Inhibitory effect of flavonoids on mutant H-Ras p^{21} protein

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Abstract :
Mutant form of H-Ras (Harvey-Ras) proteins are found in almost 10%-25% of human tumours. Mutational activation transforms it into an oncogenic form, which results in the loss of intrinsic GTPase function and therefore the protein is constitutively in the active, GTP-bound state and is continuously sending signals for cell growth and proliferation. In the present insilico study, the inhibitory effect of different flavonoid compounds on mutant H Ras protein p^{21} has been assessed. In addition, inhibitory effect of flavonoids is compared with 3 known anticancer drugs. Upon docking, it was found that flavonoids such as Naringenin, Daidzein, and Hesperetin showed highest affinity (most negative ΔG), while Rutin showed no affinity towards mutant H Ras. The 3 clinical anticancer agents (Erlotinib, Letrozole and Exemestane) showed binding energies in the range of −1.11 to −5.51 kcal/mol which is comparatively lower than the flavonoids indicating efficacy of flavonoids in the treatment of cancer with little or no cytotoxicity. Our study demonstrates that flavonoids (particularly Naringenin, Daidzein, and Hesperetin) are the effective drugs to inhibit function of mutant H-Ras P21 protein, which in turn arrests the process of cell growth and proliferation of the cancer cell.

Keywords: Docking; flavonoids; cancer; H-Ras; Raf; autodock

Background:
Ras, a family of protooncogenes (N-Ras, H-Ras and KRas) codes for small proteins of 189 amino acids with 21 kDa molecular weight [1]. To be biologically active, RAS proteins are localised to the inner face of the plasma membrane, where they effectively interact with their upstream activators and downstream targets. The RAS gene proteins exist in two states: an active state, in which GTP is bound to the molecule and an inactive state, in which GTP is hydrolysed to GDP. In physiologic conditions, the active isoform initiates cell proliferation through the RAS dependent kinase cascade. The RAS proteins possess intrinsic GTPase activity, which normally leads to their inactivation and the control of cell growth. In tumours, a point mutation resulting in loss of the intrinsic GTPase activity is associated with transforming activity of the protein, which does not stop anymore to send the signal stimulating cell proliferation [2].

Mutational activation transforms Ras into an oncogenic form, which results in the loss of intrinsic GTPase function and therefore the protein is constitutively in the active, GTP-bound state and is continuously sending signals for cell growth [3,4]. Statistics reveal that 10–25% of all human malignancies in clinics are found to harbour a variety of Ras mutations [5,6], making Ras one of the most important targets to suppress tumour cell growth [7,8]. According to the recent surveys of the WHO (www.who.int/cancer/resources/incidences/en/), bladder cancer is rated within the five most common cancers in males in North America, Northern and Western Europe, and other developed countries. Transitional cell carcinoma (TCC) is a tumour that occurs mostly in the urinary bladder and has been linked to multiple and accumulated aberrations in oncogenes (e.g. H-ras mutations) and cancer-suppressor genes (e.g. p53 inactivation) [9-11]. Mutational activation of the H-ras oncogene was first reported [12,13] in human T24 TCC cell line that played an essential role in urothelial carcinogenesis. Molecular epidemiological studies conducted within different geographic regions in different races and tumour stages/grades have revealed that up to 84% of bladder TCC carry activated HRas [14-20].The amino acid position which corresponds to effector region on the H-Ras is 32-40 [21]. 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). Such signal transducing activities are abolished by presence of mutations in the effector region Tyr32 - Tyr40. Mutations in

the effector region 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 [21]. 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. 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 [21]. 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. 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) [21].

The objective of this work is to find a suitable drug (ligand) molecule for the mutated state of H-Ras protein 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 of great potential value to anti-cancer therapy.Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. These compounds possess a common phenylbenzopyrone structure (C6-C3-C6), and they are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavanones, and flavanonols. The weight of the epidemiological evidence for a protective effect of flavonoids against cancer is impressive. A growing number of epidemiological studies suggest that high flavonoid intake may be correlated with a decreased risk of cancer [22]. This study has identified flavonoids as potential drug molecules and provides a way towards target-based drug discovery.

Methodology:
Softwares and data sources:
All softwares used for this analysis are freely available for academic use. Table 1 provides the source of availability of data used for the study.The Protein Data Bank (PDB) (www.rcsb.org) is a worldwide repository for processing and distribution of 3D biological macromolecular structure data [23]. The protein structure of H-RAS P21 mutant (521P) and Ras-binding domain (1WXM) were downloaded from Protein Data Bank. The Drug Bank (http://redpoll.pharmacy.ualberta.ca/drugbank/) database [24] is a unique bioinformatics and cheminformatics resource that combines detailed drug (i.e., chemical, pharmacological and pharmaceutical) data

with comprehensive drug target (i.e., sequence, structure, and pathway) information. The database contains nearly 4300 drug entries including >1000 FDA-approved small molecule drugs, 113 FDA-approved biotech (protein/peptide) drugs, 62 nutraceuticals and >3000 experimental drugs. Additionally, more than 6000 protein (i.e., drug target) sequences are linked to these drug entries. Each Drug Card entry contains more than 80 data fields with half of the information being devoted to drug/chemical data and the other half devoted to drug target or protein data. All the flavonoid molecules and clinical anticancer drugs were downloaded from this database **(Table 2).** PyMol (version 1.2), is a Python-based visualization software **[25]**. The PDB files of proteins obtained from the Protein Data Bank can be visualized using PyMol. Pymol has been selected for the analysis of docking results. The docking experiments were performed using the docking software AutoDock 4.2 (The Scripps Research Institute, www.scripps.edu) **[26]**, with the help AutoDockTools (ADT)—an accessory program that allows the user to interact with AutoDock from a Graphic User Interface (GUI). AutoDock is a suite of automated docking tools designed to predict how small molecules/ligands such as substrates or drug candidates, bind to a receptor/protein of known 3D structure.

Molecular docking:

Molecular docking was performed for getting the drug-receptor binding energy. The detailing of the procedure is as follows.
Preparing the ligand and macromolecule files for AutoDock The PDB files obtained from the World Wide Web repository are often far from perfect for docking study and present with potential problems like missing hydrogen atoms, multiple molecules, added waters and related problems. Using the GUI of ADT, following files were prepared:

The Macromolecule fil:

The downloaded PDB file of H Ras (PDB ID 521P) was first read in ADT, added waters removed and polar hydrogens were added. Kollman charges were added. Finally file was saved with .pdbq extension (where 'q' represent charge).

The Ligand File:

In a similar procedure, the ligand files were read in ADT, all hydrogens added, charges added and non-polar hydrogens merged and saved with .pdbqt extension. ADT then automatically determined the best root. The ligand files were then saved with .pdbqt extension (q representing charge).

Preparing The Grid Parameter File :

For the calculation of docking interaction energy, a three-dimensional box (grid) was created in which the protein molecule is enclosed. The grid volume was large enough to allow the ligand to rotate freely, even with its most fully extended conformation. The parameters required to create such a grid were stored in the Grid Parameter File with .gpf extension.

Running Autogrid :

Autogrid4 creates one map for every type of atom in the ligand. For example a molecule having C, N, O, H, maps will be created as molecule.C.map molecule.N.map, molecule.O.map, molecule.H.map. These are grid maps in ASCII format for readability by AutoDock. AutoGrid also generates corresponding output of the macromolecular file with the extension .glg.

Preparing The Docking Parameter File:

The docking parameter file, which instructs AutoDock about the ligand to move, the map files to use, and other properties defined for the ligand was created. AutoDock's search methods include the Monte Carlo simulated annealing (SA) method, the Genetic Algorithm (GA), local search (LS) and the hybrid genetic algorithm with local search (GALS). The latter is also referred to as the Lamarckian genetic algorithm (LGA) because offsprings are allowed to inherit the local search adaptations of their parents and this was the chosen algorithm for our analysis.

Running Autodock:

Finally, AutoDock was run from the GUI of ADT and the docked ligand files were used for study. The dlg files were read in ADT as well as in PyMol to calculate the binding energies in the docked ligand–protein complexes.
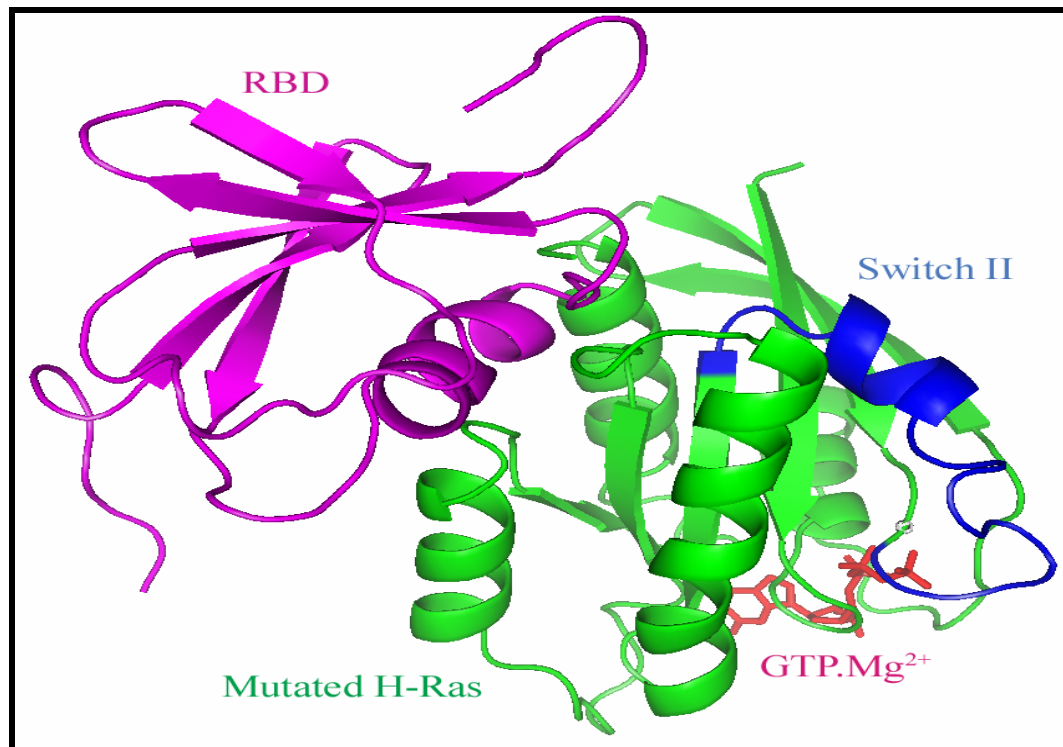

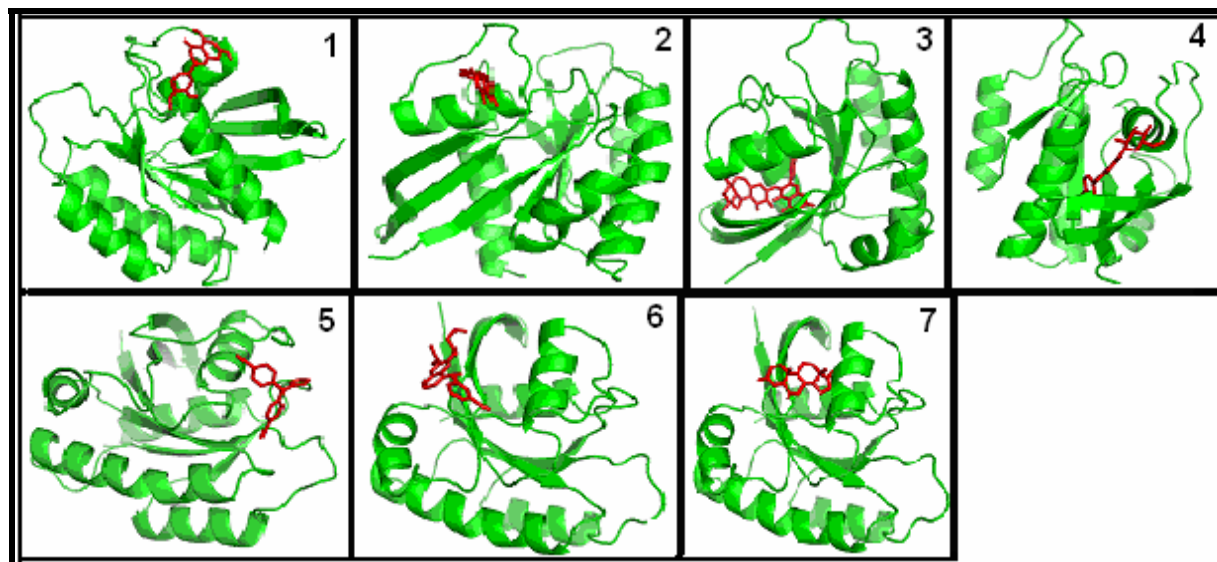**Figure 1:** Mutated H-Ras-Ras Binding Domain (RDB) Complex Structure.

Figure 2: Ligand molecules docked onto H Ras (Green) in their lowest energy docked conformation. Ligands are shown in red.
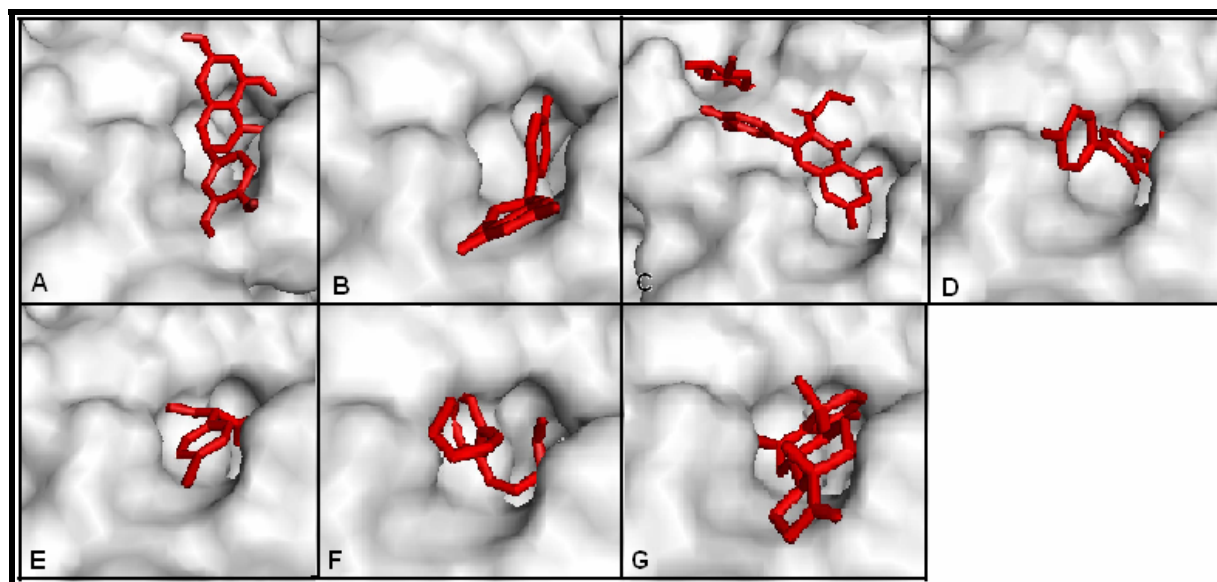

Figure 3: Ligand molecules in the active site of H Ras.

Discussion :

In order to find effective inhibitor(s) for mutant H-Ras, the binding energy of H-Ras and Raf interaction was initially predicted and it was compared with binding energies of the chosen ligands. The mutated H-Ras and RBD of Raf protein showed a minimal binding energy of +1.23 kcal/mol (**Table 3**). The detailed structure of H Ras with RBD is shown in **Figure 1** Selecting effector region (residues 32-40) of H-Ras as binding site, all the ligands were docked to it. The binding energy of mutated H-Ras into each of the ligand molecules is shown in **Table 3**. Corresponding figures mentioned in Table 3 demonstrates the receptor ligand interactions **(Figure. 2.1–2.7).**

The docked results showed that the flavonoids Naringenin, Daidzein, and Hesperetin had the highest affinity for the receptor (most negative ΔG). Rutin showed no affinity towards the receptor. Known anticancer agents (Erlotinib, Letrozole and Exemestane) showed binding energies in the range of −1.11 to −5.51 kcal/mol demonstrating efficacy of the flavonoids in the treatment of cancer with little or no cytotoxicity (**Table 3**). The

ligand interactions were stronger (energetically lesser) as compared to H-Ras-RBD interaction.

The ligand molecules also suitably acted as drug molecule 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, it was observed that all the ligand molecules except Rutin were found to obey Lipinski's rule-of-five (**Table 3**). Rutin has more than 5 hydrogen bond donors and more than 10 hydrogen bond acceptors. Many in vitro studies have been conducted on the potential anticancer activity of flavonoids in diverse cell systems. Hirano and co-workers examined anticancer efficacy of 28 flavonoids on human acute myeloid leukemia cell line HL-60, and compared differences between antiproliferative activity and cytotoxicity of these compounds with four clinical anticancer agents. Eight out of 28 flavonoids showed considerable suppressive effects on HL-60 cell growth with IC50s ranging from 10–940 ng/ml [27]. Kuntz et al. screened more than 30 flavonoids for their effects on cell proliferation and potential cytotoxicity in human colon cancer cell lines Caco-2 and HT-29. Almost all compoundds displayed

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antiproliferative activity without cytotoxicity **[28].** An array of 55 flavones having a variety of substituents was evaluated by Cushman and Nagarathnam for cytotoxicity in five cancer cell cultures, A-549 lung carcinoma, MCF-7 breast carcinoma, HT-29 colon adenocarcinoma, SKMEL-5 melanoma, and MLM melanoma. Fifteen out of the 55 flavone derivatives were significantly active against at least one of these cell cultures **[29]** In addition, seven out of the 27 examined Citrus flavonoids were observed to inhibit the proliferation of tumor cells, while less active against normal human cells **[30]**.

Conclusion :

The study concludes that flavonoids (particularly Naringenin, Daidzein, and Hesperetin) would be effective drugs in arresting the function of mutant H-Ras P21 protein. Further, these flavonoid molecules can be studied in the wet lab and incorporated into the drug development phases.

Acknowledgements :

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Supplementary material:

Table 1: Source of data used for the study

Protein or drug data	Source
H Ras	PDB ID 521P
aringenin	THE DRUG BANK (DB03467)
Rutin	THE DRUG BANK (DB01698)
Daidzein	THE DRUG BANK (DB02115)
Letrozole	THE DRUG BANK (DB01006)
Erlotinib	THE DRUG BANK (DB00530)
Exemestane	THE DRUG BANK (DB00990)

Table 2 : Structure of ligands used for the study

Hesperetin

Naringenin

Daidzein
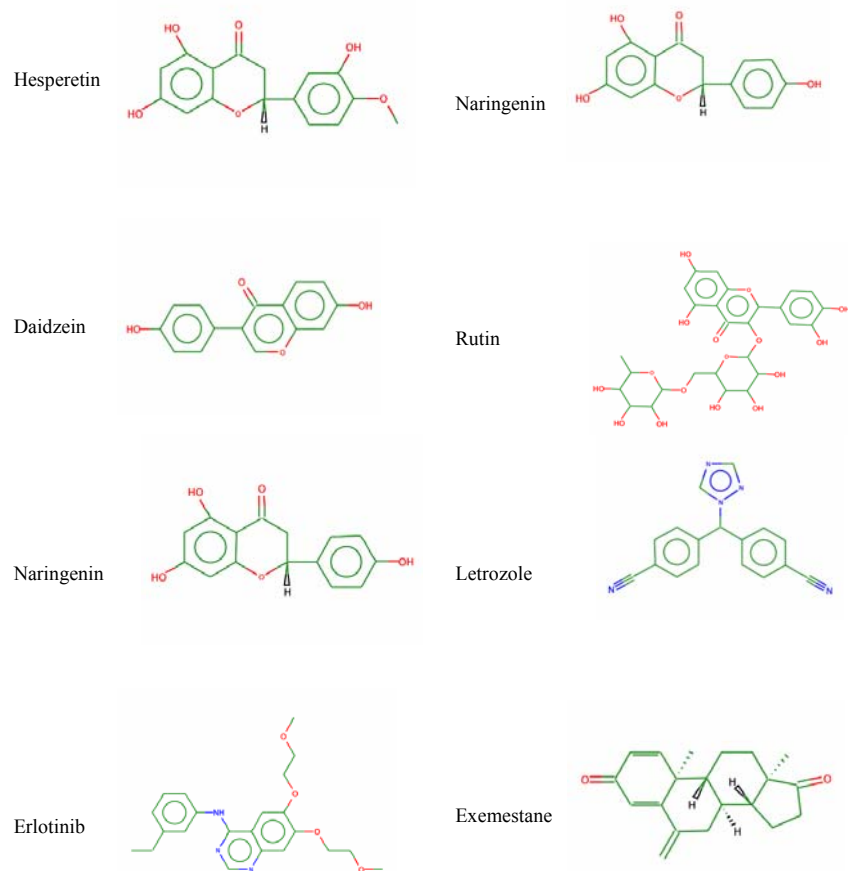
Rutin

Naringenin

Letrozole

Erlotinib

Exemestane

Table 3 : Binding Energy and Lipinski's Values of Ligand Molecules with H Ras protein

Ligand Molecules	Binding Energy (Kcal/mol)	Figure No.	Molecular Formula	Drug	Molecular weight (g/mol)	XLogP	H-Bond Donors	H-Bond Acceptors
Hesperetin	-4.59	2.1	$C_{16}H_{14}O_6$	No	302.27	2.357	3	6
Naringenin	-5.50	2.2	$C_{15}H_{12}O_5$	No	272.25	2.373	3	5
Rutin	+29.83	2.3	$C_{27}H_{30}O_{16}$	No	610.51	-1.158	10	16
Daidzein	-4.61	2.4	$C_{21}H_{20}O_9$	No	254.23	2.382	2	4
Letrozole	-2.90	2.5	$C_{17}H_{11}N_5$	Yes	285.30	2.749	0	4
Erlotinib	-1.11	2.6	$C_{22}H_{23}N_3O_4$	Yes	397.46	3.487	1	7
Exemestane	-5.51	2.7	$C_{20}H_{24}O_2$	Yes	296.40335	3.606	0	2
RBD of Raf	+1.23	1.1	-	No	-	-	-	-