Molecular docking studies of flavonoids for their inhibition pattern against β-catenin and pharmacophore model generation from experimentally known flavonoids to fabricate more potent inhibitors for Wnt signaling pathway

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ABSTRACT

Background: Canonical Wnt signaling plays a key role in tumor cell proliferation, which correlates with the accumulation of β-catenin in cell due to inactivation of glycogen synthetase kinase-3 β. However, uncontrolled expression of β-catenin leads to fibromatosis, sarcoma and mesenchymal tumor formation. Recently, a number of polyphenolic compounds of naturally occurring flavonoid family have been screened for the inhibition of Wnt signaling. **Objective:** Elucidation of the binding mode of inhibitors to β-catenin, reporting more potent inhibitors for the disease-causing protein and designing a pharmacophore model based on naturally occurring compounds, flavonoids. **Materials and Methods:** In this study, a comparative molecular docking analysis was performed to elucidate the binding mode of experimentally reported and unknown inhibitors. Based on the knowledge of geometry, binding affinity and drug score, we described a subset of novel inhibitors. **Results:** The binding energy of known inhibitors (isorhamnetin, fisetin, genistein and silibinin) was observed in a range of −5.68 to −4.98 kcal/mol, while novel inhibitors (catechin, luteolin, coumestrol and β-naphthoflavone) exhibited −6.50 to −5.22 kcal/mol. We observed good placement and strong interactions of selected compounds inside the binding pocket of β-catenin. Moreover, flavonoid family members and T cell factors 4 (TCF4) compete for β-catenin binding by sharing common binding residues. **Conclusion:** This study will largely help in understanding the molecular basis of β-catenin/TCF4 inhibition through flavonoids by exploring their structural details. Finally, the novel inhibitors proposed in this study need further attention to uncover cancer treatment and with the generated pharmacophore model, more and potent β-catenin inhibitors can be easily screened.

Key words: β-catenin inhibitors, docking studies, flavonoids, ligand optimization, pharmacophore modeling, Wnt signaling

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INTRODUCTION

The Wnt signaling pathway plays a key role in tumor cell de-differentiation and proliferation.[1] Evidence indicates that abnormal activation of Wnt pathway plays a vital role in tumor progression.[2-5] In general, tumor formation occurs due to the abnormal Wnt signaling by nuclear accumulation of β-catenin as a result of glycogen synthetase kinase-3 (GSK-3) β inactivation. Wnt signaling becomes deregulated through multiple mechanisms leading to cancer; particularly colorectal cancer, for which adenomatous polyposis coli (APC) or β-catenin are mutated in approximately 95% of tumors.[6]

Intracellularly, the best characterized mode of Wnt signaling regulation is the degradation of β-catenin which initiates with phosphorylation of β-catenin by GSK-3, subsequently its recognition and degradation by ubiquitination and proteolysis by proteasome.[7,8] Activation of Wnt signaling leads to β-catenin translocation in the nucleus resulting in expression of target genes where it binds to T cell factors/lymphoid enhancer factor (TCF/LEF) transcription factor family.[9] In case the process is uncontrolled due to inactivation of destruction complex, there will be a continuous supply of non-phosphorylated β-catenin to nucleus leading to over-expression of genes.

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β-catenin, as a co-activator, in complex with trans acting TCFs or LEF-1 is a cause of a wide variety of carcinomas.[10-12] Inhibition of this complex may lead to prevention of transcriptional activation of β-catenin/TCF target genes, thereby serving as a therapeutic agent. Uncontrolled transcription in cancer caused due to activity of β-catenin[13] can thus be avoided by prevention of β-catenin/TCF complex formation; either by inhibiting non-phosphorylated β-catenin or by introducing competitive inhibitor for TCFs to slow down its binding with β-catenin.

Naturally occurring compounds have been used for the prevention and treatment of different diseases including cancer. Natural compounds are far beneficial than synthetic compounds due to less toxicity, more accessibility and being less expensive. Flavonoids are a group of natural compounds with antiviral, anti-oxidative, anti-inflammatory and anti-tumoral effects[14] known to inhibit β-catenin/TCF complex by occupying their binding sites. Flavonoids, characterized by two or more aromatic rings (ARs), are a polyphenols subclass categorized into flavonols, flavones and isoflavones on the basis of direction of the phenyl ring and state of substitution.[15] There are various ways through which carcinogenesis is affected by flavonoids, such as the suppression of β-catenin and TCF4 interaction.[16] The physical inhibition of the interaction of β-catenin with TCF leads to the repression of TCF target genes' expression.

Compounds from the flavonoid family were thus selected for screening their inhibitory effects on β-catenin through in silico docking and for pharmacophore modeling. From Protein Data Bank (PDB) entries, 1JDH and 1JPW, it was known that β-catenin residues His260, Asn261, Lys292, Ile296, Asp299, Tyr306, Gly307, Lys312, Lys335, Lys345, Arg376, Arg386, Asn387, Asn426, Cys429, Lys435, Cys466, His470, Arg474 and Lys508 are the residues that interact with TCF4 to form a complex.[17,18]

The compounds from flavonoid family were expected to bind the TCF-binding residues of β-catenin in order to prevent the TCF/β-catenin interaction from happening and for reduction of unnecessary transcription of target genes.

MATERIALS AND METHODS

Data extraction

β-catenin 3D structure (PDB ID: 1JDH, 1.9 Å resolution) was extracted from PDB. Blind ligand-flexible docking of this protein was carried out with flavonoids. Compounds from different categories of the flavonoid family, such as flavonones, flavonols, flavones and isoflavones, were selected to be tested for their inhibitory capabilities, among which some were already experimentally known to inhibit β-catenin. The compounds were extracted from PubChem database[19] of compounds and categorized on the basis of their known inhibitory capabilities into known and novel.

Inhibition study by molecular docking analysis

Docking was performed using AutoDock 4.2.[20] Polar hydrogen atoms were added to the receptor protein, β-catenin. Ligand torsions were made rotatable in order to perform flexible-ligand docking which includes random flexible orientations and torsions for ligands. Grid with 120 × 60 × 120 $Å^3$ dimensions and points separated by 0.371 Å was set around the receptor protein so that all residues were available in an equal-opportunity zone for binding of the ligand. Grid maps were thus generated through AutoGrid. Lamarckian genetic algorithm was used with docking parameters set as follows: Number of docking runs was set to 100 with an initial population of 150, 2.5 × 10^6 energy evaluations, a maximum number of 27,000 iterations, mutation rate of 0.02 and crossover rate of 0.80. With a root mean square tolerance of 1.0 Å, AutoDock performed cluster analysis on initial docked conformations to provide final docking results.

Inhibitor selection

Docking analysis was carried out for interacting residues, binding energies and intermolecular energies. On the basis of docking results, the best hits belonging to different flavonoid sub-categories from the known and novel inhibitor categories were selected [Figure 1].

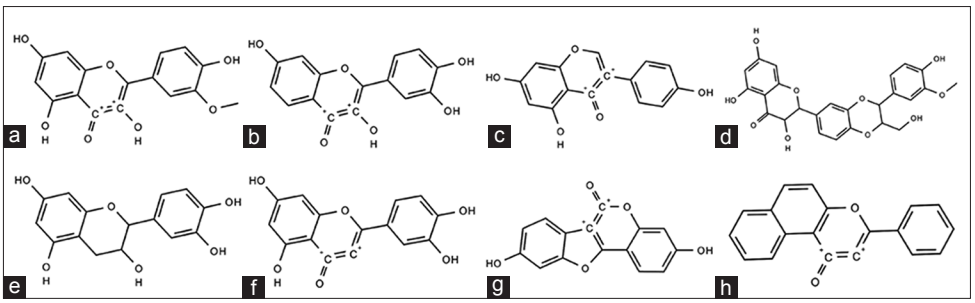

Figure 1: Hits after screening the flavonoids. (a-d) Known; (e-h) novel. (a) Isorhamnetin (flavonols); (b) fisetin (flavonols); (c) genistein (isoflavones); (d) silibinin (flavonoids); (e) catechin (flavonols); (f) luteolin (flavones); (g) coumestrol (isoflavones) and (h) β-naphthoflavone

Ligand optimization

Ligand optimization was performed on the 8 hits using HyperChem 8.0.5.[21] Compounds were re-docked to β-catenin. Docking results were analyzed for docking, binding and unbound energies. Docked complexes were saved and interaction figures were created using LigPlot + 1.3,[22] University of California, San Francisco Chimera[23] and Discovery Studio (DS) Visualizer.

Ligand-based pharmacophore generation

Four different compounds known to inhibit the Wnt signaling pathway were used to generate ligand-based pharmacophore model using LigandScout.[24] These compounds, namely isorhamnetin, fisetin, genistein and silibinin, belong to different categories of the flavonoids family. Isorhamnetin and fisetin belong to flavonols, genistein belongs to isoflavones and silibinin belongs to flavonones.

RESULTS

Parameter-based docking analysis of selected flavonoid family members against β-catenin

The flavonoid members were utilized for docking analysis against β-catenin and their interaction specificities were carefully studied. Previously, the selected compounds have been experimentally verified.[16] On the basis of binding energies and interacting residues, the best docked complexes obtained were isorhamnetin (−4.98 kcal/mol) and fisetin (−5.68 kcal/mol) belonging to flavonols, genistein (−5.44 kcal/mol) of isoflavones and silibinin (−5.32 kcal/mol) from flavonones among the known flavonoid members. In case of novel inhibitors, β-naphthoflavone (−6.50 kcal/mol) exhibited lowest binding energy value, while the other inhibitors

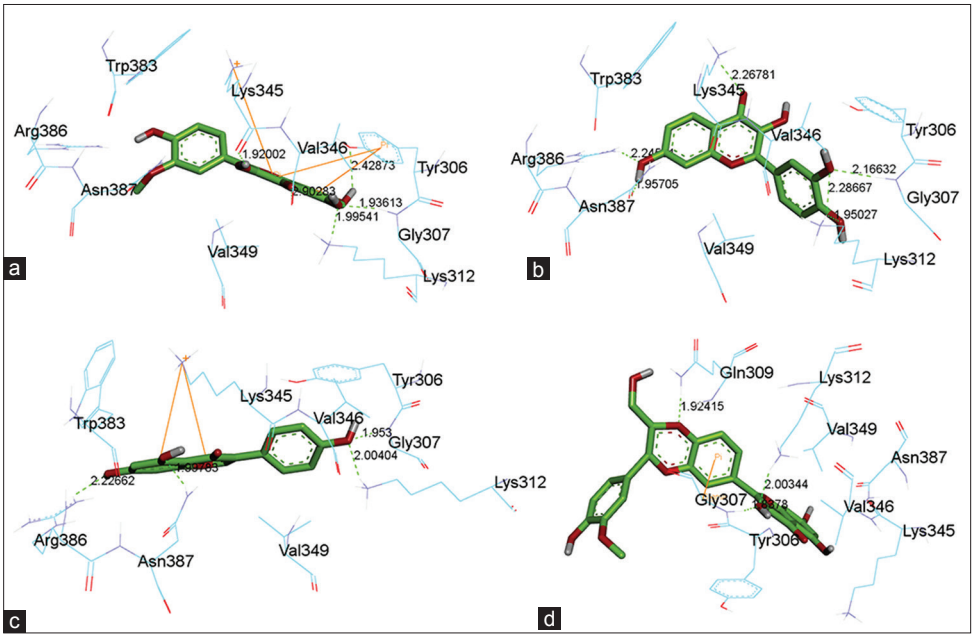

Figure 2: Interacting residues of β-catenin in complex with flavonoids as their known inhibitors: (a) Isorhamnetin; (b) fisetin; (c) genistein; and (d) silibinin. The figure is color-coded with cyan lines, β-catenin interacting residues; green sticks, flavonoid ligand; green dotted lines, H-bonds labeled with distances in Å; orange line, pi interaction; red, O-atoms; blue, N-atoms; grey, H-atoms

Table 1: Energy values and inhibition constant (Ki) of known and novel flavonoids' inhibitory action on β-catenin after geometry optimization of ligands

Flavonoids	Binding energy (kcal/mol)	Unbound energy (kcal/mol)	Intermolecular energy (kcal/mol)	Docking energy (kcal/mol)	Ki (μM)	Torsional energy (kcal/mol)
Known						
Isorhamnetin	−4.98	−1.12	−6.77	−7.89	225.20	1.79
Fisetin	−5.68	−0.41	−7.17	−7.58	68.28	1.49
Genistein	−5.44	−0.82	−6.63	−7.45	103.43	1.19
Silibinin	−5.32	−1.19	−8.01	−9.20	125.76	2.68
Novel						
Catechin	−5.22	−0.36	−7.01	−7.37	150.12	1.79
Luteolin	−5.70	−0.96	−7.20	−8.16	65.91	1.49
Coumestrol	−5.99	0.05	−6.58	−6.53	40.81	0.60
β-napthoflavone	−6.50	−0.22	−6.80	−7.02	17.09	0.30

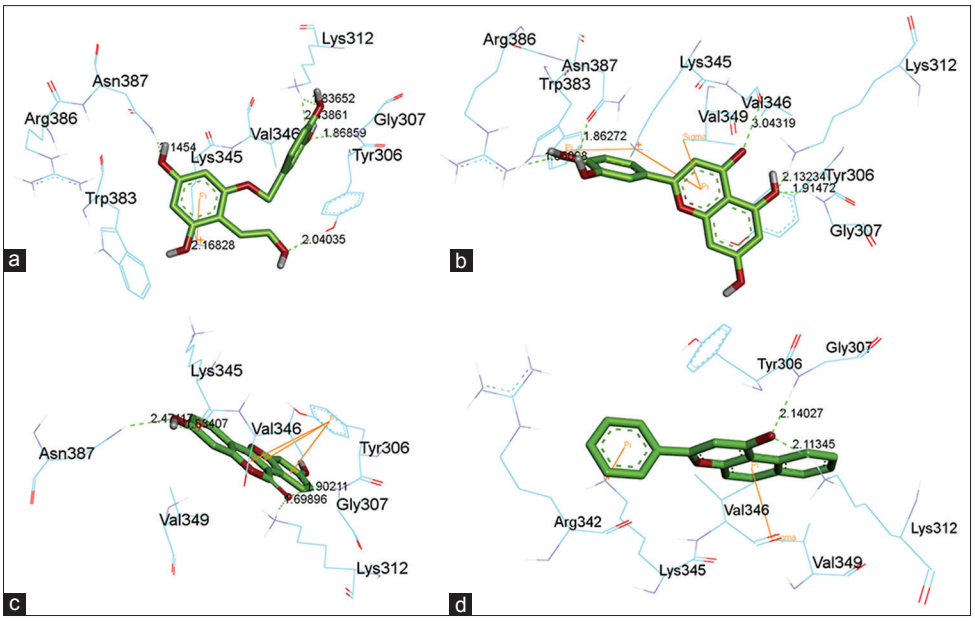

Figure 3: Interacting residues of β-catenin in complex with flavonoids as their novel inhibitors: (a) Catechin; (b) luteolin; (c) coumestrol; and (d) β-naphthoflavone. The figure is color-coded with cyan lines, β-catenin interacting residues; green sticks, flavonoid ligand; green dotted lines, H-bonds labeled with distances in Å; orange line, pi interaction; red, O-atoms; blue, N-atoms; grey, H-atoms

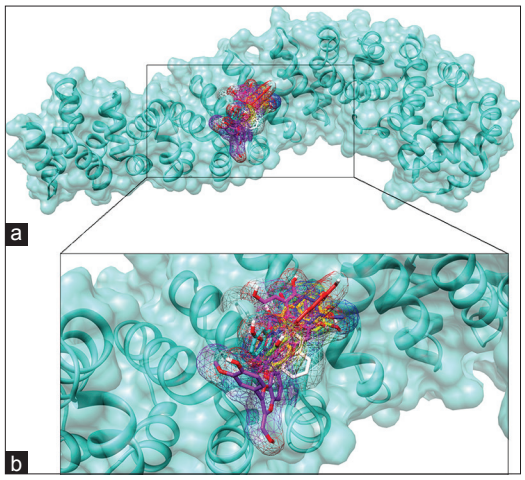

Figure 4: Binding patterns of the flavonoid inhibitors on β-catenin: (a) Binding cavity occupied by known and novel inhibitors, shown as a close-up in (b) figure is color-coded with cyan ribbon and surface, β-catenin; colored by atom type; flavonoid ligands

contained a slightly different binding energy profile of average −5.63 kcal/mol [Table 1].

As inhibition constant value (Ki) for β-naphthoflavone is 17.09 μM with better drug properties and apparently low energy parameters [Table 1], we propose that it may prove to be a more potent inhibitor for inhibition of β-catenin/TCF complex.

Docking interaction analysis

In all of the cases, Gly307 and Lys312 residues of β-catenin were found to be involved in hydrogen bonding.

Table 2: Pharmacophoric features in each compound

Ligand	HBA	HBD	AR
Isorhamnetin	4	6	1
Fisetin	4	4	1
Genistein	4	4	1
Silibinin	4	4	1

HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; AR: Aromatic ring

Isorhamnetin interacted with β-catenin forming H-bonds at TCF-interacting region involving residues Tyr306, Gly307, Lys312, Lys345 and Val346 at distances of 2.43 Å, 1.94 Å, 2.00 Å, 1.92 Å and 2.90 Å, respectively. Tyr306 formed pi-pi interactions with two rings at 4.16 Å and 5.87 Å and Lys345 was involved in cation-pi interaction at 6.11 Å with this inhibitor. β-catenin residues Val349, Trp383, Arg386 and Asn387 were involved in hydrophobic interactions [Figure 2a].

The interaction of fisetin and β-catenin involved six hydrogen bonds, two with the residue Lys312 of β-catenin at distances 1.95 Å and 2.29 Å and one each with Gly307, Lys345, Arg386 and Asn387 at distances 2.17 Å, 2.27 Å, 2.24 Å and 1.96 Å respectively. No pi-pi interaction was found, whereas hydrophobic interactions of this inhibitor were found with β-catenin residues Tyr306, Val346, Val349 and Trp383 [Figure 2b].

H-bonds formed by interaction of genistein and β-catenin involved β-catenin residues Gly307, Lys312, Arg386 and Asn387 at distances of 1.95 Å, 2.00 Å, 2.23 Å and 2.00 Å, respectively. Lys345 made cation-pi interactions with two

rings of the compound at 5.38 Å and 5.43 Å, respectively. Tyr306, Val346, Val349 and Trp383 made hydrophobic interactions with genistein [Figure 2c].

Four H-bonds were formed by interaction of silibinin with β-catenin involving the residues Gly307, Gln309 and Lys312 at distances 1.84 Å, 1.92 Å and 2.00 Å, respectively. Gly307 made sigma-pi interaction at 3.74 Å while the residues Tyr306, Lys345, Val346, Val349 and Asn387 made hydrophobic interactions with the compound [Figure 2d].

The interaction of catechin and β-catenin involved six hydrogen bonds, two with β-catenin residue Lys312 at distances of 1.84 Å and 2.44 Å and one each with Tyr306, Gly307, Lys345 and Asn387 at a distance of 2.04 Å, 1.87 Å, 2.17 Å and 2.14 Å, respectively. Lys345 made cation-pi interaction with a ring at 3.80 Å, while hydrophobic interactions of the inhibitor were found with β-catenin residues Val346, Trp383 and Arg386 [Figure 3a].

H-bonds formed by interaction of luteolin and β-catenin involved residues Gly307, Lys312, Val346, Arg386 and

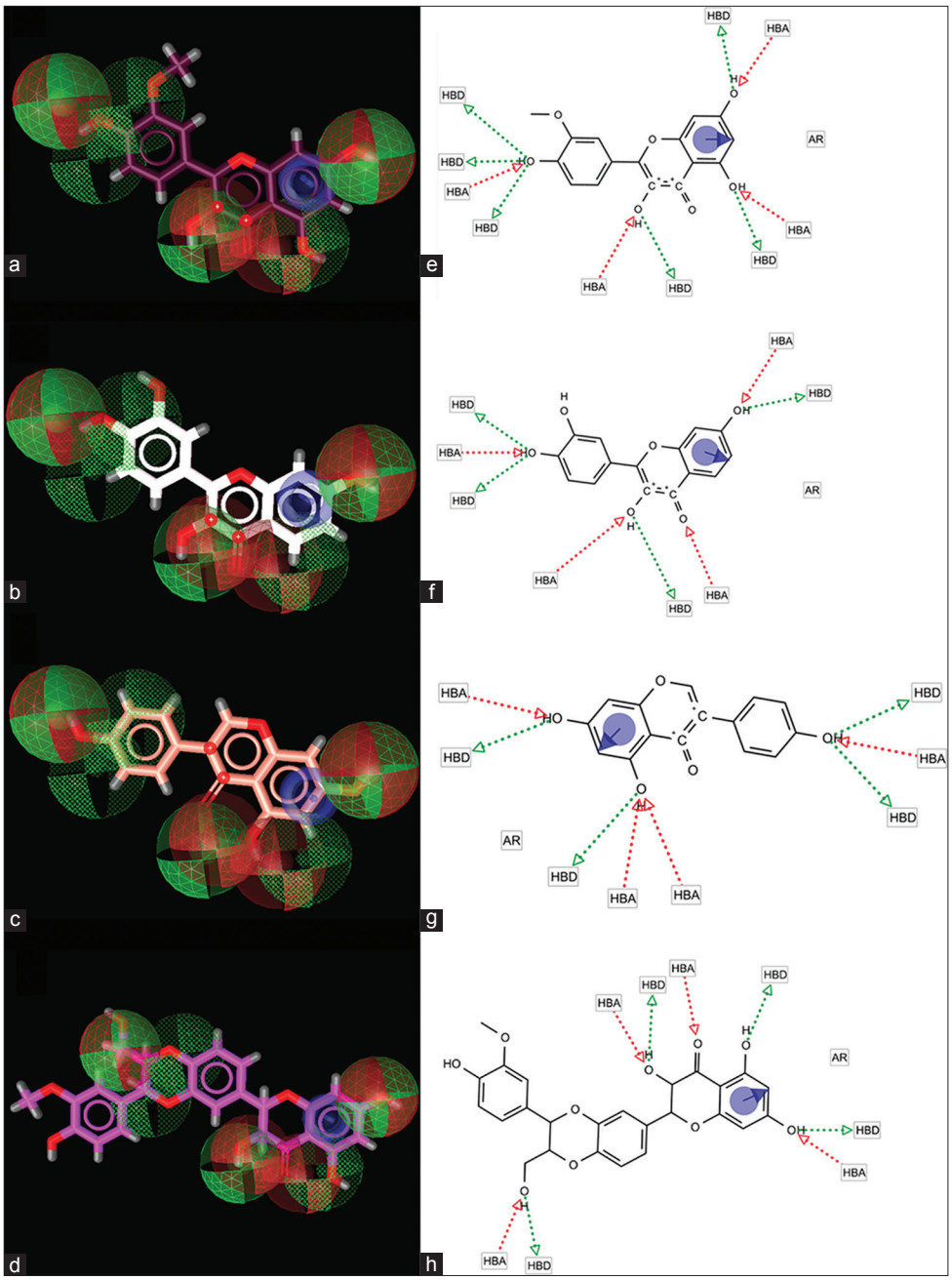

Figure 5: 2D and 3D representations of pharmacophoric features of the four compounds used in pharmacophore generation: (a and b) Isorhamnetin with 4 hydrogen bond acceptors (4 HBAs), 6 HBAs and an aromatic ring (AR); (c and d) fisetin with 4 HBAs, 4 hydrogen bond donors (4 HBDs) and an AR; (e and f) genistein with 4 HBAs, 4 HBDs and an AR; (g and h) silibinin with 4 HBAs, 4 HBDs and an AR. Pharmacophoric features are color-coded with red, HBAs; green, HBDs; blue, AR

Asn387 at distances of 1.92 Å, 2.13 Å, 3.04 Å, 1.85 Å and 1.86 Å, respectively. Cation-pi interaction was made by Lys345 at 6.64 Å and a sigma-pi interaction was made by Val349 at 3.87 Å. The residues Tyr306 and Trp383 made hydrophobic interactions with luteolin [Figure 3b].

Coumestrol interacted with β-catenin forming four H-bonds with β-catenin at TCF-interacting region involving β-catenin residues Gly307, Lys312, Lys345 and Asn387 at a distance of 1.90 Å, 1.70 Å, 1.88 Å and 2.47 Å, respectively. Tyr306 was involved in pi-pi interactions with three rings of the inhibitor at distances of 4.21 Å, 4.26 Å and 5.01 Å, respectively. β-catenin residues Val346 and Val349 were involved in hydrophobic interactions with this compound [Figure 3c].

Two H-bonds were formed by interaction of β-naphthoflavone with β-catenin involving residues Gly307 and Lys312 at distances of 2.14 Å and 2.11 Å, respectively. Lys345 showed a cation-pi interaction at a distance of 3.98 Å from the ring of this compound, while Val349 was found to be involved in a sigma-pi interaction at a distance of 3.55 Å. Hydrophobic interactions with this compound were made by β-catenin residues Tyr306, Arg342 and Val346 [Figure 3d].

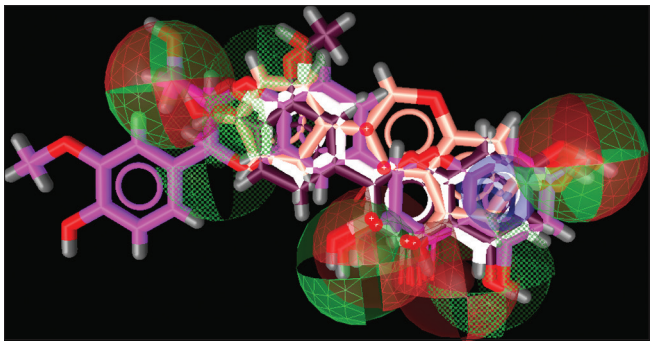

Figure 6: Common feature pharmacophore. Features are color-coded with red, hydrogen bond acceptors; green, hydrogen bond donors; blue, aromatic ring. Molecules are color-coded with purple, isorhamnetin; white, fisetin; peach, genistein; magenta, silibinin

Inhibitory effect of flavonoids on Wnt signaling pathway by targeting β-catenin is supported by the fact that the interaction pattern of these newly discovered flavonoid inhibitors of β-catenin coincides with that of the experimentally known flavonoid inhibitors as they occupy the same binding cavity as the known inhibitors [Figure 4].

Pharmacophore evaluation

Using the four experimentally known inhibitors of Wnt signaling, a pharmacophore model was generated. The generated pharmacophore showed three main features: Hydrogen bond acceptors (HBAs), hydrogen bond donors (HBDs) and AR. HBDs are shown in green, HBAs in red and ARs in blue. The representative 2D and 3D pharmacophoric features of each compound are shown in Figure 5.

Each compound constitutes individual pharmacophoric features which are summarized in Table 2.

From these individual characteristic pharmacophores, a merged pharmacophore with common features was generated, as shown in Figure 6. This common feature pharmacophore with a score of 0.8316 showed certain features: Four HBDs, four HBAs and one AR.

Distances among different features of the merged pharmacophore were calculated using DS as shown in Figure 7. Location constraints of the features were represented using sphere meshes of different colors for different features.

DISCUSSION

In case of wild type β-catenin protein, APC binds at almost the same residues as TCF[25] which means the formation of destruction complex prevents TCF4-binding by physically

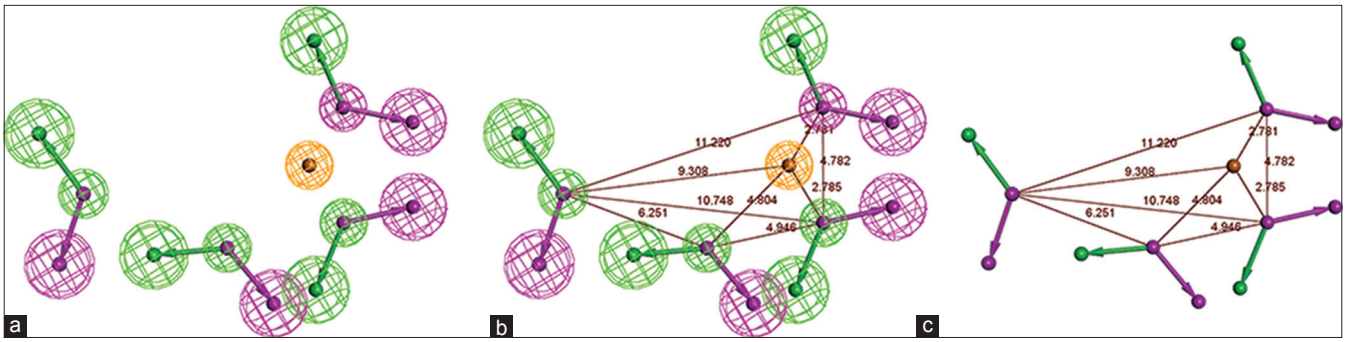

Figure 7: Pharmacophore hypothesis for β-catenin inhibitors. (a) Pharmacophore model with 9 features: 4 hydrogen bond acceptors (HBAs), 4 hydrogen bond donors (4 HBDs), 1 aromatic ring (AR); (b) Pharmacophore distance along with location constraints; (c) Pharmacophore distance without location constraints. All features in this pharmacophore model are color coded with green, HBA; magenta, HBD; orange, AR. Distance between certain features is represented in Å

blocking the TCF4-binding site. However, in case of mutated β-catenin, shifting in axin and APC binding stabilizes β-catenin for TCF4-binding, resulting in uncontrolled expression of target genes. Thus, inhibition of β-catenin/TCF4 complex in cancer cells may prevent the disease developed due to the uncontrolled transcription of its target genes.

Flavonoids are a family of naturally-occurring compounds known for the inhibition of β-catenin/TCF4 complex.[16] Apigenin,[26] fisetin,[27] isorhemnentin, genistein[16] and silibinin[28] are known to strongly suppress β-catenin/TCF-driven transcription. In this study, experimentally known inhibitors of β-catenin/TCF4 complex were docked against β-catenin to explore the binding site details which were consistent with the experimental data.[16] We observed binding of flavonoid members with similar residues involved in β-catenin and TCF4-binding. Thus this complex was evaded because if the core TCF4-binding residues in β-catenin were pre-occupied by the inhibitor, transcription of target genes blocks away.

On the basis of known information, we tested other members of the flavonoid family to monitor their comparative mode of interaction and inhibitory effects against β-catenin/TCF4 complex. Among all the docked members, catechin, luteolin, coumestrol and β-naphthoflavone had shown significant values and were chosen for detailed analysis. The binding and docking energies of the compounds were compared where β-naphthoflavone and coumestrol were found to be those with the least energy values and favorable inhibition constant values and thus, were found to be the compounds with high inhibitory effects on β-catenin/TCF4 complex.

Based on the experimentally known inhibitors, a pharmacophore model was generated with common pharmacophoric features from those compounds which will later be useful for screening of new inhibitors based on their properties.

CONCLUSIONS

The main purpose of in silico docking analysis in this study was to determine favorable binding conformations between flavonoid members and β-catenin/TCF4. A multiple number of poses were generated and evaluated on the basis of binding conformations and common interacting residues at the binding pocket. We propose that β-naphthoflavone may act as a more potent inhibitor against Wnt signaling pathway and can be used as an anti-cancer drug. Further study of proposed inhibitors needs to be carried out to explore their binding and inhibitory potential *in vivo*. Furthermore, the pharmacophore model presented can be used to screen more compounds and will thus be helpful in finding novel inhibitors to β-catenin and thus to abrupt Wnt signaling.

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