CHAPTER-4

Elucidation and Validation of Antimicrobial Potential of Chemically Synthesized Dibromotyrosine Analogues in Wet Lab and In-silico Approaches
4.1 Introduction

The top most priority and agenda in the area of biomedical research is the quest for the discovery and successive development of new antibiotics because the existing antibiotics have to face the problem of drug resistance or multi drug resistance by infective micro organisms. The current paradigm for most treatments is to either combine several smart drugs or design drugs that modulate multiple targets (multitargeted therapy), formally referred to as “dirty drugs”. Thus, “dirty drugs” are in and “smart drugs” are out [Jimeno and Hidalgo, 2006; Sams-Dodd, 2005]. However, several “dirty drugs” such as gefitinib, sorafenib etc. with inherent problem of cytotoxicity have been withdrawn from the human clinical trials [http://www.nih.gov]. Our research group has discovered various natural aglycones of glycoalkaloids (e. g. solanidine, tomatidine and solasodine) which were found to be alike dirty drugs having better anti-angiogenic, pro-apoptotic and anticancer drug leads than the standard drugs [Akhtar et al., 2011, Akhtar, S. PhD Thesis, 2012]

One of the marine natural products with the maximum potential to act as antibacterial agent is squalamine with potent antimicrobial activity, a minimum inhibition concentration (MIC) of 1.0 µg/ml against *Staphylococcus aureus* along with anti-angiogenic and antitumor properties [Rao et al., 2000].

Dibromoverongiaquinol (verongiaquinol) and Aeroplysinin-1 which are structurally simple dibromotyrosines were also selected as parent compounds for in-silico designing of new bioactive small molecules [Teeyapant et al., 1993; Ambrosio et al., 1984]. It has already been reported that dibromoverongiaquinol shows a potent antimicrobial activity against a wide spectrum of fungi and bacteria along with cytotoxicity [Koulman et al., 1996; Teeyapant et al., 1993; Ambrosio et al., 1984]. The dienone structure responsible for the formation of free
radicals forms the landmark behind the cytotoxic effect of verongiaquinol [Koulman et al., 1996].

Comparison of the structure and biological activity of secondary metabolites derived from marine habitat with terrestrial counterparts; resulted to the conclusion that marine habitat may contain efficient antifungal compounds with variable modes of action [Li H-Y et al., 1998]. Jasplakinolide is a 19-membered macrocyclic depsipeptide that targets the cytoskeletal protein actin. It showed in vitro antifungal activity against C. albicans. A 2% solution of jasplakinolide also showed in vivo topical activity against a vaginal infection of C. albicans in mice which is similar in potency to miconazole nitrate [Crews et al., 1986]. Further, epiphytic marine dinoflagellate Gambierdiscus toxicus is reported to yield the extremely potent antifungal metabolites gambieric acids. Gambieric acid A inhibited the growth of Aspergillus niger at a concentration of 10 ng/disk. The potency of gambieric acids exceeds that of the known polyene antibiotic amphotericin B by 2000-fold [Nagai et al., 1992].

Most marine natural products are not generally considered promising antibacterial and antifungal agents for clinical applications because of their cytotoxicity. In the previous chapters, we have shown that some of the dibromotyrosine analogues especially analogue 12 exhibited remarkable anticancer and antiproliferative potential with no per se genotoxicity or cytotoxicity in both the in-silico and wet lab studies. The idea which inspired this study is to explore the antimicrobial potential of these synthetic dibromotyrosine analogues because the parent compounds dibromoverongiaquinol and aeroplysinin-1 showed potent activity against multiple pathogenic bacteria and fungi. We hypothesized the use of simple in-silico protocols for screening of future drug entities as potential cost effective drug discovery technology. In this chapter also we first have carried out the in vitro wet lab studies for antimicrobial potential followed by the in-silico protocol for antimicrobial screening and
elucidation of possible mechanism(s) of action. Interestingly, these new in-silico antimicrobial screening protocols have been in significant agreement with the wet lab results for selected analogues. Further, we also would like to explore whether some of these analogues could be developed as multiple targeted “dirty” drug entity in future.

4.2 Materials and Methods

4.2.1 Collection of chemically synthesized analogues of dibromotyrosine: The analogues used in this study were chemically synthesized by our collaborator and Co-Supervisor Dr. Khalid El-Sayed, College of Pharmacy, University of Lousiana at Monroe, USA [Sallam et al., 2010]. The analogues were dissolved in DMSO and stored at a stock concentration of 100μM at 20°C.

4.2.2 Collection of bacterial and fungal strains used in this study: The bacterial and fungal strains used in this study were procured from IMTECH, Chandigarh. Bacterial strains used were *E.coli* (NCIM 2065), *S.epidermidis* (NCIM 2493), *E.aerogenes* (NCIM 5139) and *S.aureus* (NCIM 2079). Fungal strains used were *A.flavus* (NCIM 524), *A.paraciticus* (NCIM 898), *C.albicans* (ATCC 90028) and *S.cerevisiae* (RW 128). The yeast/fungal strains were cultured in Yeast Extract Peptone Dextrose 17 (YEPD) broth (BIO101, Vista, Calif.). For agar plates, 2.5% (w/v) bacto agar 18 (Difco, BD Biosciences, NJ) was added to the medium. Bacterial strains were cultured in Luria Bertini Broth (LB). All strains were stored as frozen stocks with 15% glycerol at -80°C. Before each experiment, cells were freshly revived on YEPD/LB plates from the stock.

4.2.3 Antibacterial susceptibility testing: The relative susceptibility of analogues against various bacterial strains used in this study was determined using microdilution test in Luria Bertini Broth. Cells were grown for 12 h at 37 °C to obtain single colonies which were resuspended
in a 0.9 % normal saline solution to give an optical density at 600nm (OD_{600}) of 0.1. The cells were then diluted 100-folds in bacto agar media. The diluted cell suspensions were added to round bottomed 96-well microtiter plates (100\mu l/ well) in wells containing equal volumes of medium (100\mu l/well) with different concentrations of the analogues. Analogue-free control was also included. The plates were incubated at 37\degree C for 48 h. The MIC test end point was evaluated both visually and by reading the OD_{620} in a microplate reader and is defined as the lowest analogue concentration, which gave > 50 % or (required percentage) inhibition of growth compared with the controls.

### 4.2.4 Antifungal susceptibility testing using NCCLS method M27A for C. albicans:

The relative susceptibility of analogues against various fungal strains used in this study was determined using NCCLS 27A method [Espinel-Ingroff et al., 1998; Martins and Rex, 1997; Rex et al., 1997] by modified microdilution test in [Talibi and Raymond, 1999] YEPD. Cells were grown for 48 h at 30\degree C to obtain single colonies which were resuspended in a 0.9 % normal saline solution to give an optical density at 600nm (OD_{600}) of 0.1. The cells were then diluted 100-folds in YEPD media. The diluted cell suspensions were added to round bottomed 96-well microtiter plates (100 \mu l/ well) in wells containing equal volumes of medium (100 \mu l/ well) with different concentrations of analogues [Kohli et al., 2002; Talibi and Raymond, 1999]. Analogue-free control was also included. The plates were incubated at 30\degree C for 48 h. The MIC test end point was evaluated both visually and by reading the OD_{620} in a microplate reader and is defined as the lowest analogue concentration, which gave > 50 % or (required percentage) inhibition of growth compared with the controls.

For spore forming fungi, YEPD agar was prepared and 0.5% Tween 20 was added to it. Distilled water was added to 5-6 days grown spores forming fungal plates. The distilled water containing spores was pipetted
out and was set to an optical density of 0.1. This was then spread with
cotton swabs onto YEPD agar plates. Filter Disc was prepared of
required analogue concentration and were plated onto the YEPD plates.
Antifungal susceptibility was evaluated by zone of inhibition obtained.

4.2.5 Bioassay for anti QS activity: Agar well diffusion assay: The
agar well diffusion assay was adopted to detect anti-QS activity using
standard method as described by Ahmad et al [1998]. The agar well
diffusion assay was performed by Chromobacterium violaceum (CV12472)
for determining pigment inhibition activity by plant extracts. Luria agar
plates were spread with 0.1 ml of appropriately diluted (~2.5×10⁶) freshly
grown cultures and wells of 8mm diameter were made and sealed at the
bottom by soft agar followed by treatment with 50 µM and 100 µM of
each analogue. Solvent and Luria broth were used as controls. Plates
were incubated for 18-24 h to check the inhibition of pigment production
around the well. Higher concentration was also used as control to check
growth inhibition.

4.2.6 In-silico Analysis
4.2.6.1 Ligand preparation and optimization: Analogues 1-13 were
synthesized according to Sallam et al., 2010. The chemical structures of
analogues were drawn on Chemsketch (www.acdlabs.com) followed by
generation of their PDB structures using Discovery Studio 2.5
(http://accelrys.com/products/discovery-studio/). Standard inhibitors
of different target proteins retrieved from PubChem database [Wang et al.
, 2009] and their PDB structures were generated using the online tool
[Brooks et al., 2009] was applied to analogues and standard inhibitors
and then subjected to single step minimization using smart minimiser
algorithm for 1000 steps at RMS gradient of 0.01 and optimized.
4.2.6.2 **Target protein identification:** Most favorable binding proteins of analogues 1-13 were screened from potential drug target database ([http://www.dddc.ac.cn/tarfisdock/](http://www.dddc.ac.cn/tarfisdock/)) using the reverse docking approach TarFisDock [Li *et al.*, 2006], a web-based tool for automating the procedure of searching for small molecule-protein interactions over a large repertoire of protein structures. It offers PDT (potential drug target database), a target database containing 698 protein structures covering 15 therapeutic areas and a reverse ligand protein docking proGram. In contrast to conventional ligand protein docking, reverse ligand protein docking aims to seek potential protein targets by screening an appropriate protein database. Diaminopimelate decarboxylase (PDB: 1HKV), alanine recemase (PDB: 1SFT), thymidylate synthetase (PDB: 1TSD), thymidylate synthase (PDB: 1C17), penicillopepsin (PDB: 1APU), RuvB (PDB: 1IN4), HIV-1 reverse transcriptase (1RT6) and thymidine kinase (PDB: 1VTK) were identified as potential binding proteins for analogues 1-13 through TarFisDock server. This in-silico study was performed at the School of Computational Biology and Bioinformatics, Jawaharlal Nehru University, New Delhi in consultation with Prof. N. S. Rao.

4.2.6.3 **Protein preparation and optimization:** The crystal structures of diaminopimelate decarboxylase (PDB: 1HKV), alanine recemase (PDB: 1SFT), thymidylate synthetase (PDB: 1TSD), thymidylate synthase (PDB: 1C17), penicillopepsin (PDB: 1APU), RuvB (PDB: 1IN4), HIV-1 reverse transcriptase (1RT6) and thymidine kinase (PDB: 1VTK) which were taken up in this study were extracted from protein data bank ([www.pdb.org](http://www.pdb.org)). All hetero-atoms of target proteins were removed and further subjected to two-steps energy minimization to remove the bad steric clashes using steepest descent and conjugate gradient methods for 1000 steps at RMS gradient of 0.1 and 0.05 respectively, during the energy minimization process the backbone were fixing the backbone.
4.2.6.4 Molecular docking: Docking experiments were performed using the GOLD (Genetic optimization for Ligand Docking) version 4.1.2 [Jones et al., 1997] which was set to 50 cycles of run without constraints between the ligands and the specific amino acids of the pocket in order to find the preferred binding conformations of the ligands in the receptor. The algorithm exhaustively searches the entire rotational and translational space of the ligand with respect to the receptors. The flexibility of the ligand is given by dihedral angle variations. The various solutions evaluated by a score, which is equivalent to the absolute value of the total energy of the ligand in the protein environment. The analysis of the binding conformation using a scoring function based on the free energy of binding [Huey et al., 2007]. The Active site was defined as the collection of protein residues enclosed within a 15 Å radius sphere. The annealing parameters for Van der Waals and hydrogen bonding were set to 4.0 Å and 2.5 Å respectively. Other docking parameters were set to the software’s default values. After complete execution of GOLD 10 conformations of ligand in complex with the receptor were obtained, which were finally ranked on the basis of Gold score [Jones et al., 1997]. The best conformations were visualized in the Discovery Studio Visualizer.

4.2.7 Statistical Analysis: Statistical analysis of the data was done by employing the two-tailed Student’t’ test as described by Bennet and Franklin [1967]. The effect of all the analogues at varied concentrations was repeated in triplicate. A difference at P<0.05 was considered statistically significant.
4.3 Results

4.3.1 Antibacterial potential: The designed and chemically synthesized ether and ester analogues were analyzed for their efficacy to be explored upon as antibacterial and antifungal potent drug leads against various bacterial and fungal strains. From the first set of experiments it was found out that all the analogues were ineffective against bacterial strain *E.coli* at as high a concentration as 250µM. All the analogues were found out to give similar or slightly less growth density with the treated cells at varied concentration than the growth density obtained from the untreated cells (data not shown here).

The consequence of Gram-positive bacterial strain *S.aureus* tested against all the analogues was found to show similar results as in the case of Gram negative *E.coli* except for analogue 5 which gave MIC<sub>50</sub> value of 80.55 µM as shown in figure 4.1. Further, the in-silico approach will be helpful in the exploration of the possible mechanism/molecular target by which these analogues operate in each bacterial strains finally leading to their death.
Figure 4.1: Graph showing the minimum inhibitory concentration (MIC$_{50}$) of analogue 5 (2, 6-dibromo-1-benzoyloxybenzene-4-acetamide) against bacterial strain *S. aureus*. Analogue 5 gave MIC$_{50}$ value of 80.55 µM. Inhibition values are mean ± SE for each concentration of analogue. Each value is significantly different from the respective controls at p < 0.05.

*S. epidermidis* which was the next bacterial strain used in this study has been known to possess the ability to form biofilms on plastic devices which is major virulence factor of this strain. One probable cause is surface proteins that bind blood and extracellular matrix proteins. The efficacy of five synthetic analogues from among the library of 13 completely showed significant changes in antibacterial behavior against this bacterial strain *S. epidermidis*. The noticeable MIC$_{70}$ values as obtained by these five potent analogues are depicted by the values besides each analogue: Analogue 12 (12.47µM), Analogue 2 (12.47µM), Analogue 3 (13.55µM), Analogue 11 (18.26µM) and Analogue 5
(39.25 µM), respectively, as shown in figure 4.2. Accordingly, the antibacterial potency of these analogues against *S. epidermidis* from highest to lowest is as follows: Analogue 12 ≈ Analogue 2 > Analogue 3 > Analogue 11 > Analogue 5.

![Graph showing the minimum inhibitory concentration (MIC) of Analogue 2, 3, 5, 11 and 12 against bacterial strain *S. epidermidis*. All the analogues are giving significant inhibition at micro molar concentrations. Inhibition values are mean ± SE for each concentration of analogue. Each value is significantly different from the respective controls at p<0.05.](image)

**Figure 4.2:** Graph showing the minimum inhibitory concentration (MIC) of Analogues 2, 3, 5, 11 and 12 against bacterial strain *S. epidermidis*. All the analogues are giving significant inhibition at micro molar concentrations. Inhibition values are mean ± SE for each concentration of analogue. Each value is significantly different from the respective controls at p<0.05.

*E. aerogenes* is a Gram-negative, oxidase negative, catalase positive, citrate positive, indole negative, rod-shaped bacterium. *E. aerogenes* is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections. *Enterobacter* species can also cause various community acquired infections. Some strains can become very treatment resistant, a result of their colonization within hospital environments. However, the majority are sensitive to most
antibiotics designed for this bacteria class [Chavarria et al., 2000]. The antimicrobial potency of five out of the thirteen synthetic dibromotyrosine analogues against *E. aerogenes* was found to be very promising. The MIC values of the most potent analogues are as follows: Analogue 1 (2.37µM), Analogue 3 (0.50 µM), Analogue 5 (4.20µM), Analogue 9 (9.63µM) and Analogue 10 (11.9 µM), respectively as shown in figure 4.3.

![Figure 4.3](image)

**Figure 4.3:** Graph showing the minimum inhibitory concentration (MIC<sub>70</sub>) of Analogues 1, 3, 5, 9 and 10 against bacterial strain *E. aerogenes*. The values clearly indicate the antibacterial potential of these analogues at micro molar concentrations. Inhibition values are mean ± SE for each concentration of analogue. Each value is significantly different from the respective controls at p<0.05.

**4.3.2 Anti-fungal potential:** The antifungal efficacy of these synthesized marine analogues was evaluated and they showed no significant effect against *C.albicans* wild type and efflux pumps over-expressed strains Cdr1 and CaMdr1 (data not shown here). The cell density was found out
to be similar in the un-treated well as compared to the treated wells at as high a concentration as of 100\(\mu\)M thereby bringing out the inability of all the analogues in inhibiting the growth of cells thereby decreasing the cell density. Further, the analogues were also found almost ineffective against spore forming fungal strains \textit{A.\textit{flavus}} and \textit{A.\textit{paraciticus}} at as high a concentration as of 100\(\mu\)M of all the analogues (data not shown here). Very few analogues showed antifungal activity against the other fungal \textit{S. cerivisae} giving \textit{MIC\textsubscript{70}} values as follows: Analogue 6 (67.44 \(\mu\)M), Analogue 8 (72.81 \(\mu\)M), and Analogue 12 (76.65 \(\mu\)M), respectively, as shown in figure 4.4.

\textbf{Figure 4.4:} Graph showing the minimum inhibitory concentration (MIC\textsubscript{70}) of Analogue 6, 8 and 12 against fungal strain \textit{S.cerivisae}. The antifungal efficacy is significant in these three analogues at micro molar concentrations. Inhibition values are mean ± SE for each concentration of analogue. Each value is significantly different from the respective controls at p<0.05.
4.3.3 Ability to affect Quorum Sensing: Current research has suggested that various functions in Gram negative bacteria are related to density-dependent regulation of gene expression known as quorum sensing (QS) and mediated by recognition of signaling molecules known as autoinducers. Discovery of anti-QS compounds (halogenated furanones) from Australian micro algae, Delissea pulchra has generated an interest among scientific community/fraternity to screen various natural and synthetic compounds as potential anti-QS agents [Manefield et al., 1999]. In the quest of finding new compounds for their anti-QS potential, the synthetic analogues were evaluated for their ability to do so as shown in figure 4.5. Neither ether nor ester analogues showed any anti-QS activity at concentrations of 50 μM and 100 μM, respectively. Analogues 4 and 8 showed antibacterial activity against the bacterium Chromobacterium violaceum with analogue 8’s zone of inhibition 17mm at 100 μM concentration while analogue 4 gave zone of inhibition of 19mm at 100 μM concentration.
Figure 4.5: Effect of 50 µM (a) and 100 µM (b) concentration of analogues on the anti-QS activity of the bacterium *Chromobacterium violaceum*.

4.3.4 Molecular interaction studies with selected anti-microbial targets: The mol files of all the analogues used in this study was submitted in an online server TarFisDock which gave the top 2% of the bacterial, fungal and viral candidates ranked by energy score, including their binding conformations which are shown in Table 4.1. Targets chosen in this study are on the basis of docking results with the analogues and number of times the target showed repeatedly good binding score.
Table 4.1: Top 2% of the bacterial, fungal and viral targets identified by an online server TarFisDock.

<table>
<thead>
<tr>
<th>Target Name</th>
<th>PDB ID</th>
<th>Category</th>
<th>Related Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial Targets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycobacterium Diaminopimelate Decarboxylase</td>
<td>1HKV</td>
<td>BACTERIAL INFECTIONS</td>
<td>n/a</td>
</tr>
<tr>
<td>Alanine Racemase Biosynthetic</td>
<td>1SFT</td>
<td>BACTERIAL INFECTIONS</td>
<td>Bacterial Infections, Tuberculosis, Vitamin B6 Deficiency</td>
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<tr>
<td><strong>Fungal Targets</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Thymidylate Synthetase</td>
<td>1TSD</td>
<td>FUNGAL INFECTIONS; NEOPLASTIC DISEASES; VIRAL INFECTIONS; PARASITIC INFECTION DISEASES</td>
<td>Breast Cancer, Colorectal Cancer, Fungal Diseases, Gastric Cancer, Hepatocellular Carcinoma, Ovarian \ Cancer, Pancreatic Cancer, Proliferative Diseases</td>
</tr>
<tr>
<td>Thymidylate Synthase</td>
<td>1C17</td>
<td>VIRAL INFECTIONS; FUNGAL INFECTIONS; NEOPLASTIC DISEASES; PARASITIC INFECTION DISEASES</td>
<td>Breast Cancer, Colorectal Cancer, Fungal Diseases, Gastric Cancer, Hepatocellular Carcinoma, Ovarian \ Cancer, Pancreatic Cancer, Proliferative Diseases</td>
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<td>Penicillopepsin</td>
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<td>FUNGAL INFECTIONS</td>
<td>n/a</td>
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<td><strong>Viral Targets</strong></td>
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<td>Holliday Junction DNA Helicase Ruvb</td>
<td>1IN4</td>
<td>VIRAL INFECTIONS</td>
<td>n/a</td>
</tr>
<tr>
<td>HIV-1 Reverse Transcriptase</td>
<td>1RT6</td>
<td>VIRAL INFECTIONS</td>
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</tr>
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<td>Thymidine Kinase</td>
<td>1VTK</td>
<td>VIRAL INFECTIONS</td>
<td>Bladder Cancer, Cancers</td>
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In-silico antibacterial efficacy: The first bacterial target selected by TarFisDock is mycobacterium diaminopimelate decarboxylase (PDB ID-IHKV) which is also known as DAPDC and is responsible for carrying out the final step in the bacterial lysine biosynthetic pathway and is encoded by the lysA gene. DAPDC is a vitamin B6-dependent enzyme that stereospecifically converts meso-DAP to L-lysine. Like most enzyme catalyzed decarboxylation reactions, the conversion of DAP to lysine is not reversible. Among all inhibitors (analogue 1-13) docked against bacterial target, diaminopimelate decarboxylase, DAPDC, (PDB: 1HKV), analogue 8 was found to bind with the best efficacy, Gold score 59.60, greater than its standard inhibitor of Gold score 48.4. Analogue 3 was surrounded with Ser215, Gly257, His212, His210, Arg160, Asp90, Val90, Cys92, Glu96, Lys71, Tyr404, Leu408, Arg343, Tyr347 and Arg302 amino acid residues of 1HKV in which Lys71 residue also formed 3 hydrogen bonding interactions as shown in (figure 4.6, table 4.2) given below, thereby providing stability to the binding mode of receptor ligand interactions. Analogue 5 which emerged out as the most potent antimicrobial analogue in the wet lab studies gave similar interactions with antimicrobial target DAPDC in dry lab also thereby reconfirming its efficacy to act as a potent synthetic marine antimicrobial drug lead with a Gold score of 36.4 and stability conferring 2 hydrogen bonds between O10 of the analogue with HG1 of THR175 in the macromolecule and O10 of analogue with and OG1 of THR175 in the macromolecule as shown in figure 4.6 and table 4.2. This inherent potential of synthetic analogue 5 can be further explored upon to successfully develop a possible molecule for treatment of bacterial infections.
Figure 4.6: Docked structures of analogues 8 and 5 against bacterial target DAPDC. Ligands and analogues are represented in dark green. Receptor (DAPDC) as line model, colored by element. Hydrogen bonds represented as dotted lines in green. Amino acid residues are labeled as dark blue.

Another antimicrobial target which emerged out from online tool TarFishDock is alanine racemase (PDB ID 1SFT). It is very well known that Gram-positive and Gram-negative bacteria require the D-isomer of alanine as an essential building block in the synthesis of the peptidoglycan layer of cell walls. The synthesis of the bacterial cell wall is believed to start with UDP-N acetylmuramic acid, to which assorted free amino acids are added [Adams, 1976]. UDP-N-acetylmuramyl-L Ala-D-Glu-meso-diaminopimelate-D-Ala-D-Ala is a key intermediate in the initial stage of synthesis of the peptidoglycan layer. D-alanine is added onto the growing amino acid chain as a D-alanine dipeptide. This dipeptide is produced by two enzymes, alanine racemase (EC 5.1.1.1), and D-Ala: D-Ala ligase (EC 6.3.2.4). Alanine racemase, the first enzyme in the biosynthetic pathway of peptidoglycan synthesis, racemizes the common L-isomer of alanine to the D-isomer. The D-alanine dipeptide is
then produced in a reaction catalyzed by D-Ala:D-Ala ligase, whose structure has been solved [Fan et al., 1994], and the peptide is then introduced by D-Ala-D-Ala adding enzyme (EC 6.3.2.15) to the growing UDP-\(N\)-acetylmuramyl-L-Ala-D-Glu-\textit{meso}-diaminopimelate chain. Alanine racemase is unique to bacteria, with one known exception, an alanine racemase involved in the synthesis of cyclosporin A isolated from the fungus \textit{Tolypocladium niveum} [Hoffmann et al., 1994]. Gold score of the docking simulation of all the analogues with alanine racemase showed that the binding/interaction potential of analogues was found to comparatively less and sometimes similar as compared to the standard inhibitor pyridoxal phosphate (Pubchem ID 1051) (table 4.2).

**4.3.4.2 In-silico anti-fungal efficacy:** The analogues were further evaluated for their virtual ability to act as potent anti-fungal drug hits based on their binding efficacies with antifungal targets thymidylate synthetase (PDB ID 1TSD) and thymidylate synthase, both play a key role in the synthesis of DNA by catalyzing the conversion of dUMP to dTMP. When all the analogues were docked with thymidylate synthetase (PDB ID 1TSD), analogue 8 was found to exhibit the best binding ability with 1TSD with a gold score of 53.0 as compared to that of standard inhibitor, a folate analogue (Pubchem Id 60863) giving a gold score of 54.6. Leu172, Asn177, Phe176, Glu58, Ile79, Val262, Tyr209, Asp169, Cys146, Glu165, Ser167, Arg166, His207 residues of 1TSD were found to be involved in hydrophobic interactions with analogue 8 (figure 4.7; table 4.2)
Figure 4.7: Docked structures of analogues 2 and 8 with fungal targets 1TSD/1C17. Ligands /analogues are represented in dark green. Receptors (1TSD/1C17) as line model, colored by element. Hydrogen bonds represented as dotted lines in green. Amino acid residues labelled dark blue.

Analogue 2 resulted in a gold score of 52.5 and stability conferring 2 hydrogen bonds both with H36 of the analogue and glutamate’s oxygen E1 and E2. Analogue 8 resulted in gold score of 50.6 when docked against 1TSD giving 2 hydrogen bonds ARG24:HH12 with
O12 of the analogue and H38 of the analogue with GLU63:OE1 as compared to the standard inhibitor 2-deoxyuridylic acid (Pubchem ID 65063) having a gold score of 54.63. Arg24, Glu63, Phe67, Tyr111, His 172, Asp117, Tyr237, His240, Met288, Met290, Phe204 residues of 1C17 were found out to be involved in hydrophobic interactions with analogue 2. Interactions of analogues 2 and 8 with 1C17 are shown in figure 4.7 (table 4.2) as given above.

Docking of analogues with another fungal target penicillopepsin (PDB ID 1APU) which are fungal aspartic proteinases possessing trypsinogen activating activity, showed even more significant interactions than standard inhibitor 1-chloro-3-methoxybenzene (Pubchem 17833). Analogues 2 and 8 resulted in equal gold scores of 50.4 as compared to the standard inhibitor (Pubchem 17833) with gold score of 23.3. H36 of analogue 2 was found out to be hydrogen bonded with OD2 of ASP115 of the macromolecule (1APU) while hydrophobic interactions with Glu16, Gly76, Asp77, Ser79, Phe112, Asp115, Asn118, Leu121, Thr217, Gly215 and Leu220 residues of 1APU were observed. H38 of analogue 8 was also giving 1 hydrogen bond with OD2 of ASP115. The interactions of analogue 2 and analogue 8 in the form of pictorial representation are given in the form of figure 4.8 (table 4.2).
Figure 4.8: Docked structures of Analogues 2 and 8 with fungal target 1APU. Ligands/Analogue are represented in dark green. Receptor (1APU) as line model, colored by element. Hydrogen bonds represented as dotted lines in green. Amino acid residues labelled dark blue.

4.3.4.3 In-silico antiviral efficacy: The analogues were further evaluated for their potential to act as antiviral drug leads by exploring the interaction of analogues with viral target RuvB (PDB ID 1IN4). The RuvB hexamer is the chemo-mechanical motor of the RuvAB complex that migrates Holliday junction branch points in DNA recombination and the rescue of stalled DNA replication forks. The 1.6 Å crystal structure of *Thermotoga maritima* RuvB together with five mutant structures reveal that RuvB is an ATPase associated with diverse cellular activities (AAA+ class ATPase) with a winged-helix DNA-binding domain. The RuvB-ADP complex structure and mutagenesis suggest how AAA+-class ATPases coupled nucleotide binding and hydrolysis to inter domain conformational changes and asymmetry within the RuvB hexamer implied by the crystallographic packing and small-angle X-ray scattering in solution. ATP-driven domain motion is positioned to move double stranded DNA through the hexamer and drive conformational changes...
between subunits by altering the complementary hydrophilic protein protein interfaces [Christopher et al., 2001]. Analogue 13 gave the best interaction with RuvB with a positive gold score of 40.0. There was 1 stability conferring hydrogen bond ILE143: HN interacted with O13 of the analogue as compared to the gold score of standard inhibitor 1-chloro-3-methoxybenzene (Pubchem 17833) of 32.6. Analogue 8 also gave significant interaction with RuvB with a binding interaction gold score of 26.8 and 1 hydrogen bond between HZ1 of Lysine20 with the O7 of the analogue very similar to that of the standard inhibitor. Gly141, Ile142, Ile143, Leu144, Glu145, Val17, Asn16, Lys20 residues of 1IN4 were found to be the key players of the docked complex with analogue 8. The interaction of analogue 13 and analogue 8 with RuvB is represented below in the form of figure 4.9 (table 4.2) as given below:-
Figure 4.9: Docked structures of Analogues 1, 8 and 13 with viral targets 1IN4/1RT6. Ligands /Analogues are represented in dark green. Receptors (1IN4/1RT6) as line model, colored by element. Hydrogen bonds represented as dotted lines in green. Amino acid residues labelled dark blue.

The interaction, binding ability and inherent potency to be developed as efficient antiviral drug leads of the synthesized analogues was reflected upon by their interaction studies with well known viral target HIV1.
reverse transcriptase (1RT6). HIV1 reverse transcriptase (RT) is a multifunctional enzyme that catalyzes RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H activities as well as specifically binding its physiological primer, tRNA Lys3. These functions are all required in the replication of HIV, making RT central to the virus life cycle, thus providing a primary target for anti-HIV drugs widely used in the treatment of AIDS. The interaction results of the analogues with this viral target emerged out to be even better in terms of binding or interaction potential with the target as compared with the standard inhibitor, 1-chloro-3-methoxy-benzene (Pubchem 17833). The analogue 1 and analogue 8 gave the best interaction gold scores of 48.8 and 49.0, respectively, as compared to the standard inhibitor’s score of 8.6 (table 4.2). The H31 of the analogue 1 was found to be hydrogen bonded with O of MET184 while the O22 of the analogue 8 hydrogen bonding with H of TRY183. Val90, Leu92, Gln91, Ile94, Pro157, Gln161, Phe160, Tyr115, Tyr181, Tyr183, Met230 residues of 1RT6 were found to be the key players of docked complex with analogue 8. The interaction of analogue 1 and analogue 8 with the viral target (1RT6) is represented in the form of figure 4.9 (table 4.2) as shown above.

It has been estimated that 60-95% of the world population is infected by members of the human Herpes virus family [WHO Meeting, 1985], especially by Herpes simplex virus type 1 (HSV-I) and type 2. The virus rests most of the time in a latent form in cells of the nervous system. On reactivation, it can cause a variety of diseases like the common fever blisters, genital skin lesions, blindness, and encephalitis [Whitley & Gnann, 1993]. The incidence of Herpes virus diseases in immune-compromised people such as AIDS patients is particularly high. Here, the reactivation of a latent Herpes virus infection appears to be facilitated. Numerous anti-Herpes drugs have been developed; they have been reviewed by De Clercq (1993). The initial target of these drugs is
nearly in all cases the viral thymidine kinase (TK). This enzyme is part of the thymine salvage pathway, catalyzing the transfer of the y-phosphate from ATP to the 5’-hydroxyl of dT to form dTMP. In contrast to the corresponding host cell enzyme, TK from HSV-1 is not very specific and phosphorylates its primary product, dTMP, as well as a variety of substrate analogues such as the uridine derivatives idoxuridine and brivudin or guanosine derivatives aciclovir and ganciclovir. The tolerance difference between viral and cellular thymidine kinases and the preferences of the viral DNA polymerase are the basis for the efficacy of present drugs, as well as the basis for future developments, e.g., a virally directed prodrug therapy of malignant brain tumors [Culver et al., 1992]. The therapeutic value of idoxuridine is limited, though, because it is also accepted by the cellular enzyme. A preliminary structure of TK from HSV-1 was published earlier [Wild et al., 1995], followed by an independent second structure analysis of Brown et al., 1995. The analogues showed moderate interaction with this viral target TK (PDB ID 1VTk) as compared to the standard inhibitor Adenosine Diphosphate (PUBCHEM ID 6022) which gave a positive gold interaction score of 62.7, compared to analogues 3 and 9 which resulted in gold scores of 30.9 and 29.5, respectively.
Antimicrobial Potential ............. of Analogues.  

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Table 4.2: Interaction studies of all the synthesized analogues with the various microbial targets. Interaction potential of analogues is represented in the form of positive Gold score. Targets are represented in the form of PDB ID’s and standard inhibitors in the form of PUBCHEM ID’s and analogues with their corresponding codes.

n/i = no significant interaction

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<th>Anlg 3</th>
<th>Anlg 4</th>
<th>Anlg 5</th>
<th>Anlg 6</th>
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4.4 Discussion

Marine natural products have been a focus of recent interest as potential pharmaceuticals, with sponges proving to be particularly rich in bioactive compounds. The brominated phenols and quinones isolated from Aplysina (Verongia) species include active antibacterial agents which are proposed to be derived from mono or dibromo tyrosines [Minale et al., 1976]. Attempts by other workers to demonstrate such a conversion in vivo have been unsuccessful [DeRosa et al., 1973]. The remarkable chemical diversity and biological activity of natural products found in marine sponges is often explained in terms of chemical defense of these sessile soft-bodied invertebrates against various biotic stress factors such as predation, allelopathy and biofouling [e.g. McClintock and Baker, 2001; Proksch, 1999; Uriz et al., 1991; Braekman and Daloze, 1986].

In this study, we have focused on the ability of the chemically synthesized analogues of aeroplysinin-1 and dibromoverongiaquinol to inhibit the growth of harmful bacterial and fungal strains. The inability of all the synthesized analogues to act against bacterial strain E. coli may be attributed and possibly explained by the fact that the analogues were unable to penetrate the thick lippolysaccharide covered peptidoglycan layer of the bacterial cell wall of Gram negative bacteria rendering them ineffective very similar to the results quoted by Ata et al. [2004] and Morton et al. [1998].

Analogue 5 definitely has the inherent ability to pierce upon the defense system of the Gram-positive bacterial strain S.aureus which is well known to develop resistance against a variety of antibiotics commonly used. Further, this ability of the strain can be explained by various detoxification pathways which this bacterium has in its armor to combat the effect of various agents used against them. From the figure 4.1, it is clearly evident that Analogue 5 was able to overcome the detoxification
pathways available in the armory of the bacterial strain to inhibit its growth clearly correlating the published results that PsU, PsQ, PsS, seco-PsK, PsG were the most active compounds (IC50 2.3–4.5 µM) against S.aureus [Correa et al., 2011]. The inhibition of growth may be possibly explained upon that the analogue is inhibiting the synthesis of bacterial cell wall which is easily accessible because of the absence of lipopolysaccharide layer as present in Gram negative bacteria similar to the inhibition pattern adopted by the standard antibiotic penicillin.

It is well known that S.epidermidis’s capsule which is known as polysaccharide intercellular adhesion (PIA) is made up of sulfated polysaccharide. It allows other bacteria to bind to the already existing biofilm, creating a multilayer biofilm. Such biofilms decrease the metabolic activity of bacteria within them. This decreased metabolism, in combination with impaired diffusion of antibiotics, makes it difficult for antibiotics to effectively clear this type of infection [Salyers et al., 2002].

S.epidermidis strains are often resistant to antibiotics including penicillin, amoxicillin, and methicillin. Resistant organisms are most commonly found in the intestine, but organisms living freely on the skin can also become resistant due to routine exposure to antibiotics secreted in sweat. From the MIC values of analogues 2, 3, 5, 11 and 12, it is clearly evident that these drug leads are effective against Gram positive bacterial strain S.epidermidis. These results are comparable with the activity previously reported for PsA-E, PsK, PsX and PsY isolated from specimens collected in the north Caribbean Sea [Ata et al., 2004], which were reported to have minimum inhibitory concentration (MIC) values between 4.2 and 8.8 µM [Correa et al., 2011]. This rare peculiar inherent potency of these newly synthesized marine analogues may provide an opportunity to develop them as novel drug candidates which can be of potential help in future against S.epidermidis infections.
The marked and noteworthy antibacterial activity of analogues 1,3,5,9 and 10 against Gram negative bacterial strain *E.aerogenes* doubly confirmed that these chemically synthesized ether and ester analogues have an inherent and in-built ability to kill specific bacterial strains possibly by the mechanism of blocking protein synthesis thereby providing efficient leads for treatment of specific bacterial borne diseases and infections. The antibacterial activity of the above analogues against Gram negative bacteria were in agreement with the earlier studies by Tarman et al. [2011] wherein they have reported that extract of *X.psidii* (KT30) cultivated in freshwater medium was in fact most active against Gram negative bacterial strains *E.coli* and *P.aeruginosa* with inhibition zones of 23 and 13 mm, respectively. Further studies using bioinformatics based in-silico approaches can prove to provide a better platform to elucidate the mechanism of action specific for specific bacterial strains.

The possible explanation for the inefficiency of synthesized analogues against fungal strain *C.albicans* could be derived from multi drug resistance (MDR) phenomena where the low potency of all the analogues can be attributed to the fact that the efflux pumps are active in flushing out of the drug thereby resulting in no marked or significant effect [Prasad et al., 2005]. This could further explain the mechanism that most of these analogues are acting as substrates for these efflux pumps based proteins. Further studies using in-silico approaches can be performed to check the binding efficacy of these analogues with the efflux proteins prior to concluding that these analogues are not give an opportunity to act upon these fungal strains before being effluxed out by these efflux pumps.

Analogues 6, 8 and 12 showed antifungal activity against fungal strain *S.cerevisiae* similar to the antifungal activity results shown by marine extracts [Tarman et al., 2011]. The mechanism of action of the above
mentioned analogues was further evaluated to be similar to marine sponge born Jasplakinolide which represents a new class of antifungal agent that is novel both structurally and functionally in its ability to kill the fungus at the same concentration as that required to inhibit its growth [Scott et al., 1988]. The results obtained from the analogues showed a departure from the activity seen with the imidazole antifungal agents, which are fungicidal only at high concentrations and after prolonged incubation [Plempel, 1979; Sud and Feingold, 1981]. Unfortunately the QS inhibitors reported so far have limited or no therapeutic application due to their toxicity and high reactivity to the host. The need arises for the discovery of non-toxic, broad spectrum QS inhibitors for successful exploitation in combating bacterial infections caused by drug resistant strains. The analogues as desired did not possess significant toxicity and reactivity to the host but their synthesis could be refined and modified followed by their testing in a concentration dependent manner prior to reaching a conclusion that the synthetic analogues lack anti-QS potential.

The Mycobacterium diaminopimelate decarboxylase (PDB ID-IHKV) enzyme was adopted in this study because of its importance in bacterial growth and survival. Lysine is required in protein biosynthesis and is essential for bacterial viability and development. The lysine precursor DAP itself is used as a structural cross-linking component of the peptidoglycan layer of Gram-negative, Gram-positive (except Gram-positive cocci), and mycobacterial cell walls [Cummins and Harris, 1996]. DAP cross-links provide stability to the cell wall and confer resistance to intracellular osmotic pressure [Strominger, 1962]. DAP can be synthesized by one or more of the following three different pathways: (i) the succinylase pathway, identified in all Gram negative and Gram-positive bacteria, as well as Mycobacterium tuberculosis; (ii) the dehydrogenase pathway, utilized by Bacillus sphaericus, Corynebacterium
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*glutamicum*, and *Brevibacterium* species [Misono et al., 1986]; and (iii) the acetylation pathway, which is limited to certain *Bacillus* species [Sundharadas and Gilvarg, 1967]. The presence of multiple biosynthetic pathways, at least in some bacteria, is probably an indication of the importance of DAP and lysine to bacterial survival. As the substrate and the reaction are not found in mammals, inhibitors of the enzyme may ultimately become leads for therapeutic intervention in bacterial infections [McCann and Pegg. 1992]. Analogue 5 which gave good wet lab results was found out to give similar and correlating in-silico results giving good interaction with *Mycobacterium* diaminopimelate decarboxylase (PDB ID-IHKV). The possible explanation of the mode of action of this analogue to kill *S.aureus* is because of its ability to block bacterial growth and survival by altering the stability and loss of resistance to intracellular osmotic pressure of bacterial cell leading to its demise. The explanation of the above in-silico antibacterial results can be possibly highlighted upon by the fact that the analogues are effective in blocking the synthesis of the peptidoglycan layer of bacterial cell wall probably by blocking the synthesis of Lysine in a potential and efficient manner which is essentially required in protein biosynthesis and is very much important for bacterial cell viability and development.

The approach adopted revealed that the analogues exhibit a good binding with Penicillopepsin which is a member of the aspartic proteinase family of enzymes and plays an important role in common fungal infections [Cooper, 2000]. The mechanism of action of the synthesized analogues on fungal strains was by probably blocking the DNA synthesis leading to the impaired synthesis of fungal aspartic proteinases specific for *S. cerivisae* strain of fungus.

Currently approved *anti-reverse transcriptase (RT)* drugs fall into two main classes [De Clercq, 2002]. The nucleoside analogue inhibitors (e.g., AZT, ddI, ddC, d4T, and 3TC) are incorporated into the primer strand in
their metabolically activated triphosphate forms, causing termination of DNA synthesis due to their 3'-deoxy configuration [De Clercq., 1996]. The non-nucleoside inhibitors (NNIs) are a chemically diverse set of compounds that are largely specific for HIV-1 RT and generally act as non-competitive inhibitors with respect to the nucleoside triphosphate substrates [De Clercq., 1994, 1996]. Analogue 1 and analogue 8 can be taken forward as leads for futuristic development of potential antiviral drug leads as they may act potentially as NNIs (non-nucleoside inhibitors) of the viral reverse transcriptase thereby blocking the life cycle of virus.

Finally, to explain in the above in-silico study all the comparison was made on the basis of Gold score [Jones et al., 1997], a scoring function of GOLD docking tool telling about the binding interaction of ligand receptor complexes. Higher the Gold score value, stronger the ligand binding with the target molecule. It was concluded from docking simulations that the analogue 1, 2, 5, 8 and 13 were found to be best inhibitors acting against bacterial, fungal as well as viral protein targets in order to combat different diseases caused by various organisms. Analogues 2, 8 and most importantly 5 were found to be significantly effective against microbial targets both in wet and dry lab studies. Further, in this study, the molecular docking was applied to explore the binding mechanism and to correlate its docking score with the activity of synthetic dibromotyrosine derivatives. To our knowledge, this is the first study aimed at deriving docking studies for synthetic dibromotyrosine derivatives. The docking studies provided good insights into the binding of synthetic dibromotyrosine analogues at the molecular level. Significant study between active sites and dibromotyrosine derivatives will be analyzed to propose structural changes in these analogues, with the aim of rendering them more selective and thereby leading to the
discovery of better inhibitors against bacterial, fungal and viral protein targets.

Our recent publications [Akhtar et al., 2011; Khan M.S. et al., 2011(a, b); Khan M.K.A. et al., 2011; Arif. J. M. et al., 2012(a); Siddiqui et al., 2011] further reconfirm that the wet lab data correlates with in-silico approach and can definitely be used in the preliminary screening of the unknown compounds. Our comparative observations between the wet lab and in-silico methods using model compounds/analogues and reference drugs/inhibitors opens up the hidden potential of the simple bioinformatics based quick approaches for preliminary screening of the new compounds before moving to the costly and time consuming wet lab studies.

4.5 Conclusions

In conclusion, our wet lab and dry lab results clearly suggest for the first time that the dibromotyrosine analogues (e.g. 5 and 8) remarkably possess a significant potential to be developed as state-of-art drugs against microbial infections. Further, it appears from the wet lab antimicrobial screening assays that some of the analogues especially 5 and 12 are showing a glimpse of “dirty drug leads” by acting against various bacterial and fungal strains. Interestingly, analogue 12, in the previous chapters, has already been shown to have antiproliferative and anticancer potentials against DU-145 cancer cells. This particular analogue 12 has tremendous potential to become a multiple target drug against the cancer and microbial infections.