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Original Article

IN SILICO SCREENING, SYNTHESIS AND IN VITRO EVALUATION OF SOME QUINAZOLINONE DERIVATIVES AS DIHYDROFOLATE REDUCTASE INHIBITORS FOR ANTICANCER ACTIVITY: PART-I

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ABSTRACT

Objective: The main objective of this research was to *in silico* screen, synthesize, characterize and *in vitro* evaluate some quinazolin-/e/one derivatives as dihydrofolate reductase (DHFR) inhibitors for anti-cancer activity.

Method: The present study reports a new series of Quinazoline and quinazolinone as potent inhibitors of human DHFR for anticancer activity. *In silico* screening of compound was performed on Vlife MDS 4.3 software and ADME studies were performed on PreADMET online software for prioritization of molecules for actual synthesis and *in vitro* evaluation. Prioritized molecules were synthesized by known reactions. Characterization of derivatives were done by ^{1}H NMR, C^{13} NMR, IR and melting point. *In vitro* anticancer activity was performed at ACTREC, Navi Mumbai, India for the prioritized molecules on ten human cell-lines.

Result: Molecules were prioritized with comparable docking score as compared with Methotrexate used as standard in docking. ADMET parameters such as HIA, Caco 2 cell permeability, MDCK and PPB were considered for prioritization. Prioritized synthesized molecules complied with spectroscopic assignments. Furt.her prioritized molecules were evaluated for *in vitro* anticancer activity .Molecules showed potent anticancer activity.

Conclusion: Prioritized compounds BrQSB1, BrAQ3, BAQ_{23} were highly active on A549, HeLa, SK-OV-3, KB, HCT15, SiHa, MCF7, DU145 at a concentration of < 10 μg/ml. These can further serve as templates for development of anticancer agent.

Keywords: Quinazoline, Quinazolinone, *In silico*, ADME, Molecular docking, Anticancer.

INTRODUCTION

Cancer is major cause of mortality. Amongst various methods of cancer treatment include chemotherapy with DHFR inhibitors. Inhibition of human Dihydrofolate reductase plays a major role cancer chemotherapy.

***DHFR (Dihydrofolate Reductase, E.C.1.5.1.3)*:**

DHFR inhibitors belong to the class of antimetabolite as chemotherapeutic agents. Dihydrofolate reductase catalyzes reduction of folic and dihydrofolic acids to tetrahydrofolic acid. Tetrahydrofolate and its metabolites are involved in the biosynthesis of thymidine monophosphate and purine bases hence blocking of DHFR enzyme causes termination of cell division and subsequents cell death. DHFR enzyme inhibitors are active in S- phase of cell cycle [1-3]. Compound that inhibit DHFR enzyme exhibit an important role in clinical medicine as exemplified by the use of methotrexate in anticancer agent.

Quinazolin-e/ones as DHFR inhibitors:

Research on quinazoline derivative lead to development of new anticancer agent. In order to produce innovative potent leads for anticancer drugs, a new series of quinazoline and quinazolinone analogs was designed to resemble methotrexate structure feature and fitted with functional group believed to enhance inhibition of mammalian DHFR enzyme activity, with this aim in this paper it was attempted to investigate hDHFR inhibitors by drug design [4-6]. Considering importance of hDHFR enzyme, it was chosen as target and with literature of Quinazoline and quinazolinone, these were found to be potent hDHFR enzyme inhibitor hence chosen for this research [4-8]. *In silico* prioritization of these lead moieties as hDHFR enzyme inhibitor for anticancer activity is being done by V life science MDS 4.3 drug design software. This research work reports *in silico* prioritization performed before actual synthesis, synthesis, syntheisi and *in vitro* anticancer evaluation of some quinazoline and quinazolinones for anti cancer activity.

MATERIAL AND METHODS

In silico Studies:

The in silico ADME predictions were obtained from www.bmrd.org. Docking simulations were performed on Vlife MDS4.3 Drug Design software on Windows OS. Marvin beans and Chem Draw Ultra 11.0 were used to draw the structures of the molecules and for conversion of 2-D structures into mol files.

Drug and chemicals:

All chemicals were purchased from Sigma Aldrich, SD Fine, Spectrochem and Merck. Yields refer to purified products and are not optimized. Melting points were determined on VEEGO - VMP I melting point apparatus and are uncorrected. IR spectrums were recorded on SHIMADZU spectrophotometer. ^{1}H NMR were recorded at University of Pune facility for NMR Department of Chemistry on Mercury Varian 300 MHz instrument and Brucker 400 MHz, chemical shifts (δ) are reported in parts per million (ppm) with $CDCl_3$ and DMSO as solvent for NMR. TMS was used as internal standard for NMR. Splitting of signals is represented by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplates). Thin layer chromatography (TLC) was performed on Merk GF254 precoated aluminium plate**.**

EXPERIMENTAL

I. *IN SILICO* SCREENING

1. ADME Prediction

In silico ADME parameters were obtained online from PreADMET software predicted by following parameters.

a. $Caco_2$ cell permeability

For prediction of Caco2 cell permeability in Pre ADMET, molecules were solvated in silico at pH 7.4. Caco2 cells are used to determine the apparent permeability values of compounds. The range of Caco 2 cell is 4-70 nm/sec.

b. MDCK cell permeability

MDCK cell means Madin-Darby Canine Kidney cell. MDCK cells are used to determine the apparent permeability values of compound. The range of MDCK is 25- 500 nm/sec.

c. Human Intestinal Absorption (HIA)

PreADMET can predict percent human intestinal absorption (% HIA). HIA data are the sum of bioavailability and absorption evaluated from ratio of excretion or cumulative excretion in urine, bile and faces. The range of HIA is 20- 70%.

d. Plasma Protein Binding (PPB)

Only the unbound drug is available for diffusion or transport across cell membranes and also for interaction with a pharmacological target. As a result a degree of plasma protein binding of drug influences not only the drugs action but also its disposition and efficacy. The range of PPB is about 90%. *In silico* ADME prediction are shown in Table 1.

2. Docking Study

Computer-assisted simulated docking experiments were carried out in Vlife MDS 4.2 software separately. Docking studies involves following steps:

Selection of Protein file from the database (pdb selection) [9]

Protein pdb (PDB IB: 1S3V) was selected after a comparative analysis of different pdb protein structures.

Protein Validation

Protein Validation was done using Ramchandran Plot and Errata Report. The Ramchandran plot showed 96.2% (177/184) residues in favored region; moreover 99.5% (183/184) in allowed region [10]. Figure 1 shows Ramachandran plot of protein PDB. Further this protein subjected to active site analysis, optimization and docking studies on VLife MDS 4.3 tools. Errata report was obtained from the NIH MBI sever for evaluation of protein structures and is shown in Figure 2.

Table 1: It shows *in silico* ADME Prediction data for selected compound

Compound	HIA@	Caco2 cell permeability++	MDCK+++	PPB$
BAQ_{22}	96.48	44.64	0.440	33.35
BAQ_{23}	96.62	13.56	0.085	99.19
BAQ_{24}	96.69	43.00	0.011	100.00
BAQ_{25}	96.63	15.13	0.030	98.18
BAQ_{28}	96.74	48.89	0.110	98.41
BAQ_{30}	96.74	45.62	0.119	98.01
$BrQSB_1$	96.75	38.13	0.079	100.00
$BrAQ_1$	96.54	23.95	0.410	94.80
$BrAQ_2$	93.10	20.16	0.447	48.20
$BrAQ_3$	85.63	20.24	0.237	51.25

@HIA = Human Intestinal Absorption. ++Caco 2 cell permeability = human colon adenocarcinoma and possess multiple drug transport pathways through the intestinal epithelium.+++MDCK = Madin-Darby canine kidney cell. $PPB = Plasma Protein BindinG

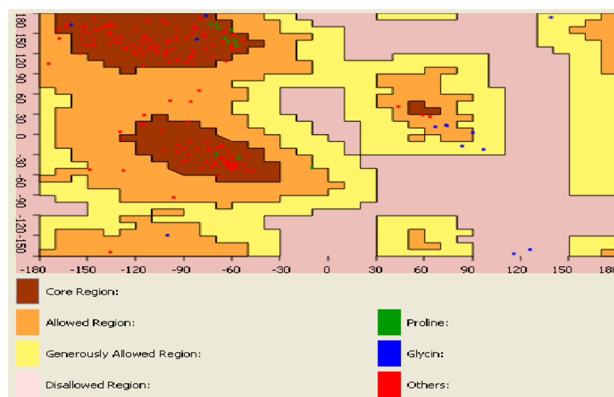


Fig.1: It shows Ramachandran plot of 1S3V.

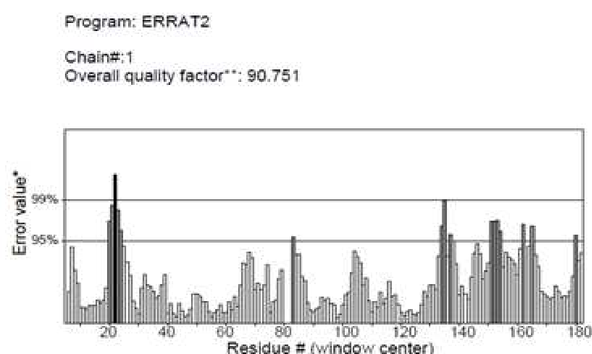
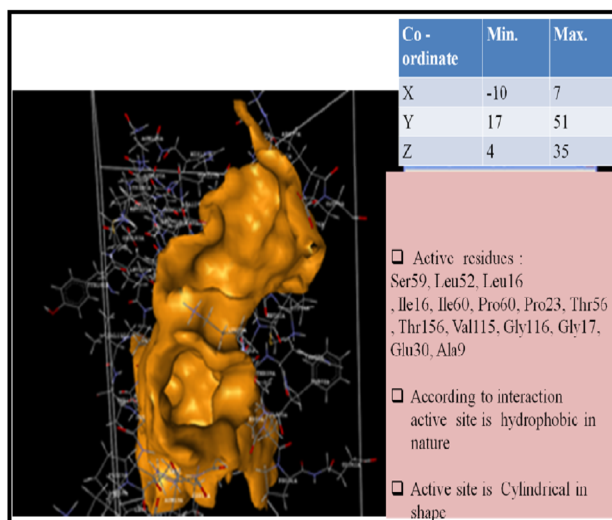


Fig. 2: It shows errata report of protein 1S3V of hDHFR

Active site analysis

It shows 3 cavities out of which cavity 1 was selected since it contained co-crystallized ligand. This site abundantly had lipophilic residues and cylindrical shape. Fig.3.



Co - ordinate	Min.	Max.
X	-10	7
Y	17	51
Z	4	35

- Active residues : Ser59, Leu52, Leu16 , Ile16, Ile60, Pro60, Pro23, Thr56 , Thr156, Val115, Gly116, Gly17, Glu30, Ala9
- According to interaction active site is hydrophobic in nature
- Active site is Cylindrical in shape

Fig. 3: It shows the co-ordinates, shape and active residues involved in 1S3V.

Protein pdb optimization

Removal of the water molecule, addition of hydrogen atoms was performed and further original ligand present in co-crystallized

structure was extracted out. Optimization of protein was done by MMFF of protein. This file was thus prepared for docking studies.

Library design and Ligand preparation

The Marvin Bean software was used to draw molecular structures of ligands and for the conversion of the 2D structure to 3D mol files. Structures of ligands were designed shown from Series 1 **(BAQ$_{1-36}$)**, Series 2 (**CAQ$_{1-7}$)**, Series 3 (**BrQSB$_{1-4}$)** and series 4 **(BrAQ$_{1-3}$)**. Library of 47 compounds was developed.

Molecules were drawn using 2D draw in Vlife MDS 4.3 tool. These ligand were further converted in to 3D and optimized using MMFF force field. General structures of these ligands are shown in Table 2 to 5.

Docking of ligands

V-Life MDS: Docking studies were performed using Biopredicta modules of VLife MDS 4.2 software. Docking was done by grid based docking. The grid based docking is a rigid and exhaustive docking method. In this method, after unique conformers of the ligand are generated, the receptor cavity of interest are chosen and grid is generated around cavity. Cavity points are found and the centre of mass of ligand is moved to each cavity point. All rotations of ligand are scanned at each cavity point where ligand is placed. For each rotation a pose of ligand is generated and corresponding bumps are checked for each pose of ligand and pose of ligand with the best score is given as output. Result of docking score, inhibition constant and binding energy are shown in Table 2 to 5.

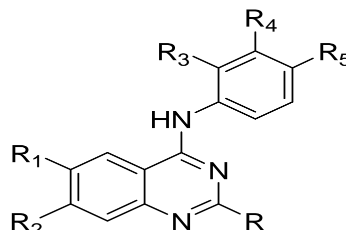


Table 2: It shows ligand of series 1 (BAQ$_{1-22}$)

S. No.	Compound	R	R$_1$	R$_2$	R$_3$	R$_4$	R$_5$	V-life score
1	BAQ$_1$	CH_3	Br	H	H	H	H	-59.88
2	BAQ$_2$	CH_3	Br	H	NO_2	H	H	-62.26
3	BAQ$_3$	CH_3	Br	H	H	H	NO_2	-65.60
4	BAQ$_4$	CH_3	Br	H	CH_3	H	H	-66.82
5	BAQ$_5$	CH_3	Br	H	H	CH_3	H	-64.59
6	BAQ$_6$	CH_3	Br	H	H	H	CH_3	-61.72
7	BAQ$_7$	CH_3	Br	H	H	H	Cl	-59.15
8	BAQ8	C_6H_5	Br	H	H	H	H	-54.16
9	BAQ$_9$	C_6H_5	Br	H	NO_2	H	H	-66.40
10	BAQ$_{10}$	C_6H_5	Br	H	H	H	NO_2	-57.32
11	BAQ$_{11}$	C_6H_5	Br	H	CH_3	H	H	-64.17
12	BAQ$_{12}$	C_6H_5	Br	H	H	CH_3	H	-67.54
13	BAQ$_{13}$	C_6H_5	Br	H	H	H	CH_3	-58.17
14	BAQ$_{14}$	C_6H_5	Br	H	H	H	Cl	-54.37
15	**BAQ$_{15}$**	**CF_3**	**Br**	**H**	**H**	**H**	**H**	**-80.83**
16	BAQ$_{16}$	CF_3	Br	H	NO_2	H	H	-49.53
17	**BAQ$_{17}$**	**CF_3**	**Br**	**H**	**H**	**H**	**NO_2**	**-73.29**
18	BAQ$_{18}$	CF_3	Br	H	CH_3	H	H	-55.39
19	**BAQ$_{19}$**	**CF_3**	**Br**	**H**	**H**	**CH_3**	**H**	**-72.35**
20	**BAQ$_{20}$**	**CF_3**	**Br**	**H**	**H**	**H**	**CH_3**	**-73.29**
21	**BAQ$_{21}$**	**CF_3**	**Br**	**H**	**H**	**H**	**Cl**	**-74.19**
22	**BAQ$_{22}$**	**H**	**Br**	**H**	**H**	**H**	**OCH_3**	**-66.26**

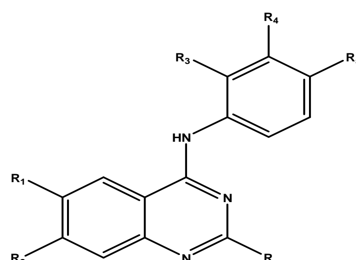
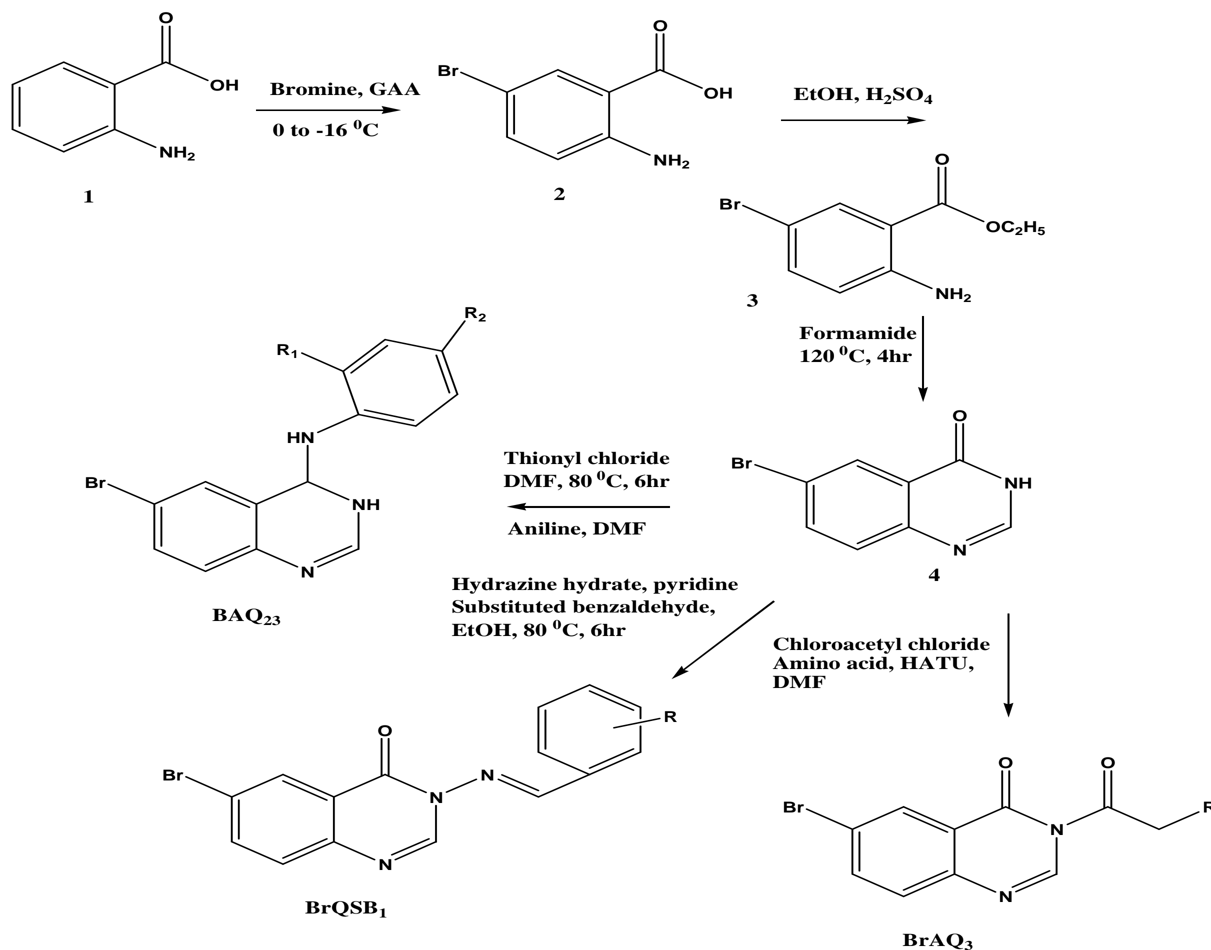


Table 3: It shows ligands of series 2 (BAQ$_{22-36}$ & CAQ$_{1-7}$)

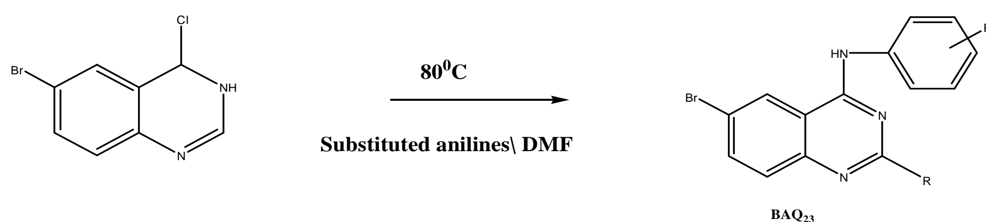
S. No	Compound	R	R$_1$	R$_2$	R$_3$	R$_4$	R$_5$	V-life score
23	**BAQ$_{23}$**	**H**	**Br**	**H**	**NO_2**	**H**	**H**	**-58.93**
24	**BAQ$_{24}$**	**H**	**Br**	**H**	**H**	**H**	**H**	**-64.05**
25	**BAQ$_{25}$**	**H**	**Br**	**H**	**H**	**H**	**NO_2**	**-66.05**
26	BAQ$_{26}$	H	Br	H	H	H	Cl	-64.77
27	BAQ$_{27}$	H	Br	H	Cl	H	NO_2	-77.23
28	**BAQ$_{28}$**	**H**	**Br**	**H**	**CH_3**	**H**	**H**	**-62.52**
29	BAQ$_{29}$	H	Br	H	H	CH_3	H	-61.53

30	**BAQ_{30}**	**H**	**Br**	**H**	**H**	**H**	**CH_3**	**-69.86**
31	BAQ_{31}	H	Br	Br	H	H	NO_2	-71.37
32	BAQ_{32}	H	Br	Br	CH_3	H	H	-63.21
33	BAQ_{33}	H	Br	Br	H	CH_3	H	-64.20
34	BAQ_{34}	H	Br	Br	H	H	CH_3	-71.37
35	BAQ_{35}	H	Br	Br	H	H	Cl	-65.63
36	BAQ_{36}	H	Br	Br	Cl	H	NO_2	-62.39
37	CAQ_1	H	Cl	H	H	H	H	-65.74
38	CAQ_2	H	Cl	H	H	H	NO_2	-66.91
39	CAQ_3	H	Cl	H	H	H	Cl	-65.67
40	CAQ_4	H	Cl	H	Cl	H	NO_2	66.96
41	CAQ_5	H	Cl	H	CH_3	H	H	-61.84
42	CAQ_6	H	Cl	H	H	CH_3	H	-70.72
43	CAQ_7	H	Cl	H	H	H	CH_3	-63.46
	Methotrexate							40.28

II. Synthesis



General Synthetic Scheme



Scheme 1

Synthesis of 6-bromo 4-(2-nitro anilino) quinazoline [11, 12]: To stirrer the solution of 6- bromo 4- chloro quinazoline (1 mol) and DMF (10 vol) was added 2-nitro aniline (1.5 mol). The reaction mixture was heated to 80°C and refluxed for 4 hrs with continuous stirring. Then the reaction mixture was poured in ice cold water and was filtered, the light yellow precipitate as 6- bromo 4- anilino quinazoline derivative separated out.

6-bromo 4-(2-nitro anilino) quinazoline (BAQ_{23}): **% Yield**: 60%, **Molecular Formula**: $C_{22}H$ $C_{14}H_9BrN_4O_2$,**Molecular wt**:345, Rf: 0.5 (Hexane:Ethyl acetate 90: 10), **Melting Point**:238-240^{0}C;**I.R. (KBr, cm^{-1})**:2900 (C-H, Str.). 1640 (Ar., C=C, Str.), 3300 (NH Str), 1530 (NO_2), 700 (C-Br, Str.), **^1H NMR (300 MHz, $CDCl_3$) δ [ppm]**:8.4 (s, H, Ar-H), 8.05 (d, H, Ar-H), 7.78 (d, H, Ar-H);8.7 (s, H, Ar-H),10.43 (s, 1H, NH); 7.4 (s, H, Ar-H); 7.6 (s, H, Ar-H); 7.76 (s, 2H, Ar-H); 7.78 (s, 2H, Ar-H).

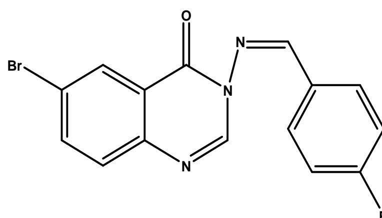


Table 4: It shows ligand of series 3 ($BrQSB_{1-4}$)

S. No.	Compound	R	V-life score
44	**$BrQSB_1$**	**Cl**	**-71.10**
45	$BrQSB_2$	H	-69.19
46	$BrQSB_3$	Br	-68.57
47	$BrQSB_4$	OCH_3	-65.66
	Methotrexate		-40.28

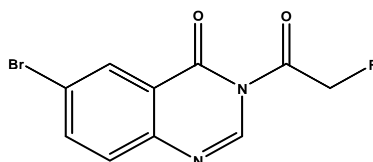
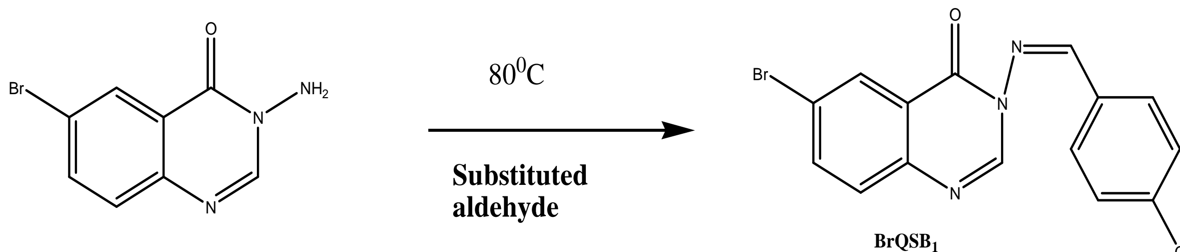
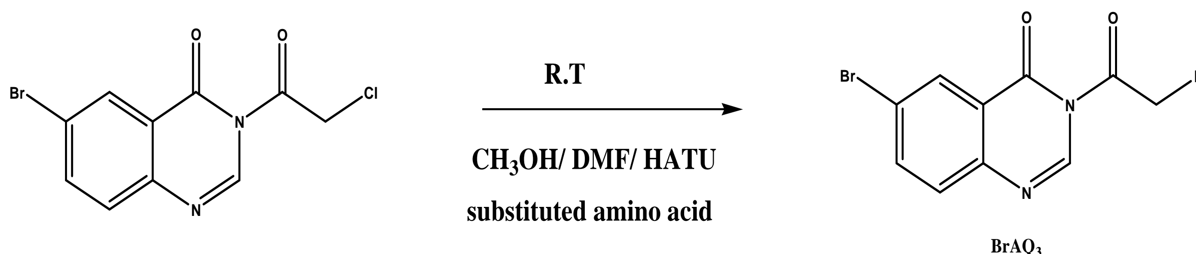


Table 5: It shows ligand of series 4 ($BrAQ_{1-3}$)

S. No.	Compound	R	V-life score
48	**$BrAQ_1$**	**Cl**	**-37.79**
49	**$BrAQ_2$**	**Glycine**	**-52.26**
50	**$BrAQ_3$**	**Glutamic acid**	**-41.54**
	Methotrexate		-40.28



Scheme 2



Scheme 3

Synthesis of 6- bromo-3-(2-substituted glutamino acetyl) quinazolin-4(3H)-one ($BrAQ_3$) [14]: The solution of 6- bromo 3-(2-chloro acetyl) quinazoline-4-one (0.017 mol) and substituted Glutamic acid (0.025 mol) was stirred for 12 hrs in methanol 10 ml, catalytic amount of DMF and HATU at room temperature. White precipitates occurs which was filtered out as 6- bromo-3-(2-substituted glutamino acetyl) quinazolin-4(3H)-one **($BrAQ_3$)**.

6- bromo-3-(2-substituted glutamino acetyl) quinazolin-4(3H)-one ($BrAQ_3$): **% Yield**: 77%, **Molecular Formula**: $C_{12}H_{10}BrN_3O_{43,}$ **Molecular wt**: 340, **Rf**: 0.3(Hexane:Ethyl acetate 70:30), **Melting Point**:210-214 ^{0}C; **I.R. (KBr, cm $^{-1}$)**:3500 (O-H, Str),3405 (N-H, Str.), 2916 (C-H, Str), 1710 (Ar., C=O, Str.), 1686 (C=N),1546 (C=C, Str.), 1317 (C-N, Str), 730 (C-Cl, Str.), 600 (C-Br, Str.), **^{1}H NMR (300 MHz, $CDCl_3$) δ [ppm]**:8.1- 8.44 (s, H, N-H); 8.3 (d, H, Ar-H); 7.6 (d, 1H, Ar-H); 4.15 (s, H, C-H); 2.97 (s, H, C-H); 2.81 (s, H, C-H).

III. *In-vitro* cytotoxicity (anticancer) assay: *In-vitro* cytotoxicity anticancer assay was done on ten human cancer cell lines i.e. A549 (lungs), SK-OV-3 (ovary), HCT15 (colon), K562 (leukemia), HeLa (cervix), KB (Nesophayngea), MCF7 (breast) and DU145 (prostate) with methotrexate used as standard. Compounds showed some comparable activity with methotrexate. The *in-vitro* cell line activity was performed at ACTREC, Navi Mumbai, India.

Assay procedure: The cells at subconfluent stage were harvested from the flask by treatment with trypsin [0.05% in PBS (pH 7.4) containing 0.02% EDTA]. Cells with viability of more than 98% as determined by trypsin blue exclusion were used for determination of cytotoxicity. The cell suspension of 1 x 10^5cells/ml was prepared in complete growth medium. Stock solutions (2 x 10^{-2} M) of compounds were prepared in DMSO. The stock solutions were serially diluted with complete growth medium containing 50 μg/ml of gentamycin to obtain working test solutions of required concentrations. *In-vitro* cytotoxicity activity of compounds was performed on ten cancer cell lines out of which three compounds BAQ_{23}, $BrAQSB_1$, $BrAQ_3$ were found to be active in *in vitro* cytotoxicity assay. The result are shown in Table 6.

Table 6: *In vitro* cytotoxicity assay of prioritized molecules on human cancer cell lines

Sr. no.	Types of cell line	Compound Code	Concentration (μg/ml)
1	A549	$BrQSB_1$	≤ 10
2	KB	BAQ_{23}	≤ 10
3	K562	$BrAQ_3$	≤ 10
4	HeLa	$BrQSB_1$	≤ 10
	Methotrexate	MTX	≤ 10

Table 7: It shows interaction of amino acids with ligands

Compound code	Hydrogen bonding	Hydrophobic bonding
BAQ_{17}		Lys55, Thr146, Gly117,Thr56
BAQ_{19}	Tyr121. Ala 9	Ala 8,Ile60
BAQ_{20}		Val8,Ile60,Pro61
BAQ_{23}	Tyr121. Ala 9	Lys 55A, The 56A, Gly 117A, Thr 146A,Ile16
$BrQSB_1$	Ser 59	Leu22A,Thr56A,Ser 59A
Methotrexate	Lys 18A, Asn 64A	Lys 55A,Thr 56A,Gly117A, Thr 146A

CONCLUSION

The active site of DHFR protein 1S3V include VAL 8, ALA 9, ILE 16, LEU 22, PHE 31, PHE 34, LYS 55, THR 56, SER 59, GLY 117 and TYR 121. Most of ligands were found to be interacting with the amino acid residues of the active sites. The present work leads to the development of quinazolinone derivatives as anticancer leads by *in silico* design. Compounds BAQ_{23}, $BrQSB_1$, and $BrAQ_3$were found to be active in *in vitro* cytotoxicity assay as compared with methotrexate used as standard in assay and can be considered as useful template for further anticancer lead development.

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