# Computer-Aided Drug Design for Cancer-Causing H-Ras $p^{21}$ Mutant Protein

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**Abstract:** GTP-bound mutant form H-Ras (Harvey-Ras) proteins are found in 30% of human tumors. Activation of H-Ras is due to point mutation at positions 12, 13, 59 and/or 61 codon. Mutant form of H-Ras proteins is continuously involved in signal transduction for cell growth and proliferation through interaction of downstream-regulated protein Raf. In this paper, we have reported the virtual screening of lead compounds for H-Ras $P^{21}$ mutant protein from ChemBank and DrugBank databases using LigandFit and DrugBank-BLAST. The analysis resulted in 13 hits which were docked and scored to identify structurally active leads that make similar interaction to those of bound complex of H-Ras $P^{21}$ mutant-Raf. This approach produced two different leads, 3-Aminopropanesulphonic acid (docked energy -3.014 kcal/mol) and Hydroxyurea (docked energy -0.009 kcal/mol) with finest Lipinski's rule-of-five. Their docked energy scores were better than the complex structure of H-Ras $P^{21}$ mutant protein bound with Raf (1.18 kcal/mol). All the leads were docked into effector region forming interaction with ILE36, GLU37, ASP38 and SER39.

**Keywords:** Molecular docking, H-Ras, Rational drug design, Ras-Raf Interaction, LigandFit, Binding affinity.

## INTRODUCTION

The Ras family of protooncogenes (*N-Ras, H-Ras* and *K-Ras*) codes for small proteins of 189 amino acids with molecular weight 21 kDa protein [1]. Ras proteins are localized in the inner plasma membrane and are involved in the transduction of external stimuli to effector molecule Raf serine/threonine kinase [2]. These proteins bind GDP/GTP and possess intrinsic GTPase activity allowing inactivation following signal transduction in the normal cellular environment [3]. Activation of point mutations in the Ras is one of the most frequent genetic alterations associated with human cancers [4]. Approximately 90% of these activating mutations occur in codons 12 and 59, identifying these codons as hot-spot targets [5]. A particular genetic alteration has been identified in a significant percentage of bladder tumors; this mutation changes a single amino acid in the H-Ras protein. Specifically, the mutation replaces the amino acid glycine with the amino acid valine at position 12 ($Ras^{G12V}$) [6]. The mutant form of $Ras^{A59T}$, which is known to undergo autophosphorylation on Thr-59 [7], shows a very strong signal that argues against the occurrence of a covalently bound phosphate [8]. As a result of these mutational changes, the mutated Ras-$p^{21}$ has a structure that disables its ability to bind with GTPase activating protein (GAP) and creation of an autophosphorylation site [9], thus keeping the Ras-$p^{21}$ in the GTP-bound, activated state contributing to a malignant cell phenotype [10, 11].

Drug target discovery involves the identification and early validation of disease-associated targets. Mutations occurring in the *Ras* gene(s) lead to uncontrolled cell growth and proliferation. In general, 30% of human tumor occurs through mutation in *Ras* gene [12]. In colorectal and pancreatic cancers, the occurrence of mutation in Ras is 50-90% [13]. When we consider treatments for cancer, they depend on the types and stages of cancer development. Chemotherapy, targeted therapies, surgery, radiation therapy, biological therapy, and hormonal therapy are the various treatments that currently exist [14]. But these types of treatment except for the target based, cannot distinguish between normal and cancerous cell. Consequently, healthy cells are commonly damaged in the process of treating the cancer, which results in side effects. In this context, target-based drug discovery is considered to be highly potential [15]. The mutated H-Ras is perceived to be an important target to fight against colorectal and pancreatic cancer. The objective of this research work is to find a suitable drug (lead) molecule for the mutated state of H-Ras protein in order to prevent complex formation with Raf protein. Antagonists of the Ras–Raf interactions that are likely to inhibit the Ras-stimulated signal transduction pathway are thus of great potential value to anti-cancer therapy [16].

The amino acid position which corresponds to effector region on the H-Ras is 32-40 [17]. The selection of this effector region as a binding site will act as potential site for docking studies. This selection has been confirmed by two following experimental evidences. Ras is known to induce activation of c-Raf-1 and MAP kinase or extracellular signal regulated kinase (ERK) [18]. Such signal transducing activities are abolished by presence of mutations in the effector region $Tyr^{32}$ - $Tyr^{40}$ [19]. Mutations in the effector region

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affect neither guanine-nucleotide binding nor GTPase activity, so the effector region is considered to be the region that interacts with the target effectors of the Ras protein [20]. In second, the experimental results of x-ray crystallographic and nuclear magnetic resonance (NMR) analyses have shown that the three-dimensional structure of the Ras protein changed upon GDP to GTP exchange [21]. In particular, the conformations of the $Asp^{30}$-$Asp^{38}$ and $Gly^{60}$-$Glu^{76}$ regions change significantly, and these regions are called switch-I and switch-II, respectively [22]. The switch I region essentially overlaps with the effector region. Some mutations in the switch I region of Ras have been reported to diminish the interaction with GAPs, Raf-1 [23]. The significant regions of Raf that bind to Ras protein have been identified as 80 amino acid N-terminal region, the so-called Ras binding domain (RBD) [24].

There has been a variety of approaches attempted for inhibiting Ras-induced activation of the Raf, ERK mitogen-activated protein kinase (MAPK) signaling cascade and inhibitors of farnesyltransferase [25-27]. In addition, two types of inhibitors namely non-steroidal drug and peptide inhibitors of Ras–Raf interaction have been developed. Sulindac sulfide, an anti-inflammatory drug was shown to decrease the Ras-induced activation of its main effector, the c-Raf-1 kinase [28] and two peptide inhibitors such as RKTFLKLA and RRFFLDIA identified from the Ras effectors GAP and c-raf-1 were shown to interfere with Ras–Raf association [29]. The peptide sequences 94-ECCAVFR-100 and 95-CCAVFRL-101 derived from the Ras-binding domain (RBD) of c-raf-1 have been shown to interfere with Ras–Raf association at least 20% at 100 microM [30]. Recently, the MCP1 compounds and its derivatives, 53 and 110 were isolated based on their ability to inhibit the activation by Ras of its downstream effectors Raf-1, MEK1 and ERK [31, 32]. However, no significant inhibition was observed when using MCP compounds to demonstrate inhibition of interaction between H-Ras and Ras-binding domain of Raf-1 [31]. As we have shown here, our approach can identify drug molecules and providing a way towards target-based drug discovery.

## MATERIALS AND METHODS

The protein structures of H-RAS P21 mutant ($Ras^{G12V, A59T}$) (521P) and of Ras-binding domain (1WXM) were downloaded from Protein Data Bank (PDB: http://www.rcsb.org/pdb/home/home.do) [33]. Two types of *in silico* methods were carried out to predict potential binding site in H-Ras. In first, rigid docking methodology was used to determine energetically favorable positions and orientations of functional groups on the surface of the complex between mutated H-Ras and a Ras-binding helix (RBH). The RBH helix (residues 78–89) contains two critical residues (Lys84 and Arg89) for Ras–Raf interaction, as mutants at these positions have been shown to abolish the Ras–Raf binding *in vivo* completely [34]. The rigid docked structure of the mutated H-Ras–RBD complex was predicted by using molecular docking software AutoDock 3.0 [35] and shown in Fig. (**1**).

When preparing the AutoDock 3.0 parameters, Kollman charges, solvation parameters, polar hydrogens were added

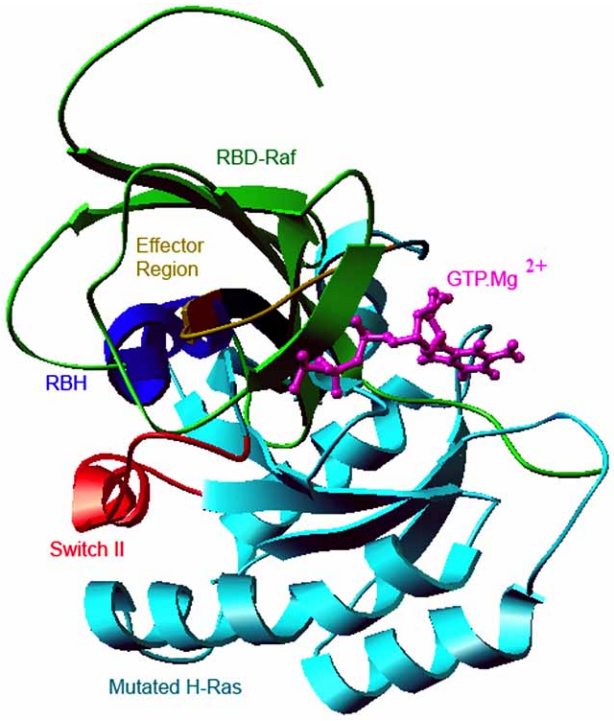


**Fig. (1).** Mutated H-Ras-Ras Binding Domain (RDB) Complex Structure; the regions of effector loop and switch II in mutated H-Ras are colored in yellow and red respectively. GTP.$Mg^{2+}$, an activator of H-Ras is colored in pink. In Raf, Ras binding domain (RBD) is colored in blue. Mutated H-Ras and Raf are represented in "Ribbon" model and GTP.$Mg^{2+}$ is in "Ball and Stick" model.

and water molecules removed from the receptor (mutated H-Ras). Kollman charges and all hydrogens were added to the RBD. The number of rotatable bonds was set to be 10. Ten docking runs were performed for mutated H-Ras-RBD interaction. The docked residues such as Ser81 and Arg89 of RBH were involved in hydrogen bond interaction with Glu37 and Try40 of the mutant H-Ras protein. In addition, Lys84 of RBH was involved in salt bridge with Try40 of mutant H-Ras, consistent with mutagenesis studies [34]. In another method, the multiple sequence alignment result of distinct sequences of Ras proteins that are involved in interactions with Raf and other candidate Ras effector targets (Fig. (**2**)) has shown that 'Core-Ras sequence domain' (residues 32-40), a part of conformational sensitive switch-I region, may represent a common interaction site for all Ras-GTP binding proteins, and may show complete identity with the equivalent sequences of Rap-1A, TC21 and RIN proteins. From the above two *in silico* studies, we concluded that selecting effector region (residue 32-40) of Ras protein as a binding site for drug interaction will be beneficial for anti-cancer therapy.

Screening of ligand molecules was carried out initially through BLAST search engine by submitting the mutated H-Ras (PDB ID: 521P) protein sequence to DrugBank database (http://redpoll.pharmacy.ualberta.ca/drugbank/drugBlast.htm). The DrugBank database is a unique Bioinformatics and cheminformatics resource that has 4300 drug entries (FDA-approved, nutraceuticals, biotech products and experimental

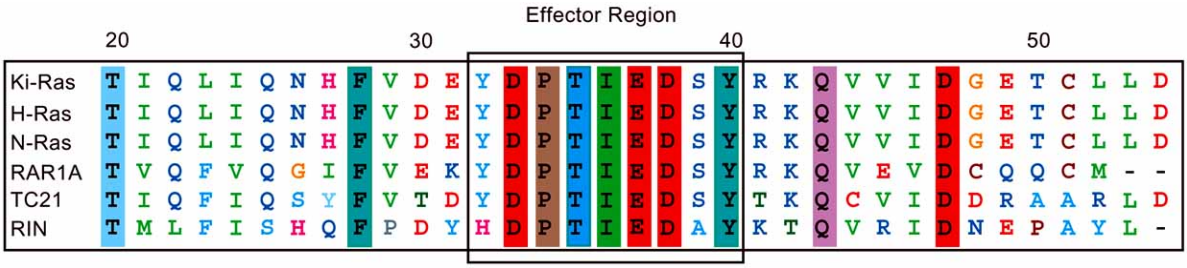


**Fig. (2).** Ras effector-interaction sequences; the multiple sequence alignment of Ras family of proteins that are involved in interactions with Raf and other candidate Ras effector targets. Effector region of Ras family shows complete identity with the equivalent sequences of Rap-1A; TC21 and RIN may represent a common interaction site for all Ras-GTP binding proteins. This multiple sequence alignment was carried out by using online ClustalW tool and the figure was generated by BioEdit.

drugs) with details of protein targets [36]. The DrugBank search showed trifluoroethanol, S-oxymethionine, and isopropanol as active ligands. In a second approach, ChemBank ligand entries were downloaded from Ligand.Info (http://ligand.info/) in SDF format. This ChemBank subset has 2344 entries of ligand and it was used for virtual screening and docking into effector region of mutated H-Ras by using Discovery Studio/LigandFit program (version 1.7, Accelrys Software Inc.) to identify active potential drugs [37]. This LigandFit docking algorithm is an interactive procedure in which random ligand conformations are generated a specified number of times, $N_{MaxTrial}$. The procedure maintains a 'Save List' in which the best-docked structures found by the algorithm were stored. The shape of each candidate ligand conformation is compared to that of the active site and if the shape similarity of the candidate conformation is worse than that of any saved structure (in the *Save List*), the candidate conformation is rejected. Otherwise, the candidate conformation is selected for docking. A Monte Carlo method was employed in the conformational search of the ligands. During the search, bond length and bond angles remained constant, only Rota table torsion angles were changeable. The binding site prediction tool of LigandFit docking program was used to predict receptor cavities and it resulted in two binding sites which comprise within effector region of mutated H-Ras protein. These two receptor cavities were having 575 atoms in binding site-I (Fig. (**3a**)) and 135 atoms in binding site-II (Fig. (**3b**)).

The virtual screened ligand molecules of BLAST, such as trifluoroethanol, S-oxymethionine, and isopropanol were used to dock into effector region of mutated H-Ras protein using AutoDock 3.0. In the receptor parameter setting, Kollman charges, solvation parameters and polar hydrogen's were added and water molecules removed, importantly grid parameter of ligand was set to the effector region of mutated H-Ras. Gasteiger charges and all hydrogens (polar and non polar) were added for ligands, and the number of rotatable bonds was set in the order of 2-5. Ten docking runs were performed for each of the virtual screened ligand molecules.

## RESULTS AND DISCUSSION

Towards finding suitable inhibitor(s) for mutant H-Ras, the binding energy of H-Ras and Raf interaction has to be initially predicted. This was done using molecular docking software AutoDock 3.0. The mutated H-Ras and RBD of Raf protein interaction was observed with minimal docked energy of 1.18 kcal/ml (Fig. (**1**)). The binding site for ligand molecules docking was selected based on core effectors region of the H-Ras corresponding to the amino acids residues 32-40 as per the experimental references and *in silico* methods described earlier. Once the ligand screening was completed, it was docked into the target site and evaluated for goodness-of-fit. In this approach, AutoDock 3.0 and LigandFit were used to dock the ligand molecules into binding site of mutated H-Ras, followed by the scoring function in order to evaluate the interaction between mutated H-Ras and of the ligand molecules. Dock energy was used to identify the cor-

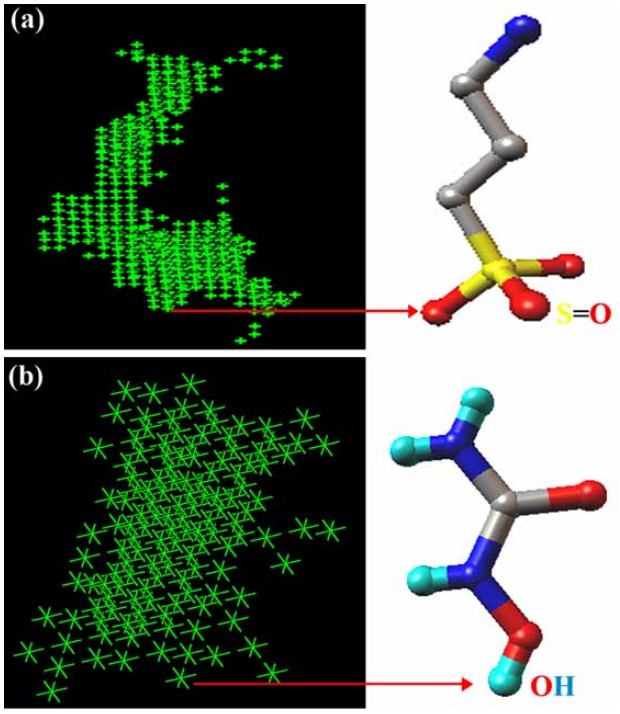


**Fig. (3).** A schematic representation of the grid systems for binding site-I and II (Green boundary, Jacks representation). (**a**) Binding site-I is having 575 atoms and (**b**) 135 atoms in binding site-II that were embraced within effector region of Ras. The ligand molecule such as 3-Aminipropanesulphonic acid was matching exactly with the position of biding site-I and hydroxyurea matching with biding site-II in grid system. These two ligand molecules were screened by LigandFit docking algorithm.

rect binding pose and then ranked the most befitting target-ligand complex based on their binding affinity. The docked energy of mutated H-Ras into each of the ligand molecules such as trifluoroethanol, S-oxymethionine, isopropanol was -3.27, -4.13, and -3.00 kcal/mol, respectively.

The LigandFit docking algorithm produced 10 different hits of ligand, such as YS035, nizatidine, leuhistin, 3-aminopropanesulphonic acid, guanidine, acetamide, methoxamine, urea, aluminum fluoride and hydroxyurea from two different binding site cavities that were encompassed in effector region of mutated H-Ras. The ChemBank ligand subsets such as YS035, nizatidine, leuhistin and 3-aminopropanesulphonic acid were used to dock into mutated H-Ras protein in the receptor cavity consisting of 575 atoms, where as the remaining ligand molecules guanidine, acetamide, methoxamine, urea, aluminum fluoride and hydroxyurea were used to dock into other receptor binding cavity that consists of 135 atoms. These two ChemBank subsets were docked using LigandFit method and the dock energies are described in Table **1**. The purpose of LigandFit docking algorithm used in ChemBank subset was to perform a virtual-screening of ligands from database and to dock with the target molecule, while in Autodock 3.0, there is no option for virtual screening of ligands. Comparison of docked energy of all ligand-H-Ras (mutated) complex into the docked energy of mutated H-Ras-RBD interaction showed that the ligand (lead) interactions were energetically lesser and more stable in biological reaction. Besides the identifying of the ligands molecules for high binding affinity and selectivity into target mutated H-Ras protein molecules, it was also investigated for their suitability to act as drug molecule. This was achieved by their adherence to the properties such as Absorption, Distribution, Metabolism, and Excretion (ADME) as per the Lipinski's "rule of five".

From the inspection of ligand molecules, we observed that all the screened ligand molecules except S-oxymethionine and YS035 were found to obey Lipinski's rule-of-five (Table **1**). These two ligand molecules have more than 5 hydrogen bond donors. It didn't fall under the Lipinski's rule of five for further drug development process. The ligand molecules such as trifluoroethanol, propan-2-ol and S-oxymethionine are solvents. These will produce only solvent effect to the target mutated H-Ras. Leaving the above, 3-aminopropanesulphonic acid (Fig. (**4a**)) was docked with energy of -0.009 kcal /mol at the binding site-I and hydroxyurea (Fig. (**4b**)) with -3.014 kcal /mol at the binding site-II. These two ligand molecules were also found to obey the Lipinski's rule of five (Table **1**) and were selected for further "re-docking" procedure with mutant H-Ras by using Autodock 3.0 instead of LigandFit to validate the docking results. In the resulted "re-docking" complexes (Fig. (**5**)), the residues, ILE36 and GLU37 of mutant H-Ras were involved in hydrogen bond formation with 3-aminopropanesulfonic acid whereas hydroxyurea was found to have hydrogen bond with ASP38 and SER39. Hence, we used both the AutoDock 3.0 and LigandFit docking procedure for all ligand molecules. It was confirmed from both the docking results that ligand molecules were involved in hydrogen bond formation into effector region of mutated H-Ras for stopping signal transduction in cell growth.

This result corroborates well with earlier experimental results [38-44] and it is evident that the identified binding conformations of these inhibitors are reliable and produce anti-tumor effects in a variety of solid tumor and leukemia. 3-aminopropanesulfonic acid is a synthetic gamma-aminobutyric acid (GABA) analog. It was reported in the transport of $Ca^{2+}$ by mitochondria isolated from rat liver and this molecule would enhance $Ca^{2+}$ uptake with a $K_m$ value of 2.63 mM. It would behave as an uncompetitive activator of $Ca^{2+}$ uptake under pathological conditions such as oxidative stress [38]. Hydroxyurea is an antineoplastic agent that produces anti-tumor effects in animals and man in a variety of solid tumor and leukemia [39, 40]. It was earlier reported

**Table 1. Docked Energy and Lipinski's Values of Ligand Molecules**

| Ligand Molecules | Molecular Formula | Docked Energy (kcal /mol) | Drug | Machine | Molecular Weight (g/mol) | XLogP | H-Bond Donors | H-Bond Acceptors |
|---|---|---|---|---|---|---|---|---|
| Trifluoroethanol | $C_2H_3F_3O$ | -3.27 | No | AutoDock 3.0 | 100.04 | 0.71 | 1 | 4 |
| Propan-2-ol | $C_3H_8O$ | -4.13 | No | AutoDock 3.0 | 60.10 | +/- 0.19 | 1 | 1 |
| S-Oxymethionine | $C_5H_{10}NO_3S$ | -3.00 | No | AutoDock 3.0 | 309.31 | -2.7 | 7 | 9 |
| YS035 | $C_{21}H_{30}NO_4$ | 34.36 | No | LigandFit | 330.24 | 4.063 | 10 | 5 |
| Nizatidine | $C_{12}H_{21}N_5O_2S_2$ | 21.232 | Yes | LigandFit | 310.28 | 0.643 | 2 | 7 |
| Leuhistin | $C_{11}H_{19}N_3O_3$ | 11.772 | No | LigandFit | 222.14 | 0.249 | 4 | 6 |
| 3-aminopropanesulphonic acid | $C_3H_9NO_3S$ | -3.014 | Yes | LigandFit | 130.1 | -0.998 | 2 | 4 |
| Guanidine | $CH_5N_3$ | -3.581 | No | LigandFit | 54.03 | -0.988 | 3 | 1 |
| Acetamide | $C_2H_5NO$ | -3.047 | No | LigandFit | 54.03 | -0.813 | 1 | 1 |
| Methoxamine | $CH_5NO$ | -1.643 | No | LigandFit | 42.02 | -0.648 | 1 | 2 |
| Urea | $CH_4N_2O$ | 0.925 | Yes | LigandFit | 56.02 | -1.043 | 2 | 1 |
| Aluminum fluoride | $ALF_3$ | -0.05 | No | LigandFit | 83.98 | 0.596 | 0 | 0 |
| Hydroxyurea | $CH_4N_2O_2$ | -0.009 | Yes | LigandFit | 72.02 | -1.055 | 3 | 2 |
| Mutant H-Ras-RBD of Raf | - | 1.18 | No | AutoDock 3.0 | - | - | - | - |

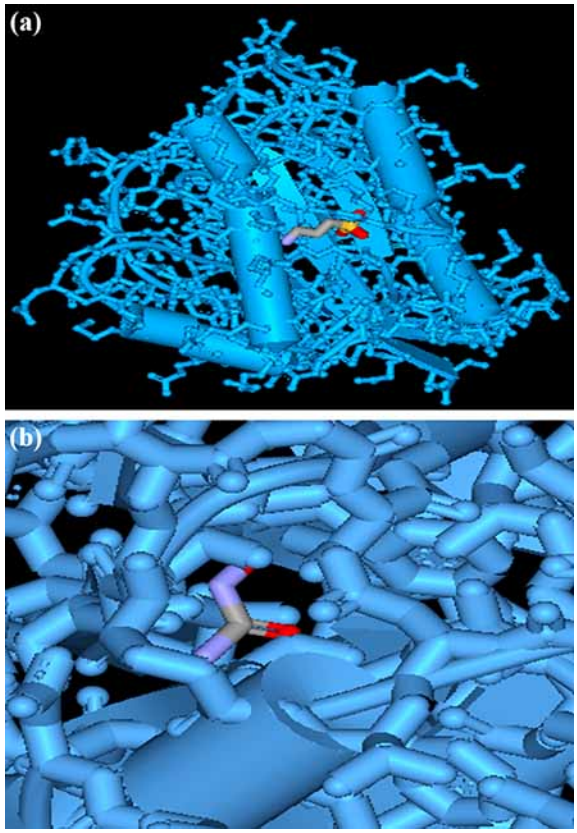


**Fig. (4).** Illustration of docked structure for (**a**) 3-aminopropanesulphonic acid in ligand binding site-I and (**b**) Hydroxyurea in ligand binding site-II of mutated H-Ras. This protein structure is represented as both "secondary structure schematics" and "ball and stick" model (Colored in Cyan) and ligands are in "Ball and Stick" model (Colored by CPK, Red-Oxygen, Yellow-Sulfur, Light grey-Carbon, Nitrogen-Blue, Hydrogen-Cyan. LigandFit docking algorithm was used for virtual-screening of ligands from ChemBank database and used to dock with the H-Ras. This figure was generated by DS Visualizer.

that when hydroxyurea was administered orally to 20 patients with chronic myelogenous leukemia, white blood cell counts decreased to normal [41]. This diagnosis was confirmed by bone marrow examination with supporting leukocyte alkaline phosphatase determinations. Stearns *et al*. [39] have found that hydroxyurea itself is effective against the standard L 1210 leukemia in mice upon intraperitoneal or oral administration at a dose of 200-400 mg./kg and shows 51-100% inhibition depends on dosage increments. It is also of interest that hydroxyurea is active against the solid tumor, LB 82T leukemia, since at a dose of 100 mg/kg it causes a 91% inhibition of tumor growth in mice. Hydroxyurea is also known to interfere with the synthesis of DNA in bacteria and animal cells [42]. Some of the experimental evidence suggests that this drug inhibits the DNA synthesis by decreasing the conversion of ribonucleotides to deoxyribonucleotides [43]. Its mechanism of action is believed to be based on its inhibition of the enzyme ribonucleotide reductase by scavenging tyrosyl free radicals [44]. This enzyme inhibitory process is required to stop the cell division in cancer cell.

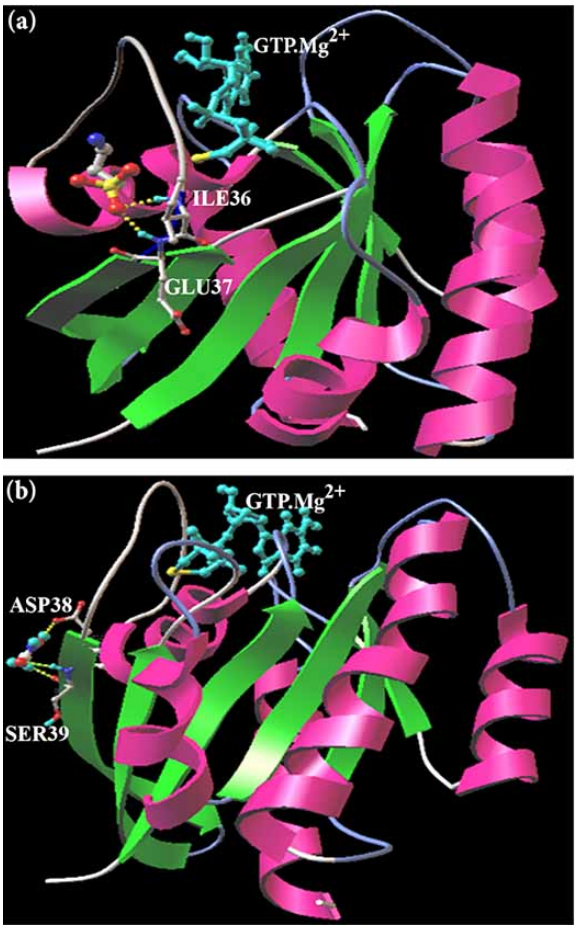


**Fig. (5).** Representation of re-docked structure for (**a**) 3-aminopropanesulphonic acid and (**b**) Hydroxyurea in effector region of mutated H-Ras. This mutated protein structure is represented as in a ribbon model (Colored by secondary structure, Helix-Pink, Sheet-Green) and ligand molecules are represented as "Ball and Stick" model (Colored by CPK, Red-Oxygen, Yellow-Sulfur, Light grey-Carbon, Nitrogen-Blue, Hydrogen-Cyan). Residues such as ILE36, GLU37 of H-Ras are involved in hydrogen bond formation with 3-aminopropanesulphonic acid whereas residues ASP38, SER39 involved in hydrogen bond with hydroxyurea. Hydrogen bonds are indicated by yellow dot line and GTP.$Mg^{2+}$ in Cyan color. These re-docking processes were carried out by using AutoDock 3.0.

## CONCLUSION

From this *in silico* study and previously reported experimental data in literature, we conclude that hydroxyurea and 3-aminopropanesulphonic acid would be an effective drug to inhibit function of mutant H-Ras $P^{21}$ protein, which will in turn arrest the process of cell growth and proliferation of the cancer cell. Further, the two ligand molecules can be incorporated into the drug development phases or clinical trial.

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## ABBREVIATIONS

PDB = Protein data Bank

GAP = GTPase activating protein

GEF = Guanine nucleotide exchange factor

RDB = Ras binding domain

RDH = Ras binding helix

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