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Research Article

IN SILICO ANALYSIS OF FUNCTIONAL SNPs OF ALOX12 GENE AND IDENTIFICATION OF PHARMACOLOGICALLY SIGNIFICANT FLAVONOIDS AS LIPOXYGENASE INHIBITORS

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ABSTRACT
Cancer is a disease affecting any part of the body and in comparison with normal cells there is an elevated level of lipoxygenase enzyme in different cancer cells. Thus generation of lipoxygenase enzyme inhibitors have suggested being valuable. Individual variation was identified by the functional effects of Single Nucleotide Polymorphisms (SNPs). 696 SNPs were identified from the ALOX12 gene, out of which 73 were in the coding non-synonymous region, from which 8 were found to be damaging. In silico analysis was performed to determine naturally occurring flavonoids such as isoflavones having the basic 3-phenylchromen-4-one skeleton for the pharmacological activity, like Genistein, Diadzein, Irilone, Orobol and Pseudobaptigenin. O-methylated isoflavones such as Biochanin, Calycosin, Formononetin, Glycitein, Irigenin, 5-O-Methylgenistein, Pratensein, Prunetin, ψ-Tectorigenin, Retusin and Tectorigenine were also used for the study. Other natural products like Aesculetin, a coumarin derivative; flavones such as ajoene and baicalein were also used for the comparative study of these natural compounds along with acteoside and nordihydroguaiaretic acid (antioxidants) and active inhibitors like Diethylcarbamazine, Zileuton and Azelastine as standard for the computational analysis. The protein-ligand interaction was studied and the docking analysis was performed by Patch Dock server using the 3D3L human arachidonate 12-lipoxygenase protein. Out of the 19 herbal constituents selected, it was found that Diadzein, Biochanin, Glycitein, Irigenin, 5-O-Methylgenistein, ψ-Tectorigenin and Tectorigenin showed significant binding affinity in inhibiting lipooxygenase enzyme as compared with standard compounds of acteoside, nordihydroguaiaretic acid, Diethylcarbamazine, Zileuton and azelastine. Naturally occurring compounds on further advancement could act as potent lipoxygenase inhibitors.
Keywords: Cancer, Lipoxygenase enzyme, SNP, In silico analysis, Docking, Drug likeness

INTRODUCTION

Computer aided Drug designing is currently playing a major role in the field of research and drug discovery. The conventional method of development of molecules by synthesis is preceded by the use of drug designing techniques computationally. Various imperative steps are involved in drug designing such as structure based and ligand based drug designing. In silico approaches based on ligand drug designing have been performed for the lead molecule development. The necessary molecular characteristics of the ligand have been generated computationally. The various interactions between the proteins and the ligand are analyzed by docking simulation with the protein target 3D3L[1,2]. The binding is measured in terms of the score based on the protein-ligand interactions. The study of other parameters such as detection of cavities was also carried out. Single nucleotide polymorphism plays an essential role in identification of individual variations and determination of mutations. Drug likeness is a parameter that helps to characterize compounds based on the varied molecular properties like hydrophobic character, electron distribution, hydrogen bond formation, size and adaptability of the molecule in conjunction with the presence of various pharmacophores. The lead molecule affects the bioavailability, protein binding property, absorption mechanism, toxicity and stability of the compound[3]. Mol inspiration was accustomed to calculate parameters like relative molar mass, log P, number of hydrogen bond donors or acceptors which are essential to eliminate non-drug like molecules. Sophisticated Bayesian statistics was employed in Mol inspiration for the calculation of the physicochemical properties of the lead molecules. Log P (octanol/water partition coefficient) and Total Polar Surface Area (TPSA) was calculated by the sum of fragment based contributions and correction factors[4]. TPSA is observed to be an indispensable descriptor influencing the drug absorption, $CaCO_2$ permeability and blood-brain barrier penetration. Molecular Volume calculation is predicted based on cluster contributions and are fully optimized by the semi empirical AM1 technique. Rule of 5 is a set of molecular descriptors used by Lipinski in formulating the Rule of 5. The rule states that most "drug-like" compounds have log P < = 5, molecular weight < = 500, number of hydrogen bond acceptors < = 10 and number of hydrogen bond donors < = 5[5]. Molecules which do not pursue more than one of these rules may have problems with bioavailability. Topological parameter used for measuring molecular flexibility is the Number of Rotatable Bonds (nrotb)[6]. Rotatable bond is defined as any single non-ring bond, bounded to non-terminal heavy (non-hydrogen) atom. The rule elucidates the molecular properties like absorption, distribution, metabolism and excretion (ADME). Lipoxygenase (LOXs EC1.13.11) are iron containing enzymes which are classified as arachidonate 5-lipoxygenase and 12-lipoxygenase in humans[7]. Lipoxygenase enzyme may initiate the synthesis of a signaling molecule or be involved in inducing structural or metabolic changes in the cell[8]. Human lipoxygenases are the enzymes participating in the metabolism of the polyunsaturated fatty acids and catalyzing their oxidation to a variety of eicosanoids acting as the secondary signal transducers playing a major role in human homeostasis[9]. Phosphorylation via Mitogen activated protein kinase-2 is critical in controlling and activation of this enzyme[10,11]. LOX are implicated in the pathogenesis of diseases like asthma[12], ulcerative colitis[13], psoriasis[14],

atherosclerosis[15] and cancer[16]. In comparison with normal tissues, significantly higher concentration of LOX intermediates have been found in breast, colon, lung, skin and prostate cancers, along with persons with both acute and chronic leukaemias[17]. Flavonoids are naturally occurring plant metabolite with numerous pharmacological activities. The focus of the research was to verify certain selected flavonoids as lipoxygenase enzyme inhibitor[18,19]. Certain isoflavones like Genistein, Diadzein, Irilone, Orobol and Pseudobaptigenin; O-methylated isoflavones such as Biochanin, Calycosin, Formononetin, Glycitein, Irigenin, 5-O-Methylgenistein, Pratensein, Prunetin, ψ-Tectorigenin, Retusin and Tectorigenin were selected for the study. The isoflavones have the basic 3-phenylchromen-4-one skeleton for the pharmacological activity. Other natural products like Aesculetin, a coumarin derivative; ajoene and baicalein, flavone; were also used for the analysis. Acteoside and nordihydroguaiaretic acid denoted as antioxidants and active inhibitors like diethylcarbamazine, Zileuton and azelastine were used as standard for the comparative computational analysis.

MATERIALS AND METHODS

The X-ray crystallographic structure of lipoxygenase domain of human arachidonate 12-lipoxygenase, 12S-type (PDB ID 3D3L) protein was obtained from RCSB protein data bank[20] at a resolution of 2.60 Å. Water molecules, ligands and other hetero atoms were removed from the protein molecule. Addition of hydrogen atoms to the protein was performed using CHARMm force field. The protein molecules were subjected to stability studies computationally. The primary and secondary structure of proteins was studied[21,22]. Parameters studied were Extinction coefficients, half-life, aliphatic index and Grand average of hydropathicity (GRAVY)[23]. The Extinction coefficients indicate how much light a protein absorbs at a certain wavelength. They are measured in units of M^{-1} cm^{-1}, at 280 nm in water. The half-life is the prediction of time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. The N-terminal of the sequence considered is M (Met). Prot Param is based on the N-end rule which correlates the half-life of a protein to the identity of its N-terminal residue[24]. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It helps to influence the thermo stability of globular proteins. The aliphatic index of a protein is based on the following formula[25] mentioned in Eq. 1.

$$\text{Aliphatic index} = X(\text{Ala}) + a * X(\text{Val}) + b * X(\text{Ile}) + X(\text{Leu}) \quad \text{Eq. 1}$$

Where X (Ala), X (Val), X (Ile), and X (Leu) are mole percent (100 X mole fraction) of alanine, valine, isoleucine, and leucine

The coefficients a and b are the relative volume of valine side chain (a = 2.9) and of Leu/Ile side chains (b = 3.9) to the side chain of alanine. The GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. Detection of the location of the enzyme was performed computationally using WoLFPSORT[26,27]. WoLFPSORT predicts the sub cellular localization sites of proteins based on their amino acid sequences. The ligand structures of naturally occurring flavonoids such as Genistein, Diadzein, Irilone, Orobol, Pseudobaptigenin, Biochanin, Calycosin, Formononetin, Glycitein, Irigenin, 5-O-Methylgenistein, Pratensein, Prunetin, ψ-Tectorigenin, Retusin, Tectorigenin, Aesculetin, ajoene, baicalein, along with the standard drugs like acteoside, nordihydroguaiaretic acid, diethylcarbamazine, Zileuton and azelastine were generated using Marvin Sketch and saved in the PDB format[28]. The various properties of the ligands were studied using Mol inspiration. The ligands and proteins were subjected to energy minimization and finally the docking analysis was performed using the Patch Dock server[29]. Patch Dock is an algorithm for molecular docking. The input for Patch Dock is molecules of any type like proteins, DNA, peptides or drugs. The output is a list of potential complexes sorted by shape complementarily criteria[30]. The corresponding gene ALOX12 of the protein 3D3L has been explored to identify the functional effect of Single Nucleotide Polymorphism (SNPs) retrieved from dbSNP database[31] and the deleterious amino acid substitution prediction by SIFT[32]. 696 SNPs have been recognized in the ALOX12 gene, out of which 73 were in the coding non-synonymous region, 47 in the mRNA UTR region, 36 in the coding synonymous region and remaining 540 SNPs in the intron region of the gene.

RESULTS

The number of amino acids of the protein was found to be 541 with the molecular weight as 61089.2 g. The theoretical pI was found to be 6.35 with the total number of negatively charged residues (Asp + Glu) as 58 and the total number of positively charged residues (Arg + Lys) as 52. The total number of atoms was found to be 8547. The Extinction coefficient was 87735 at Abs 0.1 % (= 1 g/l) 1.436, assuming all pairs of Cys residues form cystines and Ext. coefficient at 86860 with Abs 0.1 % (= 1 g/l) 1.422, assuming all Cys residues are reduced. The estimated half-life was 30 hours (mammalian reticulocytes, *in vitro*), > 20 hours (yeast, *in vivo*) and > 10 hours (*Escherichia coli, in vivo*). The instability index (II) was computed to be 48.66. The Aliphatic index was found to be 86.21. Grand average of hydropathicity (GRAVY) was computed to be -0.259. The target protein secondary structure analysis was performed. The Alpha helix (Hh) was found to be 226 (41.77 %), 3_{10} helix (Gg), Pi helix (Ii), Beta bridge (Bb) and Bend region (Ss) was 0 %. The Extended strand (Ee) was 56 (10.35 %), Beta turn (Tt) was found to be 29 (5.36 %) and Random coil (Cc) 230 as 42.51 %. The LOX enzyme was found in large quantity in cytoplasm. The ligand molecules were subjected to molecular property calculation using Mol inspiration. The properties calculated were total polar surface area (TPSA), number of atoms, molecular weight, number of hydrogen bond acceptors and number of hydrogen bond donors, nviolations and number of rotatable bonds (Table 1). The calculation of drug likeness and bioactivity scores of ligands were studied using the parameters GPCR ligands, ion channel modulators, kinase inhibitors, nuclear receptor ligands, protease inhibitors and other enzyme targets (Table 2). After the preliminary analysis, the protein molecule was subjected to the energy minimization. The minimized protein and ligands were saved in PDB format. The Accelrys Discovery studio 3.5 visualizer[33] was used for visualization of the PDB format of the protein 3D3L as shown in Figure 1. Docking analysis was performed by Patch Dock server and the energy values were computed as in Table 3 and the column chart of the Patch Dock score was represented in Figure 2. In silico analysis was also carried out to determine the damaging Single Nucleotide Polymorphisms (SNPs) which might be responsible for the variations in individuals. 696 SNPs were analyzed using the gene ALOX12 of the corresponding

protein 3D3L. It was found that 73 were in the coding non-synonymous region, 47 in the mRNA UTR region, 36 in the coding synonymous region and remaining 540 SNPs in the intron region of the gene. 8 SNPs were found to be damaging in nature with deleterious amino acid substitutions which was displayed in Table 4 with its score values.

Table 1: Drug likeness calculation of the ligand molecules

Compounds	mi Log P	TPSA	natoms	MW	nON	nOHNH	nviolations	nrotb	volume
Diadzein	2.56	70.67	19	254.24	4	2	0	1	216
Genistein	2.268	90.895	20	270.24	5	3	0	1	224.1
Irilone	2.637	89.135	22	298.25	6	2	0	1	239.9
Orobol	1.778	111.12	21	286.239	6	4	0	1	232.1
Pseudobaptigenin	2.928	68.907	21	282.251	5	1	0	1	231.9
Biochanin	2.804	79.901	21	284.267	5	2	0	2	241.6
Calycosin	2.377	79.901	21	284.267	5	2	0	2	241.6
Formononetin	3.095	59.673	20	268.268	4	1	0	2	233.6
Glycitein	2.377	79.901	21	284.267	5	2	0	2	241.6
Irigenin	2.086	118.6	26	360.318	8	3	0	4	300.7
5-*O*-Methylgenistein	2.544	79.901	21	284.267	5	2	0	2	241.6
Pratensein	2.086	100.13	22	300.266	6	3	0	2	249.6
Prunetin	2.804	79.901	21	284.267	5	2	0	2	241.6
ψ-Tectorigenin	2.283	100.13	22	300.266	6	3	0	2	249.6
Retusin	2.835	79.901	21	284.267	5	2	0	2	241.6
Tectorigenin	2.283	100.13	22	300.266	6	3	0	2	249.6
Zileuton	2.465	66.56	16	236.296	4	3	0	2	203.2
Aesculetin	1.021	70.667	13	178.143	4	2	0	0	144.6
Ajoene	1.802	17.071	13	234.411	1	0	0	8	207.9
Baicalein	2.682	90.895	20	270.24	5	3	0	1	224.1
Diethylcarbamazine	0.881	26.785	14	199.298	4	0	0	2	209.6
Nordihydroguaiaretic acid	3.476	80.912	22	302.37	4	4	0	5	287.9
azelastine	4.817	38.135	27	381.907	4	0	0	3	349.3
acteoside	-0.449	245.29	44	624.592	15	9	3	11	532.5

TPSA: total polar surface area; natoms: number of atoms; MW: molecular weight; nON: number of hydrogen bond acceptors; nOHNH: number of hydrogen bond donors; nrotb: number of rotatable bonds

Table 2: Calculation of Bioactivity scores of ligand molecule

Compounds	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
genistein	-0.22	-0.54	-0.06	0.23	-0.68	0.13
Diadzein	-0.31	-0.064	-0.2	0.04	-0.83	0.02
Irilone	-0.2	-0.67	-0.16	0.07	-0.7	0.02
Orobol	-0.17	-0.5	0.03	0.28	-0.63	0.15
Pseudobaptigenin	-0.21	-0.66	-0.13	0.01	-0.73	0.02
Biochanin	-0.23	-0.59	-0.06	0.23	-0.66	0.07
Calycosin	-0.25	-0.65	-0.08	0.06	-0.78	0.01
Formononetin	-0.3	-0.69	-0.19	0.05	-0.8	-0.02
Glycitein	-0.24	-0.66	-0.08	0.07	-0.77	0.01
Irigenin	-0.21	-0.57	0	0.02	-0.6	0.03
5-*O*-Methylgenistein	-0.21	-0.58	-0.06	0.19	-0.67	0.04
Pratensein	-0.19	-0.56	0.02	0.22	-0.65	0.09
Prunetin	-0.23	-0.59	-0.06	0.23	-0.66	0.07
ψ-Tectorigenin	-0.23	-0.4	-0.06	0.12	-0.65	0.11
Retusin	-0.29	-0.65	-0.1	0.03	-0.75	0.04
Tectorigenin	-0.22	-0.64	-0.01	0.1	-0.72	0.05
Zileuton	0.01	-0.14	-0.28	-0.56	0.15	0.74
aesculetin	-1.05	-0.61	-1.06	-0.81	-1.17	-0.22
ajoene	-0.67	-0.99	-1.32	-0.74	-0.63	0.24
baicalein	-0.12	-0.18	0.19	0.17	-0.35	0.26
diethylcarbamazine	-0.23	-0.13	-0.34	-0.76	-0.44	-0.18
nordihydroguaiaretic acid	0.03	0.11	-0.05	0.14	0.01	0.13
azelastine	0.12	-0.18	0.13	-0.56	-0.18	0.18
acteoside	0	-0.54	-0.31	-0.24	0.06	0

Table 3: Docking analysis result using Patch Dock server

Compounds	Patch dock
genistein	4190
Diadzein	5416
Irilone	4758
Orobol	4468
Pseudobaptigenin	4560
Biochanin	4832
Calycosin	4654
Formononetin	4598
Glycitein	4938
Irigenin	5472
5-*O*-Methylgenistein	5472
Pratensein	4634
Prunetin	4704
ψ-Tectorigenin	5076
Retusin	4614
Tectorigenin	4870
Zileuton	2610
aesculetin	3076
ajoene	4022
bacalein	1196
diethylcarbamazine	3908
nordihydroguaiaretic acid	4966
azelastine	5556
acteoside	7368

Table 4: In silico analysis of Single Nucleotide Polymorphisms (SNPs)

S. No	SNP	Amino acid change	Score	Median
1	rs41359946	T38N	0.04	2.77
2	rs114985038	D134H	0.05	2.78
3	rs140116643	A497V	0.05	2.78
4	rs141346813	R348	-1	-1
5	rs143493293	T364I	0.03	2.77
6	rs147000782	E40K	0.01	2.9
7	rs147158964	R404Q	0.03	2.77
8	rs148602792	K133N	0.01	2.78

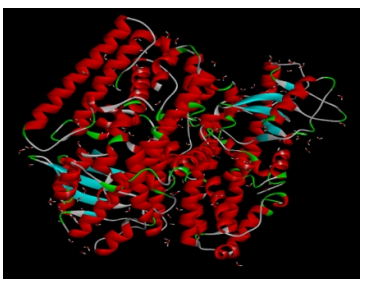

Figure 1: Minimized protein 3D3L prepared for docking analysis

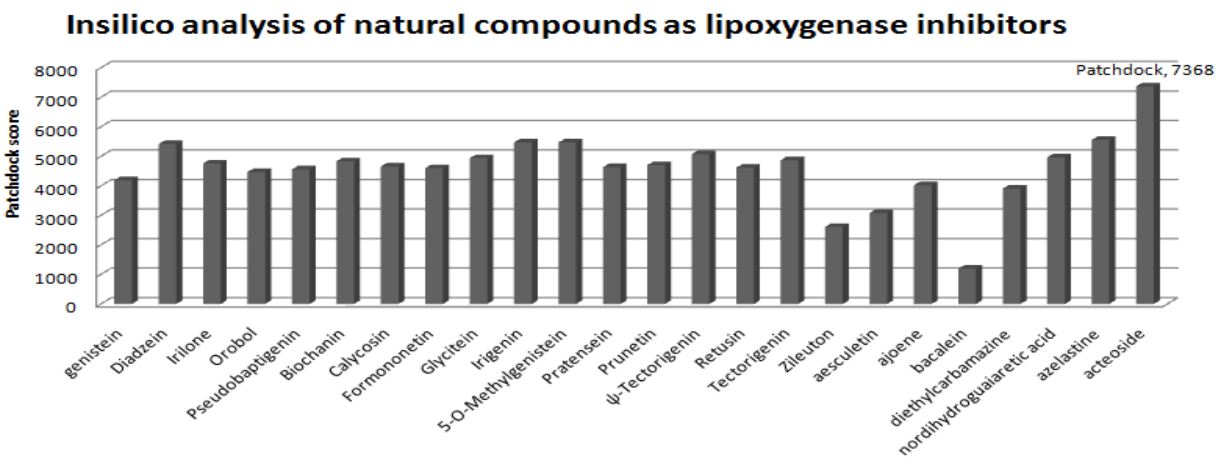

Figure 2: In silico analysis of natural compounds as Lipoxygensae inhibitors

DISCUSSION

The preliminary in silico analysis was performed to check the parameters such as the primary and secondary structure analysis of the protein 3D3L. Primary analysis involved the calculation of Extinction coefficient, estimated half-life, Instability index, Aliphatic index and Grand average of hydropathicity (GRAVY) values. The instability index (II) was computed to be 48.66 and this classifies the protein as being in an unstable state. The Aliphatic index was found to be 86.21 and therefore the protein demonstrates considerable thermo stability. GRAVY value of -0.259 indicates that the protein was hydrophilic in nature. Secondary structure analysis showed considerable stability of the protein. The LOX enzyme was found in large quantity in cytoplasm. The ligand molecules were generated using Marvin Sketch and saved in the PDB format. They were subjected to molecular property calculation using Mol inspiration. The properties calculated were total polar surface area (TPSA), number of atoms, molecular weight, number of hydrogen bond acceptors and number of hydrogen bond donors, nviolations and number of rotatable bonds. Lipinski Rule of 5 is satisfied as the log P values and molecular weight is < = 5 and < = 500 respectively, with the exception of the ligand acteoside. The ligands selected thus shows good permeability across cell membrane. TPSA is below 160 $Å^2$ and *n* violatios is < 0 it denotes compound to bind readily to receptors. Number of rotatable bond is more for Nordihydroguaiaretic acid and acteoside. The calculation of drug likeness and bioactivity scores of ligands were studied using the parameters GPCR ligands, ion channel modulators, kinase inhibitors, nuclear receptor ligands, protease inhibitors and other enzyme targets. The ligands were in agreement with the bioactivity compared to the standard drugs. After the preliminary analysis, the protein molecule was subjected to the energy minimization. The minimized protein and ligands which were saved in the PDB format were viewed with Accelryls Discovery studio 3.5 visualizer. Docking analysis was performed by Patch Dock server and the energy values were computed. Docking plays a central function in the selection of a better lead compound. It was seen that ligands Diadzein, Biochanin, Glycitein, Irigenin, 5-O-Methylgenistein, ψ-Tectorigenin, Tectorigenin were comparable with that of the standard drugs nordihydroguaiaretic acid, azelastine and acteoside. An attempt was made by in silico analysis to identify the damaging Single Nucleotide Polymorphisms (SNPs) which might be responsible for the variations in individuals. 696 SNPs were analyzed using the gene ALOX12 of the corresponding protein 3D3L. It was found that 73 were in the coding non-synonymous region, 47 in the mRNA UTR region, 36 in the coding synonymous region and remaining 540 SNPs were in the intron region of the gene. During the analysis, 8 SNPs have been predicted as damaging and 22 SNPs as tolerant in nature. The damaging SNPs with deleterious amino acid substitutions could be modified to prevent the individual variations.

CONCLUSION

The results of the *in silico* analysis indicate the selection of natural compounds such as flavonoids like Diadzein, Biochanin, Glycitein, Irigenin, 5-O-Methylgenistein, ψ-Tectorigenin and Tectorigenin show significant binding interaction in inhibiting lipooxygenase enzyme which was comparable with that of the standard drugs like nordihydroguaiaretic acid, azelastine and acteoside. The ALOX12 gene and 3D3L protein were investigated in this work by evaluating the influence of functional SNPs through computational analysis. Of a total of 696 SNPs analyzed, it was found that 73 were in the coding non-synonymous region, 47 in the mRNA UTR region, 36 in the coding synonymous region and remaining 540 SNPs were in the intron region of the gene. During the analysis, 8 SNPs have been predicted as damaging and 22 SNPs as tolerant in nature. The SNPs which were damaging could be modified to prevent the individual variations. The lipoxygenase enzyme inhibitor can prove to be effective in case of individuals with cancer. Naturally occurring compounds on further advancement could act as potent lipoxygenase inhibitors. Further investigations are essential for the development of potent lipoxygenase inhibitor chemical entities for the prevention and treatment of cancer.

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