# Evaluation of apramycin against spectinomycin-resistant and -susceptible strains of *Neisseria gonorrhoeae*

Running Title: Apramycin activity against *N. gonorrhoeae*

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## SYNOPSIS

**Background.** The emergence of *Neisseria gonorrhoeae* resistant to all currently available antimicrobial therapies poses a dire public health threat. New antimicrobial agents with activity against *N. gonorrhoeae* are urgently needed. Apramycin is an aminocyclitol aminoglycoside with broad-spectrum *in vitro* activity against multidrug-resistant Gram-negative pathogens and *S. aureus*. However, its activity against *N. gonorrhoeae* has not been described.

**Objectives.** The activity spectrum of apramycin against a collection of multi-drug resistant *N. gonorrhoeae* was assessed. Isolates tested included those both susceptible and resistant to the structurally distinct aminocyclitol, spectinomycin.

**Results.** The modal MICs for apramycin and spectinomycin were 16 mg/L and 32 mg/L, respectively. The ECOFF for apramycin was 64 mg/L. No strains among seventy-seven tested had an MIC above this ECOFF, suggesting very low levels of acquired apramycin resistance. In time-kill analysis, apramycin demonstrated rapid bactericidal activity comparable to spectinomycin.

**Conclusions.** Apramycin has broad-spectrum, rapidly bactericidal activity against *N. gonorrhoeae*. Future pharmacokinetic and pharmacodynamic studies will be needed to determine whether apramycin and/or apramycin derivatives hold promise as new therapeutics for *N. gonorrhoeae* infection.

## INTRODUCTION

*Neisseria gonorrhoeae* is a sexually transmitted pathogen, which continues to present a significant and global public health challenge. According to the data from global sexually transmitted infection surveillance networks, an estimated 78 million cases of gonorrhea are diagnosed each year.[1] With the introduction of effective antimicrobial agents in the 1940s, gonorrhea could be reliably treated; however, during the past few decades, successful treatment has become significantly more difficult due to the organism's propensity to develop resistance to antimicrobial agents typically used for treatment.[2-4]

Antimicrobial resistance (AMR) in *N. gonorrhoeae* occurs by several mechanisms: drug inactivation, alteration of antimicrobial targets, efflux pumps, and/or decreased antimicrobial uptake. Several regional and global surveillance networks for AMR in *N. gonorrhoeae* have raised concerns regarding emerging multidrug-resistance based on these mechanisms that will ultimately lead to infection that is effectively untreatable with currently available agents.[4-6]

In 2012, the WHO published its "Global Action Plan to Control the Spread and Impact of Antimicrobial Resistance in *Neisseria gonorrhoeae*".[7, 8] This plan contains three important core components: rigorous AMR surveillance; early detection of AMR and treatment failures in individual patients; and development of antimicrobials with unique mechanisms of action. In response to this threat, several new antimicrobial agents, such as solithromycin, a fluoroketolide;; eravacycline, a glycylcycline; and zoliflodacin, a spiropyrimidinetrione, are in development.[9-14] However, their potential contribution to treatment shortfalls and staying power against emerging resistance in *N. gonorrhoeae* is not yet established. Several studies have suggested that further evaluation of existing antimicrobial agents such as ertapenem, fosfomycin, and gentamicin may be warranted.[15, 16]

Aminoglycosides are potent Gram-negative agents with potential activity against *N. gonorrhoeae*.[17] Concerns about treatment-associated ototoxicity and nephrotoxicity have generally precluded their use in *N. gonorrhoeae* treatment. However, gentamicin is the first line of treatment in Malawi, based on cost, proven efficacy, and lack of obvious toxic effects after a single intramuscular injection.[18, 19] Gentamicin has been used either alone or in combination with doxycycline.[18, 19] The emergence of isolates with reduced susceptibility, but not resistance, has been variably observed in different longitudinal studies.[18-22] Interestingly, the structurally distinct aminocyclitols, spectinomycin and apramycin, are known or believed to have significantly lower risk for these side effects.[23, 24] Spectinomycin is an approved agent for *Neisseria gonorrhoeae* treatment via intramuscular injection, and resistance is rarely observed.[25] However, this agent is neither routinely available nor routinely used for human therapy.[25] It is unavailable in 30 of 38 European countries and in the United States.[26]

Apramycin is currently available as a veterinary treatment for bovine mastitis and diarrheal disease in farm animals.[27-30] It possesses an unusual bicyclic octadiose aminosugar linked to a monosubstituted 4-0-deoxystrepatamine moiety. Apramycin was originally isolated in 1967 from *Streptomyces tenebrarius* obtained isolated from a Sonora, Mexico soil sample .[31,32, 33] Apramycin is believed to bind to the 16s rRNA A-decoding site of the 30S ribosomal subunit and thereby inhibit peptide chain elongation and also lead to incorporation of noncognate amino acids through induced miscoding activity.[34] Resistance is primarily conferred by a single aminoglycoside modifying enzyme, AAC(3)-IV, which circulates at very low frequency in Gram-negative pathogens. [35, 36] Importantly, in contrast to other aminoglycosides, apramycin's activity is not blocked by circulating 1405G rRNA methylases which are found with increasing frequency in NDM-1 carbapenemase-producing Enterobacteriaceae.[27, 37-40]

Apramycin demonstrates broad-spectrum *in vitro* activity against human isolates of multidrug-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, carbapenem-resistant Enterobacteriaceae, and *Staphylococcus aureus*,[15, 16, 35, 36, 41, 42] and rapid *in vitro* bactericidal activity.[43] It has also demonstrated *in vivo* activity against *A. baumannii*, *S. aureus*, and *Mycobacterium tuberculosis* in murine models.[43, 44] Therefore, based on previously demonstrated broad-spectrum activity and other compelling properties, we evaluated *in vitro* activity of apramycin against contemporary clinical strains of *Neisseria gonorrhoeae* as a first step in assessing whether apramycin or potential derivatives of apramycin might serve as future therapeutics against this problematic pathogen.

## MATERIALS AND METHODS

### Bacterial isolates

A total of 72 clinical isolates of *Neisseria gonorrhoeae* were tested against spectinomycin and apramycin. Forty-nine isolates were obtained from the FDA-CDC Antimicrobial Resistance Isolate Bank (https://www.cdc.gov/drugresistance/resistance-bank/). Twenty-one isolates were from the CDC Gonococcal Isolate Surveillance Program (GISP) Bank at Beth Israel Deaconess Medical Center (BIDMC). and were obtained from several locations in the United States (Chicago, IL; Minneapolis, MN; New York, NY; Boston, MA; and Erie, PA). Testing of de-identified GISP isolates was approved by the Institution Review Board at BIDMC. Three spectinomycin resistant isolates were obtained from the Culture Collection University of Gothenburg (CCUG): CCUG 15821 (WHO-A); CCUG 57601 (WHO-O) and CCUG 41811. ATCC 49226/F-18 and CDC F-28 are spectinomycin susceptible and resistant quality control

114 strains, respectively. These quality control strains were tested for spectinomycin susceptibility in
115 duplicate on each day of testing, and results were consistently within the acceptable range.
116 Among the FDA-CDC Antimicrobial Resistance Isolate Bank isolates, 100%, 82%, 100%, 2%,
117 80%, and 0% were non-susceptible to penicillin, ciprofloxacin, tetracycline, ceftriaxone,
118 cefpodoxime, and spectinomycin, respectively, based on CLSI susceptibility criteria (i.e., MIC >
119 0.06, 0.06, 0.25, 0.25, 0.5, and 32 mg/L, respectively) and strain MIC data.
120
121 **Agar dilution antimicrobial susceptibility testing**
122 Spectinomycin was obtained from Sigma Aldrich (St. Louis, MO, USA) or Alfa Aesar
123 (Tewksbury, MA, USA), and apramycin was obtained from Alfa Aesar. AST was performed
124 using the agar dilution (AD) method following CLSI guidelines and the CDC's GISP protocol
125 for AST of *N. gonorrhoeae*.[45, 46] From spectinomycin stock solutions, appropriate working
126 concentrations were prepared to achieve a range of test concentrations from 0.5 to 1024 mg/L.
127 Similarly, from apramycin stock solutions, appropriate working concentrations were prepared to
128 achieve a range of test concentrations from 0.5 to 256 mg/L.
129 For inoculum preparation, colonies of all *N. gonorrhoeae* isolates (including the QC
130 strains) from a chocolate agar plate (20 to 24 h of incubation) were suspended in Mueller-Hinton
131 broth to prepare a solution adjusted to a 0.5 McFarland standard density. The agar plates were
132 inoculated with 1 to 2 μL of each suspension using a Steers inoculum-replicating apparatus. Agar
133 growth control plates (no antimicrobial agent added) were inoculated at the beginning and end of
134 every test run to ensure that there was no contamination or antimicrobial carryover during
135 inoculation. The endpoints for determining the MIC by AD testing were interpreted as no visible
136 growth on an agar plate for a specific antimicrobial concentration. The CLSI categorical

interpretive criteria of ≤ 32 mg/L, susceptible; 64 mg/L, intermediate; and ≥ 128 mg/L were applied for spectinomycin.[24]

**Time-kill studies**

Time-kill studies were performed per CLSI recommendations[47, 48] with substitution of Wade-Graver liquid medium (WGM), as previously described,[17, 49] to permit robust growth of *N. gonorrhoeae.* Antibiotic stocks were diluted in 10 mL of WGM in 25 x 150 mm glass round-bottom tubes to achieve multiples of the MIC for each strain tested. To prepare the inoculum, 100 μL of a 0.5 McFarland suspension of colonies from an overnight Chocolate Agar plate (Remel, Lenexa, KS) were added to 5 mL of WGM, and incubated at 35°C in a 5% $CO_2$ incubator for eight to ten hours until log phase (i.e., 1 to 1.5 McFarland). The culture was then adjusted to a turbidity of 1.0 McFarland, and 200 μL was inoculated into each growth tube containing antibiotic dilutions.

During incubation of tube cultures on a shaker platform at 35°C, 5% $CO_2$ atmosphere, aliquots were removed at indicated time points, and tenfold serial dilutions prepared in 0.9% sodium chloride. A 10 μL drop from each dilution was spotted on a Chocolate Agar plate (54, 55) and incubated overnight. Drops containing 3 to 30 colonies were considered "countable" and used for cfu determination. If more than one dilution was countable, the cfu of the two dilutions was averaged. If no drops were countable, consecutive drops above and below the countable range were averaged. The limit of detection was 300 cfu/mL. Antibiotic carryover effect was not observed. Bactericidal activity was defined as a ≥3 $\log_{10}$ cfu/mL reduction sustained at 24 hours of incubation at ≤ 4 times the MIC determined by agar dilution (39, 56).

## Genomic analysis

We queried the AAC(3)-IV and ApmA protein sequence against all predicted proteins from *N. gonorrhoeae* available at the National Center for Biotechnology Information (NCBI) using the BlastP[50] algorithm with an expect value (e-value) cutoff of $< 10^{-10}$. All *N. gonorrhoeae* protein sequences available in the CARD Prevalence, Resistomes, and Variant database (https://card.mcmaster.ca/download), which uses a more conservative expect value threshold of $< 10^{-30}$, were also screened for matches to all known apramycin resistance determinants.[51, 52]

## RESULTS AND DISCUSSION

A total of 72 strains of *N. gonorrhoeae* were tested. MIC distributions for apramycin and spectinomycin are shown in Fig. 1A and 1B, respectively. The modal MICs for apramycin and spectinomycin were 16 mg/L and 32 mg/L, respectively. No categorical interpretative breakpoints are available for apramycin from either EUCAST or CLSI, and therefore categorical assessment was not made. An apramycin epidemiological cutoff value (ECOFF) of 64 mg/L was assigned based on visual inspection.[53] There were no strains with an apramycin MIC above this value suggesting absence of acquired resistance in the tested strain set.

For the 68 spectinomycin-susceptible strains of *N. gonorrhoeae*, 56 isolates (82%) had identical apramycin and spectinomycin MIC values; 13 isolates (19%) had a two-fold dilution lower apramycin MIC; and 3 isolates (4%) and 4 isolates (6%) had a two-fold and four-fold dilution higher apramycin MIC, respectively. Four known spectinomycin resistant isolates were tested and confirmed to be spectinomycin resistant (MIC > 1024 mg/L). WHO-O contains the C1192T spectinomycin resistance mutation in the 16S rRNA gene.[25] WHO-A contains the T22P spectinomycin resistance mutation in the ribosomal S5 protein (encoded by the rpsE gene).[25] The

183 mutations in F-28 and CCUG.41811 have not yet been characterized. Notably, high-level
184 spectinomycin resistance in these strains did not confer detectable cross-resistance to apramycin.
185 Two of the spectinomycin resistant isolates had an apramycin MIC of 16 mg/L, and two had an
186 apramycin MIC of 32 mg/L, consistent with findings in spectinomycin susceptible strains.

187 Four representative strains were tested in time-kill analysis including the ATCC type
188 strain F-18; spectinomycin resistant F-28; and FDA-CDC Isolate Bank strains 193 and 200.
189 Rapid, sustained bactericidal activity was observed for both apramycin and spectinomycin within
190 four hours with the exception, as expected, for spectinomycin in the spectinomycin-resistant
191 strain, F-28 (Fig. 2). Time-kill results were consistent with prior observations of rapid
192 bactericidal activity of spectinomycin[17, 54] and gentamicin[17] for *N. gonorrhoeae.* Our data
193 suggest that apramycin also exhibits similar bactericidal activity.

194 In Gram-negative organisms, a single aminoglycoside modifying enzyme, AAC(3)-IV,
195 has been described that inactivates apramycin through acetylation of the *C*-3 amine on the
196 deoxystreptamine ring.[55] The presence of this resistance element is rare, even in multidrug-
197 resistant organisms such as carbapenem-resistant Enterobacteriaceae and *A. baumannii*;
198 consistent with the infrequency of organisms with MICs above the ECOFF's for these
199 pathogens.[36, 43] A BLASTP[56] search performed August 12, 2018 for AAC(3)-IV found no
200 matches to *N. gonorrhoeae* among the 451 complete genomes and other *N. gonorrhoeae*
201 sequence available in the National Center for Biotechnology Information databases (NCBI).[51]
202 Similarly, no significant homology was found with ApmA, an aminoglycoside-modifying
203 enzyme, which also inactivates apramycin, and has been described recently in two
204 staphylococcal porcine isolates.[57, 58]

Of note, apramycin remains active in strains expressing ribosomal methylases that modify 16s rRNA at position G1405, in contrast to aminoglycosides currently used for human therapy and the novel aminoglycoside, plazomicin.[59] In contrast, activity of both apramycin and the aforementioned aminoglycosides are blocked by NpmA, identified in one *E. coli* clinical isolate, and KamB, found in aminoglycoside-producing *Actinomycetales*, that methylate 16s rRNA at position A1408.[60, 61] However, again BLASTP analysis did not identify any significant homology between these proteins and available *N. gonorrhoeae* sequence. Therefore, our analysis also indicates that, currently, A1408 ribosomal rRNA methylases, that would undermine apramycin activity, must be extremely rare to absent in *N. gonorrhoeae*. Furthermore, a search of the curated CARD Prevalence, Resistomes, & Variants database (Version 3.0.2) also did not identify apramycin resistance elements in the *N. gonorrhoeae* genomic sequence. Only a single kanamycin aminoglycoside modifying enzyme, APH(3')-Ia,[62] was identified at very low prevalence (0.24%) in the *N. gonorrhoeae* sequences available in the NCBI database.

Several limitations of our study should be noted. First, spectinomycin does not achieve sufficient pharyngeal levels for effective treatment of gonococcal pharyngitis,[25] although cure of pharyngeal infection with gentamicin used in combination azithromycin appears to occur.[21] Based on these observations, it is possible that apramycin, also a highly hydrophilic aminocyclitol, may have similar limitations. Second, isolates with reduced susceptibility to gentamicin, observed in regions where gentamicin is used for primary treatment,[19, 22] were not available to us. It is possible such reduced susceptibility, potentially based on decreased bacterial permeability or acquisition of efflux pumps, could the basis for cross resistance to apramycin, an issue that warrants further study. Furthermore, it is not yet established with what frequency spontaneous apramycin resistance would arise under direct selective pressure.

Taken together, the lack of acquired resistance (i.e., strains with MIC values above the ECOFF and genetic evidence for resistance elements), rapid bactericidal activity, and putative lack of typical aminoglycoside associated toxicities,[24] highlight the potential of apramycin, either directly and/or after derivatization, for development as an alternate treatment of multidrug-resistant *N. gonorrhoeae*. However, further experimental and human pharmacokinetic and pharmacodynamic studies are needed to determine whether efficacious drug levels can be obtained at sites of infection, and whether compelling dosing strategies, such as single, high-dose administration for cure, can be established.

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**TRANSPARENCY DECLARATIONS**

None to declare.

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## FIGURE LEGENDS

**Figure 1. Apramycin and spectinomycin MIC Distribution for *N. gonorrhoeae***

**Figure 2. Time-kill analysis.** Apramycin (APR) and spectinomycin (SPT) were tested at multiples of their respective MIC values indicated in parentheses in panel titles. A no antibiotic control and a doubling dilution series of increasing concentrations tested are indicated respectively by filled circles, open squares, open triangles, open inverted triangles, open diamonds, and open circles.. Specific concentrations tested in mg/L are indicated in respective panel legends. Both APR and SPT were bactericidal.

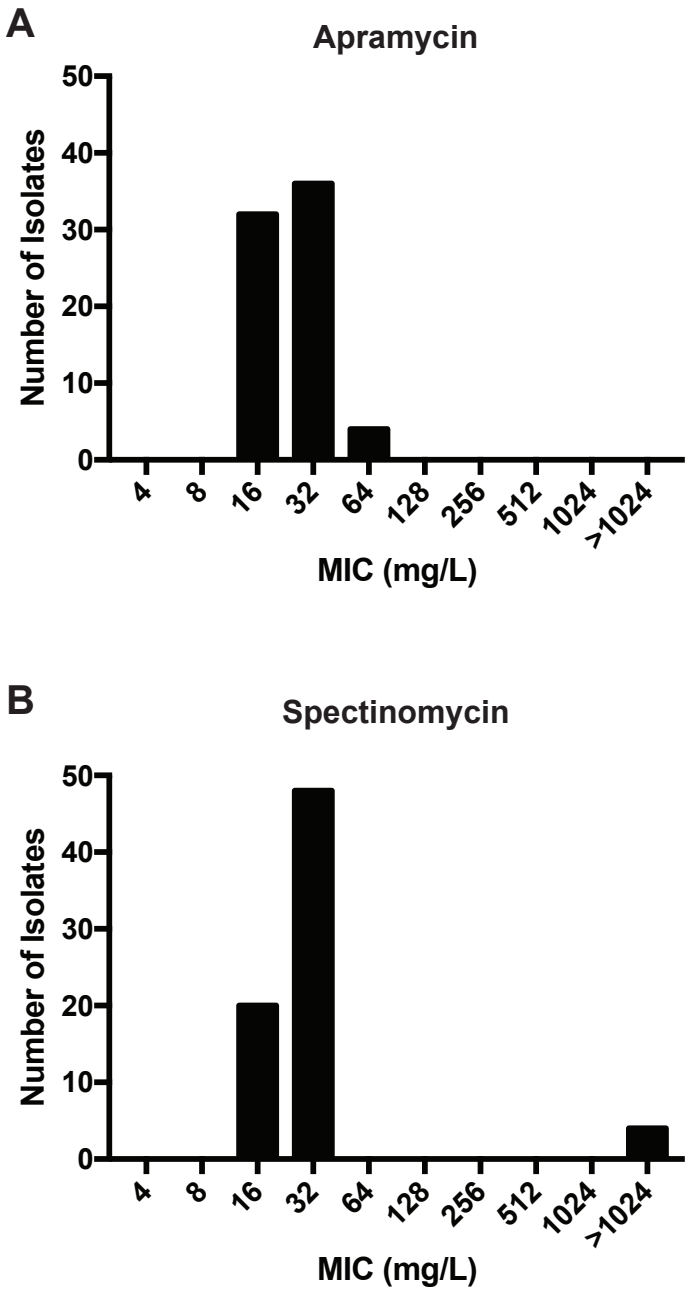
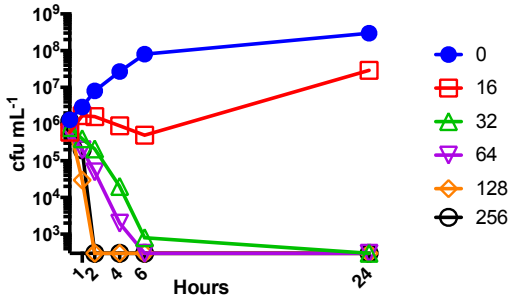
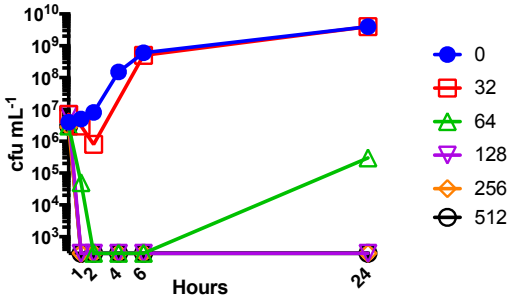



**Figure 1. Apramycin and Spectinomycin MIC distribution**

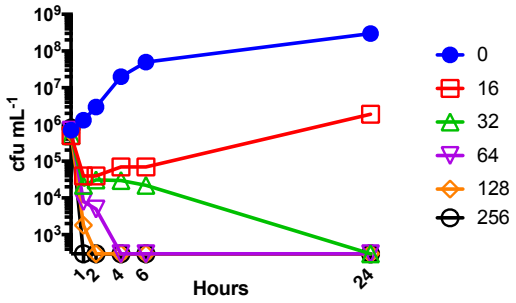
FDA-CDC-193 APR (16 mg/L)



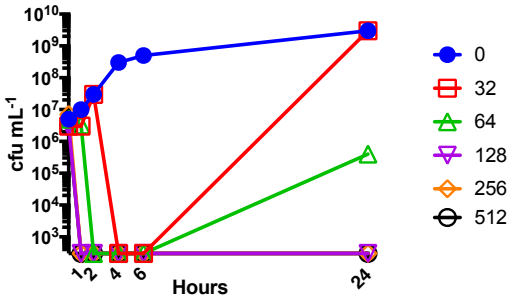

FDA-CDC-193 SPT (16 mg/L)



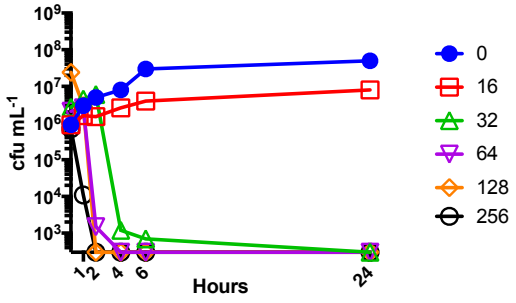

FDA-CDC-200 APR (16 mg/L)



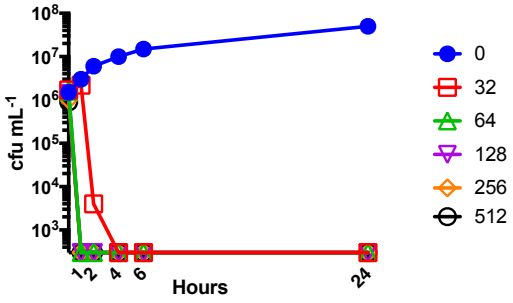

FDA-CDC-200 SPT (32 mg/L)



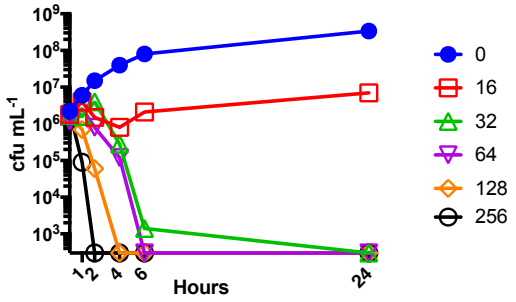

F-18 APR (32 mg/L)




F-18 SPT (32 mg/L)




F-28 APR (16 mg/L)




F-28 SPT (> 1024 mg/L)