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EXPERIMENTAL THERAPEUTICS

Repurposing Salicylamide for Combating Multidrug-Resistant *Neisseria gonorrhoeae*

Marwa Alhashimi,[a] Abdelrahman Mayhoub,[b,c] Mohamed N. Seleem[a,d]

[a]Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA
[b]Department of Pharmaceutical Organic Chemistry, College of Pharmacy, Al-Azhar University, Cairo, Egypt
[c]University of Science and Technology, Nanoscience Program, Zewail City of Science and Technology, Giza, Egypt
[d]Purdue Institute of Inflammation, Immunology, and Infectious Disease, Purdue University, West Lafayette, Indiana, USA

ABSTRACT The U.S. Centers for Disease Control and Prevention (CDC) lists *Neisseria gonorrhoeae* as one of the most urgent antibiotic-resistant threats in the United States. This is due to the emergence of clinical isolates that have developed resistance to nearly every antibiotic used to treat gonorrhea and highlights the critical need to find new therapeutics. The present study discovered salicylamide, an analgesic and antipyretic drug, has antibacterial activity against 40 different antibiotic-resistant strains of *N. gonorrhoeae* (MIC, 8 to 32 μg/ml) with low frequency of resistance $<2.4 \times 10^{-9}$. Interestingly, salicylamide did not inhibit growth of bacterial species in the vaginal microflora involved in defense against gonococcal infections, such as *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, and *Lactobacillus crispatus*. A time-kill assay revealed that salicylamide is a rapidly bactericidal drug, as it eradicated a high inoculum of *N. gonorrhoeae* within 10 h. Salicylamide was superior to the drug of choice, ceftriaxone, in reducing the burden of intracellular *N. gonorrhoeae* by 97% in infected endocervical cells. Furthermore, salicylamide outperformed ceftriaxone in reducing expression of the proinflammatory cytokine interleukin 8 (IL-8) from endocervical cells infected with *N. gonorrhoeae*. A checkerboard assay revealed that salicylamide exhibited a synergistic interaction with tetracycline and additive relationships with azithromycin, ciprofloxacin, and ceftriaxone. A more in-depth investigation of the structure-activity relationship of derivatives of salicylamide revealed the amide and hydroxyl groups are important for antigonorrheal activity. In conclusion, this study identified salicylamide as a promising candidate for further investigation as a novel treatment option for multidrug-resistant gonorrhea.

KEYWORDS IL-8, *Neisseria gonorrhoeae*, intracellular, repurposing

Neisseria gonorrhoeae is the causative agent of gonorrhea, a sexually transmitted infection that is the second most reported notifiable disease in the United States and one of the most important antimicrobial resistance threats worldwide (1, 2). If left untreated, gonorrhea can result in severe complications that include infertility, ectopic pregnancy, pelvic inflammatory disease, and increased susceptibility to HIV infections (3). The Centers for Disease Control and Prevention (CDC) estimate that over 820,000 new gonococcal infections occur annually in the United States, with an estimated total treatment cost of nearly $5 billion dollars (4). Few effective therapeutic options are available to treat gonorrhea. Current guidelines recommend treating gonorrhea with dual therapy involving azithromycin and ceftriaxone. However, increasing resistance to this treatment option has been reported, which has resulted in multidrug-resistant *N. gonorrhoeae* (i.e., super gonorrhea) becoming a critical public health concern. Compounding the problem further is that over the last 2 decades, almost no new classes of

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Address correspondence to Mohamed N. Seleem, mseleem@purdue.edu.

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antibiotic drugs have been approved (5), raising a serious concern that super gonorrhea infections may be untreatable in the near future (6, 7).

The traditional process of drug discovery is complex, time consuming, and expensive (8–10). The approach of translating a new lead natural product or synthetic molecule from the bench to the clinic takes approximately 10 to 15 years and can cost more than $2 billion dollars (11, 12). However, due to the substantial financial investment and small window of therapeutic application, there has been a marked decrease in antibiotic drug discovery and development (13). Thus, alternative approaches to discovering and developing antibacterial agents are needed.

Drug repositioning is a promising field that investigates new therapeutic opportunities for existing approved drugs with readily available information on their pharmacokinetic profile, dosages, and toxicity to humans (14–19). This approach can significantly reduce the total cost, time, and effort to develop novel antibacterial agents. To this end, we screened FDA-approved drugs and clinical molecules present in the Johns Hopkins Clinical Compound Library in search of agents that possess antibacterial activity against multidrug-resistant *N. gonorrhoeae*. Salicylamide, an over-the-counter drug with analgesic and antipyretic properties, emerged as the lead candidate against *N. gonorrhoeae*. Salicylamide was evaluated against a diverse panel of *N. gonorrhoeae* clinical isolates, both alone and in combination with antibiotics used to treat gonorrhea. In addition, we investigated the ability of salicylamide to reduce intracellular *N. gonorrhoeae* and the effect on interleukin 8 (IL-8) expression in endocervical cells infected with *N. gonorrhoeae*. Furthermore, we investigated the structure-activity relationship of salicylamide by analyzing the antibacterial activity of other structurally related derivatives against *N. gonorrhoeae*. We also investigated the activity of salicylamide and representative analogues against vaginal microbiota that inhibit *N. gonorrhoeae* colonization, such as *Lactobacillus gasseri* and *Lactobacillus crispatus*.

RESULTS

Antibacterial susceptibility testing. The antigonococcal activity of salicylamide was evaluated against a panel of 40 *N. gonorrhoeae* clinical isolates, including several multidrug-resistant strains. These strains exhibited resistance to azithromycin and ciprofloxacin. As presented in Table 1, salicylamide inhibited the growth of the 40 tested *N. gonorrhoeae* isolates at concentrations ranging from 8 to 32 μg/ml. The MIC_{50} of salicylamide was 16 μg/ml and MIC_{90} was 32 μg/ml. Moreover, salicylamide's MIC was consistent against azithromycin-resistant strains, such as *N. gonorrhoeae* strains 167, 175, 179, 181, 193, 197, 199, and 202. Azithromycin inhibited growth of azithromycin-susceptible isolates at concentrations ranging from 0.5 to 4 μg/ml. The MIC of azithromycin ranged from 8 to 256 μg/ml against azithromycin-resistant *N. gonorrhoeae* isolates (strains 167, 175, 179, 181,193, 197,199, and 202). Five strains of *N. gonorrhoeae* (167, 175, 179, 181, and 194) were utilized in agar dilution method to confirm the MIC values of tested drugs. A 1-fold increase was observed in salicylamide's MIC values in comparison with those from the microbroth method, whereas the control's MICs remained constant. The results of these assays are presented in Table 2.

Effect of salicylamide and control drugs on the vaginal microbiota. The vaginal microbiota competes with *N. gonorrhoeae* for adhesion to the urinary tract in addition to creating an acidic environment that prevents gonococcal colonization. Thus, we investigated the activity of salicylamide against different species of *Lactobacillus* that comprise the female urogenital tract microbiota. Salicylamide exhibited a strong selectivity toward *N. gonorrhoeae* without inhibiting growth of different species of *Lactobacillus* up to a concentration of 256 μg/ml (Table 2). In contrast, both drugs of choice for gonorrhea infections, azithromycin and ceftriaxone, inhibited growth of the 11 species of *Lactobacillus* tested at concentrations as low as 1 μg/ml.

Time-kill assay of salicylamide against *N. gonorrhoeae*. After confirming salicylamide's ability to inhibit growth of a large panel of antibiotic-resistant *N. gonorrhoeae* isolates *in vitro*, we moved to examine if salicylamide is a bacteriostatic or bactericidal agent. Thus, a time-kill assay was used to evaluate the ability of salicylamide and

TABLE 1 MIC of salicylamide and a control antibiotic against 40 *N. gonorrhoeae* isolates

N. gonorrhoeae strain no.	MIC (μg/ml)	
	Salicylamide	Azithromycin
167	8	16
168	8	1
171	8	0.5
176	8	1
177	8	2
185	8	1
183	8	1
187	8	2
188	16	1
195	16	1
197	16	8
165	16	1
169	16	1
170	16	2
172	16	0.5
173	16	1
174	16	1
175	16	32
178	16	1
179	16	8
180	16	0.5
182	16	0.5
186	16	1
189	16	0.5
190	16	1
191	16	1
192	16	1
193	16	4
196	16	0.5
198	16	1
199	16	4
200	16	1
211	16	1
202	16	16
214	16	0.5
194	16	0.5
204	32	0.5
181	32	256
184	32	0.5
203	32	1
MIC_{50}	16	4
MIC_{90}	32	16

azithromycin (both at 5× MIC) to reduce *N. gonorrhoeae* strain 194's CFU over the course of 24 h. Salicylamide exhibited a strong bactericidal activity *in vitro* and reduced the bacterial inoculum to below the limit of detection (100 CFU/ml) after 10 h (Fig. 1). This was comparable to azithromycin, which reduced the bacterial inoculum to below the limit of detection after 8 h.

TABLE 2 MICs of salicylamide and a control antibiotic against 5 *N. gonorrhoeae* isolates using agar dilution method

N. gonorrhoeae strain no.	MIC (μg/ml)	
	Salicylamide	Azithromycin
167	16	16
178	32	1
198	32	1
181	64	256
194	32	1

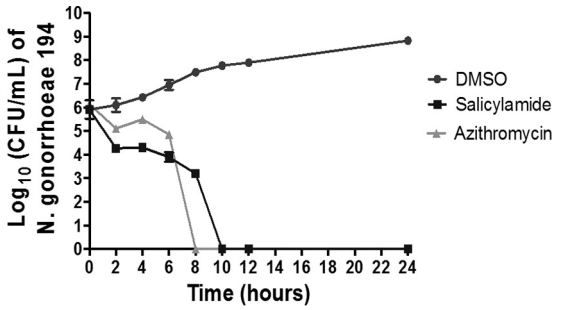


FIG 1 Time-kill assay of salicylamide and azithromycin (both at 5× MIC) against multidrug-resistant *Neisseria gonorrhoeae* strain 194 over a 24-h incubation period at 37°C. DMSO (solvent for the compounds) served as a negative control. The error bars represent standard deviation values obtained from triplicate samples used for each test agent.

Combination testing of salicylamide with azithromycin, ciprofloxacin, ceftriaxone, and tetracycline against *N. gonorrhoeae*. Because *N. gonorrhoeae* exhibits high-level resistance to numerous antibiotics, monotherapy is not recommended for the treatment of gonorrhea (20). As dual therapy is the most effective strategy for treating gonococcal infections, this prompted us to investigate the combination of salicylamide with various antibiotics. Azithromycin and ceftriaxone were chosen because they are drug of choice for the treatment of gonorrhea. Tetracycline and ciprofloxacin were also investigated, as they were previously shown to possess a synergistic relationship with derivatives of salicylamide against vancomycin-resistant enterococci (21). As presented in Table 3, salicylamide displayed an additive relationship when combined with either azithromycin, ceftriaxone, or ciprofloxacin against four different *N. gonorrhoeae* isolates (fractional inhibitory concentration indices [FICIs] ranged from 1 to 1.5). Salicylamide possessed a synergistic relationship with tetracycline against one strain (181) with an FICI of 0.5 and showed additive activity with the remaining three *N. gonorrhoeae* strains tested (FICIs ranged from 0.625 to 1).

Intracellular clearance of *N. gonorrhoeae*. *N. gonorrhoeae* is able to invade mucosal epithelia and survive intracellularly. Ceftriaxone, because of its complex and bulky structure, high molecular weight (554.58 g/mol), high hydrophilicity, and low active transport, exhibits low cellular permeability and is unable to clear intracellular *N. gonorrhoeae* infections (22–24). Salicylamide, in contrast, is a much smaller molecule (molecular weight of 137.14 g/mol). To determine if salicylamide can clear intracellular *N. gonorrhoeae*, an intracellular clearance assay was conducted. As depicted in Fig. 2, after 24 h, salicylamide (at 5× MIC) successfully reduced intracellular *N. gonorrhoeae* from infected endocervical cells (End1/E6E7) by 1.4 $\log_{10}$ (equivalent to 97% reduction in the bacterial count), relative to cells treated with dimethyl sulfoxide (DMSO). Ceftriaxone, as expected, produced a nonsignificant 0.25-$\log_{10}$ reduction in intracellular *N.*

TABLE 3 MICs of salicylamide, azithromycin, and ceftriaxone against a panel of vaginal microflora strains

Strain designation	MIC (μg/ml)		
	Salicylamide	Azithromycin	Ceftriaxone
L. gasseri HM-642	>256	≤1	≤1
L. gasseri HM-644	>256	≤1	≤1
L. gasseri HM-403	>256	≤1	≤1
L. gasseri HM-400	>256	≤1	≤1
L. crispatus HM-638	>256	≤1	≤1
L. crispatus HM-370	>256	≤1	2
L. jensenii HM-640	256	≤1	4
L. jensenii HM-105	>256	≤1	≤1
L. jensenii HM-639	>256	≤1	≤1
L. johnsonii HM-643	>256	≤1	≤1

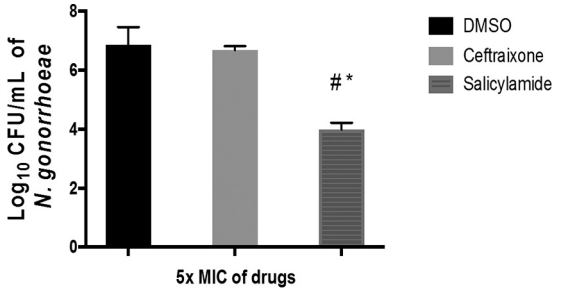


FIG 2 Effectiveness of salicylamide and ceftriaxone (both at 5× MIC) against intracellular *N. gonorrhoeae* in infected human endocervical cells (End1/E6E7). End1/E6E7 cells were infected with *N. gonorrhoeae* strain 194 for 6 h and then treated with either salicylamide or ceftriaxone for 24 h. End1/E6E7 cells were subsequently lysed and intracellular bacterial CFU was determined. Error bars represent standard deviation values from triplicate samples used for each test agent; test was conducted twice. *, $P < 0.01$ versus DMSO; #, $P < 0.01$ versus ceftriaxone by unpaired *t* test.

gonorrhoeae. This result indicates that salicylamide is able to enter endocervical cells and effectively reduce the burden of intracellular *N. gonorrhoeae*.

Effect of salicylamide on IL-8 expression. We next investigated the anti-inflammatory effect of salicylamide against human endocervical cells (End1/E6E7) infected with *N. gonorrhoeae*. IL-8 is a major proinflammatory cytokine that is produced during gonococcal infection of epithelial cervical cells (16). IL-8 was measured in the End1/E6E7 cell supernatant after establishing infection with *N. gonorrhoeae* strain 194 in the presence or absence of salicylamide and control antibiotics (at 0.5× MIC). DMSO was used as a negative control. Treatment with salicylamide resulted in a significant reduction equivalent to 37.7% of IL-8 expression in comparison to that with ceftriaxone at 3.9% (minor reduction in IL-8 concentration was observed) (Fig. 3).

Evaluation of structure-activity relationship of salicylamide. We investigated the antibacterial structure-activity relationship (SAR) of salicylamide by testing the antigonorrheal activity of 11 commercially available hydroxybenzamide derivatives and related analogues. Salicylamide's MIC_{50} value against the 11 *N. gonorrhoeae* strains was 16 μg/ml (Table 4). On the other hand, the other 3- and 4-hydroxy regioisomers (compounds 3 and 4, 3-hydroxybenzamide and 4-hydroxybenzamide, respectively) were devoid of any antigonorrheal activity ($MIC_{50} > 128$ μg/ml). Alkylation of the amino group of benzamide yielded compound 5 (*N,N*-diethylsalicylamide) with weak activity against *N. gonorrhoeae* ($MIC_{50} = 128$ μg/ml). Similarly, alkylation of the hydroxyl group furnished an inactive derivative (compound 9, anthranilamide). *para*-Alkylation of the benzene skeleton also had a negative impact on antigonorrheal potency, as the 4-methyl derivative of salicylamide possessed a MIC_{50} value of 64 μg/

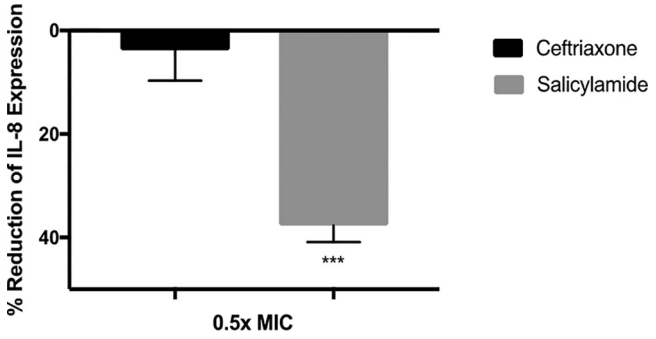


FIG 3 Effects of salicylamide and ceftriaxone on IL-8 expression in infected endocervical cells. IL-8 levels were assessed in End1/E6E7 endocervical cells infected with *N. gonorrhoeae* strain 194 in the presence and absence of 0.5× MIC of salicylamide or ceftriaxone. OD_{450} coincides with the level of IL-8 in the cell supernatant. Error bars represent standard deviation values from triplicate samples used for each test agent; test was conducted twice. ***, $P < 0.01$ versus ceftriaxone by unpaired *t* test.

TABLE 4 Fractional inhibitory concentration index range of salicylamide in combination with azithromycin, ciprofloxacin, and tetracycline against *N. gonorrhoeae* isolates

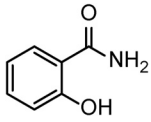
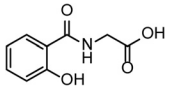
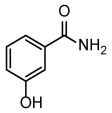
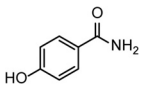
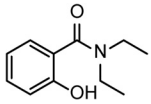
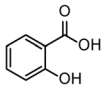
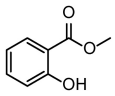
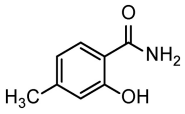
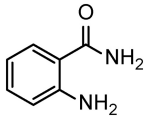
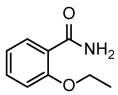
Drug(s)	Description	MIC (μg/ml) and FICI results for *Neisseria gonorrhoeae* strain no.: 175	178	181	194
Salicylamide	Alone	16	16	32	16
	Combination	8	8	32	16
Azithromycin	Alone	16	1	256	0.5
	Combination	8	0.5	64	0.25
Salicylamide-azithromycin	FICI	1	1	1.25	1.5
	Interpretation[a]	ADD			
Salicylamide	Alone	16	16	32	16
	Combination	4	8	8	4
Ciprofloxacin	Alone	0.015	16	0.015	0.015
	Combination	0.015	8	0.015	0.015
Salicylamide-ciprofloxacin	FICI	1.25	1	1.25	1.25
	Interpretation	ADD			
Salicylamide	Alone	16	16	32	16
	Combination	4	8	16	4
Tetracycline	Alone	1	8	1	1
	Combination	0.25	4	0.125	0.5
Salicylamide-tetracycline	FICI	0.5	1	0.625	0.75
	Interpretation	SYN	ADD		
Salicylamide	Alone	16	16	32	16
	Combination	16	8	16	16
Ceftriaxone	Alone	0.015	0.125	0.06	0.25
	Combination	0.015	0.06	0.06	0.25
Salicylamide-ceftriaxone	FICI	2	1	1.5	2
	Interpretation	ADD			

[a]FICI was interpreted as follows: an FICI of $\leq$0.5 is indicative of a synergistic relationship (SYN), an additive relationship (ADD) was defined as an FICI of $>$0.5 but $\leq$4, and antagonism was defined as an FICI of $>$4.

ml. In contrast to the *N*-alkylation that caused the compounds to become inactive against *N. gonorrhoeae*, replacing the amide group with a carboxylate moiety maintained the drug's antigonorrheal activity. For example, salicylic acid (compound 6) possessed the same MIC_{50} value as its corresponding amide derivative (compound 1). Again, alkylation of the carboxylate group of salicylic acid (compound 6) negatively impacted the compound's antibacterial activity (compound 7, MIC_{50} = 128 μg/ml). Further expansion of the polar part at the amide group yielded either a completely inactive compound (compound 2, 2-hydroxyhippuric acid) or a less active one (compound 11, salicylhydrazide, MIC_{50} = 32 μg/ml). Finally, expanding the lipophilic bulkiness through substitution of the benzene ring with a naphthalene ring (compound 12, 3-hydroxy-2-naphthamide) enhanced the compound's antigonorrheal activity by 1-fold (MIC_{50} of 8 μg/ml) compared to salicylamide.

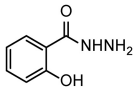
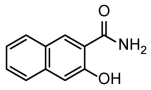
***Neisseria gonorrhoeae* spontaneous mutation frequency.** Salicylamide's ability to develop resistance was tested using the single step resistance assay as described previously (25) to calculate the frequency of spontaneous mutation. Results are reported in Table 5. After 72 h, no colonies were observed. Salicylamide's frequency of mutation was calculated as $<2.4 \times 10^{-9}$ (Table 6), a rate that is comparable to azithromycin, which is the drug of choice (26). Rifampin's frequency of mutation was higher (1.8×10^{-6}), as previously reported in other bacterial strains (27, 28).

TABLE 5 Antibacterial activity of salicylamide and structurally related analogues against four *Neisseria gonorrhoeae* isolates

Compound	MIC (μg/ml) for *N. gonorrhoeae* strain no.				
	188	181	195	194	MIC_{50}
Salicylamide (1) 	16	32	16	16	16
2-Hydroxyhippuric acid (2) 	>128	>128	>128	>128	>128
3-Hydroxybenzamide (3) 	>128	>128	>128	>128	>128
4-Hydroxbenzamide (4) 	>128	>128	>128	>128	>128
N,N-Diethylsalicylamide (5) 	128	128	128	128	128
Salicylic acid (6) 	8	16	16	16	16
Methyl salicylate (7) 	128	>128	>128	>128	128
4-Methylsalicylamide (8) 	32	64	64	64	64
Anthranilamide (9) 	128	>128	128	128	128
2-Ethoxybenzamide (10) 	32	64	64	64	64

(Continued on next page)

TABLE 5 (Continued)

Compound	MIC (μg/ml) for *N. gonorrhoeae* strain no. 188	181	195	194	MIC_{50}
Salicyl hydrazide (11) 	32	64	32	32	32
3-Hydroxy-2-naphthamide (12) 	8	16	8	16	8

DISCUSSION

N. gonorrhoeae infections have evolved into a challenging disease, as resistance has rapidly emerged to all antibiotics used for treatment. With no effective vaccines available, the development of effective antibacterial agents is a critical priority (7). Using a drug repurposing approach, we identified salicylamide as a promising new candidate for the treatment of drug-resistant gonorrhea infections. Salicylamide is an FDA-approved drug that has been extensively used in humans. Therefore, its pharmacokinetic and safety profiles in humans are well established.

The antibacterial activity of salicylamide was investigated against a panel of 40 clinical isolates of *N. gonorrhoeae* utilizing the broth microdilution method. Current CLSI guidelines recommend the use of the agar dilution method for the susceptibility testing of *N. gonorrhoeae* (29). Nevertheless, several previous studies have shown great results using the broth microdilution method (30). Salicylamide successfully inhibited the growth of the 40 clinical isolates of *N. gonorrhoeae* tested (MIC values ranged from 8 to 32 μg/ml). Salicylamide (at 5× MIC) exhibited bactericidal activity against *N. gonorrhoeae*, successfully reducing the bacterial burden below the limit of detection after 10 h. Azithromycin, the current drug of choice for gonorrhea, achieved the same effect in 8 h, which is in agreement with previous reports (31, 32). Due to the nature of gonococcal infection, a bactericidal drug is preferred over bacteriostatic drugs in quelling infection and preventing transmission.

N. gonorrhoeae is known to invade epithelial cells of the genital tract and cross the epithelial barrier into the subepithelial space (33). Previous studies have shown that *N. gonorrhoeae* can survive inside host cells and pass epithelial cell layers (a key step in causing disseminated infections). As salicylamide exhibited bactericidal activity *in vitro*, we examined if the drug could reduce the burden of intracellular *N. gonorrhoeae* present in infected endocervical cells. End1/E6E7 cells were infected with *N. gonorrhoeae* and subsequently treated with either salicylamide or ceftriaxone (both at 5× MIC) for 24 h. Salicylamide generated a 1.4-log_{10} reduction in *N. gonorrhoeae* inside infected endocervical cells while ceftriaxone, in contrast, was ineffective. The results

TABLE 6 Spontaneous mutation frequencies of salicylamide and rifampin at 10× agar MIC against *N. gonorrhoeae* 197, 202, and 206

N. gonorrhoeae strain no.	Drug	Frequency of mutation
197	Salicylamide	$<2.4 \times 10^{-9}$
	Rifampicin	1.8×10^{-6}
202	Salicylamide	$<2.4 \times 10^{-9}$
	Rifampicin	4.17×10^{-6}
206	Salicylamide	$<2.4 \times 10^{-9}$
	Rifampicin	1.2×10^{-6}

collectively indicate that salicylamide has the ability to gain entry into endocervical cells at a concentration high enough to significantly reduce intracellular *N. gonorrhoeae* at a rate that is superior to that for ceftriaxone.

Dual therapy with ceftriaxone and azithromycin is the recommended approach to treat infections caused by *N. gonorrhoeae*. The use of two antibiotics in conjunction is thought to curb the rapid emergence of resistance to either antibiotic used alone. Thus, we investigated the potential of salicylamide to be used in combination with other antibiotics against *N. gonorrhoeae*. Using a standard checkerboard assay, salicylamide was found to possess a synergetic relationship with tetracycline against one strain of *N. gonorrhoeae*. Salicylamide exhibited an additive relationship with azithromycin, ceftriaxone, and ciprofloxacin against four *N. gonorrhoeae* strains. This suggests that dual therapy involving salicylamide and one of these antibiotics may be feasible, though further investigation is needed.

To assess the potential for rapid emergence of resistance of *N. gonorrhoeae* to salicylamide, we attempted to generate a *N. gonorrhoeae* mutant that is resistant to salicylamide using a single-step mutation assay. *N. gonorrhoeae* mutants exhibiting resistance to salicylamide could not be isolated, indicating a low likelihood of rapid resistance emerging to these drugs. The inability to generate a resistant mutant of *N. gonorrhoeae* to salicylamide suggests this drug may have multiple targets.

Two mechanisms that enhance gonococcal colonization and infection are disruption of the healthy microbiota present in the genitourinary tract and release of proinflammatory cytokines by epithelial cells in the reproductive tract. Most bacteria that reside in the female genital tract are from the genus *Lactobacillus* (34). Lactobacilli are Gram-positive bacteria, in contrast to *N. gonorrhoeae*. One of the disadvantages of the first-line drugs used to treat *N. gonorrhoeae* infections is that they are nonspecific and inhibit growth of both gonococci and commensal bacteria indiscriminately (35). In contrast to the current first-line treatments, which inhibited both *N. gonorrhoeae* and *Lactobacillus* species equally (MIC values ranged from 1 to 4 μg/ml), salicylamide did not inhibit growth of *Lactobacillus* strains tested at concentrations up to 256 μg/ml. Previous studies have demonstrated the role of a disturbed vaginal microbiota in establishing and maintaining gonococcal infections (36, 37). The commensal vaginal flora decreases the vaginal pH and can directly compete with pathogens for space and adherence to the epithelial cells lining the vaginal tract (38–40). Therefore, salicylamide's specificity is a valuable characteristic in the treatment of *N. gonorrhoeae* and reducing gonococcal colonization (36).

Once *N. gonorrhoeae* has successfully colonized the genitourinary tract, infections can induce a severe inflammatory response resulting in the production of several proinflammatory cytokines that are considered a hallmark of gonococcal infections (37). Dampening this response is a potential approach to reduce the severity of the infection. As salicylamide is a nonsteroidal anti-inflammatory drug, we hypothesized it would be capable of reducing the expression of proinflammatory cytokines from host epithelial cells. Using preinfected endocervical cells, we confirmed that salicylamide significantly reduced IL-8 expression compared with ceftriaxone. Thus, salicylamide was found to possess three advantages over ceftriaxone as an alternative treatment for *N. gonorrhoeae* infections: (i) the ability to reduce the burden of intracellular *N. gonorrhoeae* in infected endocervical cells, (ii) the lack of activity against lactobacilli, and (iii) the ability to reduce expression of proinflammatory cytokines that contribute to gonococcal infections.

Salicylamide by its very simple chemical nature (low molecular weight, small number of hydrogen bond donors and acceptors, and low lipophilicity) represents a very attractive lead structure for medicinal chemists (41). In other words, after identifying a lead compound, the optimization step starts, mainly by adding substructures, to understand the SAR (Fig. 4). In this vein, the study of the antigonorrheal activity of eleven congruent benzamide derivatives provided insight into the essential functionalities. In brief, regioselectivity was found to be an essential issue, as only *ortho*-hydroxybenzamide was active and the other two *meta* and *para* isomers were inactive.

TABLE 7 Bacterial strains that were used in this study

Strain	Description
Neisseria gonorrhoeae strains	
N. gonorrhoeae 214	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 213	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 212	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 211	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 210	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 209	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 208	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 207	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 206	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 205	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 204	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 203	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 202	Resistant to azithromycin
N. gonorrhoeae 201	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 200	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 199	Resistant to azithromycin, tetracycline, and penicillin
N. gonorrhoeae 198	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 197	Resistant to azithromycin tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 196	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 195	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 194	Resistant to penicillin, not susceptible to ceftriaxone, cefixime, and cefpodoxime
N. gonorrhoeae 193	Resistant to azithromycin, tetracycline, and penicillin
N. gonorrhoeae 192	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 191	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 190	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 189	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 188	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 187	Resistant to penicillin, less susceptible to azithromycin
N. gonorrhoeae 186	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 185	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 184	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 183	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 182	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 181	Resistant to tetracycline and azithromycin
N. gonorrhoeae 180	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 179	Resistant to azithromycin
N. gonorrhoeae 178	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 177	Resistant to tetracycline, less susceptible to azithromycin
N. gonorrhoeae 176	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 175	Resistant to azithromycin
N. gonorrhoeae 174	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 173	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 172	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 171	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 170	Resistant to tetracycline, penicillin, and ciprofloxacin, less susceptible to azithromycin
N. gonorrhoeae 169	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 168	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 167	Resistant to azithromycin
N. gonorrhoeae 166	Resistant to tetracycline, penicillin, and ciprofloxacin
N. gonorrhoeae 165	Resistant to tetracycline, penicillin, and ciprofloxacin
Lactobacillus strains	
L. gasseri HM-642	Vaginal isolate from a healthy U.S. woman, obtained in 2007
L. gasseri HM-644	Vaginal mucosal isolate of a healthy U.S. woman of child-bearing age, obtained in 2007
L. gasseri HM-403	Isolated from human patient's mid-vaginal wall in March 2010 in Richmond, VA, USA
L. gasseri HM-400	Isolated from human patient's mid-vaginal wall in March 2010 in Richmond, VA, USA
L. crispatus HM-638	Vaginal isolate from a healthy Chinese woman, obtained in 2007
L. crispatus HM-370	Isolated from human patient's mid-vaginal wall in March 2010 in Richmond, VA, USA
L. jensenii HM-640	Isolated in 2007 from the vaginal mucosa of a healthy Chinese woman
L. jensenii HM-105	Human vaginal isolate obtained from Texas
L. jensenii HM-639	Isolated in 2007 from the vaginal mucosa of a healthy U.S. woman
L. johnsonii HM-643	Isolated in 2007 from the vaginal mucosa of a Chinese woman

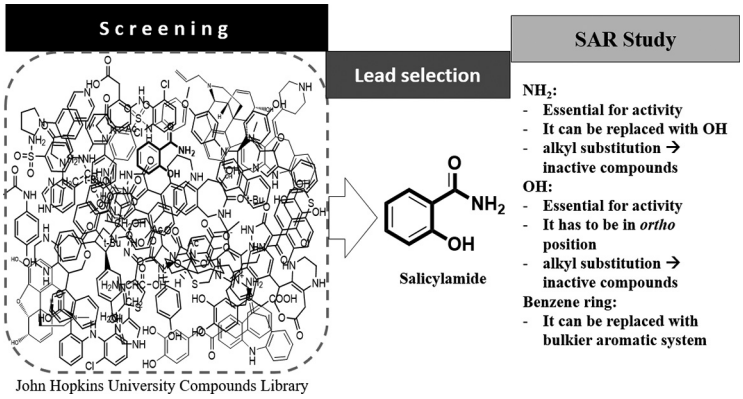


FIG 4 Diagrammatic representation of antibacterial SAR for optimization of salicylamide's antigonococcal activity.

The amino group can only be replaced with a hydroxyl group. Any *N*- or *O*-alkylation nullifies the antigonorrheal activity of the compounds. Finally, the naphthalene core exhibited the best antigonorrheal activity and provides a bulkier skeleton for further structural optimization.

In conclusion, we report that salicylamide, an FDA-approved over-the-counter drug, has potent *in vitro* antibacterial activity against *N. gonorrhoeae* without inhibiting the vaginal *Lactobacillus* spp. *N. gonorrhoeae* mutants exhibiting resistance to salicylamide could not be isolated, indicating a low likelihood of rapid resistance emerging to this drug. Furthermore, salicylamide can gain access inside endocervical cells and reduce intracellular infection by *N. gonorrhoeae* as well as reduce IL-8 expression to alleviate inflammation caused by infection. Additionally, salicylamide possesses a synergetic or additive relationship with other antibiotics that could be used for dual therapy. Further modification to salicylamide's chemical structure might be utilized to increase its potency and inhibitory activity against multidrug-resistant *N. gonorrhoeae*. The preliminary SAR analysis conducted with derivatives of salicylamide against *N. gonorrhoeae* will aid medicinal chemists in designing analogues that are more potent.

MATERIALS AND METHODS

Bacterial strains and reagents. The *N. gonorrhoeae* panel was obtained from the U.S. Centers for Disease Control and Prevention (CDC), and *Lactobacillus* strains were obtained from BEI Resources (Manassas, VA, USA) (Table 7). Salicylamide, 2-hydroxyhippuric acid, 3-hydroxybenzamide, 4-hydroxybenzamide, *N*,*N*-diethylsalicylamide, salicylic acid, methyl salicylate, 4-methylsalicylamide, anthranilamide, 2-ethoxybenzamide, salicylhydrazide, 3-hydroxy-2-naphthamide, ciprofloxacin, and tetracycline were obtained from Sigma-Aldrich (St. Louis, MO). Azithromycin (TCI America, Portland, OR), ceftriaxone, cefixime (Acros Organics, Morris Plains, NJ), and gentamicin (Thermo Fisher Scientific, Waltham, MA) were purchased from commercial vendors, as were yeast extract and dextrose (Fisher Bioreagents, Fairlawn, NJ), proteose-peptone, agarose, NAD, Triton X-100, and phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO), pyridoxal (Chem-Impex International, Wood Dale, IL), and calcium chloride (Gibco, Big Cabin, OK). Chocolate II agar (GC II Agar with hemoglobin and IsoVitaleX), *Brucella* supplemented broth base (BSB), gonococcal GC medium base (Thermo Fisher Scientific, Waltham, MA), and Difco lactobacilli MRS broth were purchased from Becton, Dickinson and Company (Cockeysville, MD). Keratinocyte medium (KSFM), bovine pituitary extract, and epidermal growth factor (Gibco, Big Cabin, OK) were purchased from commercial vendors.

Antibacterial susceptibility testing of salicylamide against *N. gonorrhoeae*. Salicylamide was tested against a panel of clinical isolates of multidrug-resistant *N. gonorrhoeae* using the broth microdilution assay as described previously (30), with slight modifications. Briefly, a 1.0 McFarland standard was prepared and diluted in BSB (*Brucella* broth was used instead of Colombia broth, and it was supplemented with yeast extract, dextrose, agarose, proteose-peptone, NAD, pyridoxal and hematin) to reach a bacterial count of 5×10^5 CFU/ml. Drugs were added and serially diluted. Plates were then incubated for 24 h at 37°C in the presence of 5% CO_2. A confirmatory test was conducted on 5 strains of *N. gonorrhoeae* using the CLSI recommended agar dilution method as described before (29) to confirm MIC values obtained from the broth microdilution assay.

Salicylamide's activity against vaginal microflora. Antimicrobial susceptibility testing against *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, and *L. crispatus* was conducted using

the broth microdilution assay in accordance with the CLSI guidelines (29) to test the activity of salicylamide against a panel of strains that are important members of the vaginal microbiome.

Time-kill assay. To determine if salicylamide is a bacteriostatic or bactericidal antibacterial *in vitro*, a time-kill assay was utilized, as described previously (42, 43), against *N. gonorrhoeae* strain 194. Briefly, *N. gonorrhoeae* was grown in BSB to logarithmic phase and further diluted to reach an initial inoculum of 5×10^6 CFU/ml. Salicylamide and azithromycin (positive control) were then added (at 5× MIC in triplicates). Bacteria exposed to DMSO (solvent of drugs) alone served as a negative control. An aliquot from each sample was serially diluted and plated onto chocolate II agar plates. Plates were incubated for 24 h at 37°C in the presence of 5% CO_2 to determine the CFU count. Test agents were categorized as exhibiting bactericidal activity if the bacterial CFU was reduced by at least 3 $\log_{10}$ over a 24-h period.

Synergetic activity of salicylamide with azithromycin, ciprofloxacin, ceftriaxone, and tetracycline. The relationship between salicylamide and four antibiotics (azithromycin, ciprofloxacin, ceftriaxone, and tetracycline) against *N. gonorrhoeae* was assessed via a standard checkerboard assay, as described previously (43, 44). Briefly, a 1.0 McFarland standard solution of bacteria was prepared and then diluted in BSB to achieve a bacterial inoculum of 5×10^5 CFU/ml. Salicylamide and antibiotics were added at the corresponding concentrations, and media containing bacteria were subsequently added. The plates were then incubated for 24 h at 37°C in the presence of 5% CO_2. Interactions where the fractional inhibitory concentration index (FICI) was $\leq$0.5 were categorized as synergistic. An FICI of $>$0.5 but $\leq$1.25 was categorized as additive. An FICI of $>$1.25 but $\leq$4 was considered indifferent, while an FICI $>$4 was categorized as antagonistic (45, 46).

Intracellular clearance assay. An intracellular bacterial clearance experiment was utilized to investigate the ability of salicylamide to enter endocervical cells and reduce the burden of intracellular *N. gonorrhoeae*, as described previously (33, 47, 48). Briefly, human endocervical cells (ATCC CRL-2615, End1/E6E7) were seeded at a density of $\sim 1 \times 10^5$ cells per well in 96-well tissue-culture-treated plates for 24 h at 37°C with 5% CO_2 before being infected with bacteria. The cells were routinely grown in serum-free keratinocyte medium (KSFM) supplemented with 0.05 mg/ml bovine pituitary extract, 0.1 ng/ml epidermal growth factor, and 44.1 mg/liter calcium chloride. Following incubation, the cells were washed once with PBS. Cells were subsequently infected with *N. gonorrhoeae* strain 194 (at a multiplicity of infection of 100:1) for 6 h at 37°C with 5% CO_2. The cells were washed three times with PBS containing 320 μg/ml gentamicin and were further incubated for 1 h to kill and wash off nonphagocytized bacteria. End1/E6E7 cells were subsequently exposed to either salicylamide or ceftriaxone, at 5× MIC, and incubated for 24 h at 37°C with 5% CO_2. DMSO (solvent for the drugs) served as a negative control. After incubation, the test agents were removed, and endocervical cells were washed with PBS and subsequently lysed using 0.01% Triton X-100 to collect intracellular bacteria. The lysate was serially diluted in PBS and plated on chocolate II agar plates. Plates were incubated at 37°C with 5% CO_2 for 24 h. Experiments were performed using triplicate samples for each treatment group, and the experiment was repeated at least twice. Data were analyzed via unpaired *t* tests using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

IL-8 cytokine expression in infected endocervical cells. To investigate the anti-inflammatory activity of salicylamide, the proinflammatory cytokine IL-8 was detected in supernatants of *N. gonorrhoeae*-infected human endocervical cells exposed to either salicylamide or ceftriaxone, as described in a previous study (35). Briefly, cells were infected with *N. gonorrhoeae* strain 194 for 2 h followed by treatment with 0.5× MIC of ceftriaxone or salicylamide (in triplicates) for 4 h. DMSO served as a negative control. Cell supernatants were collected and tested for IL-8 concentration using the Human IL-8 ELISA kit (Bosterbio, Pleasanton, CA), according to the manufacturer's instructions. Data were analyzed via unpaired *t* tests using GraphPad Prism 6.0.

Evaluation of the structure-activity relationship of the benzamide scaffold. Salicylamide is a simple benzamide derivative with only one *ortho*-hydroxyl group. To investigate the structure-antibacterial activity relationship (SAR) of salicylamide, we evaluated the antigonorrheal activity of 11 salicylamide analogs (2-hydroxyhippuric acid, 3-hydroxybenzamide, 4-hydroxybenzamide, *N*,*N*-diethylsalicylamide, salicylic acid, methyl salicylate, 4-methylsalicylamide, anthranilamide, 2-ethoxybenzamide, salicylhydrazide, and 3-hydroxy-2-naphthamide) using the broth dilution assay described above.

***Neisseria gonorrhoeae* spontaneous mutation frequency.** Three *N. gonorrhoeae* strains (197, 202, and 206) were tested against salicylamide or rifampin for a single-step mutation assay as previously described (25). Briefly, drugs were mixed with gonococcal GC medium agar supplemented with 5% horse blood to produce a 10× MIC mixture. Plates were then prepared in triplicates and allowed to dry at room temperature. A bacterial suspension was prepared and matched to a 7 McFarland standard solution; bacterial suspension was then diluted to achieve a bacterial concentration of 10^{10} CFU/ml. A 10-fold serial dilution of the inoculum was plated and incubated at 37°C (with 5% CO_2) for 72 h. Plates were routinely inspected at 24 h and 48 h. The spontaneous mutation frequency was calculated as described previously (25).

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