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A Comprehensive Modeling of Bioenhancers Docked to Transport Proteins to Enhance Bioavailability

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ABSTRACT

With pharmaceutical availability being a pertinent issue in modern medicine, the ability of bioenhancers to increase the bioavailability of a drug, thereby reducing the required dosage, can be critical for reducing treatment costs. Flavonoids, one form of bioenhancers, are metabolites that increase the availability through inhibition of key proteins in gut epithelial cells and transport proteins. Bioenhancers have the potential to inhibit proteins that limit absorption, thus increasing the amount of a target drug that can enter systemic circulation, increasing bioavailability. P-glycoprotein (P-gp) is one of the membrane transport proteins whose function is to transport drugs in and out of the cell. Human serum albumin (HSA), the most abundant protein in human plasma, is a protein that serves to transport several signals and other compounds throughout the circulatory system. This study assessed the binding of various bioenhancers (piperine, quercetin, capsaicin, naringin, genistein, lysergol, sinomenine, tangeretin) to various forms of P-gp, HSA and ABC transporters to improve drug bioavailability. We hypothesized that the bioenhancers would bind to these transport proteins, thereby inhibiting them and increasing bioavailability.An examination of the geometric shape complementarity scores in PatchDock and the binding affinities (ΔG kcal/mol) from three other web servers (Webina, DockThor, CB-Dock) showed that naringin produces the most optimal binding scores overall. Given the promising optimal binding scores, the data provides critical insight into administering bioenhancers with drugs to improve bioavailability, as well as suggesting that naringin may be a valuable compound to conduct further tests *in vitro* and *in vivo*.

Introduction

The concept of bioenhancers or bio potentiators is a relatively recent development to modern science. It was first reported by Bose in 1929, who described the increase in the antiasthmatic effects of vasaka (*Adhatoda vasica*) leaves by the addition of long pepper (*Piper longum*) to it (1). The development and consequent isolation of these molecules is considered as a scientific breakthrough. A bioenhancer is an agent capable of enhancing the bioavailability and efficacy of a drug with which it is co-administered, without any pharmacological activity of its own at the therapeutic dose used (2). They tend to decrease the dose of active drug required for the optimal endpoint of the treatment strategy, bypassing the need to use injectable routes of drug administration to a larger extent, which may help in overcoming the resistance to antimicrobials and saving precious raw materials for the manufacturing of medicines (1,2). Such fixed drug combinations (FDCs) are economically viable as well (2).

Docking at the molecular level is a process which involves conforming the ligand to its target receptor in the right pose so as to minimize binding energies. Geometric and electrostatic interactions play a critical role in quantifying the accuracy of the orientation of the ligand to the active site of its targeted receptor. As such, Coulombic and Van der Waals interactions (3), which quantify the interactions between the electrical charges of the molecules, in addition to the formation of hydrogen bonds, are summed together to form a binding score which is indicative of the binding potential between the two molecules (4). Docking softwares work by incorporating search algorithms which recursively search the orientation of the ligand until the binding energy of the ligand to the receptor is minimized (3).

Over the past few decades, theories and methodologies developed in regard to molecular docking are used as the fundamental base of operation for the majority of docking softwares (5). In essence, it ideated the concept of rigid docking in which both the ligand and the receptor are treated as rigid bodies, and in which the binding affinity score holds a proportional relationship to the geometric fit of the ligand to its targeted receptor (5). The induced-fit theory, introduced by Daniel Koshland in 1958, proposes a more flexible style of docking in which both the target and receptor make minor conformational and geometric changes to adapt to each other's core shapes in order to optimize their best fit (6). As a result of this flexibility and ability to adapt, flexible docking algorithms are able to implement higher accuracy and efficiency in predicting both binding affinities and modes in comparison to their rigid body docking counterparts (6). These individual models and methodologies of molecular docking developed over the years each highlight a specific portion of the molecular recognition process.

Methods

Ligand Preparation

In preparation for docking the ligands, we took the smiles code from pubchem, provided by ncbi. Using avogadro and orca, we DFT optimized all the molecules to make their structures more accurate.

PatchDock

PatchDock, created by the Swiss Bioinformatics Group alongside the team of the Tel Aviv Computer Science School at https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php. For the purpose of this study, along the inputted receptor and ligand, the clustering RMSD condition was left at 4.0 and the complex type was set to protein-small ligand (7). The software produces a web results page with the top 20 binding conformation these as and poses with a solutions table which incorporates a geometric shape complementarity score, desolvation energy, size of the interfaced area, and the actual rigid transformation within itself (8).

CB-Dock

CB-Dock is a web server that can be accessed at http://clab.labshare.cn/cb-dock/php/blinddock_classical.php, that performs blind docking (9). The server calculates the center and size of the docking boxes, and molecular docking is performed using AutoDock Vina. The results web page displays the Vina scores (binding affinity in kilocalories/mol) along with cavities sizes, docking centers, and docking box size (9).

DockThor

The web server DockThor, available at https://dockthor.lncc.br/v2/, is useful for docking highly flexible ligands, being able to support up to 40 rotatable bonds (10, 11, 12). DockThor employs an empirical scoring function named DockTScore, which accounts for physics-based interactions which contribute to binding energy. DockTScore utilizes a

MMFF49S force field and considers van der Waals & electrostatic energy terms, terms which are optimized to account for solvation, protein-ligand interactions which are lipophilic, and estimation of ligand torsional entropy contribution (10).

Webina

Webina 1.0.2, available at https://durrantlab.pitt.edu/webina, is AutoDock Vina ported to WebAssembly (13). The optimization algorithm that the software uses aims to rank the scoring confirmations it produces. Based on the results and ranks that they produced, the software aims to determine the binding affinity. AutoDockTools is a graphical interface that can be used for reading and writing files, calculation of charges, protonation, and specifying rotatable bonds in proteins and ligands (14). It also can be used for many other tasks with regards to preparing for and analyzing docking experiments.

Bioenhancers Used in This Study

Piperine is an alkaloid found in both black pepper (Piper nigrum) and long pepper (Piper longum) (15). Piperine is used for its number of biological properties, including anti-inflammatory activity, antipyretic activity, antifungal activity, antidiarrheal activity, anti-cancer activity, and more. Piperine is thought to work by numerous mechanisms to increase bioavailability, including alteration of membrane dynamics, inhibition of gastrointestinal and hepatic metabolism, and inhibition of P-gp and CYP3A4; P-gp and CYP3A4 play a role in the first-pass elimination of many drugs (15). Quercetin is a flavonoid found in numerous plants, including vegetables, citrus fruits, leaves, and grains. Quercetin has shown activities including antioxidant, radical scavenging, anti-inflammatory, antiatherosclerotic, anticancer, and antiviral effects (16). Quercetin's mechanisms of action include inhibition of both P-gp and CYP3A4, thereby demonstrating inhibition of MDR efflux as well as first-pass metabolism (15). Capsaicin, an alkaloid, is found as the active ingredient in chili peppers (Capsicum annum) and increases bioavailability by inhibiting P-gp mediated drug efflux (15, 16). Capsaicin serves as a temporary pain reliever for arthritis and muscle aches. This bioenhancer is also significantly used in pepper spray and pesticides (17). Naringin, a flavonoid glycoside found in plants including apples, grapefruit, tea, and onions, exhibits effects such as antioxidant activity, antiulcer activity, antiallergenic activity and anticancer activity. It has been reported to be a P-gp modulator and inhibitor of CYP3A4 (16). Genistein is a flavonoid, isoflavone and phytoestrogen. Derived from plants such as soybean (Glycine max) and kudzu (Pueraria lobata), Genistein demonstrates anticancer and anti-inflammatory activity (16). Genistein is an inhibitor of the efflux transporters MRP, BCRP, and P-gp, and also demonstrates CYP3A4 inhibition (15). The alkaloid Lysergol, a phytomolecule, is found in the Morning Glory Plant (Ipomoea spp.). It has also been isolated in higher plants such as Rivea corymbosa, Ipomoea violacea, and Ipomoea muricata, as well as lower fungi like Clavicpes, Penicillium, and Rhizopus (16). Lysergol shows psychotropic, analgesic, analeptic, hypotensive and immuno-stimulant effects. It has been shown to inhibit metabolism as well as BCRP (15). Sinomenine is an alkaloid found in the plant Sinomenium acutum. It is known to increase bioavailability by inhibiting P-gp efflux transport (15). Tangeretin, a flavone found in citrus fruits, has shown the ability to inhibit P-gp (15). In order to simulate these molecules, we used pubchem's data base, provided by NCBI.

Description of Docked Proteins

P-Glycoprotein

P-gp is a protein that is part of the ATP binding cassette transporters family (ABC Transporters). ABC transporters are a superfamily of transport proteins that use ATP hydrolysis to modulate the movement of molecules across the cell membrane. P-gp is frequently expressed on the cells of the intestinal epithelium (18). P-gp uses energy from ATP hydrolysis to pump xenobiotics out of the intestinal epithelium and into the intestinal lumen. P-gp reduces the bioavailability of drugs by preventing them from staying in the intestinal epithelium and thus preventing drugs from entering the bloodstream and target site. The binding and potential inhibition of P-gp by bioenhancers is of importance because it could increase the bioavailability of other drugs when administered simultaneously.

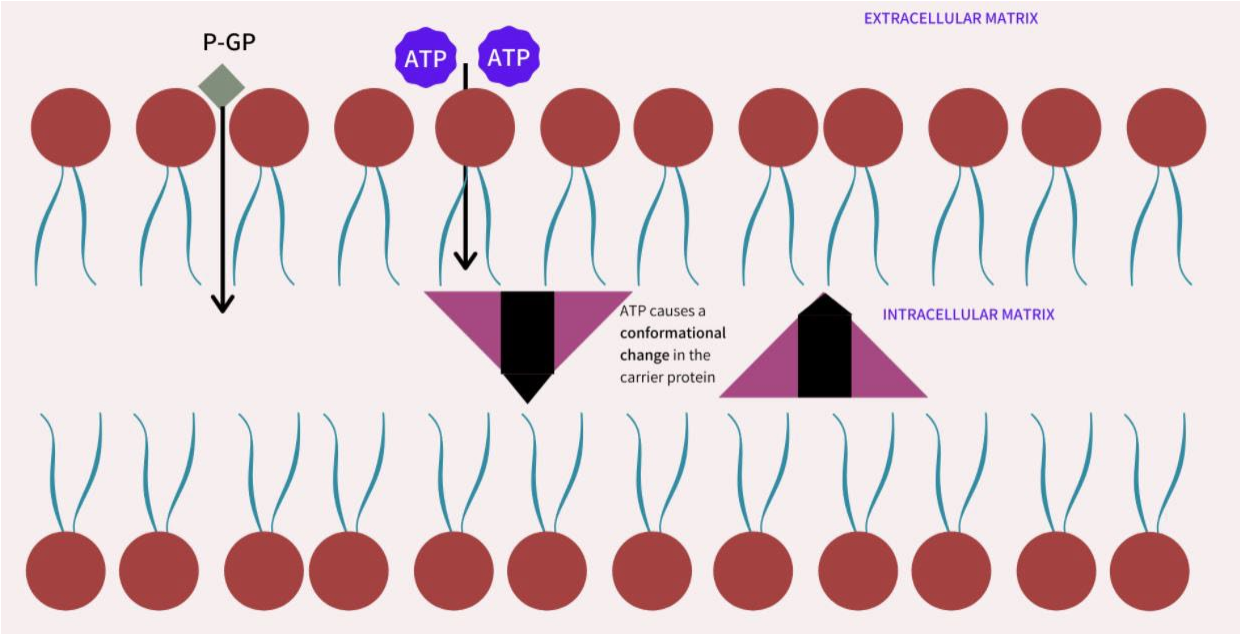

Figure 1. The model shows the mechanism of the P-glycoprotein. The ATP causes a conformational change in the carrier protein [image designed in Canva].

Human Serum Albumin

Human serum albumin (HSA) is the most commonly found protein in human plasma. (19). HSA can bind a variety of drugs, affecting their distribution and elimination as well as their active concentration (19, 20). While some degree of binding to HSA can assist in solubilizing compounds, drugs with a high affinity for HSA necessitate higher dosages to produce the desired effect. HSA is made up of three domains which are homologous, and each domain is split into two subdomains, A and B. Two primary drug binding sites are located in subdomains IIA and IIIA; the drugs that bind to these sites often have acidic or electronegative elements (19). These sites are commonly named Sudlow's sites I and II (20). Bioenhancers that can bind to HSA and potentially inhibit it are of importance as they could potentially improve the bioavailability of certain drugs with a high affinity to HSA.

Table 1. PDB Code, Protein Name and Organism Information

PDB Code	Protein Name	Organism
4F4C (18)	P-glycoprotein	Found in *C. Elegans* and plays the role of multidrug transport. Functions include absorption and distribution of several drugs. Widely used in cancer treatment.
4Q9H (21)	P-glycoprotein at 3.4 A resolution	Alpha helix ABC transmembrane protein found in *homo sapiens*. Binding to ligands allows for ATP hydrolysis due to conformational change which triggers a response.
6C0V (22)	P-glycoprotein outward-facing conformation	Transport protein found in *homo sapiens*. Removes harmful substances from the cell through the outward facing conformation controlled by ATP.
2BXQ (23)	Human Serum Albumin complexed with myristate, phenylbutazone and indomethacin.	Transport protein found in *homo sapiens* that have formed a complex with myristate (fatty acid), phenylbutazone (nonsteroidal drug) and indomethacin (anti-inflammatory drug).
3KQ0 (24)	Crystal structure of human alpha1-acid glycoprotein	An important drug-binding protein in the plasma in *homo sapiens*. Can bind hundreds of ligands including warfarin. Largely binds basic and neutral molecules.
1HA2 (25)	Human Serum Albumin Complexed with Myristic Acid and the S- (-) enantiomer of warfarin	Transport protein found in *homo sapiens*; complexed with myristic acid (fatty acid) and warfarin (a widely used anticoagulant). Warfarin shares a binding site with phenylbutazone and indomethacin.
2BXF (26)	Human serum albumin complexed with diazepam.	Transport protein found in *homo sapiens*. Well present plasma protein which exhibits the ability to bind to several drugs, therefore increasing drug availability.
6FFC (27)	Structure of an inhibitor-bound ABC transporter	Transport protein found in ATP binding cassette of *homo sapiens* that serves as an inhibitor to promote body tissues to the resistance of several drugs.

Results

DockThor						
Score Range	-6.0 to -7.0	-7.1 to -8.0	-8.1 to -9.0	-9.1-10	-10.1-11	
Ligand + Protein	2BXQ	2BXF	3KQ0	6FFC	1HA2	Average
Piperine	**-9.477**	**-7.472**	**-9.266**	**-7.089**	**-9.332**	-8.5272
Quercetin	**-6.943**	**-6.991**	**-8.006**	**-7.202**	**-6.253**	-7.079
Capsaicin	**-7.544**	**-7.506**	**-7.955**	**-7.696**	**-7.661**	-7.6724
Naringin	**-7.234**	**-8.035**	**-8.489**	**-9.502**	**-7.228**	-8.0976
Genistein	**-7.293**	**-7.155**	**-8.28**	**-9.506**	**-7.809**	-8.0086
Lysergol	**-8.481**	**-7.501**	**-8.492**	**-8.808**	**-6.958**	-8.068
Sinomenine	**-7.426**	**-8.067**	**-8.49**	**-11.016**	**-8.817**	-8.7632
Tangeretin	**-7.383**	**-7.484**	**-10.08**	**-7.089**	**-7.567**	-7.9206
Average protein score	-7.722625	-7.526375	-8.63225	-8.489975	-7.701625	

Figure 2. Results obtained from DockThor. All numbers reported in kcal/mol, with heat map coloration provided for ease of interpretation. Average binding affinity for each bioenhancer bound with all proteins as well as average binding affinity for each protein bound with all bioenhancers is provided. Docking with all bioenhancers to PDB IDs 4F4C, 4Q9H and 6C0V produced errors and were omitted from the results.

CB Dock									
Score Range	-6.0 to -7.0	-7.1 to -8.0	-8.1 to -9.0	-9.1-10	-10.1-11	>-11.0			
Ligand + Protein	4F4C	4Q9H	6C0V	2BXQ	2BXF	3KQ0	6FFC	1HA2	Average
Piperine	-7.8	-8	-9.2	-9.9	-8.8	-8.8	-9.6	-10.2	-9.0375
Quercetin	-8.7	-7.8	-8.9	-9.1	-8.4	-8.4	-8.4	-9.1	-8.6
Capsaicin	-7.1	-6.9	-7.5	-8.9	-7.7	-7.7	-10.9	-8.9	-8.2
Naringin	-10	-9	-11.8	-11.2	-9.6	-9.6	-8.3	-10.9	-10.05
Genistein	-7.8	-7	-7.6	-8.6	-7.9	-7.9	-9.1	-8.2	-8.0125
Lysergol	-7.5	-7.1	-8.1	-9.3	-7.9	-7.9	-9.3	-9.4	-8.3125
Sinomenine	-8.4	-7.1	-8.7	-7.8	-7.8	-7.8	-9	-7.9	-8.0625
Tangeretin	-7.3	-6.6	-8	-8.8	-7.8	-7.8	-9.6	-9.2	-8.1375
Average protein score	-8.075	-7.4375	-8.725	-9.2	-8.2375	-8.2375	-9.275	-9.225	

Figure 3. Results obtained from CB-Dock. All numbers reported in kcal/mol, with heat map coloration provided for ease of interpretation. Average binding affinity for each bioenhancer bound with all proteins as well as average binding affinity for each protein bound with all bioenhancers is provided.

Webina						
Score Range	< -5.9	-6.0 to -7.0	-7.1 to -8.0	-8.1 to -9.0	-9.1-10	
Ligand + Protein	4Q9H	2BXF	3KQ0	6FFC	1HA2	Average
Piperine	-5.1	-6.9	-8.1	-6.9	-8.8	-7.16
Quercetin	-5.7	-7.3	-8.0	-5.3	-7.2	-6.7
Capsaicin	-4.4	-6.5	-6.8	-1.2	-7.7	-5.32
Naringin	-5.9	-5.6	-9.4	-7	-9.6	-7.5
Genistein	-5.7	-7.3	-8.0	-6.3	-7.4	-6.94
Lysergol	-5.2	-7.1	-7.8	-6.5	-8.6	-7.04
Sinomenine	-5.4	-7.5	-7.9	-4.6	-7.4	-6.56
Tangeretin	-5.1	-6.9	-6.5	-6.9	-7.4	-6.56
Average protein score	-5.3125	-6.8875	-7.8125	-5.5875	-8.0125	

Figure 4. Results obtained from Webina. All numbers reported in kcal/mol, with heat map coloration provided for ease of interpretation. Average binding affinity for each bioenhancer bound with all proteins as well as average binding affinity for each protein bound with all bioenhancers is provided. Docking with all bioenhancers to PDB IDs 4F4C, 4Q9H and 6C0V produced errors and were omitted from the results.

PatchDock									
Score Range	3000-4000	4001-5000	5001-6000	> 6000					
Ligand + Protein	4F4C	4Q9H	6C0V	2BXQ	2BXF	3KQ0	6FFC	1HA2	Average
Piperine	4908	4428	5376	4806	5288	4272	4444	5070	4824
Quercetin	4476	4022	4552	4284	4674	3908	5084	4452	4431.5
Capsaicin	5310	4904	5932	5910	6274	4866	6436	5464	5637
Naringin	6172	5892	6896	5646	6562	5748	4152	6638	5963.25
Genistein	4310	3994	4454	4180	4414	3886	4502	4246	4248.25
Lysergol	4512	4150	4450	4280	4496	3830	5022	4546	4410.75
Sinomenine	4834	4706	5292	4512	5036	4288	5114	4974	4285.625
Tangeretin	5402	5388	6272	5354	5610	5238	4444	6180	5486
Average protein score	4990.5	4685.5	5403	4950.25	5294.25	4504.5	4899.75	5196.25	

Figure 5. Results obtained from PatchDock. All numbers reported according to the geometric shape complementary score, with heat map coloration provided for ease of interpretation. Average binding affinity for each bioenhancer bound with all proteins as well as average binding affinity for each protein bound with all bioenhancers is provided.

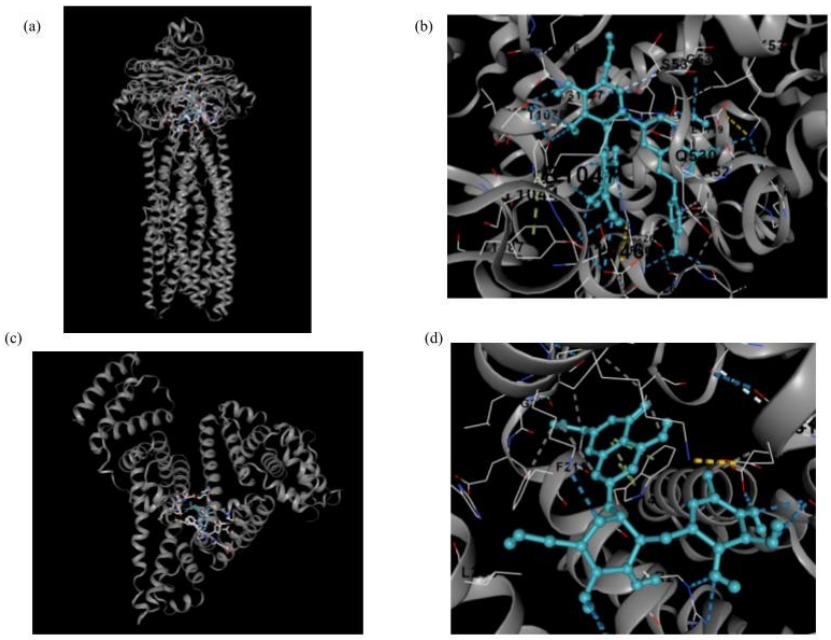

Figure 6. Select images of in silico docking. (a) Naringin bound to P-glycoprotein in *homo sapiens* (PDB ID 6C0V) using CB-Dock: -11.8 kcal/mol (zoomed out). (b) Naringin bound to P-glycoprotein in *homo sapiens* (PDB ID 6C0V) using CB-Dock: -11.8 kcal/mol (zoomed in). (c) Naringin bound to human serum albumin complexed with myristate, phenylbutazone and indomethacin (PDB ID 2BXQ) using CB-Dock: -11.2 kcal/mol (zoomed out). (d) Naringin bound to human serum albumin complexed with myristate, phenylbutazone and indomethacin (PDB ID 2BXQ) using CB-Dock: -11.2 kcal/mol (zoomed in).

The rationale for this experiment was based on the potential of bioenhancers to act as inhibitors of transport proteins, thereby increasing the amount of a drug that can reach systemic circulation and have an active pharmacological effect. If the bioenhancers studied here can inhibit the transport proteins which commonly prevent drugs from reaching their targets (such as P-gp, HSA, and ABC transporters), then the bioavailability of those drugs will increase. This inhibition is quantified through how well the bioenhancer and protein bind; a more negative binding affinity or higher geometric shape complementarity score correlates with better binding. In this study, all eight bioenhancers were tested with all eight proteins in four docking web servers (Patch Dock, CB-Dock, Webina, DockThor), and the binding affinity or geometric shape complementarity score (depending on which the server utilized) was analyzed. Four docking servers were utilized in order to account for variability in scores reported between different servers.

In Webina, naringin produced the most optimal score when docked to all proteins (compared to the other bioenhancers) with the exception of 2BXF. Naringin docked to 3KQ0 produced a score of -9.4 kcal/mol, and naringin docked to 1HA2 produced a score of -9.6 kcal/mol. These were the two highest scores produced in all of the docking jobs performed by Webina.

In CB-Dock, naringin produced the best scores overall with an average of -10.05 kcal/mol for all the proteins it was docked to. Naringin also produced the highest score with each protein it was bound to, except for 6FFC, where capsaicin produced a score of -10.9 kcal/mol while naringin produced a score of -8.3 kcal/mol. Specifically, the transport proteins that produced the prime scores when comparing all docking jobs performed by CB-Dock were 6COV and 2BXQ, with scores of -11.8 kcal/mol and -11.2 kcal/mol respectively.

In PatchDock, naringin produced the best scores with an average of 5963.25 compared with other bioenhancers. Specifically, naringin produced the most optimal score with six of the eight proteins. Naringin docked to 6C0V produced the best score of 6896. Capsaicin produced more favorable binding scores in comparison to naringin when docked to both 2BXQ (5910 vs. 5646). However, when docked to 6FFC, the remaining bioenhancers were significantly more efficient than naringin. Generally, sinomenine produced the least optimal scores in comparison to all the other bioenhancers with the average being 4285.625.

In DockThor, sinomenine had the best average score (-8.7632 kcal/mol), while naringin (-8.0976 kcal/mol) ranked third after piperine (-8.5272 kcal/mol). Naringin never produced a top score.

Discussion

After conducting the study, it was found that naringin consistently had the best binding score. In Patch Dock and CB-Dock, with an exception of 2BXQ and 6FFC respectively, naringin produced the best binding score in seven out of the eight proteins it was docked to. In general, Webina produced higher, therefore poorer, scores in comparison to the other docking servers utilized, likely due to differences in the methodology of AutoDock Vina. Additionally, in Webina, the protein 4Q9H itself served as a determining factor for the proteins it was docked to, meaning that all bioenhancers bound to the protein produced binding affinities in the same range; these binding affinities were significantly poorer.

In CB-Dock, naringin performed the best, specifically when docked to 6COV with a score of -11.8 kcal/mol. Genistein was the poorest bioenhancer with an average score of -8.0125 kcal/mol. In terms of the proteins, bioenhancers bound to 4Q9H produced the poorest score on average (compared with other proteins) of -7.435 kcal/mol. DockThor produced errors for proteins 4F4C, 4Q9H and 6C0V, likely because DockThor does not accept protein files that have more than one thousand residues per chain. The result of naringin performing as the top bioenhancer is not as confidently seen in DockThor.

The overall pattern of evidence is clear in supporting the assertion that naringin is the most efficient bioenhancer. These scores were the best in comparison to all the other bioenhancers that naringin was docked to in Webina.

All the bioenhancers that were docked to 4Q9H produced the poorest score in comparison to the other proteins, however naringin still produced the best score of -5.9 kcal/mol. There was not one bioenhancer that did poorly in all the docking softwares, and essentially, there was variability depending on the protein it was docked to.

Conclusion

After conducting the study, all docking softwares that was used resulted in naringin having one of the best binding scores. Generally, naringin produced the most optimal binding scores in the in-silico trials conducted, consistently resulting in highly negative ΔG values. The data found in this study is highly promising in terms of the future potential applications of naringin but, further in vitro studies would be required to develop a better understanding of its pharmacokinetic properties to improve its delivery mechanisms.

Limitations

One limitation of this study was the variance in the way scores were reported, namely, the difference in the scores of PatchDock against the other softwares. This made it difficult to compare softwares. PatchDock expresses results in terms of the geometric shape complementary score of the protein-ligand complex, while other servers give results in terms of binding affinity, measured in kilocalories per mol (kcal/mol). Ultimately, this makes scores from PatchDock more difficult to compare to those of other web servers.

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