# Investigating FDA-Approved Drugs for Treatment of Multidrug-Resistant *Neisseria gonorrhoeae*

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## Abstract

*Neisseria gonorrhoeae*, the causative agent of gonorrhea, is the second most prevalent sexually transmitted infection that leads to substantial morbidity and economic burden worldwide. Improperly treated or untreated gonorrhea can lead to severe and life-threatening complications, including abortion, infertility, pelvic pain, and maternal death. *Neisseria gonorrhoeae* has developed resistance to the formally and currently used antibiotics. The Centers for Disease Control and Prevention (CDC) have listed multi-drug resistant *N. gonorrhoeae* as an urgent threat that promptly requires the development of novel therapeutic agents.

Traditional drug discovery and development is a time-consuming and costly process associated with high risks. To address the dire need to replenish the dry pipeline of anti-gonorrhea medications, drug repurposing is a promising approach. In this study, an FDA-approved drug library was screened, and 14 drugs were found to exhibit promising anti-gonococcal activity. Interestingly, three extremely potent and narrow-spectrum novel candidates, itraconazole, isavuconazole, and ravuconazole, are azole antifungals, and their activities were further investigated *in vitro*.

Of the three azoles, ravuconazole displayed the most potent activity against *N. gonorrhoeae* clinical isolates. The time-kill assay revealed that the three azoles showed bactericidal activity. All three azole drugs showed a low frequency of resistance. Besides, isavuconazole and ravuconazole have a longer post-antibiotic effect than azithromycin. All three azoles cleared the burden of intracellular *N. gonorrhoeae* completely, which is superior to ceftriaxone.

In conclusion, itraconazole, isavuconazole, and ravuconazole merit future investigation for the development of anti-gonorrheal therapeutics. This study provided unexplored avenues and promising opportunities that can be further evaluated to combat *N. gonorrhoeae* infection.

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## General Audience Abstract

*Neisseria gonorrhoeae*, the causative agent of gonorrhea, is the second most prevalent sexually transmitted infection that leads to substantial morbidity and economic burden worldwide. Improperly treated or untreated gonorrhea can lead to severe and life-threatening complications, including abortion, infertility, pelvic pain, and maternal death. Due to the increasing prevalence of drug resistance against the formally and currently used antibiotics, the Centers for Disease Control and Prevention (CDC) have classified multi-drug resistant *N. gonorrhoeae* as an urgent-threat pathogen. Therefore, the discovery of new anti-gonorrheal therapeutics is an urgent need.

Drug repurposing is the process of discovering new therapeutic uses for approved or investigational drugs that go beyond the original medical indication. To address the dire need to replenish the dry pipeline of anti-gonorrheal drugs, repurposing FDA-approved drugs is a promising approach as it significantly reduces the time and expense associated with traditional drug development. By screening an FDA-approved drug library, 14 drugs were found to display promising anti-gonococcal activity. Interestingly, three (itraconazole, isavuconazole, and ravuconazole) out of 14 identified drugs were azole antifungal drugs, and their activities were further investigated *in vitro*.

All three azole drugs showed bactericidal activity, meaning that they killed bacteria, had a low propensity to develop resistance, and completely cleared the burden of intracellular *N. gonorrhoeae*. Besides, our findings suggested that isavuconazole and ravuconazole possessed exceptional activity in the suppression of bacterial growth following brief antibiotic exposure. In conclusion, the three azole drugs exhibited potent anti-gonococcal activity and merited further investigation. This study provided unexplored avenues and promising opportunities that can be further evaluated to combat multidrug-resistant *N. gonorrhoeae*.

*Dedicated to my mother, father, grandparents, and significant other for their unconditional love and support*

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# Table of contents

# List of Figures

# List of Tables

# List of Abbreviations

| | |
|---|---|
| ADD | Additive |
| AMR | Antimicrobial resistance |
| ANOVA | Analysis of variances |
| ATCC | American Type Culture Collection |
| BEI Resources | Biodefense and Emerging Infections Research Resources Repository |
| BSB | Brucella supplemented broth |
| Carboxy-H2DCFDA | 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate |
| CDC | Centers for Disease Control and Prevention |
| CFU | Colony forming unit |
| CLSI | The Clinical & Laboratory Standards Institute |
| COVID-19 | Coronavirus disease |
| DHPS | Dihydropteroate synthetase |
| DMSO | Dimethyl sulfoxide |
| ECDC | European Centre for Disease Prevention and Control |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| FICI | Fractional inhibitory concentration index |
| GC | Gonococcal |
| HIV | Human immunodeficiency virus |
| $H_2O_2$ | Hydrogen peroxide |
| IND | Indifferent |
| MRS | deMan, Rogosa and Sharpe |
| MIC | Minimum inhibitory concentration |
| NCCLS | National Committee for Clinical Laboratory Standards |
| *N. gonorrhoeae* | *Neisseria gonorrhoeae* |
| PAE | Post-antibiotic effect |
| PBP | Penicillin-binding protein |
| PBS | Phosphate-buffered saline |
| PHAC | Public Health Agency of Canada |
| PPNG | Penicillinase-producing *Neisseria gonorrhoeae* |
| ROS | Reactive oxygen species |
| SNP | Single nucleotide polymorphism |
| STI | Sexually transmitted infection |
| SYN | Synergistic |
| VRE | Vancomycin-resistant *Enterococcus* |
| WHO | World Health Organization |

# Chapter 1. Introduction

## 1.1 Antimicrobial Resistance

Disease emergencies are not new occurrences; they have posed a threat to human survival throughout recorded history. Infectious diseases were a major cause of morbidity and mortality before the beginning of the 20th century. For example, the Black Death killed between 30% and 60% of all Europeans; the Spanish flu wiped out more people than in World War I; and the Japanese smallpox epidemic killed approximately one-third of the entire Japanese population[1-4]. Not to mention that the recent coronavirus disease (COVID-19) pandemic has shown how deadly emerging infectious illnesses may be to human life. Pandemics cause significant social, human, and economic crises worldwide. A recent study indicated that 33 clinically significant bacterial pathogens were involved in 13% of global deaths in 2019, which ranks as the second leading cause of death globally, behind ischemic heart disease (16%)[5,6]. Despite having access to advanced medical care nowadays, the threat of infectious diseases to human society has not been reduced much.

In 1928, the legendary discovery of penicillin by Alexander Fleming marked the beginning of the golden era of antibiotic research and development [7]. The introduction of antibiotics revolutionized the treatment of infectious diseases worldwide. However, only a few years after the implementation of penicillin for clinical use, penicillin-resistant bacteria were observed as early as the 1940s[8]. Throughout the subsequent decades, resistant bacterial strains emerged in conjunction with the launch of innovative medicines. Misuse and overuse of antimicrobials are the leading causes of the emergence of drug-resistant bacteria. Consequently, the World Health Organization (WHO) has ranked antimicrobial resistance (AMR) as one of the top 10 worldwide public health problems[9]. Bacterial AMR contributed to an estimated 1.27 million deaths and was associated with nearly 5 million deaths globally in 2019[10]. In addition, COVID-19 exacerbates existing AMR problems due to the dramatic disruption of healthcare systems. According to the Centers for Disease Control and Prevention (CDC), about 2.8 million cases of antibiotic-resistant infections occur annually in the U.S., resulting in over 35,000 deaths[11]. The financial burden of multidrug-resistant pathogens costs more than $4.6

billion annually in the U.S.[12]. Without intervention, drug-resistant illnesses might kill 10 million people annually by 2050[13].

AMR is a worldwide health threat that requires urgent action. In this thesis, a drug-resistant bacterium, *Neisseria gonorrhoeae*, is targeted. The CDC classified *Neisseria gonorrhoeae* as one of the top five urgent threat-level pathogens. The antimicrobial resistance of *N. gonorrhoeae* is discussed below.

### 1.2 Antimicrobial Resistance in *Neisseria gonorrhoeae*

Gonorrhea, caused by the Gram-negative diplococci bacterium *Neisseria gonorrhoeae*, is the second most prevalent sexually transmitted infection (STI). WHO estimated that more than one million STIs were acquired globally every day in 2020[14]. Gonococcal infections represent 82.4 million (~20%) of the estimated 374 million new cases of STIs that occur annually worldwide[15]. In the U.S., one in five people has an STI, with nearly 68 million infections and $16 billion in direct lifetime medical costs[16]. However, due to asymptomatic gonococcal infections, these numbers are likely underestimated.

Notably, gonorrhea can be transmitted even if the infected person does not experience any symptoms. Many people carrying *N. gonorrhoeae* transmit the infection to their sexual partners without their knowledge. Gonorrhea is primarily transmitted through vaginal, anal, or oral sex with an infected person, especially among young people ages 15 to 24. An infected pregnant woman can also transmit the infection to the baby during childbirth. *N. gonorrhoeae* infections can cause disease in the genitals, rectum, and throat in both men and women. Improperly treated or untreated gonorrhea can lead to severe and long-term life-threatening complications such as tubal scarring, infertility, pelvic inflammatory disease, ectopic pregnancy, maternal death, first-trimester abortion, long-term pelvic pain, and eye infections in the newborn[17-20]. In addition, untreated gonorrhea could increase the risk of contracting and transmitting HIV fivefold in both sexes[20].

Gonorrhea is the most antibiotic-resistant STI. Alarmingly, the number of drug-resistant *N. gonorrhoeae* strains increased by 124% in 2019 compared to 2013[21]. *N. gonorrhoeae* has developed resistance to each class of antibiotics deployed against it,

including penicillin, tetracyclines, spectinomycin, fluoroquinolones, macrolides, and cephalosporins[22]. Fifty percent of all gonococcal infections are resistant to at least one antibiotic. Ceftriaxone, a third generation extended-spectrum cephalosporin, is the only remaining option for empirical first-line treatment currently, but high-level gonococcal clinical resistance to ceftriaxone has been documented[23,24]. Because vaccines for the prevention of *N. gonorrhoeae* are unavailable[25], effective gonorrhea control relies solely on antibiotics. Altogether, the need for the development of new antibacterial agents for the treatment of gonorrhea is critical.

Antibiotics have been the only reliable method for treating of gonorrhea, but their effectiveness is now in doubt. Given that *N. gonorrhoeae* is competent for transformation and can modify its genome via mutations, *N. gonorrhoeae* possesses an extraordinary capability to alter its genetic materials. Therefore, *N. gonorrhoeae* can adapt and evolve rapidly to acquire resistance to antimicrobials. Bacteria develop AMR primarily through the following molecular mechanisms: alteration of antibiotic targets, decreased influx of antibiotics into the cell, expression of multi-drug efflux pumps, and expression of antibiotic degrading enzymes. *N. gonorrhoeae* has developed a wide variety of resistance determinants across all these routes, making it resistant to treatment with almost every class of antibiotic. Table 1.1 summarizes the resistance mechanisms of AMR in *N. gonorrhoeae*[26,27].

Overall, antimicrobial resistant *N. gonorrhoeae* is a serious and growing issue that requires immediate attention. The rise of multidrug-resistant gonorrhea strains threatens to hamper decades of progress in combating this STI. To mitigate the spread of drug-resistant *N. gonorrhoeae*, continuous surveillance programs, enhanced international collaborative actions, and the development of vaccines and new antimicrobials are necessary[28].

**Table 1.1 Resistance mechanisms in *Neisseria gonorrhoeae* for previously or currently recommended treatment of gonorrhea**

| Antimicrobial class | Antimicrobial mechanism | Resistance mechanisms |
|---|---|---|
| **Sulfonamides** | Competitive substrate and structural analogs of p-aminobenzoic acid in the synthesis of folic acid. | • Increased production of competitive substrate *p*-aminobenzoic acid. |

| Antimicrobial class | Antimicrobial mechanism | Resistance mechanisms |
|---|---|---|
| | | • Mutations in *folP* reduce target affinity. *folP* encodes the sulfonamides target dihydropteroate synthetase (DHPS) |
| **Penicillins** | Inhibition of cell wall synthesis by binding of the β-lactam ring to penicillin-binding proteins (PBPs) | • Mutations in *penA* and *ponA* reduce the PBP acylation rate of β-lactam antibiotics and PBP binding. *penA* encodes PBP2, while *ponA* encodes PBP1.<br>• Mutations in *mtrR* increase efflux. MtrR is a repressor of mtrCDE efflux pump expression.<br>• Mutations in *porB* (*penB*) reduce influx. *porB* encodes major outer membrane porin.<br>• A single nucleotide polymorphism (SNP) in *pilQ* reduces influx. *pilQ* encodes the pore-forming secretin PilQ of the type IV pili.<br>• Penicillinase-producing *Neisseria gonorrhoeae* (PPNG) carried plasmid-mediated β-lactamase (bla) gene type TEM (bla-TEM). |
| **Tetracyclines** | Inhibition of protein synthesis by binding to the bacterial 30S ribosomal subunit. | • A SNP in *rpsJ* reduces target affinity. *rpsJ* encodes 30S ribosomal protein S10.<br>• *mtrR* mutations.<br>• *penB* mutations.<br>• A SNP in *pilQ*.<br>• Plasmid-mediated tetracycline resistance of TetM-encoding plasmids. TetM binds to the 30S ribosomal subunit and prevents tetracycline binding. |

| Antimicrobial class | Antimicrobial mechanism | Resistance mechanisms |
|---|---|---|
| **Spectinomycin** | Inhibition of protein synthesis by binding to the bacterial 30S ribosomal subunit. | • Mutations in *rpsE* reduce target binding. *rpsE* encodes the 30S ribosomal protein S5.<br>• A 16S rRNA SNP reduces target affinity. |
| **Quinolones** | Inhibition of DNA synthesis by targeting topoisomerase II (DNA gyrase) and topoisomerase IV. | • *gyrA* SNPs reduce quinolone binding to DNA gyrase.<br>• *parC* reduces quinolone binding to topoisomerase IV. |
| **Macrolides** | Inhibition of protein synthesis by binding to the bacterial 50S ribosomal subunit. | • *erm* genes (*ermB*, *ermC*, and *ermF*) block macrolides binding. *erm* genes encode rRNA methylases that methylate nucleotides in the 23S rRNA target.<br>• 23S rRNA SNPs reduce target affinity.<br>• *mtrR* mutations.<br>• MacAB efflux pump<br>• mef-encoded efflux pump |
| **Cephalosporins** | Inhibition of cell wall synthesis by binding of the β-lactam ring to PBPs. | • Mosaic *penA* alleles reduce PBP2 acylation rate.<br>• *mtrR* mutations.<br>• *penB* mutations. |

### 1.3 Current Treatments

In response to the increasing antimicrobial resistance of *N. gonorrhoeae*, international guidelines have been revised regularly (Table 1.2). The Public Health Agency of Canada (PHAC) adapts combination therapy to enhance treatment efficacy and prevent or delay the emergence and spread of antimicrobial-resistant gonorrhea. The recommended combination therapy includes cephalosporin (250 mg of intramuscular ceftriaxone or 800 mg of oral cefixime) with 1 g oral azithromycin for anogenital and pharyngeal infections[29].

In the past, the CDC recommended dual therapy of 500 mg intramuscular ceftriaxone along with 1 g oral of azithromycin for the treatment of gonorrhea until 2020. However, azithromycin is no longer recommended because the growing resistance to azithromycin could hinder its ability to treat other common bacterial infections. The current CDC guidelines advise administering a single 500 mg intramuscular dose of ceftriaxone for uncomplicated gonococcal infection of the cervix, urethra, rectum, or pharynx[30].

Similarly, the European Centre for Disease Prevention and Control (ECDC) supports the use of a dual therapy of 1 g intramuscular ceftriaxone with 2 g oral azithromycin or a monotherapy of 1 g intramuscular ceftriaxone as the preferred therapy for uncomplicated *N. gonorrhoeae* infections of the urethra, cervix, and rectum[31]. A single dose of 1 g intramuscular ceftriaxone is recommended only in well-controlled settings when recent local susceptibility testing has shown no resistance to ceftriaxone, a test of cure is mandatory, the likelihood of the patient returning for a test of cure is high, and doxycycline 100 mg twice a day for seven days is given to cover *Chlamydia trachomatis* co-infection. In comparison to PHAC and CDC, the ECDC recommended higher doses of ceftriaxone and azithromycin.

The WHO recommends a combination of 250 mg intramuscular ceftriaxone or 400 mg oral cefixime plus 1 g oral azithromycin as the primary treatment for genital and anorectal gonococcal infections. Single therapy such as ceftriaxone, spectinomycin, or cefixime is an alternative option when surveillance data support its feasibility[32].

In summary, the current antimicrobial therapy recommended for gonococcal infections across Europe, Canada, and by the WHO is a dual therapy of intramuscular ceftriaxone and oral azithromycin with different doses, while the CDC has updated its guidelines and now recommends a single 500 mg dose of intramuscular ceftriaxone. *N. gonorrhoeae* has developed resistance to sulfonamides, penicillins, tetracycline, ciprofloxacin, macrolides (e.g., azithromycin), and cephalosporins (e.g., ceftriaxone), rendering it a candidate for causing an untreatable disease. As the threat of antimicrobial resistance continues to grow, it is essential to adopt the latest treatment guidelines and invest in the development of novel antimicrobial agents.

**Table 1.2 Recommended guidelines for gonococcal infection**

| Region/ organization | Therapy | Alternative regimes | Year guideline published /updated | Reference |
|---|---|---|---|---|
| Canada (PHAC) | CRO 250 mg IM + AZM 1 g PO or CFX 800 mg PO + AZM 1 g PO | If there is a contraindication to macrolide use: CFO 250 mg + DOX 100 mg PO or CFX 800 mg PO+ DOX 100 mg PO | 2022 | [29] |
| U.S. (CDC) | CRO 500 mg IM | If CRO is unavailable: GEN 240 mg IM + AZM 2 g PO or CFX 800 mg PO | 2021 | [30] |
| Europe (ECDC) | CRO 1 g IM + AZM 2 g PO or CRO 1 g IM | If AZM is unavailable: CRO 1 g IM | 2020 | [31] |
| WHO | CRO 250 mg IM + AZM 1 g PO or CFX 400 mg PO + AZM 1 g PO | If recent local data confirm susceptibility to one of the following antimicrobials: CRO 250 mg IM or CFX 400 mg PO or SPC 2 g IM | 2016 | [32] |

CRO: ceftriaxone, GEN: gentamicin, AZM: azithromycin, CFX: cefixime, DOX: doxycycline, SPC: spectinomycin, IM: intramuscular, PO: per os (oral administration)

### 1.4 New Treatments in Development

The emergence of antibiotic-resistant strains of *N. gonorrhoeae* highlights the critical need for developing novel drugs to combat the challenges. Several promising agents have the potential to address this global health problem (Table 1.3). Three of these antibiotics have progressed to Phase III clinical trials. Many of them were discovered through drug repurposing.

Solithromycin, gepotidacin, and zoliflodacin are three new antibiotics in Phase III clinical trials. Solithromycin is a macrolide antibiotic that targets the 50S subunit of bacterial ribosomes and inhibits protein synthesis. The *in vitro* activity of solithromycin was more effective against gonococcal isolates than azithromycin and several other classes of antibiotics[33,34]. Besides, a phase II clinical trial using two oral doses (1000 mg and 1200 mg) of solithromycin for the treatment of uncomplicated gonorrhea was conducted. All infected patients were cured at the genital, oral, and rectal sites of

infection at both dosages[35]. The Phase III clinical study comparing a single dose of oral solithromycin (1000 mg) to ceftriaxone (500 mg) and azithromycin (1000 mg) dual therapy for the treatment of uncomplicated genital gonorrhea failed to demonstrate non-inferiority with a 4.0% lower difference in eradication rate[36]. This suggests a single 1000 mg dose of solithromycin is not an ideal alternative to ceftriaxone and azithromycin dual therapy. Additional studies are needed to evaluate the efficacy and dose adjustments of solithromycin as an alternative first-line therapy for *N. gonorrhoeae* infections.

Gepotidacin (GSK2140944) is a novel triazaacenaphthylene topoisomerase inhibitor that displays a broad spectrum of antibacterial activity *in vitro*, including against *N. gonorrhoeae*[37,38]. It targets DNA gyrase and topoisomerase IV with a mechanism of action that is distinct from all currently approved antibiotics. The new mechanism may be able to circumvent the mechanisms of antibiotic resistance that have rendered conventional treatments ineffective against *N. gonorrhoeae*. Gepotidacin has demonstrated potent *in vitro* activity against a wide range of multidrug-resistant *N. gonorrhoeae* strains with a low propensity for resistance development[37,39]. In the Phase II clinical study, 1500 mg or 3000 mg of oral gepotidacin once a day for seven days demonstrated a high cure rate of 96% for uncomplicated urogenital gonorrhea[40]. The trial outcomes supported the advancement of gepotidacin into Phase III clinical trials, which are evaluating the efficacy and safety of oral gepotidacin versus oral nitrofurantoin in the treatment of uncomplicated urinary tract infections.

Zoliflodacin (AZD0914 or EXT0914) is a novel spiropyrimidinetrione antibiotic that targets the GyrB subunit in DNA gyrase[41]. Studies have shown zoliflodacin demonstrated potent activity against multidrug-resistant *N. gonorrhoeae* with equal or lower $MIC_{50}$ and $MIC_{90}$ than every previously used antimicrobial[42,43]. Moreover, zoliflodacin exhibited a low rate of resistance frequency and lack of cross-resistance[44]. A Phase II clinical trial to evaluate the efficacy of zoliflodacin for the treatment of uncomplicated gonorrhea has been completed. A single dose of 2 g and 3 g of oral zoliflodacin exhibited 100% treatment success rates for rectal gonorrhea, 96% for urogenital gonorrhea, and 50% and 82% for pharyngeal infection, which is slightly less effective than a single 500 mg intramuscular dose of ceftriaxone (100%) in all cases[45]. Apart from pharyngeal gonorrhea, zoliflodacin seems to be effective and promising.

Zoliflodacin progressed to a Phase III clinical trial to evaluate the safety and efficacy of a 3 g oral dose of zoliflodacin compared to a combination of ceftriaxone and azithromycin for the treatment of uncomplicated gonorrhea. There are other upcoming antibiotics in the early stages of drug development for antigonococcal agents including lefamulin, SMT-571, aminoethyl spectinomycins, closthioamide, and tebipenem[46-51].

Several drugs with indications other than antibacterial agents have been studied by utilizing drug repurposing against *N. gonorrhoeae*. For example, salicylamide, fenamic acid derivatives (tolfenamic, flufenamic, and meclofenamic acid), and auranofin are analgesic and antipyretic, nonsteroidal anti-inflammatory, and antirheumatic drugs, respectively[52-54]. They all demonstrated a low frequency of resistance, bactericidal effect, and superior intracellular clearance activity *in vitro*. Notably, auranofin was investigated in a female murine model of genital[55]. A 1.4 $\log_{10}$ reduction (96%) was observed five days after treatment. However, most of these repurposed drugs were mainly assessed for their antibacterial activity against *N. gonorrhoeae in vitro*. Further evaluations *in vivo* are required to examine the use of the drugs in practice.

**Table 1.3 Drugs of interest for *Neisseria gonorrhoeae* antibiotic development**

| Name | Category | Target | Notes | References |
|---|---|---|---|---|
| Solithromycin (T-4288) | Antibiotic | 50S subunit of the bacterial ribosome | Ketolide, phase III clinical trial | 33-36 |
| Gepotidacin | Antibiotic | Type II topoisomerase (DNA gyrase) and type IV topoisomerase | Triazaacenaphthylene, phase III clinical trial | 40,56 |
| Zoliflodacin (ETX0914) | Antibiotic | Bacterial type II topoisomerase (GyrB) | Spiropyrimidinetrione, phase III clinical trial | 41,45,57,58 |
| Lefamulin | Antibiotic | Inhibiting bacterial translation by binding to the peptidyl transfer center of the bacterial ribosome | Pleuromutilin class | 46,59 |

| Name | Category | Target | Notes | References |
|---|---|---|---|---|
| SMT-571 | Antibiotic | Bacterial cell division | Oral antimicrobial | [47] |
| DIS-73285 | Antibiotic | Electron transfer proteins | | [60] |
| Aminomethyl spectinomycins | Antibiotic | Protein synthesis | Semisynthetic analogs of spectinomycin | [48] |
| Closthioamide | Antibiotic | Bacterial DNA gyrase | Polythioamide class | [49] |
| Tebipenem | Antibiotic | Penicillin-binding proteins (PBPs) | New carbapenem antibiotic | [50] |
| Irrestin-16 | Antibiotic | Bacterial membrane disruption and folate metabolism | Derivative of SCH-79797 | [61] |
| Cethromycin | Antibiotic | 23S rRNA of the 50S ribosomal subunit | Ketolides class | [62] |
| Phenelfamycin B | Antibiotic | EF-Tu translation factor | Natural product, linear polyketide | [63] |
| Sitafloxacin | Antibiotic, Drug repurposing | DNA gyrase and topoisomerase IV | Fluoroquinolone, originally used for respiratory infections | [64,65] |
| Aztreonam | Antibiotic, Drug repurposing | Cell wall synthesis by blocking peptidoglycan crosslinking | Monobactam class | [66-68] |
| Nitroxoline | Antibiotic, Drug repurposing | Chelation of divalent cations required for bacterial RNA polymerase | Urinary antibacterial agent | [69] |

| Name | Category | Target | Notes | References |
|---|---|---|---|---|
| Mupirocin | Antibiotic, Drug repurposing | Bacterial protein synthesis by binding to bacterial isoleucyl transfer RNA synthetase | Topical antibacterial agent treats skin infections caused by bacteria | [70] |
| Apramycin | Antibiotic, Drug repurposing | Blocking translocation | Aminoglycoside, veterinary medicine, produced by *Streptomyces tenebrarius* | [71] |
| Enacyloxin IIa | Antibiotic, Drug repurposing | Protein synthesis | Polyketides | [72] |
| Gladiolin | Antibiotic, Drug repurposing | RNA polymerase | Polyketides macrolide isolated from *Burkholderia gladioli* BCC0238 | [72] |
| Moenomycin A | Antibiotic, Drug repurposing | Cell wall synthesis | Natural product | [73,74] |
| Auranofin | Drug repurposing | Cell wall, DNA, and bacterial protein synthesis | Gold Compounds, antirheumatic drug | [54,55,75] |
| Salicylamide | Drug repurposing | | Analgesic and antipyretic drug | [52] |
| Fenamic acids (tolfenamic, flufenamic, and meclofenamic acid) | Drug repurposing | | Nonsteroidal anti-inflammatory drugs | [53] |
| Carbamazepine | Drug repurposing | Complement receptor 3 | Anticonvulsant | [76] |
| Methyldopa | Drug repurposing | Complement receptor 3 | Antihypertensives | [76] |
| Cannabidivarin | Drug repurposing | | Phase II trial for seizure | [77] |

In conclusion, there are only three viable candidates in phase III clinical trials for the development of gonorrhea treatments. Drugs that are used to treat other illnesses may also be repurposed as treatment options for *N. gonorrhoeae*. Nevertheless, these drugs are mostly in the early stages of development. Additional studies are required to evaluate the feasibility of the proposed treatments before they enter clinical trials. Considering the lengthy timeline and high failure rate in clinical trials, it is essential to continuously uncover and develop alternative antigonococcal drugs to effectively combat this global health challenge.

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# Chapter 2. Screening FDA-Approved Drug Library against *Neisseria gonorrhoeae*

## 2.1 Abstract

*Neisseria gonorrhoeae*, the second most common bacterial cause of sexually transmitted infections, is listed as an urgent-threat pathogen by the Centers for Disease Control and Prevention (CDC). Due to the growing prevalence of resistance development against first-line treatment and several classes of antibiotics, the discovery of new anti-gonorrheal therapeutics is an urgent need. Drug repurposing significantly reduces the time and expense associated with traditional drug development. Herein, utilizing a drug repurposing approach, we screened 3,802 FDA-approved and clinical drugs against *N. gonorrhoeae*. A total of 14 non-antibiotic drugs with significant antigonococcal activity were identified in the screening. Remarkably, some of the active compounds have never been reported before. The antibacterial activity of the 14 compounds was further examined against a panel of clinical isolates of *N. gonorrhoeae*. Several compounds demonstrated potent antigonococcal activity against all the tested *N. gonorrhoeae* strains, including ravuconazole, broxyquinoline, clioquinol, and auranofin, with MICs ranging from 0.008 to 0.125 μg/ml. Furthermore, all 14 compounds except auranofin and niclosamide had minimal to no effect on the growth of commensal *Lactobacillus spp*. that comprise the healthy female genital microbiota. Overall, this study provides the foundation for future gonorrhea therapeutic research.

## 2.2 Introduction

The rise of antibiotic resistance is one of the biggest threats to global health. Our capacity to treat bacterial infections is compromised in the absence of the discovery of novel antimicrobials. *N. gonorrhoeae*, the causative agent of gonorrhea, is the second most common sexually transmitted infection (STI) worldwide. According to the Centers for Disease Control and Prevention (CDC), drug-resistant *N. gonorrhoeae* results in 550,000 infections (~48%) each year from a total of 1.14 million infections annually in the U.S.[1]. The World Health Organization (WHO) estimated that 82.4 million people were newly infected with gonorrhea globally in 2020[2]. *N. gonorrhoeae* is a fastidious Gram-negative bacterium that often causes urogenital diseases in both sexes. Untreated or improperly treated gonorrhea can lead to serious health problems including pelvic inflammatory disease, ectopic pregnancy, infertility, and long-term

pelvic pain. Antibiotics to treat gonorrhea have been around for decades, but *N. gonorrhoeae* has developed resistance to nearly every drug ever used to treat it. The CDC currently advises a single 500 mg dose of intramuscular ceftriaxone. However, the first high-level ceftriaxone-resistant gonococcal strain was documented in Japan in 2009[3]. Since then, more and more gonococcal strains with resistance to ceftriaxone have been reported from many countries[4-6]. Given growing antibiotic resistance, the lack of an available vaccine, and a dry pipeline of anti-gonorrhea medications, the development of new drugs for efficacious gonorrhea treatment is a priority and necessary.

Strategies that are currently being pursued include traditional drug discovery, developing target-based agents, and repurposing existing antibiotics[7,8]. Traditional drug discovery and development is a time-consuming and costly process. The average cost of research and development to bring a new drug to market may cost more than $985 million and take between 10 and 17 years[9-11]. However, owing to the high financial investment and narrow therapeutic window, antibiotic research and development have decreased significantly[12,13]. Therefore, more productive strategies are required to discover antibacterial agents.

Drug repurposing, also termed drug repositioning, reprofiling, or retasking, is a strategy for identifying new uses for existing approved or investigational medications. It is an attractive approach to reduce the expense and expedite the timeline of drug development as the toxicology, formulation, and pharmacological features of an existing drug are better understood than newly discovered compounds[10,11,14]. This approach has been implemented for many conditions, such as cancer, viral, parasitic, and bacterial infections[13,15,16]. To this end, we screened a library of 3802 compounds containing clinical and Food and Drug Administration (FDA)-approved drugs to identify new compounds with antigonococcal activity. The antibacterial susceptibility testing against vaginal microbiota was also performed to investigate the selectivity of hits toward *N. gonorrhoeae*.

## 2.3 Materials and Methods

### 2.3.1 Bacterial strain and reagents

*N. gonorrhoeae* strains were obtained from the CDC, American Type Culture Collection (ATCC) (Manassas, VA, USA), and Microbiologics (Saint Cloud, MN) (Table 2.5S). *Lactobacillus* strains were obtained from the Biodefense and Emerging Infections Research

Resources Repository (BEI Resources) (Manassas, VA) (Table 2.6S). The Drug Repurposing Compound Library (HY-L035P) was purchased from MedChemExpress (Monmouth Junction, NJ). All chemicals and media used in this study were obtained from the vendors below: phosphate-buffered saline (PBS) (Corning, Manassas, VA), BD BBL chocolate II agar (GC II agar with hemoglobin and IsoVitaleX), BD BBL brucella broth and BD Difco lactobacilli deMan, Rogosa and Sharpe (MRS) (Becton Dickinson and Company, Sparks, MD), dextrose, yeast extract and dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA), 96 well plates (CELLTREAT Scientific Products, Pepperell, MA), broxyquinoline (Alfa Aesar, Ward Hill, MA), clioquinol, itraconazole, ketoconazole, azithromycin and ceftriaxone (TCI America, Portland, OR), auranofin (Chem-Impex Int'l Inc., Wood Dale, IL), nifurtimox, niclosamide, isavuconazole and ravuconazole (Cayman Chemical Company, Ann Arbor, MI), hematin porcine (MilliporeSigma, St. Louis, MO), tween 80 (RPI, Mt Prospect, IL), nicotinamide adenine dinucleotide (NAD) and pyridoxal hydrochloride (Chem-Impex Int'l Inc., Wood Dale, IL), isavuconazonium sulfate (Ambeed, Arlington Hts, IL), baloxavir (AstaTech, Bristol, PA), menadione and terconazole (Sigma, St. Louis, MO), proteose peptone #3 (Hardy Diagnostics, Santa Maria, CA), fosravuconazole L-lysineethanolate (MedKoo Bioscienes, Inc., Morrisville, NC), and agar (VWR International, Solon, OH).

For Brucella supplemented broth (BSB), 947.5 ml distilled water was mixed with 35 g of Brucella broth base, 5 g glucose, 5 g yeast extract, 2 g proteose peptone, 30 ml of 0.05% hematin, and 5 ml of 10% Tween 80 before being autoclaved at 121 ◦C for 15 min. After the broth cooled down, 6 ml of filter-sterilized 0.1% pyridoxal solution, 1.5 ml of filter-sterilized 1% NAD solution, and 10 ml of Kellogg's supplements were combined with the broth to make a 1 L BSB medium. Components of Kellogg's supplements were purchased from the vendors below: dextrose (Thermo Fisher Scientific, Waltham, MA), L-glutamine and thiamine pyrophosphate chloride (TCI America, Portland, OR), and iron (III) nitrate (Ward's Science, Rochester, NY). Kellogg's supplements were prepared as previously described[17].

### 2.3.2 Compounds and library

The Drug Repurposing Compound Library, containing 3802 compounds, was purchased from MedChemExpress (HY-L035). The compounds were supplied in 30 µl of DMSO, water, or EtOH at a concentration of 10 mM, 2 mM, or 3 mg/ml in 96-well plates. The library compounds

were diluted in their corresponding solvents to obtain a concentration of 1 mM or 1 mg/ml prior to the screening. Plates were stored at -80°C until used. Broxyquinoline, clioquinol, auranofin, nifurtimox, isavuconazonium sulfate, baloxavir, menadione, terconazole, niclosamide, ketoconazole, fosravuconazole L-lysineethanolate, itraconazole, isavuconazole, and ravuconazole were purchased independently from commercial suppliers to validate the screening findings of the library.

### 2.3.3 Screening assay

The Drug Repurposing Compound Library was screened at concentrations of 16 µM or 16 µg/ml against *N. gonorrhoeae* FA1090 to assess the efficacy of FDA-approved and clinical drugs. *N. gonorrhoeae* FA1090 was cultivated overnight on GC agar plates at 37°C with 5% $CO_2$. A bacterial culture was prepared and adjusted in PBS to the turbidity of McFarland 1.0 and 120 µl of bacterial culture was transferred to 12 ml of BSB broth with 120 µl of Kellogg's supplement to achieve approximately $5\times10^5$ CFU/ml. Each well of 96-well plates containing 1.6 µl library compounds received 100 µl of the bacterial suspension. The plates were incubated at 37°C with 5% $CO_2$ for 24 h. Compounds that inhibited bacterial growth visually were considered hits. Hits were purchased from commercial suppliers to confirm their antigonococcal activity against *N. gonorrhoeae* FA1090. Non-active commercial drugs were excluded from further study.

### 2.3.4 Antibacterial susceptibility of the library hits against a panel of *Neisseria gonorrhoeae* strains

Screening hits were subjected to examine the minimum inhibitory concentrations (MIC) against *N. gonorrhoeae* strains using the broth microdilution method, as previously described. *N. gonorrhoeae* strains were cultivated on GC chocolate agar plates and incubated with 5% $CO_2$ at 37°C for 24 h. The tested agents and control antibiotics were added to the first row of the 96-well plates to achieve a final concentration of 64 µg/ml. *N. gonorrhoeae* was suspended in PBS and adjusted to a turbidity of 1.0 McFarland standard. The bacterial suspension was diluted 1:100 in BSB with 1% Kellogg's supplement, then serially diluted in the 96-well plates. Plates were incubated at 37°C with 5% $CO_2$ for 24 h prior to determining the MIC values. The MIC was defined as the lowest concentration of the drugs that inhibited bacterial growth after a 24 h incubation period.

### 2.3.5 Antibacterial susceptibility testing of library hits against genitourinary tract normal microbiota

The MICs of 14 library hits and control antibiotics were determined against 15 *Lactobacillus* spp. using the broth microdilution method according to Clinical & Laboratory Standards Institute (CLSI) guidelines [18]. *Lactobacillus* spp. were cultured on MRS agar plates at 37°C with 5% $CO_2$ two days prior to the experiment. A *Lactobacillus* suspension, equivalent to the 0.5 McFarland standard, was prepared in PBS to achieve approximately $1 \times 10^6$ CFU/ml. Tested agents were placed in the 96-well plates in duplicate and serially diluted along the plates. Plates were incubated at 37°C with 5% $CO_2$ for 48 h before determining the MICs.

## 2.4 Results

### 2.4.1 Screening of FDA-approved drug library

The MCE Drug Repurposing Compound Library, containing 3802 FDA-approved and clinical drugs, was screened against *N. gonorrhoeae* FA1090 for antigonococcal agents at 16 µM. A few plates that were supplied with 3 mg/ml were screened at 16 µg/ml. Drugs that visibly inhibited bacterial growth after incubation were considered hits. Our primary focus in this study was on non-antimicrobial and non-antineoplastic drugs with antimicrobial effects. The initial screening revealed 272 hits from the library. Following the exclusion of 204 antibacterial, antiseptic, and antineoplastic agents, the remaining hits were further purchased from commercial vendors to verify the screening results (Table 2.1). Several drugs showed antigonococcal activity when screened straight from the library, but these drugs exhibited no antibacterial activity when ordered commercially. Due to identification concerns, these drugs were eliminated from this study. Finally, the antigonococcal activity of 14 compounds was identified and confirmed (Table 2.2). The MIC values of hits ranged between 0.008 and 8 µg/ml against *N. gonorrhoeae* FA1090.

Out of the 14 hits, half are antifungal agents (ravuconazole, isavuconazole, itraconazole, isavuconazonium, terconazole, ketoconazole, and fosravuconazole), four are antiparasitic agents (broxyquinoline, clioquinol, nifurtimox, and niclosamide), one is an anti-inflammatory (auranofin), one is an antiviral (baloxavir), and one is an antifibrinolytic agent (menadione). Interestingly, all the active antifungal compounds are azole antifungal drugs. Six out of seven active azole antifungals are triazoles (ravuconazole, isavuconazole, itraconazole,

isavuconazonium, terconazole, and fosravuconazole), while ketoconazole is an imidazole. Of the 14 hits, the most potent compound was ravuconazole with a MIC value of 0.008 μg/ml.

**Table 2.1 Two hundred and four initial screening hits (antibacterial, antiseptic, and antineoplastic) from the MCE Drug Repurposing Compound Library**

| No. | Product Name | Indication |
|---|---|---|
| 1 | Solithromycin | Antibacterial |
| 2 | Bemcentinib | Antineoplastic |
| 3 | Zoliflodacin | Antibacterial |
| 4 | Radezolid | Antibacterial |
| 5 | Ceftizoxime | Antibacterial |
| 6 | Incyclinide | Antineoplastic |
| 7 | Elesclomol | Antineoplastic |
| 8 | Evofosfamide | Antineoplastic |
| 9 | AFN-1252 | Antibacterial |
| 10 | Tebipenem | Antibacterial |
| 11 | Irinotecan (hydrochloride) | Antineoplastic |
| 12 | Gepotidacin | Antibacterial |
| 13 | Onatasertib | Antineoplastic |
| 14 | Lefamulin (acetate) | Antibacterial |
| 15 | Retapamulin | Antibacterial |
| 16 | Clarithromycin | Antibacterial |
| 17 | Idarubicin (hydrochloride) | Antibacterial |
| 18 | Garenoxacin (Mesylate hydrate) | Antibacterial |
| 19 | Azithromycin | Antibacterial |
| 20 | Bleomycin (hydrochloride) | Antibacterial |
| 21 | Demeclocycline (hydrochloride) | Antibacterial |
| 22 | Bleomycin (sulfate) | Antibacterial |
| 23 | Cefditoren (sodium) | Antibacterial |
| 24 | Bardoxolone | Antineoplastic |
| 25 | Rifaximin | Antibacterial |
| 26 | Cefditoren (Pivoxil) | Antibacterial |
| 27 | Cefoselis (sulfate) | Antibacterial |
| 28 | Cefotaxime | Antibacterial |
| 29 | Zabofloxacin (hydrochloride) | Antibacterial |
| 30 | Cefuroxime axetil | Antibacterial |
| 31 | Cefazolin | Antibacterial |
| 32 | Ceftaroline fosamil | Antibacterial |

| No. | Product Name | Indication |
|---|---|---|
| 33 | Methicillin (sodium salt) | Antibacterial |
| 34 | 18β-Glycyrrhetinic acid | Antineoplastic |
| 35 | MGB-BP-3 | Antibacterial |
| 36 | Faropenem daloxate | Antibacterial |
| 37 | Cephalothin (sodium) | Antibacterial |
| 38 | Piperacillin (sodium) | Antibacterial |
| 39 | Nafcillin (sodium monohydrate) | Antibacterial |
| 40 | Mezlocillin (sodium) | Antibacterial |
| 41 | Lenampicillin (hydrochloride) | Antibacterial |
| 42 | Doxorubicin (hydrochloride) | Antineoplastic |
| 43 | Bacampicillin (hydrochloride) | Antibacterial |
| 44 | Moxalactam (sodium salt) | Antibacterial |
| 45 | Norfloxacin | Antibacterial |
| 46 | Nitrofurazone | Antibacterial |
| 47 | Sulfisoxazole | Antibacterial |
| 48 | Oxacillin (sodium monohydrate) | Antibacterial |
| 49 | Cefotaxime (sodium) | Antibacterial |
| 50 | Erythromycin estolate | Antibacterial |
| 51 | Davercin | Antibacterial |
| 52 | Spiramycin | Antibacterial |
| 53 | Valnivudine | Antibacterial |
| 54 | Cefodizime (sodium) | Antibacterial |
| 55 | Carindacillin (sodium) | Antibacterial |
| 56 | Pemetrexed | Antineoplastic |
| 57 | Valemetostat (tosylate) | Antineoplastic |
| 58 | Cefodizime | Antibacterial |
| 59 | Gamithromycin | Antibacterial |
| 60 | Cefathiamidine | Antibacterial |
| 61 | Delamanid | Antibacterial |
| 62 | Sulopenem | Antibacterial |
| 63 | Gatifloxacin (hydrochloride) | Antibacterial |
| 64 | Linezolid | Antibacterial |
| 65 | Cefazedone | Antibacterial |
| 66 | Epetraborole (hydrochloride) | Antibacterial |
| 67 | Epirubicin (hydrochloride) | Antibacterial |
| 68 | Finafloxacin | Antibacterial |
| 69 | β-Lapachone | Antineoplastic |
| 70 | Meisoindigo | Antineoplastic |

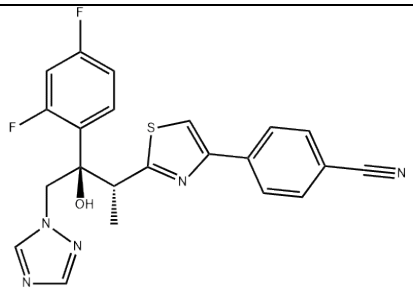
| No. | Product Name | Indication |
|---|---|---|
| 71 | Meropenem (trihydrate) | Antibacterial |
| 72 | SQ109 | Antibacterial |
| 73 | Tedizolid | Antibacterial |
| 74 | Ozenoxacin | Antibacterial |
| 75 | Omadacycline (hydrochloride) | Antibacterial |
| 76 | Delafloxacin (meglumine) | Antibacterial |
| 77 | Brilacidin (tetrahydrochloride) | Antibacterial |
| 78 | Dalbavancin (hydrochloride) | Antibacterial |
| 79 | Furagin | Antibacterial |
| 80 | Faropenem sodium | Antibacterial |
| 81 | Clavulanate (lithium) | Antibacterial |
| 82 | Cephapirin (sodium) | Antibacterial |
| 83 | Nifuratel | Antibacterial |
| 84 | Prulifloxacin | Antibacterial |
| 85 | Plicamycin | Antineoplastic |
| 86 | Telithromycin | Antibacterial |
| 87 | Nitrofurantoin | Antibacterial |
| 88 | Flucloxacillin sodium | Antibacterial |
| 89 | Ofloxacin | Antibacterial |
| 90 | Aztreonam | Antibacterial |
| 91 | Cefdinir | Antibacterial |
| 92 | Pefloxacin (mesylate) | Antibacterial |
| 93 | Doripenem (monohydrate) | Antibacterial |
| 94 | Bestatin (hydrochloride) | Antineoplastic |
| 95 | Tigecycline (tetramesylate) | Antibacterial |
| 96 | Cefaclor | Antibacterial |
| 97 | Chloroxine | Antibacterial |
| 98 | Cefoperazone | Antibacterial |
| 99 | Rifapentine | Antibacterial |
| 100 | Rifampicin | Antibacterial |
| 101 | Erythromycin | Antibacterial |
| 102 | Oxytetracycline | Antibacterial |
| 103 | Sulfadiazine (sodium) | Antibacterial |
| 104 | Oxytetracycline (hydrochloride) | Antibacterial |
| 105 | Cefoperazone (sodium salt) | Antibacterial |
| 106 | Sitafloxacin (hydrate) | Antibacterial |
| 107 | Prothionamide | Antibacterial |
| 108 | Ciprofloxacin (hydrochloride monohydrate) | Antibacterial |

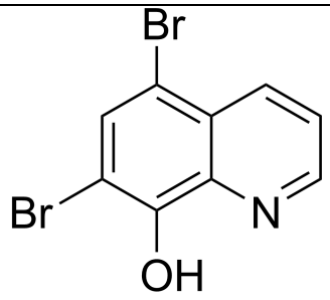
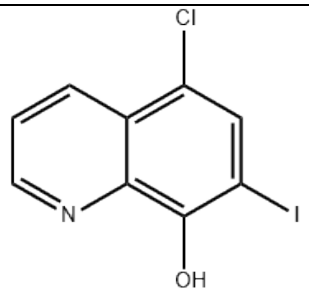
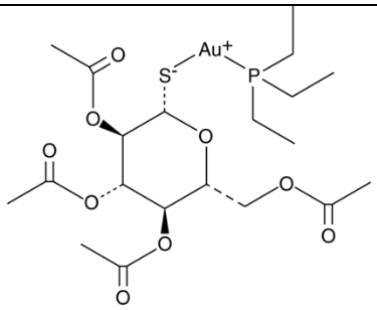
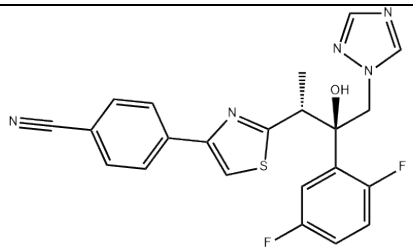
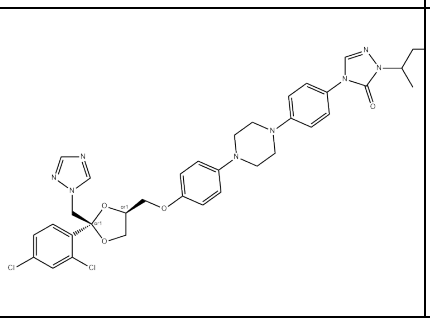
| No. | Product Name | Indication |
|---|---|---|
| 109 | Levofloxacin | Antibacterial |
| 110 | Sulbactam (sodium) | Antibacterial |
| 111 | Nalidixic acid | Antibacterial |
| 112 | Sulbactam | Antibacterial |
| 113 | Hydroxyurea | Antineoplastic |
| 114 | Levofloxacin (hydrate) | Antibacterial |
| 115 | Tebipenem pivoxil | Antibacterial |
| 116 | Sarafloxacin (hydrochloride) | Antibacterial |
| 117 | Cefepime (Dihydrochloride Monohydrate) | Antibacterial |
| 118 | Ceftazidime (pentahydrate) | Antibacterial |
| 119 | Ampicillin (sodium) | Antibacterial |
| 120 | Azlocillin (sodium salt) | Antibacterial |
| 121 | Zinc Pyrithione | Antibacterial |
| 122 | Dirithromycin | Antibacterial |
| 123 | Ceftazidime | Antibacterial |
| 124 | Cefmenoxime (hydrochloride) | Antibacterial |
| 125 | Pazufloxacin (mesylate) | Antibacterial |
| 126 | Ceftibuten (dihydrate) | Antibacterial |
| 127 | Cefozopran (hydrochloride) | Antibacterial |
| 128 | Cefoxitin (sodium) | Antibacterial |
| 129 | Triclosan | Antibacterial |
| 130 | Benzethonium chloride | Antineoplastic |
| 131 | Cefazolin (sodium) | Antibacterial |
| 132 | Gemifloxacin (mesylate) | Antibacterial |
| 133 | Mupirocin | Antibacterial |
| 134 | Erythromycin Ethylsuccinate | Antibacterial |
| 135 | Clofazimine | Antibacterial |
| 136 | Cefuroxime (sodium) | Antibacterial |
| 137 | Cefonicid (sodium) | Antibacterial |
| 138 | Cefamandole (sodium) | Antibacterial |
| 139 | Ceforanide | Antibacterial |
| 140 | Ticarcillin (disodium) | Antibacterial |
| 141 | Cetylpyridinium (chloride monohydrate) | Antibacterial |
| 142 | Pipemidic acid | Antibacterial |
| 143 | Nitroxoline | Antibacterial |
| 144 | Thonzonium (bromide) | Antibacterial |
| 145 | Cefmetazole (sodium) | Antibacterial |
| 146 | Chlorhexidine | Antibacterial |

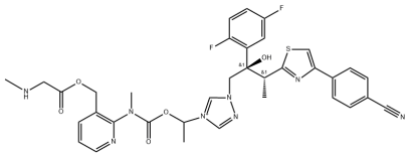
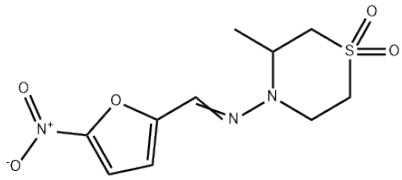
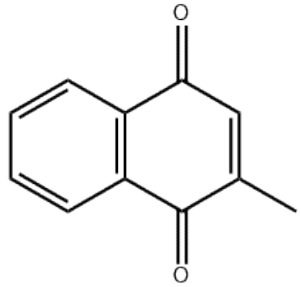
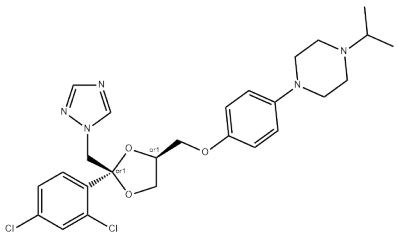
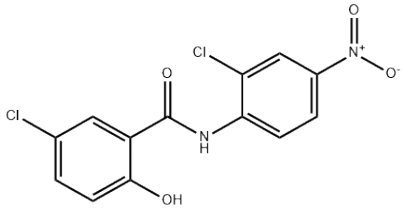
| No. | Product Name | Indication |
|---|---|---|
| 147 | Chlorhexidine (dihydrochloride) | Antibacterial |
| 148 | Fusidic acid | Antibacterial |
| 149 | Fusidic acid (sodium salt) | Antibacterial |
| 150 | Chlorquinaldol | Antibacterial |
| 151 | Florfenicol | Antibacterial |
| 152 | Cetylpyridinium (chloride) | Antibacterial |
| 153 | Nifuroxazide | Antineoplastic |
| 154 | Meclocycline (Sulfosalicylate Salt) | Antibacterial |
| 155 | Domiphen (bromide) | Antiseptic |
| 156 | Tazobactam | Antibacterial |
| 157 | Furazolidone | Antibacterial |
| 158 | Cefixime | Antibacterial |
| 159 | Puromycin (dihydrochloride) | Antibacterial |
| 160 | Cefetamet pivoxil (hydrochloride) | Antibacterial |
| 161 | Cefoxitin | Antibacterial |
| 162 | Midecamycin | Antibacterial |
| 163 | Tosufloxacin (tosylate hydrate) | Antibacterial |
| 164 | Josamycin | Antibacterial |
| 165 | Doxycycline | Antibacterial |
| 166 | Doxycycline (hyclate) | Antibacterial |
| 167 | Calcimycin | Antibacterial |
| 168 | Penicillin G Procaine | Antibacterial |
| 169 | Penicillin G benzathine (tetrahydrate) | Antibacterial |
| 170 | Ceftezole (sodium) | Antibacterial |
| 171 | Cefotetan | Antibacterial |
| 172 | Sultamicillin (tosylate) | Antibacterial |
| 173 | Cefpodoxime Proxetil | Antibacterial |
| 174 | Cadazolid | Antibacterial |
| 175 | Rifabutin | Antibacterial |
| 176 | Minocycline (hydrochloride) | Antibacterial |
| 177 | Sulbenicillin (disodium) | Antibacterial |
| 178 | Cefminox (sodium) | Antibacterial |
| 179 | Biapenem | Antibacterial |
| 180 | Dicloxacillin (Sodium hydrate) | Antibacterial |
| 181 | Colistin (sulfate) | Antibacterial |
| 182 | Lomefloxacin (hydrochloride) | Antibacterial |
| 183 | Ampicillin | Antibacterial |
| 184 | Chlortetracycline (hydrochloride) | Antibacterial |

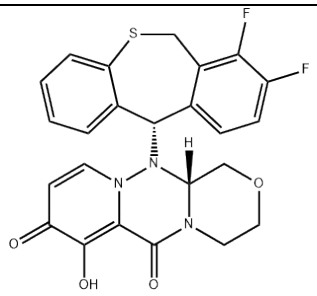
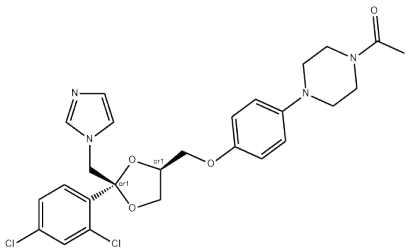
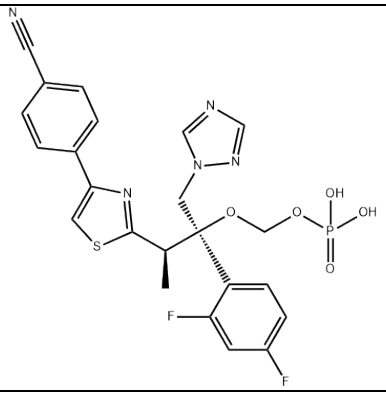
| No. | Product Name | Indication |
|---|---|---|
| 185 | Octenidine (dihydrochloride) | Antibacterial |
| 186 | Polymyxin B (Sulfate) | Antibacterial |
| 187 | Rifalazil | Antibacterial |
| 188 | Ciprofloxacin | Antibacterial |
| 189 | Rufloxacin hydrochloride | Antibacterial |
| 190 | Sparfloxacin | Antibacterial |
| 191 | Gatifloxacin | Antibacterial |
| 192 | Enoxacin (hydrate) | Antibacterial |
| 193 | Amoxicillin (sodium) | Antibacterial |
| 194 | Cloxacillin (sodium monohydrate) | Antibacterial |
| 195 | Tetracycline (hydrochloride) | Antibacterial |
| 196 | Methacycline (hydrochloride) | Antibacterial |
| 197 | Novobiocin (Sodium) | Antibacterial |
| 198 | Roxithromycin | Antibacterial |
| 199 | Clindamycin (hydrochloride) | Antibacterial |
| 200 | Amoxicillin (trihydrate) | Antibacterial |
| 201 | Thiamphenicol | Antibacterial |
| 202 | Nadifloxacin | Antibacterial |
| 203 | Amoxicillin | Antibacterial |
| 204 | Danofloxacin (mesylate) | Antibacterial |

**Table 2.2 Minimum inhibitory concentration (MIC in µg/ml) of library hits and control antibiotics against *N. gonorrhoeae* FA1090**

| No. | Drug name | Chemical structure | MIC (µg/ml) | Indication |
|---|---|---|---|---|
| 1 | Ravuconazole |  | 0.008 | Antifungal |

| No. | Drug name | Chemical structure | MIC (µg/ml) | Indication |
|---|---|---|---|---|
| 2 | Broxyquinoline |  | 0.06 | Antiparasitic |
| 3 | Clioquinol |  | 0.06 | Antiparasitic |
| 4 | Auranofin |  | 0.06 | Anti-inflammatory |
| 5 | Isavuconazole |  | 0.125 | Antifungal |
| 6 | Itraconazole |  | 0.125 | Antifungal |

| No. | Drug name | Chemical structure | MIC (µg/ml) | Indication |
|---|---|---|---|---|
| 7 | Isavuconazonium (sulfate) |  | 0.25 | Antifungal |
| 8 | Nifurtimox |  | 0.5 | Antiparasitic |
| 9 | Menadione |  | 1 | Antifibrinolytic |
| 10 | Terconazole |  | 1 | Antifungal |
| 11 | Niclosamide |  | 1 | Antiparasitic |

| No. | Drug name | Chemical structure | MIC (μg/ml) | Indication |
|---|---|---|---|---|
| 12 | Baloxavir |  | 4 | Antiviral |
| 13 | Ketoconazole |  | 8 | Antifungal |
| 14 | Fosravuconazole (L-lysineethanolate) |  | 8 | Antifungal |

### 2.4.2 Antibacterial activity of hits against a panel of *Neisseria gonorrhoeae* strains

*N. gonorrhoeae* has a high rate of developing antibiotic resistance. Different strains of *N. gonorrhoeae* may have various antibiotic susceptibilities. To identify which hits are most effective against a variety of *N. gonorrhoeae* strains, MIC assays were conducted to determine the spectrum of inhibitory effects of the 14 hits against a panel of *N. gonorrhoeae* strains. As shown in Table 2.3, the most potent hits were ravuconazole, broxyquinoline, clioquinol, and auranofin, with MIC values ranging from 0.008 to 0.125 μg/ml, which outperformed azithromycin (0.125 to 8 μg/ml) and were comparable to ceftriaxone (0.0005 to 1 μg/ml). The MIC values of itraconazole and isavuconazole ranged from 0.125 to 8 μg/ml, similar to azithromycin. Isavuconazonium and fosravuconazole are the prodrugs of isavuconazole and ravuconazole respectively; therefore, less antibacterial potency compared to active drugs is

expected. Menadione and niclosamide exhibited moderate antigonococcal activity with MIC values ranging from 1 to 4 µg/ml. Although nifurtimox, terconazole, baloxavir, and ketoconazole showed moderate antigonococcal activity (0.5 to 8 µg/ml) against *N. g* FA1090, some variances of MICs were observed across the six tested strains (0.5 to >64 µg/ml). The mechanisms of the variances are not clear and are outside the scope of this study.

**Table 2.3 Minimum inhibitory concentration (MIC in µg/ml) of the 14 hits and control antibiotics against a panel of *N. gonorrhoeae* clinical isolates**

| No. | Drug name | *N. gonorrhoeae* strains | | | | | |
|---|---|---|---|---|---|---|---|
| | | **FA1090** | **WHO-P** | **WHO-X** | **WHO-Y** | **WHO-Z** | **F-28** |
| 1 | Ravuconazole | 0.008 | 0.125 | 0.03 | 0.015 | 0.06 | 0.06 |
| 2 | Broxyquinoline | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.03 |
| 3 | Clioquinol | 0.06 | 0.125 | 0.06 | 0.06 | 0.06 | 0.06 |
| 4 | Auranofin | 0.06 | 0.06 | 0.06 | 0.03 | 0.125 | 0.125 |
| 5 | Isavuconazole | 0.125 | 4 | 0.5 | 0.5 | 1 | 1 |
| 6 | Itraconazole | 0.125 | 8 | 1 | 0.5 | 2 | 2 |
| 7 | Isavuconazonium (sulfate) | 0.25 | 16 | 2 | 1 | 4 | 4 |
| 8 | Nifurtimox | 0.5 | 32 | 32 | 4 | 32 | 0.5 |
| 9 | Menadione | 1 | 2 | 1 | 1 | 2 | 1 |
| 10 | Terconazole | 1 | 32 | 4 | 4 | 8 | 8 |
| 11 | Niclosamide | 1 | 4 | 2 | 2 | 2 | 2 |
| 12 | Baloxavir | 4 | >64 | >64 | >64 | >64 | 4 |
| 13 | Ketoconazole | 8 | >64 | 64 | 16 | 64 | 64 |
| 14 | Fosravuconazole (L-lysineethanolate) | 8 | >64 | 32 | 32 | 32 | 16 |
| | Azithromycin | 0.125 | 8 | 1 | 1 | 1 | 0.125 |

| No. | Drug name | *N. gonorrhoeae* strains | | | | | |
|---|---|---|---|---|---|---|---|
| | | **FA1090** | **WHO-P** | **WHO-X** | **WHO-Y** | **WHO-Z** | **F-28** |
| | Ceftriaxone | 0.004 | 0.002 | 1 | 0.5 | 0.5 | 0.0005 |

### 2.4.3 Antibacterial susceptibility testing of library hits against genitourinary tract normal microbiota

The colonization of the reproductive system is crucial to *N. gonorrhoeae* pathogenesis. A healthy microbiome is one of the natural obstacles to such colonization. The vaginal microflora competes with *N. gonorrhoeae* for attachment to the urinary tract by providing an acidic environment. Thus, an ideal *N. gonorrhoeae* treatment should not inhibit the growth of healthy vaginal microbiota[19,20]. Here, we evaluated the activities of the 14 hits against 15 *Lactobacillus* species that are present in the microbiota of the urogenital tract.

Unlike antibiotics azithromycin and ceftriaxone inhibited *Lactobacillus* spp. at low concentrations ranging from ≤0.5 to 4 µg/ml, 10 out of the 14 hits (ravuconazole, broxyquinoline, clioquinol, isavuconazole, itraconazole, isavuconazonium, nifurtimox, terconazole, ketoconazole, and fosravuconazole) were inactive at concentrations of 128 µg/ml or up to 256 µg/ml against all tested *Lactobacillus* species. Menadione and baloxavir inhibited *Lactobacillus* spp. growth at a slightly lower concentration between 8 to 64 µg/ml. Niclosamide and auranofin inhibited normal genitourinary tract microbiota at low concentrations between ≤0.5 to 4 µg/ml (Table 2.4).

**Table 2.4 Minimum inhibitory concentration (MIC in µg/ml) of the 14 hits and control antibiotics against normal genitourinary tract microbiota**

| **Drug name** | | **Genitourinary tract normal microbiota strains** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***L. crispatus* HM-103** | ***L. crispatus* HM-371** | ***L. crispatus* HM-638** | ***L. gasseri* HM-407** | ***L. gasseri* HM-642** | ***L. gasseri* HM-644** | ***L. gasseri* HM-647** | ***L. jensenii* HM-105** | ***L. jensenii* HM-372** | ***L. jensenii* HM-374** | ***L. jensenii* HM-639** | ***L. jensenii* HM-640** | ***L. jensenii* HM-645** | ***L. jensenii* HM-646** | ***L. johnsonii* HM-643** |
| Broxyquinoline | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Clioquinol | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |

| **Drug name** | | **Genitourinary tract normal microbiota strains** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***L. crispatus* HM-103** | ***L. crispatus* HM-371** | ***L. crispatus* HM-638** | ***L. gasseri* HM-407** | ***L. gasseri* HM-642** | ***L. gasseri* HM-644** | ***L. gasseri* HM-647** | ***L. jensenii* HM-105** | ***L. jensenii* HM-372** | ***L. jensenii* HM-374** | ***L. jensenii* HM-639** | ***L. jensenii* HM-640** | ***L. jensenii* HM-645** | ***L. jensenii* HM-646** | ***L. johnsonii* HM-643** |
| Ravuconazole | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Auranofin | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 |
| Isavuconazole | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Nifurtimox | >256 | 128 | 128 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Itraconazole | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Isavuconazonium (sulfate) | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Niclosamide | 2 | 2 | 2 | 4 | 4 | 4 | 4 | 2 | 2 | 1 | 1 | 2 | 2 | 2 | 2 |
| Menadione | 16 | 8 | 16 | 8 | 32 | 64 | 64 | 16 | 32 | 32 | 32 | 16 | 64 | 64 | 32 |
| Terconazole | >256 | 128 | >256 | 128 | >256 | >256 | 256 | >256 | 256 | 256 | >256 | >256 | >256 | >256 | >256 |
| Baloxavir | 16 | 16 | 16 | 16 | 64 | 64 | 32 | 32 | 32 | 32 | 32 | 32 | 64 | 32 | 32 |
| Ketoconazole | >256 | >256 | >256 | 256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Fosravuconazole (L-lysineethanolate) | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Azithromycin | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | 1 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | 1 | ≤0.5 | 1 | 1 | ≤0.5 |
| Ceftriaxone | 2 | ≤0.5 | 4 | ≤0.5 | 1 | 1 | ≤0.5 | ≤0.5 | 1 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 | ≤0.5 |

## 2.5 Discussion

*Neisseria gonorrhoeae* is a global public health concern and remains the main cause of sexually transmitted diseases. It has developed resistance to all antimicrobials formerly or currently used for empirical first-line therapy worldwide. New drugs are urgently needed for the treatment of *N. gonorrhoeae* infections. Drug repurposing strategies have been employed for many infectious diseases. This study uncovered active drugs for the treatment of gonorrhea by screening a library consisting of FDA-approved and clinical drugs.

By implementing a drug repurposing approach, we initially found 272 hits out of 3802 compounds. The majority of hits (91%) were antimicrobial drugs; hence, their antigonococcal activity is not surprising. Many of these antimicrobials (e.g., penicillin, tetracycline, and ciprofloxacin) were the recommended drugs of choice for gonorrhea in the past. Notably, some antimicrobials discovered in the initial screening, such as SQ109 and MGB-BP-3, have not been evaluated for their potential use against gonorrhea and thus may be worth additional investigation.

After excluding antibacterial, antiseptic, and antineoplastic agents and performing confirmation tests, 14 non-antimicrobial hits were identified as possessing antigonococcal activity. Some of these hits were identified in a previous screening study including clioquinol, niclosamide, nifurtimox, and auranofin[7]. Nevertheless, we found several promising anti-gonorrhea candidates, that had not been reported before such as azole antifungal drugs.

Interestingly, all seven antifungal hits contain an azole scaffold. Among seven azole antifungals, ravuconazole was the most potent compound with the MIC value of 0.008 µg/ml followed by isavuconazole (0.125 µg/ml), itraconazole (0.125 µg/ml), isavuconazonium (0.25 µg/ml), terconazole (1 µg/ml), ketoconazole (8 µg/ml), and fosravuconazole (8 µg/ml) against *N. gonorrhoeae* FA1090. Many studies have suggested that azoles exhibit anticancer, antiviral, and antibacterial activity, but the antigonococcal activity of azoles has never been reported[21-23]. The antigonococcal properties of azole antifungals deserve further attention.

Four antiparasitic drugs were identified during the screening. Notably, broxyquinoline and clioquinol share an 8-hydroxyquinoline scaffold, demonstrating potent antibacterial activity in all the tested *N. gonorrhoeae* clinical isolate strains. However, both drugs are poorly absorbed in the gastrointestinal tract. Besides, clioquinol is associated with neurotoxicity. Nifurtimox, the treatment of Chagas disease caused by *Trypanosoma cruzi*, has been identified as a potential

antigonococcal agent in a previous screening study[7]. Further investigation is required to evaluate the potential use of nifurtimox in gonorrhea treatment. Niclosamide is used to treat beef, pork, fish, and dwarf tapeworm infections. Antibacterial activity of niclosamide has been reported in Gram-positive bacteria such as vancomycin-resistant *Enterococcus* (VRE), but not in *N. gonorrhoeae*[24,25].

The last group of hits consisted of compounds from scattered therapeutic classes. Auranofin, an FDA-approved drug for the treatment of rheumatoid arthritis, has shown potential therapeutic use in many other diseases including cancer, parasitic, fungal, and bacterial infections[26-30]. The antibacterial activity of auranofin against *Mycobacterium tuberculosis*, *Clostridioides difficile*, VRE, methicillin-resistant *Staphylococcus aureus*, and *N. gonorrhoeae* has been reported[8,31-38]. Menadione, namely vitamin K3, is used for the treatment of hypoprothrombinemia and as a nutritional supplement. Although menadione displayed potent antibacterial activity against clinical multidrug-resistant *N. gonorrhoeae* isolates, the suggested drug dosage for hypoprothrombinemia is very low (10 mg), and high doses have been reported to cause brain damage[39]. Baloxavir, the treatment for influenza virus, has never been tested against *N. gonorrhoeae*; however, its antibacterial activity against multidrug-resistant *N. gonorrhoeae* is not consistent.

A healthy microbiome in the female reproductive system may decrease the risk of sexually transmitted illnesses by inhibiting pathogen colonization [20]. The normal vaginal flora contains *Lactobacillus crispatus* and *Lactobacillus jensenii*. Previous studies have shown that *Lactobacillus* inhibits the growth of *Gardnerella vaginalis* and *N. gonorrhoeae* [19,40]. However, antibiotics with a broad spectrum are known to disturb the healthy microbiota. New treatments that maintain a beneficial microbiome while treating the target infection selectively would be desired. Here, we test the MICs of the 14 hits against a panel of *Lactobacilli* spp. to assess whether these hits disrupt vaginal flora. Unlike ceftriaxone and azithromycin, all of the hits except auranofin and niclosamide did not inhibit the growth of *Lactobacillus* strains tested at concentrations below 8 µg/ml or even up to 256 µg/ml.

In conclusion, we successfully identified 14 compounds that possess antibacterial activity against *N. gonorrhoeae* by screening the MCE drug repurposing compound library. We revealed several promising anti-gonorrhea candidates, including ravuconazole, isavuconazole, and itraconazole, that were not previously reported. The antibacterial activity of all 14 compounds

was tested against the genitourinary tract microbiota. All 14 compounds except auranofin and niclosamide had minimal to no effect on the vaginal flora. Overall, the findings from this study provide a foundation for future gonorrhea therapeutic research.

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## 2.7 Supplementary material

**Table 2.5S *Neisseria gonorrhoeae* strains used in this study**

| Strains | Description |
|---|---|
| *N. gonorrhoeae* FA1090 | Resistant to streptomycin. Isolated from a female patient with disseminated gonococcal infection. |
| *N. gonorrhoeae* WHO-P | Resistant to azithromycin and tetracycline. |
| *N. gonorrhoeae* WHO-X | Resistant to cefixime, ceftriaxone, ciprofloxacin, penicillin, and tetracycline. Isolated from a female pharynx specimen in Kyoto, Japan, 2009. |
| *N. gonorrhoeae* WHO-Y | Resistant to cefixime, ceftriaxone, ciprofloxacin, and tetracycline. Isolated from a urethral specimen of a 50 years old male in Quimper, France, 2010. |
| *N. gonorrhoeae* WHO-Z | Resistant to cefixime, ceftriaxone, ciprofloxacin, penicillin, and tetracycline. Isolated from a female genital swab in Australia, 2013. |
| *N. gonorrhoeae* CDC F-28 | Resistant to spectinomycin. |
| All susceptibility/resistance breakpoints are based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2023). EUCST notes that MIC ≥1.0 µg/ml for azithromycin is defined as the epidemiological cutoff value. | |

**Table 2.6S *Lactobacillus* strains used in this study**

| Strains | Description |
|---|---|
| *L. crispatus* HM-103 | Isolated from normal human vaginal flora. |
| *L. crispatus* HM-371 | Isolated from a human mid-vaginal wall in Richmond, Virginia in March 2010. |
| *L. crispatus* HM-638 | Vaginal isolate from a healthy Chinese woman in 2007. |
| *L. gasseri* HM-407 | Isolated from a human mid-vaginal wall in Richmond, Virginia in March 2010. |
| *L. gasseri* HM-642 | Vaginal isolate from a healthy US woman in 2007. |
| *L. gasseri* HM-644 | Isolated from the vaginal mucosa of a healthy U.S. woman of child-bearing age in 2007. |
| *L. gasseri* HM-647 | Isolated from human vaginal mucosa in 2007. |
| *L. jensenii* HM-105 | Human isolate from Texas. |
| *L. jensenii* HM-372 | Isolated from a human mid-vaginal wall in Richmond, Virginia in March 2010. |

| | |
|---|---|
| *L. jensenii* HM-374 | Isolated from a human mid-vaginal wall in Richmond, Virginia in March 2010. |
| *L. jensenii* HM-639 | Isolated from the vaginal mucosa of a healthy woman in the United States in 2007. |
| *L. jensenii* HM-640 | Isolated from the vaginal mucosa of a healthy Chinese woman in 2007. |
| *L. jensenii* HM-645 | Isolated from human vaginal mucosa in 2007. |
| *L. jensenii* HM-646 | Isolated from human vaginal mucosa in 2007. |
| *L. johnsonii* HM-643 | Isolated from the vaginal mucosa of a Chinese woman in 2007. |

# Chapter 3. Repurposing Azole Drugs as Antigonococcal Agents

## 3.1 Abstract

*Neisseria gonorrhoeae*, the second most common bacterial cause of sexually transmitted infections (STIs), is listed as an urgent-threat pathogen by the Centers for Disease Control and Prevention (CDC). Due to the growing prevalence of resistance development against first-line treatment and several classes of antibiotics, the discovery of new anti-gonorrhea therapeutics is an urgent need. In this study, we discovered that three antifungals, itraconazole, isavuconazole, and ravuconazole, exhibited potent antigonococcal activities against *N. gonorrhoeae* clinical isolates. The minimal inhibitory concentrations (MICs) of the three azoles range from 0.002 to 4 μg/ml. All three azole drugs showed a low frequency of spontaneous mutations. The time-kill assay revealed that the three azoles showed bactericidal activity at 5 × and 10 × MIC. Besides, isavuconazole and ravuconazole have a prolonged PAE effect (8-16 h) compared with azithromycin (4-8 h). All three tested azole drugs cleared the burden of intracellular *N. gonorrhoeae* to below the limit of detection, which is superior to ceftriaxone. Furthermore, all three azoles showed indifferent or additive activity in combination with the drugs of choice for gonococcal infections, azithromycin or ceftriaxone, suggesting their potential to be used as dual therapy. Next, we investigated the mechanism of action of the three azoles. No significantly elevated DNA levels were found in the supernatant of the *N. gonorrhoeae* treated group (itraconazole, isavuconazole, and ravuconazole), suggesting that the mechanism of action was not disruption of the cell membrane. Finally, we evaluated the effect of three azoles on reactive oxygen species (ROS) production. None of the azoles significantly increased ROS production at 1 × or 0.5 × MIC compared to the control group. In conclusion, our findings suggest three azole antifungals as potential antigonococcal therapeutic candidates and warrant further investigation.

## 3.2 Introduction

Gonorrhea, caused by the Gram-negative bacterium *Neisseria gonorrhoeae*, is the second most prevalent sexually transmitted infection (STI)[1]. Gonococcal infections account annually for 82 million of the estimated 374 million new cases of STIs that occur worldwide, with an estimated yearly health care cost of $162.1 million[2,3]. More alarmingly, the United States Centers for Disease Control and Prevention (CDC) reported a 124% increase in gonorrhea cases in 2019 compared to 2013. However, due to asymptomatic gonococcal infections, these numbers are likely underestimated. *N. gonorrhoeae* can infect the genitals, rectum, and throat. Invasive infections with *N. gonorrhoeae*, such as disseminated gonococcal infection, endocarditis, and meningitis, may cause severe morbidity.

Gonorrhea, when improperly treated or untreated, can result in serious and life-threatening complications, including tubal scarring, infertility, pelvic inflammatory disease, ectopic pregnancy, maternal death, first-trimester abortion, chronic pelvic pain, and newborn eye infections[4-7]. In addition, untreated gonorrhea could increase the risk of contracting and transmitting HIV fivefold in both sexes[7]. Due to the inherent ability of *N. gonorrhoeae* to develop resistance to antibiotics, gonorrhea treatment has become a burden on human health and the medical system. As a result, the CDC has classified *N. gonorrhoeae* as an urgent multidrug-resistant superbug mandating the development of novel therapeutic agents[8].

*N. gonorrhoeae* has developed resistance to all classes of antibiotics used against it since 1940, including penicillin, tetracyclines, spectinomycin, fluoroquinolones, macrolides, and cephalosporins [9,10]. Currently, ceftriaxone is the last remaining option for empirical first-line treatment, but high-level gonococcal clinical resistance to ceftriaxone has been documented[11,12]. Due to the absence of vaccines for the prevention of *N. gonorrhoeae* infections[13], effective gonorrhea control relies solely on these available antibiotics. At present, only three antibiotic candidates, solithromycin, gepotidacin, and zoliflodacin, are in the drug pipeline for the treatment of gonorrhea[14]. Altogether, the need for the development of new antibacterial agents for gonorrhea is urgently needed.

The development of novel, effective, and safe antimicrobials is a slow, time-consuming, and expensive process. To bypass the expense and time involved with conventional drug discovery and development, drug repurposing of FDA-approved medications provides an appealing strategy for uncovering new antibacterial agents. By employing a drug repurposing approach, we identified three extremely potent and narrow-spectrum novel candidates, itraconazole, isavuconazole, and ravuconazole, as potential antigonococcal agents. Itraconazole and isavuconazole are FDA-approved drugs that belong to the triazole class of antifungals. The antigonococcal properties of the three azole antifungals have never been reported. Thus, the objective of this study was to investigate the *in vitro* activities of the three azoles (itraconazole, isavuconazole, and ravuconazole) against *N. gonorrhoeae*.

## 3.3 Materials and Methods

### 3.3.1 Bacterial strains, cell line, and reagents

*N. gonorrhoeae* strains were obtained from the CDC, American Type Culture Collection (ATCC) (Manassas, VA), and Microbiologics (Saint Cloud, MN) (Table 3.5S). ME-180 cell line (ATCC HTB-33) was obtained from ATCC (Manassas, VA).

Chemicals, reagents, and supplies used in this study were purchased from the listed manufacturers below: cefixime (Acros Organics, Morris Plains, NJ), doxycycline monohydrate (Alfa Aesar, Ward Hill, MA), BD Difco gonococcal (GC) medium base, BD BBL hemoglobin, BD BBL brucella broth, BD BBL chocolate II agar (GC II agar with hemoglobin and IsoVitaleX) and BD BBL IsoVitaleX enrichment (Becton Dickinson and Company, Sparks, MD), AZD 0914 (Zoliflodacin) (Biosynth Carbosynth, Staad, Switzerland), isavuconazole and ravuconazole (Cayman Chemical Company, Ann Arbor, MI), 96 well plates, 96 well tissue culture plates, 100 x 15 mm Petri dishes and 50 ml centrifuge tubes (CELLTREAT Scientific Products, Pepperell, MA), nicotinamide adenine dinucleotide (NAD), pyridoxal hydrochloride and gentamicin sulfate (Chem-Impex Int'l Inc., Wood Dale, IL), phosphate-buffered saline (PBS) (Corning, Manassas, VA), fetal bovine serum (FBS) (Corning, Woodland, CA), ciprofloxacin (Enzo Life Sciences, Farmingdale, NY), proteose peptone #3 (Hardy Diagnostics, Santa Maria, CA), 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA) (Invitrogen, Waltham, MA), McCoy's 5A medium (Quality Biological, Gaithersburg, MD), potassium phosphate monobasic and potassium phosphate dibasic (Macron, Center Valley, PA), gepotidacin (MedChemExpress, Monmouth Junction, NJ), hematin porcine (MilliporeSigma, St. Louis, MO), tween 80 (RPI, Mt Prospect, IL), triton X-100, tetracycline, erythromycin, chloramphenicol, rifampicin and 30% hydrogen peroxide ($H_2O_2$) solution (Sigma-Aldrich, St. Louis, MO), corn starch (Spectrum Chemical Mfg. Crop., New Brunswick, NJ), azithromycin, ceftriaxone, itraconazole, saponin, L-glutamine and thiamine pyrophosphate chloride (TCI America, Portland, OR), dimethyl sulfoxide (DMSO), dextrose, yeast extract, sodium chloride, L-shaped cell spreaders, 96 well black plates and NanoDrop One/One$^c$ spectrophotometer (Thermo Fisher Scientific, Waltham, MA), microcentrifuge tubes (VWR International, Radnor, PA), iron (III) nitrate (Ward's Science, Rochester, NY), and sheep whole blood (Innovative Research, Novi, MI).

For supplemented Brucella broth (BSB), 947.5 ml distilled water was mixed with 35 g of Brucella broth base, 5 g glucose, 5 g yeast extract, 2 g proteose peptone, 30 ml of 0.05% hematin, 5 ml of 10% Tween 80 and autoclaved at 121 ◦C for 20 min. After the broth cooled down, 6 ml of filter-sterilized 0.1% pyridoxal solution, 1.5 ml of filter-sterilized 1% NAD solution, and 10 ml of Kellogg's supplements were combined with the broth to make a 1 L BSB medium. Components of Kellogg's supplements were purchased from the vendors below: dextrose (Thermo Fisher Scientific, Waltham, MA), L-glutamine and thiamine pyrophosphate chloride (TCI America, Portland, OR), and iron (III) nitrate (Ward's Science, Rochester, NY). Kellogg's supplements were prepared as previously described[15]. GC broth was prepared according to ATCC medium 814: GC Agar/Broth Medium.

### 3.3.2 Antibacterial susceptibility testing of azole drugs against *N. gonorrhoeae*

*N. gonorrhoeae* isolates were grown on GC chocolate II agar plates at 37°C with 5% $CO_2$ for 24 h. A bacterial suspension equivalent to a 1.0 McFarland standard was diluted in BSB with 1% Kellogg's supplement to achieve a bacterial concentration of approximately $5 \times 10^5$ CFU/ml. WHO-V, AR-0965, and AR-0969 gonococcal strains were diluted in GC broth due to their poor growth in BSB media. Tested agents itraconazole, ravuconazole, isavuconazole, gepotidacin, zoliflodacin, and control antibiotics (azithromycin and ceftriaxone) were added to the 96-well plates and serially diluted along the plates. Plates were incubated at 37° C with 5% $CO_2$ for 20-24 h. The minimum inhibitory concentration (MIC) is the lowest drug concentration that inhibits bacterial growth visually.

### 3.3.3 Agar dilution

The CLSI-recommended agar dilution method was conducted to validate the MIC values obtained from the broth microdilution method, with slight modifications [16]. Briefly, GC agar plates were prepared with 1% Kellogg's supplement and 0.5 % agar. Serial concentrations of tested agents were incorporated thoroughly into the GC agar base and poured into 100 x 15 mm Petri dishes. *N. gonorrhoeae* strains were prepared in PBS to reach a 0.5 McFarland standard. Drug-containing and drug-free GC agar plates were inoculated with $10^4$ CFU per spot. All plates were incubated at 37° C with 5% $CO_2$ for 24 h before reading the MICs. The MIC is the lowest concentration of drug required to inhibit the growth of $10^4$ CFU in a bacterial spot.

### 3.3.4 Time-kill assay

A time-kill assay was performed to determine whether three azole drugs are bactericidal or bacteriostatic. *N. gonorrhoeae* FA1090 was grown in BSB with 1% Kellogg's supplement until reaching the logarithmic phase and then diluted to achieve a starting inoculum of ~$10^6$ CFU/ml. Itraconazole, ravuconazole, isavuconazole, and control antibiotics (azithromycin and ceftriaxone) were added at 5 × and 10 × MIC in duplicate on 96-well plates. DMSO, the solvent of azole drugs, served as a negative control. At times 0, 2, 4, 6, 8, 12, and 24 h, each sample was serially diluted in PBS and plated onto GC chocolate II agar plates. Viable counts were determined after 24 h incubation at 37° C with 5% $CO_2$. Bactericidal activity was defined as a reduction of at least 3 $\log_{10}$ CFU/ml relative to the initial inoculum after 24 h, whereas bacteriostatic activity corresponds to <3 $\log_{10}$ CFU/ml relative to the initial inoculum after 24 h.

### 3.3.5 Post-antibiotic effect

The post-antibiotic effect (PAE) refers to the length of time after the complete removal of a drug in which an antibiotic effect is still observed. PAE was determined as previously described [17,18]. Briefly, *N. gonorrhoeae* strains were cultivated in BSB with 1% Kellogg's supplement to attain the logarithmic phase and further diluted to reach an initial inoculum of about $10^6$ CFU/ml. Tested agents (10 × MIC, in duplicate) were added to each microcentrifuge tube containing bacteria. All tubes are incubated at 37°C on an orbital shaker at 250 rpm for 2 h. DMSO served as growth control. Thereafter, drugs were removed by diluting each tube 1:1000 in fresh BSB with 1% Kellogg's supplement, and tubes were incubated at 37°C with 5% $CO_2$ for 24 h. Samples were collected from each tube, serially diluted, and plated on GC chocolate II agar plates at the corresponding times. The PAE was calculated using this equation: PAE = T − C, where T is the time taken by the bacterial culture treated with a drug to increase by one $\log_{10}$, and C is the time required for the negative control (DMSO) to increase by one $\log_{10}$.

### 3.3.6 Checkerboard assay

A checkboard assay was used to examine the influence of the combination of two drugs in comparison to their separate activities[19]. Each azole drug was combined with either azithromycin or ceftriaxone. Briefly, *N. gonorrhoeae* strains were grown on GC chocolate II agar plates overnight. A bacterial suspension equivalent to a 1.0 McFarland standard was prepared and diluted in BSB with 1% Kellogg's supplement, to achieve a bacterial concentration of about $1 \times 10^6$ CFU/ml. Afterward, bacteria were combined with a drug mixture in pairs that had been serially diluted in the same 96-well plates, vertically for one compound and horizontally for the other. The combinatorial inhibitory concentration is indicated by the fractional inhibitory concentration index (FICI): FICI = $MIC_A$ combination/$MIC_A$ alone + $MIC_B$ combination/$MIC_B$ alone. Interactions with FICI values of ≤ 0.5 will be considered synergistic, while those with FICI values of 0.5-1.25 are considered additive. FICI values of 1.25-4 are categorized as indifferent, while interactions with FICI >4 are considered antagonistic[20].

### 3.3.7 Single-step mutation assay

The resistance frequency of the three azole drugs was tested against *N. gonorrhoeae* FA1090 as previously described with slight modifications [21]. Rifampicin served as a positive control [22,23]. To prepare drug-containing plates, the GC agar base was autoclaved at 121°C for 20 min. After being slightly cooled, whole sheep blood was incorporated into the medium to reach the final concentration of 5%. The mixture was heated up until it turned brown, and the mixture was removed from a hotplate. IsoVitaleX was added to reach a final concentration of 1%. Tested agents were combined with the medium agar to produce a 10 × MIC molten agar-drug mixture. The mixtures were poured into 100 x 15

mm Petri dishes and left to solidify at room temperature. Approximately $10^{10}$ CFU/ml of overnight *N. gonorrhoeae* FA1090 was prepared in PBS. Total viable counts were determined by the plate count method using drug-free GC plates. The plates were inverted and incubated at 37°C with 5% $CO_2$ for 72 h. At 24, 48, 72 h, plates were manually examined for growth, and colony counts were obtained. The counts at 72 h were used to determine the frequency of spontaneous mutations using the following formula: the number of colonies on a drug-containing plate divided by the total number of inoculums. When there were no colonies on the drug-containing plates, the spontaneous mutation frequency was computed as 1/inoculum count, indicating that it was below the detection limit (1 CFU).

### 3.3.8 Multi-step mutation assay

To assess the tendency of resistance development to each azole drug in gonococcus, *N. gonorrhoeae* FA1090 was exposed to three azole drugs and control antibiotics for 14 consecutive passages, as previously described [24]. On day 1, the broth microdilution assay was used to determine the initial MIC for itraconazole, isavuconazole, ravuconazole, azithromycin, ceftriaxone, ciprofloxacin, and spectinomycin in duplicate. Beginning on day 2, 2 µl of bacterial culture from a sub-MIC (sub-inhibitory concentration of the drug) well with comparable turbidity to untreated control wells was diluted 1:500 in BSB with 1% Kellogg's supplement to determine the MIC of each drug for the subsequent passage. The procedures were carried out for 14 successive days. Plates were incubated at 37°C in the presence of 5% $CO_2$ for 24 h before the MIC was determined visually. The MIC fold change was defined as $(MIC_{day\#} - MIC_{day1})/MIC_{day1}$.

### 3.3.9 Intracellular clearance assay

Intracellular clearance activity of *N. gonorrhoeae* FA1090 was conducted to explore the ability of the azoles to enter cervical cells and clear the gonococcus burden as described previously with slight modifications [25-27]. The human cervix carcinoma cell line ME-180 (ATCC HTB-33) was grown in McCoy's 5A medium supplemented with 10% FBS. ME-180 cells were seeded in a 96-well tissue-culture-treated plate at $5\times10^5$ cervical cells/well and incubated at 37°C with 5% $CO_2$ until confluence. The cells were washed three times with PBS before infection. *N. gonorrhoeae* FA1090 was grown on a GC plate at 37°C with 5% $CO_2$ overnight and prepared in PBS. ME-180 cells were infected at a multiplicity of infection of 100:1 and incubated at 37°C in a 5% $CO_2$ for 12 h. The cells were washed three times with PBS to remove non-adherent bacteria. McCoy's 5A supplemented medium containing 200 µg/ml gentamycin was added to each well for 2 h to remove extracellular bacteria. ME-180 cells were washed with PBS three times and subsequently treated with itraconazole, isavuconazole,

ravuconazole, azithromycin, and ceftriaxone at 3 × MIC at 37°C with 5% $CO_2$ for 24 h. DMSO, the solvent of the azoles, served as a negative control. To quantify the number of intracellular bacteria, cells were lysed with 1.5% saponin. The lysate was serially diluted in PBS, plated on GC agar plates, and incubated at 37°C with 5% $CO_2$ for 24 h. Each treatment group is carried out in quadruplicate with two independent experiments. Data were analyzed via one-way ANOVA using GraphPad Prism 8.0.0.

### 3.3.10 DNA leakage assay

To investigate the potential mechanism of the three azole drugs in *N. gonorrhoeae*, we sought to test if the three azole drugs could permeabilize the bacterial cell membrane. *N. gonorrhoeae* FA1090 was grown in a 50 ml centrifuge tube with GC broth supplemented with 1% IsoVitaleX and incubated in a shaking incubator at 250 rpm at 37°C for 12 h. The bacterial culture was washed twice with PBS at 4000 rpm for 10 min and resuspended in PBS. One ml of suspended bacterial culture was transferred to 2 ml microcentrifuge tubes. Itraconazole, isavuconazole, ravuconazole, azithromycin, and ceftriaxone were added to the tubes at 10 × MIC respectively. Each group performed in triplicate. The remaining suspended bacterial culture was washed again in PBS at 4000 rpm for 10 min. The tube was re-suspended in 0.1% sterile Triton X-100 in the same volume as the remaining suspended bacterial culture. One ml of a 0.1% Triton X-100 mixture was distributed into three microcentrifuge tubes. All the tubes were incubated in a shaking incubator at 37°C for 30 min. Subsequently, all bacterial cultures in tubes were spun down at 18000 r.c.f. for 5 min. Double-strand DNA concentrations in supernatants were measured by Nanodrop. Data were analyzed via ordinary one-way ANOVA using GraphPad Prism 8.0.0.

### 3.3.11 Reactive oxidative species (ROS) detection

The formation of ROS by *N. gonorrhoeae* FA1090 upon treatment with three azole drugs was assessed by carboxy-H2DCFDA dye, as previously described [28,29]. *N. gonorrhoeae* FA1090 was cultivated in BSB broth with 1% Kellogg's supplement and incubated in a shaking incubator at 250 rpm at 37 °C. Logarithmic phase culture was washed in PBS three times, adjusted to a 1.0 McFarland standard, and diluted in PBS to reach $10^6$ CFU/ml. Cells were incubated in a 96-well black plate in the dark with either 1 × or 0.5 × MIC of itraconazole, isavuconazole, ravuconazole, azithromycin, ceftriaxone, $H_2O_2$ (positive control), and DMSO at 37°C with 5% $CO_2$ for 30 min. After treatment, a final concentration of 50 µM DCFH-DA was added, and the samples were incubated at 37°C with 5% $CO_2$ for 30 min. *N. gonorrhoeae* cultures that were not stained served as a negative control. The emission of DCFH-DA fluorescence was measured using a BioTek Synergy H1 Hybrid Microplate

Reader with excitation/emission wavelengths of 495/529 nm. The background fluorescence of PBS and the autofluorescence of the bacterial cells cultured without the dye were evaluated to determine the net fluorescence emitted. The experiment was conducted in triplicate.

### 3.3.12 Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA). Multiple t-tests and one-way ANOVA were used to compute P-values, and P-values <0.05 were defined as significant. The data are shown as means and standard deviations.

## 3.4 Results

### 3.4.1 Antibacterial susceptibility testing against *N. gonorrhoeae* isolates

Antigonococcal activity of the three azoles (itraconazole, isavuconazole, and ravuconazole) was assessed against clinical isolates of *N. gonorrhoeae* (Table 3.6S). The *N. gonorrhoeae* strains have a variety of resistance characteristics, with the majority being multi-drug resistant. As depicted in Table 3.1, itraconazole inhibited *N. gonorrhoeae* growth ranging from 0.03 to 4 µg/ml with $MIC_{50}$ of 1 µg/ml and $MIC_{90}$ of 2 µg/ml. Isavuconazole is slightly more potent than itraconazole. The MIC of isavuconazole ranges from 0.015 to 2 µg/ml with $MIC_{50}$ of 0.5 µg/ml and $MIC_{90}$ of 1 µg/ml. Ravuconazole is the most potent agent among the three azole drugs. The MIC of ravuconazole ranges from 0.002 to 0.25 µg/ml with both $MIC_{50}$ and $MIC_{90}$ of 0.06 µg/ml. Ceftriaxone and azithromycin are treatment options for gonorrhea. The MIC of azithromycin against a wide panel of *N. gonorrhoeae* strains is between 0.03 to >512 µg/ml, while ceftriaxone is between 0.001 to 1 µg/ml. Gepotidacin and zoliflodacin are currently in phase III clinical trials for gonorrhea treatment. The MIC of gepotidacin ranges from 0.03 to 8 µg/ml with $MIC_{50}$ 1 µg/ml and $MIC_{90}$ 4 µg/ml. The MIC of zoliflodacin ranges from 0.004 to 0.5 µg/ml with $MIC_{50}$ 0.06 µg/ml and $MIC_{90}$ 0.125 µg/ml.

**Table 3.1 Antibacterial susceptibility testing of *N. gonorrhoeae* isolates**

| | **Number of strains** | **MIC range (µg/ml)** | **$MIC_{50}$ (µg/ml)** | **$MIC_{90}$ (µg/ml)** |
|---|---|---|---|---|
| Itraconazole | 97 | 0.03-4 | 1 | 2 |
| Isavuconazole | 97 | 0.015-2 | 0.5 | 1 |
| Ravuconazole | 97 | 0.002-0.25 | 0.06 | 0.06 |
| Azithromycin | 97 | 0.03->512 | 1 | 4 |
| Ceftriaxone | 97 | 0.001-1 | 0.03 | 0.06 |
| Gepotidacin | 62 | 0.03-8 | 1 | 4 |
| Zoliflodacin | 62 | 0.004-0.5 | 0.06 | 0.125 |

### 3.4.2 Agar dilution

Currently, CLSI recommends agar dilution methods to perform antibacterial susceptibility testing in *N. gonorrhoeae*. Here, we tested 16 strains of *N. gonorrhoeae* using the agar dilution method according to CLSI guidelines (Table 3.2). The MICs of isavuconazole and ravuconazole in the agar dilution method are relatively similar to their corresponding MICs using broth microdilution with an approximately 4-fold MIC increase. About a 16-fold MIC increase was observed in itraconazole among the tested strains. The observed increased MICs in the agar dilution method may be due to the poor solubility of the three azole drugs. Moreover, it is well-known that protein binding can have a significant impact on MIC values. Proteins such as hemoglobin that are present in the GC agar plates can result in higher MIC values. The MICs of azithromycin and ceftriaxone remained constant when compared with reference MICs with some strains demonstrating a 2-fold increase or decrease[30,31]. Gepotidacin and zoliflodacin also showed more consistent MICs in broth microdilution and agar dilution methods with a 2 to 4-fold decrease in gepotidacin and a 2 to 4-fold increase in zoliflodacin. Remarkably, ravuconazole was still the most potent drug among all the tested agents, as the $MIC_{50}$ and $MIC_{90}$ values were comparable to zoliflodacin and outperformed azithromycin, ceftriaxone, and gepotidacin.

**Table 3.2 Minimum inhibitory concentration (MIC in µg/ml) of azole drugs and control antibiotics against *N. gonorrhoeae* isolates by agar dilution method**

| No. of strains | *N. gonorrhoeae* strains | Itraconazole | Isavuconazole | Ravuconazole | Azithromycin | Ceftriaxone | Gepotidacin | Zoliflodacin |
|---|---|---|---|---|---|---|---|---|
| 1 | FA1090 | 8 | 0.5 | 0.06 | 0.06 | 0.002 | 0.06 | 0.06 |
| 2 | WHO-L | 16 | 4 | 0.25 | 0.5 | 0.5 | 4 | 0.25 |
| 3 | WHO-X | 16 | 2 | 0.25 | 0.25 | 2 | 0.5 | 0.25 |
| 4 | WHO-Y | 16 | 2 | 0.25 | 0.25 | 1 | 0.5 | 0.25 |
| 5 | WHO-Z | 16 | 4 | 0.25 | 0.5 | 0.5 | 0.25 | 0.25 |
| 6 | CDC-166 | 16 | 4 | 0.25 | 0.5 | 0.125 | 0.5 | 0.25 |
| 7 | CDC-169 | 16 | 4 | 0.25 | 0.5 | 0.125 | 0.5 | 0.25 |
| 8 | CDC-171 | 16 | 4 | 0.25 | 0.5 | 0.125 | 0.5 | 0.125 |
| 9 | CDC-173 | 16 | 4 | 0.25 | 0.5 | 0.125 | 0.5 | 0.25 |

| No. of strains | *N. gonorrhoeae* strains | Itraconazole | Isavuconazole | Ravuconazole | Azithromycin | Ceftriaxone | Gepotidacin | Zoliflodacin |
|---|---|---|---|---|---|---|---|---|
| 10 | CDC-174 | 32 | 4 | 0.25 | 0.5 | 0.25 | 1 | 0.25 |
| 11 | ATCC 49981 | 2 | 0.25 | ≤0.03 | 0.03 | 0.008 | ≤0.03 | 0.008 |
| 12 | ATCC 43070 | 4 | 0.5 | ≤0.03 | 0.06 | 0.004 | 0.125 | 0.125 |
| 13 | MS11 | 32 | 4 | 0.25 | 0.25 | 0.06 | 0.5 | 0.25 |
| 14 | SPL-4 | 16 | 4 | 0.25 | 0.25 | 0.125 | 0.25 | 0.125 |
| 15 | SPJ-15 | 32 | 8 | 0.5 | 2 | 0.015 | 0.5 | 0.5 |
| 16 | P681E | 16 | 2 | 0.125 | 0.03 | 0.008 | 0.125 | 0.06 |
| | $MIC_{50}$ | 16 | 4 | 0.25 | 0.25 | 0.125 | 0.5 | 0.25 |
| | $MIC_{90}$ | 32 | 4 | 0.25 | 0.5 | 1 | 1 | 0.25 |

### 3.4.3 Time-kill assay

A time-kill assay was performed to characterize the bactericidal or bacteriostatic activity of an antimicrobial agent. Itraconazole, isavuconazole, ravuconazole, azithromycin, and ceftriaxone were tested at both 5 × and 10 × MIC against *N. gonorrhoeae* FA1090. In 5 × MIC, ravuconazole decreased the bacterial inoculum to below the limit of detection in 12 h, the same as ceftriaxone. At 5 × MIC, itraconazole and isavuconazole almost cleared all gonococcus in 12 h, and no colonies were observed after 24 h (Figure 3.1a). At 10 × MIC, itraconazole, isavuconazole, ravuconazole, and ceftriaxone reduced the bacterial inoculum to below the limit of detection in 12 h (Figure 3.1b). Azithromycin, which is known for its bactericidal activity, decreased the bacterial inoculum to below the limit of detection in 6 h at both 5 × and 10 × MIC. All three azole drugs are bactericidal at both 5 × and 10 × MIC, as they all reduced CFU/mL by more than 3 $\log_{10}$ fold after 24 h, which is comparable to ceftriaxone.

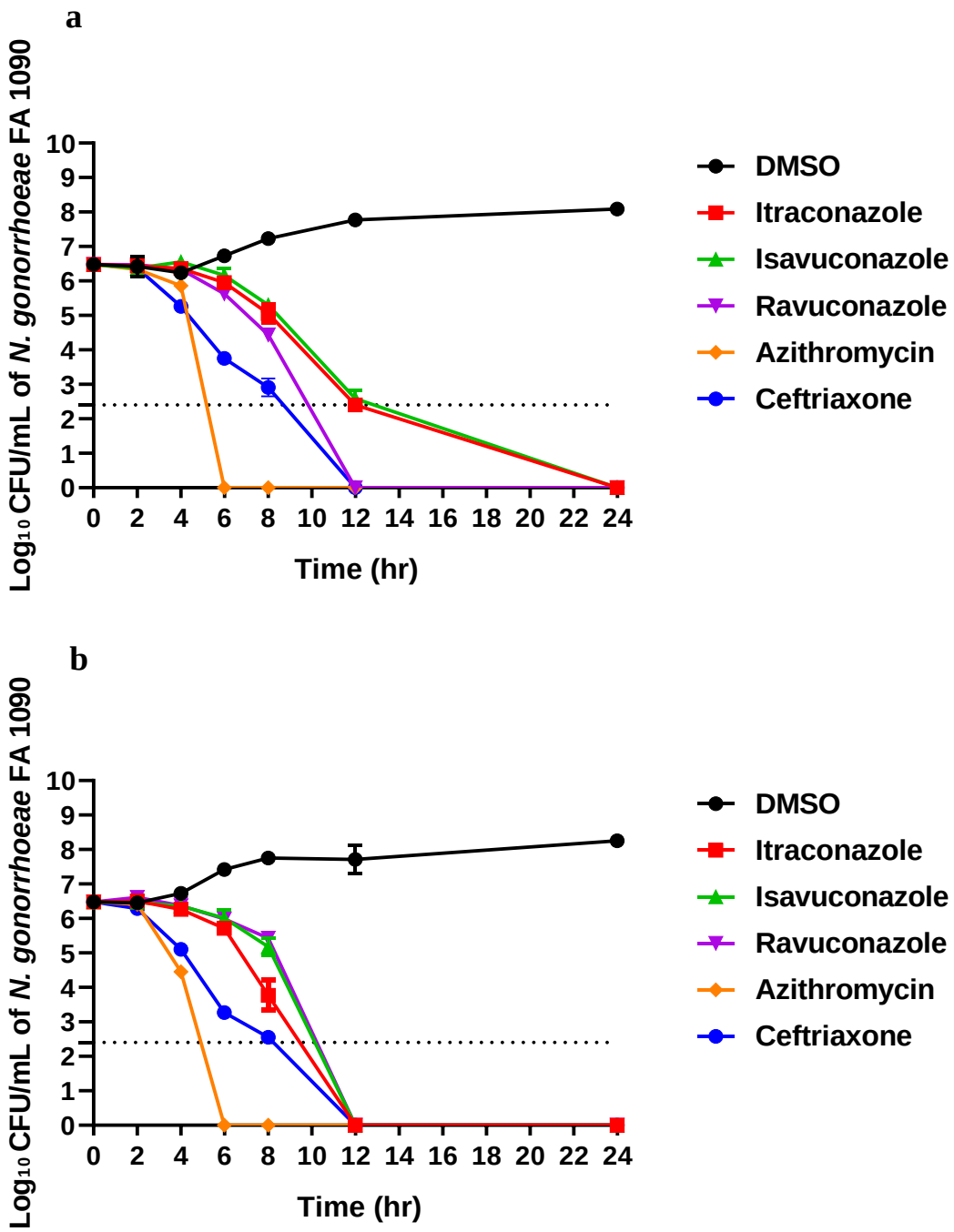


**Figure 3.1 Time-kill assay of itraconazole, isavuconazole, ravuconazole, and control antibiotics at (a) 5 × MIC and (b) 10 × MIC against *N. gonorrhoeae* FA1090 for a 24 h incubation period.** DMSO, the solvent for the azoles, served as a negative control. Samples were plated for bacterial counts at the indicated time points. The error bars represent standard deviation values for each tested agent.

### 3.4.4 Post-antibiotic effect

The capacity of azole drugs to exert a prolonged inhibitory effect after a limited exposure time was examined in *N. gonorrhoeae* FA1090, WHO-P, and WHO-Y by the PAE experiment. Following a 2

h exposure to 10 × MIC of azoles and azithromycin, tested agents were removed, and bacteria were plated on GC plates for up to 24 h. As depicted in Table 3.3, ravuconazole displayed the longest PAE (12 to 16 h) against three *N. gonorrhoeae* strains compared with all the tested agents, followed by isavuconazole (8 to 12 h) and itraconazole (4 h). Isavuconazole and ravuconazole showed a longer PAE effect than azithromycin, which is the drug of choice but only showed a PAE of 4 to 8 h.

**Table 3.3 Post-antibiotic effect of azole drugs against *N. gonorrhoeae* strains**

| | **PAE (h) of azole drugs against *N. gonorrhoeae* stains** | | |
|---|---|---|---|
| | **FA1090** | **WHO-P** | **WHO-Y** |
| Itraconazole | 4 | 4 | 4 |
| Isavuconazole | 12 | 8 | 10 |
| Ravuconazole | 12 | 12 | 16 |
| Azithromycin | 4 | 8 | 4 |

### 3.4.5 Single-step mutation assay

A single-step resistance assay was performed to determine the frequency of spontaneous mutations to each azole drug against *N. gonorrhoeae* FA1090. Plates were inspected for growth at 24, 48, and 72 h. No growth was observed in the plates containing any of the three azoles at a concentration of 10 × MIC, resulting in a low resistance frequency of $<1.1 \times 10^{-10}$, which is comparable to azithromycin and ceftriaxone (Table 3.4). *N. gonorrhoeae* developed resistance rapidly to the positive control, rifampin, which exhibited a high resistance frequency of $2.18 \times 10^{-7}$, which is similar to previously reported in other bacteria strains[32,33].

**Table 3.4 Single-step resistance assay of azole drugs, azithromycin, ceftriaxone, and rifampin at 10× MIC against *N. gonorrhoeae* FA1090**

| **Drugs** | **Frequency of mutation** |
|---|---|
| Itraconazole | $<1.1 \times 10^{-10}$ |
| Isavuconazole | $<1.1 \times 10^{-10}$ |
| Ravuconazole | $<1.1 \times 10^{-10}$ |
| Azithromycin | $<1.1 \times 10^{-10}$ |
| Ceftriaxone | $<1.1 \times 10^{-10}$ |
| Rifampicin | $2.18 \times 10^{-7}$ |

### 3.4.6 Multi-step mutation assay

To study whether gonococcus can develop resistance to the three azole drugs over time, *N. gonorrhoeae* FA1090 was serially exposed to sub-MIC levels of the tested agents for 14 days. There

was only a 1-fold increase in MIC for the three azoles after 14 days (Figure 3.2). This result is the same with ceftriaxone, the first-line treatment for gonorrhea. The common gonorrhea treatment timeline is seven days. Therefore, we performed this experiment for 14 days. Rifampicin is well-known for causing rapid resistance emergence; however, it was not observed in this experiment. Continuous passages may be performed to observe the elevating fold-change in MIC.

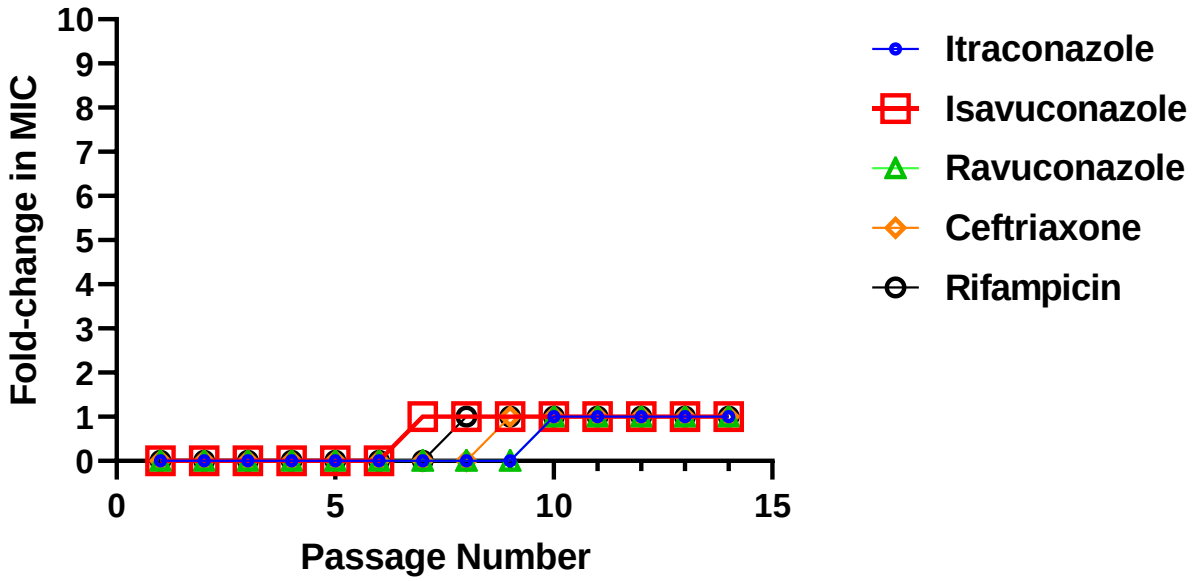


**Figure 3.2 Multi-step resistance assay of azole drugs against *N. gonorrhoeae* FA1090.**

### 3.4.7 Intracellular clearance assay

*N. gonorrhoeae* can infect and invade cervix epithelial cells [29,34]. An intracellular clearance assay was performed to evaluate whether azole drugs can eradicate intracellular *N. gonorrhoeae* from infected endocervical cells. *N. gonorrhoeae* FA1090 infected the human cervical cancer cell line ME-180 for 12 h, followed by groups of treatment at 3 × MIC for 24 h. All three tested azole drugs significantly decreased the load of intracellular *N. gonorrhoeae* to below the limit of detection (250 CFU/ml), and azithromycin had the same effect (Figure 3.3). Ceftriaxone, the currently recommended regimen for gonococcal infection, did not have a significant difference from DMSO (negative control).

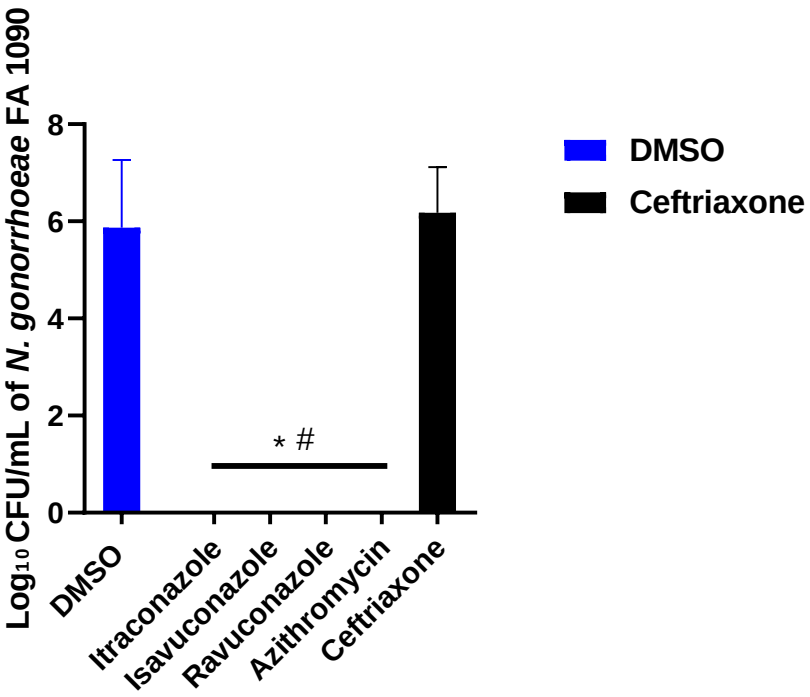


**Figure 3.3 Effectiveness of azoles at 3 × MIC against intracellular *N. gonorrhoeae* FA1090 in infected human endocervical cells (ME-180).** Intracellular clearance assay of itraconazole, isavuconazole, ravuconazole, azithromycin, ceftriaxone, and DMSO at 3 × MIC against *N. gonorrhoeae* FA1090 in ME-180 cervical cells. Error bars represent the standard deviation of quadruplicate samples from two independent experiments. An asterisk (*) denotes statistical significance (P<0.0001) compared to the control DMSO. A pound sign (#) denotes statistical significance (P<0.0001) compared to ceftriaxone. P values <0.0001 were analyzed by one-way ANOVA using Dunnett's test for multiple comparisons.

### 3.4.8 Checkerboard assay

Dual treatment is an effective approach to combat *N. gonorrhoeae*. Dual therapy of azithromycin and ceftriaxone was recommended by the CDC before 2020 to treat gonorrhea until the emerging resistance to azithromycin. For people who are allergic to cephalosporins, an alternative regimen of gentamicin plus azithromycin can now be considered. Therefore, we investigate whether azole drugs have the potential to be implemented as dual treatments. Itraconazole, isavuconazole, and ravuconazole were combined with either azithromycin or ceftriaxone using the checkerboard assay. Azole drugs in combination with drugs of choice, azithromycin or ceftriaxone, exhibited an indifferent or additive effect with a fractional inhibitory concentration index (FICI) ranging from 0.75 to 2 against *N. gonorrhoeae* FA1090, WHO-P, and WHO-Y (Table 3.5).

**Table 3.5 Fractional inhibitory concentration index range of azole drugs in combination with azithromycin or ceftriaxone against *N. gonorrhoeae* strains**

| **Drugs** | | **FA1090** | **WHO-P** | **WHO-Y** |
|---|---|---|---|---|
| | | **MIC (µg/ml)** | | |
| Itraconazole | Alone | 0.25 | 8 | 1 |
| | Combination | 0.25 | 2 | 1 |
| Azithromycin | Alone | 0.125 | 8 | 1 |
| | Combination | 0.125 | 4 | 1 |
| | **FIC Index** | **2** | **0.75** | **2** |
| | **Interpretation** | **IND** | **ADD** | **IND** |
| Isavuconazole | Alone | 0.25 | 2 | 0.5 |
| | Combination | 0.25 | 1 | 0.5 |
| Azithromycin | Alone | 0.125 | 8 | 1 |
| | Combination | 0.125 | 4 | 1 |
| | **FIC Index** | **2** | **1** | **2** |
| | **Interpretation** | **IND** | **ADD** | **IND** |
| Ravuconazole | Alone | 0.008 | 0.25 | 0.03 |
| | Combination | 0.008 | 0.125 | 0.03 |
| Azithromycin | Alone | 0.06 | 8 | 1 |
| | Combination | 0.06 | 4 | 1 |
| | **FIC Index** | **2** | **1** | **2** |
| | **Interpretation** | **IND** | **ADD** | **IND** |
| Itraconazole | Alone | 0.25 | 8 | 1 |
| | Combination | 0.25 | 2 | 0.5 |
| Ceftriaxone | Alone | 0.002 | 0.002 | 0.5 |
| | Combination | 0.002 | 0.001 | 0.25 |
| | **FIC Index** | **2** | **0.75** | **1** |
| | **Interpretation** | **IND** | **ADD** | **ADD** |
| Isavuconazole | Alone | 0.25 | 2 | 0.5 |
| | Combination | 0.25 | 1 | 0.25 |
| Ceftriaxone | Alone | 0.002 | 0.002 | 0.5 |
| | Combination | 0.002 | 0.001 | 0.25 |
| | **FIC Index** | **2** | **1** | **1** |
| | **Interpretation** | **IND** | **ADD** | **ADD** |
| Ravuconazole | Alone | 0.008 | 0.25 | 0.03 |
| | Combination | 0.008 | 0.125 | 0.015 |
| Ceftriaxone | Alone | 0.002 | 0.002 | 0.5 |
| | Combination | 0.002 | 0.001 | 0.25 |
| | **FIC Index** | **2** | **1** | **1** |
| | **Interpretation** | **IND** | **ADD** | **ADD** |

The FIC Index was interpreted as follows: a synergistic relationship (SYN) was defined as a FICI of ≤ 0.5, an additive relationship (ADD) was defined as a FICI value between 0.5-1.25, an indifferent

relationship (IND) was defined as a FICI value between 1.25-4, and antagonism was defined as a FICI of >4.

### 3.4.9 DNA leakage assay

Next, we investigated whether the mechanism of action of the three azole drugs is cell membrane disruption. Each drug was incubated with *N. gonorrhoeae* FA1090 for 30 min, then the bacterial cultures were spun down and the DNA concentrations present in the supernatants were measured by Nanodrop. When a drug permeabilizes the cell membrane, DNA will be released into the supernatant. Therefore, an increased level of DNA concentration would be observed compared to the untreated group. Triton X-100, which served as a positive control, is a surfactant that permeabilizes cell membranes. As shown in Figure 3.4, Triton X-100 showed a significant increase in DNA levels. No significant difference was observed in all three azoles, azithromycin, or ceftriaxone.

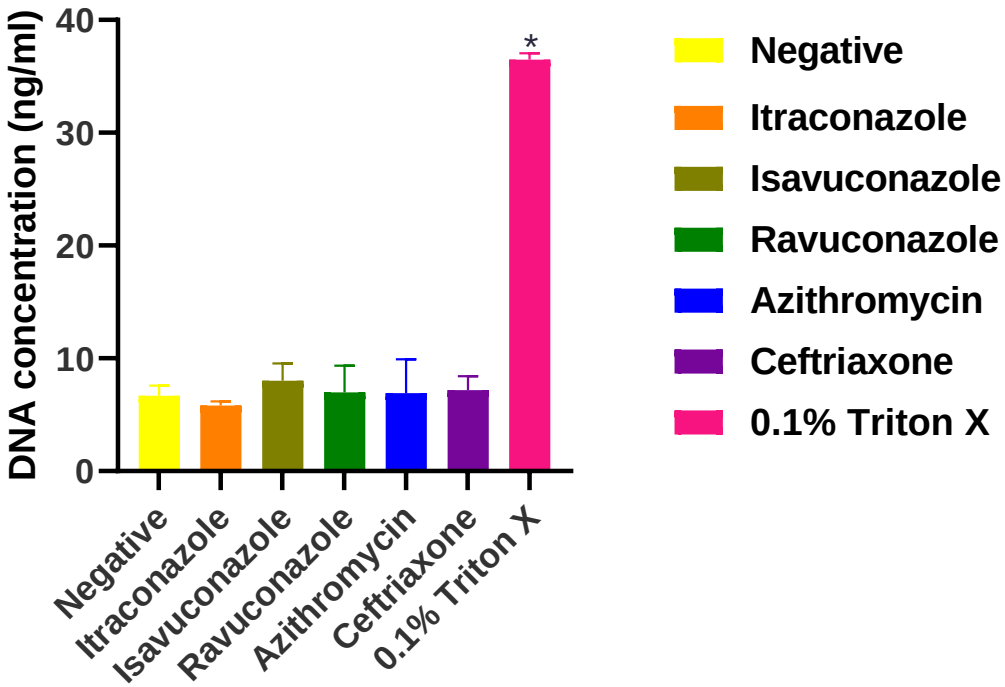


**Figure 3.4 DNA leakage assay of azoles, azithromycin, ceftriaxone, and 0.1% Triton X at 10 × MIC against *N. gonorrhoeae* FA1090.** DNA concentrations were measured by Nanodrop. Error bars represent the standard deviation from triplicate samples for each tested agent. An asterisk denotes a significant difference between bacterial cells treated with azoles, azithromycin, ceftriaxone, and $H_2O$ compared to cells treated with 0.1% Triton X. $P<0.0001$ analyzed by one-way ANOVA using Dunnett's multiple comparisons test.

### 3.4.10 Reactive oxidative species (ROS) detection

A previous study showed that imidazole induces reactive oxygen species (ROS) in *Mycobacterium tuberculosis* [35]. This finding encouraged us to explore whether itraconazole, isavuconazole, and ravuconazole can increase ROS production in *N. gonorrhoeae*. To assess whether azole drugs induce ROS in *N. gonorrhoeae*, *N. gonorrhoeae* FA1090 was treated with 1 × or 0.5 × MIC of three azole drugs for 30 min, then ROS activity was measured by carboxy-H2DCFDA staining. As shown in Figure 3.5, none of the azoles significantly increased ROS production at 1 × or 0.5 × MIC compared to the negative control, DMSO. Azithromycin and ceftriaxone also did not affect ROS production in *N. gonorrhoeae* FA1090.

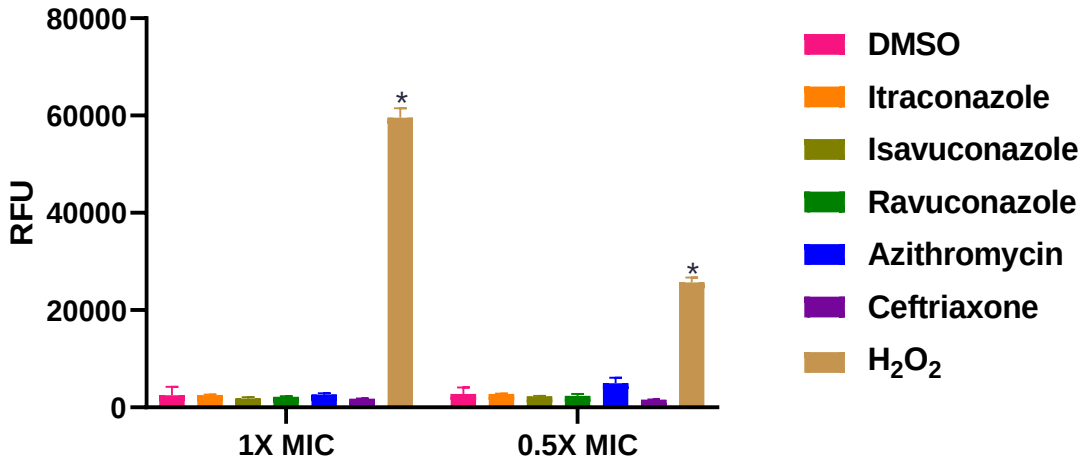


**Figure 3.5 Effect of azoles on ROS levels in *N. gonorrhoeae* FA1090.** Fluorescent were measured by staining carboxy-H2DCFDA after treatment with azoles, azithromycin, ceftriaxone, DMSO, or $H_2O_2$ (positive control) for 30 min. Asterisks (*) indicate statistical significance ($P < 0.001$) between DMSO and the treated groups. $P<0.0001$ analyzed by one-way ANOVA using Dunnett's multiple comparisons test.

## 3.5 Discussion

*Neisseria gonorrhoeae* is a global public health concern and remains the main cause of sexually transmitted diseases. It has developed resistance to all antimicrobials formerly or currently used for empirical first-line therapy worldwide. Due to the growing prevalence of gonococcal resistance, limited treatment options, and lack of effective vaccines, developing new therapeutic options is a top priority. Repurposing drugs for additional indications could be a useful method of expanding the variety of accessible therapeutic alternatives. Drug repurposing strategies have been employed for many infectious diseases and cancers. By implementing a drug repurposing strategy, we identified itraconazole,

isavuconazole, and ravuconazole as promising candidates for treating multidrug-resistant gonorrhea infections.

Itraconazole, ravuconazole, and ravuconazole are triazoles, a class of azole antifungal agents. Itraconazole is the first generation of FDA-approved azole drugs used to treat serious fungal or yeast infections, including aspergillosis, blastomycosis, and histoplasmosis. Isavuconazole and ravuconazole are the second-generation azoles. Isavuconazole is an FDA-approved drug for the treatment of invasive aspergillosis and mucormycosis. Ravuconazole is authorized for the treatment of onychomycosis in Japan. Besides, itraconazole, isavuconazonium sulfate, and fosravuconazole are orally available azoles. Of note, isavuconazonium sulfate shows 98% oral bioavailability. The good oral bioavailability suggests that these azoles can reach infection sites and treat *N. gonorrhoeae*.

In this study, we evaluated the antibacterial activity of the three azole antifungals against a panel of multidrug-resistant *N. gonorrhoeae* clinical isolates using broth dilution and agar dilution techniques. Itraconazole, isavuconazole, and ravuconazole inhibited all the tested gonococci with MICs in the range of 0.002-4 µg/ml. Of these three azoles, ravuconazole was the most potent drug that showed the lowest $MIC_{90}$ value compared to itraconazole, isavuconazole, azithromycin, gepotidacin, and zoliflodacin. Ravuconazole exhibits $MIC_{50}$ and $MIC_{90}$ values of 0.06 µg/ml and 0.06 µg/ml, respectively, which are similar to ceftriaxone. The MIC range for ravuconazole (0.002-0.25 µg/ml) outperformed ceftriaxone (0.001-1 µg/ml), with its highest MIC value (0.25 µg/ml) being lower than ceftriaxone (1 µg/ml). Isavuconazole demonstrated better $MIC_{50}$ and $MIC_{90}$ values (0.5 µg/ml and 1 µg/ml respectively) than gepotidacin (1 µg/ml and 4 µg/ml respectively), as well as a more favorable MIC range (0.015-2 µg/ml) compared to gepotidacin (0.03-8 µg/ml). Itraconazole was less potent with $MIC_{50}$ and $MIC_{90}$ values of 1 µg/ml and 2 µg/ml, respectively, which $MIC_{90}$ being less than the value for azithromycin (4 µg/ml). The MIC range for itraconazole (0.03-4 µg/ml) was superior to gepotidacin (0.03-8 µg/ml).

In addition to potent antigonococcal activity, all three azoles (at 5 × and 10 × MIC) exhibited bactericidal activity against *N. gonorrhoeae* FA1090. All three azoles (at 10 × MIC) successfully reduced the bacterial burden below the limit of detection (250 CFU/ml) in 12 h, and the same effect was observed in ceftriaxone. Because gonorrhea is often asymptomatic and can cause disseminated gonococcal infection, bactericidal drugs are favored over bacteriostatic ones for gonorrhea treatment to eliminate infection, minimize the period of therapy, prevent its spread, and potentially reduce the emergence of resistance[36,37].

Determining the PAE of a new antimicrobial is an important step for dosing and has clinical importance[38-40]. Antimicrobials with an extended PAE possess several advantages, including reduced

costs of the drug, limited toxicity, and better patient compliance[40-42]. The capacity of the azoles to display a prolonged inhibitory effect against *N. gonorrhoeae* after a limited exposure time (2 h at 10 × MIC) was examined in a PAE experiment[26,43,44]. Ravuconazole and isavuconazole exhibited a long PAE that ranged from 8 to 16 h, which was longer than both ceftriaxone and azithromycin (Table 3.3). The PAE for gepotidacin against *N. gonorrhoeae* was reported to range from 0.5 to >2.5 h[45], while the PAE for zoliflodacin against *Staphylococcus aureus* was reported to be 1.65 to 2.4 h[46]. These results provide valuable evidence that *N. gonorrhoeae* is very slow to recover after exposure to ravuconazole with irreversible potent target engagement.

Given the excellent activities of the three azoles in terms of their potency, bactericidal effect, and prolonged PAE, we assessed the ability of *N. gonorrhoeae* to develop resistance to the azoles. *N. gonorrhoeae* exhibits an extraordinary ability to adapt and acquire resistance to antibacterial agents. To tackle the challenge of the rapid emergence of antibiotic resistance, we examined the potential emergence of spontaneous resistance in *N. gonorrhoeae* to the azoles using a single-step mutation assay. As shown in Table 3.4, no mutants were observed when *N. gonorrhoeae* was exposed to any of the three azole drugs at 10 × MIC, indicating the probability of resistance developing against the three azoles is low. In contrast, a high frequency of spontaneous mutations was observed in rifampicin, which agrees with previous reports in other bacteria[32,33]. Next, we performed a multi-step mutation assay to investigate the potential for resistance development against the azoles in the longer term. *N. gonorrhoeae* FA1090 was serially passaged at sub-MIC levels of tested agents for 14 consecutive days. As depicted in Figure 3.2, no resistance was observed in the three azoles after 14 serial passages (only a one-fold increase in MIC). Collectively, the results indicate a low probability of rapid resistance development in *N. gonorrhoeae* to the three tested azoles.

*N. gonorrhoeae* can invade and replicate inside mucosal epithelial cell layers in the urogenital tract, rectum, and pharynx. *N. gonorrhoeae* is also capable of transmigrating across the mucosal epithelia following the invasion and can cause disseminated infections [25,47,48]. Ceftriaxone, due to its high polarity and poor cellular permeability, is unable to clear intracellular *N. gonorrhoeae* [26,43,49]. Alternative drugs with the ability to penetrate infected cells to clear the infection are a major advantage. We used the gentamicin protection assay to examine the ability of the azoles to reduce the burden of intracellular *N. gonorrhoeae* present in infected endocervical cells[25,26,43,44,47]. As presented in Figure 3.3, the azoles were superior to ceftriaxone and cleared intracellular *N. gonorrhoeae* within infected endocervical cells. The results collectively indicate that azoles can enter endocervical cells and significantly reduce intracellular *N. gonorrhoeae* burden at a rate that is superior to ceftriaxone.

A dual therapy of azithromycin plus ceftriaxone was previously recommended treatment for *N. gonorrhoeae* infection in the U.S. until 2020 before azithromycin was removed due to its rapid emergence of resistance. On the other hand, the European Centre for Disease Prevention and Control (ECDC) and the Public Health Agency of Canada (PHAC) support combination treatments of azithromycin and ceftriaxone[50,51]. The primary advantages of dual therapy are the use of antimicrobials with distinct mechanisms of action to potentially mitigate the spread of antimicrobial resistance and enhance treatment efficacy. Therefore, the relationship between the three azoles and the drug of choice, azithromycin or ceftriaxone, was assessed by checkerboard assay. The checkerboard assay confirmed that no antagonism effect was observed in any of the combinations. The results suggested that the three azole drugs can not only be used as monotherapy but also in combination treatments, which broadens their potential therapeutic applications.

Finally, we sought to identify the target of the three azoles. We examined whether the mode of action of the three azoles involved disruption of the *N. gonorrhoeae* cell membrane. A cell leakage assay confirmed that the azoles do not compromise the bacterial membrane integrity in the same way as the positive control, Triton X. The finding suggests that the mode of action of all three azoles is not via cell membrane disruption. A previous study demonstrated that imidazoles are bactericidal in *Mycobacterium tuberculosis*, accompanied by a significant elevation in ROS levels in the treated bacteria[35]. It has been suggested that bactericidal antibiotics function through the induction of ROS[52]. Thus, we investigated the effect of the three azoles on ROS production. The base level of ROS production in wild-type *N. gonorrhoeae* treated with DMSO was quantified. No significant increase in ROS levels was observed in response to groups treated with any of the three azoles (Figure 3.5). The precise mechanism of action of the azoles against *N. gonorrhoeae* requires additional study.

In conclusion, these *in vitro* studies demonstrate the promising properties of the three azole drugs as novel antigonococcal agents, including potency, bactericidal activity, low frequency of resistance, and outstanding intracellular clearance activity. Future investigations of these three azoles are warranted for their potential use in treating *N. gonorrhoeae* infections.

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## 3.7 Supplementary material

**Table 3.6S *Neisseria gonorrhoeae* clinical isolates used in this study**

| Strains | Description |
|---|---|
| *N. gonorrhoeae* FA1090 | Resistant to streptomycin. Isolated from a female patient with disseminated gonococcal infection. |
| *N. gonorrhoeae* WHO-F | Isolated in Canada, 1991. |
| *N. gonorrhoeae* WHO-G | Resistant tetracycline and ciprofloxacin. Isolated in Thailand, 1997. |
| *N. gonorrhoeae* WHO-K | Resistant to cefixime, ciprofloxacin, penicillin, and tetracycline. |
| *N. gonorrhoeae* WHO-L | Resistant to ceftriaxone, ciprofloxacin, penicillin, and tetracycline. Isolated in Asia, 1996. |
| *N. gonorrhoeae* WHO-M | Resistant to ciprofloxacin, penicillin, and tetracycline. Isolated in the Philippines, 1992. |
| *N. gonorrhoeae* WHO-N | Resistant to ciprofloxacin, penicillin, and tetracycline. Isolated in Australia, 2001. |
| *N. gonorrhoeae* WHO-O | Resistant to penicillin, tetracycline, and spectinomycin. Isolated in Canada, 1991. |
| *N. gonorrhoeae* WHO-P | Resistant to azithromycin and tetracycline. |
| *N. gonorrhoeae* WHO-U | Resistant to azithromycin and tetracycline. Isolated from a female pharynx specimen in Sweden, 2011. |
| *N. gonorrhoeae* WHO-V | Resistant to ciprofloxacin, penicillin, tetracycline, and azithromycin. Isolated from a urethral specimen from a case of urethritis in Karlstad, Sweden, 2012. |
| *N. gonorrhoeae* WHO-W | Resistant to cefixime, ciprofloxacin, penicillin, and tetracycline. Isolated from Hong Kong, 2007. |
| *N. gonorrhoeae* WHO-X | Resistant to cefixime, ceftriaxone, ciprofloxacin, penicillin, and tetracycline. Isolated from a female pharynx specimen in Kyoto, Japan, 2009. |
| *N. gonorrhoeae* WHO-Y | Resistant to cefixime, ceftriaxone, ciprofloxacin, and tetracycline. Isolated from a urethral specimen of a 50 years old male in Quimper, France, 2010. |
| *N. gonorrhoeae* WHO-Z | Resistant to cefixime, ceftriaxone, ciprofloxacin, penicillin, and tetracycline. Isolated from a female genital swab in Australia, 2013. |
| *N. gonorrhoeae* CDC-165 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-166 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-167 | Resistant to azithromycin. |

| | |
|---|---|
| *N. gonorrhoeae* CDC-168 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-169 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-170 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-171 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-172 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-173 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-174 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-175 | Resistant to azithromycin. |
| *N. gonorrhoeae* CDC-176 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-177 | Resistant to azithromycin and tetracycline. |
| *N. gonorrhoeae* CDC-178 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-179 | Resistant to azithromycin. |
| *N. gonorrhoeae* CDC-180 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-181 | Resistant to azithromycin and tetracycline. |
| *N. gonorrhoeae* CDC-182 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-183 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-184 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-185 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-186 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-187 | Resistant to azithromycin, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-188 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-189 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |

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|---|---|
| *N. gonorrhoeae* CDC-190 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-191 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-192 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-193 | Resistant to azithromycin, tetracycline, and penicillin. |
| *N. gonorrhoeae* CDC-195 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-196 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-197 | Resistant to azithromycin, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-198 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-199 | Resistant to tetracycline and penicillin. |
| *N. gonorrhoeae* CDC-200 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-201 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-202 | Resistant to azithromycin and tetracycline. |
| *N. gonorrhoeae* CDC-203 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-204 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-205 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-206 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-207 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-208 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-209 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-210 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-211 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-212 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |

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|---|---|
| *N. gonorrhoeae* CDC-213 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* CDC-214 | Resistant to cefixime, tetracycline, ciprofloxacin, and penicillin. |
| *N. gonorrhoeae* AR-0933 | Resistant to tetracycline. |
| *N. gonorrhoeae* AR-0934 | Resistant to tetracycline. |
| *N. gonorrhoeae* AR-0935 | - |
| *N. gonorrhoeae* AR-0936 | Resistant to tetracycline and ciprofloxacin. |
| *N. gonorrhoeae* AR-0937 | Resistant to penicillin, tetracycline, and ciprofloxacin. |
| *N. gonorrhoeae* AR-0938 | Resistant to penicillin, tetracycline, and ciprofloxacin. |
| *N. gonorrhoeae* AR-0963 | - |
| *N. gonorrhoeae* AR-0964 | Resistant to ciprofloxacin. |
| *N. gonorrhoeae* AR-0965 | - |
| *N. gonorrhoeae* AR-0966 | - |
| *N. gonorrhoeae* AR-0967 | - |
| *N. gonorrhoeae* AR-0968 | - |
| *N. gonorrhoeae* AR-0969 | - |
| *N. gonorrhoeae* AR-0970 | - |
| *N. gonorrhoeae* AR-0971 | Resistant to ciprofloxacin. |
| *N. gonorrhoeae* AR-0972 | - |
| *N. gonorrhoeae* AR-0973 | - |
| *N. gonorrhoeae* AR-0974 | Resistant to ciprofloxacin. |
| *N. gonorrhoeae* AR-0975 | Resistant to ciprofloxacin. |
| *N. gonorrhoeae* AR-0976 | Resistant to ciprofloxacin. |

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|---|---|
| *N. gonorrhoeae* ATCC 19424 | - |
| *N. gonorrhoeae* ATCC 31426 | Resistant to penicillin. |
| *N. gonorrhoeae* ATCC 43069 | Isolated from Illinois, USA. |
| *N. gonorrhoeae* ATCC 43070 | Isolated from cervix. |
| *N. gonorrhoeae* ATCC 49226 | National Committee for Clinical Laboratory Standards (NCCLS)-recommended quality control strain |
| *N. gonorrhoeae* ATCC 49981 | - |
| *N. gonorrhoeae* CDC-10328 | Resistant to penicillin and ciprofloxacin |
| *N. gonorrhoeae* CDC-10329 | Resistant to penicillin, tetracycline, and ciprofloxacin. |
| *N. gonorrhoeae* P681E | Resistant to penicillin and tetracycline. |
| *N. gonorrhoeae* SPL-4 | Resistant to penicillin, tetracycline, ceftriaxone, and ciprofloxacin. |
| *N. gonorrhoeae* SPJ-15 | Resistant to azithromycin. |
| *N. gonorrhoeae* F-28 | Resistant to spectinomycin. |
| *N. gonorrhoeae* MS11 | Isolated from a patient with uncomplicated anterior urethritis. |
| All susceptibility/resistance breakpoints are based on EUCAST (2023). EUCST notes that MIC ≥1.0 µg/ml for azithromycin is defined as the epidemiological cutoff value. | |

**Table 3.7S Minimum inhibitory concentration (MIC in µg/ml) of azole drugs and control antibiotics against *N. gonorrhoeae* isolates by broth microdilution method**

| No. of strains | *N. gonorrhoeae* strains | Itraconazole | Isavuconazole | Ravuconazole | Azithromycin | Ceftriaxone | Gepotidacin | Zoliflodacin |
|---|---|---|---|---|---|---|---|---|
| 1 | FA1090 | 0.25 | 0.125 | 0.008 | 0.125 | 0.002 | 0.25 | 0.03 |
| 2 | WHO-F | 1 | 2 | 0.06 | 0.25 | 0.002 | 1 | 0.06 |
| 3 | WHO-G | 1 | 1 | 0.06 | 0.25 | 0.004 | 4 | 0.125 |
| 4 | WHO-K | 1 | 1 | 0.06 | 0.5 | 0.03 | 1 | 0.125 |
| 5 | WHO-L | 1 | 1 | 0.06 | 1 | 0.125 | 8 | 0.125 |

| No. of strains | *N. gonorrhoeae* strains | Itraconazole | Isavuconazole | Ravuconazole | Azithromycin | Ceftriaxone | Gepotidacin | Zoliflodacin |
|---|---|---|---|---|---|---|---|---|
| 6 | WHO-M | 2 | 1 | 0.06 | 0.5 | 0.015 | 4 | 0.03 |
| 7 | WHO-N | 2 | 2 | 0.125 | 0.25 | 0.004 | 0.5 | 0.125 |
| 8 | WHO-O | 1 | 0.5 | 0.03 | 0.5 | 0.015 | 1 | 0.06 |
| 9 | WHO-P | 4 | 2 | 0.25 | 4 | 0.002 | 4 | 0.25 |
| 10 | WHO-U | 2 | 1 | 0.125 | 4 | 0.001 | 0.25 | 0.06 |
| 11 | *WHO-V | 1 | 2 | 0.125 | >512 | 0.06 |  | 0.125 |
| 12 | WHO-W | 1 | 0.5 | 0.03 | 0.5 | 0.03 | 0.5 | 0.06 |
| 13 | WHO-X | 0.5 | 0.5 | 0.03 | 0.5 | 1 | 1 | 0.06 |
| 14 | WHO-Y | 0.5 | 0.25 | 0.03 | 1 | 1 | 1 | 0.06 |
| 15 | WHO-Z | 1 | 1 | 0.06 | 1 | 0.5 | 1 | 0.06 |
| 16 | CDC-165 | 2 | 1 | 0.06 | 1 | 0.03 |  | 0.06 |
| 17 | CDC-166 | 2 | 1 | 0.06 | 1 | 0.06 | 1 | 0.125 |
| 18 | CDC-167 | 0.5 | 0.25 | 0.015 | 16 | 0.004 | 0.5 | 0.125 |
| 19 | CDC-168 | 1 | 0.5 | 0.06 | 1 | 0.03 | 1 | 0.06 |
| 20 | CDC-169 | 1 | 1 | 0.06 | 1 | 0.06 | 2 | 0.125 |
| 21 | CDC-170 | 1 | 1 | 0.06 | 1 | 0.03 | 2 | 0.06 |
| 22 | CDC-171 | 0.5 | 0.25 | 0.03 | 1 | 0.03 | 2 | 0.06 |
| 23 | CDC-172 | 1 | 1 | 0.06 | 1 | 0.03 | 1 | 0.06 |
| 24 | CDC-173 | 2 | 1 | 0.06 | 2 | 0.06 | 2 | 0.125 |
| 25 | CDC-174 | 1 | 1 | 0.06 | 1 | 0.06 | 0.25 | NT |
| 26 | CDC-175 | 0.25 | 0.125 | 0.015 | 16 | 0.008 | 0.125 | NT |
| 27 | CDC-176 | 1 | 1 | 0.06 | 1 | 0.06 | 0.25 | NT |
| 28 | CDC-177 | 0.25 | 0.25 | 0.015 | 2 | 0.008 | 0.125 | NT |
| 29 | CDC-178 | 2 | 1 | 0.06 | 2 | 0.06 | 0.5 | NT |

| No. of strains | *N. gonorrhoeae* strains | Itraconazole | Isavuconazole | Ravuconazole | Azithromycin | Ceftriaxone | Gepotidacin | Zoliflodacin |
|---|---|---|---|---|---|---|---|---|
| 30 | CDC-179 | 0.25 | 0.125 | 0.015 | 16 | 0.002 | 2 | 0.06 |
| 31 | CDC-180 | 1 | 0.5 | 0.06 | 1 | 0.03 | 1 | 0.125 |
| 32 | CDC-181 | 2 | 1 | 0.06 | >512 | 0.03 | 1 | 0.125 |
| 33 | CDC-182 | 1 | 0.5 | 0.03 | 1 | 0.03 | 0.25 | NT |
| 34 | CDC-183 | 1 | 1 | 0.06 | 1 | 0.06 | 0.125 | NT |
| 35 | CDC-184 | 1 | 1 | 0.06 | 1 | 0.03 | 0.5 | NT |
| 36 | CDC-185 | 1 | 0.5 | 0.06 | 1 | 0.03 | 0.5 | NT |
| 37 | CDC-186 | 1 | 0.5 | 0.03 | 1 | 0.06 | 4 | 0.125 |
| 38 | CDC-187 | 2 | 2 | 0.125 | 4 | 0.03 | 0.125 | NT |
| 39 | CDC-188 | 1 | 0.5 | 0.03 | 1 | 0.03 | 0.25 | NT |
| 40 | CDC-189 | 1 | 0.5 | 0.03 | 1 | 0.03 | NT | NT |
| 41 | CDC-190 | 1 | 1 | 0.06 | 2 | 0.06 | 4 | 0.06 |
| 42 | CDC-191 | 2 | 2 | 0.06 | 1 | 0.06 | NT | 0.06 |
| 43 | CDC-192 | 1 | 0.5 | 0.06 | 1 | 0.06 | NT | NT |
| 44 | CDC-193 | 1 | 0.5 | 0.03 | 2 | 0.03 | NT | NT |
| 45 | CDC-195 | 2 | 1 | 0.06 | 2 | 0.06 | NT | 0.06 |
| 46 | CDC-196 | 1 | 0.5 | 0.06 | 2 | 0.03 | 0.25 | NT |
| 47 | CDC-197 | 1 | 0.5 | 0.03 | 4 | 0.03 | 1 | 0.06 |
| 48 | CDC-198 | 0.5 | 0.5 | 0.03 | 1 | 0.06 | 2 | 0.06 |
| 49 | CDC-199 | 0.5 | 0.25 | 0.03 | 1 | 0.015 | NT | NT |
| 50 | CDC-200 | 0.5 | 0.5 | 0.03 | 0.5 | 0.06 | 1 | 0.125 |
| 51 | CDC-201 | 1 | 0.5 | 0.06 | 1 | 0.06 | 2 | 0.125 |
| 52 | CDC-202 | 0.5 | 0.25 | 0.03 | 16 | 0.015 | 0.125 | NT |
| 53 | CDC-203 | 0.5 | 0.5 | 0.03 | 0.5 | 0.06 | 2 | 0.125 |

| No. of strains | *N. gonorrhoeae* strains | Itraconazole | Isavuconazole | Ravuconazole | Azithromycin | Ceftriaxone | Gepotidacin | Zoliflodacin |
|---|---|---|---|---|---|---|---|---|
| 54 | CDC-204 | 2 | 1 | 0.06 | 1 | 0.06 | NT | NT |
| 55 | CDC-205 | 1 | 0.5 | 0.06 | 1 | 0.06 | NT | NT |
| 56 | CDC-206 | 1 | 0.5 | 0.06 | 1 | 0.03 | NT | NT |
| 57 | CDC-207 | 1 | 0.5 | 0.06 | 1 | 0.03 | NT | NT |
| 58 | CDC-208 | 1 | 0.5 | 0.06 | 1 | 0.03 | NT | NT |
| 59 | CDC-209 | 1 | 0.5 | 0.06 | 1 | 0.03 | NT | NT |
| 60 | CDC-210 | 1 | 0.25 | 0.03 | 0.5 | 0.03 | NT | NT |
| 61 | CDC-211 | 1 | 1 | 0.06 | 1 | 0.06 | 0.25 | NT |
| 62 | CDC-212 | 0.5 | 0.25 | 0.03 | 1 | 0.03 | NT | NT |
| 63 | CDC-213 | 0.5 | 0.25 | 0.03 | 0.5 | 0.03 | NT | NT |
| 64 | CDC-214 | 1 | 0.5 | 0.03 | 1 | 0.06 | 2 | 0.125 |
| 65 | AR-0933 | 0.125 | 0.125 | 0.008 | 0.25 | 0.008 | 0.06 | NT |
| 66 | AR-0934 | 1 | 1 | 0.06 | 0.25 | 0.03 | NT | 0.03 |
| 67 | AR-0935 | 0.5 | 0.25 | 0.015 | 0.125 | 0.008 | NT | 0.03 |
| 68 | AR-0936 | 1 | 0.5 | 0.03 | 2 | 0.015 | NT | 0.03 |
| 69 | AR-0937 | 1 | 1 | 0.06 | 0.25 | 0.008 | 0.5 | NT |
| 70 | AR-0938 | 1 | 1 | 0.125 | 0.125 | 0.008 | 0.25 | NT |
| 71 | AR-0963 | 0.5 | 0.5 | 0.03 | 1 | 0.008 | 0.125 | NT |
| 72 | AR-0964 | 0.5 | 0.5 | 0.06 | 0.5 | 0.008 | NT | 0.06 |
| 73 | *AR-0965 | 1 | 0.5 | 0.03 | 0.5 | 0.03 | NT | 0.06 |
| 74 | AR-0966 | 0.5 | 0.25 | 0.015 | 512 | 0.008 | 0.125 | NT |
| 75 | AR-0967 | 2 | 2 | 0.06 | 0.25 | 0.001 | NT | 0.06 |
| 76 | AR-0968 | 0.5 | 0.25 | 0.015 | 0.25 | 0.001 | NT | 0.06 |
| 77 | *AR-0969 | 0.25 | 0.125 | 0.008 | 0.125 | 0.008 | 0.25 | NT |

| No. of strains | *N. gonorrhoeae* strains | Itraconazole | Isavuconazole | Ravuconazole | Azithromycin | Ceftriaxone | Gepotidacin | Zoliflodacin |
|---|---|---|---|---|---|---|---|---|
| 78 | AR-0970 | 0.5 | 0.25 | 0.015 | 0.125 | 0.004 | NT | 0.015 |
| 79 | AR-0971 | 1 | 0.5 | 0.03 | 0.5 | 0.004 | NT | 0.03 |
| 80 | AR-0972 | 1 | 1 | 0.06 | 2 | 0.002 | NT | 0.125 |
| 81 | AR-0973 | 0.5 | 0.25 | 0.015 | 0.125 | 0.002 | NT | 0.015 |
| 82 | AR-0974 | 1 | 1 | 0.06 | 2 | 0.06 | NT | 0.125 |
| 83 | AR-0975 | 1 | 0.5 | 0.03 | 0.5 | 0.004 | NT | 0.03 |
| 84 | AR-0976 | 2 | 1 | 0.06 | 0.25 | 0.03 | NT | 0.125 |
| 85 | ATCC 19424 | 0.25 | 0.25 | 0.015 | 0.125 | 0.001 | NT | 0.03 |
| 86 | ATCC 31426 | 0.5 | 0.25 | 0.015 | 0.25 | 0.008 | NT | 0.06 |
| 87 | ATCC 43069 | 0.5 | 0.25 | 0.015 | 0.125 | 0.002 | NT | 0.015 |
| 88 | ATCC 43070 | 0.03 | 0.015 | 0.002 | 0.03 | 0.002 | 0.06 | NT |
| 89 | ATCC 49226 | 0.25 | 0.25 | 0.008 | 0.5 | 0.03 | 0.25 | 0.125 |
| 90 | ATCC 49981 | 0.125 | 0.06 | 0.004 | 0.06 | 0.015 | NT | 0.004 |
| 91 | CDC-10328 | 0.06 | 0.06 | 0.008 | 0.06 | 0.002 | 1 | 0.008 |
| 92 | CDC-10329 | 1 | 1 | 0.125 | 1 | 0.004 | 8 | 0.06 |
| 93 | P681E | 0.5 | 0.5 | 0.03 | 0.125 | 0.004 | 1 | 0.06 |
| 94 | SPL-4 | 1 | 1 | 0.06 | 1 | 0.03 | 1 | 0.06 |
| 95 | SPJ-15 | 2 | 1 | 0.125 | 8 | 0.004 | 2 | 0.25 |
| 96 | F-28 | 4 | 2 | 0.06 | 0.06 | 0.004 | 0.03 | 0.5 |
| 97 | MS11 | 2 | 1 | 0.125 | 1 | 0.015 | 2 | 0.125 |

**N. gonorrhoeae* strains WHO-V, AR-0965, and AR-0969 were tested in GC broth due to their poor growth in BSB broth. NT: Non-tested