow rate of 250 μl min<sup>-1</sup>. All plasma and blood samples including control plasma and blood were thawed at room temperature. The analytes were diluted with EtOH to a concentration of 1 mM and eight standard concentrations (0.131–21 000 nM) in duplicate plus a control sample per compound were produced using a Tecan robot (Tecan) and precipitated with six parts of MeCN/1% HOAc containing an internal standard (Dexamethasone 0.5 μM) and then centrifuged at 2500 × g for 10 min. Supernatant, 50 μl, was diluted with five parts (250 μl) of Milli-Q water and 20 μl of resultant solution was injected into the LC-MS/MS system. Accuracy ranged from 85 to 115% and precision ±15% for the standard concentrations except for the lower limit of quantification where accuracy was 80–120% and precision ±20%.

All the samples were evaluated using the area ratio from the standard samples (curve fit: power, weighting: ln y).

#### Pharmacokinetic modeling

Non-compartment analysis of plasma/whole-blood concentration was performed using the Microsoft Excel-based program PKSolver.<sup>19</sup> Area under the zero moment (AUC<sub>0-t</sub>) and first moment curves (AUMC<sub>0-t</sub>) from 0 to last time *t* were calculated using the linear trapezoidal method. The terminal

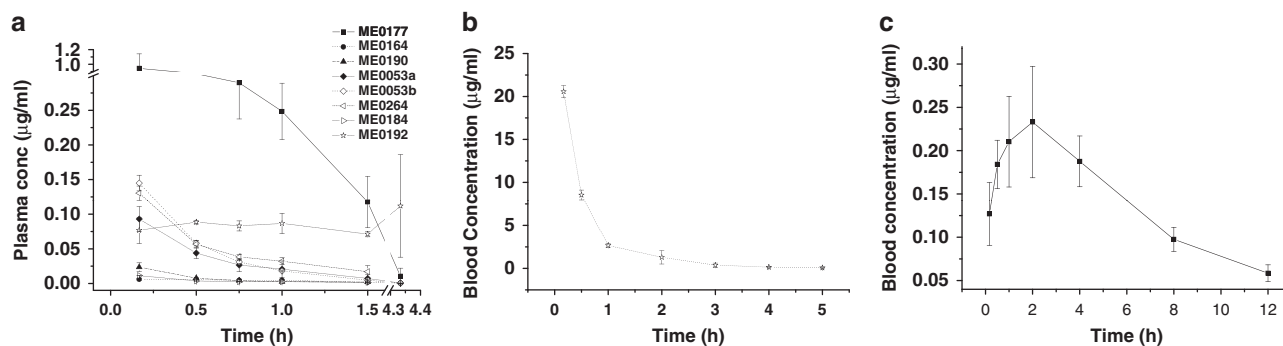
**Table 1** Minimal inhibitory concentration (MIC) of the salicylidene acylhydrazides for *Chlamydia trachomatis* and *Chlamydia pneumoniae* and percent cell viability at 25 and 50  $\mu\text{M}$ 

ID	Structure	MIC <i>C. trachomatis</i> $\mu\text{M}/\mu\text{g ml}^{-1}$	MIC <i>C. pneumoniae</i> $\mu\text{M}/\mu\text{g ml}^{-1}$	% Cell viability 50 $\mu\text{M}$	% Cell viability 25 $\mu\text{M}$
ME0164		25/8.2	12.5/4.1	78 ± 0.9	94 ± 0.1
ME0177		50/17.7	12.5/4.4	66 ± 2	84 ± 0.4
ME0179		25/9.0	25/9.0	90 ± 7	94 ± 5
ME0180		50/11.9	> 50 / > 11.9	96 ± 7	98 ± 1
ME0184		50/18.4	12.5/4.6	80 ± 3	92 ± 3
ME0190		50/18.6	12.5/4.6	72 ± 2	79 ± 3
ME0192		12.5/4.8	3/1	65 ± 2	67 ± 4
ME0259		50 <sup>a</sup> /17.7	n.d.	68 ± 2	95 ± 2
ME0260		25 <sup>a</sup> /8.7	50/17.5	70 ± 1	87 ± 2
ME0261		50 <sup>a</sup> /16.7	> 50 / > 16.7	87 ± 8	100 ± 2
ME0263		50 <sup>a</sup> /15.5	50/15.5	93 ± 1	97 ± 2
ME0264		50 <sup>a</sup> /15.2	12.5/3.8	83 ± 1	88 ± 2
ME0053		25/7.7	12.5/3.9	55 ± 2	70 ± 2

Abbreviation: n.d., not determined.

Cell viability was assessed using resazurin and is indicated as percent viability (mean ± standard deviation of triplicates) compared to controls treated with solvent (0.5% DMSO).

<sup>a</sup>Data from reference.<sup>15</sup>



**Figure 1** Plasma and blood concentrations over time. (a) Plasma concentration for seven salicylidene acylhydrazides administered intraperitoneally in two cassettes (each compound 15–19 µg, 0.7–1 mg kg<sup>-1</sup>) to two mice per cassette. Filled symbols represent compounds in cassette 1 and open symbols cassette 2. ME0053 was included in both cassettes. Blood samples were collected 10, 30, 45, 60, 90 and 240 min after administration of compounds. Blood concentrations over time for (b) ME0177 after 1 mg (52–60 mg kg<sup>-1</sup>) dose and for (c) ME0192 after 0.5 mg (28–30 mg kg<sup>-1</sup>) dose administered intraperitoneally to seven mice per compound. Five mice were sampled at each time point, 10, 30, 60, 120, 180, 240, and 300 min after administration for ME0177 and 10, 30, 60, 120, 240, 480 and 720 min after administration of ME0192.

**Table 2** Calculated pharmacokinetic parameters (mean ± s.d.) after intraperitoneal administration of ME0177 (1 mg, 55–60 mg kg<sup>-1</sup>) and ME0192 (0.53 mg, 28–30 mg kg<sup>-1</sup>)

Pharmacokinetic parameter	ME0177	ME0192
$t_{1/2}$ (h)	0.66 ± 0.08	5.04 ± 1.06
$T_{max}$ (h)	0.26 ± 0.15	1.36 ± 0.58
$C_{max}$ (µg ml <sup>-1</sup> )	17.08 ± 5.5	0.24 ± 0.05
AUC 0-inf_obs (µg ml <sup>-1</sup> h)	12.80 ± 3.5	2.15 ± 0.27
MRT 0-inf_obs (h)	0.75 ± 0.14	7.39 ± 1.63
$V_z/F_{obs}$ ((µg)/(µg ml <sup>-1</sup> ))	80.99 ± 25.6	2046.13 ± 424.28
$Cl/F_{obs}$ ((µg)/(µg ml <sup>-1</sup> ) h <sup>-1</sup> )	86.17 ± 29.9	283.12 ± 37.15

elimination slope ( $\lambda_z$ ) was estimated using regression with largest  $R^2$  on the points prior to peak plasma concentration ( $C_{max}$ ). The elimination half-life ( $t_{1/2}$ ), volume of distribution ( $V_d/F$ ), clearance ( $Cl/F$ ) and mean residence time parameters were also determined. Peak plasma drug concentrations ( $C_{max}$ ) and the times to reach the peak concentration ( $T_{max}$ ) values were derived from the plasma concentration versus time profiles.

### Animal infection model

A mouse model was used to test the ability of ME0192 to attenuate a vaginal infection by *C. trachomatis* serovar D.<sup>20</sup> All the experiments were approved by the University of California Irvine Institutional Animal Care and Use Committee. Seven-to-eight-week-old female C3H/HeJ (H-2<sup>k</sup>) mice (Jackson Laboratories, Sacramento, CA, USA) received two subcutaneous doses of medroxyprogesterone acetate (SICOR Pharmaceuticals, Irvine, CA, USA) on days 10 and 3 before a vaginal challenge with  $1 \times 10^3$  inclusion forming units 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 with 0.05 ml of 1 mM ME0192 in SPG, 25% DMSO, 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. Control mice were treated the same but received SPG with DMSO without ME0192. Vaginal swabs were collected 4, 7, 11, 14, 17, 21, 24 and 29 days after infection and were cultured, stained and evaluated as previously described.<sup>20</sup> The experiment was repeated three times with 5–10 mice per experimental group. Statistical analysis of the vaginal swab cultures was performed using SigmaStat 3.5 software (SYSTAT Software Inc, Chicago, IL, USA) and the significance level was <0.05. Fisher's exact test was used to analyze the number of infected mice in the two groups and Mann-Whitney *U*-test was used to analyze the infectious burden, the number of inclusion forming unit recovered, in the two groups.

### Stability of ME0192 in a vaginal simulant

Vaginal simulant fluid was prepared over a pH range of 4.5–6.5.<sup>21</sup> ME0192 dissolved in DMSO was added to the simulant to achieve a final concentration of 250 µM and incubated at room temperature for 15 min. To test for compound activity, the mixture was diluted in cell culture medium to a final ME0192 concentration of 25 µM and added to cell cultures newly infected with *C. trachomatis*. Control cultures were treated with DMSO alone. Primary cultures were incubated at 37 °C for 48 h, after which they were washed twice with SPG, sonicated in 0.5 ml SPG and this was used to infect HeLa cell monolayers as described previously.<sup>20</sup> Upon transfer and after 48 h of incubation, cultures were fixed, stained and the inclusion forming units were counted.

### Effect of ME0192 on bacteria in the vaginal flora

Overnight cultures of *L. jensenii*, *L. crispatus* strain 6G and *E. coli* were diluted to  $10^5$  colony forming units (cfu) ml<sup>-1</sup> in Brain Heart Infusion (BD) containing serial dilutions of ME0192 and incubated in 96-well microtitre plates 24 h at 37 °C in 5% CO<sub>2</sub>. The MIC was defined as the lowest concentration at which no visible growth was detected and the MBC was determined by subculturing the wells without visible growth and the first well with growth to establish the lowest concentration which contained <99% of the inoculum.

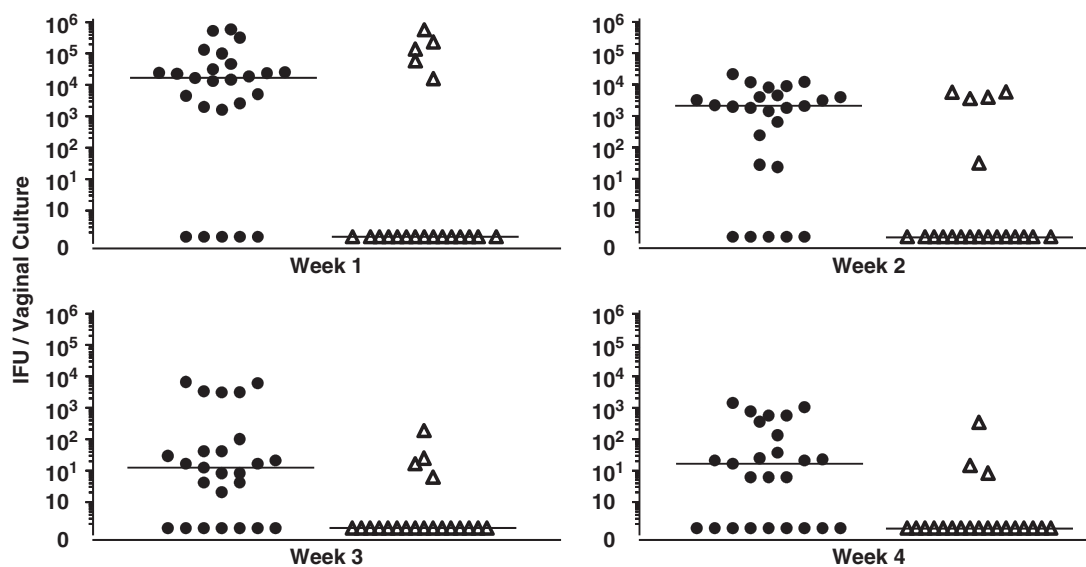
## RESULTS

### Selection of non-toxic compounds that inhibit *Chlamydia* growth

MIC values  $\leq 50$  µM for *C. trachomatis* was observed for 22/58 compounds. (chemical names and structures of all 58 compounds are found in Dahlgren *et al.*<sup>15</sup>) The effect on HeLa cell viability was assessed by resazurin and 12/22 compounds were considered as non-toxic at 50 µM as defined by  $\geq 65\%$  cell viability compared with solvent-treated controls. Eleven of the non-toxic compounds were also tested for growth inhibitory effect with *C. pneumoniae* and 9/11 had a MIC  $\leq 25$  µM, which was generally lower than that obtained for *C. trachomatis*. One compound ME0192 had a notably lower MIC compared with the other compounds, 12.5 µM for *C. trachomatis* and 3 µM for *C. pneumoniae*. The results are summarized in Table 1.

### Pre-clinical pharmacokinetics in mice

Compounds with the lowest *C. pneumoniae* MIC, 3–12.5 µM (Table 1), were chosen for evaluation of pharmacokinetic parameters in mice. The plasma concentrations over time after cassette dosing of 50 nmol (0.7–1 mg kg<sup>-1</sup>) of each compound are shown in Figure 1a.



**Figure 2** The number of inclusion forming unit (IFU) of *C. trachomatis* in vaginal cultures from mice treated intravaginally with ME0192 (open triangle,  $n=20$ ) or vehicle without compound (filled circles,  $n=25$ ) during the four week observation period after infection. The median IFU are indicated by horizontal lines and was 0 in the ME0192-treated group during the whole experiment.

**Table 3** The inhibitory effect of ME0192 is not reduced by pre-incubation of the compound in vaginal fluid simulant (VFS) at pH 4.5–6.5. Number of *C. trachomatis* inclusion forming units (IFU) are indicated in the table

Experiment	Compound	No simulant	VFS		
			pH 4.5	pH 5.5	pH 6.5
1	Vehicle	3 509 000	3 908 300	3 920 400	4 428 600
	ME0192	240	40	30	80
2	Vehicle	n/a	5 239 700	n/a	3 683 800
	ME0192		40		40
	ME0192		20		40

Abbreviation: n/a, not available.

The calculated PK parameters showed that ME0177 had up to 100 times higher  $C_{max}$  ( $0.94 \pm 0.20 \mu\text{g ml}^{-1}$ , average  $\pm$  s.d.) as compared with other compounds ( $0.01$ – $0.12 \mu\text{g ml}^{-1}$ ). ME0192 showed the longest plasma half-life (10.57 h) compared with other compounds (0.58–0.98 h). The reference ME0053 behaved similarly in the two sets of mice. Whole-blood concentration time profiles of ME0177 and ME0192 after intraperitoneal administration of higher doses are shown in Figures 1b and c, respectively. The calculated PK parameters of the two compounds are compared in Table 2.  $C_{max}$  for ME0177 was almost four times as high as the MIC value in *C. pneumoniae* and at the MIC for *C. trachomatis*. The AUC divided by MIC for *C. pneumoniae* was around three. For ME0192, the blood concentrations after the higher dose were far below the MIC of this compound in both *Chlamydia* species. The half-life of ME0192 in this analysis was variable and shorter compared with the cassette analysis, whereas the results for ME0177 were similar in the two experiments. ME0177 and ME0192 were also administered intravenously to two mice each. Intraperitoneal and intravenous routes of administration gave comparable PK parameters for ME0177 but were variable for ME0192 (data not shown).

#### ME0192 inhibited a *C. trachomatis* genital infection in mice

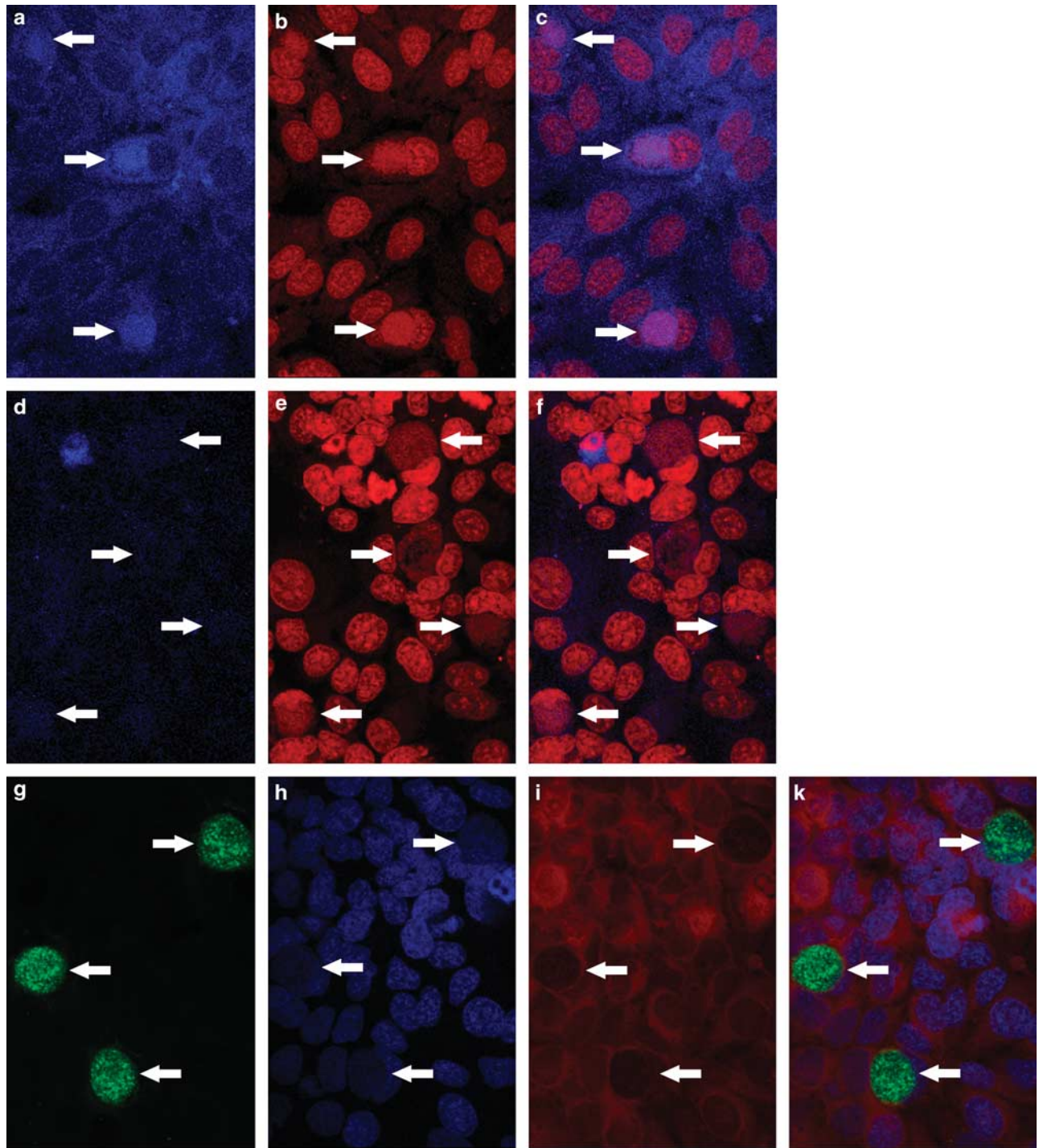
ME0192 had the highest *in vitro* anti-chlamydial activity of the compounds but poor pharmacokinetic properties after intraperitoneal and intravenous administration. Therefore, the effectiveness *in vivo* was tested by topical administration. Mice inoculated intravaginally with *C. trachomatis* were treated with ME0192 intravaginally prior to, at the time of infection and 5 days after infection. The infectious burden, as quantified by the number of inclusion forming unit in vaginal swab cultures, was significantly ( $P < 0.05$ ) lower in the ME0192-treated mice (Figure 2). At all the time points examined, the number of infected mice was significantly lower in the ME0192-treated group compared with the control group, 25% (5/20) and 84% (21/25), respectively.

#### ME0192 is stable in vaginal fluid and does not inhibit the normal vaginal flora

Incubation of ME0192 in vaginal simulant fluid at pH 4.5–6.5 did not reduce the inhibitory effect of the compound toward *C. trachomatis* (Table 3). In addition, the MIC and MBC of ME0192 toward *L. jensenii*, *L. crispatus* and *E. coli*, bacteria normally found in the vaginal flora, were  $> 100 \mu\text{M}$ , the highest concentration of ME0192 tested.

#### Visualization of ME0192 in *Chlamydia* inclusions

ME0192 was found to have weak autofluorescence in the blue spectrum. When added to *C. trachomatis*-infected HeLa cells during the last 8 h of a 48-h infection, ME0192 was observed to accumulate in the *Chlamydia* inclusions (Figures 3a–c). The fluorescence was weak and subjected to rapid quenching during illumination. High gain had to be applied, resulting in the background seen in the DMSO control (Figures 3d–f). Double staining with *Chlamydia*-specific antibodies obscured the autofluorescence of ME0192 and thus immunostaining of *Chlamydia* is shown on separate coverslips processed in parallel (Figures 3g–k).



**Figure 3** Accumulation of the auto fluorescent compound ME0192 in *C. trachomatis*-infected HeLa cells. *Chlamydia* inclusions, indicated by arrows, can be distinguished by their morphology in nucleic acid staining (b) and (e) (SYTOX orange) and (h) (DAPI). (a–c) Cells incubated with ME0192. The blue fluorescent ME0192 accumulates in *Chlamydia* inclusions (a). (c) is a merge of (a) and (b). (d–f): Cells incubated with solvent (1% DMSO), only background in the blue channel (d). (f) is a merge of (d) and (e). (g–k) Immunostaining: green *Chlamydia* inclusions (g), DAPI (h), Evans blue staining of cells (i) and merge (k).

## DISCUSSION

Salicylidene acylhydrazides inhibit bacterial T3S and thus have the potential to be formulated into drugs against infections caused by *Chlamydiae* and gastrointestinal pathogens.<sup>1,2</sup> In this study, we investigated 58 salicylidene acylhydrazides to identify anti-chlamydial lead compounds. Twelve non-toxic compounds with MICs to *C.*

*trachomatis* and *C. pneumoniae* between 3 and 50  $\mu\text{M}$  were identified (Table 1). In general, most of the compounds had lower MICs to *C. pneumoniae* than to *C. trachomatis*, in agreement with published data.<sup>11,12</sup> The most potent compound ME0192 had an MIC to *C. pneumoniae* four times lower than the MIC for *C. trachomatis*, thus suggesting that it may be easier to reach therapeutic concentrations

*in vivo* for the treatment of *C. pneumoniae* infections. MIC values for antibiotics are similar for *C. pneumoniae* and *C. trachomatis*,<sup>22</sup> and the observed difference in sensitivity to salicylidene acylhydrazides between the two species may be related to a different affinity for the molecular target. Recently, it was suggested that the salicylidene acylhydrazides target regulation of T3S in *E. coli* O157 and *Yersinia pseudotuberculosis* by the interaction with several proteins.<sup>23</sup> The mode of action in *Chlamydia* still remains to be elucidated.

Cassette dosing can be applied to PK screening in early drug discovery and was adopted in this pilot study to evaluate the pharmacokinetic properties of the most potent compounds of the salicylidene acylhydrazide class. The cassettes were designed with doses under 1 mg kg<sup>-1</sup> and only four compounds per cassette, one common in both cassettes, conditions that increase the reliability of the results.<sup>24</sup> ME0177 gave the highest C<sub>max</sub> and was subsequently subject to individual PK analysis after a 60 mg kg<sup>-1</sup> dose. C<sub>max</sub> was then four times the MIC of *C. pneumoniae*. The effect on *Chlamydia* infections remains even after removal of the compound<sup>10,12</sup> and one can speculate that the effect *in vivo* may be concentration-dependent, meaning that the ratio between AUC and MIC is the most important factor in predicting efficacy of treatment. After the 60 mg kg<sup>-1</sup> dose, the AUC/MIC ratio for ME0177 was roughly three. For concentration-dependent antibiotics, a AUC/MIC ratio well above 10 is often needed.<sup>25</sup> Further, development of ME0177 as a systemic agent would require administering the compounds in higher doses.

All the compounds except ME0192 had a short half-life, <1 h, indicating rapid metabolism/elimination or tissue distribution. Because of its longer half-life, ME0192 was tested individually at higher dose (30 mg kg<sup>-1</sup>). Very low plasma concentrations and high variability between individual mice indicated problems with compound stability and solubility after parenteral administration. ME0192 had poor pharmacokinetic properties and therefore was chosen for topical administration because it had the lowest MIC of all the evaluated compounds. Recently, topical treatment with another salicylidene acylhydrazide was shown to reduce the infectious burden in a mouse model of vaginal *C. trachomatis* infection<sup>20</sup> without disturbing the normal vaginal flora or damaging the vaginal tissue.<sup>26</sup> ME0192 may be even more efficient in the vaginal treatment of *C. trachomatis* infections due to its low MIC value. Incubation of ME0192 in vaginal simulant fluid did not reduce the effect of the compound and the compound did not affect representatives of the normal vaginal bacterial flora, *Lactobacilli* and *E. coli*, suggesting that ME0192 may be developed for intravaginal use.

The 58 compounds used in this study were previously designed, synthesized and tested for inhibition of T3S in *Y. pseudotuberculosis* and quantitative structure–activity relationships were computed. Eight of the compounds were tested in *C. trachomatis* with good congruence with the *Y. pseudotuberculosis* results.<sup>15</sup> We have now tested the remaining 50 compounds and 7 compounds inhibited *Chlamydia* growth. In all, 5 of them also inhibited T3S in *Yersinia*, whereas 13 compounds active in *Yersinia* did not inhibit *Chlamydia* or were toxic to HeLa cells. The discrepant results between the species may be explained by factors such as variation in target affinity and membrane permeability. In order to affect *Chlamydia*, the compounds need to penetrate into the eukaryote host cell and reach the intracellular *Chlamydia* inclusion. Additionally, compound efflux and metabolism may reduce the efficacy. From the currently available data, chemical modifications enhancing the efficacy and PK properties of this class of compounds are hard to predict.

There are few examples of inhibitors of bacterial virulence that reduce infections in animal models.<sup>27–29</sup> Salicylidene acylhydrazides have been shown to arrest the intracellular development of *Chlamydia*.<sup>9–11</sup> Although the exact mechanism by which these compounds inhibit *Chlamydia* is not known, they have been shown to directly or indirectly involve iron acquisition.<sup>12</sup> Here we demonstrated accumulation of the fluorescent compound ME0192 in *C. trachomatis* inclusions.

*C. trachomatis* and *C. pneumoniae* cause common human infections in the reproductive organs, ocular and respiratory systems, respectively.<sup>13</sup> Novel drugs for treatment of *Chlamydia* infections would reduce the use of antibiotics such as macrolides and tetracyclines, which have the potential to select for resistant strains.<sup>30</sup> Topical use of anti-chlamydial drugs intravaginally or in ocular formulations may prevent infections after exposure, which would reduce the risk for post-infectious complications such as infertility and blindness. We have identified two salicylidene acylhydrazides as lead compounds for potential drug development against *Chlamydia* infections; ME0177, which may be explored for systemic administration in higher doses, and ME0192, which is highly active against *Chlamydia* growth and may be developed for topical administration.

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