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N-Acylated Derivatives of Sulfamethoxazole and Sulfafurazole Inhibit Intracellular Growth of *Chlamydia trachomatis*

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Antibacterial compounds with novel modes of action are needed for management of bacterial infections. Here we describe a high-content screen of 9,800 compounds identifying acylated sulfonamides as novel growth inhibitors of the sexually transmitted pathogen *Chlamydia trachomatis*. The effect was bactericidal and distinct from that of sulfonamide antibiotics, as para-aminobenzoic acid did not reduce efficacy. Chemical inhibitors play an important role in *Chlamydia* research as probes of potential targets and as drug development starting points.

Chlamydia trachomatis causes sexually transmitted disease that can lead to infertility (1) and increased susceptibility to other sexually transmitted pathogens such as HIV (2). *Chlamydophila pneumoniae* is a respiratory pathogen that can cause pneumonia (1). These common infections are treated with broad-spectrum antibiotics such as doxycycline and azithromycin which can select for resistant strains (3). Specific treatments would affect the normal bacterial flora less and reduce the use of these important antibiotics. High-content screening (HCS) uses cell-based assays, automated microscopy, and image analysis to collect complex information (4) and is well suited to screen for compounds for use against the obligate intracellular bacterium *Chlamydia* (5). Inhibitors of *Chlamydia* have been used to investigate the *Chlamydia* life cycle (6–11) and have been suggested for prevention of transmission (12–14).

HCS of 9,800 compounds gave 12 hits that inhibited *C. trachomatis* growth without visual changes in morphology of HeLa cell nuclei. The compound collection (Chembridge Corporation, San Diego, CA) was selected based on chemical diversity and drug similarities. In 96-well plates, 10^4 HeLa 229 cells (CCL-2.1; ATCC) were infected with 3,000 CFU *C. trachomatis* serovar L2 (VR-902B; ATCC) in 30 μl Hanks balanced salt solution (HBSS) (14). After 1 h, HBSS was replaced with 100 μl RPMI cell culture medium with 50 μM test compounds or 1% dimethyl sulfoxide (DMSO) and incubated for 18 h. DAPI (4′,6-diamidino-2-phenylindole) was used for staining together with fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR)-conjugated purified serum IgG (Melon Gel IgG Spin Purification kit; Thermo Scientific) from rabbit (Agrisera AB, Vännäs, Sweden) immunized with formalin-fixed *C. trachomatis* L2 elementary bodies (15). Photomicrographs were generated (20× objective), and the number and area of *Chlamydia* inclusions were determined (spot detection method) using an ArrayScan VTi HCA Reader (Thermo Fisher Scientific, Pittsburgh, PA). Artifacts and cellular toxicity were judged visually. Six hits were excluded due to the lack of a dose response or visible toxicity to the host cells. Two potent hits, compounds 1 and 2, were selected for further investigation (Table 1).

Statistical molecular design (16), cherry-picking, and chemical synthesis (see the supplemental material) were used to select 28 and 44 analogs of compounds 1 and 2, respectively. MICs for *C. trachomatis* were determined for all compounds (14). No potent analogs of compound 2 were identified (data available upon request), while analogs of compound 1 had a range of MIC values (Table 1). Basic structure-activity relationships for these acylated sulfonamides demonstrated that the 5-methyl-3-isoxazolyl group is preferred to the 3,4-dimethyl-5-isoxazolyl group (*cf.* compounds 1 and 19, 3 and 21, and 17 and 18). Truncation as in the case of compounds 7 and 10 or introduction of furane as in the case of compounds 8, 9, 22, and 25 was not beneficial. Compound 18 with a benzothiophene-2-carboxamide group was the most potent inhibitor (MIC, 6 μM). The antichlamydial effect was not caused by general toxicity to the host cells. HeLa cell viability after 24 to 48 h at 50 μM was >70% for most compounds as determined using an XTT cell proliferation assay kit (ATCC) and uncolored Dulbecco's modified Eagle's medium (DMEM) (Table 2). A parahalogenated aryl ring in place of the isoxazole ring (compounds 26 to 29) was associated with cytotoxicity.

The 50% inhibitory concentration (IC_{50}) in *C. trachomatis* and *C. pneumoniae* was determined for well-tolerated compounds with MIC < 25 μM (Table 2). *C. pneumoniae* T45 (17) was grown in HEp-2 cells (CCL-23; ATCC) for 70 h in the presence of 0.5 μg/ml cycloheximide (14). Inclusion counts were logarithmized and normalized. Nonlinear regression was used, and the IC_{50}s were derived from fitted curves (18). Dose-dependent activity was demonstrated for *C. trachomatis* (see Fig. S2 and S3 in the supplemental material) and less prominent with *C. pneumoniae*, indicating lower specificity in *C. pneumoniae* and differences in the molecular targets. Compounds listed in Table 2 were bacterial, as formation of infectious progeny was completely inhibited. No inclusions were detected by immunostaining after passage of *C. tra-*

Received 16 September 2013 **Returned for modification** 16 October 2013
Accepted 17 February 2014

Published ahead of print 24 February 2014

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02015-13.

doi:10.1128/AAC.02015-13

TABLE 1 Structures and MICs against *C. trachomatis* for hit 1 and hit 2 and for the analogs of hit 1, compounds 3 to 30

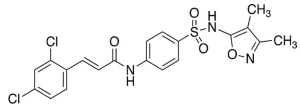
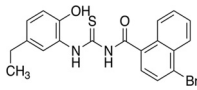
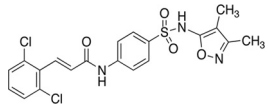
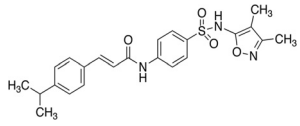
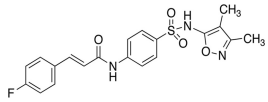
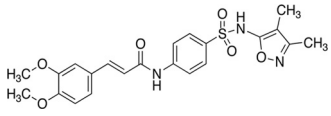
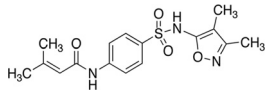
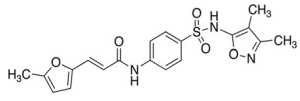
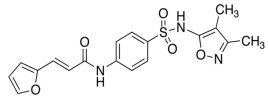
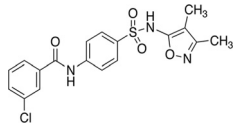
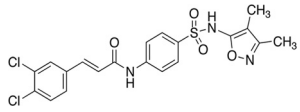
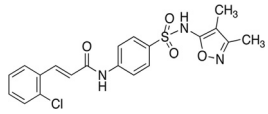
Compound	Structure	MIC (μM)
1		25
2		50
3		50
4		12
5		50
6		>50
7		>50
8		>50
9		>50
10		>50
11		25
12		25

TABLE 1 (Continued)

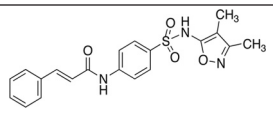
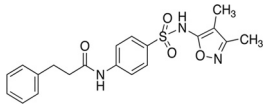
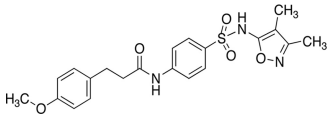
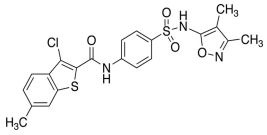
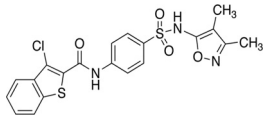
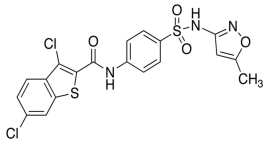
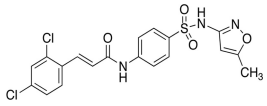
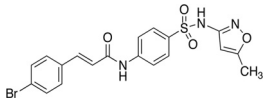
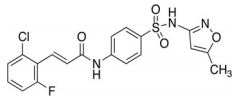
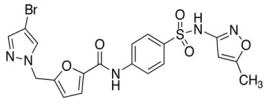
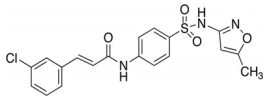
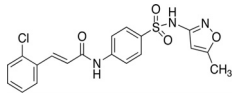
Compound	Structure	MIC (μM)
13		>50
14		>50
15		50
16		25
17		50
18		6
19		12
20		12
21		25
22		>50
23		12
24		12

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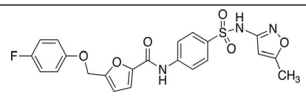
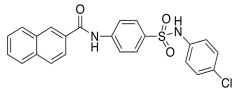
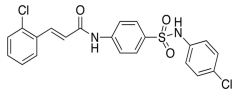
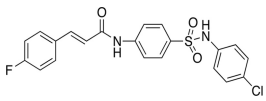
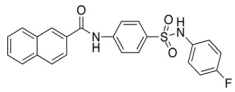
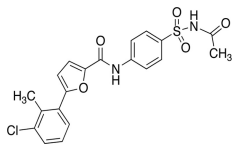
TABLE 1 (Continued)

Compound	Structure	MIC (μM)
25		>50
26		12
27		25
28		25
29		25
30		25

chomatis grown 44 h with 25 μM compound and harvested by freeze-thawing in sterile water.

All compounds follow Lipinski's rules of five (19) with minor exceptions for compounds 22 and 25. Even though the aqueous solubility was low, good oral absorption was predicted *in silico* (20) mainly due to favorable cellular permeability (see Table S2 in the supplemental material). The predicted numbers of metabolites (0 to 5) are comparable to those of average drugs (21). Acylated sulfonamides share the structural core with the orally bioavailable sulfonamide antibiotics sulfafurazole and sulfamethoxazole. The acylated sulfonamides are, however, more lipophilic and might concentrate more readily in chlamydial inclusions.

Sulfonamide antibiotics inhibit folate synthesis by competing with the substrate para-aminobenzoic acid (PABA) (22). Chlamydiae are capable of synthesizing folate, and levels of susceptibility to sulfonamide antibiotics have been reported to differ among species and strains (23–25). Compounds 1, 18, and 23 had identical MICs in DMEM with or without 10 μM PABA (50, 12.5, and 25 μM, respectively), while sulfamethoxazole inhibited *C. trachomatis* growth only in PABA-free DMEM (25% inhibition at 100 μM). Our compounds did not inhibit growth of *Escherichia coli* and *Straphylococcus aureus* (in-house strains), except compound 14, which inhibited *E. coli* 80% at 100 μM. Overnight growth curves were recorded in Mueller-Hinton broth with 100 to 1.56 μM test compounds or 1% DMSO (600 nm absorbance) (Tecan Safire microplate reader; Tecan, Männerdorf, Switzerland), and sulfamethoxazole IC_{50}s were 12 and 6 μM for *E. coli* and *S. aureus*, respectively. These data suggest that acylated sul-

TABLE 2 The effect of compound 1 and its most promising analogs against *C. trachomatis* and *C. pneumoniae* and on host cell viability

Compound	*C. trachomatis*		*C. pneumoniae*		% (SD) HeLa cell viability at indicated time (h) and compound concn			
					24		48	
	IC_{50} (μM)	MIC (μM)	IC_{50} (μM)	MIC (μM)	25 μM	50 μM	25 μM	50 μM
1	11.7	25	7.4	>50	93 (3)	86 (5)	95 (4)	96 (4)
4	4.9	12	3.2	50	94 (7)	99 (4)	98 (3)	97 (4)
18	2.8	6	ND[a]	>50	94 (7)	93 (4)	91 (9)	82 (12)
19	6.0	12	7.8	50	98 (8)	86 (8)	95 (6)	90 (3)
20	5.5	12	4.7	>50	91 (5)	86 (5)	94 (3)	89 (3)
23	4.2	12	4.7	25	96 (7)	87 (5)	94 (2)	88 (3)
24	6.5	12	7.7	>50	96 (5)	85 (6)	97 (2)	92 (3)

[a] ND, not determined.

fonamides do not affect folate synthesis. The different mechanisms of action are likely due to the fact that acylated derivatives lack the free amine of sulfonamide antibiotics that fits the active site in dihydropteroate synthetase (22).

Acylated and benzylidene sulfonamides with structural similarity to compound 10 in this study have recently been reported to inhibit mycobacteria and staphylococci (26). However, 10 completely lacked antichlamydial and antistaphylococcal activities. Except for compound 14, our compounds did not inhibit growth of representative extracellular bacteria, and further investigation is needed to determine if the antimicrobial spectrum is narrow.

The compounds presented here are promising starting points for development of novel antichlamydial drugs. Specific treatments for these common infections would reduce the risks of disturbing the normal flora or spreading antibiotic resistance. The mode of action is unknown but may be identified by selection for resistant mutants and subsequent whole-genome sequencing (9, 27). Novel antichlamydial compounds may also be of importance as probes to validate potential drug targets and may thereby reveal new insights into *Chlamydia* biology.

ACKNOWLEDGMENTS

David Andersson is acknowledged for computational assistance.

This work was supported by Laboratories for Chemical Biology Umeå, Chemical Biology Consortium Sweden, the Swedish Government Fund for Clinical Research (ALF), the Scandinavian Society for Antimicrobial Chemotherapy foundation (for Å.G.), and the Swedish Research Council, the Swedish Governmental Agency for Innovation Systems (VINNOVA), the Knut & Alice Wallenberg foundation, and the Carl Trygger foundation (for M.E.).

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