Research Article



Novel Glycoconjugate of 8-Fluoro Norfloxacin Derivatives as Gentamicin-resistant *Staphylococcus aureus* Inhibitors: Synthesis and Molecular Modelling Studies

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Antibiotic resistance has been the subject of interest in clinical practice due to high prevalence of antibioticresistant pathogenic organisms. In view of the prevalence of lesser resistance in antibiotics belonging to aminoglycoside class of compounds viz. Food and Drug Administration-approved gentamicin for the treatment of Staphylococcus infections, which also has instances of resistance in the clinical isolates of Staphylococcus aureus, a series of novel glycoconjugates of 8-fluoro norfloxacin analogues with high regio-selectivity by employing copper (I)-catalyzed 1, 3-dipolar cycloaddition of 1-O-propargyl monosaccharides has been synthesized and evaluated for the antibacterial activity against gentamicin resistance Staphylococcus aureus. Among these compounds, the compound 10g showed better antibacterial activity (MIC = 3.12 μ g/ml) than gentamicin (Escherichia coli (12.5 µg/ml), Staphylococcus aureus (6.25 µg/ml) and Klebsiella pneumonia (6.25 µg/ml), including gentamicin resistant (>50 µg/ml) strain in vitro). The docking studies suggest DNA gyrase of Staphylococcus aureus as a probable target for the antibacterial action of compound 10g.

Key words: drug discovery, molecular modeling, molecular recognition, therapeutic target

Received 26 August 2014, revised 9 December 2014 and accepted for publication 12 December 2014

Gentamicin (GM) is an aminoglycoside antibiotic used in the treatment of several Gram-positive and Gram-negative bacterial infections (1). Gentamicin acts by linking the ribosomal subunits 30S and 50S and blocking the translation of mRNA in the initial phase of protein synthesis, through initiation of non-functional proteins in prone micro-organisms (2). The action of gentamicin and other bactericides inside the bacterial cell takes place in two phases by an active transport mechanism. In the first phase, entrance into the cell depends on the transmembrane potential produced by aerobic metabolism, while the previous union of the aminoglycoside to the bacterial ribosome favours the second phase. Certain conditions reduce the electrical potential of the membrane, such as low pH or anaerobic status of the medium and thus decreasing the accumulation of these compounds into the bacterial cytoplasm (3).

Although GM has been effectively used against most bacterial infections including Staphylococcus spp., Klebsiella pneumoniae, Escherichia coli, Serratia marcescens, Citrobacter spp., Enterobacteriaceae spp. and Pseudomonas spp, nevertheless, its clinical use suffered a major limitation due to the emergence of resistance in 1974 (4,5). Resistance to gentamicin and the related aminoglycosides amikacin, kanamycin, netilmicin, sisomicin and tobramycin is mediated by AAD(6') and APH(2") activities catalyzed by a single bifunctional protein encoded by aacA-aphD (6). This gene is carried by the IS256-flanked composite transposon Tn4001, found on large staphylococcal multiresistance plasmids such as pSK1 and pSK41, or on the chromosome (7). There are three mechanisms of GM resistance: (i) reduced uptake due to decreased cell permeability, (ii) alterations at the ribosomal binding sites and (iii) production of aminoglycoside-modifying enzymes (8). Some strains of Pseudomonas aeruginosa and other bacteria exhibit aminoglycoside resistance due to a transport defect or membrane impermeabilization. This mechanism is likely to be chromosomally mediated and results in cross-reactivity to all aminoglycosides (9,10). The protein channels termed as permeases which is found on the bacterial cell wall is specific in the recognition and transport of selective chemical substances including carbohydrates (11). The interactions between carbohydrates and proteins have determining roles in many biological processes including infection mechanisms, inflammation, immunological processes, signal transduction and cell differentiation. Most of the biogenic proteins are also glycosylated, and the glycosyl part of these proteins



influences their biological activity, although the molecular basis for such processes is still uncertain (12-14). As the cell membrane of bacteria recognizes the carbohydrates during the transmembrane transport, hence, improvement of cell permeability by incorporating carbohydrate moiety to conventional small ligands may provide a rational to prevent permeability-related resistance of aminoglycosides. In this context, we have selected guinolone class of molecules due to their inherent influence in the area of antimicrobial chemotherapy. These molecules potentially offer a wide range of advantages including broad spectrum of activity, combined with high potency, good bioavailability, oral and intravenous formulations, high serum levels, large volume of distribution representing ample concentration in tissues and low incidence of side-effects (15,16). So we have designed a prototype molecule (1) incorporating carbohydrate moieties on guinolone nucleus using molecular hybridization approach and 'click chemistry'. The application of click chemistry has been explored in all aspects of drug discovery, ranging from lead discovery through combinatorial chemistry and target-template in situ chemistry, to proteomics and DNA research using bioconjugation reactions (17). The usage of click chemistry in the discovery of new medicinally important molecules delivers a means for the fast exploration of chemical space, assisting lead optimization by structure-activity relationship (SAR) through the generation of analogue libraries (Figure 1) (18, 19).

Several carbohydrate conjugates of 1-ethyl-6,8-difluoro-4oxo-7-(1H-1,2,3-triazol-1-yl)-1,4-dihydroquinoline-3-carboxylic acid have been synthesized and biological evaluated for their antibacterial activity in comparison with gentamicin. The antibacterial effect of quinolones is mediated through inhibition of DNA gyrase; hence, we have performed docking studies of our synthesized carbohydrate conjugates with DNA gyrase to reveal important binding interactions.

Experimental

General chemistry

Reagent grade solvents were used for the extraction and flash chromatography. All the reagents and chemicals were purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA), Lancaster, and were used directly without further purification. The progress of reactions was checked by analytical thin-layer chromatography (TLC,



Figure 1: The structure of prototype molecule.

Gentamicin-resistant S. Aureus Inhibitors

Merck silica gel 60F-254 plates). The plates were visualized first with UV illumination followed by charring with 10% H_2SO_4 in CH₃OH. Flash column chromatography was performed using silica gel (230-400 mesh). The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. All glasswares were dried in an open flame before use in connection with an inert atmosphere. Solvents were evaporated under reduced pressure. Tetramethylsilane (0.0 p.p.m.) was used as an internal standard in ¹H NMR, and CDCl₃ (77.0 p.p.m.) was used in ¹³C NMR. The abbreviations used to indicate the peak multiplicity were as follows: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; g, guartet; m, multiplet; Hz, Hertz. FAB MS was recorded on Jeol (Japan)/SX-102/DA 6000. Infrared spectrum was taken with KBr on Perkin-Elmer RX-1, Waltham, MA, USA. Melting points were determined on a Buchi 535 digital melting point apparatus and were uncorrected. Elemental analysis was performed on a Perkin-Elmer 2400 C, H, N analyzer, and values were within $\pm 0.4\%$ of the calculated values.

Bioevaluation methods

The susceptibility testing was performed by standard broth micro-dilution method as per Clinical and laboratory Standard Institute (CLSI)(20) guidelines using RPMI 1640 Medium buffered with MOPS [3-(N-morpholino) propane-sulphonic acid] for fungal cultures and Mueller-Hinton Broth (Difco, Franklin Lakes, NJ, USA) for bacterial cultures in 96-well microtitre plates. The maximum concentration tested was 50 μ g/ml, and the inoculum load in each test well was in the range of $1-5 \times 10^3$ cells. The plates were incubated for 24–48 h for yeasts, 72–96 h for mycelial fungi at 35 °C and 24 h for bacteria at 37 °C and read visually as well as spectrophotometrically (Spectra max) at 492 nm for determination of minimal inhibitory concentrations (MICs).

Modelling studies

Ligand and protein preparation

The protein preparation was performed using Protein Preparation Wizard (21) implemented in Schrödinger 9 suite^a. The 3D structures of the ligands were sketched in the Maestro workspace using the drawing tools in maestro window. The 3D structures were geometrically optimized by clean up geometry, and subsequent ligand preparation was performed utilizing the LIGPREP module^b in MAESTRO (version 9.0).

Molecular Docking and Scoring

The molecular docking of the compounds was executed using the GLIDE^c XP (extra precision) module implemented in Schrödinger 9 suite^a. The receptor grid was generated using the ligand ciprofloxacin present in the crystal structure with pdb id 2XCT^c. Visualization of the docked conformation was performed in PyMOL.

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Chemistry

The required key intermediate quinolone 6,7,8 trifluoro quinolone-3–carboxylate (**7**) was synthesized according to the reported method (22), starting from 1,2,3 trifluoro aniline (**2**) and diethyl 2-(ethoxymethylene) malonate (EMME, **3**), which on condensation followed by the thermal cyclization in diphenyl ether of the condensed product **4** thus formed to give ester **5**. The N alkylation of **5** with ethyl iodide by K₂CO₃ in DMF yielded the N-ethylated derivative **6**. The substitution of C-7 F in quinolone ester **6** by azide was carried out by NaN₃ (3 equivalent) in DMF at 120 °C (Figure 2).

The azide **7** was then subjected to hydrolysis in basic condition using 2N NaOH, which resulted in insoluble mass, so the acidic hydrolysis by HCl was carried out which yielded the acid **8** in excellent yield. The acid **8** was kept in airtight container because it turns orange-red when left in open air.

After synthesizing the azide counter part of 1, the alkyne part of the prototype molecule (1) was planned for synthesis. The alkyne counterpart was synthesized from commercially available carbohydrates (D-arabinose, D-galactose, D-glucose, D-mannose, N-Ac-glucosamine, D-ribose, D-xylose and L-rhamnose) in excess of propargyl alcohol using silica-supported sulphuric acid (SiO₂-H₂SO₄) as an acid catalyst (23). The glycosylated sugar was further acetylated by the same sulphuric acid (SiO₂-H₂SO₄) in excess of acetic anhydride without further purification. This glycosylation reaction completed with --selectivity in most of the cases. The coupling reaction between azide and alkyne was performed in tert-butanol water (1:1) using copper sulphate and ascorbic acid as a reducing agent. The reaction was monitored by TLC, and after completion of the reaction, the compounds were isolated and purified by the flash chromatography. These compounds were characterized by their IR, NMR and mass spectra (Figure 3).



Figure 2: Reagents and conditions: (i) neat, 110–120 °C, 2 h; (ii) Ph₂O, 250 °C, 8 h; (iii) C₂H₅I, K₂CO₃, DMF, 95 °C, 10 h; (iv) NaN₃, DMF, 90–95 °C, 8 h; (v) 2 (N) HCl, reflux, 8 h; (vi) CuSO₄.5H₂O, NaASc, *t-But*OH:H₂O (1:1).

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Biological activity

The evaluation of antibacterial activities of compounds **10a-h** against various strains of the bacteria, for example, E. coli (ATCC 9637), Pseudomonas aeruginosa (ATCC BAA-427), Staphylococcus aureus (ATCC 25923), Staphylococcus aureus (ATCC ATCC 700699 methicillin resistant). Staphylococcus aureus (ATCC 29213). Staphylococcus aureus (ATCC 33592 gentamicin resistant), Klebsiella pneumoniae (ATCC 27736), was carried out according to the broth micro-dilution technique described by NCCLS. The minimum inhibitory concentration (MIC) of each compound was determined against test isolates using this technique. The MICs of standard antibacterial drug gentamicin were determined using Muller-Hinton broth.

The antibacterial activity was compared with gentamicin which was used as positive control. In all tests, the MIC values are expressed in μ g/mL. The Compounds **10a**, **10b**, **10f** and **10h** (MIC 12.5 mg/mL) had same activity when compared with gentamicin (MIC 12.50 μ g/mL), while the compound **10g** (MIC 6.25 μ g/mL) showed better antibacterial activity than gentamicin (MIC 12.50 μ g/mL) against *E. coli*. The Compound **10g** (MIC 3.25 μ g/mL) also exhibited excellent antibacterial activity than gentamicin to GM (Table 1). The Compound **10g** (MIC 3.25 mg/mL) also exhibited antibacterial activity than GM against *Klebsiella pneumoniae* (Table 1).

Docking studies

Bacterial type IIA topoisomerases (DNA gyrase and topoisomerase IV (or topo IV)) are the targets of the quinolone antibiotics that have been in practice since 1962 (24). However, direct evidences regarding the interaction of quinolones with DNA gyrase or DNA were available after



Figure 3: Structure of synthesized compounds.

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Table	1:	Antibacterial	activity	of	the	synthesized	com	poun	ds
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Compound	Minimum inhibitory concentration (MIC) in μg/ml against BACTERIA								
	10a	12.5	>50	6.25	>50	6.25	6.25	6.25	
10b	12.5	>50	6.25	>50	6.25	6.25	6.25		
10c	>50	>50	>50	>50	>50	>50	>50		
10d	>50	>50	>50	>50	>50	>50	>50		
10e	50	>50	12.5	>50	12.5	12.5	25		
10f	12.5	>50	6.25	>50	6.25	6.25	6.25		
10g	6.25	>50	3.12	>50	3.12	3.12	3.12		
10h	12.5	>50	12.5	>50	12.5	12.5	25		
Gentamicin	12.5	3.12	6.25	>50	0.78	>50	6.25		

1. E. coli (ATCC 9637), 2. Pseudomonas aeruginosa (ATCC BAA-427), 3. Staphylococcus aureus (ATCC 25923), 3a. Staphylococcus aureus (ATCC 700699 methicillin resistant), 3b. Staphylococcus aureus (ATCC 29213), 3c. Staphylococcus aureus (ATCC 33592 gentamicin resistant), 4. Klebsiella pneumoniae (ATCC 27736).

the discovery of crystal structure that provided insights about inhibitory function of quinolones. To reveal the important interactions of our synthesized compounds at the target site, we have performed docking studies that may suitably establish the structure activity relationship between these compounds. The co-crystalized structure of protein in complex with ciprofloxacin (pdb id **2XCT)** was used for molecular docking (25). The fluoroquinolones such as ciprofloxacin and norfloxacin exert their antibacterial effect by acting on DNA gyrase. The

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synthesized compounds are sugar conjugates of norfloxacin; hence, we have selected DNA gyrase as the target for these molecules (26,27). The results of the docking studies revealed that the most active compound **10g** having the norfloxacin moiety resided at the ciprofloxacin site but in an inverted conformation with the acidic moiety incorporated within DNA strands and the xylose portion incorporated in DNA gyrase.

The negative fluorine atom in **10g** forms hydrogen bond with **SER 1084,** and the oxygen atom in the ring of xylose sugar interacted through hydrogen bond forma-

tion with cytosine base pair of DNA. Besides having hydrogen bond contact with DNA base, the sugar portion in **10g** was found to maintain contact with **GLU 1088, GLY 1117, ALA 1118** and **ALA 1120** (site A) as depicted in Figure 4A. The least active molecule **10c** has its norfloxacin moiety aligned in a similar conformation as found in **10c,** but the conjugated glucose moiety failed to attain similar contact with DNA gyrase like the xylose sugar in **10g** (Table 2). The crystal structure of ciprofloxacin depicts electron density map near **SER 1084,** implicating it to be an important residue for binding (Figure 5).

Table 2: The glide docking score for the most and least active compound

Compound	Glide score	Glide evdw	Glide ecoul	Glide energy	XP Hbond
10g	-6.19	-41.490	19.57	-61.08	-0.7
10c	-3.26	-73.90	4.21	-78.12	-0.67
Ciprofloxacin	-5.66	-30.45	2.20	-32.65	-0.65



Figure 4: (A) The docked conformation of the most active compound **10g**; (B) the docked conformation of the least active compound **10c**; (C) the aligned conformation of the most and the least active compound at the binding site; (D) the aligned conformation of the most active compound **10g** and ciprofloxacin at the binding site.



Figure 5: The 2D ligand protein interaction for (A) most active compound **10g** and (B) least active compound **10c** without DNA with major interactions with DNA gyrase.

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The fluorine atom in the norfloxacin moiety occupied a position similar to the carboxylic moiety in ciprofloxacin (Figure 4D). Hence, the hydrogen bond interaction of the fluoro moiety in the most active compound **10g** with **SER 1084** and hydrophobic contact of the xylose moiety may be a possible reason for DNA gyrase inhibition.

Conclusion

We report the synthesis of a novel series of compounds by click products of carbohydrate alkyne and guinolone azide. Among the novel glycoconjugates (10a-h), the compounds in the series (10a, 10b and 10f) showed promising activity profile (MIC = 6.25 μ g/ml) in gentamicinresistant S. aureus (MIC > 50 μ g/ml) and are equipotent to gentamicin in E. coli, S. aureus (ATCC 25923) and K. pneumoniae strains. The compound 10g having xylose sugar conjugated with norfloxacin moiety showed better antibacterial activity (MIC = 3.12 μ g/ml) than gentamicin (E. coli (12.5 µg/ml), S. aureus (6.25 µg/ml) and K. pneumonia $(6.25 \ \mu g/ml)$, including gentamicin resistant (>50 µg/ml) strain in vitro). The binding conformation of 10g at the S. aureus DNA gyrase has been proposed by docking studies that provide fundamental insights into understanding the probable mode of action of these molecules.

Acknowledgments

C.S.A. and S.S.B. acknowledge CSIR, New Delhi for the award of a SRF. The authors also acknowledge SAIF-CDRI for providing spectral and analytical data. CDRI communication number: 8871.

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Notes

^aSCHRÖDINGER, version 9.0; Schrödinger, LLC: New York, 2009.

^bLIGPREP, version 2.3, Schrödinger, LLC, New York, NY, 2009.

 $^{\rm c}_{\rm GLIDE,}$ version 5.5; Schrödinger: LLC: New York, 2009.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. General information and Experimental procedure.

Appendix S2. Materials and Methods for biological evaluation.

Figure S1. ¹HNMR and ¹³CNMR spectra of selected compounds.

