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Discovery of Hydrazinecarboxamide or Hydrazinecarbothioamide Bearing Small Molecules as Dual Inhibitor of Ras Protein and Carbonic Anhydrase Enzyme as Potential Anticancer Agent Using Validated Docking Study and *In-silico* ADMET Profile.

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ABSTRACT

Here we present a new class of anticancer agent as hydrazinecarboxamide or hydrazinecarbothioamide derivatives (R1-R9) acting as Ras protein and Carbonic anhydrase inhibitor. This design was justified by molecular docking using AUTODOCK 4.1 aganist 3KKP (Ras protein) and 4KP5 as carbonic anhydrase enzyme (CA XII), which was validated by cluster analysis with 2Å RMSD value. As well as predict the insilico ADMET and biological activity through ADMET predictor and Molinspiration server. The outcomes reveal that R4 was the best molecule as per the docking study aganist 3KKP and 4KP5 receptor as comparing to standard Kobe 2601. Except R1, R3 all the molecules follow Lipinski rule and R5, R6 with best activity profile by enzyme inhibition, GPCR- ligand and kinase inhibition. More or less the designed ligands with less developmental toxiciy and mutagenicity.

Keywords: Ras Protein, Carbonic Anhydrase Enzyme, Molecular Docking Study, *Insilico* ADMET Property, *Insilico* Biological Property.

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INTRODUCTION

Ras proteins act as on–off switches that regulate signal transduction pathways controlling cell growth, differentiation and survival. Ras is the most frequently mutated oncogene in human tumors and oncogenic mutations. The frequency of mutated Ras genes and the type of the mutated Ras gene (H- Ras, K- Ras or N- Ras) varies widely depending on the tumor type. However, K- Ras is the most frequently mutated gene, with the highest incidence detected in pancreatic (90%) and sporadic colorectal carcinomas (50%) [1]. The contribution of aberrant Ras function to human malignancies is likely to be higher than that indicated by Ras mutation status, as the overexpression of many tyrosine kinase growth factor receptors also leads to increased Ras -dependent signaling [2]. Interconversion between the two forms, which mainly involves the conformational changes of two flexible regions called switch I (residues 32–38) and switch II (residues 60–75), is reciprocally catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Kobe 2601 [3], MDC-1016 and Tipifarnib were the most potent Ras inhibitors. Human carbonic anhydrases (EC 4.2.1.1) IX (CA IX) and XII (CA XII) are two tumor-associated proteins, being overexpressed in many tumors and involved in critical processes associated with cancer progression and response to therapy [4-6].

MATERIALS

Experimental Data

Structural modification and Receptor consideration

Here the structural modification done by enacting hydrazinecarbothioamide moiety of Kobe 2601, which was previously docked against 3KKP receptor procured from protein data bank [7]. Bioisosteric replacement of Kobe 2601 ligand hydrazinecarbothioamide group by hydrazinecarboxamide as well as addition of nitro group, amino group and trifluromethyl group inginite the molecule to bind with active Switch II region and bioisosteric replacement of fluorine and addition of substituted naphthyl, substituted biphenyl group needed for hydrophobic attachment with 3KKP receptor feature [2], which was diagrammatized in Figure 1. Then as per HITPICK server [8], designed ligands (R1-R9) were suggested carbonic anhydrase receptor (CA XII) association with 53.3% precision value. To confirm this state, using BLAST 3KKP receptor (associated with Ras Protein) and 4KP5 (associated with carbonic anhydrase enzyme) FASTA sequence, comes out 26% similarity index value and E value 7.0, reported in Figure 2 [9]. Why we choose 4KP5? From protein databank it was revealed that three CA XII family receptor exist as 4KP5, 4KP8, 4HT2 but among them the co crystallized ligand of 4KP5 as E1F has some structural similarity with the designed ligands as well as Kobe2601.

Molecular Docking Study against 3KKP and 4KP5

Molecular docking study was performed using AUTODOCK 4.1 software against 3KKP and 4KP5 receptor with 2Å RMSD value and simultaneously checked their dock sore and

surrounding residues suggest the receptor active site residues features. In case of 3KKP, SER75 and in case of 4KP5, SER123 were used as flexible residue respectively. [10-13]

Prediction of *in silico* physicochemical ADME -Toxicity & Lipinski' Rule of Five

The *insilico* ADME properties of designed ligands (R1-R9) were tested by means of Human Intestinal Absorption, Log P_{app} cm/s, P-glycoprotein Substrate, CYP450 3A4 Substrate, CYP450 1A2 Inhibitor, CYP450 2C9 Inhibitor, CYP450 2D6 Inhibitor, CYP450 2C19 Inhibitor and CYP450 3A4 Inhibitor. The toxicity profile of R1-R9 were tested by means of developmental toxicity, mutagenicity and oral LD_{50} values by using OECD TEST software [14-15]. As well as the Lipinski' rule of five was estimated by the following characteristics as: The rule states, that most "drug-like" molecule have log P <= 5, molecular weight <= 500, number of hydrogen bond acceptors <= 10, and number of hydrogen bond donors <= 5. Molecules violating more than one of these rules may have problems with bioavailability [16].

Prediction of *in silico* biological activity

By using the molinspiration online software the mode of activity of the designed ligands (R1-R9) were predicted such as G-Protein Coupled Receptor (GPCR) type, Kinase inhibitor type, Nuclear receptor type, Protease inhibitor and Enzyme inhibitor type with respect to the standard Kobe2601 molecule [17].

RESULTS AND DISCUSSION

As per the comparative molecular docking results (Table 1) of the designed ligands (R1-R9) with comparison of Kobe2601 as standard reveals that R4 was the best conformer, in case of 3KKP receptor -9.04 Kcal/mol whereas Kobe2601 with -7.55 Kcal/mol and in case of 4KP5 -10.87 Kcal/mol whereas Kobe2601 with -9.24Kcal/mol. The surrounding residues of R4 were GLY25, GLY22, VAL24, LYS26 and Kobe2601 ARG138, LYS166, ASN163, GLU154, ILE153, TYR152 on 3KKP respectively. The surrounding residues of R4 were ASN64, THR199, LYS69, GLN89, THR198, LEU197 and Kobe2601 ASN64, VAL119, LEU197, TRP4, THR198 on 4KP5 respectively, the docking score was diagrammatized in Figure 3. All other docking parameter was reported in (Table 2). The cluster analysis of docking results (Figure 4) reveal a new story that in case of 3KKP receptor among all of docked structure only R2, R4, R8, R9 were situated in the same voxel of Kobe2601 and remaing all were outlayered; whereas in case of 4KP5 all docked structure as well as Kobe2601 were situated in same voxel. Human intestinal absorption prediction reveals that R1-R9 have better absorption profile than the standard among them R5, R6, R7 have highest LogPapp values. Not a single molecule were P-glycoprotein substrate, so there was least chance of efflux from gut. R1-R9 were non-substrate of CYP450 3A4, so metabolism was not navigate by CYP4503A4 microsomal enzyme. R2, R3 were inhibitor of CYP450 2C19 whereas R4, R5, R6, R8, R9 were inhibitor of CYP450 1A2 and rest of the molecules were non-inhibitor of all the above mentioned microsomal enzyme so least chance of drug-drug interaction with other simultaneous drug application in poly therapy and all the results were reported in (Table 3). R5, R6, R7, R8 has less developmental toxicity than standard

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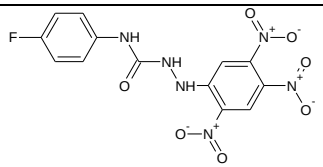
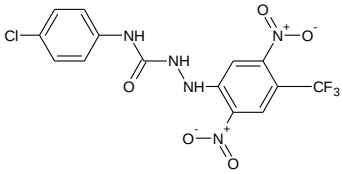
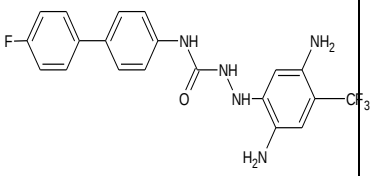
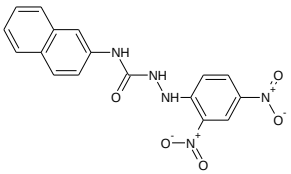
and R2, R5, R6, R7 has less mutagenic than standard Kobe 2601. All the molecules follow Lipinski rule of five except R1, R3 showed violation of rule and all the results were reported in (Table 4). Whereas Table 6 reveals that all the molecules have better enzyme inhibitor, GPCR-ligand and kinase inhibitor than Kobe 2601 and among them R5, R6 was the best molecule for the above mention target.

Table 1: Comparative docking score of R1-R9 using 3KKP and 4KP5 as receptors

SN	Structure Code	Structure	IUPAC Name	Docking Score on 3KKP Kcal/mol	Surrounding Residue on 3KKP receptor	Docking Score on 4KP5 Kcal/mol	Surrounding Residue on 4KP5 receptor
1.	R1		*N*-(4-fluorophenyl)-2-(2,4,5-trinitrophenyl)hydrazinecarboxamide	-5.76	ASN149,ILE150 SER118,PRO120, GLN178.	-9.0	TRP4, ASN64, THR198, LYS69, GLN89, VAL119
2.	R2		*N*-(4-chlorophenyl)-2-[2,5-dinitro-4-(trifluoromethyl)phenyl]hydrazinecarboxamide	-7.32	HIS169,ARG173, LYS166,ASP165, ASN163,HIS169.	-9.12	ASN64,VAL119, TRP4, GLN89,HIS66,
3.	R3		2-[2,5-diamino-4-(trifluoromethyl)phenyl]-*N*-(4'-fluorobiphenyl-4-yl)hydrazinecarboxamide	-7.01	THR68, ASP67, SER27,TYR42, PRO44,ALA69.	-7.27	THR199, SER133, PRO200, GLN89, VAL205, LEU197
4.	R4		2-[2,5-diamino-4-(trifluoromethyl)phenyl]-*N*-(4'-fluorobiphenyl-4-yl)hydrazinecarboxamide	-9.04	GLY25,GLY22, VAL24, LYS26.	-10.87	ASN64, THR199, LYS69, GLN89, THR198, LEU197

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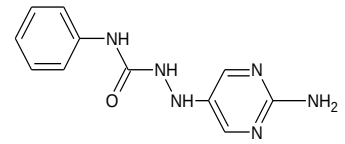
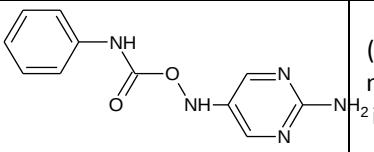
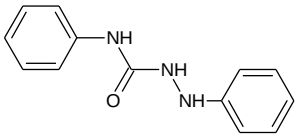
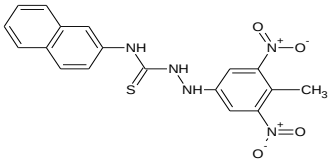
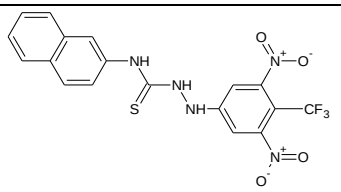
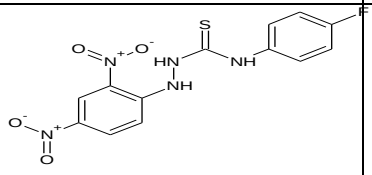
5.	R5		2-(2-aminopyrimidin-4-yl)-*N*-phenylhydrazinecarboxamide	-6.99	PRO120,PHE119 HIS107,SER118, TYR148, ILE150.	-6.5	ASN64, THR199,GLN 89, VAL119, LEU197, TRP208, THR198
6.	R6		N^4-(phenylcarbamoyl)oxy]pyrimidine-2,4-diamine	-6.60	TYR148,VAL174, ILE150, PHE119, SER118.	-6.62	ASN64, THR199, GLN89, SER67, TRP4, HIS 66
7.	R7		*N*,2-diphenylhydrazinecarboxamide	-6.94	LYS166, PRO151, TYR152, ARG138, ASP170.	-6.98	ASN64, GLN89, TRP4, THR199, VAL119, TRP208
8.	R8		2-(4-methyl-3,5-dinitrophenyl)-*N*-naphthalen-2-ylhydrazinecarbothioamide	-7.54	GLN177, ASP170, LYS166, GLN178, GLN177	-9.42	GLN89, TRP208, LEU197, SER130
9.	R9		2-[3,5-dinitro-4-(trifluoromethyl)phenyl]-*N*-naphthalen-2-ylhydrazinecarbothioamide	-7.59	LYS26, GLY22, GLY25, VAL39, LYS26.	-10.03	ASN64, THR199,GLN 89, VAL119, LEU197, TRP208, THR198
10.	Kobe 2061		2-(2,4-dinitrophenyl)-*N*-(4-fluorophenyl) hydrazinecarbothioamide	-7.55	ARG138, LYS166, ASN163, GLU154, ILE153, TYR152.	-9.24	ASN64, VAL119, LEU197, TRP4, THR198

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Table 2: Docking Parameter of R1-R9 using 3KKP and 4KP5

SN	Structure Code	Docking Parameter associated with 3KKP					Docking Parameter associated with 4KP5				
		Binding Energy[a]	Intermol Energy[b]	Electrostat Energy	Total Energy	Torsional Energy	Binding Energy[a]	Intermol Energy[b]	Electrostat Energy	Total Energy	Torsional Energy
1.	R1	-5.76	-7.1	0.7	-0.08	1.37	-9.0	-10.09	-2.29	-0.19	1.37
2.	R2	-7.32	-7.78	-0.8	-0.74	1.37	-9.12	-10.22	-252	-0.67	1.37
3.	R3	-7.01	-8.25	-0.03	-0.61	1.65	-7.27	-8.36	0.09	-1.16	1.65
4.	R4	-9.04	-10.55	-2.6	0.23	1.1	-10.87	-11.98	-3.93	-0.08	1.1
5.	R5	-6.99	-7.3	-0.37	-0.51	0.55	-6.5	-6.72	-0.45	-0.63	0.55
6.	R6	-6.6	-7.55	-0.35	-0.65	1.1	-6.62	-7.44	-0.62	-0.78	1.1
7.	R7	-6.94	-7.02	-0.07	-0.48	0.55	-6.98	-8.41	-0.02	-1.12	2.2
8.	R8	-7.54	-10.33	-1.21	-0.42	2.2	-9.42	-11.74	-1.83	0.18	2.2
9.	R9	-7.59	-9.6	-1.59	-1.39	2.47	-10.03	-11.87	-2.03	-1.25	2.47
10.	Kobe2601	-7.55	-9.71	-2.64	-0.22	2.2	-9.24	-11.1	-3.94	-0.45	2.2

[a]The predicted binding energy (Kcal/mol) is the sum of intermolecular energy and torsional free energy
[b] Intermolecular energy is sum of Vdw-hb-desolv-energy and electrostatic-energy

Table 3: Predicted ADME profile of R1-R9

Structure Code	Human Intestinal Absorption (P)	Log P_{app} cm/s (P)	P-glycoprotein Substrate (P)	CYP450 3A4 Substrate (P)	CYP450 1A2 Inhibitor (P)	CYP450 2C9 Inhibitor (P)	CYP450 2D6 Inhibitor (P)	CYP450 2C19 Inhibitor (P)	CYP450 3A4 Inhibitor (P)
R1	HIA+ (0.6436)	0.7786	Non-substrate (0.7703)	Non substrate (0.5912)	Non-Inhibitor (0.5310)	Non inhibitor (0.5846)	Non inhibitor (0.8867)	Non inhibitor (0.5433)	Non inhibitor (0.7870)
R2	HIA+ (0.6451)	0.7292	Non-substrate (0.7039)	Non substrate (0.5555)	Inhibitor (0.5362)	Non inhibitor (0.5816)	Non inhibitor (0.8758)	Inhibitor (0.5610)	Non inhibitor (0.7174)
R3	HIA+ (0.9632)	0.9474	Non-substrate (0.6954)	Non substrate (0.6552)	Inhibitor (0.7212)	Non inhibitor (0.6544)	Non inhibitor (0.8749)	Inhibitor (0.6900)	Non inhibitor (0.5789)
R4	HIA+ (0.7860)	0.6614	Non-substrate (0.6606)	Non substrate (0.5842)	Inhibitor (0.5610)	Non inhibitor (0.6451)	Non inhibitor (0.8991)	Non inhibitor (0.7106)	Non inhibitor (0.9199)
R5	HIA+ (0.9719)	1.0210	Non-substrate (0.6834)	Non substrate (0.7383)	Inhibitor (0.6167)	Non inhibitor (0.9528)	Non inhibitor (0.9234)	Non inhibitor (0.8107)	Non inhibitor (0.8300)
R6	HIA+ (0.9818)	1.2650	Non-substrate (0.7760)	Non substrate (0.6993)	Inhibitor (0.5441)	Non inhibitor (0.8220)	Non inhibitor (0.8667)	Non inhibitor (0.7903)	Non inhibitor (0.5969)
R7	HIA+ (0.9527)	1.3224	Non-substrate (0.7010)	Non substrate (0.7385)	Non Inhibitor (0.5310)	Non Inhibitor (0.7602)	Non Inhibitor (0.9325)	Non Inhibitor (0.6452)	Non Inhibitor (0.8762)
R8	HIA+ (0.6888)	0.5392	Non-substrat (0.6393)	Non substrate (0.5885)	Inhibitor (0.6604)	Non Inhibitor (0.6029)	Non Inhibitor (0.8043)	Inhibitor (0.5356)	Non Inhibitor (0.6502)

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R9	HIA+ (0.6888)	0.5392	Non substrate (0.6393)	Non substrate (0.5885)	Inhibitor (0.6604)	Non Inhibitor (0.6029)	Non Inhibitor (0.8043)	Inhibitor (0.5356)	Non Inhibitor (0.6502)
Kobe 2601	HIA- (0.6650)	0.7311	Non-substrate (0.7804)	Non substrate (0.6216)	Inhibitor (0.5377)	Non inhibitor (0.5388)	Non inhibitor (0.8264)	Non inhibitor (0.5000)	Non inhibitor (0.6449)

Table 4: Predicted Toxicity profile of R1-R9

Structure Code	Structure Code	Developmental Toxicity(+/-)	Mutagenicity(+/-)	Oral LD_{50} value(mg/kg)
1	R1	1.24 (+)	0.98 (+)	577.42
2	R2	1.07 (+)	0.52 (+)	51.99
3	R3	1.23 (+)	1.32 (+)	766.33
4	R4	1.11 (+)	1.00 (+)	518.82
5	R5	0.56 (+)	-0.16 (-)	109.60
6	R6	0.64 (+)	0.77 (+)	10005.35
7	R7	0.37 (+)	-0.03 (-)	834.56
8	R8	0.93 (+)	1.06 (+)	171.42
9	R9	0.98 (+)	0.85 (+)	11.29

Table 5: Drug Like Property of R1-R9

Structure Code	Log P	TPSA	MW	nON	nOHNH	Nviolation	Nrotb	MV
R1	2.799	190.6	380.24	13	3	1	6	286.576
R2	4.249	144.8	419.70	10	3	0	6	303.144
R3	5.53	144.8	479.34	10	3	1	7	365.948
R4	3.931	144.8	367.32	10	3	0	5	302.303
R5	0.926	104.9	244.25	7	5	0	3	214.619
R6	1.103	102.1	245.24	7	4	0	4	211.201
R7	2.902	53.15	227.26	4	3	0	3	211.643
R8	4.212	127.7	397.4	9	3	0	7	327.742
R9	4.658	127.7	451.3	9	3	0	8	342.478
Kobe 2601	2.815	127.7	351.31	9	3	0	7	272.12

Table 6: Predicted Bioactivity Score of R1-R9

Structure Code	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
R1	-0.23	-0.28	-0.21	-0.76	-0.32	-0.22
R2	-0.14	-0.17	-0.17	-0.59	-0.28	-0.21
R3	-0.08	-0.13	-0.06	-0.41	-0.20	-0.13
R4	-0.16	-0.29	-0.17	-0.80	-0.27	-0.15
R5	-0.07	-0.14	0.17	-1.07	-0.35	0.16
R6	0.13	0.10	0.19	-0.46	-0.04	0.48
R7	-0.38	-0.26	1.28	1.26	-0.50	-0.15
R8	-0.60	-0.51	-0.50	0.80	-0.56	-0.35
R9	-0.47	-0.30	-0.33	0.66	-0.39	-0.27
Kobe 2601	-0.65	-0.62	-0.60	-1.11	-0.69	-0.43

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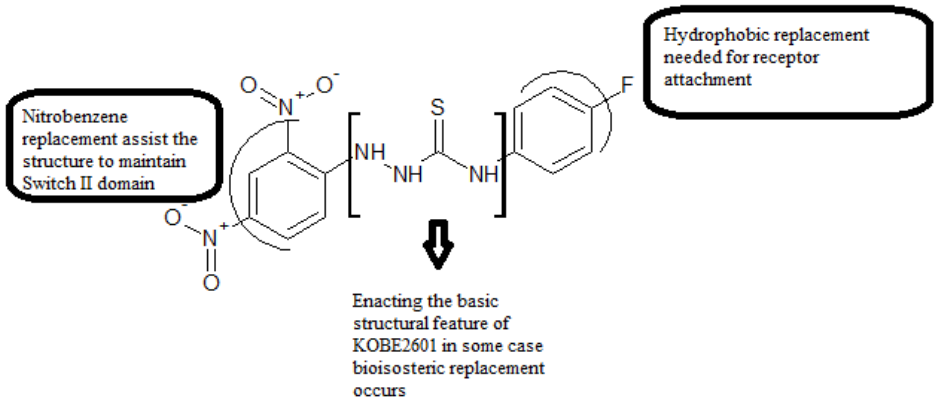


Figure 1: Justification behind structural modification of Kobe2601

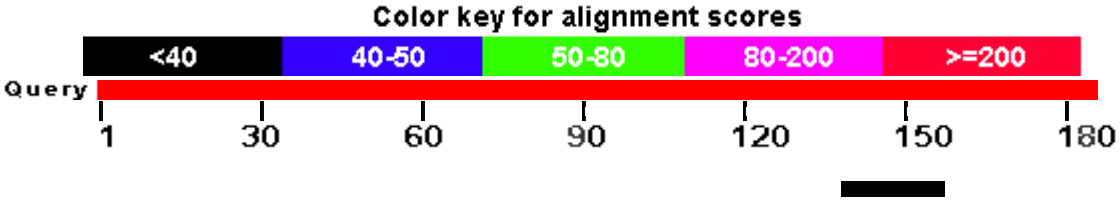
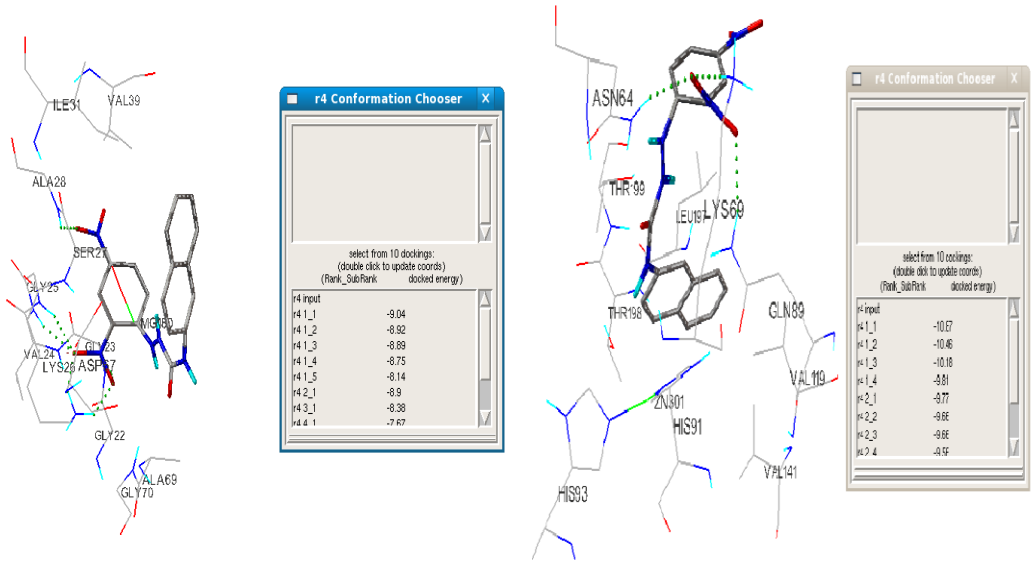


Figure 2: BLAST of 3KKP and 4KP5 FASTA sequence



Green Dots reflect Hydrogen Bond Interaction

Figure 3: Docking Score of R4 (best conformer) on 3KKP (left) and 4KP5 receptor (Right)

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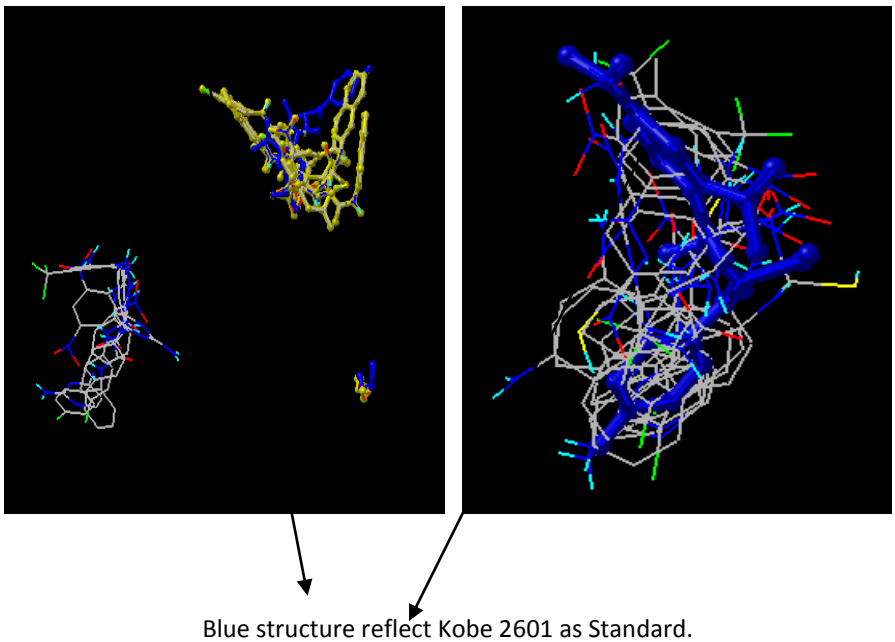

Figure 4: Clustering analysis of R1-R9 docked structure on 3KKP (left) and 4KP5 receptor (Right)

CONCLUSIONS

In this present study, the designed ligands were tested *in silico* way as molecular docking study, ADME-Toxicity profiling and bioactivity scoring. The results reveal that R4 was the best molecule as per the docking study aganist 3KKP and 4KP5 receptor and among them in the case of 4KP5 all the designed ligands within the same clustering as standard Kobe 2601. Except R1, R3 all the molecules follow Lipinski rule and R5, R6 with best activity profile by enzyme inhibition, GPCR- ligand and kinase inhibition. More or less the designed ligands with less developmental toxiciy and mutagenicity. So if the designed ligands were synthesized specially R4, R2, R8, R9 then it act as an potent anticancer agent by following inhibition Ras protein mutation and carbonic anhydrase enzyme.

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