ABSTRACT

Neisseria gonorrhoeae is a gram-negative diplococci bacterium and a causative agent of gonorrhoeae, the second most prevalent sexually transmitted disease. The emergence of multiple drug-resistant 'super gonorrhoea' complicates the management and treatment of *Neisseria gonorrhoeae* infections. The antimicrobial-resistant (AMR) traits acquired by the pathogen are attributed to the cumulative mutations in the bacterial proteome, either in the direct targets of antibiotics or in the additional factors involved in AMR. A continuous evaluation of bacterial proteome is necessary to understand the global mutational landscape and identify conserved molecular targets and their respective inhibitors for therapeutic applications.

In this study, a comparative genomic analysis of the *N. gonorrhoeae* genes (*penA, ponA, 23s rRNA, rpoB, gyrA, parC, mtrR and porB*) proposed to confer resistance to drugs like ceftriaxone, cefixime, azithromycin and ciprofloxacin, was done. The Shannon entropy data of this analysis disclosed highly uncertain amino acid positions in the antibiotic target genes. When a similar analysis was performed on the whole proteome of *N. gonorrhoeae* MDR and XDR isolates, two proteins, the L-asparaginase and the HtpX, emerged as highly conserved targets. Interestingly, in the drug-susceptible isolates, these two proteins also showed complete conservation, emphasising their importance in the growth and survival of the pathogen. L-asparaginase is an amino hydrolase previously indicated to provide survival benefits to intracellular pathogens. HtpX is a putative metalloprotease, which is suggested to be involved in proteolytic control of membrane protein quality during stress. Thus, when functionally compromised using specific inhibitors, both NgA and NgHtpX are likely to act as duel targets.

Therefore, both NgA and NgHtpX were cloned in E. coli, expressed and purified to homogeneity and their biophysical and biochemical properties were evaluated. NgA showed

high stereospecificity and affinity for L -Asp, its natural substrate. Based on modelled structure, high-throughput screening and MD simulations, three FDA-approved drugs, pemirolast, thalidomide, and decitabine, displayed strong binding to NgA. The binding energies of the drugs were -20.14, -19.67, and -16.47 kcal/mol, respectively, compared to -6.82 kcal/mol for L-Asn. Subsequently, fluorescence quenching confirmed the high binding affinity of pemirolast thalidomide and decitabine to NgA with dissociation constants (K_d) 1.19 µM, 1.61 µM and 0.90 µM, respectively.

Similarly, in the case of NgHtpX, the zinc-binding potential was established using fluorescence quenching (K_d =0.4 µM), and E141 was identified as a crucial residue required for zinc binding. A composite high throughput screening followed by MD identified pemirolast (-15.18 kcal/mol) and thalidomide (-19.13 kcal/mol) as highly efficient ligands. The binding was verified through fluorescence quenching, where pemirolast and thalidomide displayed binding affinities with dissociation constants (K_d) of 3.47 µM and 1.04 µM, respectively. Together, the pemirolast and thalidomide appeared to have a high binding affinity towards both NgA and NgHtpX targets. Conclusive evidence came from the cell viability assays where these drugs were found to impede the growth of *N. gonorrhoeae* culture effectively. Overall, our results provide high entropy positions in the targets of presently used antibiotics, which can be further explored to understand the AMR mechanism. Additionally, NgA, NgHtpX and their specific inhibitors identified can be explored further in combination therapy or dual therapy for managing gonococcal infections.