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NOVEL PHENOTHIAZINE COMPOUNDS AS THIRD GENERATION BCR-ABL TYROSINE KINASE INHIBITORS

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ABSTRACT

Several 3(rd) generation inhibitors are being developed for the treatment of patients with Chronic myelogenous leukemia (CML). The present work mainly aims to discover novel small molecular inhibitors against important molecular target T3151 Abl mutant involved in leukemia. Docking study was carried out and the binding affinity of the proteins with the phenothiazine compounds 3a-g and 7a-c was measured. The docking scores of the N-acylated compounds 7a-c are higher than that of the unacylated compounds. The compounds were tested for drug like molecules, used the Lipinski's rule of five. All the phenothiazine compounds (3a, 3c, 3d and 7a-c) were employed for inducing antiproliferative effect by trypan blue and MTT assay and induction of apoptosis in K562 cells. In general all the N-acylated compounds and in particular NPHC-3 with a chloro substituent in the para position of the phenyl ring appeared to be most potent molecule with an IC_{50} value of 32.44 and 24.01(µg/ml) by trypan blue and MTT assay respectively.Further a dose- dependent increase in LDH release was observed, confirming the antiproliferative potential of the compounds.

KEYWORDS: **Chronic myelogenous leukemia, Phenothiazine, Synthesis, characterization, Anti-proliferative activity.**

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INTRODUCTION

In the post-genomic era, rational anti-cancer drug discovery aims to discover or design small molecules that modulate the activity of key therapeutic targets pivotal for carcinogenesis[1]. Computer- aided or *in silico* design is being utilized to expedite and facilitate the lead molecule identification. It reduces the size of chemical space and thereby allows focus on more promising candidates for lead discovery and optimization. Chronic myelogenous leukemia (CML) is a hematological stem cell disorder caused by increased and unregulated growth of myeloid cells in the bone marrow[2], and the accumulation of excessive white blood cells. Abelson tyrosine kinase (ABL) is a non-receptor tyrosine kinase involved in cell growth and proliferation and is usually under tight control[3]. However, 95% of CML patients have the ABL gene from chromosome 9 fused with the breakpoint cluster (BCR) gene from chromosome 22, resulting in a short chromosome known as the Philadelphia chromosome. This Philadelphia chromosome was responsible for the production of BCR-ABL, a constitutively active tyrosine kinase that causes uncontrolled cellular proliferation[4]. An ABL inhibitor, imatinib, is currently used as first line therapy. However, a high percentage of clinical relapse has been observed due to long term treatment with imatinib. A majority of these relapsed patients have several point mutations at and around the ATP binding pocket of the ABL kinase domain in BCR-ABL[4]. In order to address the resistance of mutated BCR-ABL to imatinib, 2(nd) generation inhibitors such as dasatinib, and nilotinib were developed[5]. These compounds were approved for the treatment of CML patients who are resistant to imatinib[6,7]. All of the BCR-ABL mutants are inhibited by the 2(nd) generation inhibitors with the exception of the T315I mutant. Several 3(rd) generation inhibitors were being developed to target the T315I mutation. This prompted us to try some of the phenothiazine derivaties which are synthesized in our laboratory in order to address the resistance of mutated BCR-ABL to imatinib, 2(nd) generation inhibitors such as dasatinib, and nilotinib. If the present work is encouraging it is anticipated that physicians will have additional drugs at their disposal for the treatment of patients with the mutated BCR-ABL-T315I. The success of these inhibitors will have greater implication not only in CML, but also in other diseases driven by kinases where the mutated gatekeeper residue plays a major role.

MATERIALS AND METHODS

Molecular Docking

The catalytic sites of tyrosine kinase Receptor along with area and volume of binding pocket was carried out with Meta Pocket 2.0 Finder program.[8,9] The three dimensional crystal structure of the T3151 Abl mutant in comp-lex with the aura kinase inhibitor was retrieved from the Protein Data Bank.[10,11] 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. The phenothiazine compound structures were drawn using Chem draw 12.0 software and converted to Pymol format with the standard settings and further used for docking studies. The molecular docking program Aurgus Lab 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 the 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.

Synthesis of N-acyl Phenothiazine Chalcones (7a-c)
General procedure

Phenothiazine chalcones were prepared and characterised as per the procedure reported from our laboratory. 0.001 Mole of chalcone[12] (PHC-1 to PHC-3) was dissolved in 25ml dichloromethane, and 0.001 mole of Acetic anhydride was added followed by catalytic amount of acetic andride was added and stirred with heating by using magnetic stirrer for 6 hrs. The reaction was monitored by TLC. After completion of the reaction, the reaction mixture was kept as such for evaporation. Later the product formed was dried and purity of the compound was checked by TLC using the solvent system Chloroform[13].

3-(4-phenyl)-1-(10H-phenothiazin-2-yl)prop-2-en-1-one (7a).

Yield: 64%; m.p: 182ºC, UV λ max (nm): 438.00, 312.00, 247.00; IR (KBr) cm^{-1}: 3336 (NH), 1680, 1658 (C=O), 1597 (C=C), 1554, 1508, 1473, 1392, 1327, 1253, 1172, 1083, 983, 891, 829, 794, 740; ^{1}H-NMR (400MHz $CDCl_3$) δ: 7.30 (1H, d, J=1.6Hz, H-1'), 7.09 (1H, d, J=7.8Hz, H-4'), 6.93 (1H, d, J=7.8Hz, H-6'), 6.78-6075 (1H, t, J=7.8Hz, H-7'), 7.02-6.98 (1H, td, J=7.8, 1.6Hz, H-8'), 6.65 (1H, d, J=7.8Hz, H-9'), 7.81-7.69 (2H, q, J=16Hz, H-2,3), 7.87-7.84 (2H, m, H-3'',5''), 7.47-7.45 (3H, m, H-2'', 6'', 3'), 7.63 (1H, d, J=8Hz, H-4''), 2.31 (3H, s, $COCH_3$); ^{13}C-NMR: 112.92 (C-1'), 134.66 (C-2'), 122.08 (C-3'), 130.57 (C-4'), 128.90 (C-6'), 114.57 (C-7'), 126.11 (C-8'), 115.19 (C-9'), 141.10 (C-9a'), 114.57 (C-5a'), 142.12 (C-10a'), 122.52 (C-4a'), 172.32, 188.02 (C = O), 122.52 (C-2), 143.73 (C-3), 136.85 (C-1''), 126.24 (C-2'', 6''), 127.97 (C-3'', 5''), 128.75 (C-4''), 31.26 ($COCH_3$); MS $[M+1]^+$: 371.23.

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3-(4-chlorophenyl)-1-(10H-phenothiazin-2-yl)prop-2-en-1-one (7b)

Yield: 65%; m.p: 190ºC, UV λ max (nm): 342.00, 282.00; IR (KBr) cm^{-1}: 3336 (NH), 1678, 1658 (C=O), 1597 (C=C), 1554, 1508, 1473, 1392, 1327, 1253, 1172, 1083, 983, 891, 829, 794, 740; ^{1}H-NMR (400MHz $CDCl_3$) δ: 7.29 (1H, d, *J*=1.6Hz H-1'), 7.64-7.61 (1H, dd, *J*=8,1.6Hz, H-3'), 7.09 (1H, d, *J*=8Hz, H-4'), 6.93-6.91 (1H, dd, *J*=8, 1.6Hz, H-6'), 6.78-6.74 (1H, td, *J*=8, 1.6Hz, H-7'), 7.02 (1H, td, *J*=8, 1.6Hz, H-8'), 6.67-6.65 (1H, dd, *J*=8, 1.6Hz, H-9'), 7.67 (1H, d, *J*=16Hz, H-2), 7.83 (1H, d, *J*=16Hz, H-3), 7.89 (2H, d, *J*=8Hz, H-2",6"), 7.53 (2H, d, *J*=8Hz, H-3",5"), 2.32 (3H, s, $COCH_3$) ; ^{13}C-NMR: 112.86 (C-1'), 136.75 (C-2'), 122.64 (C-3'), 130.45 (C-4'), 135.04 (C-6'), 114.58 (C-7'), 126.10 (C-8'), 115.16 (C-9'), 141.07 (C-9a'), 114.58 (C-5a'), 142.13 (C-10a'), 123.75 (C-4a'), 171.98, 187.92 (C =O), 122.09 (C-2), 142.24 (C-3), 126.24 (C-1"), 127.98 (C-2", 6"), 128.94 (C-3", 5"), 133.65 (C-4"), 31.12 ($COCH_3$); MS $[M+1]^+$: 404.254.

1-(10H-phenothiazin-2-yl)-3-p-tolylprop-2-en-1-one (7c)

Yield: 63%; m.p: 179ºC, UV λ max (nm): 432.50, 315.50, 248.50; IR (KBr) cm^{-1}: 3336 (NH), 1677, 1658 (C=O), 1593 (C=C), 1558, 1523, 1465, 1435, 1396, 1350, 1323, 1188, 972, 929, 852, 798, 740, 663; ^{1}H-NMR (400MHz $CDCl_3$) δ: 7.30 (1H, d, *J*=1.6Hz, H-1'), 7.61-7.58 (1H, dd, *J*=8, 1.6Hz, H-3'), 7.08 (1H, d, *J*=8Hz, H-4'), 6.93-6.91 (1H, dd, *J*=8, 1.6Hz, H-6'), 6.79-6.75 (1H, td, *J*=8, 1.6Hz, H-7'), 7.03-6.98 (1H, *J*=8, 1.6Hz, td, H-8'), 6.67-6.65 (1H, dd, *J*=8, 1.6Hz, H-9'), 7.75-7.69 (4H, dd, *J*=16, 8Hz H-2, 3, 2",6"), 7.28 (1H, d, *J*=8Hz, H-3",5"), 2.35 (3H, s, CH_3), 2.29 (3H, s, $COCH_3$); ^{13}C-NMR: 112.93 (C-1'), 140.66 (C-2'), 120.79 (C-3'), 131.93 (C-4'), 129.53 (C-6'), 114.57 (C-7'), 126.23 (C-8'), 115.23 (C-9'), 141.12 (C-9a'), 114.57 (C-5a'), 142.11 (C-10a'), 122.41 (C-4a'), 167.34, 187.93 (C = O), 122.07 (C-2), 143.81 (C-3), 126.08 (C-1"), 127.94 (C-2", 6"), 128.76 (C-3", 5"), 136.97(C-4"), 21.05 (CH_3), 30.61 ($COCH_3$); MS $[M+1]^+$: 387.4124.

Determination of invitro antiproliferative effect of the compounds on cultured K562 cells

K562 cells were purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°c in 5 % CO2 (NBS, EPPENDORF, GERMANY) in a humidified atmosphere in a CO2 incubator. Compounds were added to grown cells at concentrations of 6.25 µg, 12.5µg,25 µg,50 µg and 100µg from a stock of 1mg/ml 0.1% DMSO and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation.

Trypan Blue Exclusion Assay

Trypan blue was a vital stain used to selectively colour dead tissues or cells blue. The live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. So a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. A 1:1 dilution of the culture cell suspension with a 0.4% trypan blue solution (1;1 dilution in PBS) was charged on the counting chamber of a heamocytometer and counted at 40x (Olympus CH 20). Compounds were added to grown cells at concentrations of 6.25 µg, 12.5µg,25 µg,50 µg and 100µg from a stock of 1mg/ml 0.1% DMSO and incubated for 24 hours. Stained cells and total cells were counted to percentage of viable cells was calculated. Control was also treated in the same manner[14].

% of viability = [A/ (A+B) ×100
A = number of viable cells
A+B = total number of viable and dead cell

MTT assay

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (Himedia) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells[15,16]. The cell culture suspension was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at 37^0C for 3 hours. MTT was removed by washing with 1x PBS and 200µlof DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in an ELISA reader (LISASCAN, Erba).

% viability = (OD of Test/ OD of Control) X 100

Lactate Dehydrogenase Leakage Assay

Lactate dehydrogenase (LDH) released is an indicator of membrane integrity and thus resulting in cell injury. LDH assay was performed to evaluate the LDH release to the media following treatment with the 3a-c and 7a-c (6.25, 12.5, 25, 50 and 100 µg) on K562 for 24 h and it was measured using standard protocols [3, 23]. The intracellular LDH was determined after lysing the cells by rapid freezing and thawing in liquid nitrogen. The LDH release was measured at an absorbance of 490 nm. The percentage of LDH release was calculated as: (LDH activity in media) / (LDH activity in media þ intracellular

LDH activity) X100%. Results are presented as percentage of LDH release subtracting the control values from treated ones. All the experiments were performed in triplicate and yielded similar results[17,18].

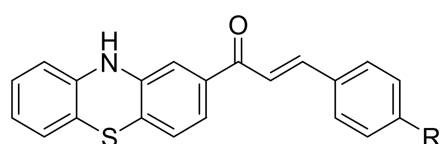
RESULTS AND DISCUSSION

These compounds have a similar mechanism as Bcr-Abl kinase inhibitors.

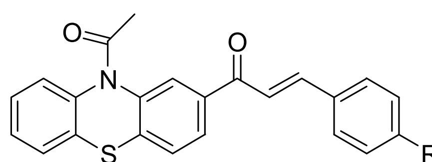
From medicinal chemistry perspective, phenothiazines are important groups of condensed three ring heterocycles. Phenothiazine derivatives and their hetero analogues containing 1, 4-thiazine structural fragment showed diverse biological activities. A phenothiazine derivative that contains amino alkyl substituents at the thiazine nitrogen atom are used as antipsychotic and antihistamine drugs. Extensive search has been conducted regarding the synthesis of potentially useful phenothiazine derivatives having pharmacological activity. From our ongoing anticancer research programme we have selected 3a-g for the present work to explore the possibility of finding an effective T315I mutated Bcr-Abl kinase inhibitor. To start with docking analysis on the compounds 3a-g with T315I mutated Bcr-Abl kinase protein was carried out.

Molecular docking

The T3151 Abl mutant was selected as the receptor protein in the ABL kinase domain in BCR-ABL and is present in the K562 leukemic cancer lines. The protein structures was derived from PDB and used as a target for docking simulation. Docking was performed using Argus lab software, the crystal structure of protein T315I mutated Bcr-Abl kinase was refined from the crude PDB structure and then saved as mole file to be used for docking simulation. Compounds 3a-g were constructed on ChemDraw 8.0. Structure and the 2D structure of the selected compounds were converted to their 3D form, and then energy minimized and saved as mol. The observed negative value for the docking score energy (Table 1) indicated the binding affinity of these compounds into T315I mutated Bcr-Abl kinase , this may give a reasonable explanation for their high activity. Docking analysis in Table 1 demonstrated the lower binding energy of inhibitors 3a-g (Scheme-1) with T315I mutated Bcr-Abl kinase under the participation of Van der Waals force, hydrogen bonds and hydrophobic interaction during the process of enzyme-inhibitor interaction. The detection of ligand-binding sites is often the starting point for protein function identification and drug discovery. In our study, meta pocket active site Finder predicted active site of the proteins. The active site comprises of hydrophobic amino acid residues. So they are the main contributors to the receptor-ligand interaction. The binding affinity of the protein with the PHC 1-8 were measured by kcal/mol. The table shows the docking scores, H-bonded interactions and the amino acids surrounding the ligand. All the phenothiazine chalcones (3a-g) showed good docking scores indicating a good affinity between the ligands and the protein tested. 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 score -11.0186, 11.6544 and 12.220 k cal/mol. These three molecules were selected for further research. In the above molecules there is only one H-bonding donor(NH) and one H-bonding acceptor(C=O). In order to find the difference in activities when the -NH group was replaced by an acyl group we selected three more molecules 7a to 7c and the docking analysis was carried out.



3a; H
3b:CH_3
3c: Cl
3d; Br
3e; CHO
3f; F
3e; NO_2
3g; OCH_3



N-acetyl-Phenothiazine chalcones

7a; H
7b; CH_3
7c; Cl

Scheme 1

Table 1
The docking scores, phenothiazine chalcones and N-acyl derivatives of phenothiazine chalcones.

S. No.	Compound	Score Kcal/mole	S. No.	Compound	Score Kcal/mole
1.	**3a**	-11.0186	7.	**3g**	-10.7513
2.	**3b**	-11.6544	8.	**3h**	-10.9388
3.	**3c**	-12.222	9.	**7a**	-11.382
4.	**3d**	-10.7513	10.	**7b**	-12.3588
5.	**3e**	-11.0018	11.	**7c**	-12.1095
6.	**3f**	-11.8987			

Table 2
H-bonding interactions and surrounding amino acids in the binding pocket for the 3a-c and 7a-c

Compound	Score Kcal/mole	H-bond interaction	Surrounding amino acids in the binding pocket
3a	-11.0186	ILE-314,315,LEU-302	LEU-266,302,ILE-242,314,315,GLY-302,GLU-236,238,THR-240,267,TRP-261,ASP-241,ARG-239,ALA-269,MET-237,SER-265,VAL-268
3b	-11.6544	ALA-269,LEU-302,ILE-314,315,GLY-303	GLU-316,258,GLY-321,303,249,250,VAL-270,268,LEU-301,248,266,PHE-307,LYS-247,ILE-314,THR-269,264,257,SER-265,MET-237,
3c	-12.226	ILE-313,314,GLY-250,251	VAL-270,256,268,GLY-249,250,251,252,321,LYS-245,271,LEU-248,ILE-313,314,ALA-269
7a	-11.382	Gly 383	ARG-362, ASP-360, TYR-353, GLY-254, ARG-380, LEU-387, VAL-378, PHE-401, ALA394
7b	-12.3588	PHE-317, ALA-239, GLY 321, ALA 269	GLY-250, PHE-317, LEU-248, HIS-246, LYS-247, LEU-370, GLY-321, THR-319, LEU-290, LYS-247, MET-318, ALA-269, GLY-321.
7c	-12.1095	No H-bonding	GLY-250, ALA-269, PHE-317, VAL-262, VAL-2, LYS-271, VAL-256, GLY-249, TYR-25, LEU-24, VAL-299, GLN-30, GLY-321.

It was learned from the table that compounds 7a-c with H, methyl & chloro substituents respectively exhibited a reasonably high scores -12.3558, 11.382 and 12.1096 kcal/mol when compared with the unacylated compounds. The compounds 3a-c form hydrogen bonds with ILE 314, ILE 315, LEU 302 and the surrounding amino acids available for hydrophobic interactions are mainly ALA-269,MET-237,SER-265. In case of 7a-c, the compounds form H-bonds with GLY-383, PHE-317, ALA 239, GLY 321 and ALA 269 and the surrounding aminoacids vary depends upon the molecules. The difference in the H-bonding interactions is due to the difference in the number of H-bonding donors and acceptors present in the molecules.

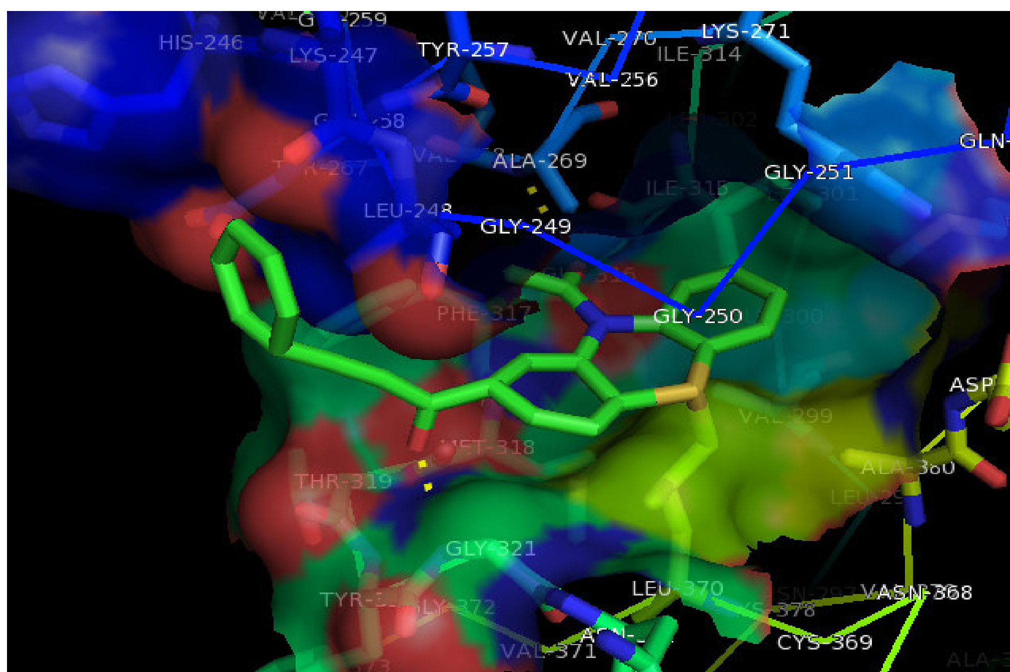


Figure 1
Binding of 7a in to the 2V7A binding site

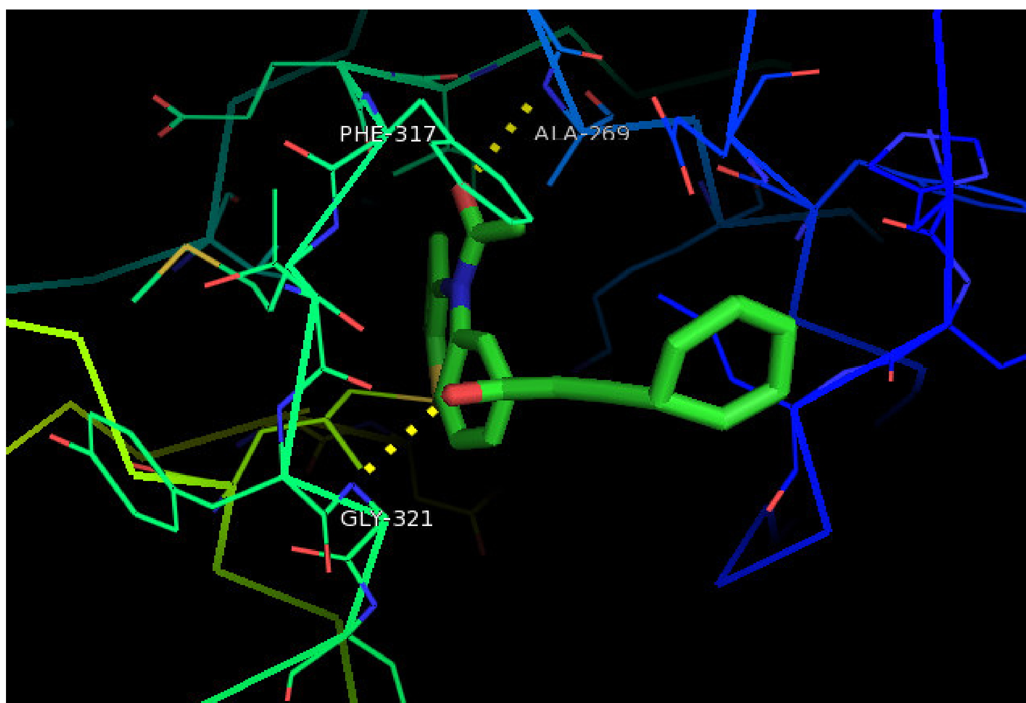


Figure 2
H-bond intraction of 7a with 2V7a protine

Molecular docking of 7c against 2V7A(CML protein)
Since all the selected ligands have shown reasonable scores in the docking studies indicating a good affinity towards the T3151 Abl mutant suggests that they can behave as third generation tyrosinase inhibitors for the CML. The results were useful for the design and development of novel compounds having better inhibitory activity against several type of cancer. To study structural activity relationship between the synthesized ligands against T3151 Abl mutant, we have selected three derivatives from the phenothiazine chalcones(3a-g) and their corresponding N-acyl derivatives (7a-c) for synthesis. The molecules were selected in such a way that two are with phenyl ring without substituents 3a and 7a, two are with an electron donating substituent in the phenyl ring system (3d and 7c) and two are with an electron withdrawing substituent (3c and 7c). To proceed further the above six compounds were selected as potential drug candidates for validation in the wet lab studies for its proper function.

Table 3
Lipinski's rule of five parameters for 3a-c and 7a-c

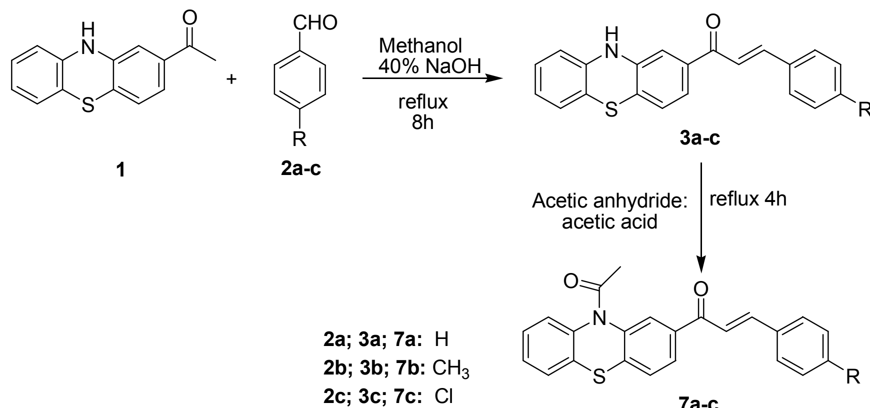
Compound	Molecular weight	Molecular formula	No. of H-donar	No. of H-acceptor	Rotatable bonds	Log p
3a	329.41	$C_{21}H_{15}NOS$	1	1	3	4.84
3b	343.44	$C_{22}H_{17}NOS$	1	1	3	5.38
3c	363.86	$C_{21}H_{14}ClNOS$	1	1	3	3.63
7a	371.1	$C_{23}H_{17}NO_2S$	0	2	2	4.84
7b	385.11	$C_{24}H_{19}NO_2S$	0	2	2	5.33
7c	405.06	$C_{23}H_{16}ClNO_2S$	0	2	2	5.40

To identify whether the above selected ligands are drug like molecules the Lipinski's rule of five parameters were applied and analysed. The results were shown in the Table 3. All the parameters are well within the rule except the Log P value where it is acceptable for 3a, 3c and 7a. The values for the other three ligands 3b, 7b and 7c are marginally on the higher side. Based on the majority of the acceptable parameters all the six compounds were selected and synthesised.

Synthesis of Chalcone
The chalcones 3a-c were prepared by Claisen-Schmidt condensation (Scheme-2) of equimolar quantities of phenothiazinyl methyl ketone 1 with substituted aryl aldehydes 2a-c in the presence of alcoholic alkali. The analytical data, reaction conditions and the yield of the product and characterization were already reported. The chalcones (3a-c) were treated with acetic anhydride and stirred in a magnetic stirrer to yield the respective N-acyl phenothiazine chalcones7a-c in Scheme-2.

Characterization of Chalcones 3a-c and 7a-c

The UV spectra of 3a-c exhibited characteristic peaks at 400nm and 260nm for chalcones. The IR spectra showed characteristic absorption bands to shows the presence of two carbonyl groups at1678 and 1651cm^{-1} and C=C at 1600cm^{-1}. In ^{1}HNMR spectrum of compound 3a H-2 and H-3 were found to be in the trans position and appeared as doublets at δ 7.27 and 7.72 with a coupling constant 16 Hz. The protons present in the phenothiazine ring are explained as follows. H-1' appeared as doublet with *J*=2Hz at δ 7.11, H-3' appeared as doublet of doublet with *J*=8, 1.6 Hz between δ 7.38-7.36. H-4' appeared as doublet with *J*=8Hz between δ 6.98 - 6.96. H-6' appeared as doublet of doublet with *J*=8, 1.6Hz at δ 6.76, H-8', 9' appeared as multiplet at δ 7.52. The proton present in the aromatic ring H-2", 6", 3", 5" appeared as multiplet between δ 6.89-6.85. The N acyl group appeared as singlet at δ 2.30. In ^{13}C-NMR spectrum, the signals were assigned based on their positions and intensities. For compound 7c, the signals were in good agreement with the proposed structure. The carbonyl groups (C=O) appeared at δ 189.00, and 170.00, C-2 appeared at δ 122.80 and C-3 appeared at δ 144.62. All the aromatic carbon atoms appeared between δ 113.46 to 161.75.



Scheme 2
Synthesis of N-substituted derivatives of Phenothiazine

IN VITRO ANTICANCER ACTIVITIES
Trypan Blue Exclusion Assay

In order to evaluate the antiproliferative effect of 3a, 3c, 3d , 7a, 7b and 7c on K562 cell leukemic cell line, we used trypan blue assay. K562 cells were treated with 6.25, 12.5, 25, 50 or 100 µg/ml of 3a-c and 7a-c. Since the com pounds were dissolved in DMSO, the cells with DMSO were used as vehicle control. Following addition of the compound, cells were counted after 24 h. Results showed that cell growth was affected with increase in concentration of the compound (Fig.). 3a-c and 7a-c induces antiproliferative effect in K562 leukemic cell line in a dose dependent manner. The effect was limited in case of 3a-c whereas in case of 7a-c the effect was appreciable. In case of 7c there was significant reduction in the cell number even when 12.5 µg/ml was used for the study. However, concentrations of 100 µg/ml resulted in significant cell death in all the compounds. The IC50 of 3a, 3c, 3b and 7a-c was approximately 76.17, 73.24, 214.30, 61.16, 66.73 and 32.44 respectively. In phenothiazine chalcones 3a-c and in N-acyl phenothiazine chalcones the effect was found to be more when there was substituent in the 4th position of the benzene ring. The effect was more in the compounds with the electron withdrawing group (chloro). These results suggest that 3a-c and 7a-c induce antiproliferative effect in human leukemic cells in a dose-dependent manner. Interestingly for 7c IC_{50} value was 32.44 µg/ml and found to be more effective when compared to all other compounds.

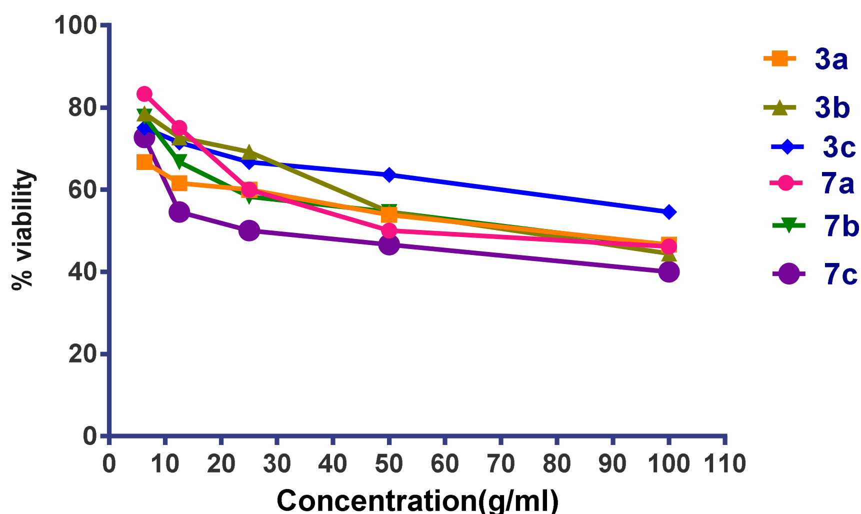


Figure 3
Trypan blue assay % viability of K562 cell with effect of 3a-c and 7a-c

MTT Assay
The effect of 3a-c and 7a-c on proliferation of K562 leukemic cells was further verified using MTT assay. K562 cells were treated with 6.25, 12.5, 25, 50 and 100 µg/ml of compounds and were subjected to MTT assay after 48 h (Fig. 2). Results showed that cell viability was affected at higher concentration in case of the phenothiazine chalcones 3a-c while the effect was more pronounced in the case of the N-acyl Phenothiazine chalcones 7a-c. In case of 7c cell viability was affected significantly as low as 6.25 µg/ml itself. The IC_{50} values of the compounds 3a-c and 7a-c were 95.84, 91.20, 58.38, 51.63, 45.33, and 24.01 respectively. Based on the both Trypan blue and MTT assays (Table 4), we find that potency of 7c (IC_{50} value of 32.44 and 24.01 µg/ml) is 2 to 3 fold higher than all other compounds.

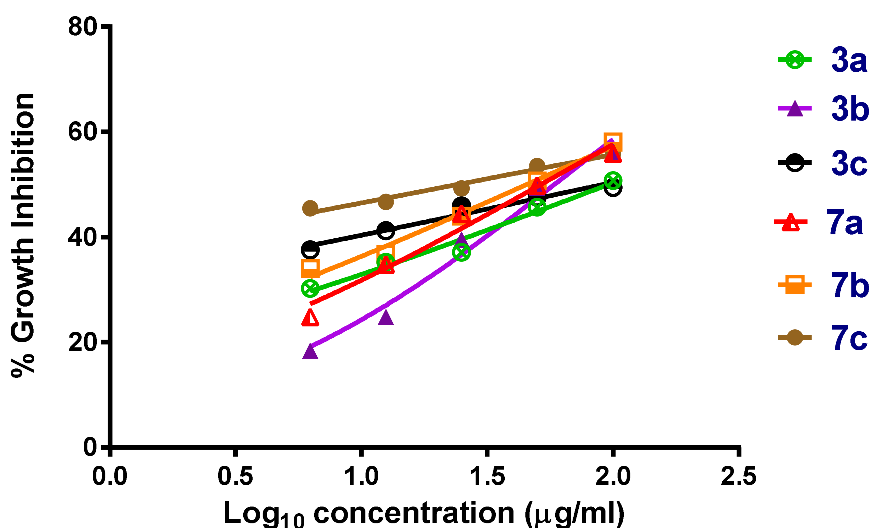


Figure 4
Growth inhibition K562 cell effect ogof 3a-c and 7a-c

Table 4
IC_{50} Values of 3a-c and 7a-c against K562 cell

S.No	Compound	IC_{50}(μg/ml)	Compound	IC_{50}(μg/ml)
	Trypen Blue assay		MTT assay	
1	3a	76.17	3a	95.84
2	3b	214.3	3b	91.2
3	3c	73.24	3c	58.38
4	7a	61.16	7a	51.63
5	7b	66.73	7b	45.33
6	7c	32.44	7c	24.01

When all the phenothiazine compounds (3a-c and 7a-c) were employed for inducing cytotoxic activity and induction of apoptosis in K562 cells, the 7c with a chloro substituent in the para position of the phenyl ring appeared to be most potent followed by the methyl substituted phenothiazine and the unsubstitued phenothiazine compounds. The electron withdrawing chloro group may be the reason to have an affinity towards the receptor which may enhance the binding of this molecule. Further the enhanced activity of the N-acyl compounds may be due to the tendency of the carbonyl group to form hydrogen bonds with the proteins. Both the trypan blue assay and MTT assay showed that the N-acylated phenothiazine ring system (7a-c) shows higher activity when compared with the non-acylated phenothiazine system (3a-c). Further presence of substituents like methyl and chloro(7b and 7c, 3c, 3b) increases the activity of the pehnothiazine ring system. Among these two substituents the electron withdrawing chloro group was better than the methyl substituent. It seems that the ring system requires electron with drawing or electron donating groups to polarize the amino acids and attract the T3151 protein molecules for interaction. Further the presence of the N-acyl group being polar in nature is used to form H-bonding and for other interactions, which may be the reason to show a higher activity. The docking scores also complement the above observations that the scores with the N-acylated phenothiazine ring system (7a-c) are higher when compared with the non-acylated phenothiazine system (3a-c). Further presence of substituents like methyl and chloro (7b and 7c, 3b, 3c) showed higher score than the unsubstituted pehnothiazine ring system.

Lactate Dehydrogenase Leakage Assay

LDH release assay was performed to test the cell membrane damage induced by the compounds 3a-c and 7a-c . For this, K562 cells were cultured with 6.25, 12.5, 25, 50 and 100 μg/ml of 3a-c and 7a-c and LDH released was measured at 48 h. Consistent with above results, we observed a dose- dependent increase in LDH release, further confirming the cytotoxic potential of 3a-c and 7a-c

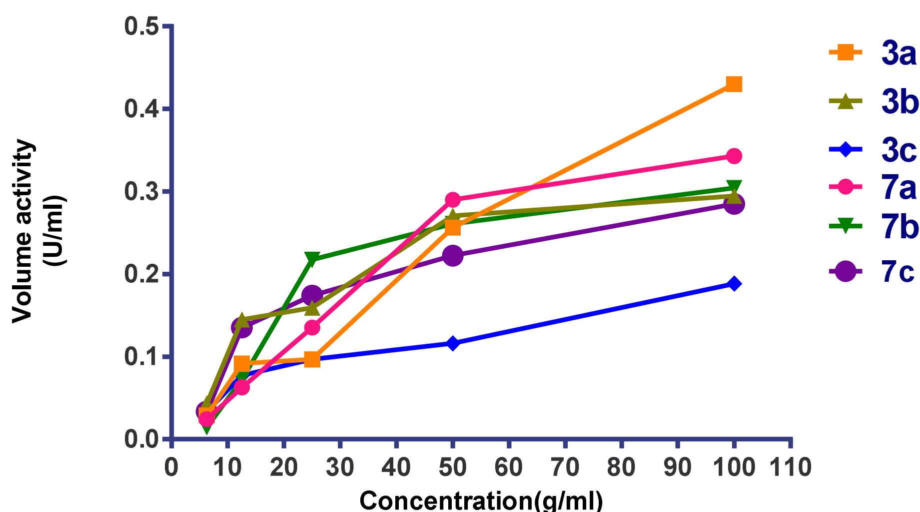


Figure 5
LDH release of K562 cells on the effect of 3a-c and 7a-c

CONCLUSION

This work mainly aims to discover novel small molecular inhibitors against important molecular targets involved in leukemia. The T3151 Abl mutant was selected as the receptor protein in the ABL kinase domain in BCR-ABL and is present in the K562 leukemic cancer lines. Docking studies were carried out and the binding affinity

of the proteins with the phenothiazine compounds 3a-g and 7a-c was measured. The docking scores of the N-acylated compounds 7a-c (12.3558, 11.382 and 12.1096 k cal/mol) are higher than that of the unacylated compounds 3a, 3b and 3c (11.0186, 11.6544 and 12.220 k cal/mol). The above six molecules were tested for drug like molecules, using the Lipinski's rule of five. Among the phenothiazine compounds 3a-g and 7a-c were employed for inducing antiproliferative effect by trypan blue and MTT assay and induction of apoptosis in K562 cells, 7c with a chloro substituent in the para position of the phenyl ring appeared to be most potent. The electron withdrawing chloro group may be the reason to have an affinity towards the receptor which may enhance the binding of this molecule. Further the enhanced activity of the N- acyl compounds may be due to the tendency of the carbonyl group to form hydrogen bonds with the proteins. LDH release assay was performed to test the cell membrane damage induced by the compounds. We observed a dose-dependent increase in LDH release, further confirming the antiproliferative effect of the compounds

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