Type II Fatty Acid Synthesis Is Essential for the Replication of Chlamydia trachomatis

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Background: Chlamydia trachomatis has a phospholipid composition that resembles its eukaryotic host, but it contains branched-chain fatty acids of chlamydial origin.

Results: The inhibition of the enoyl-acyl carrier protein reductase (FabI) in chlamydial fatty acid synthesis blocks C. trachomatis replication.

Conclusion: Bacterial FASII is required for C. trachomatis proliferation.

Significance: FabI is a therapeutic target against C. trachomatis.

Chlamydia trachomatis is a Gram-negative, obligate intracellular bacterial parasite Chlamydia trachomatis are the same as its eukaryotic host except that they also contain chlamydiamade branched-chain fatty acids in the 2-position. Genomic analysis predicts that C. trachomatis is capable of type II fatty acid synthesis (FASII). AFN-1252 was deployed as a chemical substrate to specifically inhibit the enoyl-acyl carrier protein reductase (FabI) of C. trachomatis to determine whether chlamydial FASII is essential for replication within the host. The C. trachomatis FabI (CtFabI) is a homotetramer and exhibited typical FabI kinetics, and its expression complemented an Escherichia coli fabI(Ts) strain. AFN-1252 inhibited CtFabI by binding to the FabI-NADH complex with an IC₅₀ of 0.9 μM at saturating substrate concentration. The x-ray crystal structure of the CtFabI-NADH-AFN-1252 ternary complex revealed the specific interactions between the drug, protein, and cofactor within the substrate binding site. AFN-1252 treatment of C. trachomatis-infected HeLa cells at any point in the infectious cycle caused a decrease in infectious titers that correlated with a decrease in branched-chain fatty acid biosynthesis. AFN-1252 treatment at the time of infection prevented the first cell division of C. trachomatis, although the cell morphology suggested differentiation into a metabolically active reticulate body. These results demonstrate that FASII activity is essential for C. trachomatis proliferation within its eukaryotic host and validate CtFabI as a therapeutic target against C. trachomatis.

The major phospholipid classes of the obligate intracellular bacterial parasite Chlamydia trachomatis are the same as its eukaryotic host except that they also contain chlamydiamade branched-chain fatty acids in the 2-position. Genomic analysis predicts that C. trachomatis is capable of type II fatty acid synthesis (FASII). The atomic coordinates and structure factors (code 4Q9N) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: EB, elementary body; ACP, acyl carrier protein; FabI, enoyl-ACP reductase; CtFabI, enoyl-ACP reductase of C. trachomatis; SoFabI, enoyl-ACP reductase of S. aureus; FASII, bacterial type II fatty acid synthesis; LOS, lipoisogaschiride; RB, reticulate body; MOMP, chlamydial major outer membrane protein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
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cells found that a decrease in cPLA₂ activity has no effect on C. trachomatis growth (5). In mouse cells, cPLA₂ knockdown increases chlamydial titers, suggesting that cPLA₂ is more likely involved in intracellular immunity (5). One function of FASII is to generate the 3-hydroxy fatty acids for lipopolysaccharide synthesis in Gram-negative bacteria (6). The outer membrane of C. trachomatis is decorated with a similar lipooligosaccharide (LOS) that contains 18 and 20 carbon 3-hydroxy fatty acids (7–9). The inhibition of LOS biosynthesis in C. trachomatis does not block RB proliferation but does prevent the differentiation of RB into EB, resulting in the accumulation of non-infectious RBs within the chlamydial inclusion (10). Thus, one function of FASII would be to provide these 3-hydroxy fatty acids for LOS synthesis in C. trachomatis, but it is unknown whether FASII is needed for phospholipid synthesis.

We used AFN-1252 as a chemical biology tool to specifically inhibit the enoyl-acyl carrier protein (ACP) reductase (FabI) (11–13) and assess the role of FASII in C. trachomatis replication. C. trachomatis is predicted to encode a FASII system that depends on FabI (Fig. 1). AFN-1252 inhibits C. trachomatis FabI (CtFabI) by forming a tight ternary CtFabI-NADH-AFN-1252 complex visualized at 1.8 Å resolution. AFN-1252 treatment arrests the replication of C. trachomatis when added at any time during the infectious cycle, and when administered at the beginning of the infection, it blocks development at a single cell RB-like state. These data show that FASII is required for C. trachomatis replication and validate CtFabI as a suitable target for antichlamydial therapy.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The fabI gene (CT104) of C. trachomatis strain D/UW-3/Cx (NCBI Microbial Genomes Database) was optimized for expression in *Escherichia coli* through GeneArt gene synthesis (Invitrogen). A NdeI cleavage site was engineered at the 5’-end of the gene with a start codon in the NdeI site, whereas a 6-histidine tag, stop codon, and an EcoRI cleavage site, whereas a 6-histidine tag, stop codon, and an EcoRI cleavage site were sequentially engineered at the 3’-end of the gene. The fabI sequence was cloned into the plasmids pET21a (Novagen) and pPJ131, pBluescript plasmid (Stratagene) with a modified multiple cloning site (14), via the NdeI and EcoRI (New England Biolabs) cloning sites. The pET21a-CtFabI expression plasmid was transformed in BL21 Tuner cells (Novagen) for protein expression and purification.

The pPJ131-CtFabI plasmid was transformed into the fabI temperature-sensitive *E. coli* strain JP1111 to determine complementation. The JP1111 strain is viable at 30 °C but nonviable at 42 °C without fabI gene complementation. The JP1111 cells were transformed with the pPJ131-CtFabI plasmid, the pPJ131 parent plasmid, and pBluescript plasmids expressing fabI from *E. coli* and *Bacillus anthracis* and then plated on Luria-Bertani (LB) plates at 30 °C with 100 µg/ml carbenicillin. The transformed cells were restreaked onto LB plates with 100 µg/ml carbenicillin and grown at 30 or 42 °C to determine whether the *C. trachomatis fabI* complements the *E. coli fabI* activity.

**CtFabI Expression and Purification**—BL21 Tuner cells harboring the pET21a-CtFabI plasmid were grown in LB medium with 100 µg/ml carbenicillin at 37 °C and 225 rpm shaking until *A*₆₀₀ reached 0.6–0.8. The culture was then induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside and grown at 17 °C and 225 rpm shaking overnight. Cells were pelleted and washed twice with 20 mM Tris, pH 8.0, and finally resuspended in 20 mM Tris, pH 8.0 (40 ml/liter of culture). Cells were lysed via a cell disruptor, and the C-terminal 6-histidine-tagged CtFabI was purified via nickel chelation chromatography. Briefly, the cell lysate was centrifuged at 20,000 × g to remove the cell debris. The resulting supernatant was poured over a nickel-nitritoltri-acetic acid resin column (4 ml of resin/liter of cell culture) to bind the protein to the column. The column was washed with 5 column volumes of 20 mM Tris, pH 8.0, and 20 mM imidazole, followed by 5 column volumes of 20 mM Tris, pH 8.0, 500 mM NaCl, and 50 mM imidazole. The protein was eluted from the column with 20 mM Tris pH 8.0 and 250 mM imidazole. The fractions containing protein, as determined by the Bradford reagent, were collected and dialyzed against 20 mM Tris, pH 8.0, 10 mM EDTA, and 150 mM NaCl at 4 °C overnight. A pure protein (≥95%) running at ~32 kDa (theoretical average mass of 32,783.49 with N-terminal methionine) was observed on a NuPAGE 10% BisTris gel. Over 25 mg of purified CtFabI was obtained per liter of culture.

**Analytical Ultracentrifugation**—Analytical ultracentrifugation experiments and analysis were carried out as described previously by the St. Jude Molecular Interaction Analysis Facility (15, 16). Experiments were performed in 20 mM Tris, pH 7.5, 10 mM EDTA, 150 mM NaCl buffer. Chlamydial FabI has a calculated molecular mass of 32,783 Da.

**Enzymology**—FabI activity was determined by monitoring the optical density at 340 nm to measure the conversion of NAD(P)H to NAD(P)⁺. The enzyme reactions were 100 µl in volume and were performed and monitored in Costar UV half-area 96-well plates with a SpectraMax 340 instrument taking 340-nm readings at 10-s intervals at 30 °C. The substrate NADH and NADPH were obtained from Sigma-Aldrich. The substrate crotonyl-ACP from *S. aureus* was synthesized as described previously (17). For velocity measurements, the CtFabI enzyme was added to 100 µM crotonyl-ACP and a 250 µM concentration of either NADPH or NADH in 20 mM Tris, pH 8.0. Upon finding that CtFabI prefers NADH over NADPH by over 1000-fold, NADH was used for future measurements. For determination of *Kₘ* of crotonyl-ACP, 100 nM CtFabI was added to 200 µM NADH and 3, 6, 12, 24, 36, or 48 µM crotonyl-ACP. For determination of *Kₘ* of NADH, 100 nM CtFabI was added to 50 µM crotonyl-ACP and 5, 10, 15, 20, 30, 50, and 75 µM NADH. The reaction was mixed for 10 s by the mix function on the plate reader, and data were acquired at 10-s intervals for 5 min. The initial velocity was calculated from the linear phase of the progress curve, and the initial velocity data were fit using a standard Michaelis-Menten equation to determine the apparent *Kₘ*. The IC₅₀ of AFN-1252 and triclosan against CtFabI was measured as above at saturating substrate concentrations (50 µM crotonyl-ACP and 200 µM NADH) against different concentrations of AFN-1252 and triclosan (0, 0.156, 0.312, 0.625, 1.25, 2.5, 5, and 10 µM). All kinetic experiments were run in triplicate.

**Protein Thermal Shift Analysis**—Protein thermal shift analysis was conducted to determine the mode of binding of AFN-1252 to CtFabI. Solutions (30 µl) of CtFabI (10 µM), CtFabI (10
µM) + AFN-1252 (10 µM), CtrFabI (10 µM) + NADH (100 µM), CtrFabI (10 µM) + NAD (100 µM) + AFN-1252 (10 µM), CtrFabI (10 µM) + NADH (100 µM) + AFN-1252 (10 µM), CtrFabI (10 µM) + NAD+ (100 µM), and CtrFabI (10 µM) + NAD+ (100 µM) + AFN-1252 (10 µM) in a final buffer of 100 µM Tris, pH 7.0, 0.5% DMSO, and 2.5× Sypro Orange Dye (Sigma-Aldrich) were added to wells of ThermoGrid optically clear PCR plates (Denville Scientific). The plates were centrifuged for 5 min at 1000 × g to remove air bubbles and then subjected to thermal shift analysis in the ABI 7300 real-time PCR system. The temperature was ramped from 25 to 95 °C at 1 °C/min with the fluorescence read six times at each temperature ramp. The resulting data were fit to a Boltzmann sigmoidal equation to determine the melting point of the CtrFabI under each particular ligand combination. Each ligand condition was replicated six times, and each replicate was subjected to independent analysis to determine the thermal melting point. The melting points of the six independent runs per ligand condition were averaged to determine the thermal melting point for each ligand condition. Representative thermal shift runs along with the average thermal melting points are presented.

Crystallization and Structure Determination—The CtrFabI protein was incubated for 1 h with 0.4 mM NADH and 0.25 mM AFN-1252 at 2 mg/ml and then concentrated to 7 mg/ml for crystallization. Initial trials were performed at 18 °C against the PEGs Suite and PEGs II Suite (Qiagen), and several conditions produced small crystals that were subsequently optimized. Crystals suitable for X-ray analysis were eventually obtained in 28% (w/v) polyethylene glycol 300, 0.1 M HEPES (pH 7.5), and 0.2 M potassium formate. Crystals were flash-frozen directly from mother liquor in liquid nitrogen and diffracted to 1.8 Å in a crystallographic cell with space group P43 with unit cell dimensions a = 96.2 Å, b = 96.2 Å, c = 263.0 Å. Diffraction data were collected at the SER-CAT beam line ID22 at the Advanced Photon Source and processed using HKL2000 (18). The CtrFabI structure was solved by molecular replacement using the program Phaser (19) and the coordinates of apo-FabI from Brassica napus (Protein Data Bank accession code 1ENO) as the search model. The structure was completed by iterative rounds of refinement using Phenix (20) and manual rebuilding using Coot (21). The refinement was monitored by following the refinement using Phenix (20) and manual rebuilding using Coot (21). The structure was completed by iterative rounds of refinement using Phenix (20) and manual rebuilding using Coot (21). The structure was completed by iterative rounds of refinement using Phenix (20) and manual rebuilding using Coot (21). The structure was completed by iterative rounds of refinement using Phenix (20) and manual rebuilding using Coot (21). The structure was completed by iterative rounds of refinement using Phenix (20) and manual rebuilding using Coot (21).
lipids via the Bligh and Dyer protocol (24). Radioactive incorporation was measured by counting the lipid extracts on an LS6500 multipurpose scintillation counter. Measurements were made in triplicates, and the averages with S.E. were reported. For the HeLa cell controls, a mock infection (same infection protocol with no C. trachomatis) was performed on the cells, and the same labeling procedure was performed. Cell viability was measured with the NucleoCounter (New Brunswick Scientific) following the manufacturer’s protocol.

RESULTS

C. trachomatis FASII—A bioinformatics analysis of the C. trachomatis genome suggests that it encodes a complete FASII (Fig. 1). Branched-chain fatty acids arise from the utilization of 2-methylbutyryl-CoA by FabH to prime FASII (25). 2-Methylbutyryl-CoA is generated from isoleucine by the branched-chain α-ketoacid dehydrogenase complex. The structure and mechanism of the branched-chain α-ketoacid dehydrogenase complex is similar to the pyruvate dehydrogenase complex. The genes encoding both the branched-chain α-ketoacid dehydrogenase complex (CT245 to CT268) were identified in the C. trachomatis genome. This bioinformatics analysis predicts that C. trachomatis has the gene set for the synthesis of branched-chain and hydroxy fatty acids that cannot be obtained from the host. Branched-chain fatty acids are incorporated into chlamydial phospholipids (26), and the straight-chain 3-hydroxy fatty acids are found in the LOS of this organism (27–29). The gene(s) for unsaturated fatty acid synthesis are absent in C. trachomatis. Long-chain acyl-ACP is used for phospholipid synthesis, and long-chain 3-hydroxyacyl-ACP is used for LOS synthesis.

FabI Is an Enoyl-ACP Reductase—The enoyl-ACP reductase plays a determinant role in establishing the rate of FASII (30, 31). The predicted FabI sequences from different chlamydial strains have greater than 99% amino acid identity and are homologous to the E. coli FabI (31% identity, 49% similarity). The function of CtFabI in FASII was assessed by determining whether CtFabI expression complemented the growth phenotype of an E. coli fabI(Ts) mutant. The E. coli codon-optimized fabI gene from C. trachomatis D/UW-3/Cx was cloned into the pBluescript expression vector and transformed into E. coli strain JP1111 (fabI(Ts)) (32). At the permissive temperature (30 °C), strain JP1111 and all its transformants exhibited robust growth (Fig. 2A). At the non-permissive temperature (42 °C), strain JP1111 was unable to grow without a complementing fabI gene being present in the plasmid (Fig. 2B). The E. coli, B. anthracis, and C. trachomatis fabI genes all complemented growth at the non-permissive temperature, confirming that the C. trachomatis fabI gene product functioned as an enoyl-ACP reductase in the heterologous E. coli FASII.

CtFabI was expressed, and the protein was purified by affinity chromatography (Fig. 3A). CtFabI sedimented as a 128-kDa complex in analytical centrifugation experiments (Fig. 3B), which was consistent with CtFabI as a homotetramer (131 kDa expected), like other characterized FabI proteins (33). The enzymatic reaction of CtFabI was assessed by measuring NADH/NADPH conversion to NAD+/NADP+ at 340 nm. The CtFabI catalyzed the turnover of NADH (Km = 22.93 ± 3.16 μM) (Fig. 3C) into NAD+ in the presence of crotonyl-ACP.
(K_m = 18.18 ± 2.67 μM) (Fig. 3D). NADPH was >1000-fold less efficient than NADH in supporting the reaction, consistent with CtFabI preferring NADH like most bacterial FabIs, with the sole exception of SaFabI (30, 34). The k_cat of CtFabI with NADH and crotonyl-ACP was 15.6 ± 0.05 min⁻¹, which is similar in velocity to other characterized FabI enzymes (35).

These biochemical experiments confirmed that CtFabI was an enoyl-ACP reductase.

AFN-1252 Inhibition of CtFabI—The chemical biology approach was validated by determining the affinity and the mode of inhibition of AFN-1252 for CtFabI. AFN-1252 exhibited an IC₅₀ of 0.95 ± 0.21 μM against CtFabI at saturating substrate concentrations (Fig. 4A). The IC₅₀ against the prototypical FabI inhibitor, triclosan, was also determined (0.32 ± 0.08 μM). AFN-1252 inhibited SaFabI by forming a FabI-NADPH-AFN-1252 complex (12, 17). The formation of this ternary complex on CtFabI was demonstrated by examining the effects of AFN-1252 on the CtFabI melting temperature using a protein thermal denaturation assay (Fig. 4B). CtFabI had a melting temperature of 48 °C in the absence of any ligand. The addition of AFN-1252 did not shift the melting temperature, indicating the absence of a CtFabI-NAD⁺-AFN-1252 complex. These data indicated that AFN-1252 inhibited CtFabI by binding to the CtFabI-NAD⁺ complex. Thus, AFN-1252 is a sharp tool to study the effects of FASII inhibition on C. trachomatis replication in light of its selectivity, its binding affinity, and the absence of off-target effects on human cells (11, 36).

The FabI-NADH-AFN-1252 Ternary Complex—A 1.8 Å crystal structure of the ternary complex was solved to validate the biochemical characterization (Table 1). There were eight molecules of the ternary complex in the crystal asymmetric unit that represented two copies of the biological tetramer. The protomers were all structurally similar with a root mean square deviation between all main chain atoms of 0.17 Å. The electron densities of the co-factor NADH and the small molecule inhibitor in each of the eight active sites were well resolved. The comparison of the overall fold of CtFabI with that of SaFabI shows that both proteins have a seven-stranded parallel β-sheet flanked by 11 α-helices and several loops that are typical for FabIs (Fig. 5) (33). The active site contains the signature catalytic tyrosine and lysine residues (Tyr-188 and Lys-195) directly

FIGURE 3. Purification and biochemistry of CtFabI. A, CtFabI was purified by affinity and gel filtration chromatography. Gel electrophoresis shows the purity of CtFabI. Lane 1, standards; lane 2, CtFabI (32.7 kDa). B, the sedimentation velocity profiles (fringe displacement) were fitted to a continuous sedimentation coefficient distribution model c(s). CtFabI sedimented as a 128-kDa tetramer (131 kDa theoretical mass). C, initial velocities were determined in triplicate as a function of NADH concentration. D, initial velocities were determined in triplicate as a function of crotonyl-ACP concentration. The data points were fit to the Michaelis-Menten nonlinear regression fit in GraphPad Prism version 5. The fitted line is shown on the graph. The fitted K_m values with the S.E. values (error bars) are reported.
adjacent to the bound cofactor (33). CtfFabI has two additional loops comprising residues 56–69 and 84–104, but they are located at the surface and do not impact the conserved FabI fold or the active site (Fig. 5). AFN-1252 consists of oxotetrahydro-naphthyridine and 3-methylbenzofuran groups linked by a cis-amide. The carbonyl oxygen atom of the cis-amide makes hydrogen bond interactions with the 2’-hydroxyl of the nicotinamide ribose and the hydroxyl of Tyr-188, whereas the pyridyl nitrogen and the N-acyl hydrogen of the oxotetrahydro-naphthyridine moiety make hydrogen bonds with the peptide backbone carbonyl and amide of Ser-130, respectively (Fig. 6A). The 3-methylbenzofuran group forms an edge-to-face π-interaction with the side chain of Phe-235. FabI is characterized by a flexible substrate-binding loop, called the “flipping loop,” that “closes” to orient the substrate with respect to the cofactor nicotinamide ring for reduction (37–39). This loop (residues 228–240) adopts the “closed” conformation that has been observed with other FabI-inhibitor complexes (12, 40, 41), and the cis-amide stacks onto the nicotinamide ring to apparently mimic the targeted double bond of the bound substrate (33).

The CtfFabI and SaFabI (Protein Data Bank accession code 4FS3) structures are very similar, with a root mean square deviation on main chain atoms of 1.4 Å, and the modes of binding of the cofactor and inhibitor are practically identical (Fig. 6). One difference is that Phe-204 on the SaFabI substrate-binding loop (residues 195–207) is replaced by Ile-236 in CtfFabI; however, the adjacent Phe-235 of CtfFabI remains positioned to make the edge-to-face π-interaction with the 3-methylbenzofuran group of the inhibitor. FabI proteins have a relatively unconserved and conformationally flexible substrate-binding α-helical loop that creates a “lid” over the active site following the binding of the cofactor and substrate (17, 40). In SaFabI, Met-99 within this segment (residues 97–102) extends into the active site pocket toward the oxotetrahydro-naphthyridine group of AFN-1252, and this key hydrophobic interaction is critical for the high-affinity binding of AFN-1252 to SaFabI (Fig. 6B) (17). Comparison of the CtfFabI and SaFabI structures revealed not only that CtfFabI contains a two-residue deletion, including this methionine, but that the loop (residues 130–133) was apparently more flexible and lacked the ordered α-helical segment found in SaFabI (Fig. 6). Most significantly, the loop is flipped away from the inhibitor-binding site and, as a result, no residues of CtfFabI are positioned to replace the lost hydrophobic interaction. In addition, the movement of this loop reduces the binding surface of AFN-1252 such that the oxotetrahydro-naphthyridine moiety occupies a solvent-exposed cavity (Fig. 6A). In comparison, the lid

### TABLE 1

X-ray crystallographic data and refinement statistics for CtfFabI-NADH-AFN1252 complex (Protein Data Bank entry 4Q9N)

| Crystal | CtfFabI-NADH-AFN1252 |
|---------|-----------------------|
| Data collection | P43 |
| Space group | |
| Wavelength (Å) | 1.0 |
| Unit cell dimensions | |
| a (Å) | 96.15 |
| b (Å) | 96.15 |
| c (Å) | 263.08 |
| α, β, γ (degrees) | 90, 90, 90 |
| Molecules/ASU | 8 |
| Resolution (Å) | 1.8 (1.85–1.80) |
| Completeness (%) | 98.8 (95.1) |
| Rejection % | 6.3 (3.4) |
| No. of total reflections | 335,128 |
| No. of unique reflections | 221,426 |
| I/σ(I) | 15.5 (1.57) |
| Rsym | 12.2 (61.6) |

| Refinement statistics | |
| Resolution (Å) | 1.8 |
| No. of reflections | 218,306 |
| Rwork/Rfree (%) | 0.1863/0.2292 |
| No. of atoms | 19,232 |
| Protein | 17,700 |
| Ligand/ion | 576 |
| Water | 956 |
| B-Factors (Å²) | |
| Protein | 25.4 |
| Ligand/ion | 22.7 |
| Water | 28.2 |
| Root mean square deviations | |
| Bond length (Å) | 0.006 |
| Bond angle (degrees) | 1.057 |

| Ramachandran analysis | |
| Most favored (%) | 97.5 |
| Allowed (%) | 3.3 |
| Disallowed (%) | 0.2 |

* Asymmetric unit.
* Values in parentheses are for the highest resolution shell.
* $R_{\text{work}} = \Sigma |I-I_o|/\Sigma I_o$, where $I$ is the observed intensity, and $I_o$ is the average intensity of multiple observations of symmetry-related reflections.
* $R_{\text{free}} = \Sigma |I-I_o|/\Sigma I_o$.
* $R_{\text{free}}$ is calculated from 5% of the reflections excluded from refinement.
adopts a more tightly closed conformation around the inhibitor in the SaFabI structure (Fig. 6B). These differences provide a structural explanation for why AFN-1252 exhibits lower affinity against CtFabI compared with SaFabI. However, the key interactions between AFN-1252 and the peptide backbone and cofactor are maintained in the CtFabI/H18528/NADH/H18528 AFN-1252 complex, offering opportunities for the structure-based design of modified AFN-1252 derivatives that can exploit the larger substrate pocket in CtFabI.

AFN-1252 Inhibits Fatty Acid Synthesis—The effect of AFN-1252 on branched-chain fatty acid synthesis was determined to verify that the drug blocked bacterial FASII. The branched-chain fatty acids are synthesized by initiating FASII with a short-chain branched-chain acyl-CoA, followed by five rounds of elongation using malonyl-ACP derived from acetyl-CoA (Fig. 1) (25). Straight-chain fatty acids are built from acetyl-CoA molecules entirely (25, 42). Isoleucine is the precursor to 2-methylbutyryl-CoA via the bacterially encoded branched-chain α-ketoacid dehydrogenase, whereas glucose is a precursor to acetyl-CoA in both mammalian and bacterial metabolic schemes. Incorporation of the radioactivity from [U-14C]glucose into the lipid fraction indicated total fatty acid synthesis activity in both the host and the parasite. Because the eukaryotic host cannot make branched-chain fatty acids, incorporation of radioactive [U-14C]isoleucine into the lipid fraction primarily reflected the FASII activity of C. trachomatis. One caveat was that isoleucine is broken down into acetyl-CoA by the mammalian host (43), which can then be incorporated into fatty acids derived from both host and parasite. Therefore, we also measured the amount of [U-14C]isoleucine incorporated into uninfected HeLa cells to serve as an estimate for the extent of this metabolic conversion.

Cell viability and [U-14C]glucose and [U-14C]isoleucine incorporation into the uninfected HeLa cells when treated with 50 μM of AFN-1252 were determined to ensure that AFN-1252 does not exhibit off-target effects on the host cell. Treatment of the host cell with 50 μM AFN-1252 did not affect radioactive incorporation from [U-14C]glucose (Fig. 7A) or cell viability (>95%). Infected HeLa cells were pulse-labeled for 4 h with [U-14C]glucose 20 h following the initial infection to measure
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FIGURE 6. FabI-NADH-AFN ternary complex structure. A, CifFabI-NADH-AFN-152 ternary complex (Protein Data Bank accession code 4Q9N). B, SaFabI-NADPH-AFN-1252 ternary complex (Protein Data Bank accession code 4F33). The color schemes are the same in A and B. AFN-1252 is shown in yellow, and NADPH/NADH is shown in green. The flexible, substrate-binding flipping loop is colored slate, and the poorly conserved lid loop is colored chartreuse. Black dashes show the conserved hydrogen bonding network for AFN-1252. In SaFabI, the substrate lid adopts a more ordered, helical structure and orients Met-99 into the active site toward the inhibitor. In contrast, in CifFabI, the active site is more open, due to the less structured conformation of the lid. C, active site of the CifFabI-NADH-AFN-1252 ternary complex. AFN-1252 binds within a hydrophobic pocket composed of Tyr-178, Phe-235, and Ile-236, with Phe-235 positioned for 

n-interactions with the 3-methylbenzofuran of AFN-1252. The 2'-hydroxyl of NADH and the hydroxyl of Tyr-188 make hydrogen bonds with the carbonyl of the linking cis-amide, and the Ser-130 peptide backbone carbonyl and amide make hydrogen bonds with the pyridyl nitrogen and the N-acyl hydrogen of the oxotetrahydroanaphthrydine of AFN-1252. α-weighted 2Fo-Fo electron density is shown for AFN-1252 and NADH contoured at 1.5 σ.

the effect of AFN-1252 on the total fatty acid biosynthetic activity in the infection model. The infected cell population incorporated 7.5 times more radioactivity compared with uninfected HeLa cells (Fig. 7A). AFN-1252 treatment reduced [U-14C]glucose incorporation by over 70% compared with the untreated infected cells. Similar labeling experiments with [U-14C]isoleucine revealed that infected HeLa cells incorporated over 6 times more radioactivity into the lipid fraction than did uninfected HeLa cells, consistent with an increased synthesis of branched-chain fatty acids in the infected cells (Fig. 7B). AFN-1252 treatment of infected HeLa cells caused a dose-dependent decrease in [U-14C]isoleucine incorporation (Fig. 7C). In cells treated with 50 μM AFN-1252, [U-14C]isoleucine incorporation was the same as in uninfected cells (Fig. 7, B and C), demonstrating that AFN-1252 blocked the increase in [U-14C]isoleucine incorporation resulting from the infection. The incorporation of [U-14C]isoleucine into infected cells and the residual incorporation in AFN-1252-treated cells were attributed to the conversion of isoleucine to acetyl-CoA (43), which was used for host fatty acid synthesis. These results demonstrated that AFN-1252 inhibited C. trachomatis fatty acid synthesis in the infection model.

AFN-1252 Inhibits C. trachomatis Replication—The role of endogenous fatty acid synthesis in regulating C. trachomatis growth was determined by treating the Chlamydia-HeLa cell infection model with AFN-1252. HeLa cells infected with C. trachomatis serovar L2 were incubated with increasing concentrations of AFN-1252 for 48 h. The number of inclusions in an infected monolayer (Fig. 8A) and the number of infectious units (EBs) formed (Fig. 8B) were both decreased in a dose-dependent manner by AFN-1252. At >6 μM AFN-1252, no infectious particles were generated. AFN-1252 effectively decreased the infectious titers when added as late as 32 h postinfection, consistent with a critical role for FASII throughout the entire developmental cycle (Fig. 8C). The number of chlamydial genomes per cell decreased over time when infected cells were treated with 10 μM AFN-1252, suggesting that AFN-1252 arrested the replication of C. trachomatis, and the host cell cleared the organisms that had been internalized (Fig. 8D).

The stage in the developmental cycle where AFN-1252 arrested chlamydial growth was determined by double-staining individual cells with a mouse monoclonal antibody directed against chlamydial Hsp60 (cytoplasmic marker, green) and Hoechst (blue) to mark host cell nuclei. AFN-1252 treatment caused a dose-dependent decrease in the size of the chlamydial inclusion in HeLa cells infected with C. trachomatis serovar L2 (Fig. 9A). At concentrations exceeding 4 μM AFN-1252, only a single bacterium was detected in each infected cell (Fig. 9A). The effect of AFN-1252 on C. trachomatis serovar D was also examined to verify that the ability of AFN-1252 to arrest C. trachomatis replication was not strain-dependent (Fig. 9B). In the D serovar, at AFN-1252 concentrations exceeding 4 μM, only a single bacterium was detected in each infected cell. These data also showed that AFN-1252 prevented the first cell division of C. trachomatis following infection. Double staining with Hsp60 (red) and a polyclonal antibody against the chlamydial MOMP (green) was performed to determine whether EBs differentiated into RBs in AFN-1252-treated cells. EBs were characterized by
a small area of Hsp60 staining completely enclosed by MOMP indicated by a yellow cytoplasmic dot surrounded by green (Fig. 9C). Treatment of C. trachomatis-infected cells with rifampicin (RNA synthesis inhibitor) or chloramphenicol (protein synthesis inhibitor) arrested cells in an EB-like state with no change in size or pattern of MOMP or Hsp60 staining compared with EBs (Fig. 9C), consistent with RNA and protein synthesis being required to support differentiation into an RB. In the normal

FIGURE 7. AFN-1252 inhibits C. trachomatis FASII. A, [U-14C]glucose incorporation into the lipids of uninfected HeLa cells, uninfected HeLa cells treated with 50 μM AFN-1252, infected HeLa cells, and infected HeLa cells treated with AFN-1252. B, [U-14C]isoleucine incorporation into the lipids of uninfected HeLa cells, uninfected HeLa cells treated with AFN-1252, infected HeLa cells, and infected HeLa cells treated with AFN-1252. C, [U-14C]isoleucine incorporation into the lipids of infected HeLa cells as a function of increasing concentrations of AFN-1252. HeLa cells were infected at a multiplicity of infection of 5 at time 0. All labeling experiments were conducted by adding AFN-1252 to the cell cultures for 30 min at 20 h postinfection, followed by radioactive pulse labeling for 4 h with the indicated radiochemical. Error bars, S.E.

FIGURE 8. AFN-1252 inhibits C. trachomatis replication. A, number of chlamydial inclusions observed per ×40 field at 42 h postinfection was determined as a function of increasing AFN-1252 concentrations added immediately after the infection of HeLa cells with C. trachomatis serovar L2. B, number of infectious units of C. trachomatis serovar L2 generated in the presence of increasing AFN-1252 concentrations added immediately after the initial infection. C, number of infectious units of C. trachomatis serovar L2 produced when 6 μM AFN-1252 was added at 0, 8, 16, 24, and 32 h after the initiating infection. The number of infectious particles was counted at the end of 48 h in all cases. Six measurements were made in each of two experiments. Significance was determined using Student’s t test: *, p < 0.05; **, p < 0.01; ***, p < 0.001. D, number of C. trachomatis serovar L2 genomes during the course of an infection cycle in control and cells treated with 10 μM AFN-1252. Error bars, S.E.
Fatty Acid Synthesis in C. trachomatis

Our results show that FASII is essential for the replication of C. trachomatis within its eukaryotic host. FASII is essential for the synthesis of two major membrane constituents in free-living Gram-negative bacteria. First, FASII supplies the fatty acids required for the synthesis of membrane phospholipids, and second, FASII generates the 3-hydroxy fatty acids required for LOS synthesis. Previous work showed that the selective inhibition of LOS synthesis at the LpxC step permitted the proliferation of C. trachomatis within its cytoplasmic inclusion but blocked the terminal differentiation of RBs into EBs (10). If FASII is only required for generating 3-hydroxy fatty acids for LOS synthesis and the bacterium obtains its phospholipids from the host, then AFN-1252 inhibition would have the same effect on the infection cycle as a LOS inhibitor. However, blocking FASII arrests cell replication whenever it is added over the entire developmental cycle, which is consistent with phospholipid synthesis using products derived from FASII as essential for C. trachomatis replication. Inhibitors of RNA synthesis (rifampicin) and protein synthesis (chloramphenicol) blocked C. trachomatis development at the EB stage, showing that these processes are necessary for EB to RB conversion. C. trachomatis had an enlarged, RB-like morphology in AFN-1252-treated cells, indicating that FASII inhibition arrested development after the differentiation from EB to RB is initiated but before the first cell division. The phospholipid classes of C. trachomatis reflect the host cell composition (26). Previous work suggests that C. trachomatis obtains its phospholipids from the host and modifies a subset of these lipids by introducing a branched-chain fatty acid into the 2-position (3, 26). Additional work is needed to determine whether C. trachomatis synthesizes an important class of phospholipids de novo, using the products of FASII, or whether the acylation/deacylation pathway is critical for the modification of host phospholipids with branched-chain fatty acids. Nonetheless, it is clear from this study that FASII products are critical for cell division in this parasitic bacterium.

This work identifies FASII, and FabI in particular, as a therapeutic target in C. trachomatis. As an intracellular pathogen that acquires vital nutrients from the host, C. trachomatis lacks many biochemical pathways that are traditional antibacterial drug targets (44, 45). Although previous work suggested that fatty acids and phospholipids of chlamydial membranes are obtained from the host (45), our study shows that FASII is nonetheless essential for chlamydial replication. AFN-1252 is effective in cell culture against C. trachomatis and has pharmacological properties that make it an effective agent against S. aureus infections (36, 46). However, its potency may have to...
be improved in order for it to be effectively deployed to treat *C. trachomatis*. Our CrFabI-NADH-AFN-1252 crystal structure shows that AFN-1252 inhibits CrFabI by making similar contacts to the backbone amides and nucleotide cofactor as SaFabI (12). Our structure characterization also clearly points to the absence of the Met-99 side chain interaction with the oxetetra-hydronaphthyridine ring of AFN-1252 as the primary reason for the lower affinity of AFN-1252 binding to CrFabI compared with SaFabI. This interaction with Met-99 in SaFabI is established to be critical for high-affinity AFN-1252 binding in SaFabI based on the finding that a missense mutation giving rise to a M99T mutation in FabI accounts for acquired resistance to AFN-1252 in *S. aureus* (17). Structure modifications to AFN-1252 that compensate for this interaction should allow for the design of a potent and selective CrFabI inhibitor.

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