Structure-activity relationship studies for inhibitors for vancomycin-resistant Enterococcus and human carbonic anhydrases

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ABSTRACT
Vancomycin-resistant enterococci (VRE), consisting of pathogenic Enterococcus faecalis and E. faecium, is a leading cause of hospital-acquired infections (HAI). We recently repurposed the FDA-approved human carbonic anhydrase (CA) inhibitor acetazolamide (AZM) against VRE agent with the likely mechanism of action for the molecules being inhibition of one, or both, of the bacterial CA isofoms expressed in VRE. To elucidate how inhibitor binding to the enzymes relates to MIC, we further characterised the inhibition constants (Ki) against the E. faecalis α-CA (Efα-CA) and γ-CA (Efγ-CA), as well as against human CA I (hCAI) and human CA II (hCAII) to assess selectivity. We have also utilised homology modelling and molecular dynamics (MD) simulations to gain a better understanding of the potential interactions the molecules are making with the targets. In this paper, we elaborate on the SAR for the AZM analogs as it pertains to MIC and Ki for each CA.

1. Introduction
Vancomycin-resistant enterococci (VRE) is a member of the notorious group of drug-resistant ESKAPE pathogens and is considered a serious threat to public health by the Centres for Disease Control and Prevention. VRE encompasses a host of Enterococcus species, facultative anaerobic Gram-positive bacteria that are able to withstand harsh conditions and colonise surfaces in healthcare settings. The earliest report of VRE from 1899 revealed it as a leading cause of infective endocarditis. Later studies also indicated that VRE is a leading cause of pelvic, neonatal and urinary tract infections (UTIs). In the 1970s, VRE primarily consisted of Enterococcus faecalis, which accounted for over 90% of clinical enterococcal isolates during the first wave of VRE infections. However, since the 1990s, the leading causative agent of VRE has been Enterococcus faecium with now more than 70% of isolated strains being resistant to vancomycin. Furthermore, VRE was responsible for a 10% mortality rate among nearly 55,000 reported cases in 2017. However, the mortality rate climbs for those with resistant VRE strains being resistant to vancomycin. Furthermore, VRE was the leading cause of hospital-acquired infections (HAIs). We recently repurposed the FDA-approved human carbonic anhydrase (hCA) inhibitor acetazolamide (AZM) possessed anti-enterococcal activity with a minimum inhibitory concentration (MIC) of 0.5 μg/mL. Our team then carried out a hit-to-lead optimization campaign centred on the AZM scaffold and generated a series of AZM analogs, many of which displayed significant potency against VRE strains. However, the mechanisms of action were not fully understood. It was speculated that the molecules may be targeting the E. faecium α- and/or γ-carbonic anhydrases (Efα-CA and Efγ-CA, respectively) inside the bacteria. Carbonic anhydrases (CAs) are ubiquitous metalloenzymes that exist in prokaryotes and eukaryotes. The role of CAs is to catalyse the conversion of carbon dioxide to bicarbonate, most often mediated by a Zn2+ ion, in order to maintain the pH homeostasis of the biological system. There are four main CA families that are
genetically independent from each other: $\alpha$, $\beta$, $\gamma$, and $\delta$-CAs\textsuperscript{12}, while new families are still being discovered\textsuperscript{13}. The $\alpha$-CAs are widely distributed among vertebrates, bacteria, algae, and plants and is the only isomorph expressed in humans, while $\beta$, $\delta$- and $\gamma$-CAs are distributed across different kingdoms, including in archaea and bacteria\textsuperscript{14,15}. It is yet to be established whether either Ef$\alpha$-CA and/or Ef$\gamma$-CA are essential in VRE, but CAs have been demonstrated to be essential in other pathogens such as Helicobacter pylori\textsuperscript{16,17} and Neisseria gonorrhoeae\textsuperscript{18,19}. We hypothesise based on the efficacy of our known human CA inhibitor scaffold that one or both of these genes may be essential for VRE growth as well. However, the inhibitory activity of these molecules against either Ef$\alpha$-CA or Ef$\gamma$-CA has never been characterised. Therefore, our group set out to fill this gap in knowledge and to quantify the inhibitory activity of the reported anti-VRE compounds against both VRE-CA isofoms. We then assessed the structure-activity-relationship (SAR) for the series against both VRE-CA, compared to the activity against human isofoms hCA I and hCA II, and also investigated possible protein-ligand interactions in Ef$\alpha$-CA that could explain binding affinity using molecular dynamics (MD) simulations. The results from these studies are reported herein.

2. Materials and methods

2.1. Expression and purification of VRE CAs

Both Ef$\alpha$-CA and Ef$\gamma$-CA were recombinantly expressed and purified as follows. A pHis2 plasmid (GenScript) containing either the Ef$\alpha$-CA sequence or Ef$\gamma$-CA sequence was used for the expression of hexa-histidine tagged protein in a bacterial culture. The plasmid was transformed into competent BL21 (DE3) E. coli cells (Novagen, catalog no. 70953) according to manufacturer recommendation. Starter cultures were grown at 37°C with shaking at 225rpm overnight. One litre of autoclaved Luria-Bertani broth containing 100 µg/mL ampicillin and 1 mM ZnCl$_2$ was inoculated with a 10-ml aliquot of the starter culture and grown at 37°C with shaking at 225 rpm to an OD of 0.8 before being cold shocked and induced with 500 µL of IPTG (1 M). The induced cultures were grown for 16h at 17°C with shaking at 225 rpm. Bacterial cell pellets were spun down at 4000 g for 20 min and resuspended in 1× PBS containing 0.5 mM TCEP, 5% glycerol, pH = 7.4 to which 5 mg lysosome was added to aid in lysis. The resuspended bacterial cells were incubated on ice for 20 min and then lysed via sonication. Lysed bacterial cells were pelleted by centrifugation at 14,000 g for 1 h to remove cellular debris, and the supernatant was loaded onto a nickel-NTA column equilibrated with 1x PBS containing 0.5 mM TCEP, 5% glycerol, pH = 7.4. Once flowthrough was collected, the protein was eluted using a 0–500 mM imidazole stepwise gradient in the same equilibration buffer, and fractions were collected. Fractions that contained the desired protein, as determined by SDS-PAGE were concentrated, flash frozen, and placed in −80°C for future use.

2.2. Carbonic anhydrase CO$_2$ hydration catalytic assay and $K_i$ determination

The assay was performed according to previously published protocols\textsuperscript{19–24}. Recombinant Ef$\alpha$-CA and Ef$\gamma$-CA was obtained as described above and 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 IC$_{50}$ values into the Cheng–Prusoff equation$^{25}$ for $K_i$ from catalytic inhibition constants.

2.3. Protein preparation and ligand docking

All computational protein and ligand preparation and docking was performed using programs available within the Maestro interface of the Schrödinger Small Molecule Drug Discovery Suite (Schrödinger, LLC, New York, NY, software release 2021–2). The Ef$\alpha$-CA homology model built from previous work\textsuperscript{13} was utilised for protein preparation. The homology model was processed using the Protein Preparation Wizard from the Schrödinger platform. During the pre-processing step, bond orders were assigned, the CCD database was used, hydrogens were added, zero-order bonds to metals were created, disulphide bonds were created, and heteroatom states of pH 7.0 ± 2.0 were generated with Epik. The structure was then refined by sampling water orientations using PROPKA pH 7.4, followed by the removal of waters 3.0 Å beyond heteroatoms and with fewer than 3 hydrogen bonds to non-waters. The last step of refinement involved restrained minimisation by converging heavy atoms to an RMSD of 0.3 Å using the OPLS4 forcefield.

To prepare for ligand docking, AZM was placed in the known catalytic binding site near the Zn$^{2+}$ ion. A grid was generated with the Receptor Grid Generation program in Glide using AZM as the workspace reference ligand and setting the Zn$^{2+}$ ion as a metal coordination constraint. All other parameters were set to default settings. Ligands were prepared using LigPrep with default ligand preparation settings. After ligand preparation, AZM and the ligands of interest were docked into the binding site with Glide using Extra Precision (XP) and default docking settings. The ligand poses with the best Glide-XP scores that formed a coordination between the sulphonamide moiety and the Zn$^{2+}$ ion were carried forward into the molecular dynamic simulations.

2.4. Molecular dynamics simulations of ligand binding

All MD simulations were performed using the Desmond (D. E. Shaw Research) program available in the Maestro interface of the Schrödinger platform (software release 2021–2). In preparation for the MD simulations, a solvation model was built with System Builder powered by Desmond using the following parameters. The solvent model was predefined as TIP3P with boundary conditions set to orthorhombic. The box size calculation was set to buffer with box volume minimised. Ion and salt placement was excluded within 5 Å of the ligand, and ions were placed to neutralise the calculated charge. Additionally, 0.15 M NaCl was added to the model.

Once the solvation model was built, molecular dynamics simulations were run using Desmond. Each simulation time was set to 72 ns with recording intervals of 10 ps. Ensemble class was set to
3. Results and discussion

3.1. Structure-activity relationship data

To further understand the structure-activity relationship, we have collected the inhibition constants \( K_i \) against \( EfxCA \), \( EF_2CA \), hCA I and hCA II (Table 1) for all the analogs developed against VRE reported in our previous studies11.

Compared with AZM, the addition of branched alky1 bulk adjacent to the amide carbonyl position generally improved potency against both \( EfxCA \) and \( EF_2CA \) with one exception. Progression from methyl (AZM) to ethyl (1) to iso-propyl (2) provided stepwise improvement against \( EfxCA \) with \( K_i \) values of 56.7, 37.5 and 23.7 nM, respectively. A similar trend was observed against \( EF_2CA \), as AZM displayed a \( K_i \) of 322.8 nM and was improved to 218.4 nM in the iso-propyl derivative 2. Interestingly, the tert-butyl analog 3 maintained similar potency against \( EfxCA \) (\( K_i = 29.8 \) nM) compared to 2 but led to a 2-fold reduction of potency against \( EF_2CA \) (\( K_i = 440.8 \) nM) compared to 2. When the alkyl group was linearly extended an additional methylene from the amide carbonyl, the series of analogs with increased alkyl branching (4–6) was generally less potent against both EFCAIs compared to the series with the branch point adjacent to the carbonyl. Conversely, extending the linear alkyl chain to an n-hexyl substituent in analog 7 increased the \( K_i \) values against \( EfxCA \) and \( EF_2CA \) to 78.8 nM and 631.7 nM, respectively.

A set of cycloalkyl derivatives provided additional, tractable SAR data points against both \( EfxCA \) and \( EF_2CA \). The first cohort of matched molecular pairs in which the cycloalkane branch point was directly adjacent to the amide carbonyl followed a similar trend against \( EfxCA \) as was observed for the branched alkanes. For example, expansion of the ring from three to four to five carbons improved potency for the series with the cyclopentane analog 10 displaying the most potent \( K_i \) (9.8 nM) of any analog thus far. However, similarly to what was observed for the branched alkanes, there appears to be a potency limit with respect to increased ring size as the cyclohexane derivative 11 was the least potent of this group (\( K_i = 49.3 \) nM), followed by the quaternary 1-methyl substituted 12 (\( K_i = 44.5 \) nM). Placing the methyl substituent at the 4-position in 13 slightly improved \( EfxCA \) binding (\( K_i = 29.6 \) nM) but not to the level observed for the cyclopentane derivative 10. Interestingly, \( EF_2CA \) preferred the cycloalkane modifications. Contrarily to the \( EfxCA \) binding data, the cyclohexane derivative 11 was the most potent of the series with a \( K_i \) of 131.1 nM. Increasing the alkyl branching on the cyclohexane ring at either the 1- or the 4-position as in analogs 12 and 13, respectively, reduced \( EF_2CA \) binding to \( K_i \) values over 200 nM.

The second set of cycloalkane derivatives (15–18), in which a methylene linker is inserted between the carbonyl and the ring generally improved potency against \( EfxCA \) across the board with all analog \( K_i \)s ranging from 11–22 nM. The cyclopentane analog 17 did display about 2-fold reduced potency compared to its matched molecular paired analog 10, but this cohort generally was the best performing as a group against \( EfxCA \). However, as observed for the branched alkane analogs (4–7), extension of the linker was detrimental to \( EF_2CA \) activity for analogs 15–18 with \( K_i \) values consistently less potent compared to the nearest neighbour counterparts not containing the methylene insertion. Adding a second methylene to the linker for the cyclopentane (19) and cyclohexane (20) derivatives reduced potency against both isoforms compared to the single methylene derivatives.

Two sets of matched molecular pairs compare the saturated cyclohexane (11 and 20) to a phenyl substitution (14 and 21) with varying linker length from the carbonyl. For the cyclohexane to phenyl substitution attached directly to the carbonyl (11 and 14) the activity was essentially equipotent against \( EfxCA \), while the phenyl derivative was more active against \( EF_2CA \) (\( EfxCA \) \( K_i \) = 131.1 nM, \( EF_2CA \) \( K_i \) = 94.8 nM). This activity difference between isoforms was more pronounced with respect to the matched molecular pair containing a two-methylene linker (20 and 21). In this case, the phenyl-containing derivative was superior to the cyclohexane against both EFCAIs, with a 10-fold increase in activity against \( EfxCA \) with a \( K_i \) value of 6.4 nM and a 2-fold improvement against \( EF_2CA \) with a \( K_i \) value of 148.4 nM. One takeaway from this data is that an aromatic pendant group seems to be preferred for \( EF_2CA \) activity, but only in some instances for \( EfxCA \) activity.

Another general trend that was observed for EFCA activity involves the polar pendant group in analogs 22–25. Addition of heteroatoms into the cycloalkane ring system were generally tolerated for \( EfxCA \) with \( K_i \) values ranging from approximately 20–80 nM with the least potent being the N-methylpiperezine analog 25 and most potent being the morpholine derivative 24. However, this cohort consistently outperformed all other analogs with respect to \( EF_2CA \) with \( K_i \) values ranging from 56–110 nM suggesting a preference for polarity in the active site of \( EF_2CA \). Analog 24 was the most potent in this series against both \( EfxCA \) and \( EF_2CA \) with \( K_i \) values of 20.1 nM and 56.4 nM, respectively.

The final set of analogs were designed with targeted modifications to remove different atoms in the scaffold to determine their essentiality for binding the EFCAIs. In analogs 26–28, the amide carbonyl was removed, leaving three analogs with simply an amine linkage. This resulted in a reduction of binding affinity by more than 2- to 3-fold for both \( EfxCA \) and \( EF_2CA \) across the set when compared to the carbonyl-containing counterparts (AZM, 11, and 14, respectively). Modification of the thiazolene central ring to the thiazole by removing the nitrogen directly adjacent to the sulphonamide in 29 resulted in a 6-fold reduction in binding affinity against \( EfxCA \) and a 3.5-fold reduction against \( EF_2CA \), indicating that this particular nitrogen is important for binding against both isoforms. Modification of the central thiazole to the benzenesulphonamide in 30 also resulted in reduction of binding and antimicrobial activity against both isoforms.

In general, modifications that improved activity against the \( EfxCA \) also improved activity against the human isoform hCA II (an \( \alpha \text{-CA} \) isoform), while improving selectivity over hCA I. For example, the extension of the alkyl linker to two methylene units improved hCA II activity but was detrimental to hCA I activity, while maintaining potency against \( EfxCA \). Analog 21 with the phenyl moiety was among the most potent against \( EfxCA \) with the widest selectivity window over hCA I of more than 100-fold. The data also suggest the polar pendant groups were well tolerated among all CAs tested with the morpholine derivative providing sub-56 nM \( K_i \) values across all four CAs tested.

Another point to note is that no clear correlative trends were observed between EFCA \( K_i \) values and MIC potency, at least when considering the most potent antibacterial analogs 6, 7, and 20 as these analogs (MIC values of 0.015, 0.015, and 0.007 \( \mu \)g/mL, respectively, full data set published by Kaur et al.11) generally lagged behind the rest in terms of EFCA binding. Alternatively, for the molecules that displayed significantly reduced antibacterial activity.
Table 1. Inhibition constants (K<sub>i</sub>) against the α-EfCA, γ-EfCA, hCAI and hCAII.

| Cmpd | Structures | K<sub>i</sub> (nM) | Efα-Ca | Efγ-Ca | hCA I<sup>a</sup> | hCA II<sup>a</sup> |
|------|------------|-------------------|--------|--------|-----------------|------------------|
| AZM  | [Structures] | 56.7 | 322.8 | 250 | 12.5 |
| 1    | [Structures] | 37.5 | 250.1 | 235.4 | 37.2 |
| 2    | [Structures] | 23.7 | 218.4 | 179.7 | 30.9 |
| 3    | [Structures] | 29.8 | 440.8 | 167.3 | 9.5 |
| 4    | [Structures] | 50.1 | 325.3 | 212.5 | 22.3 |
| 5    | [Structures] | 62.7 | 279 | 215.1 | 55.6 |
| 6    | [Structures] | 69.6 | 389.5 | 230.9 | 7.3 |
| 7    | [Structures] | 78.8 | 631.7 | 327.5 | 22.7 |
| 8    | [Structures] | 36.2 | 192.7 | 156.9 | 24.6 |
| 9    | [Structures] | 27.2 | 285.9 | 125.2 | 41.5 |
| 10   | [Structures] | 9.8 | 194.9 | 76.5 | 26.2 |
| 11   | [Structures] | 49.3 | 131.1 | 63.3 | 20.2 |
| 12   | [Structures] | 44.5 | 239.6 | 117.3 | 47.7 |
| 13   | [Structures] | 29.6 | 345.3 | 151.9 | 26 |
| 14   | [Structures] | 56.2 | 94.8 | 108.6 | 29 |
| 15   | [Structures] | 11.7 | 310.5 | 372.4 | 7.6 |
| 16   | [Structures] | 18.4 | 408.4 | 77.8 | 4.9 |
| 17   | [Structures] | 21.9 | 232.8 | 86.3 | 19.4 |
| 18   | [Structures] | 14.5 | 304.8 | 58.7 | 24.5 |
| 19   | [Structures] | 39.3 | 244.6 | 190.2 | 10.2 |
| 20   | [Structures] | 66.9 | 345.9 | 945.9 | 0.32 |
| 21   | [Structures] | 6.4 | 148.4 | 855.3 | 8.1 |
| 22   | [Structures] | 38.1 | 92.8 | 64.7 | 58.1 |
| 23   | [Structures] | 29.5 | 110.6 | 53.4 | 20.9 |
| 24   | [Structures] | 20.1 | 56.4 | 14 | 0.9 |
| 25   | [Structures] | 81.5 | 64.7 | 9.6 | 1.6 |
| 26   | [Structures] | 165.2 | 504.5 | 701 | 47.2 |
| 27   | [Structures] | 117.3 | 398 | 1135 | 78.4 |
| 28   | [Structures] | 163.5 | 282 | 1623 | 103.8 |
| 29   | [Structures] | 374.7 | 1129 | 465.2 | 97.2 |
| 30   | [Structures] | 179.7 | 528.6 | 1331 | 67.7 |

*Ki data for analogs against hCA isoforms previously reported<sup>21</sup>.

(continued)
activity (26–30) there were tangible reductions in EfCA activity that could, at least in part, explain the reduced antibacterial activity. However, additional variables may confound the comparisons between EfCA and antimicrobial activity such as permeability of the molecules to reach the EfCA within the bacterial cell, differential essentiality between the two EfCAs for bacterial survival, or the possibility of additional targets. At this point more information is necessary to draw clear correlative conclusions about the relationship between EfCA inhibition and antibacterial activity.

3.2. MD simulation

MD simulations were performed in an attempt to elucidate binding interactions of particular analogs with Efz-CA that could explain their observed inhibition constants. The data shown for AZM and 20 was reported in previous work11. These MD simulations were run using the GPU-accelerated Desmond (D. E. Shaw Research) software package accessed through the Maestro interface of the Schrodinger platform (software release 2021–2). The MD simulation data further supported the experimental data that the amide bond of the acetazolamide scaffold is crucial for ligand binding to the Efz-CA active site. In Figure 1, the amide nitrogen of AZM is shown to be forming a hydrogen bond with P181 during 68% of simulation, whereas 26 lacks this hydrogen bond. This could be due to the additional rotatable bond introduced in 26, thereby increasing the flexibility of this analog. The primary interaction present in the simulation data for 26 was a hydrogen bond formed between one sulphonamide oxygen and the T179 backbone present for 80% of the simulation time. Likewise, MD simulation data suggested that the thiazolidone nitrogen nearest to the sulphonamide moiety is necessary for ligand binding. When this nitrogen is missing in 29, the thiazolidone core is flipped relative to its position in AZM, and there is an overall lack of ligand interactions with binding site residues which could explain the 7-fold increase in \( K_i \) observed in the binding data for Efz-CA (Table 1). The only interactions reported were water-mediated hydrogen bonds which occurred less than 30% of the duration of the simulation.

MD Simulations were also utilised to analyse the differences in binding interactions between 20 and 21 to elaborate on the 10-fold difference in \( K_i \) observed in the binding assays (Table 1). In Figure 2, the relative poses of 20 and 21 at convergence are quite distinct despite only differing by the presence of aromaticity in the end of the hydrophobic moiety. The MD simulation reports revealed that the primary interaction observed for 20 was that of a hydrogen bond formed between T180 and the amide oxygen that occurred for 88% of the simulation. However, in the case of 21, this interaction was only present 20% of the time. Instead, water molecules were present in the binding site, resulting in water-mediated hydrogen bonds between the sulphonamide and R216 as well as between the sulphonamide and E102 (not pictured due to low prevalence at convergence), all of which were observed for 24–47% of the duration of the simulation. A protein RMSF analysis revealed that there was a much higher flexibility of the T179 and T180 residues in the simulation of 21 (3.2 Å) compared to the simulation of 20 (0.6 Å)11. As a result of this flexibility and presence of water molecules, 21 was positioned nearer to N125, forming a hydrogen bond between the residue and the amide nitrogen of 21 for 44% of the MD simulation. Additionally, the phenyl ring in 21 was angled towards W9 (not shown) forming a hydrophobic interaction for 25% of the simulation duration, an interaction not observed in 20. This could be due to increased protein flexibility in the N-terminus for the 21 MD simulation which was not observed in that of 20. Why the 21 MD simulation suggested presence of water molecules in the binding site and increased residue flexibility compared to 20 when the ligand structures are highly similar remains unclear. The final observation from the MD simulation is that the predicted pose for the hydrophobic tail containing 21 indicates that the thiazolidone heterocycle is flipped in the active site by approximately 180° when compared to AZM. This was consistent with our previous observation for the same alternative orientation of the thiazolidone for 2013 indicating this may be a feature of this scaffold with the hydrophobic tail extended.

4. Conclusion

This report documents pharmacological inhibition of both Efz-CA and Efz-CA for the first time. The AZM-based thiazolidone CAIs previously reported as potent anti-VRE inhibitors were screened for their ability to inhibit both Efz-CA and Efz-CA in the CO₂ hydration assay. It was observed that the scaffold was generally more potent

![Figure 1](image-url)
against the Efα-CA compared to the Efγ-CA isoform. Increase of alkyl branching up to a tertiary alkane was generally preferred for inhibitors activity against Efα-CA. Linker length also played a role in Efα-CA inhibition with the single-methylene linker being preferred over no-linker and the di-methylene linker when comparing nearest neighbour analogs. Some SAR diverged for Efγ-CA. For example, the cycloalkane substituted analogs with no linker between the carbonyl and ring were among the most potent of the hydrophobic derivatives against this isoform. The polar pendant groups displayed the best combination of potency and the only analogs with consistent Ki values below 110 nM against both EFICA isoforms. Finally, targeted modifications such as removal of the carbonyl or alteration of the thiadiazole core had detrimental effects on potency against both isoforms suggesting that these moieties are beneficial for inhibition. Overall, there were no correlative trends with regard to EFICA inhibition and improved antibacterial potency towards VRE; however, molecules that exhibited highly-reduced antimicrobial activity did display 2.5- to 6-fold reduced EFICA inhibition compared to AZM. MD simulations further supported the essentiality of particular scaffold elements such as the amide oxygen and thiadiazole nitrogen adjacent to the sulphonamide by demonstrating an overall lack of direct binding interactions of the Efα-CA homology model with analogs missing these structural elements. In summation, the data presented provides an initial assessment of SAR and the first reported inhibitory activity against Efα-CA and Efγ-CA.

Author contributions

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

Disclosure statement

Dr. CT Supuran is Editor-in-Chief and Dr. Flaherty is on the Editorial Board of the Journal of Enzyme Inhibition and Medicinal Chemistry. Neither author was involved in the assessment, peer review, or decision-making process of this paper. The authors have no relevant affiliations of financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No potential conflict of interest was reported by the author(s).

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