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Article

Acylpyrazoline-Based Third-Generation Selective Antichlamydial Compounds with Enhanced Potency

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Cite This: *ACS Omega* 2023, 8, 6597−6607

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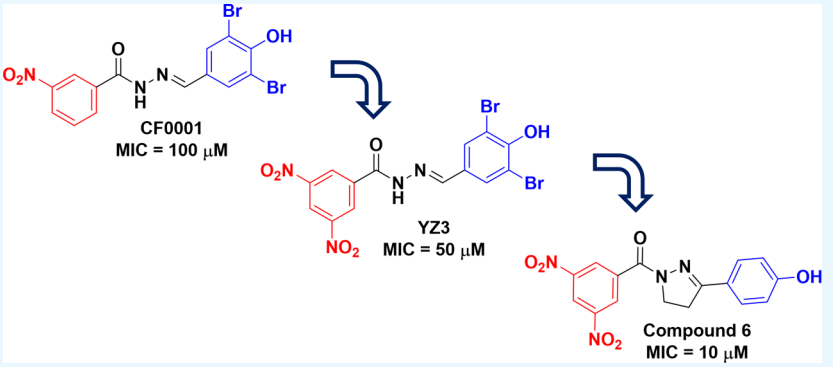


ABSTRACT: Chlamydiae are obligate intracellular Gram-negative bacteria and widespread pathogens in humans and animals. Broad-spectrum antibiotics are currently used to treat chlamydial infections. However, broad-spectrum drugs also kill beneficial bacteria. Recently, two generations of benzal acylhydrazones have been shown to selectively inhibit chlamydiae without toxicity to human cells and lactobacilli, which are dominating, beneficial bacteria in the vagina of reproductive-age women. Here, we report the identification of two acylpyrazoline-based third-generation selective antichlamydials (SACs). With minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of 10−25 μM against *Chlamydia trachomatis* and *Chlamydia muridarum*, these new antichlamydials are 2- to 5-fold more potent over the benzal acylhydrazone-based second-generation selective antichlamydial lead **SF3**. Both acylpyrazoline-based SACs are well tolerated by *Lactobacillus*, *Escherichia coli*, *Klebsiella*, and *Salmonella* as well as host cells. These third-generation selective antichlamydials merit further evaluation for therapeutic application.

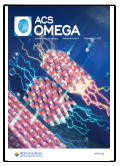
INTRODUCTION

Chlamydiae are obligate intracellular bacterial pathogens that are widespread in humans and animals.[1] *Chlamydia pneumoniae* is a causative agent of pneumonia and bronchitis in both children and adults. It is also a possible risk factor for atherosclerosis[2] and late-onset dementia.[3,4] *C. trachomatis* is the most common sexually transmitted bacterial pathogen globally.[5,6] In the United States, *C. trachomatis* infection consistently comprises most of all sexually transmitted diseases reported to the CDC since the infection was first classified as a reportable infection in 1994.[7] Certain *C. trachomatis* serovars also cause ocular infection and are the leading infectious microbes associated with blindness in various developing countries and parts of some industrialized countries.[8,9] Numerous *Chlamydia* species with animals as natural hosts may cause zoonotic infections.[10,11] Human psittacosis, which is due to the avian pathogen *Chlamydia psittaci*, has a particularly high mortality rate if left undiagnosed and untreated.[10,11]

Several broad-spectrum antibiotics are used to treat diagnosed chlamydial infection.[12] However, most infected individuals do not seek medical treatment because they are mostly asymptomatic.[13] Significantly, without proper antibiotic treatment, nearly one-third of infected women, particularly younger women, may develop severe complications, including pelvic inflammatory syndrome, infertility, ectopic pregnancy, and spontaneous abortion.[13]

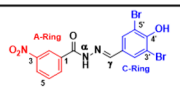
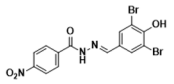
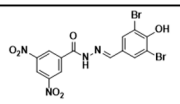
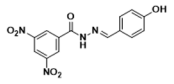
Existing broad-spectrum antibiotic therapies for chlamydial infection are associated with several concerns. First, they may cause dysbiosis in the genital tract and other systems.[14,15] In addition, *C. trachomatis* may develop resistance,[16] for example, by acquiring a tetracycline- and doxycycline-resistant gene from the swine pathogen *Chlamydia suis* because most *C. suis* strains are resistant to these drugs[17−22] and *C. suis* and *C. trachomatis* or *C. pneumoniae* could coinfect humans.[16]

Received: November 1, 2022
Accepted: January 30, 2023
Published: February 8, 2023



https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607

Table 1. First- and Second-Generation Benzal Acylhydrazone-Based Selective Antichlamydials[a]

Compound	Structure	MW (g/mol)	MIC vs. *C. trachomatis* (μM)	MIC vs. *Lactobacillus crispatus* (μM)
CF0001 (1)		443	100	>100
CF0002 (2)		443	100	>100
SF3		488	50	>75
YZ3		330	*IND	*ND

[a]*IND, indeterminable (due to **YZ3** being insoluble at 12.5 μM). ND, not determined.

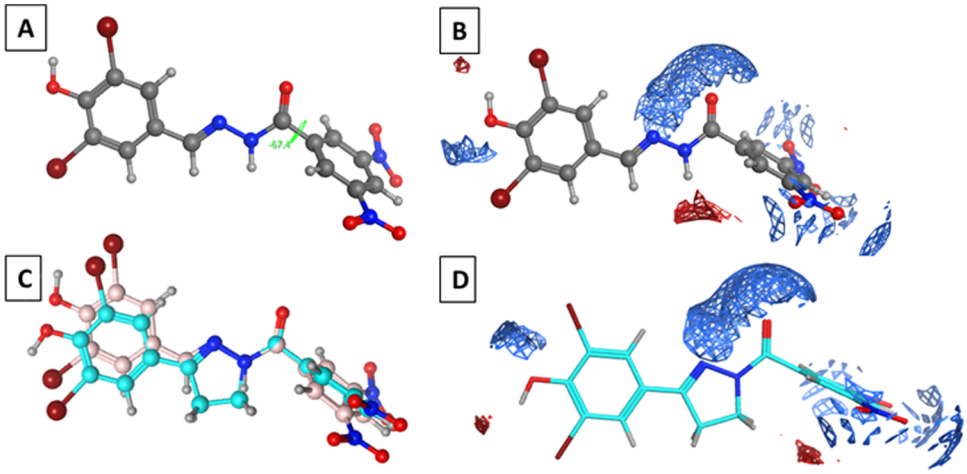


Figure 1. (A) Gas-phase conformation of **SF3**. (B) Electrostatic map of **SF3**. (C) Superposition of **SF3** (pink) and designed compound **5** (blue). (D) Electrostatic map of compound **5**.

Furthermore, the use of broad-spectrum antibiotics for chlamydial infection could drive the emergence of resistance in standing-by pathogens.[23] These concerns highlight a critical need for developing new and selective antichlamydials.

We reported the first generation of selective antichlamydials in 2014.[24] Among benzal acylhydrazone-based compounds, **CF0001** (**1**) and **CF0002** (**2**) were found to inhibit *C. trachomatis*, *C. pneumoniae*, and *Chlamydia muridarum* with MICs of approximately 100 μM, while having no detectable toxicity to either host cells or vaginal lactobacilli (Table 1).[24] We subsequently developed a second-generation selective antichlamydial, **SF3** (Table 1), derived from **CF0001**. **SF3** exhibited more potent antichlamydial activity (with an MIC of 50 μM), while still maintaining a lack of toxicity to lactobacilli as well as human and animal cells.[25]

Early SAR studies of the benzal acylhydrazones established that the 3,5-dinitrobenzhydrazide was best for the A-ring, while the 3,5-dibromo-4-hydroxybenzal was best for the C-ring (Table 1).[25] Despite having selective inhibitory effects vs *Chlamydia*, the first- and second-generation benzal acylhydrazone-containing antichlamydials are associated with poor pharmaceutical properties,[24] due to hydrolytic instability, conformational flexibility, *E* and *Z* photocatalyzed isomerism,[26] and pan-assay interference (PAINS).[27] These deficiencies make them unsuitable leads for further development. To mitigate these issues, replacements for the central acylhydrazone group were sought based on examples existing in the literature.[25] Here, we report the identification of two novel acylpyrazoline-based selective antichlamydials. While maintaining a lack of toxicity to host cells and other bacteria, these third-generation selective antichlamydials exhibit 2- to 5-fold greater potency relative to the second-generation selective antichlamydial **SF3**.

RESULTS AND DISCUSSION

Identification of Acylpyrazoline-Based Antichlamydials. Molecular modeling of **SF3** using the Molecular Operating Environment (MOE)[28] and the MMFF94 force field allows us to predict the lowest-energy conformation (Figure 1A) and electrostatic map (Figure 1B) of the molecule. This analysis suggested that we could keep the conformation of the molecule fixed and increase the hydrolytic stability of the molecule by attaching the a-NH to the g-CH via an ethylene linker to form an acylpyrazoline (**compound 5**) (Figure 1C)

https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607

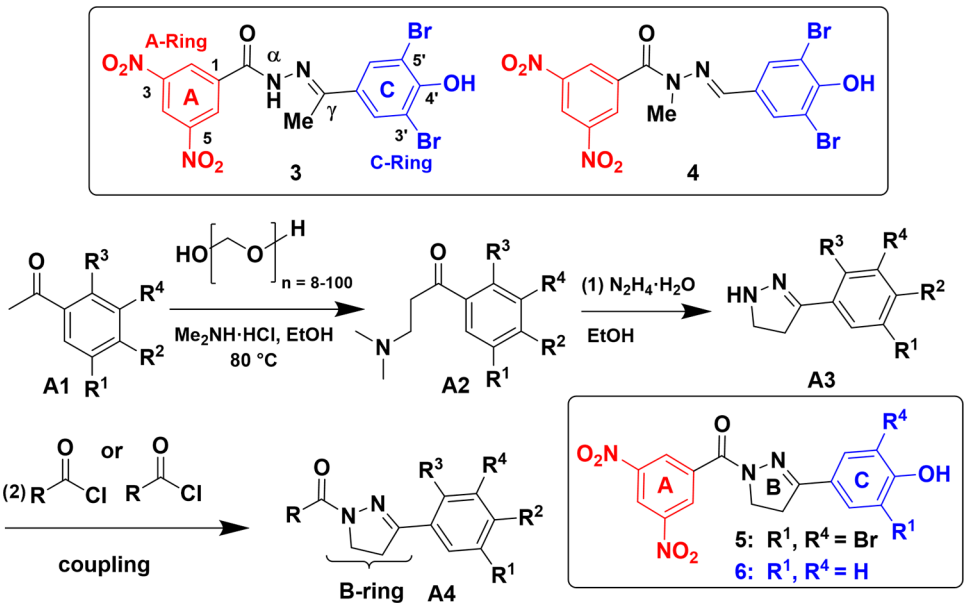
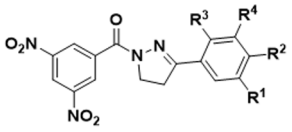


Figure 2. Compounds **3** and **4** and general synthesis scheme for the acyl pyrazoles, with compounds **5** and **6** being given as examples.

Table 2. Molecular Weights (MW) and Solubilities of Newly Developed Compounds and Their Minimal Inhibitory Concentrations (MIC) vs *Chlamydia trachomatis*



#	R^1	R^2	R^3	R^4	M_W (g/mol)	observed solubility[a] (HM)	*c* log S[b]	MIC (μM)
3	See Figure 2				502	>200	−6.6	100
4					502	>200	−6.3	100
5	Br	OH	Br	H	514	>200	−6.6	>200
6	H	OH	H	H	355	25	−4.3	10
7	H	OMe	H	H	370	50−100	−4.6	>50
8	H	OCH_2CH_2OH	H	H	400	12.5−25	−4.4	>12.5
9	H	OCH_2COOEt	H	H	442	50−100	−4.8	>50
10	H	OCH_2COOH	H	H	414	25−100	−4.4	>25
11	Cl	OCH_2CH_2-*N*-morpholino	H	H	504	12.5−25	−4.9	>12.5
12	H	OH	Me	H	370	25−50	−4.9	>25
13	Br	OH	H	H	435	50−100	−5.2	50
14	Cl	OH	H	H	391	50−100	−5.1	50
15	F	OH	H	H	374	50	−4.6	25

[a]Solubility was determined with a cell culture medium containing 1% DMSO. [b]Predicted solubility at pH 7.5 and 25 °C (DataWarrior).[29]

with a similar conformation and electrostatic map to **SF3** (Figure 1D). We first prepared methylated analogues of **SF3**, compound **3** (γ-Me), and compound **4** (α-Me) (Figure 2), which retained some activity (Table 2).[29] The general scheme for the synthesis of the acylpyrazolines used acetophenone (**A1**) paraformaldehyde and dimethylamine hydrochloride to give the Mannich product (**A2**) (Figure 2). Treatment of **A2** with hydrazine hydrate provided the pyrazoline **A3**, which after coupling with acid or acid chloride yielded the acylpyrazolines **A4** (Figure 2). In the case where R^2=OH, the phenol was alkylated with a variety of chains with the hope of improving the solubility and activity to give compounds of type **A5**. While azithromycin, chloramphenicol, and doxycycline demonstrated MICs of 0.25, 2, and 0.031 μM respectively, against *C. trachomatis*, compound **5** (Figure 2) was devoid of antichlamydial activity (Table 2). However, the des-bromo analogue **6** (Figure 2) was surprisingly active (Table 2), more so than what we would have expected from the activity of **YZ3** (Table 1), which was probably limited by its low solubility. Predicted and observed solubilities clearly show that compound **6** is much more soluble than **YZ3** (Table 2).

Modifications of the C-ring were made with the goal of further improving the activity and physical properties of lead compound **6**. Capping the phenol or adding solubilizing chains removed activity (compounds **7**−**11** in Table 2). Adding a methyl at R^3 to change the orientation of the C-ring (**12**) also reduced activity, while decreasing solubility. Mono-halo phenols (**13**−**14**) had better solubility than parent **6**, but their antichlamydial activities were only as good as the previous lead **SF3**. However, the mono-halo phenol **15** demonstrated improved antichlamydial activity over **SF3** (Table 2).

https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607

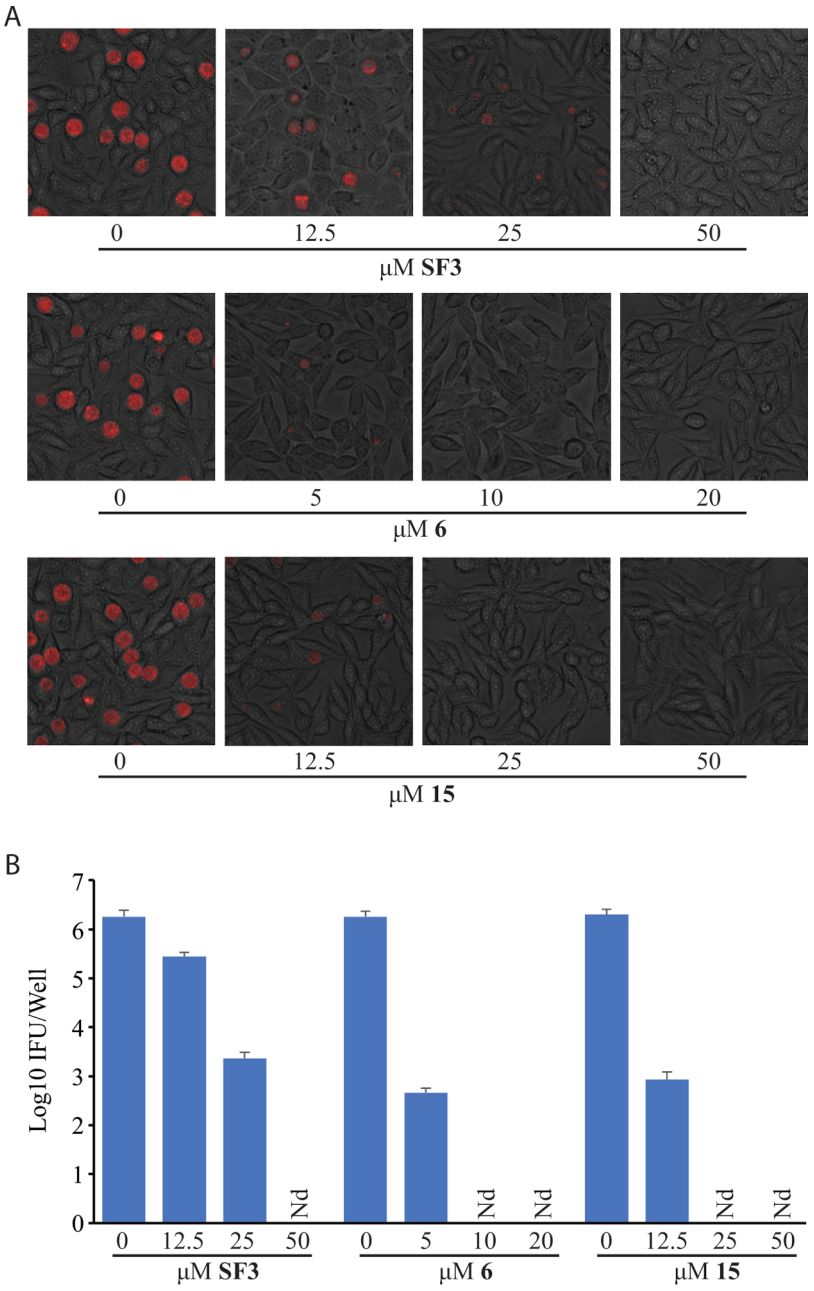


Figure 3. Dose-dependent inhibition of *C. trachomatis* L2 growth by **SF3** and the newly identified lead compounds **6** and **15**. L929 cells were infected with RFP/CtL2 and cultured in the presence of the indicated compound concentrations. (A) Images of chlamydial inclusions emitting red fluorescence signals and cellular images under bright light were acquired at 30 h and overlaid. (B) Progeny chlamydiae were quantified by harvesting infected cultures at 40 h, inoculating onto new L929 monolayers following limiting dilution, and scoring inclusions in the secondary cultures. Data are averages ± standard deviations of biological triplicates. Nd, none detected.

Figure 3 shows the dose-dependent inhibition of *C. trachomatis* growth by lead compounds **6** and **15** relative to **SF3**. While **SF3** was only able to prevent *C. trachomatis* from forming inclusions at a concentration of 50 μM, **6** and **15** prevented inclusion formation at concentrations of only 10 and 25 μM, respectively (Figure 3A). In addition, 10 μM **6** and 25 μM **15** completely blocked *C. trachomatis* from forming progeny EBs, while **SF3** was not able to achieve this inhibitory effect at concentrations below 50 μM (Figure 3B). Thus, judged by both MIC (Figure 3A) and MBC (Figures 3B), **6** is 5 times more potent than **SF3**, and **15** is twice more potent.

R51G Mutation in GrgA Confers a Low Level of Tolerance to 6 and 15. Previous studies have shown that the antichlamydial activities of the first- and second-generation selective antichlamydials are affected by the genotype of *grgA*, which encodes the *Chlamydia*-specific transcription factor GrgA.[24,25,30] Specifically, *C. muridarum* with a point mutation in *grgA* leading to the R51G substitution in the GrgA protein exhibits low-level resistance to **CF0001**, **CF0002**, and **SF3**.[24,25,30] We compared the inhibition activities of **6** and **15** against two isogenic *C. muridarum* variants r4s9 and r8s6, which express wild-type GrgA and R51G mutant GrgA, respectively. The MBCs of **6** and **15** against both *C. muridarum* variants were 10 and 15 μM, respectively (Figure 4). When partial killing concentrations were tested, both **6** and **15** exhibited statistically significant higher inhibition activity vs

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ACS Omega 2023, 8, 6597−6607

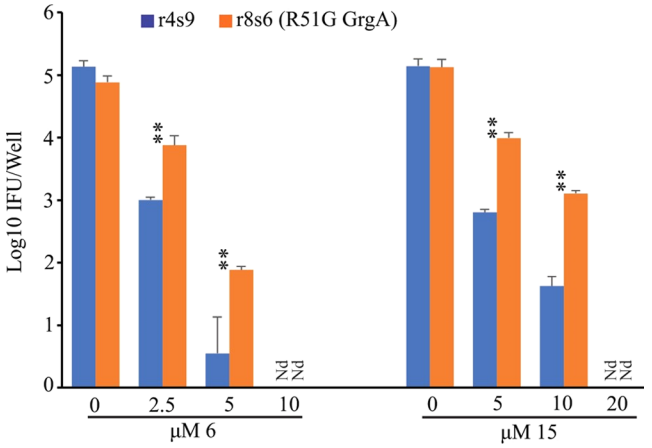

Figure 4. Impact of lead compounds **6** and **15** on progeny formation in *C. muridarum* expressing wild-type or mutant GrgA. When cultured with sublethal concentrations of the third-generation lead compounds **6** and **15**, *C. muridarum* r8s6, which expresses R51G GrgA, forms significantly more progeny than isogenic strain r4s8, which expresses wild-type GrgA. Infected L929 cells were cultured in the presence of the indicated compound concentrations. Progeny *Chlamydiae* were quantified by harvesting infected cultures at 30 h postinoculation, inoculating onto new L929 monolayers following limiting dilution, and scoring inclusions in the secondary cultures following immunostaining. Data are average ± standard deviation of biological triplicates. Nd, none detected.

r4s9 compared to r8s6. These results suggest that **6** and **15** inhibit *Chlamydia* through a GrgA-dependent mechanism, similar to the first- and second-generation benzal acylhydrazone-based selective antichlamydials.

Compounds 6 and 15 Are Well Tolerated by Host Cells. To determine the effects, if any, of **6** and **15** on host cells, we cultured immortalized but nonmalignant opossum kidney tubule epithelial cells (OK) with media containing either compound starting with low cell confluency. MTT assays, which quantitatively measure the metabolic activity of cells and are predictive of cell viability, showed no adverse effects of **6** at 25 μM, which is 2.5 times higher than its MIC and MBC against both *C. trachomatis* and *C. muridarum* (Figure 5). Compound **15** exhibited no host cell toxicity at 50 μM, which is 2−2.5 times higher than its MIC and MBC vs *Chlamydia* species (Figure 5). These findings suggest that **6** and **15** are well tolerated by host cells. However, weak and species-specific cytotoxicity have been reported for nitrobenzene and dinitrobenzene, respectively,[31,32] and further safety studies of **6** and **15**, which both carry a dinitrobenzene moiety, are needed.

Compounds 6 and 15 Are Not Toxic to Beneficial *Lactobacillus crispatus*. Lactobacilli are Gram-positive bacteria that account for over 90% of vaginal microbes in most reproductive-age women.[33] By producing lactic acid and modulating mucosal innate immunity, lactobacilli protect women from sexually transmitted infections.[33−37] Lactobacilli are also beneficial in the gastrointestinal tract.[38,39] We determined the impact of **6** and **15** on the growth of *L. crispatus*, one of the most protective *Lactobacillus* species in the human vagina.[38,40,41] As shown in Figure 6, *L. crispatus* 33197 was able to grow in the presence of 25 μM **6** and 50 μM **15**. These concentrations are 2−2.5 times of the antichlamydial MBCs of the compounds, suggesting that the novel antichlamydials are well tolerated by beneficial lactobacilli.

Compounds 6 and 15 Are Not Toxic to Gram-Negative Bacteria. We next determined the effects of **6** and **15** on the growth of several Gram-negative bacteria. The ability of *Escherichia coli* ATCC11775, *Klebsiella pneumoniae* ATCC13883, and *Salmonella enterica* ATCC13076 to form colonies on LB agar were not affected by **6** or **15** at 50 μM (Figure 7). The MICs of azithromycin, chloramphenicol, and doxycycline against *E. coli* ATCC11775 were 6.25, 5, and 0.1 μM, respectively; their MICs against *K. pneumoniae* ATCC13883 were 25, 10, and 4 μM, respectively; their MICs against *Salmonella enterica* ATCC13076 were 12.5, 10, and 4 μM, respectively. These results indicate that the acylpyrazoline-based lead compounds are highly selective antichlamydials with little or no adverse effects on Gram-negative bacteria.

In summary, we have developed two acylpyrazoline-based lead antichlamydials. These novel compounds are 2- to 5-fold more potent than the second-generation benzal acylhydrazone-based selective antichlamydial **SF3** against *Chlamydia*, while lacking detectable toxicity to host mammalian cells. They do not affect beneficial probiotic Gram-positive lactobacilli that

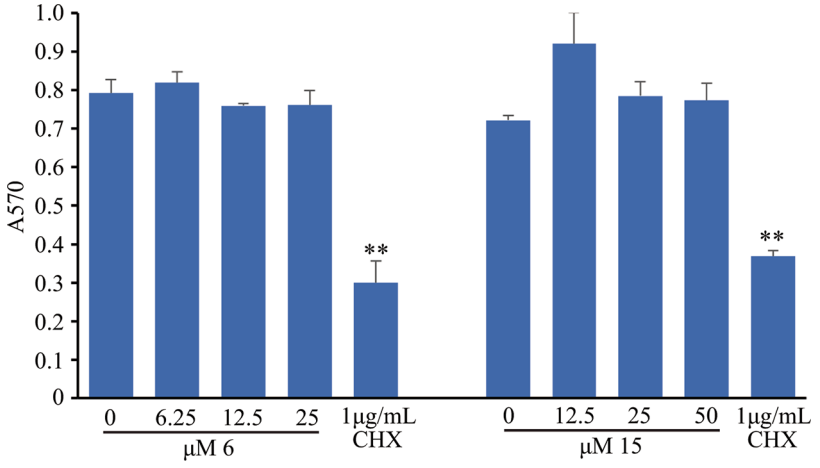

Figure 5. Lead compounds **6** and **15** are not toxic to mammalian cells. OK cells were seeded at 30% confluency and cultured in media containing the indicated compound concentrations. MTT assays were performed 40 h after the initiation of treatment. Cycloheximide (CHX), a eukaryotic protein synthesis inhibitor, was used as a toxicant control. Double asterisks indicate statistically significantly decreased cell viability ($P < 0.01$).

https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607

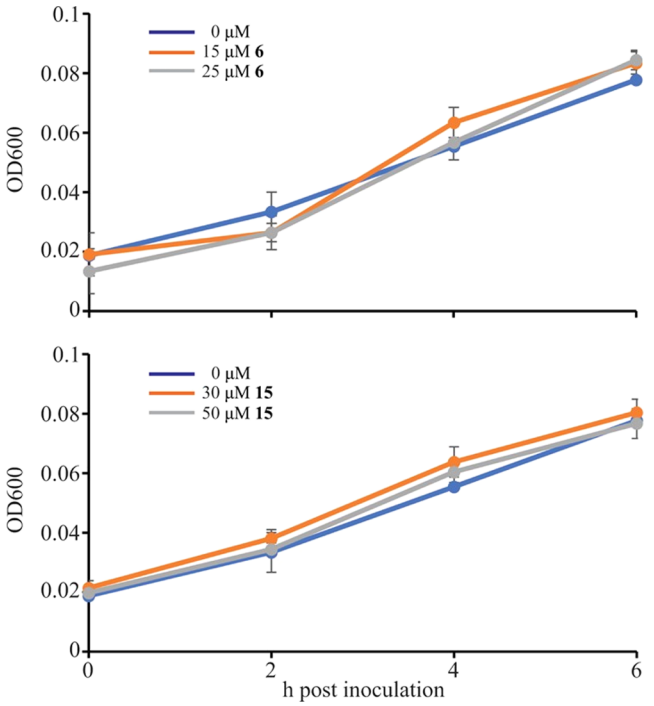

Figure 6. Lead compounds **6** and **15** do not adversely impact the growth of beneficial *Lactobacillus crispatus*. Growth of *L. crispatus* ATCC33197 was monitored by measuring the OD_{600} of cultures containing the indicated compounds at the indicated culture times.

dominate the vaginal microbiota in reproductive-age women or Gram-negative *E. coli*, an important component of the human gastrointestinal microbiota. They are also devoid of adverse effects on both Gram-negative pathogens analyzed, *K. pneumoniae* and *S. enterica*. These third-generation acylpyrazoline-based selective antichlamydials merit further evaluation for therapeutic applications.

MATERIALS AND METHODS

Chemicals. Azithromycin, chloramphenicol, and doxycycline were purchased from Sigma-Aldrich. Compound **SF3** was prepared using the published procedure.[24] Compounds **1**−**15** were synthesized and purified at Rutgers University.

*3-(Dimethylamino)-1-(4-hydroxyphenyl)propan-1-one hydrochloride (Figure 2, **A2**, R^1, R^3, R^4=H, R^2=OH). Method A.* To a solution of 4-hydroxyacetophenone (1.78 g, 13 mmol, 1.0 equiv) and paraformaldehyde (1.62 g, 54 mmol, 4 equiv) in ethanol (2 M, 6.5 mL) was added dimethylamine hydrochloride (2.13 g, 26 mmol, 2 equiv). After stirring the reaction for 24 h at 80 °C, 4 N hydrochloric acid in dioxane (0.1 mL, 0.4 mmol, 0.03 equiv) was added dropwise to the reaction mixture and stirred at 80 °C until completion as observed by LC/MS. The reaction was cooled to room temperature, and the precipitate was filtered off and washed with diethyl ether to afford a white solid (2.45 g, 10.6 mmol, 82% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.87 (d, J = 8.5 Hz, 2H), 6.88 (d, J = 8.5 Hz, 2H), 3.48 (m, 2H), 3.34 (m, 2H), 2.77 (s, 6H); $[M + H]^+$ calcd for $C_{11}H_{15}NO_2$, 194.11; found, 194.1.

*3-(Dimethylamino)-1-(3-fluoro-4-hydroxyphenyl)propan-1-one (**A2**, R^1=F, R^3, R^4=H, R^2=OH).* 3-(Dimethylamino)-1-(3-fluoro-4-hydroxyphenyl)propan-1-one was prepared from 1-(3-fluoro-4-hydroxyphenyl)ethan-1-one using **method A**, yield 21%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.78 (m, 1H), 7.71 (m, 1H), 7.09 (m, 1H), 3.46 (m, 2H), 3.35 (m, 2H), 2.78 (s, 6H); $[M - H]^-$ calcd for $C_{11}H_{14}FNO_2$, 210.1; found, 210.0.

*3-(Dimethylamino)-1-(4-hydroxy-2-methylphenyl)-propan-1-one (**A2**, R^1, R^4=H, R^3=Me, R^2=OH).* 3-(Dimethylamino)-1-(4-hydroxy-2-methylphenyl)propan-1-one was prepared from 1-(4-hydroxy-2-methylphenyl)ethan-1-one using **method A**, yield 85%. ^{1n}H NMR (500 MHz, DMSO-d_6) δ 10.33 (s, 1H), 7.84 (d, J = 8.75 Hz, 1H), 6.72 (dd, J = 8.75, 2.5 Hz, 1H), 6.68 (d, J = 2.5 Hz, 1H), 3.43(m, 2H), 3.32 (m, 2H), 2.77 (s, 6H), 2.42 (s, 3H); $[M - H]^-$ calcd for $C_{12}H_{17}NO_2$, 206.13; found, 206.0.

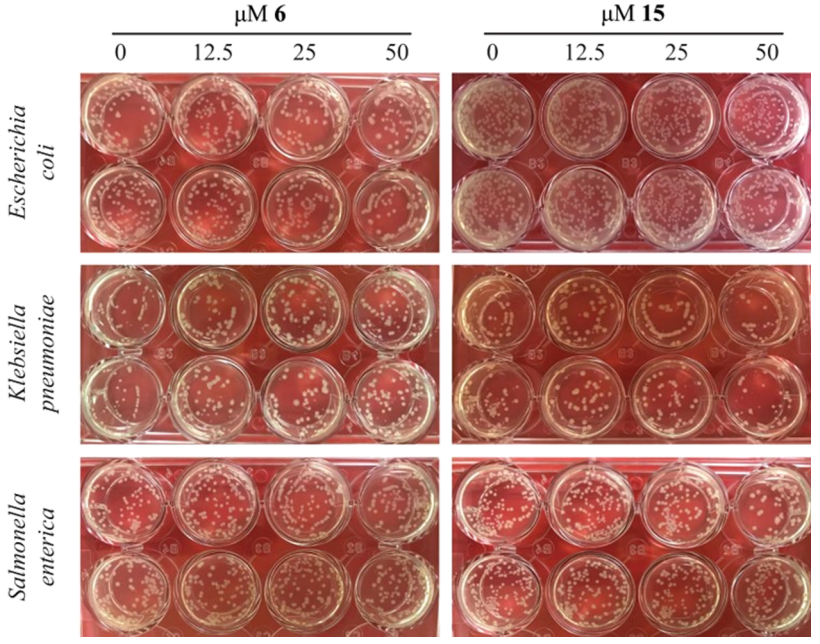

Figure 7. Lead compounds **6** and **15** do not impact the growth of Gram-negative bacteria. *E. coli* 11775, *K. pneumoniae* ATCC13883, and *S. enterica* ATCC13076 were inoculated onto LB agar plates containing the indicated compound concentrations or 3% DMSO vehicle and cultured at 37 °C. Plates were photographed 16−18 h postinoculation.

https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607

*1-(4-Bromophenyl)-3-(dimethylamino)propan-1-one (**A2**, R^1, R^3, R^4═H, R^2═Br).* 1-(4-Bromophenyl)-3-(dimethylamino)propan-1-one was prepared from 1-(4-bromophenyl)ethan-1-one using **method A**, yield 89%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.93 (d, J = 8.75 Hz, 2H), 7.78 (d, J = 8.75 Hz, 2H), 3.60 (m, 2H), 3.37 (m, 2H), 2.77 (s, 6H); $[M + H]^+$ calcd for $C_{11}H_{14}BrNO$, 256.03; found, 256.1.

Acylpyrazoline Synthesis Method B. *(3,5-Dinitrophenyl)(3-(4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone (**6**).* To a suspension of 3-(dimethylamino)-1-(4-hydroxyphenyl)propan-1-one hydrochloride (200 mg, 0.8 mmol, 1 equiv) in ethanol (0.2 M, 4 mL) was added hydrazine hydrate (0.2 mL, 200 mg, 4 mmol, 5 equiv) dropwise. The reaction was heated overnight at 95 °C until completion as observed by LC/MS. The reaction was concentrated under a stream of nitrogen gas, then tetrahydrofuran (0.2 M, 4 mL) was added to dissolve the crude solid, followed by the addition of 3,5-dinitrobenzoyl chloride (435 mg, 1.8 mmol, 2.1 equiv). The reaction was stirred at 22 °C overnight. The reaction was quenched with MeOH (1 mL). The solvent was removed under reduced pressure. HPLC purification provided an off-white solid (62 mg, 20% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.08 (s, 1H, OH), 9.08 (d, J = 2.2 Hz, 2H), 8.95 (t, J = 2.2 Hz, 1H), 7.64−7.57 (m, 2H), 6.87−6.81 (m, 2H), 4.15 (dd, J = 10.3, 9.0 Hz, 2H), 3.40−3.33 (m, 2H); HR-MS $[M + H]^+$ calcd for $C_{16}H_{12}N_4O_6$, 357.0830; found, 357.0834.

*(3,5-Dinitrophenyl)(3-(4-hydroxy-2-methylphenyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone (**12**). Method B.* Yellow solid. (31 mg, 17% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.91 (s, 1H), 9.04 (s, 2H), 8.93 (s, 1H), 7.42 (d, J = 9.5 Hz, 1H), 6.70−6.67 (m, 2H), 4.07 (m, 2H), 3.39 (m, 2H), 2.37 (s, 3H). $[M + H]^+$ calcd for $C_{17}H_{14}N_4O_6$, 371.09; found 371.2.

*(3,5-Dinitrophenyl)(3-(3-fluoro-4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone (**15**). Method B.* Yellow solid. (10 mg, 5% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.05 (s, 2H), 8.94 (s, 1H), 7.51 (d, J = 8.75 Hz, 1H), 7.39 (d, J = 8.5 Hz, 1H), 7.01 (dd, J = 8.75, 8.5 Hz, 1H), 4.15 (m, 2H), 3.33 (m, 2H). HR-MS $[M + H]^+$ calcd for $C_{16}H_{11}N_4O_6F$, 375.0735; found 375.0738.

*(3-(3,5-Dibromo-4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,5-dinitrophenyl)methanone (**5**).* To a solution of 3-chloro-1-(3,5-dibromo-4-hydroxyphenyl)propan-1-one (150 mg, 0.44 mmol, 1 equiv) in ethanol (4 mL), hydrazine monohydrate (0.2 mL, 6.24 mmol, 14.2 equiv) was added and stirred at 80 °C overnight. The solvent was removed under reduced pressure. The crude sample was used in the next step without purification. To a suspension of 2,6-dibromo-4-(4,5-dihydro-1H-pyrazol-3-yl)phenol (70 mg, 0.22 mmol, 1 equiv) in THF (4 mL), TEA (44.3 mg, 60 μL, 0.44 mmol, 2 equiv) was added, followed by 3,5-dinitrobenzoyl chloride (50.4 mg, 0.22 mmol, 1 equiv). The reaction was stirred at room temperature for 2 h. Quenched with MeOH (1 mL), concentration gave an oil. HPLC purification provides a white solid (6.8 mg, 14% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.65 (s, 1H), 9.11 (d, J = 2.2 Hz, 2H), 8.96 (t, J = 2.2 Hz, 1H), 7.90 (s, 2H), 4.18 (dd, J = 10.5, 9.1 Hz, 3H), 3.37 (dd, J = 10.6, 9.0 Hz, 3H); $[M - H]^-$ calcd for $C_{16}H_{10}Br_2N_4O_6$, 512.89; found, 513.1

*(3,5-Dinitrophenyl)(3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone (**7**).* To a suspension of 3-chloro-1-(4-methoxyphenyl)propan-1-one (200 mg, 1.10 mmol, 1 equiv) in ethanol (4 mL), hydrazine monohydrate (0.2 mL, 200 mg, 6.24 mmol, 6.2 equiv) was added dropwise. The reaction was heated at 95 °C overnight. After cooling, the solvent was removed under reduced pressure. The residue was used in the next step without purification. To a solution of 3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole (177.6 mg, 1.01 mmol, 1 equiv) in THF (10 mL), TEA (0.42 mL, 305.96 mg, 3.02 mmol, 3 equiv) was added and stirred for 5 min. 3,5-Dinitrobenzoyl chloride (348.55 mg, 1.51 mmol, 1.5 equiv) was added and stirred at room temperature for 1 h, diluted with EtOAc (15 mL), filtered, and washed with EtOAc (5 mL × 2). The solid was collected and dried on vacuum overnight to provide a yellow solid. (314 mg, 84% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.07 (s, 2H), 8.94 (s, 1H), 7.69 (d, J = 9 Hz, 2H), 7.02 (d, J = 9 Hz, 2H), 4.16 (m, 2H), 3.80 (s, 3H), 3.37 (m, 2H). $[M + H]^+$ calcd for $C_{17}H_{14}N_4O_6$, 371.09; found 371.1.

*(3-(3-Bromo-4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,5-dinitrophenyl)methanone (**13**).* To a solution of 4-[1-(3,5-dinitrobenzoyl)-4,5-dihydro-1H-pyrazol-3-yl]phenol (**6**) (50 mg, 0.14 mmol, 1 equiv) in DMF (4 mL), NBS (24.98 mg, 0.14 mmol, 1 equiv) was added and stirred at room temperature for 4 h. HPLC purification gave a yellow solid (12 mg, 20% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.08 (s, 2H), 8.94 (s, 1H), 7.84 (s, 1H), 7.56 (d, J = 8.25 Hz, 1H), 6.96 (d, J = 8.25 Hz, 1H), 4.14 (m, 2H), 3.33 (m, 2H). HR-MS $[M + H]^+$ calcd for $C_{16}H_{11}N_4O_6Br$, 434.9935; found 434.9942.

*(3-(3-Chloro-4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,5-dinitrophenyl)methanone (**14**).* To a solution of 4-[1-(3,5-dinitrobenzoyl)-4,5-dihydro-1H-pyrazol-3-yl]phenol (**6**) (40 mg, 0.11 mmol, 1 equiv) in DMF (4 mL), NCS (14.99 mg, 0.11 mmol, 1 equiv) was added and stirred at room temperature overnight. Preparative HPLC provides a light-yellow solid. (30 mg, 68% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.07 (s, 2H), 8.94 (s, 1H), 7.70 (s, 1H), 7.54 (d, J = 8.75 Hz, 1H), 7.02 (d, J = 8.75 Hz, 1H), 4.15 (m, 2H), 3.34 (m, 2H). $[M + H]^+$ calcd for $C_{16}H_{11}ClN_4O_6$, 391.04; found 391.2.

*(3,5-Dinitrophenyl)(3-(4-(2-hydroxyethoxy)phenyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone (**8**).* To a solution of 4-[1-(3,5-dinitrobenzoyl)-4,5-dihydro-1H-pyrazol-3-yl]phenol (**6**) (40 mg, 0.11 mmol, 1 equiv) in MeCN (4 mL), 2-chloroethanol (20 μL, 0.22 mmol, 2 equiv), K_2CO_3 (77.58 mg, 0.56 mmol, 5 equiv), and NaI (5 mg) were added and heated at 65 °C overnight, diluted with EtOAc (150 mL), washed with water (50 mL × 1), dried with Na_2SO_4, and concentrated to give an oil. Preparative HPLC provided a yellow solid (21 mg, 46.7% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.07 (s, 2H), 8.94 (s, 1H), 7.67 (d, J = 8.75 Hz, 2H), 7.02 (d, J = 8.75 Hz, 2H), 4.87 (m, 1H), 4.16 (m, 2H), 4.03 (m, 2H), 3.70 (m, 2H), 3.35 (m, 2H). $[M + H]^+$ calcd for $C_{18}H_{16}N_4O_7$, 401.10; found 401.2.

*Ethyl 2-(4-(1-(3,5-dinitrobenzoyl)-4,5-dihydro-1H-pyrazol-3-yl)phenoxy)acetate (**9**).* To a solution of 4-[1-(3,5-dinitrobenzoyl)-4,5-dihydro-1H-pyrazol-3-yl]phenol (**6**) (40 mg, 0.11 mmol, 1 equiv) in DMF (4 mL), ethyl 2-bromoacetate (120 μL, 1.12 mmol, 10 equiv), K_2CO_3 (77.58 mg, 0.56 mmol, 5 equiv), and NaI (catalytic amount, 5 mg) were added and heated at 80 °C overnight, diluted with EtOAc (150 mL), washed with water (50 mL × 1), dried with Na_2SO_4, and concentrated to give an oil. Flash column chromatography (50% EtOAc/Hex) provided a yellow solid

https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607

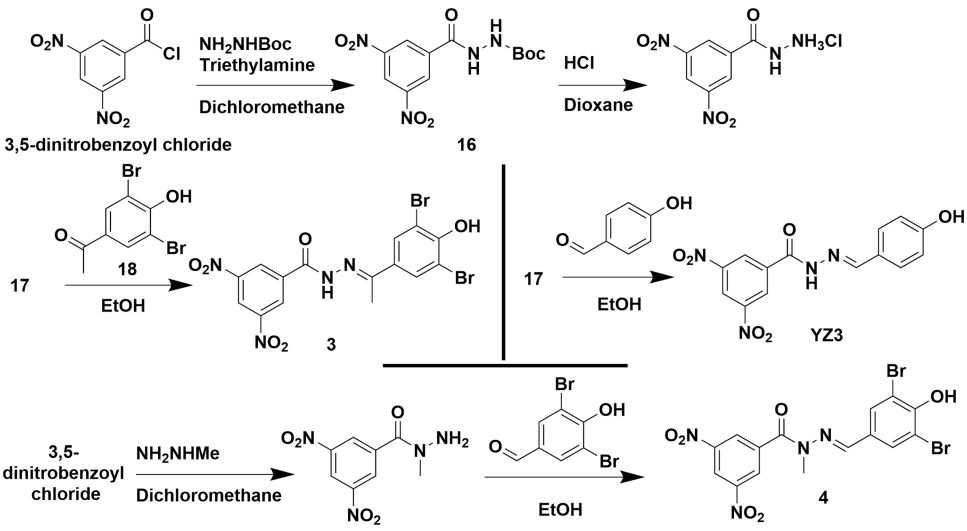

Figure 8. Synthetic route for compounds **3**, **YZ3**, and **4**.

(34 mg, 68% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.06 (s, 2H), 8.94 (s, 1H), 7.68 (d, J = 9.25 Hz, 2H), 7.02 (d, J = 9.25 Hz, 2H), 4.85 (s, 2H), 4−18−4.12 (m, 4H), 3.37 (m, 2H), 1.19 (m, 3H). $[M + H]^+$ calcd for $C_{20}H_{18}N_4O_8$, 443.11; found 443.3.

*2-(4-(1-(3,5-Dinitrobenzoyl)-4,5-dihydro-1H-pyrazol-3-yl)-phenoxy)acetic acid (**10**).* To a solution of ethyl 2-(4-[1-(3,5-dinitrobenzoyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenoxy)acetate (**9**) (20 mg, 0.05 mmol, 1 equiv) in MeOH (4 mL), NaOH (2 M, 50 μL) was added and stirred at room temperature overnight. The pH was adjusted to pH = 7 by the addition of 2 M $KHSO_4$. The solvent was removed under reduced pressure. Preparative HPLC provides a yellow solid (10 mg, 53% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.07 (s, 2H), 8.93 (s, 1H), 7.62 (d, J = 8.75 Hz, 2H), 6.86 (d, J = 8.75 Hz, 2H), 4.22 (s, 2H), 4.14 (m, 2H), 3.35 (m, 2H). $[M + H]^+$ calcd for $C_{18}H_{14}N_4O_8$, 415.08; found 415.2.

*(3-(3-Chloro-4-(2-morpholinoethoxy)phenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,5-dinitrophenyl)methanone (**11**).* To a solution of 2-chloro-4-[1-(3,5-dinitrobenzoyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenol (**14**) (20 mg, 0.05 mmol, 1 equiv) in DMF (4 mL), 4-(2-chloroethyl)morpholine (76.58 mg, 0.51 mmol, 10 equiv), K_2CO_3 (77.58 mg, 0.56 mmol, 5 equiv), and NaI (catalytic amount, 5 mg) were added and heated at 80 °C overnight. Preparative HPLC provided a yellow solid (9 mg, 35% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.07 (s, 2H), 8.94 (s, 1H), 7.78 (s, 1H), 7.66 (d, J = 8.75 Hz, 1H), 7.27 (d, J = 8.75 Hz, 1H), 4.23 (m, 2H), 4.17 (m, 2H), 3.55 (m, 4H), 3.37 (m, 2H), 2.73 (m, 2H). 2.48 (m, 4H). $[M + H]^+$ calcd for $C_{22}H_{22}ClN_5O_7$, 504.12; found 504.3.

*Synthesis of N′-(1-(3,5-Dibromo-4-hydroxyphenyl)-ethylidene)-3,5-dinitrobenzohydrazide (3), (E)-N′-(4-Hydroxybenzylidene)-3,5-dinitrobenzohydrazide (YZ3), and N′-(3,5-Dibromo-4-hydroxybenzylidene)-N-methyl-3,5-dinitrobenzohydrazide (**4**).* 3,5-Dinitrobenzoyl chloride (1.02 g, 4.4 mmol, 1 equiv) in anhydrous dichloromethane (0.9 M, 5 mL) was added to a solution of *tert*-butyl carbazate (1.77 g, 13.4 mmol, 3 equiv) and triethylamine (1.85 mL, 1.34 g, 13.2 mmol, 3 equiv) dissolved in anhydrous dichloromethane (5 mL). The reaction was stirred at room temperature for 24 h and then extracted between water (10 mL) and dichloromethane (3 × 10 mL). The organic layer was washed with brine, dried over sodium sulfate, and concentrated under vacuum. The resulting residue was purified by column chromatography on silica using 1:4 ethyl acetate/hexanes as the eluent to afford *tert*-butyl 2-(3,5-dinitrobenzoyl)hydrazine-1-carboxylate (**16**, Figure 8) as a white solid (1.13 g, 78% yield); $[M - H]^-$ calcd for $C_{12}H_{14}N_4O_7$, 325.09; found, 325.4

To a mixture of **16** (212 mg, 0.65 mmol, 1 equiv) in anhydrous dichloromethane (0.2 M, 3 mL) and dioxane (0.4 M, 1.5 mL) was added 4 N hydrochloride in dioxane (1.6 mL, 6.5 mmol, 10 equiv). The reaction was stirred at room temperature for 24 h and concentrated under vacuum to afford 3,5-dinitrobenzohydrazide hydrochloride (**17**) as a white hydrochloride salt (180 mg, 93% yield); $[M - H]^-$ calcd for $C_7H_6N_4O_5$, 225.03; found, 225.3

3,5-Dinitrobenzohydrazide **17** (105 mg, 0.35 mmol, 1 equiv) was added to a solution of 3,5-dibromo-4-hydroxyacetophenone (**18**, 106 mg, 0.36 mmol, 1.02 equiv) in ethanol (0.5 M, 1 mL). The reaction was stirred overnight. The product was filtered from the reaction mixture and rinsed with methanol, dichloromethane, and diethyl ether to afford *N′*-(1-(3,5-dibromo-4-hydroxyphenyl)ethylidene)-3,5-dinitrobenzohydrazide (**3**) as an off-white solid (147 mg, 84% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.56−11.38 (m, 1H, NH), 10.52−10.20 (m, 1H, OH), 9.09−8.90 (m, 3H), 8.07−7.73 (m, 2H), 2.38−2.29 (m, 3H). Note: Peaks have complex splitting from multiple conformational isomers. $[M + H]^+$ calcd for $C_{15}H_{10}Br_2N_4O_6$, 502.89; found, 503.0.

*(E)-N′-(4-Hydroxybenzylidene)-3,5-dinitrobenzohydrazide (**YZ3**).* To a solution of 3,5-dinitrobenzohydrazide (**17**) (100 mg, 0.44 mmol, 1 equiv) in EtOH (10 mL), 4-hydroxybenzaldehyde (54 mg, 0.44 mmol, 1 equiv) was added, followed by AcOH (10 μL), and stirred at 60 °C overnight. After cooling, it was filtered, washed with cool EtOH, and dried on high vacuum for 4 h to provide a yellow solid. (115 mg, 79% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 12.21 (s, 1H), 10.00 (s, 1H), 9.10 (s, 2H), 8.97 (s, 1H), 8.39 (s, 1H), 7.60 (d, J = 6.75 Hz, 2H), 6.84 (d, J = 6.75 Hz, 2H). HR-MS $[M + H]^+$ calcd for $C_{14}H_{10}N_4O_6$, 331.0673; found 331.0679.

*N′-(3,5-Dibromo-4-hydroxybenzylidene)-N-methyl-3,5-dinitrobenzohydrazide (**4**).* 3,5-Dinitrobenzoyl chloride (375 mg, 1.6 mmol, 1 equiv) was added to a solution of methylhydrazine (0.2 mL, 150 mg, 3.25 mmol, 2 equiv) in

https://doi.org/10.1021/acsomega.2c06992
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anhydrous dichloromethane (0.4 M, 4 mL) at −78.5 °C. The reaction was stirred for 24 h at room temperature and then diluted with 10 mL of water. The product was extracted with ethyl acetate (3 × 10 mL), and the resulting organic layers were dried over sodium sulfate and concentrated. The resulting residue was purified by column chromatography on silica using a gradient from dichloromethane to 2% methanol/dichloromethane to afford *N*-methyl-3,5-dinitrobenzohydrazide (**18**) as a white solid (272 mg, 70% yield); $[M + H]^+$ calcd for $C_8H_8N_4O_5$, 241.05; found, 241.0.

N-Methyl-3,5-dinitrobenzohydrazide **18** (84 mg, 0.35 mmol, 1 equiv) was added to a solution of 3,5-dibromo-4-hydroxybenzaldehyde (98 mg, 0.35 mmol, 1 equiv) in ethanol (0.09 M, 4 mL). The reaction was stirred overnight. The product was filtered from the reaction mixture and rinsed with diethyl ether to afford *N*′-(3,5-dibromo-4-hydroxybenzylidene)-*N*-methyl-3,5-dinitrobenzohydrazide (**4**) as an off-white solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.43 (s, 1H, OH), 8.97 (t, J = 2.2 Hz, 1H), 8.88 (d, J = 2.2 Hz, 2H), 8.03 (s, 1H), 7.69 (s, 2H), 3.51 (s, 3H); $[M + H]^+$ calcd for $C_{15}H_{10}Br_2N_4O_6$, 502.89; found, 502.9.

Host Cells and Culture Conditions. Mouse fibroblast L929 cells were used as host cells for *Chlamydia* cultures. The cells were maintained as adherent cultures using Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 20 μg/mL gentamicin. They were cultured in 37 °C incubators with humidified air supplemented with 5% CO_2.

Bacterial Strains. mKate/L2 strain was derived by transforming *C. trachomatis* L2 434/BU with the shuttle vector pASK/mKate2-L2[41] as previously described.[42] The transformation resulted in the constitutive expression of mKate, a red fluorescence protein (RFP). Isogenic *C. muridarum* strains r4s9 and r8s6 were obtained through lateral gene transfer.[30] EB stocks were raised from L929 cells and purified with ultracentrifugation through MD-76 gradients.[43]

E. coli strain ATCC11775, *K. pneumoniae* strain ATCC13883, and *S. enterica* strain ATCC13076 were purchased from American Type of Culture Collection. They were cultured with Luria−Bertani broth or Agar plates.

***Chlamydia* Inhibition Tests.** Potential antichlamydial activities in chemical compounds were evaluated by determining their effects on chlamydial inclusions formation and/or progeny EBs yield as previously reported.[23,24,30,43,44] At the time of inoculation, L929 cells were about 70% confluent. The multiplicity of infection was 0.2 inclusion-forming unit (IFU) per cell. Chemical treatment was initiated by replacement of the culture medium with fresh medium containing desired concentrations of an inhibitor or the vehicle DMSO (final concentration: 1%) at 1 h postinoculation. Cultures were observed to determine the formation of RFP-positive mKate/L2 inclusions 28 h postinoculation under an Olympus IX51 fluorescence microscope through the red fluorescence channel.[45] Lowest concentration of a chemical that resulted in the apparent absence of chlamydial inclusion formation was defined as the minimal inhibition concentration (MIC).

To determine the minimal bactericidal concentration (MBC) of compounds, media were removed from cultures with the MIC, a concentration above the MIC as well as a concentration below at 48 h postinoculation. Cells were then scraped off the plastic, collected into 500 μL of fresh media, and disrupted by sonication. Cell lysates were centrifuged at 500*g* for 10 min. The supernatants were inoculated onto new L929 cells at about 90% confluency on 24-well plates. The inoculated plates were centrifuged at 900*g* for 20 min to maximize the efficiency of infection. Cycloheximide was added into cultures to a final concentration of 1 μg/mL to maximize chlamydial growth. All cells in the well were carefully observed under the fluorescence microscope 48 h after inoculation to determine if progeny inclusions existed. The lowest concentration of a compound that resulted in full abrogation of progeny EB formation was defined as MBC.

To compare inhibition efficiencies of compounds in *C. muridarum* strains, media were aspirated at 30 h post-inoculation. Cells were harvested as described above. Supernatants of cell lysates were 1:10 serially diluted and inoculated to L929 monolayers in 96-well plates. Infected L929 cells were cultured in medium containing 1 μg/mL cycloheximide for 24 h. Cells were fixed with cold methanol and reacted sequentially with polyclonal mouse anti-*C. muridarum* at 1:2,000 dilution and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Sigma-Aldrich). Inclusions were numerated under an Olympus IX51 microscope.

Determination of Host Cell Toxicity. Host cell toxicity of antichlamydials was assessed using MTT assay as previously described.[24] Briefly, OK cells were seeded at 30% confluency. After 3 h incubation in a tissue culture incubator, culture medium was replaced with 90 μL (per well) of phenol-red-free DMEM containing 10% fetal bovine serum and indicated concentration of antichlamydials or 1 μg/mL cycloheximide, a eukaryotic growth inhibitor. The final concentration of DMSO in all cultures was 0.25%. After 40 h incubation, 10 μL of a 12 mM MTT stock solution prepared in phosphate-buffered saline was added into each well. Cells were cultured for an additional 4 h and lysed by the addition of 100 μL 10% (W/V) sodium dodecyl sulfate containing 10 mM HCl. After another 4 h incubation at 37 °C, plates were placed on an orbital shaker for 5 min and OD_{570} values were obtained using a plate reader.

Determination of Tolerance by *Lactobacillus*. *L. crispatus* strain ATCC33197 was cultured with the MRS Lactobacilli broth (Sigma) in a humidified 5% CO_2 incubator. For testing the effects of antichlamydials on lactobacilli, an overnight culture was diluted 100-fold with MRS Lactobacilli broth (Sigma). The fresh culture was incubated at 37 °C in a humidified 5% CO_2 incubator until the OD_{600} reached 0.5.[44] It was 50-fold diluted in the MRS broth containing the indicated concentration of compounds or 3% DMSO as vehicle control on 96-well plates. OD_{600} values were obtained at 2, 4, and 6 h post treatment using a plate reader.

Determination of Tolerance by *E. coli*, *K. pneumoniae*, and *S. enterica*. LB agar was autoclaved, cooled to 50 °C, aliquoted into test tubes containing desired antichlamydials, and poured onto 6- or 12-well plates. Overnight LB cultures were diluted 100-fold. When the fresh culture was incubated until OD_{600} reached 0.5, the culture was diluted 10,000-fold. A 10 μL sample of the diluted bacterial suspension was inoculated into each well of the agar plates. Growth of colonies was observed the following day.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c06992.

^{1}H NMR spectra for compounds **3**−**15** (PDF)

https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Drs. Wurihan Wurihan and Yehong Huang for helpful discussions. This work was supported by grants from the National Institutes of Health (Grant # AI 154305 to H.F.), New Jersey Health Foundation (PC 98−20 to H.F. & J.Y.R.), Rutgers Aresty Research Center (to E.R.P.), and National Natural Sciences Foundation of China (Grant # 82072306 to X.W.).

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https://doi.org/10.1021/acsomega.2c06992
ACS Omega 2023, 8, 6597−6607