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In Vitro Assessment of Dual Drug Combinations To Inhibit Growth of *Neisseria gonorrhoeae*

Kevin Pettus, Samera Sharpe, John R. Papp

Centers for Disease Control and Prevention, Atlanta, Georgia, USA

The development of resistance to first-line antimicrobial therapies has led to recommendations for combination therapies for the treatment of gonorrhea infection. Recent studies have shown the success of combination therapies in treating patients, but few have reported on the *in vitro* activities of these drug combinations. An *in vitro* assessment of azithromycin in combination with gentamicin demonstrated inhibition of growth and suggests that clinical trials may be warranted to assess the utility of this combination in treating gonorrhea infections.

Clinical management of *Neisseria gonorrhoeae*, a common sexually transmitted bacterial pathogen, is complicated by the ability of the organism to become resistant to antibiotics through genetic mutations and/or acquisition of resistance conferring genes (1). Antimicrobial resistance in *N. gonorrhoeae* strains occurs as chromosomally mediated resistance to a variety of antimicrobial agents, including penicillin, tetracycline, spectinomycin, and fluoroquinolones, or high-level plasmid-mediated resistance to penicillin and tetracycline (2). The Centers for Disease Control and Prevention (CDC) currently recommends that uncomplicated gonococcal infections be treated with ceftriaxone in combination with azithromycin or doxycycline (3). Combination therapy is hypothesized to lessen the survivability of *N. gonorrhoeae* strains that become resistant to one or the other antibiotic. The combination of ceftriaxone or cefixime with azithromycin was shown to effectively inhibit *N. gonorrhoeae* growth *in vitro* through independent actions (4). However, the effect of drug combinations involving azithromycin on *N. gonorrhoeae* growth has not been well studied.

In vitro drug activity against *N. gonorrhoeae* strains can be determined by either diffusion or dilution methods. The agar plate dilution method is considered to be the standard reference method and is used in the CDC's Gonococcal Isolate Surveillance Project (5), which monitors susceptibility trends in the United States to inform treatment recommendations. The method is most appropriate for assessing drug combinations since the dilutions are incorporated directly into the agar and not prone to differences in diffusion if disk or strip tests are used. In the present study, combinations of azithromycin and gentamicin, azithromycin and rifampin, and azithromycin and ciprofloxacin were tested against a panel of clinical *N. gonorrhoeae* isolates to determine if there was any synergistic or antagonist effect on bacterial growth.

A total of 99 *N. gonorrhoeae* clinical isolates, collected as part of the Gonococcal Isolate Surveillance Project from 2005 to 2007 from sexually transmitted disease (STD) clinics across the United States (5), were selected based on having a range of susceptibilities to azithromycin and ciprofloxacin. Isolates were stored frozen at −70°C in tryptic soy broth containing 20% glycerol from the time of initial testing. Stock solutions of azithromycin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), rifampin (Sigma-Aldrich), and ciprofloxacin (Sigma-Aldrich) were prepared and serial dilutions incorporated into GC II agar base medium (Difco) supplemented with 1% IsoVitaleX (Fisher) maintained at 50°C. The final concentrations of azithromycin were 0 to 8.0 μg/ml in combination with 0 to 32 μg/ml of gentamicin. A similar range of azithromycin dilutions was used in combination with 0 to 64 μg/ml of ciprofloxacin and 0 to 8 μg/ml of rifampin. Following the addition of antibiotic dilutions, the medium was poured into square 100-mm by 100-mm petri dishes. The medium was allowed to solidify, and the plates were stored at 4°C for up to 1 month prior to use. The overnight growth of an isolate on chocolate agar was

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Address correspondence to John R. Papp, jwp6@cdc.gov.

All authors contributed equally to this article.

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TABLE 1 MICs of *Neisseria gonorrhoeae* isolates with single and combination antibiotics

	MIC (μg/ml) data[a]											
	MIC_{50}		MIC_{90}		MIC_{50}		MIC_{90}		MIC_{50}		MIC_{90}	
Test	AZI	GEN	AZI	GEN	AZI	CIP	AZI	CIP	AZI	RIF	AZI	RIF
Single antibiotic test	0.5	4	4	4	0.5	4	4	32	0.5	2	4	8
	AZI-GEN		AZI-GEN		AZI-CIP		AZI-CIP		AZI-RIF		AZI-RIF	
Dual antibiotic test	0.25/4		2/4		0.5/4		4/16		0.5/8		4/8	

[a] AZI, azithromycin; GEN, gentamicin; CIP, ciprofloxacin; RIF, rifampin.

TABLE 2 MICs and FICIs of gentamicin-azithromycin against *Neisseria gonorrhoeae* isolates

Gentamicin plus azithromycin	No. of isolates	MIC (μg/ml) (median [range]) data for[a]:				FICI	FICI interpretation[b]
		MIC_{GEN}	$MIC_{GEN\ (with\ AZI)}$	MIC_{AZI}	$MIC_{AZI\ (with\ GEN)}$		
Stratified by gentamicin MIC							
Gentamicin, MIC 1–2 μg/ml	39	2 (1–2)	4 (0.5–4)	0.25 (0.016–4)	0.25 (0.008–4)	2.5 (1.5–3)	Indifference
Gentamicin, MIC 4 μg/ml	60	4 (4)	8 (2–8)	0.5 (0.125–8)	0.425 (0.06–8)	2.5 (2.1–3)	Indifference
Stratified by azithromycin MIC							
Azithromycin, MIC ≤ 0.25 μg/ml	27	2 (1–4)	4 (0.5–8)	0.25 (0.016–0.25)	0.125 (0.008–0.5)	2.5 (2–3)	Indifference
Azithromycin, MIC 0.5–1 μg/ml	55	4 (2–4)	8 (2–8)	0.5 (0.5–1)	0.25 (0.06–1)	2.5 (1.5–3)	Indifference
Azithromycin, MIC 2–8 μg/ml	17	4 (2–4)	8 (2–8)	8 (2–8)	2 (0.25–8)	2.25 (2.13–3)	Indifference
Overall total	99	4 (1–4)	8 (0.5–8)	0.5 (0.16–8)	0.25 (0.008–8)	2.5 (1.5–3)	Indifference

[a] MIC_{GEN}, gentamicin MIC; $MIC_{GEN\ (with\ AZI)}$, MIC of gentamicin in combination with azithromycin; MIC_{AZI}, azithromycin MIC; $MIC_{AZI\ (with\ GEN)}$, MIC of azithromycin in combination with gentamicin.
[b] The FICI was interpreted as drug synergy (FICI ≤ 0.5), indifference (FICI > 0.5 to 4.0), or drug antagonism (FICI > 4.0).

suspended in Mueller-Hinton broth (Becton Dickinson) to a concentration of 10^8 CFU. The bacterial suspensions were inoculated onto the antibiotic-containing agar plates using a Steers replicator that allows for up to 36 isolates to be tested on one plate. The MIC (all isolates inhibited), MIC_{50}, and MIC_{90} values of single drugs and drug combinations were determined after 20 to 24 h incubation at 36.5°C under 5% CO_2. For analysis, results were arbitrarily stratified by MIC values into low, medium, and elevated groupings to assess differences between these values. There were no medium rifampin MIC values for isolate grouping. A fractional inhibitory index (FICI) was calculated as previously described (4, 6). The FICI was used to assess synergy (FICI ≤ 0.5), indifference (FICI = 0.5 to 4.0), or antagonism (FICI > 4.0) of the antibiotic combinations. The accuracies of the antibiotic dilutions were assessed using *N. gonorrhoeae* quality control strains F18, F28, P681E, 10328, 10329, SPJ-15, and SPL-4. Only results from quality control-validated test runs that had expected MIC values for azithromycin, gentamicin, rifampin, and ciprofloxacin were used in the analysis.

The MIC_{50} and MIC_{90} values of antibiotic combinations with respect to the growth of *N. gonorrhoeae* isolates were compared to values from single-antibiotic dilutions (Table 1). There were 1-dilution decreases of the MIC_{50} and MIC_{90} values of azithromycin when used in combination with gentamicin. Azithromycin plus ciprofloxacin resulted in a 1-dilution decrease for the MIC_{90} but not the MIC_{50} of ciprofloxacin. There were no effects on the azithromycin MIC values in combination with ciprofloxacin. In contrast, the MIC_{50} of rifampin increased by 2 dilutions when azithromycin was added. However, the MIC_{90} values of rifampin combined with azithromycin were the same as those from plates with one antibiotic. There were slight differences in the reproducibility of single MIC values for azithromycin when tested for each antibiotic combination, but these values did not differ by more than 1 dilution. This was primarily noted when testing the combination of azithromycin and ciprofloxacin when the single MIC values for some isolates altered the number of isolates within the arbitrary groupings.

There was no antibiotic synergy or antagonism when azithromycin was paired with either gentamicin or ciprofloxacin (Tables 2 and 3). Although the overall FICI interpretation for rifampin and azithromycin was indifference (Table 4), antagonism was observed with 23 isolates. The azithromycin and rifampin MIC values for these 23 isolates ranged from 0.125 to 8 μg/ml and 0.015 to 8 μg/ml, respectively. There was no stratification by single-drug MIC values for those isolates in which antagonism was noted between rifampin and azithromycin. Comparing the genomic sequences of isolates that had drug antagonism to those without it may elucidate the mechanisms responsible for these observations. These *in vitro* data suggest that rifampin should not be considered for use in combination with azithromycin for the treatment of gonorrhea. However,

TABLE 3 MICs and FICIs of ciprofloxacin-azithromycin against *Neisseria gonorrhoeae* isolates

Rifampin plus azithromycin	No. of isolates	MIC (μg/ml) (median [range]) data for[a]:				FICI	FICI interpretation[b]
		MIC_{CIP}	$MIC_{CIP\ (with\ AZI)}$	MIC_{AZI}	$MIC_{AZI\ (with\ CIP)}$		
Stratified by ciprofloxacin MIC							
Ciprofloxacin, MIC ≤ 0.25 μg/ml	34	0.03 (0.03–0.25)	0.023 (0.016–0.125)	0.5 (0.125–0.5)	0.25 (0.06–0.5)	1.14 (0.34–3)	Indifference
Ciprofloxacin, MIC 1–8 μg/ml	21	4 (1–8)	2 (0.25–1)	0.5 (0.25–1)	0.25 (0.125–0.5)	1 (0.75–2)	Indifference
Ciprofloxacin, MIC 16–64 μg/ml	44	32 (16–64)	16 (8–32)	2 (0.5–8)	1.5 (0.25–8)	1.25 (0.38–2)	Indifference
Stratified by azithromycin MIC							
Azithromycin, MIC ≤ 0.25 μg/ml	21	0.03 (0.03–4)	0.03 (0.016–2)	0.25 (0.125–0.25)	0.125 (0.06–0.25)	1.5 (1.03–3)	Indifference
Azithromycin, MIC 0.5–1 μg/ml	44	2 (0.03–32)	1 (0.008–32)	0.5 (0.5–1)	0.38 (0.125–1)	1.13 (0.34–2)	Indifference
Azithromycin, MIC 2–8 μg/ml	34	32 (16–64)	16 (8–32)	4 (2–8)	2 (1–8)	1 (0.38–2)	Indifference
Overall total	99	4 (0.03–64)	4 (0.008–32)	0.5 (0.125–8)	0.5 (0.06–8)	1.03 (0.34–3)	Indifference

[a] MIC_{CIP}, ciprofloxacin MIC; $MIC_{CIP\ (with\ AZI)}$, MIC of ciprofloxacin in combination with azithromycin; MIC_{AZI}, azithromycin MIC; $MIC_{AZI\ (with\ CIP)}$, MIC of azithromycin in combination with ciprofloxacin.
[b] The FICI was interpreted as drug synergy (FICI ≤ 0.5), indifference (FICI > 0.5 to 4.0), or drug antagonism (FICI > 4).

TABLE 4 MICs and FICIs of rifampin-azithromycin against *Neisseria gonorrhoeae* isolates

Rifampin plus azithromycin	No. of isolates	MIC (μg/ml) (median [range]) data for[a]: MIC_{RIF}	$MIC_{RIF\ (with\ AZI)}$	MIC_{AZI}	$MIC_{AZI\ (with\ RIF)}$	FICI	FICI interpretation[b]
Stratified by rifampin MIC							
Rifampin, MIC ≤ 0.06 μg/ml	42	0.06 (0.002–0.06)	0.125 (0.002–0.25)	0.5 (0.03-≥8)	0.5 (0.008–1)	3.08 (2–17.63)	Indifference
Rifampin, MIC 0.125–4 μg/ml	12	1.5 (0.125–4)	4 (0.125–8)	0.5 (0.03–1)	0.5 (0.016–0.5)	2.25 (1.12–9)	Indifference
Rifampin, MIC 8 μg/ml	45	8 (8)	8 (8)	0.5 (0.06–8)	0.5 (0.03–8)	2 (1.06–5)	Indifference
Stratified by azithromycin MIC							
Azithromycin, MIC ≤ 0.25 μg/ml	41	0.125 (0.002–8)	0.25 (0.002–8)	0.25 (0.03–0.25)	0.25 (0.008–0.5)	3 (1.12–17.63)	Indifference
Azithromycin, MIC 0.5–1 μg/ml	40	1 (0.03–8)	4.25 (0.125–8)	0.5 (0.5–1)	0.5 (0.25–1)	2.58 (1.5–5.17)	Indifference
Azithromycin, MIC 2–8 μg/ml	18	8 (0.016–8)	8 (0.06–8)	8 (2–8)	8 (0.25–8)	2 (1.06–4.25)	Indifference
Overall total	99	8 (0.016–8)	8 (0.002–8)	0.5 (0.03–8)	0.5 (0.008–8)	2.58 (1.06–17.63)	Indifference

[a] MIC_{RIF}, rifampin MIC; $MIC_{RIF\ (with\ AZI)}$, MIC of rifampin in combination with azithromycin; MIC_{AZI}, azithromycin MIC; $MIC_{AZI\ (with\ RIF)}$, MIC of azithromycin in combination with rifampin.

[b] The FICI was interpreted as drug synergy (FICI ≤ 0.5), indifference (FICI > 0.5 to 4.0), or drug antagonism (FICI > 4).

there was no reported antagonism when rifampin was combined with ceftriaxone or cefixime (6).

Dual therapy is currently recommended by the CDC for the treatment of uncomplicated *N. gonorrhoeae* infections (3), and the assessment of drug antagonism is important when new combinations are considered. Combining a macrolide with either an aminoglycoside or fluoroquinolone effectively inhibited growth, with MIC values that were similar to single-antibiotic usage. The combination appeared to be additive rather than synergistic. Since *N. gonorrhoeae* has developed and retained resistance to ciprofloxacin in the United States (7), adding azithromycin in combination would likely be ineffective in treating isolates that are resistant to ciprofloxacin. However, there were no antagonistic outcomes with either of the antibiotics when used to assess the *in vitro* growth of *N. gonorrhoeae*, and this may be an important observation for using fluoroquinolones other than ciprofloxacin in combination with azithromycin. A recently completed clinical trial demonstrated 100% and 99.5% successes in patients with uncomplicated gonorrhea infections receiving azithromycin combined with gentamicin or gemifloxacin, respectively (8). *In vitro* assessments with antibiotic combinations were not performed in that study, but our data support the observed clinical utility of these antibiotic combinations.

Resistance to therapeutic agents continues to evolve in *N. gonorrhoeae*, leaving scant antibiotic choices for the treatment of uncomplicated gonococcal infections. As these evolutionary bacterial trends continue, new antibiotics are needed. Until new antibiotics are developed, evaluated, and approved for treatment, investigating existing antibiotics used in combination may be therapeutically relevant.

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