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Original Citation:

Structure-Activity Relationship Studies of Acetazolamide-Based Carbonic Anhydrase Inhibitors with Activity against Neisseria gonorrhoeae / Hewitt C.S., Abutaleb N.S., Elhassanny A.E.M., Nocentini A., Cao X., Amos D.P., Youse M.S., Holly K.J., Marapaka A.K., An W., Kaur J., Krabill A.D., Elkashif A., Elgammal Y., Graboski A.L., Supuran C.T., Seleem M.N., Flaherty D.P.. - In: ACS INFECTIOUS DISEASES. -

Availability:

The webpage <https://hdl.handle.net/2158/1262679> of the repository was last updated on 2022-03-27T16:52:55Z

Published version:

DOI: 10.1021/acsinfecdis.1c00055

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(Article begins on next page)



Published in final edited form as:

ACS Infect Dis. 2021 July 09; 7(7): 1969–1984. doi:10.1021/acsinfecdis.1c00055.

Structure-activity relationship studies of acetazolamide-based carbonic anhydrase inhibitors with activity against *Neisseria gonorrhoeae*

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Abstract

Neisseria gonorrhoeae is an urgent threat to public health in the United States and around the world. Many of the current classes of antibiotics to treat *N. gonorrhoeae* infection are quickly becoming obsolete due to increased rates of resistance. Thus, there is a critical need for alternative antimicrobial targets and new chemical entities. Our team has repurposed the FDA-approved carbonic anhydrase inhibitor scaffold of acetazolamide to target *N. gonorrhoeae* and the bacteria's essential carbonic anhydrase, NgCA. This study established both structure-activity and structure-property relationships that contribute to both antimicrobial activity and NgCA activity. This ultimately led to molecules **20** and **23**, which displayed minimum inhibitory concentration values as low as 0.25 µg/mL equating to an 8- to 16-fold improvement in anti-gonococcal activity compared to acetazolamide. These analogs were determined to be bacteriostatic against the

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

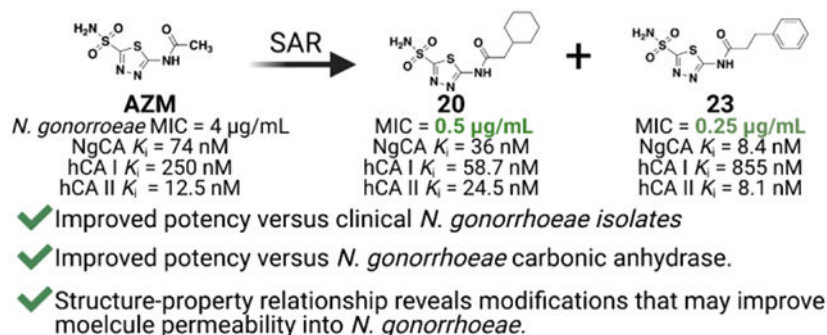
Supporting information

Supporting information is available free of charge at <https://pubs.acs.org>.

pathogen and likely on-target against NgCA. Additionally, they did not exhibit any detrimental effects in cellular toxicity assays against both a human endocervical (End1/E6E7) cell line or colorectal adenocarcinoma cell line (Caco-2) at concentrations up to 128 $\mu\text{g/mL}$. Taken together, this study presents a class of anti-gonococcal agents with the potential to be advanced for further evaluation in *N. gonorrhoeae* infection models.

Graphic:

Structure-activity relationship studies for acetazolamide-based carbonic anhydrase inhibitors with activity against *Neisseria gonorrhoeae*.



Keywords

Carbonic anhydrase inhibitors; *Neisseria gonorrhoeae*; antibiotics; drug discovery

Gonorrhea is a sexually transmitted disease caused by the bacterial pathogen *Neisseria gonorrhoeae* that colonizes urogenital, anal, and nasopharyngeal tissues. The World Health Organization (WHO) estimated there were 87 million new cases of gonorrhea in adults worldwide in 2016.¹ In the United States specifically, the Centers for Disease Control and Prevention (CDC) reported a 67% increase in gonorrhea cases between 2013 – 2018 with a record 583,404 cases reported in 2018 alone.^{2,3} However, this is believed to be an underestimation as gonorrhea can present as both symptomatic and asymptomatic. It is projected that asymptomatic colonization makes up more than half of the infected individuals at any one time, and it is this version that greatly promotes transmission of the pathogen.⁴ Both versions wreak havoc on world health care systems causing pelvic inflammatory disease, infertility and ectopic pregnancies.⁵ The bacteria can also be transmitted from mother to child during birth and lead to blindness.⁶ If left untreated, *N. gonorrhoeae* can cause blood infection known as gonococcemia resulting in disseminated gonococcal infection which can lead to a variety of clinical symptoms including skin infection, arthritis or endocarditis.^{7,8}

Pathogenic *N. gonorrhoeae* strains are increasingly resistant to common front-line antibiotics. The WHO surveillance program reports resistance to available antibiotics including β -lactams, tetracyclines and quinolones.⁹ Since 2010 the CDC has recommended treatment with a combination of oral azithromycin and intramuscular injection of ceftriaxone, to which resistance has been documented as well.¹⁰ For this reason, along with

potential negative affects the dual therapy has on commensal microbiota, the CDC has recently recommended using only a single 500 mg injection of ceftriaxone, removing azithromycin from the treatment regimen, for uncomplicated *N. gonorrhoeae* infection.¹¹ This recommendation leaves no effective oral therapeutic option. Rampant resistance has caused the CDC and the WHO each to classify *N. gonorrhoeae* as a superbug¹² and a future with an untreatable gonococcal infection is a real possibility.¹³ Therefore, the CDC has listed drug-resistant *N. gonorrhoeae* at the highest possible threat level to public health.¹⁴ Additionally, the WHO identifies *N. gonorrhoeae* as a high priority pathogen and has called for an international collaborative effort to combat the drug-resistant gonorrhea.¹⁰ Work to develop a vaccine toward *N. gonorrhoeae* is still in the discovery phase and questions remain regarding the effectiveness of immune response in mucosal membranes¹⁵. Furthermore, existing antibiotics such as delafloxacin and the clinical molecule solithromycin both were investigated against gonorrhea in clinical trials, but neither met the criteria for non-inferiority relative to current treatment options.^{16,17} There is some progress being made as the oral agents zoliflacin¹⁸ and gepotidacin¹⁹ have each passed Phase 2 clinical trials for treatment of uncomplicated gonorrhea and are entering Phase 3. Nonetheless, there remains an inadequate number of both antibacterial agents and molecular targets for treating gonorrhea and underscores the critical unmet need for safe and effective oral alternatives.

Carbonic anhydrases (CAs) are a group of zinc-metalloenzymes that consist of α , β , and γ -sub-families, among others, found in all kingdoms of life. CAs catalyze the essential reaction of converting carbon dioxide (CO_2) and water to bicarbonate, HCO_3^- , and a proton.²⁰ In humans there are 16 α -CA isoforms with broad tissue distribution that carry out this reaction. The reaction is relevant in many physiological processes such as transport of CO_2 from metabolizing tissues to excretion in the lungs,²¹ maintaining pH and CO_2 homeostasis in various tissues,²² and regulating electrolyte secretion in various tissues and organs.^{23–25} These roles have made CAs prime drug targets as FDA-approved carbonic anhydrase inhibitors are used to treat various disorders including glaucoma,^{26,27} as a diuretic for kidney health,²⁸ treatment for congestive heart failure,²⁹ and recently certain isoforms have gained momentum as promising cancer targets.^{30–32}

Aside from human targets, CAs have been identified in a handful of parasites and pathogenic bacteria,^{33,34} including *Vibrio cholera*,³⁵ *Burkholderia spp*,^{36,37} and *N. gonorrhoeae*. In bacteria, altering bicarbonate homeostasis was revealed to perturb the proton motive force and reduce bacterial fitness.³⁸ It was first observed in 1967 that *Neisseria spp*, including *N. gonorrhoeae*, were susceptible to the FDA-approved CAs acetazolamide and ethoxzolamide (**AZM** and **EZM**, Figure 1).³⁹ Thirty years later the *N. gonorrhoeae* CA (NgCA) was first cloned⁴⁰ and a crystal structure was published a year later.⁴¹ NgCA was further characterized enzymatically⁴² and genomic data identified NgCA as an essential enzyme in *N. gonorrhoeae* in 2014.⁴³ NgCA is classified as an α -CA that resides may reside in the periplasmic space, is required for maintaining CO_2 and pH homeostasis for the organism and has been suggested to be a valuable new drug target to treat gonorrhea.³³ However, until now, there have been no reported efforts to target NgCA as a means to combat this pathogen. Thus, building on our previous work to target carbonic anhydrases in vancomycin-resistant enterococci (VRE),⁴⁴ we report carbonic anhydrase inhibitors that display sub-nanomolar

activity against NgCA and potency of $< 1 \mu\text{g/mL}$ toward various *N. gonorrhoeae* clinical isolates. Herein, our efforts for evaluation of this carbonic anhydrase inhibitor scaffold for activity against *N. gonorrhoeae* and NgCA, off-target activity at human CAs, mechanism of action, and human cell toxicity are reported.

RESULTS

Chemistry

Analogues in this study (**1** – **31**) were synthesized previously as part of our efforts to develop inhibitors for VRE.⁴⁴ In brief, **AZM** was used as the starting reagent for this series of molecules. The acetamide group was cleaved under acidic conditions to provide the free amine. This amine then served as a diversification point for amide coupling or reductive amination reactions to yield the final analogues for testing in the various assays (Scheme S1 and S2). Analogues **32** and **33** were purchased from commercial vendors and characterized to confirm identity and purity. Details are provided in supporting information.

For the purposes of a fluorescence polarization (FP) assay to assess inhibitor binding to NgCA and human carbonic anhydrases (hCAs), a fluorescein-linked **AZM** probe (**FITC-AZM**, Figure 2A) was designed. This molecule was synthesized starting with intermediate **1** and coupling with 6-azidohexanoic acid to form the azide intermediate **35** (Scheme S3). This intermediate was then reduced using H_2 and Pd/C and the corresponding free amine was coupled with fluorescein isothiocyanate (5-isomer) to provide the desired **FITC-AZM** probe utilized in the FP assays.

Carbonic anhydrase fluorescence polarization assay

Analogues were assessed in two orthogonal assays to determine inhibitory constants (K_i) against NgCA: 1) a fluorescence-polarization (FP) competition assay and 2) a catalytic CO_2 hydration activity assay.^{45,46} The FP assay provides a more accessible means to assess K_i compared to the catalytic CO_2 hydration assay, which requires stopped-flow instrumentation. However, it should be noted that the K_i determined from FP is from competition of a fluorophore-labeled tracer while the K_i from the hydration assay is generated from inhibition data. Nonetheless, both assays were performed in parallel to understand how analogues bind to or inhibit NgCA *in vitro*.

The dissociation constants (K_d s) for **FITC-AZM** to NgCA, as well as human carbonic anhydrase I (hCA I) and II (hCA II), were determined by titration of protein while maintaining the concentration of probe and polarization values were recorded according to published protocols.^{47,48} The titrations for NgCA (Figure 2B, top panel) and hCA II (Figure 2C, top panel) were fit to a one site – specific binding non-linear regression model and dissociation constants were calculated (hCA I shown in Figure S1). These K_d values were used to select optimal conditions to maximize dynamic range for a competitive FP assay (**FITC-AZM** and protein concentrations shown in Table S1). Next, analogues were tested in dose-response to determine an IC_{50} value for displacement of **FITC-AZM** from each carbonic anhydrase with data for **AZM** shown in Figure 2B and C (bottom panels; hCA I shown in Figure S1). The K_i values for all analogues were determined by entering the resulting

IC₅₀ values, probe K_i s, and protein concentrations into the FP K_i equation developed by Nikolovska-Coleska et al⁴⁹ and are presented in Table 1. The IC₅₀ values, 95% confidence intervals and K_i s for all CAs tested for each analog are provided in Table S2.

Structure-activity relationship studies

Analogues were tested for antimicrobial activity versus the azithromycin-resistant clinical *N. gonorrhoeae* strain CDC 181 to determine minimum inhibitory concentrations (MICs) and the aforementioned NgCA FP and CO₂ hydration assay in support of structure-activity relationship studies. **AZM** displayed an initial MIC value of 4 µg/mL and K_i values of 24 nM and 74.1 nM for the FP and CO₂ assays, respectively (Table 1). Removal of the acetamide group in **1** provided no change in antimicrobial activity or CO₂ hydration K_i , but did reduce the FP K_i significantly. The K_i s for this analog reinforce the caveats related to the FP assay when compared to the catalytic assay as one assay is measuring the competition with **FITC-AZM** while the other is measuring the competition with CO₂. Increase of lipophilic bulk in place of the methyl group on **AZM** reduced activity versus *N. gonorrhoeae* as the substituents were ranked in terms of potency in the order of methyl (**AZM**) > ethyl (**2**) > *iso*-propyl (**3**) > *tert*-butyl (**4**). Interestingly, the opposite trend was observed for the K_i values as the methyl and ethyl derivatives were the least potent, particularly in the CO₂ hydration assay. Extending the alkyl chain away from the carbonyl by a single carbon for analogs **5** - **8** did not result in a discernible change in antimicrobial potency; however, these analogs were observed to be more active against NgCA. The *n*-hexyl derivative **9** displayed an improvement over **AZM** with an MIC value of 2 µg/mL and this was the first molecule that paired improved antimicrobial activity with improved activity against NgCA (CO₂ hydration K_i = 8.5 nM). Among this first set of analogs it was observed that linear alkyl chains were preferred over branched alkane counterparts for anti-gonococcal activity; however, the branched alkane generally outperformed the nearest neighbor linear alkane analogs in the NgCA K_i assays.

The next set of analogs featured cyclic alkanes and provided an interesting trend as both the smallest ring-size, cyclopropyl (**10**), and largest ring size, cyclohexyl (**13**), displayed MIC values of 2 µg/mL. The intermediate ring sizes lagged in activity with the cyclopentyl (**12**) MIC at 8 µg/mL and the cyclobutyl (**11**) at 16 µg/mL. The K_i values for the CO₂ hydration assay also followed this general trend as the cyclobutyl analog **11** was 3- to 4-fold less potent against NgCA compared to the other ring sizes while the cyclohexyl **13** was the most potent (CO₂ K_i values of 42.3 and 9.8 nM, respectively). The same antimicrobial trend (cyclopropyl = cyclohexyl > cyclopentyl > cyclobutyl) was observed again for the nearest neighbor analogs in which a methylene was inserted between the cyclic alkane and the carbonyl (**17** – **20**). This latter set of cycloalkane substituted analogs yielded the most potent of the study to this point as both the cyclopropyl (**17**) and cyclohexyl (**20**) containing derivatives displayed MIC values of 0.5 µg/mL each, an 8-fold improvement over **AZM**. The SAR regarding the *in vitro* NgCA activity was relatively flat for this set as most remained in the 30 – 35 nM range, with the exception of the cyclobutyl derivative **18**, which was the most potent in the CO₂ hydration assay but the least potent in the FP assay.

The trend of alkyl branching that led to reduced antimicrobial activity was again observed for analogs **13** – **15**. The cyclohexamide derivative **13** exhibited an MIC value of 2 µg/mL and was among the most potent against NgCA (CO₂ K_i = 9.8 nM) while installation of a methyl at the 1-position carbon to provide a quaternary carbon directly adjacent to the carbonyl (**14**) resulted in reduction of both antimicrobial and NgCA activities (MIC > 64 µg/mL; K_i = 75.8 nM), although NgCA potency was still comparable to **AZM**. Moving the methyl to the 4-position on the cyclohexane ring (**15**) ring also resulted in an 8-fold reduction of antimicrobial activity compared to **13** but improved NgCA potency to K_i = 8.7 nM. These modifications generally maintained *in vitro* activity against NgCA and suggest the presence of a quaternary carbon may have an effect on *N. gonorrhoeae* permeability. Meanwhile, substitution of an aromatic phenyl group (**16**) in place of the cyclohexane provided a two-fold reduction of antimicrobial activity and 7-fold reduction of NgCA activity. However, the opposite trend was observed when the pendant group was extended away from the carbonyl by two methylenes as the phenyl derivative **23** outperformed the cyclohexyl containing **22** by 4-fold; thus, yielding the most potent anti-gonococcal agent in this study with an MIC value of 0.25 µg/mL. Three of the most potent analogs in terms of antimicrobial activity (**21** – **23**) were also among the most potent with regards to K_i against NgCA.

Interestingly, insertion of heteroatoms, such as nitrogen and oxygen, into the cyclic alkane ring systems retained activity versus NgCA with the K_i range of 30 – 60 nM. However, these changes abolished antimicrobial activity as the polar pendant groups such as pyrrolidine (**24**), piperidine (**25**), morpholine (**26**), and piperazine (**27**) all had MICs values > 64 µg/mL. The importance of the carbonyl on the amide for antimicrobial activity was also noted as three analogs (**28** – **30**) containing an amine linkage, rather than amide, all exhibited significant reduction of antimicrobial activity with MIC values of > 64 µg/mL. Yet again, these analogs all displayed NgCA inhibitory activity comparable to **AZM** and other active analogs. This set of derivatives emphasizes the disconnect that often may arise between *in vitro* inhibitory data and whole-cell bacterial efficacy in Gram-negative pathogens, suggesting the molecules may have reduced permeability properties in *N. gonorrhoeae*.

To round out the SAR observations with respect to antimicrobial activity three analogs were tested with modifications to other regions of the scaffold (Figure 3). The first substituted a sulfone (**31**) in place of the sulfonamide and this molecule was inactive against *N. gonorrhoeae*. The next two analogs contained alterations to the central thiadiazole heterocyclic core of the scaffold. Analog **32** replaced the 4-nitrogen directly flanking the sulfonamide substituent with a carbon resulting in a thiazole scaffold and led to an 8.5-fold reduction of NgCA potency in the CO₂ hydration assay and a complete loss of anti-gonococcal activity. The same was true for analog **33** in which the thiadiazole was replaced with a phenyl aromatic core.

To summarize the observed SAR, there was a clear trend that increased alkyl branching, particularly directly adjacent to the amide carbonyl, was detrimental to the anti-gonococcal activity. This effect was diminished as the branched carbon was extended away from the carbonyl. Cyclic alkanes provided mixed results with the cyclopropyl and cyclohexyl derivatives being preferred. Extending these cyclic alkanes away from the carbonyl

improved activity by approximately 4-fold. The best performing analogs in combined antimicrobial and NgCA activity were **20**, **22**, and **23**, of which each contained at least one-methylene linker with either a cyclohexyl or phenyl pendant group. Insertion of nitrogen or oxygen into the cyclic alkane rings was not tolerated for antimicrobial activity and the carbonyl was also shown to be essential for potency, even though these modifications had no impact on NgCA activity.

Anti-gonococcal activity against additional drug-resistant and -sensitive *N. gonorrhoeae* strains

The activity of two of the best performing analogs from the SAR study, **20** and **23**, as well as **AZM** and azithromycin were assessed against a broad panel of 30 *N. gonorrhoeae* clinical isolates (strain information in Table S3). As shown in Table 2, analog **20** was the most potent overall displaying MIC values ranging from 0.06 – 2 µg/mL, inhibiting 50% of the isolates tested (MIC₅₀) at the concentration of 0.5 µg/mL and 90% of the tested isolates (MIC₉₀) at 2 µg/mL. Analog **23** and **AZM** provided a range of MICs from 0.5 µg/mL to 4 µg/mL, with a lone strain for **23** dropping to 0.25 µg/mL. Both molecules displayed the same MIC₅₀ and MIC₉₀ values of 2 and 4 µg/mL, respectively. Azithromycin inhibited the tested strains at concentrations ranging from 0.125 µg/mL to 16 µg/mL, with a lone strain not exhibiting an MIC > 64 µg/mL. Azithromycin displayed MIC₅₀ and MIC₉₀ (1 µg/mL and 4 µg/mL, respectively) values comparable to **AZM**, **20** and **23**. Although the MIC₅₀ and MIC₉₀ values for **20** are modest improvements over azithromycin, the analog showed the potential to outperform azithromycin against many isolates by 2 – 16-fold. These results also indicate the molecules exhibited similar potencies against azithromycin-sensitive and -resistant strains.

Mechanism of action studies

Previous studies have suggested **AZM**'s antimicrobial properties against *N. gonorrhoeae* may be dependent on inhibition of the essential α -carbonic anhydrase, NgCA.³⁹ CO₂ is the substrate for carbonic anhydrases; therefore, high levels of the gas will out compete an inhibitor of NgCA and the bacteria should exhibit reduced susceptibility.^{39,50} To confirm that the new analogs maintained on-target activity at NgCA we performed MIC assays for *N. gonorrhoeae* in both standard lab culture conditions in ambient atmosphere and in conditions that contain 5% CO₂. This assay was performed against two strains of *N. gonorrhoeae* and the results for representative analogs are presented in Table 3. MIC values for multiple strains of *N. gonorrhoeae* in presence and absence of CO₂ are presented in Table S4.

It was observed that in two strains of *N. gonorrhoeae*, one susceptible to azithromycin and one resistant, that the efficacy of **AZM** and analogs **20** and **23** was significantly reduced in the presence of CO₂. As a control to ensure that the CO₂ conditions did not cause unintended drug-resistance to drugs with a different mechanism of action, azithromycin was also tested and displayed no change in efficacy against *N. gonorrhoeae* strain CDC 178 when cultured in CO₂ conditions compared to normal ambient air. The analogs did show weak antimicrobial activity (range of 16 – 32 µg/mL) in the CO₂ conditions against a few strains (Table S4), however, the difference between ambient and CO₂ conditions was still an 8- to 16-fold reduction in potency. It is too early to determine if this result indicates a

secondary target and further testing is taking place to understand the difference in carbonic anhydrase inhibitor susceptibility in these strains. Nonetheless, these results suggest the primary intracellular target for the sulfonamide scaffold is likely NgCA.

The analogs next were assessed in a killing kinetics assay to determine whether the scaffold exhibits bacteriostatic or bactericidal activity against *N. gonorrhoeae* ATCC 70085 strain. The positive control azithromycin displayed bactericidal activity consistent with previous reports (Figure 4).^{51–53} The carbonic anhydrase inhibitors all displayed bacteriostatic properties against *N. gonorrhoeae* over the time course of the experiment and were found to have significantly reduced the bacterial burden compared to DMSO (negative control). After 24 hours, analogs **20** and **23** reduced *N. gonorrhoeae* load by 1.9- and 2.6- $\log_{10}$ units, respectively. At the 24-hour time point, **20** and **23** were found to reduce the bacterial CFU count by 3- and 3.8- $\log_{10}$, respectively as compared to DMSO. The analogs **20** and **23** also outperformed **AZM** reducing the bacterial burden by 2.4- and 3.1- $\log_{10}$ -reduction, respectively as compared to **AZM**. It is worth noting that the CFU count for analog **20** slightly increased at 24 hours. We isolated the colonies and performed MIC assay on these isolates and did not find any shift in the MIC values. Consequently, this rebounding at 24 hours could be attributed to the concentration of **20** potentially becoming diminished below the MIC at that timepoint and suggests frequent dosing for analog **20**.

Inhibition of human carbonic anhydrases

Humans express at least 16 α -carbonic anhydrase (hCA) isoforms in various tissues.⁵⁴ Given that these enzymes are ubiquitous throughout the human body, it is critical to gain an understanding for how the new analogs inhibit these enzymes to assess potential drug distribution and toxicity liabilities. To do this, we measured the K_i values for all analogs versus two representative hCAs; hCA I and hCA II. These hCAs were chosen as they are widely distributed in various tissues including in erythrocytes. This factor may affect systemic distribution of the molecules *in vivo* as hCA I and hCA II have been shown to act as a sink and partition carbonic anhydrase inhibitors into erythrocytes as opposed to plasma.⁵⁵ NgCA shares 26.7% sequence identity with hCA I and 25.6% identity with hCA II; however, there is high identity of residues within the active sites (alignment shown in Figure S2).

In general, most analogs maintained nanomolar range activity against both hCAs. **AZM** was found to be almost 6-fold more selective for hCA II over NgCA while it was less selective for hCA I (Table 4). Although the selectivity window was able to be narrowed to approximately equipotent values between NgCA and hCA II, rarely was it observed that the new analogs were more potent against NgCA. In fact, only four analogs (**9**, **12**, **13**, and **15**) exhibited greater than 1.5-fold selectivity for NgCA over hCA II. Analog **22** and **23**, among the most potent in terms of antimicrobial activity, were also among the most potent against both NgCA and hCA II, with **22** reaching sub-nanomolar potency against each.

An increase in the selectivity window was able to be achieved over hCA I. The initial K_i value for **AZM** versus hCA I was 250 nM, equating to 3.3-fold selectivity for NgCA. Analog **22** and **23** each exhibited reduced hCA I activity (hCA I K_i = 945 and 855 nM,

respectively). This, coupled with improved activity toward NgCA, provided selectivity windows in favor of NgCA that were > 1,300-fold for **22** and 100-fold for **23**.

Several analogs provided carbonic anhydrase targeting data points even though they were inactive against the bacteria. It is interesting to note that the polar analogs **24** – **27** were essentially equipotent against NgCA but became increasingly more potent against hCA I and II. To this point, the *N*-methylpiperazine analog **27** was the least active against NgCA (K_i = 63.6 nM) but had single-digit nanomolar potency toward the human isoforms (K_i values for hCA I and II of 9.6 and 1.6 nM, respectively). The effect of the amide carbonyl on K_i also differed among the three carbonic anhydrases. Removal of the amide carbonyl provided little change in K_i (3-fold or less) versus NgCA across three analogs **28** – **30** when compared to the carbonyl containing counterparts. Removal of the carbonyl had a greater effect versus the human carbonic anhydrases with **30** representing the least potent analog against both hCA I and II (K_i values of 1,623 and 104 nM, respectively). This amounts to a reduction of activity against hCA I by almost 15-fold compared to the matched molecular pair analog **16**.

Two analogs explored modification to the central thiadiazole core. Molecule **32**, in which a nitrogen is replaced with a carbon to provide a thiazole core, displayed an 8.5-fold reduction in NgCA activity compared to **AZM** with a K_i value of 632 nM. This also led to a 7.7-fold decrease in potency against hCA II (K_i = 97.2 nM). The modification had less effect on hCA I with only about 1.8-fold decrease in activity. Finally, replacing the heterocyclic core with a phenyl core (**33**) was still not preferred for any of the carbonic anhydrases with approximately 5-fold loss in K_i across all three carbonic anhydrases.

In summation, the SAR trends generally aligned for NgCA and hCA II while there was greater flexibility for improvement of selectivity against hCA I. The propensity for analog potency to align more between NgCA and hCA II is likely attributed to the conservation of active site threonine residue, Thr204 in NgCA and Thr200 hCA II. This residue was been shown to participate in polar interactions with the ligand, particularly the 4-nitrogen of the thiadiazole as shown in the **AZM** complex with hCA II⁵⁶ (PDB: 3HS4). Alternatively, hCA I has a histidine at that corresponding residue location (PDB: 1AZM) that is shown to participate in π – π stacking interactions with **AZM** that are weaker than hydrogen-bonds.⁵⁷ Thus, the likely conservation of the hydrogen bond between ligands and the threonine accounts for the observation NgCA and hCA II potency tracks together. Future work will incorporate structure-based design to build in selectivity against the human carbonic anhydrases.

Assessment of human cell toxicity for analogs **20** and **23**

To be considered as viable leads the molecules should display little-to-no toxicity against relevant human cell lines at concentrations greater than the MIC values. Thus, to prepare for future *in vivo* experiments an assessment of cell toxicity using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was performed in two human cell lines. *N. gonorrhoeae* is known to invade epithelial cells of the genital tract and cross the epithelial barrier into the subepithelial space⁵⁸; therefore, one cell line used in the assay was endocervical End/E6E7 cells. The second cell line used were human

colorectal adenocarcinoma epithelial cells (Caco-2) to determine potential for detrimental effects on the gastrointestinal tract upon oral dosing for future *in vivo* efficacy assays. Cells were dosed at concentrations of 8 – 128 µg/mL and assessed for viability. Compounds **20** and **23** displayed no toxic effects against either End1/E6E7 endocervical or Caco-2 cells compared to DMSO control at doses up to 128 µg/mL after 24 hours of incubation at 37 °C (Figure 5). These concentrations represent more than 256-fold and 64-fold higher values than the respective MIC₅₀s for **20** and **23**. It should be noted that Caco-2 cells have high endogenous expression of human CA XIII⁵⁹, to which **AZM** has a *K_i* value of 16 nM.⁶⁰ Literature search could not identify the human CA isoforms present in the End1/E6E7 cell line; however, a report suggests a related cell line studied for endocervical cancer expresses human CA IX.⁶¹ Regardless, the molecules derived in this study showed no adverse cell toxicity at the highest doses tested.

DISCUSSION

An investigation of a set of carbonic anhydrase inhibitors for efficacy against *N. gonorrhoeae* was carried out. Previous studies had reported that **AZM** possessed antimicrobial activity against *N. gonorrhoeae* and it was hypothesized that the intracellular target was the α-carbonic anhydrase, NgCA.³⁹ With this in mind, our team assessed a set of carbonic anhydrase inhibitors developed by our lab for activity against *N. gonorrhoeae*. It was shown that these molecules have anti-gonococcal properties with MICs ranging from 0.06 µg/mL – 32 µg/mL. Analog **23** displayed the greatest potency against the azithromycin-resistant *N. gonorrhoeae* strain CDC 181 followed by analogs **17** and **20**. The antimicrobial effects generated by these molecules are significantly reduced when higher levels of CO₂ are present within the culture suggesting the activity is likely mediated primarily via NgCA inhibition. Even with this hypothesis we must note that sulfonamide containing drugs used to be a common therapeutic option to treat gonorrhea in the 1930's through mid-1940's and they were determined to inhibit bacterial dihydropteroate synthase (DHPS);⁶² however, by the late-1940's >90% of gonococcal strains were resistant to sulfonamides.^{63–65} If a second target was potentially contributing to the antimicrobial activity of the molecules presented herein, like DHPS, we would expect analogs to maintain an appreciable level antimicrobial activity in higher CO₂ levels, as in the case of azithromycin. Further, in order to investigate the possibility of DHPS inhibition as a target for our analogs we tested the antibacterial activity of sulfamethoxazole, sulfadoxine, and sulfathiazole against three *N. gonorrhoeae* strains. As depicted in Table S5, sulfa drugs did not inhibit the bacterial strains tested, under both CO₂ and non-CO₂ conditions at concentrations up to >64 µg/mL while CAIs – **AZM**, **20**, and **23** – inhibited the test strains under non-CO₂ conditions with MIC values ranging from 0.125 µg/mL to 1 µg/mL.

Preliminary evidence based on previous literature and the reduced susceptibility to the molecules in CO₂ conditions suggests that the intracellular target for these molecules is likely NgCA. Specific analog SAR observations may also support this hypothesis. First, the observation that the modification of the sulfonamide to a sulfone abrogates both antimicrobial activity as well as *in vitro* activity against all three CAs tested supports this claim. Structural and mechanism of action studies for CAIs have shown that inhibition is mediated primarily through coordination of the active site Zn²⁺ by a chelating moiety,^{66–68}

in this case a sulfonamide. Our data shows that modification of this functional group (as in analog **31**) eliminates both the ability of the molecules to bind the target NgCA (Figure 6), and in turn reduces antimicrobial activity, supporting the hypothesis that the intracellular target is NgCA.

Another SAR data point that supports this hypothesis is that modification of central thiadiazole ring reduced activity in both assays. Analog **32** investigated the role of the 4-nitrogen in the thiadiazole ring on both *in vitro* CA and antimicrobial activity. The nitrogen is documented to participate in a hydrogen bond with the side chain of Thr200 in hCA II⁵⁶ and this threonine is conserved in NgCA (Figure S2). Modification to the thiazole ring in **32** reduced activity in both NgCA and hCA II by 5–6-fold compared to **AZM**, suggesting the change had the same detrimental effect on each CA and that these analogs may interact with NgCA similar to how CAIs bind to hCA II. This resulted in **32** displaying the second highest K_i in the CO₂ hydration assay behind the sulfone derivative described above. If the concurrent loss in NgCA potency and antimicrobial activity are related it further bolsters the claim that NgCA is the intracellular target. However, this analysis does not account for any potential changes to *N. gonorrhoeae* compound permeability that could also affect the antimicrobial potency. Studies are ongoing to quantify bacterial permeability of analogs in *N. gonorrhoeae* and to confirm on-target inhibition in a cellular context.

Observed SAR on the amide functionality for antimicrobial activity (Figure 6) generally preferred linear alkanes with reduced steric bulk. The impact of alkyl branching was most noticeable on analogs with the branched carbon directly next to the carbonyl. This is illustrated by the congeneric series of analogs ranking in order of activity of H (**1**) = methyl (**AZM**) > ethyl (**2**) > *iso*-propyl (**3**) > *tert*-butyl (**4**) with increased alkyl branching trending to poorer MIC values. A modification that appears to have a large impact on antimicrobial efficacy is the presence of a quaternary carbon. Three analogs that possess quaternary carbons (**4**, **8**, and **14**) were all less potent in antimicrobial assays than their counterparts that contained reduced alkyl branching. For example, when comparing the cyclohexyl containing analog **13** (MIC = 2 µg/mL) there was a steep decrease in activity for the quaternary carbon containing nearest analog **14** (MIC > 64 µg/mL). With respect to *in vitro* NgCA activity the increase in alkyl bulk generally improved inhibition of the enzyme. Among the linear alkane analogs the *tert*-butyl representatives, **4** and **8**, displayed the most potent K_i values (30.5 and 9.1 nM, respectively) within their respective congeneric series yet were the least potent in antimicrobial assays. This trend for *in vitro* activity was not observed with the cyclohexyl containing matched molecular pair **13** and **14**. The quaternary carbon containing analog **14** was less potent against NgCA compared to **13**. However, **14** still maintained NgCA activity (K_i = 75.8 nM) comparable to **AZM**, and many other analogs, yet the molecule was inactive in antimicrobial assays at concentrations up to 64 µg/mL. Conversely, the tertiary carbon derivative **13** displayed an MIC value of 2 µg/mL. It would seem the divergence in NgCA and antimicrobial activity among these analogs indicates that increased alkyl branching at these positions may be detrimental to the permeability of the molecules to access the intracellular target.

Interestingly, the hypothesis that increased alkyl branching could reduce permeability may be further supported by SAR observations against Gram-positive VRE for this same set of

analogs. As previously reported for this series the trend of increasing alkyl branching steadily improved antimicrobial activity against the *Enterococcus faecium*.⁴⁴ Direct comparison of MIC values between the two pathogens are shown in Table 5. For example, against *E. faecium* strain HM-965 the two congeneric series that increased alkyl branching yielded stepwise improvements to anti-enterococcal activity, while the opposite was observed in *N. gonorrhoeae* as described above. These analogs are hypothesized to inhibit the α -CA in *E. faecium* (α -EfCA) and *in vitro* data collection against this enzyme is ongoing. Therefore, there are currently no *in vitro* potency comparisons that can be made for the analogs between NgCA and α -EfCA, nonetheless, based on sequence data alignment (Figure S2) the sequence identity within the two active sites is 67% with key residues known for CAI interaction, the threonine in particular, remaining intact. Thus, we would suspect similar *in vitro* potency trends to be observed for these analogs in both NgCA and α -EfCA. If this assumption is indeed shown to be true then the variance in antimicrobial potency between the Gram-positive and Gram-negative pathogens may be less due to difference in target inhibition and more so due to differences in accessibility to the intracellular targets. The physicochemical properties that drive permeability of molecules into *N. gonorrhoeae* is an active arm of study within this project and may illuminate the properties necessary for improved permeability.

Another observation may link cyclization of alkyl functional groups to improved *N. gonorrhoeae* permeability. In general, if a tertiary carbon was branched as part of a linear alkane the antimicrobial activity was less potent than if that tertiary center was incorporated within a cycloalkyl ring. For example, comparing the *iso*-propyl analog **3** (MIC = 16 μ g/mL) with cyclized matched molecular pairs listed in order of increasing ring-size: **10**, **11**, **12**, and **13** were comparable or much improved in antimicrobial activity compared to the *iso*-propyl counterpart all while displaying similar NgCA activity. This observation suggests alkane cyclization may improve permeability compared to the linear alkanes and will be investigated further.

The discrepancy between whole-cell antibacterial activity and *in vitro* data against NgCA, particularly for the molecules that are not active against the pathogen, suggests a physicochemical property of these inactive molecules may cause reduced permeability into the Gram-negative bacteria. We attempted to rationalize this difference in activity by applying the eNTRY rules for Gram-negative *Escherichia coli* bacteria developed by Richter et al⁶⁹. All molecules were submitted for analysis using the online portal associated with the Richter manuscript, www.entry-way.org. Almost all molecules analyzed fit at least two of the three criteria that are correlated with improved Gram-negative permeability, those being globularity < 0.25 and < 5 rotatable bonds. However, none of the analogs contain a primary amine and there were no clear trends for any metrics that would correlate with antimicrobial activity (spreadsheet with values provided in supporting information). The antimicrobial active sulfonamide analogs in the present work lack a primary amine, or any positive charge for that matter. In fact, the sulfonamide of **AZM** has a reported pKa of 7.2⁷⁰ while the proton on the amide has a predicted pKa of 6.6 (MarvinSketch version 18.28, ChemAxon, <https://chemaxon.com>). These values indicate the majority of molecules in solution would carry a net negative charge at physiological pH and this, in theory, may limit their ability to

enter through the such porins. However, it is important to note that these rules were developed in *E. coli* as the model organism and there likely may be differences in the porins between *E. coli* and *N. gonorrhoeae* that could limit the applicability of the eNTRY rules to *N. gonorrhoeae*. For example, the current drug of choice to treat gonococcal infection is ceftriaxone, which is marketed as a di-sodium salt, and would be predominantly anionic at physiological pH. Additionally, previous reports have demonstrated negatively-charged molecules are capable of flux through the two porins associated with *N. gonorrhoeae*,⁷¹ although these studies were performed in liposomal systems with recombinantly express porins and not in a whole-cell context. Nonetheless, these studies and ours indicate the presence of a primary amine and avoidance of a negatively charged analog may not be as important for permeability in *N. gonorrhoeae*.

Even though the scaffold may not fully adhere to the eNTRY rules it does appear to have an intrinsic ability to permeate the Gram-negative outer membrane even while lacking a positive charge. Previous work by O'Shea and Moser looked into the effect both cLogD_{7.4} and total polar surface area (tPSA) had on Gram-negative active FDA-approved antibiotics.⁷² To investigate whether these metrics have an effect on the activity of our analogs we used the program QikProp (Meastro release 2020-4; Schrödinger, LLC; New York, NY) to generate the predicted octanol/water partition coefficient (QLogPo/w) and the tPSA for each analog. We then plotted these metrics in a 3-dimensional scatter plot with tPSA on the X-axis, QLogPo/w on the Y-axis and MIC value on the Z-axis (Figure 7). We introduced a 4th dimension in bubble size that corresponds to the K_i values for each analog against NgCA in the CO₂ hydration assay. We then added a 5th dimension by color coding the bubbles into cohorts according to the following: green = analogs with MIC < 64 µg/mL and NgCA K_i < 100 nM; red = polar pendant groups (**24** – **27**); yellow = contain quaternary carbon (**4**, **8**, and **14**); gray = no carbonyl on the amide (**28** – **30**); and violet = NgCA K_i > 400 nM (**32** and **33**). Analog **31** was left out of the analysis as the sulfone is known to abrogate carbonic anhydrase activity and it was inactive against the bacteria. We also colored **AZM** black as a point of reference from the hit molecule.

What was observed is that molecules maintaining physicochemical properties of QLogPo/w between 0 and -2 and tPSA between 120 and 135 Å² (Figure 7, green spheres) as well as NgCA K_i < 100 nM generally maintained antimicrobial activity against *N. gonorrhoeae* at MIC values ≤ 16 µg/mL. Exceptions to this trend were analogs **4**, **8** and **14** (yellow spheres), which contained quaternary carbons and maintained sub-100 nM K_i versus NgCA but displayed MICs of 32, 16, and >64 µg/mL, respectively. In fact, analog **8** (K_i = 9.1 nM) was among the most potent of the entire study against NgCA but had an MIC of only 16 µg/mL; however, within this congeneric series the MIC values did correlate to NgCA potency. These observations, along with those discussed prior, suggest that perhaps the quaternary carbon limits molecule permeability into *N. gonorrhoeae*. Exceptions were also noted for a few analogs containing a tertiary carbon, but these analogs never had MIC values worse than 16 µg/mL and reduced antimicrobial potency was not applicable to all tertiary carbon containing analogs.

In the O'Shea and Moser analysis, the authors noted that for sulfa-class antibacterials the average tPSA was 112 Å², which would suggest that the tPSA lower limit for Gram-negative

permeability for this scaffold is likely $< 120 \text{ \AA}^2$. Five analogs in our set had tPSA values below this threshold but were still inactive against *N. gonorrhoeae*. Digging deeper, two of these analogs, **32** and **33** (purple spheres), had steep reductions in NgCA activity ($K_i > 400 \text{ nM}$) that may explain the reduced antimicrobial activity. The other three analogs below $120 \text{ \AA}^2$ with no antimicrobial activity were observed to lack a carbonyl on the amide (**28 – 30**, gray spheres). These molecules maintained potent NgCA activity but did not have antibacterial effect. It is unclear if the removal of the carbonyl reduces permeability; however, this modification would increase both the rotatable bond count and flexibility of the analogs, two trends that would be detrimental to Gram-negative permeability according to the eNTRY rules.

Finally, four analogs containing polar pendant groups (**24 – 27**) are presented in red spheres. These analogs also were active against NgCA in the CO_2 hydration assays at comparable levels to the rest of the set; however, they were inactive against the bacteria. In observing where these molecules reside in terms of physicochemical property space, they are the most polar with QPLogPo/w < -1.4 and tPSA $> 135 \text{ \AA}^2$. Thus, it is reasonable to presume that the increased polarity of these molecules has crept outside of the range necessary for *N. gonorrhoeae* permeability. While this initial analysis offers clues to the properties that may provide entry into *N. gonorrhoeae*, a more exhaustive study to correlate structure-property relationship with permeability into the bacteria is necessary.

The analog set maintained potent activity against the two representative human carbonic anhydrases, hCA I and hCA II. At this point of the project, it is unclear whether this would be a liability for the class. Generally, FDA-approved carbonic anhydrase inhibitors are relatively non-toxic and require high doses before they exhibit side-effects.⁷³ For example, a phase 1 trial showed that 90% of patients tolerated $> 1 \text{ g/day}$ of **AZM** for 6 months with 45% tolerating up to 4 g/day over the same time frame.⁷⁴ Moreover, when side effects are observed in the clinic they tend to be attributed to age^{75,76} or drug-drug interaction with salicylate.⁷⁷ Furthermore, a common length of an antimicrobial drug dosing regimen would likely be in the range of days to a few weeks at most. In theory, this acute dosing timeline could reduce the impact of any negative side-effects associated with inhibition human carbonic anhydrases. Even though inhibition of human carbonic anhydrases may not prove to be a liability in terms of toxicity, it could have a profound effect on molecule distribution *in vivo*. Carbonic anhydrase inhibitors are known to partition quite readily into red blood cells that contain hCA I and hCA II, which effectively forms a sink to sequester the drug.^{55,78} Thus, our ongoing project will be focused on reduction of binding to human isoforms as this would likely lead to improved pharmacokinetics and decrease the size of the dose needed for any *in vivo* effect.

In additional antimicrobial testing, the carbonic anhydrase inhibitors showed potential that was on par or better than azithromycin. Analog **20** consistently outperformed azithromycin against a panel of 30 *N. gonorrhoeae* isolates and posted improved MIC_{50} and MIC_{90} values compared to the positive control. While azithromycin was bactericidal, **AZM**, **20** and **23** were found to possess bacteriostatic properties over the course of a 24-hour incubation and reduced CFU load of *N. gonorrhoeae* by least 3-log units compared to the DMSO treated controls. When tested for cytotoxicity in human cells the scaffold was shown to be non-toxic

against the human endocervical cell line End1/E6E7 and Caco-2 cell lines at concentrations up to 128 µg/mL. Moreover, both **20** and **23** also retain favorable drug-like physicochemical properties with molecular weights around 300 Da and low predicted QPLogPo/w's. While these molecules are yet to be assessed in *in vitro* ADME experiments, the structurally similar analog **22** has been tested in various assays. This molecule exhibited solubility > 30 µM in PBS, was highly permeable across a Caco-2 monolayer, and was detected in plasma of mice after 10 mg/kg oral dosing.⁴⁴ It did show a propensity to be modified in human liver microsome assay with 62% of the molecule remaining after 1 hr. Analogs **20** and **23** occupy similar chemical property space compared to **22** so we would expect these molecules to possess a similar *in vitro* ADME and *in vivo* pharmacokinetic profile to **22**, although these experiments are planned to confirm this. Nonetheless, the favorable physicochemical properties of the analogs in this study may translate to reduced risk for triage due to toxicity and/or solubility as they are advanced beyond pre-clinical studies.

Even though these molecules represent promising starting points for validating carbonic anhydrase inhibition as a viable strategy to target *N. gonorrhoeae* there are improvements still to be made. Future work will include investigation of properties that drive permeability into the bacteria as well as structure-guided design of new inhibitors to improve NgCA activity, reduce hCA activity and limit sites of metabolic liability. Ultimately, these future directions will advance the scaffold to experiments to assess the molecule for *in vivo* efficacy animal models for *N. gonorrhoeae* infection.

CONCLUSION

We report a new class of sulfonamide-based carbonic anhydrase inhibitors with antimicrobial efficacy against the drug-resistant pathogen *N. gonorrhoeae*. The original hit **AZM** displayed an MIC of 4 µg/mL and subsequent analogs produced a range of activity against the bacteria with **20** and **23** rising above the rest to exhibit MIC values as low as 0.25 µg/mL. This translated to an MIC₅₀ value of 0.5 µg/mL for **20** against a panel of 30 clinical isolates and it outperformed azithromycin with respect to antimicrobial activity. It differed from azithromycin in terms of killing kinetics as the carbonic anhydrase inhibitors were found to be bacteriostatic while azithromycin is bactericidal. Moreover, we hypothesize the intracellular target for the molecules is likely the α-carbonic anhydrase, NgCA. Several SAR trends were observed in both MIC and *in vitro* inhibition assays and at times these SAR trends diverged in opposite directions when comparing antibacterial activity to NgCA potency. Additional analysis of structure-property relationship relative to antimicrobial activity also highlighted the potential roles polarity and structural modifications, such as quaternary carbons and lack of an amide carbonyl, may have on permeability across *N. gonorrhoeae* outer membrane. Ultimately, analogs that lacked a quaternary carbon and maintained tPSA < 135 Å² trended toward increased potency in both antimicrobial and NgCA assays. Analogs were also assessed against two representative human carbonic anhydrases and were shown to maintain appreciable activity against those enzymes. Finally, molecules **20** and **23** were shown to be non-toxic against two relevant human cell lines. The data provided highlights the potential of the class of molecules to be added to the arsenal of effective therapeutics for the treatment of *N. gonorrhoeae*.

METHODS

Chemistry

All analogs tested for activity in antimicrobial assays and *in vitro* binding assays were previously synthesized as part of a related project reported by Kaur et al.⁴⁴ All synthetic procedures can be found in that publication. Characterization and purification data for all analogs, as well as the synthetic procedure for **FITC-AZM**, are provided in the supporting information of this manuscript. Spectral data for **FITC-AZM** is also provided.

Biological Evaluation

Bacterial strains, media and chemicals—*N. gonorrhoeae* strains (Table S3) used in the study were clinical isolates obtained from the CDC and the American Type Culture Collection (ATCC). Drugs used in this study were purchased from chemical vendors: acetazolamide (Sigma-Aldrich, MO) and azithromycin (TCI America, OR). Compounds were synthesized in our lab and prepared in stock concentrations in DMSO. Media and reagents were purchased commercially: brucella broth, IsoVitaleX and chocolate II agar (Becton, Dickinson and Company, MD), yeast extract and dextrose (Fisher Bioreagents, NJ), protease peptone and nicotinamide adenine dinucleotide (NAD) (Sigma-Aldrich, MO), hematin, tween 80 and pyridoxal (Chem-Impex International, IL) and phosphate buffered saline (PBS) (Fisher Scientific, MA).

Antibacterial activity of synthesized compounds against *N. gonorrhoeae* strains—The minimum inhibitory concentrations (MICs) of acetazolamide and analogs were determined using the broth microdilution assay, as previously described.^{51–53} Briefly, *N. gonorrhoeae* strains were grown overnight on chocolate II agar plates. Bacterial cells were then suspended in phosphate-buffered saline (PBS) to achieve a turbidity equivalent to a 1.0 McFarland standard which was diluted in brucella broth supplemented with yeast extract, dextrose, protease-peptone, NAD, pyridoxal, hematin and IsoVitaleX to reach a bacterial count of about 1×10^6 CFU/mL. Drugs and compounds were added and serially diluted along the plates. Media containing bacteria (without test agents) were included in the assays as a control. Plates were then incubated aerobically, and in presence of 5% CO₂ for 24 hours at 37 °C prior to recording the MIC as observed visually. MICs reported are the minimum concentrations of the compounds and drugs that completely inhibited the visual growth of bacteria.

Transformation of plasmid into *E. coli*—The His-NgCA plasmid in a pET-15b vector was ordered from GenScript and transformed into BL21(DE3) competent *E. coli* cells (New England Biolabs, catalog no. C2527I) This was plated onto an ampicillin agar plate and grown overnight at 37 °C. Colonies were picked from the agar plates and grown overnight in LB media at 37 °C with shaking at 250 rpm. These cultures were used to make glycerol stocks by mixing 20% glycerol and 80% culture and placed in –80 for future protein expressions.

Recombinant expression and purification of NgCA—His-NgCA starter cultures were made in autoclaved LB media containing 100 µg/mL ampicillin from the glycerol stock

(made as described above) and grown at 37 °C with shaking overnight at 250 rpm. An aliquot of 10 mL of the starter culture was inoculated into each liter of autoclaved LB media containing 100 µg/mL ampicillin and 1 mM $ZnCl_2$. These cultures were grown at 37 °C and shaken at 250 rpm. Upon reaching an OD_{600} of 0.4 – 0.8, the cultures were induced with 300 µL of 1.0 M isopropyl β -D-thiogalactopyranoside (IPTG) and then grown overnight at 16 °C with shaking at 150 rpm. Cultures were spun down at 4000 x g for 20 minutes. Bacterial pellets were resuspended in lysis buffer (1 x PBS containing 1 mM DTT). The bacterial cells were then lysed by sonication. Lysed cells were pelleted by centrifuging them at 14000 x g for 1 hour. The supernatant of the pelleted His-NgCA sample was loaded onto a nickel-NTA column pre-equilibrated with 50 mM Tris, 150 mM NaCl, and 1 mM DTT pH = 7.4. His-NgCA was eluted from the column using a gradient of 0-500mM imidazole in the same equilibration buffer. Fractions were collected and analyzed by SDS-PAGE to determine which fractions containing the His-NgCA. These fractions were combined and dialyzed at 4 °C against 50 mM Tris, 150 mM NaCl containing 1mM DTT pH = 7.4. NgCA was concentrated using Amicon Ultra Centrifugal Filters and further purified through size-exclusion chromatography with an S100 column using running buffer [50mM Tris, 150mM NaCl, and 1mM DTT (pH 7.4)].

Determination of the K_d of FITC-AZM to NgCA, hCA I, and hCA II—All fluorescence polarization binding assays were performed in 384-well black bottom plates using an assay buffer consisting of 50 mM Tris, 150 mM NaCl, and 1mM DTT (pH 7.4). His-NgCA was purified as described above and hCA I and hCA II were ordered from Millipore Sigma (hCA I Catalog# C4396-5MG; hCA II Catalog # C6624-500UG) and diluted in assay buffer to concentrations used in the assay in assay buffer. The initial K_d of the probe to His-NgCA, hCA I, and hCA II were determined by titrating the respective proteins (at 2 x concentration) into 25 µL solution containing 20 nM probe for final concentration in the well of 10 nM **FITC-AZM** and protein concentrations ranging in 14 dilutions ranging from 0 – 2.0 µM. Each concentration of each protein was performed in technical triplicate. Each K_d determination assay plate was covered in foil and incubated together with shaking for 15 minutes at room temperature. After 15 minutes, an additional incubation of 15 minutes was completed without shaking. Assay plates were then read on a Synergy Neo 2 (BioTek) using FP settings (excitation wavelength 485 nm and emission wavelength of 530 nm) with autogain setting on probe alone control wells (autogain polarization values set to 20 and intensity set to 10,000). After a baseline correction analysis, the polarization values were plotted against the protein concentrations and a one-site specific binding model (Prism 9.0.0) was fit to the data to determine the K_d . The protein concentration utilized in the competitive-FP assay was determined at ~70% asymptotic polarization values in this initial binding experiment.

Competitive Fluorescence Polarization Assay with Carbonic Anhydrase Inhibitors—The competitive FP assay was performed with final protein assay concentrations of 50 nM His-NgCA or 50 nM hCA I or 125 nM hCA II (20 µL of 2.5x concentrations were first added to the wells, along with necessary controls as detailed below. 20 µL of 25 µM probe was then added to the protein in a dark room. Subsequently, 10 µL of a titration of carbonic anhydrase inhibitor was added in a dark room. Controls for this assay

consisted of 1) probe alone, 2) probe + protein, 3) highest concentration of inhibitor alone, and 4) highest concentration of inhibitor + probe. Each concentration of inhibitor was performed in technical triplicate. Controls 3 and 4 were used to determine if there were any internal fluorescence/polarization effects of the inhibitor or if any quenching of the probe was occurring and none was observed. Experimental data was input into Prism 9.0.0 software. Concentrations were transformed to log₁₀ based values and the control 1 and 2 (listed above) were normalized to 0 and 100% probe bound to protein, respectively. The data was then fit to non-linear response (log [inhibitor] vs. normalized response) from which the IC₅₀s were derived. Estimations of K_i s were completed by inputting the IC₅₀ values for each inhibitor, protein concentration, **FITC-AZM** concentration and K_d into the FP K_i calculation derived by Nikolovska-Coleska et al.⁴⁹

Carbonic anhydrase CO₂ hydration catalytic assay and K_i determination—The assay was performed according to previously published protocols.^{46,79–81} Recombinant hCA I and hCA II were purchased from Millipore Sigma (hCA I Catalog# C4396-5MG; hCA II Catalog # C6624-500UG). K_i values were determined from inputting the IC₅₀ values into the Cheng-Prusoff⁸² equation for K_i from catalytic inhibition constants.

Killing kinetics assay—In order to determine the mode of killing of carbonic anhydrase inhibitors and analogs a standard time kill kinetics assay was performed against *N. gonorrhoeae* ATCC 700825 as described previously.^{83–85} *N. gonorrhoeae* was grown in brucella supplemented broth to logarithmic phase and further diluted to reach an initial inoculum of 6×10^6 CFU/mL. Acetazolamide, analogs and azithromycin (positive control) were then added (at $10 \times$ MIC in triplicates), and further incubated in the ambient air at 37°C for 24 hours. Bacteria exposed to DMSO (solvent of drugs) alone served as a negative control. An aliquot from each sample was collected from each treatment after the corresponding times of incubation and subsequently serially diluted and plated onto chocolate II agar plates. Plates were incubated for 24 h at 37°C before viable CFU/mL was determined.

Cytotoxicity assessment against human endocervical End1/E6E7 and Caco-2 cell lines.—Compounds were assayed (at concentrations of 8, 16, 32, 64 and 128 µg/mL) against human endocervical (End/E6E7) cells to determine their potential toxic effect to the human endocervix cells *in vitro*. Briefly, cells were cultured in serum-free keratinocyte medium (KSFM) supplemented with 0.05 mg/ml bovine pituitary extract, 0.1 ng/ml epidermal growth factor, and 44.1 mg/liter calcium chloride, at 37 °C with CO₂ (5%). The cells were incubated aerobically with the compounds (in triplicates) in a 96-well plate at 37 °C for 24 hours. Control cells received DMSO (the solvent of the compounds) alone at a concentration equal to that in drug-treated wells to determine the baseline measure of the cytotoxic impact of the compounds. The assay reagent MTS 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, WI, USA) was subsequently added and the plate was incubated for three hours. Absorbance readings (at OD₄₉₀) were recorded using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA).

For human colorectal adenocarcinoma (Caco-2) cell line toxicity the assay went as described previously.⁴⁴ Briefly, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids (1X), penicillin-streptomycin at 37 °C with CO₂ (5%). Control cells received DMSO (the solvent of the compounds) alone at a concentration equal to that in drug-treated wells to determine the baseline measurement of the cytotoxic impact of the compounds. The cells were incubated with the compounds (in triplicates) in a 96-well plate at 37 °C with 5% CO₂ for 24 hours. The assay reagent MTS (Promega, Madison, WI, USA) was subsequently added and the plate was incubated for four hours. Absorbance readings (at OD₄₉₀) were recorded using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA).

For each cell line, the quantity of viable cells after treatment with each compound was expressed as a percentage of the viability relative to DMSO-treated control cells (average of triplicate wells $\pm$ standard deviation). A two-way ANOVA with post-hoc Dunnett's test for multiple comparisons determined no statistically significant difference between treatments with **20** or **23** as compared to DMSO-treated cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank Jason Scott for assistance in computing QikProp metrics. The research program was partially funded by a Purdue Institute for Drug Discovery Programmatic Grant (M.N.S and D.P.F.) and NIH/NIAID 1R01AI148523 (M.N.S and D.P.F.). This work was also supported by the Italian Ministry for University and Research, grant FISR2019_04819 BacCAD (C.T.S.). Figures created using [Biorender.com](https://biorender.com).

Dr. Flaherty also dedicates this work to his mentor Professor Jonathan L. Vennerstrom, who has demonstrated an unwavering commitment to combating neglected tropical disease with passion and humility, on the occasion of his 65th birthday.

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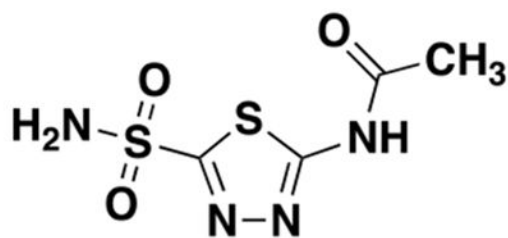
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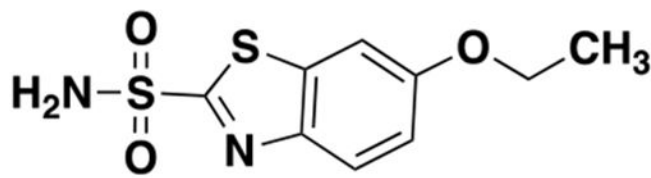
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Synopsis:

Hewitt et al. report the characterization of a set of carbonic anhydrase inhibitors based on the FDA-approved scaffold of acetazolamide for activity against the Gram-negative pathogen *Neisseria gonorrhoeae*. The in vitro inhibition constants against the *N. gonorrhoeae* carbonic anhydrase and two human carbonic anhydrase isoforms are reported. The article provides insight into the structure-activity relationship for this class of antimicrobial agents and identifies structural modifications that may reduce permeability into the Gram-negative pathogen.



Acetazolamide (AZM)



Ethoxzolamide (EZM)

Figure 1.
FDA-approved carbonic anhydrase inhibitors with antimicrobial activity against *N. gonorrhoeae*.

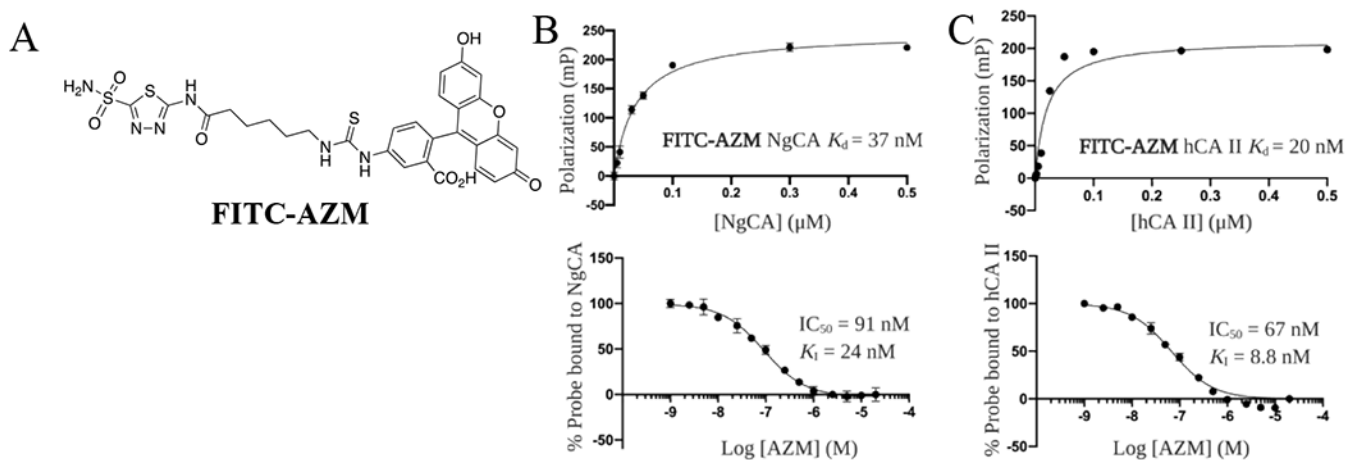
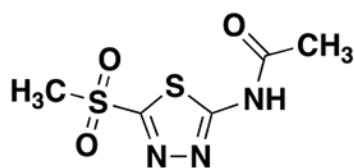
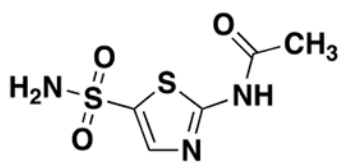


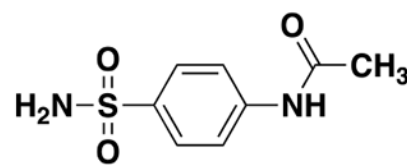
Figure 2. Fluorescent probe and FP assay development. **(A)** Structure of **FITC-AZM** fluorescent probe. **(B)** Titration curve of NgCA with 10 nM **FITC-AZM** (top), Competition assay for **AZM** to displace **FITC-AZM** probe from NgCA (bottom). **(C)** Titration curve of hCA II with 10 nM **FITC-AZM** (top), Competition assay for **AZM** to displace **FITC-AZM** probe from hCA II (bottom).

**31**

MIC > 64 $\mu\text{g/mL}$
FP K_i = >10,000 nM
CO₂ Hyd K_i = n.t.

**32**

MIC > 64 $\mu\text{g/mL}$
FP K_i = 2,410 nM
CO₂ Hyd K_i = 632 $\pm$ 41 nM

**33**

MIC > 64 $\mu\text{g/mL}$
FP K_i = 3,610 nM
CO₂ Hyd K_i = 439 $\pm$ 25 nM

Figure 3.

Analogs **31** – **33** against *N. gonorrhoeae* CDC 181 and NgCA K_i values.

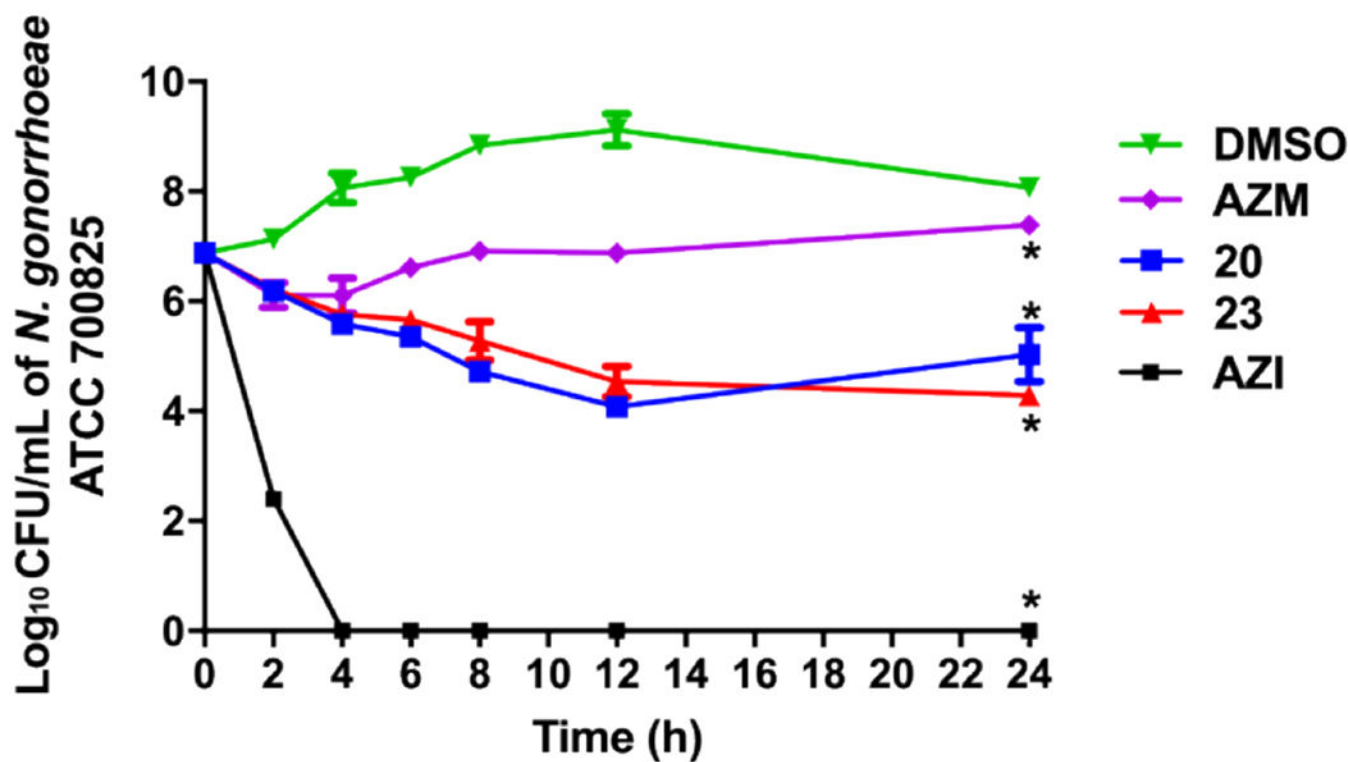


Figure 4.

Time-kill assay of carbonic anhydrase inhibitors and azithromycin (tested in triplicates, at $10 \times$ MIC) against *N. gonorrhoeae* ATCC 700825. DMSO (vehicle) served as a negative control. The error bars represent standard deviation values for each test agent studied. The data were analyzed via a two-way ANOVA with post-hoc Dunnett's test for multiple comparisons. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) between treatment with drugs/compounds compared to DMSO treatment (negative control).

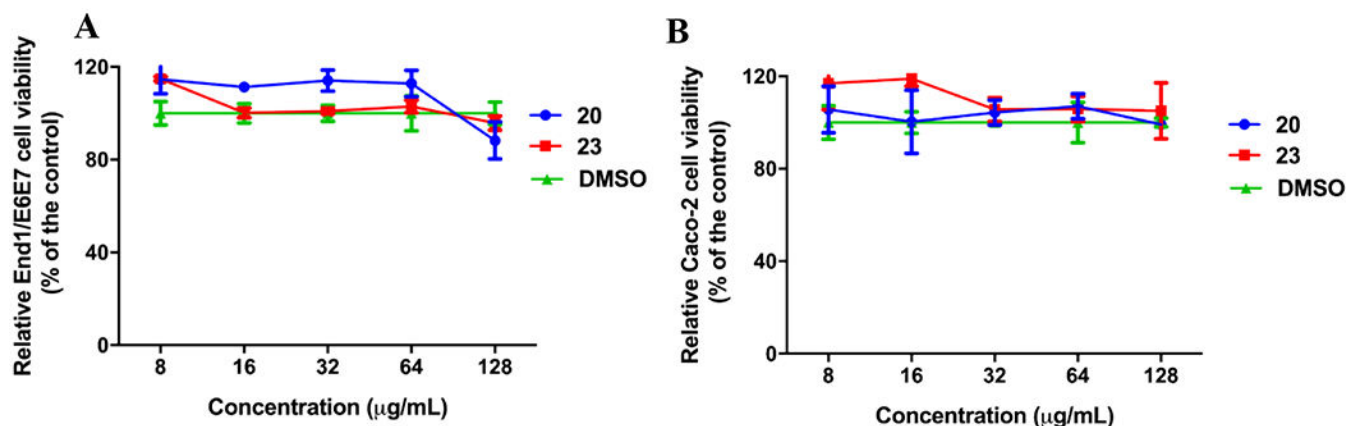


Figure 5.

Human cell toxicity of carbonic anhydrase inhibitor analogs **20** (blue) and **23** (red) compared to DMSO control (green). **(A)** Cell viability in human endocervical (End1/E6E7) cells using the MTS; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. **(B)** Cell viability in human colorectal adenocarcinoma (Caco-2) cells. Results are presented as percent viable cells relative to DMSO (negative control to determine a baseline measure for the cytotoxic impact of each compound). The absorbance values represent an average of three samples analyzed for each compound. Error bars represent standard deviation values. Data were analyzed via a two-way ANOVA with post hoc Dunnett's test for multiple comparisons.

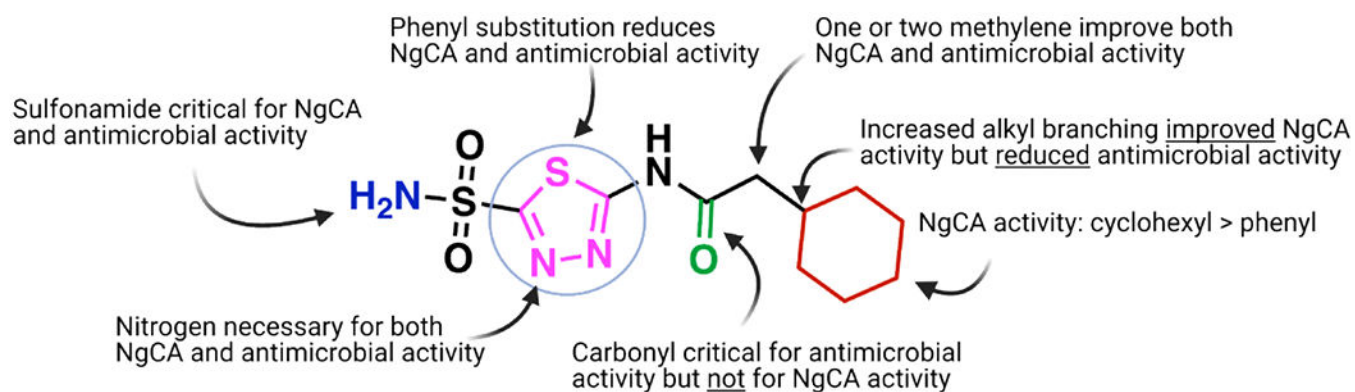


Figure 6.
Summary of SAR observations as they relate to either NgCA activity and antimicrobial activity.

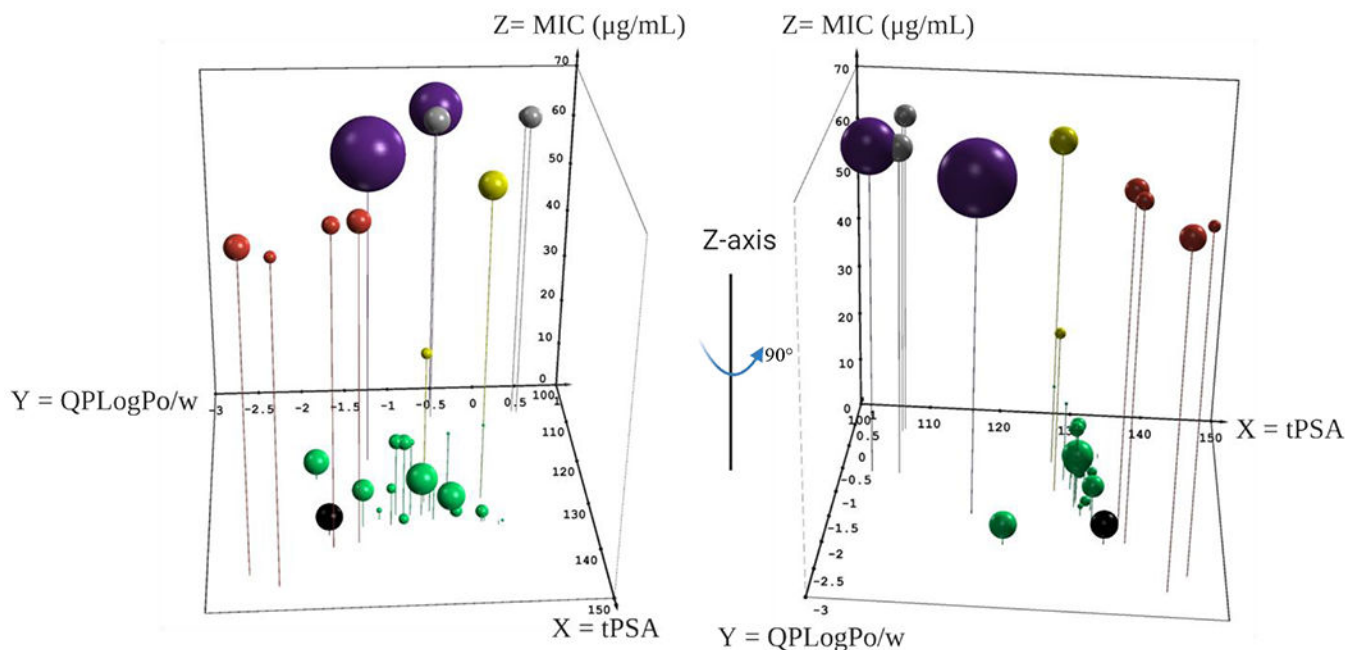


Figure 7.

Three-dimensional scatterplot to assess the relationship between physicochemical properties and antimicrobial activity. QikProp metrics tPSA ($\text{\AA}$, X-axis), QLogPo/w (Y-axis) and MIC ($\mu\text{g/mL}$, Z-axis). Size of bubble corresponds to K_i (nM) versus NgCA as determined by CO_2 hydration assay. Analogs with: $< 64 \mu\text{g/mL}$ and $< 100 \text{ nM } K_i$ (green); contain quaternary carbon (yellow); polar pendant groups (red); no carbonyl (gray); and reduced NgCA activity (violet). **AZM** is shown in black. Plot was made in Excel using the 5dchart add-in (www.5dchart.com).

Table 1.

Minimum inhibitory concentrations (MICs) and NgCA K_i values for AZM-based analogs against *N. gonorrhoeae* 181

NgCA K_i (nM)					NgCA K_i (nM)				
Cpd	R	MIC ^a	FP ^{b,c}	CO ₂ Hyd ^d	Cpd	R	MIC ^a	FP ^{b,c}	CO ₂ Hyd ^d
AZI	-	>64	n.t.	n.t.	15		16	n.d.	8.7 ± 0.7
AZM		4	24	74.1 ± 3.2	16		4	673	78.6 ± 5.2
1		4	602	73.2 ± 3.9	17		0.5	56.1	29.3 ± 2.1
2		8	35.2	59.8 ± 2.1	18		8	106	5.8 ± 0.3
3		16	15.5	41.0 ± 3.0	19		1	n.d.	34.6 ± 2.5
4		32	21.6	30.5 ± 1.1	20		0.5	n.d.	36.6 ± 2.0
5		8	12.6	27.5 ± 1.8	21		1	n.d.	6.7 ± 0.4
6		8	n.d.	87.9 ± 5.0	22		1	n.d.	0.70 ± 0.05
7		16	1027	17.7 ± 0.9	23		0.25	n.d.	8.3 ± 0.4
8		16	n.d.	9.1 ± 0.6	24		>64	557	45.2 ± 3.0
9		2	n.d.	8.5 ± 0.4	25		>64	89.4	61.4 ± 4.9
10		2	19.4	15.2 ± 0.6	26		>64	35.3	29.9 ± 1.4
11		16	20.4	42.3 ± 1.6	27		>64	62.0	63.6 ± 2.4
12		8	7.8	14.1 ± 0.8	28		> 64	615	73.9 ± 3.0
13		2	63.2	9.8 ± 0.4	29		> 64	944	42.1 ± 2.9
14		> 64	1,594	75.8 ± 5.0	30		> 64	243	62.0 ± 3.9

AZI = azithromycin, AZM = acetazolamide.

^aMIC values against *N. gonorrhoeae* strain CDC 181 in µg/mL.

^bFluorescence-polarization competition K_i values determined from the mean of one experiment performed in triplicate and IC₅₀ values input into equation from Nikolovska-Coleska et al⁴⁹. Based on the calculation theory and assay parameters the values obtained have a reliable lower limit of 33 nM for IC₅₀. Any IC₅₀ value < 33 nM unable to generate accurate K_i and thus is listed as not determined, n.d.

^cIC₅₀ values obtained from FP competition assay presented in Table S2 and expressed as both mean and as 95% confidence interval to demonstrate precision.

^dCatalytic CO₂ hydration assay K_i determined from the mean of one experiment in triplicate and IC₅₀ values entered into Cheng-Prusoff equation⁸². Values reported are ± standard error of the mean.

Table 2.MICs for Selected Analogs against a panel of *N. gonorrhoeae* clinical isolates

<i>N. gonorrhoeae</i> strains	MIC (µg/mL)			
	AZM	20	23	AZI
CDC 165	4	1	4	2
CDC 166	4	1	2	2
CDC 167	2	0.5	4	4
CDC 168	4	0.5	4	1
CDC 169	4	0.25	2	1
CDC 170	1	0.125	0.5	1
CDC 171	2	0.5	2	1
CDC 173	1	0.125	0.5	0.5
CDC 175	2	0.25	2	4
CDC 176	1	0.25	1	0.5
CDC 177	1	0.5	2	0.5
CDC 178	2	1	0.5	2
CDC 179	4	2	4	4
CDC 180	2	0.5	2	0.5
CDC 181	2	0.5	0.25	>64
CDC 182	4	0.5	2	1
CDC 183	2	0.25	2	1
CDC 184	2	0.25	2	1
CDC 185	4	0.5	2	1
CDC 187	2	0.25	2	4
CDC 189	2	0.06	1	0.5
CDC 190	2	0.125	1	1
CDC 197	4	2	4	2
CDC 202	4	2	4	16
CDC 211	1	2	4	1
ATCC 700825	0.5	0.5	0.5	0.25
MS11	0.5	0.125	1	0.25
WHO-K	1	0.25	1	0.25
WHO-X	1	0.25	1	0.25
WHO-W	2	0.25	1	0.125
^a MIC ₅₀	2	0.5	2	2
^b MIC ₉₀	4	2	4	4

AZI = azithromycin, AZM = acetazolamide.

^aMIC₅₀: minimum inhibitory concentration at which the compound/drug inhibited 50% of the tested strains.^bMIC₉₀: minimum inhibitory concentration at which the compound/drug inhibited 90% of the tested strains.

Table 3.MICs of molecules under normal and CO₂ conditions

Cpd	N. gonorrhoeae CDC 181 ^a		N. gonorrhoeae CDC 178 ^a	
	normal ^b	CO ₂ ^c	normal ^b	CO ₂ ^c
AZM	4	>64	2	>64
AZI	>64	>64	2	2
20	0.5	>64	1	>64
23	0.25	>64	0.25	>64

AZM = acetazolamide; AZI = azithromycin.

^a MIC values in µg/mL.^b indicates standard conditions in ambient air.^c indicates incubation in presence of 5% CO₂.

Table 4.

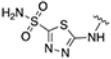
Inhibitory constants for analogs against NgCA and hCAs

Cpd	CO ₂ Hydration K_i (nM) ^a			Cpd	CO ₂ Hydration K_i (nM) ^a			Cpd	CO ₂ Hydration K_i (nM) ^a		
	NgCA	hCA I	hCA II		NgCA	hCA I	hCA II		NgCA	hCA I	hCA II
AZM	74.1 ± 3.2	250 ± 11	12.5 ± 0.8	12	14.1 ± 0.8	76.5 ± 3.6	26.2 ± 1.4	24	45.2 ± 3.0	64.7 ± 4.5	58.1 ± 2.2
1	73.2 ± 3.9	8,600 ^b ± 300	60 ^b ± 3	13	9.8 ± 0.4	63.3 ± 3.7	20.2 ± 1.5	25	61.4 ± 4.9	53.4 ± 2.8	20.9 ± 1.6
2	59.8 ± 2.1	235 ± 14	37.2 ± 1.9	14	75.8 ± 5.0	117 ± 9.4	47.7 ± 3.2	26	29.9 ± 1.4	14 ^c ± 0.9	0.9 ^c ± 0.04
3	41.0 ± 3.0	180 ± 6	30.9 ± 1.3	15	8.7 ± 0.7	152 ± 13	26 ± 1.8	27	63.6 ± 2.4	9.6 ^c ± 0.8	1.6 ^c ± 0.1
4	30.5 ± 1.1	167 ± 6.9	9.5 ± 0.8	16	78.6 ± 5.2	109 ± 7	29.0 ± 1.5	28	73.9 ± 3.0	701 ± 34	47.2 ± 4.1
5	27.5 ± 1.8	213 ± 14	22.3 ± 1.9	17	29.3 ± 2.1	372 ± 19	7.6 ± 0.6	29	42.1 ± 2.9	1,135 ± 98	78.4 ± 4.7
6	87.9 ± 5.0	215 ± 17	55.6 ± 3.7	18	5.8 ± 0.3	77.8 ± 6.9	4.9 ± 0.3	30	62.0 ± 3.9	1,623 ± 126	104 ± 9.1
7	17.7 ± 0.9	367 ± 14	23.6 ± 1.4	19	34.6 ± 2.5	86.3 ± 5.9	19.4 ± 1.1	31	>10,000	n.d.	n.d.
8	9.1 ± 0.6	231 ± 15	7.3 ± 0.60	20	36.6 ± 2.0	58.7 ± 4.1	24.5 ± 1.7	32	632 ± 41	465 ± 11	97.2 ± 11
9	8.5 ± 0.4	328 ± 14	22.7 ± 1.8	21	6.7 ± 0.4	190 ± 18	10.2 ± 0.7	33	439 ± 41	1,331 ± 11	67.7 ± 11
10	15.2 ± 0.6	157 ± 13	24.6 ± 1.3	22	0.70 ± 0.05	945 ± 37	0.32 ± 0.02				
11	42.3 ± 1.6	125 ± 11	41.5 ± 2.0	23	8.3 ± 0.4	855 ± 76	8.1 ± 0.70				

AZM = acetazolamide;

^aCatalytic CO₂ hydration assay K_i determined from the mean of one experiment performed in triplicate and IC₅₀ values entered into Cheng-Prusoff equation. Values reported are ± standard error of the mean.^bPreviously reported by Di Cesare Mannelli et al.⁸¹^cPreviously reported by Turkmen et al.⁸⁰

Table 5.MIC comparison between *N. gonorrhoeae* and *E. faecium*

									
Cpd	AZM	2	3	4	5	6	8	13	14
<i>N. gonorrhoeae</i> CDC 181 ^a	4	8	16	32	8	8	16	2	>64
<i>E. faecium</i> HM-965 ^{a,b}	2	1	1	0.25	0.5	0.125	0.015	0.25	0.25

^a MIC values in µg/mL.^b Values reported by Kaur et al.⁴⁴