Activation of Exogenous Fatty Acids to Acyl-Acyl Carrier Protein Cannot Bypass FabI Inhibition in Neisseria*

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Neisseria is a Gram-negative pathogen with phospholipidic composed of straight chain saturated and monounsaturated fatty acids, the ability to incorporate exogenous fatty acids, and lipopolysaccharides that are not essential. The FabI inhibitor, AFN-1252, was deployed as a chemical biology tool to determine whether Neisseria can bypass the inhibition of fatty acid synthesis by incorporating exogenous fatty acids. Neisseria encodes a functional FabI that was potently inhibited by AFN-1252. AFN-1252 caused a dose-dependent inhibition of fatty acid synthesis in growing Neisseria, a delayed inhibition of growth phenotype, and minimal inhibition of DNA, RNA, and protein synthesis, showing that its mode of action is through inhibiting fatty acid synthesis. Isotopic fatty acid labeling experiments showed that Neisseria encodes the ability to incorporate exogenous fatty acids into its phospholipids by an acyl-acyl carrier protein-dependent pathway. However, AFN-1252 remained an effective antibacterial when Neisseria were supplemented with exogenous fatty acids. These results demonstrate that extracellular fatty acids are activated by an acyl-acyl carrier protein synthetase (AasN) and validate type II fatty acid synthesis (FabI) as a therapeutic target against Neisseria.

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§The abbreviations used are: FASII, bacterial type II fatty acid synthesis; ACP, acyl carrier protein; PE, phosphatidylethanolamine; D4-16:0, [7,7,8,8-D4]palmitic acid; D3-16:0, [methyl-13C]palmitic acid; D3-14:0, [methyl-13C]myristic acid; D3-12:0, [methyl-13C]lauric acid; acyl-PO4, acyl-phosphate; MIC, minimal inhibition concentration; SJ-GC, Shockley-Johnston-modified gonococcal; GW, Graver-Wade.

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enoyl-ACP reductase (FabI) (23–26). FabI catalyzes the reduction of trans-2-enoyl-ACP, a rate-controlling step required to complete each round of elongation in fatty acid synthesis (27). AFN-1252 (23, 28–33) is a FabI therapeutic that has completed Phase II clinical trials and proved efficacious against *Staphylococcus aureus* infections in humans. We selected AFN-1252 as a chemical probe to test whether exogenous fatty acids can bypass FabI (FASII) inhibition. Inhibition of *Neisseria* FabI by AFN-1252 cannot be bypassed by exogenous fatty acid supplementation. This work establishes an acyl-ACP synthetase pathway for fatty acid uptake in *Neisseria* and validates FabI inhibitors as a viable strategy for the development of *Neisseria* therapeutics.

**Experimental Procedures**

Materials—Cell culture supplies were from BD Biosciences, and chemicals were from Sigma-Aldrich or Fisher unless otherwise indicated. Radiochemicals were from American Radiochemicals.

**Neisseria Growth and Propagation**—Briefly, chocolate blood agar plates were made by suspending and autoclaving 7.2 g of Difco GC Medium base in 100 ml of water. To this solution was added 100 ml of sterile 2% hemoglobin solution (BBL hemoglobin, bovine, freeze-dried) and 2 ml of IsoVitaleX supplement. The Shockley-Johnston-modified gonococcal (SJ-GC) medium was used for the planktonic growth of *N. gonorrhoeae*. The SJ-GC medium was made by combining three separate solutions. Solution 1 contained 15 g of proteose peptone, 3.4 g of KH₂PO₄, 1 g of K₂HPO₄, 5 g of NaCl, 5 g of casamino acids, and 1 g of soluble potato starch in 1 liter of distilled water. Solution 2 contained 22 g of dextrose and 3 g of MgSO₄ in 50 ml of distilled water. Solution 3 contained 0.0001 g of thiamine pyrophosphate, 0.05 g of glutamine, and 0.2 g of cysteine hydrochloride. Solution 1 was autoclaved for 15 min at 120 °C, whereas solutions 2 and 3 were filter-sterilized. The three solutions were combined once they reached room temperature to make the SJ-GC medium. The SJ-GC medium contained potato starch, a known drug binder, so the chemically defined Graver-Wade (GW) medium was used for MIC determination for both *N. gonorrhoeae* and *N. meningitidis*. The GW medium was made as described (34). Bovine serum albumin (10 mg/ml) was added to the GW medium to increase the fatty acid binding capacity of the medium. *N. gonorrhoeae* strain NCTC 12700 and *N. meningitidis* strain ATCC 13077 were streaked from 10-s intervals at 37 °C. The substrate crotonyl-ACP was synthesized and monitored in Costar UV half-area 96-well plates (96-well plate containing 100 μl of GW medium with an added 7.5 g of agarose for plates) at 30 °C with 100 μg/ml of carbenicillin. The transformed cells were restreaked onto Luria-Bertani plates with 100 μg/ml carbenicillin and grown at 30 or 42 °C to determine whether the *N. gonorrhoeae* fabI complements the *E. coli* fabI activity.

**NgFabI Protein Expression and Purification**—BL21AI cells harboring the pET21a-NgFabI were grown in Luria-Bertani medium with 100 μg/ml of carbenicillin at 37 °C with 225-rpm shaking until the *A₅₆₀* reached 0.6–0.8. The culture was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside and 0.2% arabinose (w/v) and grown at 17 °C with 225-rpm shaking overnight. Cells were pelleted and washed twice with 20 mM Tris, pH 8.2, and finally resuspended in 20 mM Tris, pH 8.2 (20 μl/milliter of culture). Cells were lysed via a cell disruptor and the C-terminal His₆-tagged NgFabI was purified via standard nickel chelation chromatography (24).

**Enzymology**—The NgFabI enzymatic activity was determined by measuring the conversion of NAD(P)H to NAD(P)⁺ at 340 nm. The enzyme reactions were 100 μl in volume and 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 37 °C. The substrate crotonyl-ACP was synthesized as described previously using *E. coli* ACP (23). The velocity of the NgFabI enzyme (200 nM) was measured by adding 50 μM crotonyl-ACP and either 200 μM NADPH or NADH in 20 mM Tris, pH 8.0. Upon finding that NADH, but not NADPH, catalyzed the NgFabI reaction, NADH was used for future measurements. The apparent *Kₘ* of crotonyl-ACP was determined by adding 200 nM NgFabI to 200 μM NADH and 3, 6, 12, 25, 50, 100, or 150 μM crotonyl-ACP. The apparent *Kₘ* of NADH was determined by adding 200 nM NgFabI to 50 μM crotonyl-ACP and 5, 10, 20, 40, 80, 160, and 320 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. Initial velocity was calculated from the linear phase of the progress curve and fit using a standard Michaelis-Menten equation to determine the
The apparent $K_m$ of AFN-1252 against $NgFabI$ was measured as above at saturating substrate concentrations (50 $\mu$M crotonyl-ACP and 200 $\mu$M NADH) against different concentrations of AFN-1252 (0, 7.8, 15.6, 31.2, 62.5, 125, 250, and 500 nM). All kinetic experiments were run in duplicates. Under standard steady state conditions, inhibitors are kept at concentrations 10-fold or more above the concentration of the enzyme, so that the formation of the enzyme inhibitor complex does not alter the concentration of free inhibitors. However, AFN-1252 had affinity values in the nanomolar range for the $NgFabI$, and nanomolar concentrations of $NgFabI$ are necessary to generate a detectable signal in our kinetic assay. Thus, the data were fitted to the Morrison quadratic equation for fitting tight binding inhibitors (23, 36), which allows for the determination of affinity in terms of free and bound concentrations of enzyme and inhibitor, accounting for the impact of enzyme inhibitor binding on the free concentration of inhibitor. The resulting $K_i$ was the apparent dissociation constant of AFN-1252 to the $NgFabI$.

$[^{14}C]$Acetate Labeling—$N$. gonorrhoeae was grown in SJ-GC medium until $A_{600}$ reached 0.5, and then aliquoted to 10-ml cultures. Increasing concentrations of AFN-1252 (DMSO only, 1, 2, 4, 8, and 16 $\mu$M) were added to each culture and incubated for 15 min at 37 °C with 125-rpm shaking. Next, 10 $\mu$Ci of $[^{1-14}C]$acetate was added to each culture and incubated for 45 min at 37 °C with 125-rpm shaking. The cells were harvested via centrifugation (4,000 × g) and washed twice with phosphate-buffered saline. The lipid fraction of the cells was extracted via the Bligh and Dyer method (37), and the total radioactive incorporation relative to the DMSO-treated cells and fitted via a standard IC$_{50}$ equation. Data were plotted from two biological replicates.

Pathway Labeling—$N$. gonorrhoeae was grown in SJ-GC medium until $A_{600}$ reached 0.5, and then aliquoted to 10-ml cultures. AFN-1252 (4 $\mu$M) or DMSO (untreated) was added to each culture and incubated for 15 min at 37 °C with 125-rpm shaking. Next, the cell cultures were labeled for 45 min at 37 °C with 125-rpm shaking. The cells were harvested via centrifugation (4,000 × g) and washed twice with phosphate-buffered saline. The lipid fraction of the cells was extracted via the Bligh and Dyer method (37), and the total radioactive incorporation was measured via liquid scintillation counting. The distribution of radioactivity into the phospholipid fraction was determined by thin-layer chromatography on Analtech Silica Gel G plates developed with chloroform/methanol/acetic acid (98/2/1, v/v/v). The percentage of incorporation into phospholipids was multiplied by the total radioactive incorporation to determine the total radioactive incorporation into phospholipids. Data were plotted as fractional incorporation relative to the DMSO-treated cells and fitted via a standard IC$_{50}$ equation. Plotted data were derived from two biological replicates.

Isotopic Labeling and Phospholipid Molecular Species Analyses—$N$. gonorrhoeae was grown in SJ-GC medium until $A_{600}$ reached 0.25 and then aliquoted to 10-ml cultures. DMSO control, 100 $\mu$M [7,7,8,8-D4]palmitic acid (D4-16:0, Cambridge Isotope Laboratory), or 100 $\mu$M [methyl-D3]lauric acid (D3-12:0, Cambridge Isotope Laboratory) was added to the culture and incubated for 2 h. The cells were harvested and washed twice in phosphate-buffered saline, extracted via the Bligh and Dyer method (37), and analyzed via molecule species profiling.

Phospholipid molecular species profiling for PE was performed as described previously (38). Phospholipid molecular species fingerprints were determined using direct infusion electrospray ionization-mass spectrometry technology (39, 40). Mass spectrometry analysis was performed using a Finnigan$^\text{TM}$ TSQ$^\text{TM}$ Quantum (Thermo Electron, San Jose, CA) triple quadrupole mass spectrometer. The instrument was operated in positive ion mode for PE analysis. Acyl chain lengths were assigned from the mass based on product scans of the particular mass peak or predictions from LipidMaps for previously identified peaks. Minor isobaric species may be present at each mass, but the major molecular species represented at each major mass peak is labeled on the figures. Two biological replicates were analyzed for each condition, and representative spectra are shown in the figures.

Exogenous Fatty Acid Incorporation—The pPJ131-NGO0530 and pPJ131-NGO1213 plasmids were transformed into strain LCH30 (aas-1 fadD88) along with the parent pPJ131 plasmid (empty vector), pPJ131 plasmid expressing the Chloramphenicol acetyltransferase (Cm-acetyltransferase) or the β-lactamase gene of E. coli. Cultures expressing the respective constructs were grown to $A_{600}$ = 0.25 in 10 ml of Luria Bertani medium (41) with 100 $\mu$g/ml carbenicillin at 37 °C, and then 100 $\mu$g D3-14:0 was added to the medium. The cultures were grown for an additional 1.5 h and then harvested. The cells were washed twice, the lipids were extracted, and the incorporation of D3-14:0 into PE was analyzed by mass spectrometry.

Results

$Neisseria$ Enoyl-ACP Reductase ($NgFabI$)—Enoyl-ACP reductase catalyzes the reduction of trans-2-enoyl-ACP into acyl-ACP and is a rate-determining step in FASII (42, 43). The predicted FabI sequences from the different species of the genus $Neisseria$ have >98% amino acid sequence identity and are homologous to the $E$. coli FabI (60% identity, e-value = 1e$^{-90}$). Whether the predicted $Neisseria$ FabI was a bona fide enoyl-ACP reductase was assessed by determining whether expression of the $Neisseria$ FabI complemented the growth of an $E$. coli fabI(Ts) mutant. The $E$. coli codon-optimized fabI gene
from *N. gonorrhoeae* strain FA 1090 was cloned into the pJP131 expression vector and then transformed into *E. coli* strain JP1111 (*fabI*Ts) (44). Strain JP1111 was able to grow without *fabI* gene complementation at the non-permissive temperature (Fig. 1A). However, strain JP1111 was not able to grow without *fabI* gene complementation at the non-permissive temperature (Fig. 1B). The *E. coli*, *S. aureus*, and *N. gonorrhoeae fabI* genes all complemented growth at the non-permissive temperature (Fig. 1B), demonstrating that the *N. gonorrhoeae fabI* functioned as an enoyl-ACP reductase in the heterologous *E. coli* FASII system.

NgFabI was expressed, and the protein was purified (Fig. 2A). The enzymatic activity of NgFabI was determined by measuring conversion of NADH to NAD\(^+\) at 340 nm arising from reduction of crotonyl-ACP to butyryl-ACP. NgFabI exhibited an apparent \(K_m\) of 116.2 ± 18.3 \(\mu\)M for NADH (Fig. 2B) and an apparent \(K_m\) for crotonyl-ACP of 7.2 ± 1.1 \(\mu\)M (Fig. 2C). NADPH did not support the enzymatic reaction (data not shown), consistent with FabI enzymes from other Gram-negative bacteria (38, 42). The \(k_{cat}\) of NgFabI was 25.2 ± 2.8 min\(^{-1}\), similar to the velocities of other characterized FabI enzymes (38, 45). The complementation and biochemical characterization experiments showed that the predicted NgFabI functioned as an enoyl-ACP reductase.

**AFN-1252 Inhibits *N. gonorrhoeae* Growth through NgFabI**—AFN-1252 was a tight binding inhibitor of the NgFabI, meaning that the dissociation constant for AFN-1252 was lower than the concentration of NgFabI enzyme necessary to get a reliable signal in the spectrophotometric assay. Therefore, the Morrison quadratic equation for tight binding inhibitors was used to calculate the \(K_{app}\) for AFN-1252 (36). This treatment of the data takes into account the change in the concentration of the inhibitor due to its binding to the enzyme. The \(K_{app}\) of AFN-1252 for NgFabI at saturating concentrations of substrates was 5 ± 2 nM (Fig. 3A).

The effect of increasing concentrations of AFN-1252 on the growth of *N. gonorrhoeae* was determined. Bacteria usually continue to grow for one generation following the inhibition of fatty acid synthesis, causing a characteristic delayed growth arrest phenotype when fatty acid synthesis is inhibited (46). This growth phenotype was observed in the growth curves of *N. gonorrhoeae* treated with increasing concentrations of AFN-1252 (Fig. 3B). *N. gonorrhoeae* was able to grow with minimal decrease in growth rate for 45 min (corresponding to another generation) after AFN-1252 was added to the culture, regardless of the concentration of AFN-1252 added. Increasing concentrations of AFN-1252 exerted increasing inhibitory effects after this initial growth phase, with 4 and 8 \(\mu\)M AFN-1252 causing a complete cessation of growth. These data showed that AFN-1252 was able to block *Neisseria* growth with a phenotype that was consistent with AFN-1252 acting through inhibiting fatty acid synthesis.

The effect of increasing concentrations of AFN-1252 on the rate of fatty acid biosynthesis in *N. gonorrhoeae* was determined. *N. gonorrhoeae* cultures were treated with increasing concentrations of AFN-1252 for 15 min and then labeled with \[^{14}C\]acetate for 45 min. Incorporation of \[^{14}C\]acetate into the phospholipid fraction was determined. AFN-1252 caused a dose-dependent decrease in the incorporation of \[^{14}C\]acetate, with greater than 95% inhibition achieved at 16 \(\mu\)M AFN-1252 (Fig. 3C). Metabolic labeling in the presence and absence of 4 \(\mu\)M AFN-1252 was used to assess its effect on the four major pathways of macromolecular biosynthesis (Fig. 3D). AFN-1252 caused a 75% inhibition of lipid biosynthesis, with less than 5% inhibition of protein, DNA, or RNA biosynthetic pathways in *N. gonorrhoeae*. This experiment showed that AFN-1252 selectively inhibited lipid biosynthesis. Together, the growth inhibition, pathway labeling, and biochemical analysis of FabI inhibition demonstrated that AFN-1252 blocked the growth of *N. gonorrhoeae* through its on-target inhibition of the FabI component of FASII.

**Incorporation of Exogenous Fatty Acids into Phospholipids**—The ability of *N. gonorrhoeae* to utilize exogenous fatty acids was determined by examining the incorporation of isotopic labeled exogenous fatty acids into the *N. gonorrhoeae* phospholipids. The major phospholipid class in *N. gonorrhoeae* was PE, comprising greater than 70% of the total phospholipids (47). Molecular species profiling was used to determine the acyl chain composition of the *N. gonorrhoeae* PE. The major PE molecular species of *N. gonorrhoeae* had a molecular mass of 690.52, corresponding to PE containing a 16:0 fatty acid in the 1-position and a 16:1 fatty acid in the 2-position (Fig. 4A). The 16:1 fatty acid is known to be palmitoleic acid (16:1\(\Delta9\)), which has a cis double bond between the 9- and 10-carbons (48). Smaller amounts of 16:0/18:1, 16:1/18:1, 16:1/16:1, 14:0/16:1, 16:0/14:0, 14:0/14:0, and 18:1/18:1 PE molecular species were also detected. A growing culture of *N. gonorrhoeae* was labeled with 100 \(\mu\)M D4-16:0. Two new prominent molecular peaks at \(m/z = 694.62\), corresponding to D4-16:0/16:1, and \(m/z = 700.64\), corresponding to D4-16:0/D4-16:0, appeared (Fig. 4B). The D4-16:0 was also incorporated into other, minor molecular species where 16:0 was normally found. This experiment showed that *N. gonorrhoeae* was able to incorporate exogenous D4-16:0 into both acyl chain positions. Next, cells were labeled with D3-12:0 to determine whether exogenous fatty acids were elongated by *Neisseria*. Cells grown in the presence of D3-12:0 had new PE molecular species corresponding to the elongation and incorporation of D3-12:0 (Fig. 4C). The most prominent peak occurred at \(m/z = 693.57\), corresponding to D3-16:0/16:1,
and a number of smaller peaks containing D3-12:0, D3-14:0, and D3-16:0 were observed. These experiments showed that N. gonorrhoeae was able to elongate D3-12:0 prior to incorporating the fatty acid into phospholipid. This result means that Neisseria encoded an enzyme or pathway that produced acyl-ACP from exogenous fatty acids.

Characterization of NGO0530 and NGO1213—N. gonorrhoeae have two genes (NGO0530 and NGO1213) that encode members of the superfamily of proteins containing the AFD-class_I domain, which corresponds to the binding site for the acyl-adenylate intermediate in acyl-ACP and acyl-CoA synthetases (12, 49). The functions of the two potential acyl-CoA/ACP synthetase genes were determined by their expression in E. coli and isotopic fatty acid labeling. E. coli strain LCH30 (aas-1 fadD88) lacks both acyl-ACP synthetase (aas) and acyl-CoA synthetase (fadD) and was unable to incorporate exogenous

FIGURE 2. Purification and biochemistry of NgFabI. A, NgFabI was purified by affinity chromatography. Gel electrophoresis shows the purity of NgFabI. Lane 1, standards; lane 2, NgFabI (28.5 kDa). B, initial velocities were determined in duplicate as a function of NADH concentration. C, initial velocities were determined in duplicate as a function of crotonyl-ACP concentration. FabI assays were performed as described under “Experimental Procedures.” The data points were fit to the Michaelis-Menten nonlinear regression equation using GraphPad Prism version 5 software, and the fitted line is shown on the graph. The apparent $K_m$ values are shown along with the S.E. (error bars).

FIGURE 3. Inhibition of N. gonorrhoeae growth is through on target inhibition of NgFabI. A, fractional activity of NgFabI versus increasing concentrations of AFN-1252 as compared with no inhibitor. Duplicate data sets were fit via the Morrison equation for tight binding inhibitors to determine the $K_i$ (36). The line on the graph is the fitted equation. B, effect of increasing concentrations of AFN-1252 on the growth of N. gonorrhoeae in SJ-GC medium. Cells were grown for 120 min prior to the addition of AFN-1252 at the indicated time. Data shown are a representative example from duplicate biological experiments. C, AFN-1252 inhibition of $[^{14}C]$acetate incorporation into the phospholipids of N. gonorrhoeae in SJ-GC medium. Data shown are from two different biological experiments and the fractional $[^{14}C]$acetate incorporation compared with no inhibitor control. D, the effect of AFN-1252 on the major biosynthetic pathways was measured by treating growing N. gonorrhoeae with 4 μM AFN-1252 compared with untreated cells. Metabolic labeling with $[^{14}C]$acetate was used for lipid biosynthesis, a $[^3H]$-labeled amino acid mixture was used to measure protein biosynthesis, $[^3H]$thymidine incorporation measured DNA biosynthesis, and $[^3H]$uracil measured stable RNA biosynthesis, as described under “Experimental Procedures.” Error bars, S.E.
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fatty acids into phospholipid (50). Thus, when strain LCH30 harboring the empty pPJ131 expression vector was grown in the presence of D3-14:0, there was no detectable incorporation of D3-14:0 into the phospholipids (Fig. 5A). Strain LCH30 expressing the E. coli acyl-CoA synthetase (FadD) did incorporate D3-14:0 into phospholipid, but there was no elongation of the fatty acid (Fig. 5B). Acyl-CoA can be used for phospholipid synthesis in E. coli but cannot be converted to acyl-ACP and elongated. In contrast, strain LCH30 expressing the C. trachomatis acyl-ACP synthetase (aa5C) converted D3-14:0 to D3-16:0 prior to incorporation into phospholipid (Fig. 5C), consistent with the formation of acyl-ACP that can either be used for phospholipid synthesis or enter the FASII elongation cycle (49). Strain LCH30 expressing NGO0530 incorporated D3-14:0 both as D3-14:0 and elongated D3-14:0 to D3-16:0, demonstrating that NGO0530 functioned as an acyl-ACP synthetase (Fig. 5D). Thus, we have named the NGO0530 gene aasN for Neisseria acyl-ACP synthetase. Strain LCH30 expressing NGO1213 incorporated D3-14:0 only as D3-14:0, demonstrating that NGO1213 functioned as an acyl-CoA synthetase (Fig. 5E).

The exogenous fatty acid labeling experiments conducted in N. gonorrhoeae showed that D3-12:0 was incorporated into the phospholipids overwhelmingly as the elongated D3-14:0 and D3-16:0 fatty acids (Fig. 4C). The existence of AasN accounts for the activation and elongation of exogenous fatty acids into the N. gonorrhoeae phospholipids. The acyl-CoA synthetase, NGO1213, did not appear to have a role in the incorporation of exogenous fatty acids into the N. gonorrhoeae phospholipids.

**AFN-1252 Blocks Exogenous Fatty Acid Incorporation**—AFN-1252 blockade at the FabI step in S. aureus results in cellular ACP being converted to short-chain enoyl-ACP and prevents the incorporation of exogenous fatty acids by tying up free ACP needed to ligate the incoming fatty acids. However, N. gonorrhoeae also encoded an acyl-CoA synthetase, which raised the possibility that exogenous fatty acids were converted into acyl-CoA that would bypass FabI inhibition by acting as the acyl donor for the acyltransferases. Therefore, the effect of AFN-1252 on the incorporation of exogenous fatty acid in N. gonorrhoeae was characterized via fatty acid labeling experiments to determine whether exogenous fatty acid incorporation was affected by FabI inhibition. A growing culture of N. gonorrhoeae was treated with increasing concentrations of AFN-1252 for 15 min and then labeled with [13C]16:0 for 45 min. AFN-1252 caused a concentration-dependent decrease in the incorporation of [13C]16:0 into the phospholipids of N. gonorrhoeae, with >90% inhibition achieved at 16 μM AFN-1252 (Fig. 5F). This result showed that the N. gonorrhoeae exogenous fatty acid incorporation mechanism was linked to the inhibition of endogenous fatty acid synthesis.

**Exogenous Fatty Acids Cannot Overcome Growth Inhibition by AFN-1252**—The SJ-GC medium contains potato starch, which is a known drug binder, so the potency of AFN-1252 against N. gonorrhoeae was tested in the chemically defined GW medium. Long-chain fatty acids have potent inhibitory effects against N. gonorrhoeae (51), so conditions to deliver the 16:0 and 16:1Δ9 fatty acids, the major components of N. gonorrhoeae phospholipids, without inhibiting N. gonorrhoeae growth were examined. N. gonorrhoeae was refractory to 100 μM 16:0 in SJ-GC medium, whereas the unsaturated 16:1Δ9 fatty acid had an MIC of 12.5 μM. N. gonorrhoeae was refractory to a 100 μM concentration of either 16:0 or 16:1Δ9 in SJ-GC medium containing 10 mg/ml BSA, showing that SJ-GC medium containing BSA was an acceptable method to deliver the fatty acids (Table 1).

AFN-1252 demonstrated a minimal inhibitory concentration of 1.25 μM against N. gonorrhoeae in SJ-GC medium (Table 2). Adding 10 mg/ml BSA to the medium shifted the MIC to 5 μM. Supplementing the medium with 50 μM 16:0 and 50 μM 16:1Δ9 did not change the AFN-1252 MIC. The cofactor lipoic acid is an important product of FASII, and adding 1 μM lipoic acid in addition to the fatty acids did not change the MIC, showing that exogenous FASII products cannot complement FabI inhibition by AFN-1252 in N. gonorrhoeae. Similar MIC experiments were performed on N. meningitidis. Unlike N. gonorrhoeae, neither 16:0 nor 16:1Δ9 inhibited N. meningitidis growth (Table 1). AFN-1252 demonstrated a minimal inhibitory concentration of 1.25 μM against N. meningitidis in SJ-GC medium that shifted to 5 μM when 10 mg/ml BSA was present in the medium (Table 2). However, fatty acid and lipoic acid supplementation did not increase the MIC. Lipoic acid was provided because it is an essential cofactor for ketoacid dehydrogenases and is derived from octanoyl-ACP arising from FASII (52–54). S. aureus lacking FASII require exogenous lipoate in addition to fatty acids to support maximum growth (35, 55). These experiments showed that exogenous fatty acids cannot bypass the inhibition of FabI by AFN-1252 in both N. gonorrhoeae and N. meningitidis.

**N. gonorrhoeae Is Refractory to the Inhibition of LPS Synthesis**—One product of FASII in Gram-negative bacteria is 3-hydroxyacyl-ACP, which is used in the synthesis of LPS. LPS synthesis is essential for E. coli (15) but is nonessential for N. meningitidis (16–18). The LpxC inhibitor CHIR-090, which has nanomolar affinity against the Neisseria LpxC (56), was tested against N. gonorrhoeae to determine whether LPS was also nonessential in N. gonorrhoeae and whether the effects of FabI inhibition were through inhibiting 3-hydroxy fatty acid synthesis. CHIR-090 demonstrated potent activity (MIC = 78 nm) against E. coli, but N. gonorrhoeae was refractory to up to 20 μM CHIR-090 (Fig. 6). This result was consistent with LPS being nonessential in Neisseria, and therefore the growth-
arresting effects of AFN-1252 were through inhibiting fatty acid synthesis required for phospholipids and not LPS.

Discussion

This work identifies an acyl-ACP synthetase (AasN) as a key participant in the activation of extracellular fatty acids in Neisseria (Fig. 7). Following their conversion to acyl-ACP, the fatty acid is either converted to acyl-PO₄ by PIsX and esterified to the 1-position of sn-glycerol phosphate by PIsY, utilized directly by the PIsC acyltransferase, or enters the elongation cycle of FASII (Fig. 7). Thus, Neisseria differ from prototypical Gram-negative bacteria, which activate extracellular fatty acid to acyl-CoA for the PIsB/PIsC acyltransferase system. This flow of exogenous fatty acids into phospholipid is distinct from the fatty acid

FIGURE 5. Neisseria encodes for an acyl-ACP synthetase and an acyl-CoA synthetase. E. coli strain LCH30 (aas-1 fadD88) completely lacks the ability to incorporate exogenous fatty acids due to the inactivation of both the acyl-ACP (aas) and the acyl-CoA (fadD) synthetase (50). Strain LCH30 expressing a series of fatty acid activation genes was labeled with 100 μM D3-14:0, and the PE molecular species profiles were determined. Major PE species arising from the incorporation of exogenous fatty acids are labeled in red. Spectra shown are representative examples from two biological replicates. A, PE molecular species profile of strain LCH30 harboring the empty pPJ131 plasmid. B, PE molecular species profile of strain LCH30 expressing E. coli fadD, an acyl-CoA synthetase. C, PE molecular species profile of strain LCH30 expressing the C. trachomatis aasC, an acyl-ACP synthetase. D, PE molecular species profile of strain LCH30 expressing the NGO0530 gene. E, PE molecular species profile of strain LCH30 expressing the NGO1213 gene. F, a growing culture of N. gonorrhoeae was preincubated with increasing concentrations of AFN-1252 for 15 min and then labeled with 5 μCi of [¹⁴C]16:0 for 45 min. The incorporation of [¹⁴C]16:0 into the phospholipids was determined and plotted as a fraction relative to the DMSO-treated cells. Error bars, S.E.
Fatty acid synthesis in Gram-positive bacteria is regulated by a phospholipid synthesis pathway that converts fatty acids to acyl-ACP by PlsX (20, 21). Acyl-ACP synthetase is phosphorylated by fatty acid kinase and are either used by PlsY or the FASII or the PlsB/PlsC acyltransferases (57, 58). The first acyl-ACP synthetase discovered is a subunit of 2-acyl-lyso-phosphatidyl-ACP synthetase of *E. coli*; however, this enzyme produces only enzyme-bound acyl-ACP that is not available to FASII or the other acyltransferases (57, 58). *Vibrio harveyi* expresses an acyl-ACP synthetase capable of releasing acyl-ACP that is used by FASII or the PlsB/PlsC acyltransferases (59, 60). *C. trachomatis* also expresses an acyl-ACP synthetase that produces acyl-ACP for FASII and the PlsE/PlsC acyltransferase system (49). The function(s) for an acyl-CoA synthetase in *N. gonorrhoeae* is not clear. In *E. coli*, acyl-CoAs are consumed by fatty acid β-oxidation, but *Neisseria* do not encode the genes necessary to break down acyl-CoA via this pathway. Furthermore, whereas both *E. coli* PlsB and PlsC acyltransferases are able to utilize acyl-CoA as acyl donors, the characterized PlsX/PlsY/PlsC acyltransferases cannot use acyl-CoA (19, 61). The observed elongation and incorporation of exogenous fatty acids in *Neisseria* is explained by AasN activation of exogenous fatty acids to acyl-ACP because exogenous fatty acids are elongated and used by the acyl-PO₄-acyl-ACP-dependent PlsX/PlsY/PlsC acyltransferase system. The acyl-CoA synthetase does not appear to function in exogenous fatty acid incorporation in our experiments. The observations that exogenous fatty acids are elongated and that AFN-1252 blocks fatty acid incorporation into phospholipid support the conclusion that acyl-ACP is the key intermediate in *Neisseria* and not acyl-CoA. The presence of AasN is sufficient to account for the elongation and incorporation of exogenous fatty acids in *Neisseria*, and the role of acyl-CoA synthetase in *Neisseria* physiology remains to be determined.

The therapeutically relevant aspect of the study is that it establishes FASII inhibitors as effective therapeutics against *Neisseria*. All bacteria studied to date are capable of incorporating exogenous fatty acids into membrane phospholipids (7). Thus, it is important to experimentally determine whether extracellular fatty acid supplementation can overcome a blockade in FASII (7–9). *Neisseria* is different from the prototypical Gram-negative *E. coli* model system in that it uses AasN to activate exogenous fatty acids and uses the acyl-PO₄ pathway for phospholipid synthesis, and LPS is not essential for survival. These properties suggest that *Neisseria* may be able to escape FASII inhibitors by obtaining straight-chain saturated and unsaturated fatty acids, as seen in *V. harveyi* (60).

**Table 1**

Fatty acid sensitivity of *N. gonorrhoeae* and *N. meningitidis*

| Fatty acid | BSA | *N. gonorrhoeae* | *N. meningitidis* |
|-----------|-----|-----------------|------------------|
| 16:0      | −   | >200            | >200             |
| 16:0      | +   | >200            | >200             |
| 16:1Δ9    | −   | 12.5            | >200             |
| 16:1Δ9    | +   | >200            | >200             |

**Table 2**

AFN-1252 sensitivity of *N. gonorrhoeae* and *N. meningitidis*

| Supplement | Strain | BSA | FA | Lipoate | *N. gonorrhoeae* | *N. meningitidis* |
|-----------|-------|-----|----|--------|-----------------|------------------|
| −         | −     | −   | 1.25 | 1.25   | *N. gonorrhoeae* | *N. meningitidis* |
| +         | −     | +   | 5   | 5      | *N. gonorrhoeae* | *N. meningitidis* |
| +         | +     | +   | 5   | 5      | *N. gonorrhoeae* | *N. meningitidis* |
unsaturated fatty acid from the host. However, our experiments establish that *Neisseria* cannot circumvent FabI inhibition by scavenging fatty acids from the environment. In *S. aureus*, FabI inhibition blocks the uptake of fatty acids due to the depletion of cellular ACP as the initiation module of FASII continues to feed acyl-ACP into the elongation cycle and the enoyl-ACP intermediates accumulate at the inhibited step (35). AFN-1252 also inhibits the incorporation of extracellular fatty acids into *Neisseria* consistent with the blockade at the FabI step triggering the same imbalance in fatty acid metabolism in both organisms. Although there remains more to understand about regulation of fatty acid metabolism in *Neisseria*, this work validates FASII, and specifically FabI, as a therapeutic target for the development of critically needed new *Neisseria* therapeutics.

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