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Synthesis, *In-Vitro* Antibacterial Activity and Molecular Docking Studies of Novel Phenothiazine Derivatives

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ABSTRACT

Invasive microbial infections are major problems around the world, especially in immune-compromised patients and there is a critical need for new antimicrobial agents which will be more selective, potent and less toxic. In the present work, MurD ligase has been chosen as the target for the development of new classes of antibacterial agents. Phenothiazine derivatives 3a-3g and 5a-5g were used conduct in-silico studies with MurD ligase using Aurgus Lab software and their scores determined. All the compounds exhibited good scores showing the effective interaction of this group of ligands with the target. Lipinski's rule of five has been applied on these compounds to determine the drug likeliness of these compounds. The compounds were synthesized in the laboratory and characterized by spectral methods. The antibacterial activity against three gram positive and three gram negative bacteria has been carried out by zone inhibition method using gentamycin as the control. The results are comparable with the control and thereby the mechanism was studied.

KEY WORDS: Mur D ligase, In- silico studies, Chaclcone, Synthesis, Anti-bacterial activity.

1. INTRODUCTION

Human beings have been in constant exposure to pathogens for many decades. Invasive microbial infections are major problems around the world, especially in immuno- compromised patients. The expansion of antimicrobial drug research has happened because there is a critical need for new antimicrobial agents which will be more selective, potent and less toxic compared to the existing drugs to treat these life threatening infections (Livermore, 2009; Davies and Davies, 2010; Fauci and Morens, 2012). With antibiotic resistance mechanisms increasing in diversity and spreading among bacterial pathogens, the development of new classes of antibacterial agents against judiciously chosen targets is a high priority task. The biochemical pathway for peptidoglycan biosynthesis is one of the best sources of antibacterial targets. Within this pathway are the Mur ligases were described as highly suitable target for the development of new classes of antibacterial agents (Lugtenberg, 1972; Lugtenberg, 1972; Lugtenberg, 1973). The amide ligases MurC, MurD, MurE and MurF function with the same catalytic mechanism and share conserved amino acid regions and structural features that can conceivably be exploited for the design of inhibitors that simultaneously target more than one enzyme. This would provide multi-target antibacterial weapons with minimized likelihood of target mediated resistance development (Imene Kouidmi, 2014).

It has been suggested that the MurD reaction is the step at which PG synthesis is regulated to maintain the relative thickness of the PG layer in Gram-negative bacteria. As MurD displays extremely high specificity for its D-amino acid substrate (Horton, 2003; Tomasic, 2009), it has been identified as one of the most promising new targets for the discovery of selective antibacterial agents (Bertrand, 1999; Walsh, 1999; Van Heijenoort, 2001). In the post-genomic era, rational drug discovery aims to discover or design small molecules that modulate the activity of key therapeutic targets pivotal for infections. This work mainly aims to discover novel small molecular inhibitors against important molecular targets involved in microbial infections (Zhang, 2011; Modugno, 2007). This prompted us to undertake the present work to try some of the phenothiazine derivaties which are synthesized in our laboratory in order to address the antibiotic resistance. Computer- aided or in silico design is being utilized to expedite and facilitate hit identification. If the present work is encouraging it is anticipated that physicians will have additional drugs as antibiotics. The success of these inhibitors will have greater implication not only as antibiotics but also in other diseases driven by MurD ligases (Barreteau, 2012).

2. MATERIALS AND METHODS

2.1 Molecular Docking: The catalytic sites of MurD ligase Receptor along with area and volume of binding pocket was carried out with Meta Pocket 2.0 Finder program. The three dimensional crystal structure of the MurD ligase was retrieved from the Protein Data Bank. The complexes bound to the receptor molecule, all the heteroatoms and the non-essential water molecules were removed and finally hydrogen atoms were merged to the target receptor molecule using Argus Lab Version 4.0.1.

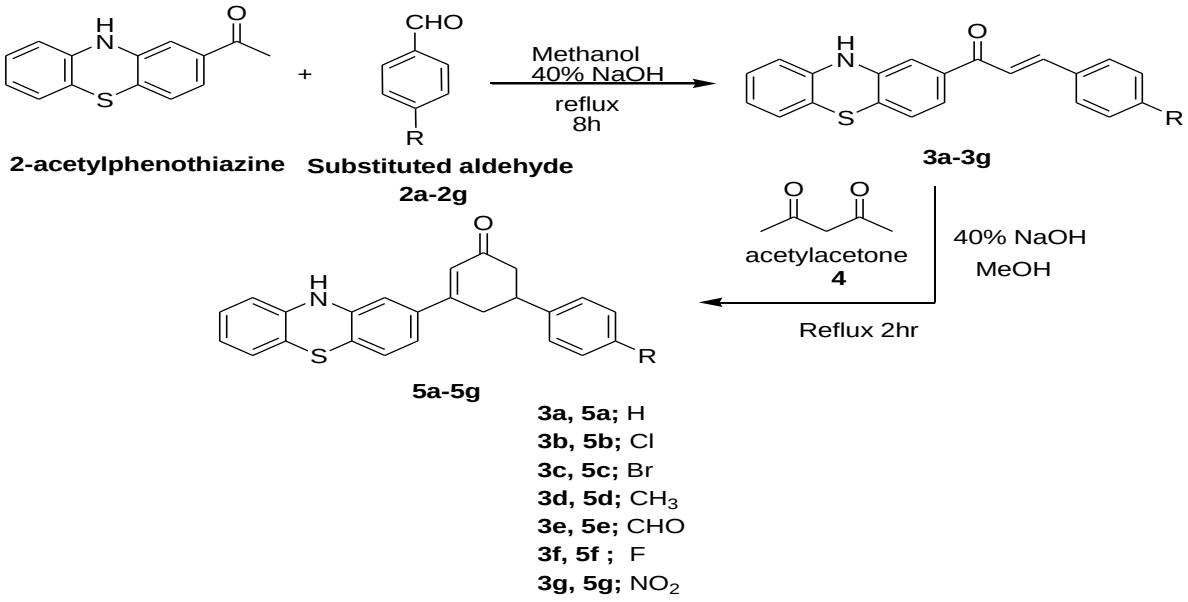
The molecular docking program Aurgus Lab software which is most commonly available software was used to perform the virtual screening. The receptor was defined from the crystallographic coordinates of the ligand. The binding site atoms were further defined from a file listing the cavity atoms. Dockings were performed under 'Standard default settings' mode. All the parameters used in Argus lab docking were selected by default. Calculation type was set to "dock" mode and "flexible mode" was selected for the ligand. The docking results were analyzed using PYMOL (TM) software, which allows visualization of the protein-ligand docking and

calculation of several descriptors such as feasible hydrogen bonding between the protein and the ligand. The scores were calculated and presented in the table. Least energy indicated the easy binding character of ligand and receptor.

2.2 Experimental

2.2.1. Synthesis of Phenothiazine derivatives of Chalcones 3a-g: 0.001 mole of 2-acetyl phenothiazine 1 was dissolved in 25 ml methanol and 0. 001 mole of different substituted benzaldehydes 2a-c were added, followed by the addition of 5 ml aqueous solution of NaOH (5%) and heated for 6 hrs with constant stirring in a magnetic stirrer. The reaction was monitored by TLC. After completion of the reaction, the reaction mixture was poured into ice-cold water, neutralized with con.HCl and left over night in a refrigerator. The resulted precipitate was filtered, dried and the purity of the compound was checked by TLC using chloroform as the solvent. The compounds 3a-g were purified by column chromatography using silica gel (60-120 mesh) (Saranya and Ravi, 2013).

2.2.2. Synthesis of Cyclohexenones derivatives of Chalcones 5a-g: 0.001 mole of chalcones 3a-g was dissolved in 25ml methanol, and 0.001 mole of ethyl acetoacetate 4a was added followed by 5ml aqueous solution of NaOH (5%) was added and kept under refluxtion for 2 hrs. The reaction was monitored by TLC. After completion of the reaction, the reaction mixture was poured into ice-cold water, neutralized by con.HCl and left over night in a refrigerator. Later the precipitate formed was filtered, dried and purity of the compound (5a-g) was checked by TLC using the solvent system chloroform (Saranya and Ravi, 2014).



Scheme.1.Synthesis of phenothiazine derivatives 3a-g and 5a-g

2.3 Antibacterial activity: *In vitro* antibacterial activity of chalcones 3a-g and cyclohexenones 5a-g was studied against six bacterial strains by agar well diffusion method against three gram positive bacteria *Bacillus cereus, Staphylococcus aureus, Bacillus subtilis* and three gram negative bacteria *Eschericia coli, Klebsiella pneumoniae, Proteus mirabilis* (Parekh and Chanda., 2007). Muller Hinton agar no 2 (Hi- Media, India) was used as a bacteriological medium. The compounds were diluted in 100% dimethyl sulphoxide (DMSO) at different concentrations of 5mg/mL and 2.5mg/mL. The antibacterial activity was evaluated at two different concentrations viz 500µg/well and 250µg/well. The Muller Hinton agar was melted and cooled to 48-50°C and a standardized inoculums (1.5×108 CFU, 0.5 McFarland) was then added aseptically to the molten agar poured into sterile Petri dishes to give a solid plate. The plates left at room temperature for solidification. A well of 6mm diameter was made using a sterile cork borer. The standard drug and test sample were placed in 6mm diameter well. Antibacterial activity assay plates were inoculated at 37°C. The antibacterial activity of each test sample was determined for the bacterial species in terms of zone sizes around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercial control antibiotics namely Gentamicin (5mg/mL). For each bacterial strain controls were maintained where pure solvents were used. The experiment was performed in triplicates to minimize the errors.

3. RESULT AND DISCUSSION

3.1 Molecular docking study: The 3D structure of the MurD ligase is retrieved from the protein data bank (PDB id 2X50) **Figure 1**. In the binding pocket the Active site residues of MurD ligase are determined by data base Meta pocket 2.0 active site finder software. The active sites are

THR 297 PHE 303 GLU 304 VAL 305 LEU 299 ARG 302 GLY 298 LYS 262 THR 117 HIS 267 ASN 268 ASP 317 LEU 263 SER 264 PHE 296 ASN 113 GLY 114 SER 325 GLY 324 ALA

328 ALA 329 LYS 115 ASN 271 PRO 300 GLY 332 LEU 333 HIS 334 GLY 265 ASN 331 LEU 330 GLN 266 GLU 327 ARG 313 ILE 315 MET 406.

The Lipinski's rule of five parameters like molecular weight, molecular formulae, No. of hydrogen bond donor atoms, Number of H-bond acceptor atoms, number of rotatable bonds and Log p values were calculated for the ligands (3a-3g) and (5a-5g) to asses whether the above ligands are drug like molecules. The results are tabulated.

All the phenothiazine chalcones (3a-3g) and cyclohexanone derivatives of phenothiazine chalcones (5a-5g) showed good docking scores. The results are tabulated in the Table-1. The table shows the docking scores, H-bonded interactions and the amino acids surrounding the ligand. The Fig shows the hydrophilic and hydrophobic interactions of the ligands with the protein. From the table it was learned that compounds 3a, 3b and 3c with H, chloro and bromo substituents respectively exhibited a reasonably good scores -10.5551, -11.5293and -11.2137 kcal/mol. Similarly, compounds 5a, 5b and 5c with H, chloro and bromo substituents respectively exhibited reasonably good scores -10.7046, -11.0346 and -11. 4298 kcal/mol. All the parameters are well within the rule except the Log P value where it is acceptable for 3b and 3g and for 5f and 5e.

3.2 Antibacterial activity: The antimicrobial activity of phenothiazines has been known since the time of Paul Ehrlich. The first such compound, chlorpromazine (CPZ), was made available in 1953 by Rhone-Poulenc (Charpentier, 1952) and, because of its extensive use; its antibacterial property was soon evident. However, since it is a golden age of antibiotics, there was no need for CPZ, or any of its derivatives, to be considered as antimicrobial agents. Furthermore, because of the side effects due to the prolonged use of CPZ, and only *in vitro* antimicrobial activity was reported at clinically irrelevant concentrations, CPZ or other phenothiazines were not seriously considered as potential sources of new antibiotics, even when they have desired antimicrobial effects *in-vivo* (Amaral and Kristiansen, 2001). However, notwithstanding the problem of resistance, dictates that phenothiazines are now seriously considered where other drugs have failed.

A series of 14 compounds were synthesized, with chalcone and phenothiazine moiety 3a-3g and cyclohexanone with phenothiazine moiety 5a-5g. They are expected to have a synergetic effect in exhibiting their antibacterial activity. The results are shown in the Table.

It was observed that Gram negative bacteria *E. coli, K.pneumoniea, P. mirabilis* are more susceptible to phenothiazines and the gram positive bacteria such as *B.cereus, S.aureus, B.subtilis are* less susceptible. It is important to note that an electron withdrawing substituents in the aromatic ring showed that good activity when compared to electron donating groups. With the introduction of substituents in the aromatic ring system the activity of the compounds increases. Among the chalcones prepared with all the substituents it showed less activity for *Staphylococcus aureus*, however it worked well with all the other bacteria. In general, most of the compounds had shown less activity when compared with the control compound gentamicin. Conversion of chalcones 3a-g into the respective cyclohexenones (5a-5g) reduced the antibacterial activity. When the antibacterial activity was considered for all the compounds synthesized, the activity of chalcones is more when compared with the activity of the cyclohexenones.

The above result suggests that all the compounds 3a-3g and 5a-5g inhibits the function of the MurD ligase and thereby exhibits antibacterial activity. It was well known that the cell wall of the bacteria is an excellent starting point for designing of antibacterial drug, as the most of the drugs like bacitracin, vancomycin, penicillins and cephalosporins act by inhibiting the late enzymatic steps of bacterial peptidoglycan biosynthesis. The early intracellular steps catalysed by a series of Mur enzymes has been unexploited as antibacterial targets. Since the compounds 3a-3g and 5a-5g had shown very good docking scores because of the hydrogen bonding interactions and hydrophobic interactions, these compounds may serve as starting point for the development of antibacterial agents.

Table.1.Docking result of 3a-g and 5a-g against MurD ligase protein

Compound	Score Kcal/mole	Compound	Score Kcal/mole
3a	-10.5111	**5a**	-10.7046
3b	-11.5293	**5b**	-11.0346
3c	-11.2137	**5c**	--11. 4298
3d	-10.9915	**5d**	-11.2747
3e	-10.6992	**5e**	-10.7544
3f	-10.3914	**5f**	-11.6745
3g	-11.2909	**5g**	-11.9888

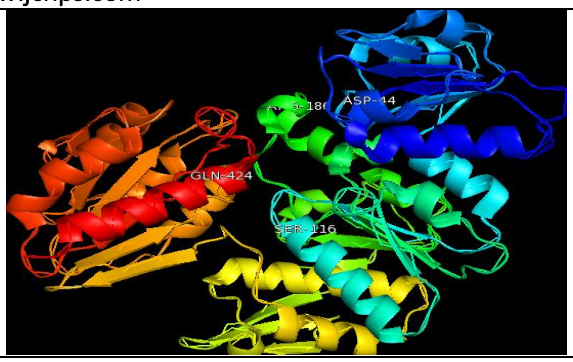


Fig.1.3D structure of the MurD ligase is retrieved from the protein data bank (PDB id:2X50)

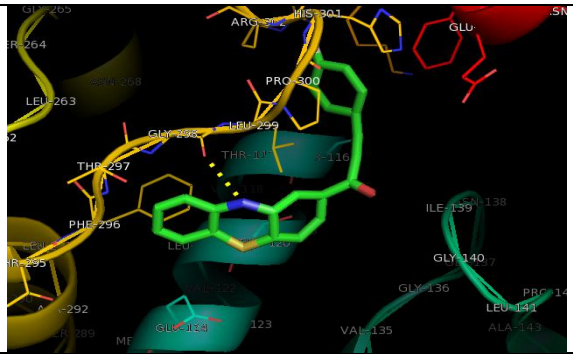


Fig.2.Intraction of Phenothiazine derivaties 3b with 2X50

Table.2.Lipinski's rule of five parameters for 3a-3g and 5a-5g

Compound	Molecular Weight	Molecular Formula	No. of H-Donar	No.of H-Acceptor	Rotatable Bonds	Log P
3a	329.41	$C_{21}H_{15}NOS$	1	1	3	4.89
3b	363.86	$C_{21}H_{14}ClNOS$	1	1	3	3.63
3c	408.31	$C_{21}H_{14}BrNOS$	1	1	3	5.72
3d	343.44	$C_{22}H_{17}NOS$	1	1	3	5.38
3e	372.46	$C_{23}H_{18}NO_2S$	1	2	3	4.21
3f	347.41	$C_{21}H_{14}FNOS$	1	2	3	5.05
3g	374.41	$C_{21}H_{14}N_2O_3S$	1	4	3	-
5a	383.51	$C_{25}H_{21}NOS$	1	1	2	4.94
5b	382.5	$C_{25}H20_{NOS}$	1	1	2	5.5
5c	462.4	$C_{25}H_{20}BrNOS$	1	1	2	5.77
5d	397.53	$C_{26}H_{23}NOS$	1	1	2	5.43
5e	411.52	$C_{26}H_{21}NO_2S$	1	2	2	4.69
5f	401.5	$C_{25}H_{20}FNOS$	1	2	2	5.1
5g	428.5	$C_{25}H_{20}N_2O_3S$	1	3	2	-

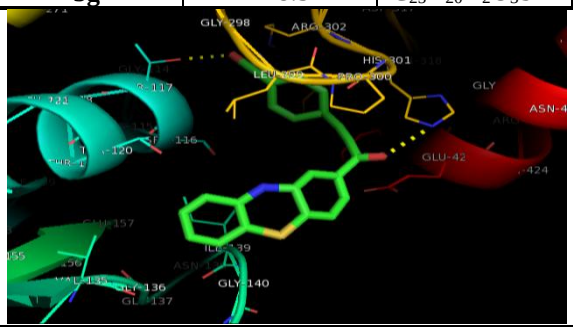


Fig.3.Intraction of Phenothiazine derivaties 3g with MurD ligase 2X50

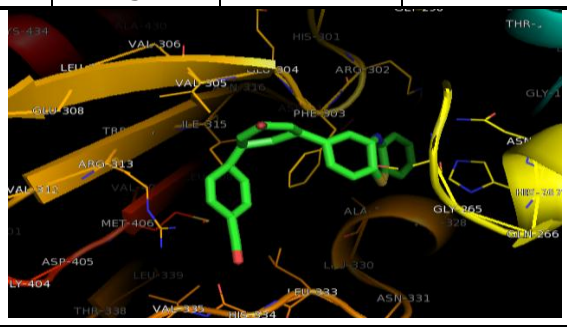


Fig.4.Intraction of Phenothiazine derivatie 5g with MurD ligase 2X50

Table.3.Docking scores, H-bond interations and surrounding aminoacids of the 3b, 3g, 5f and 5g ligands with MurD ligase

Compound	Score Kcal/mole	H-bond interaction	Surrounding amino acids in the binding pocket
3b	-11.5293	SER 318, ASP 31,SER 318, ALA 320, HIS3 01	SER 364, LEU 330, LEU 343, ASN 4 ASP 372, GLY 370, PRO 41, SER 410, HIS 301, ASP 325
3g	-11.2909	ALA 196, ALA 197, HIS 301, GLY 111, ILE 270	ARG 200, ASP 182, GLY 222, PRO 21 VSV 50, TYR202, GLY127, ILE 109, THR 110
5f	-11.6745	GLU 304, HIS 301,PRO 300, ILE 315, ASN 316, SER 318, ASP 317	ASN 322, VAL 323, SER 325, GLU 327 ASP 185, SER 160, GLY 324, SER 264 LEU 333, LEU 330, ALA 412
5g	-11.9888	PHE 189, GLY 190, GLN 193, SER 349, GLN 192, LEU 101, ASP 185	ILE 201, ARG 225, GLU 181, THR 114 LEU 183, ILE 216, ILE 109, VAL 107

Table.4.Antibacterial activity of Phenothiazine derivaties 3a-g and 5a-g

Compound	Zone of nhibition (mm)					
	B.cereus	***S.aureus***	***B.subtilis***	***E.coli***	***K.pneumoniae***	***P.mirabilis***
3a	11	9	10	14	13	16
3b	8	15	17	22	18	19
3c	16	12	19	21	14	13
3d	18	10	21	14	19	17
3e	7	14	8	21	24	20
3f	17	8	15	22	23	15
3g	19	17	18	23	21	20
5a	12	12	10	19	14	17
5b	13	17	17	13	18	10
5c	10	9	12	26	14	11
5d	11	16	15	19	23	16
5e	19	28	14	9	8	12
5f	16	10	22	23	15	9
5g	12	11	16	23	21	20
Gentamicin	19	17	20	24	26	15

4. CONCLUSION

Compounds 3a-3g and 5a-5g were docked with MurD ligase enzyme and their scores determined. All the compounds exhibited good scores showing the effective interaction of this group of ligands with the target. Lipinski's rule of five has been applied on these compounds to determine the drug likeliness of these compounds. All the compounds have drug like properties. All the compounds were synthesized in the laboratory and characterized by spectral methods. The antibacterial activity against three gram positive and three gram negative bacteria has been carried out by zone inhibition method. All the compounds synthesized with chalcone and phenothiazine moiety 3a-3g and cyclohexanone with phenothiazine moiety 5a-5g have shown good antibacterial activity and the results are comparable with the control. The activity may be due to the synergetic effect of the different functionalities chalcone and phenothiazine moiety3a-3g and cyclohexanone with phenothiazine moiety 5a-5g. Further the mechanism of action of these compounds may be due to the inhibition of the MurD ligase.

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