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Evaluation of diflunisal a difluoro-derivative of salicylic acid as an inhibitor of human serum albumin using molecular docking tools

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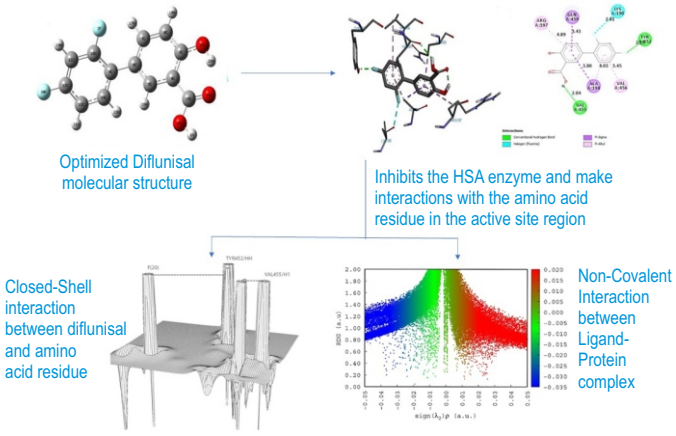
Abstract

Aim: To evaluate the binding interaction of diflunisal at active site of Human Serum Albumin (HSA), an important enzyme responsible for osteoarthritis disease.

Methodology: The gas phase molecule was optimized with B3LYP/6-311G** basis set, while a single point energy calculation was carried out for the molecule lifted from the active site. Bader's theory of atoms in molecules (AIM) was used to determine the electron density and Laplacian of electron density. The protein was assigned with polar hydrogens and Kollman charges. Iterated local search procedure was performed during docking, during this, both protein and ligand were considered as rigid. Among the ten poses, lowest binding energy pose was considered for further ligand protein interaction analysis and QTAIM studies.



Results: A close observation of the results shows that diflunisal has interactions in the binding sites IIA and IIIA of HSA as reported earlier. Two strong classical H-bonding interactions, three hydrophobic contacts with the amino acids VAL456, ALA194 andARG197, two close fluorine interactions, and two Pi-Sigma interactions have been observed. Apart from the H-bond interactions, the stability of HAS-Diflunisal complex was further empathized by five hyrdrophobic interactions framed between hydroxyl benzoic acid ring with ALA194, GLN459 and ARG197amino acid residue and difluorobenzene ring with ALA194 and VAL456.

Interpretation: The results of drug-likeness study showed that diflunisal is highly reactive and less toxic.

Key words: Binding affinity, Diflunisal, Human Serum Albumin, Inflammation, Molecular docking, Pain

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Introduction

Millions of people all over the globe are affected by osteoarthritis, a condition occurring due to the damage of bone cartilage. The major symptom of osteoarthritis include pain in hands, knees, hip, and spine joints on movement. This pain may persist even at rest, especially at night as the disorder worsens. Although complete reversal to normalcy is not possible, pain can be relieved by treatment with certain Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), like ibuprofen and naproxen sodium at prescribed doses. Among the NSAIDs, diflunisal (2',4'-difluoro-4-hydroxy-3-biphenylcarboxylic acid), a difluoro-derivative of salicylic acid is effective in the management of osteoarthritis pain (Snetkov *et al.,* 2021). This well-tolerated drug works by inhibiting the production of prostaglandins, the hormones involved in inflammation and pain (Farid *et al.,* 2021).

The binding profile of Human Serum Albumin (HSA) to a variety of endogenous and exogenous compounds has been immensely well-established in literature (Johansson *et al.*, 2020). Consequently, studying interactions of drug diflunisal with HSA is important in developing new drugs with improved potential (Gokara *et al.,* 2010). The protein contains three structurally similar alpha-helical domains (I–III), each of which is divided into subdomains A and B, which are composed of six and four alpha-helices, respectively (Wang *et al.,* 2020). The versatility and flexibility of binding sites in HSA challenge the clear understanding of binding sites of various drug molecules. Recent docking analyses of HSA–ligand complexes have revealed the majority of NSAID drugs bind to IA, IB, IIA, IIB, IIIA and IIIB subdomains (Amézqueta *et al.*, 2021). Prostaglandin J2 (Yamaguchi *et al.,* 2010) and Bilurubin (Zunszain *et al.,* 2008) makes strong interactions in subdomain IB. The dansylated amino acids (Garcia *et al.,* 2021) and other seventeen drugs (Ghuman *et al.,* 2005) were repored to have high binding affinity in two primary drug-sites of IIA and IIIA subdomain. Also, the Propofol, Thyroxine, Hemin, Ibuprofen (Varshney *et al.,* 2010) and several small molecules (Curry, 2009) reveal the prime binding site in IIIB, IA, IB, and IIB subdomains.

A meticulous search retrieved a few works on the interaction between diflunisal and HSA. Verbeeck *et al.* (1980) studied the interactions of diflunisal with HSA by equilibrium dialysis. Davilas *et al.* (2006) reported *in-vitro* competitive binding of diflunisal and uraemic toxins to serum albumin and human plasma using a potentiometric ion-probe technique. Yang *et al.* (2013) have designed an HSA-based diflunisal prodrug and studied the acetylated diflunisal-HSA interaction through structural, chemical, and biochemical methods. Ghuman *et al.* (2005) investigated the interaction between diflunisal and HSA by crystallographic analysis. Nevertheless, theoretical studies on diflunisal-HSA interaction have not been carried out to date. The present study is an attempt to understand the interactions of Diflunisal, a difluoro-derivative of salicylic acid with Human Serum Albumin using charge density analysis, reactivity descriptors, and docking analysis which will throw light into the pharmaceutical activity of drug.

Materials and Methods

Optimization of compounds: Gaussian09 program package was used to carry out the density functional calculations of the gas phase molecule and the molecule lifted from the active site. The gas phase molecule was optimized with B3LYP/6-311G** basis set (Frisch *et al.,* 2009; Smith andSutcliffe,1996), while a single point energy calculation was carried out for the molecule lifted from the active site. The optimizations converged at the threshold values; Maximum force: 0.000011 au, 0.000048 au, RMS force: 0.000003 au, 0.000074 au. Bader's theory of atoms in molecules (AIM) was used to determine the electron density and Laplacian of electron density (Bader,1990) using *ext94b* routine implemented in AIMPAC software (Keith, 2009). The deformation densities were plotted by *wfn2plots* and XD (Koritsanszky *et al.,* 2007). The Laplacian of electron density was drawn using AIMPAC. The structure of diflunisal was prepared using the PDB format.

Molecular docking: The 3-D structure of HSA was acquired from the Brookhaven Protein Data Bank (2BXE) (Ghuman *et al.,* 2005). The graphical user interface program AutoDock tools (Huey *et al.,* 2012) was used for protein and ligand preparation. The protein was assigned with polar hydrogens and Kollman charges. The grid box of size 80 x 80 x 80 xyz points was prepared with AutoGrid module. The docking of ligand with protein was performed by AutoDock along with the grid box properties. Iterated local search procedure was performed during docking, during this, both protein and ligand were considered as rigid. Among the ten poses, the lowest binding energy pose was considered for further ligand protein interaction analysis and QTAIM studies. The diflunisal-HSA complex was viewed using Biovia Discovery Studio 2017R2 . (Biovia, 2017)

Results and Discussion

To get needful insights such as binding site and binding affinity, the molecular docking study was carried out and suitable conformation of the diflunisal molecule was optimized using DFT at B3LYP/6-311G (d,p) level of theory. Among the 10 different conformers of diflunisal generated by Auto Dock, (Table. 1) conformer 1, with the least docking score of -4.52 kcal/mol and an affinity constant of K = 0.483×10^{5}mol l^{-1} was chosen to be the best suitable ligand for binding with HSA (Fig. 1). The types of interaction and their corresponding distances is listed in Table. 2 A close observation of the results showed that diflunisal interacted with in the binding sites IIA and IIIA of HSA as reported by (Amézqueta *et al.,* 2021).

Two strong classical H-bonding interactions (O···VAL455/H1 and F(20)···TYR452/HH), three hydrophobic contacts with the amino acids VAL456, ALA194 andARG197, two close fluorine interactions, and two Pi-Sigma interactions (aromatic ring···GLN459/CG and aromatic ring···ALA194/CB) have been observed in the present study (Table. 2). Fig. 2 (a, b)

Table. 1: The lowest binding energies (kcal mol^{-1}) and inhibition constant (μm) of 10 different conformers of compounds.

Confirmation	lowest binding energies (kcal mol^{-1})	K (μm)
1	-4.52	0.48
2	-4.07	1.03
3	-3.98	1.20
4	-3.93	1.32
5	-3.74	1.82
6	-3.74	1.82
7	-3.72	1.86
8	-3.71	1.90
9	-3.62	2.22
10	-3.53	2.57

Table. 3: Bond topology of diflunisal molecule.

	Gas phase		Active site	
Bonds	$\rho_{bcp}(r)$	$\nabla^2(\rho)$	$\rho_{bcp}(r)$	$\nabla^2(\rho)$
C(4)-C(5)	2.038	-19.731	2.039	-19.753
C(15)-O(24)	2.045	-12.113	2.045	-12.151
C(3)-C(4)	2.027	-19.576	2.024	-19.513
C(5)-H(16)	1.928	-23.946	1.846	-22.029
C(6)-C(9)	1.768	-15.523	1.766	-15.494
C(6)-C(5)	2.102	-20.836	2.103	-20.846
C(4)-C(15)	1.87	-17.404	1.87	-17.401
H(17)-F(2)	0.072	1.097	0.073	1.101
C(8)-C(3)	2.089	-21.129	2.088	-21.096
C(7)-C(6)	2.025	-19.628	2.026	-19.647
H(17)-C(7)	1.929	-24.016	1.846	-22.089
O(23)-C(3)	2.06	-10.141	2.064	-10.147
C(8)-C(7)	2.131	-21.505	2.136	-21.614
F(2)-C(10)	1.696	3.201	1.697	3.209
C(9)-C(10)	2.088	-20.739	2.087	-20.73
C(10)-C(11)	2.136	-21.716	2.136	-21.708
C(11)-C(12)	2.138	-21.811	2.137	-21.784
C(11)-H(19)	1.898	-23.292	1.812	-21.338
C(12)-F(20)	1.715	3.288	1.715	3.234
C(9)-C(14)	2.042	-19.721	2.045	-19.772
C(13)-C(12)	2.141	-21.973	2.142	-21.972
C(14)-C(13)	2.086	-20.627	2.085	-20.598
O(25)-C(15)	2.719	-7.516	2.719	-7.531
H(18)-C(8)	1.905	-23.396	1.827	-21.592
C(13)-H(21)	1.902	-23.349	1.82	-21.48
H(22)-C(14)	1.914	-23.612	1.836	-21.801
H(1)-O(24)	2.429	-60.639	2.43	-60.673
O(23)-H(26)	2.317	-58.191	2.315	-58.123
H(26)…O(25) [IntramolecularInteraction]	0.285	3.215	0.285	3.213
O(24)H1···VAL455/O	-	-	1.6	2.41
F(20)···TYR452/OH	-	-	0.72	1.08

Table. 2: Interactionof diflunisal with HSA

Interacting species	Type	Distance (Å)
O(24)H1···VAL455/O	Conventional H-bond	2.04
F(20)···TYR452/OH	Conventional H-bond	2.15
aromatic ring···VAL456/CG	Pi-Alkyl	5.45
aromatic ring···ALA194/CB/	Pi-Alkyl	4.03
aromatic ring···ARG197/CG/	Pi-Alkyl	4.89
aromatic ring···GLN459/CG	Pi-Sigma	3.43
aromatic ring···ALA194/CB	Pi-Sigma	3.80
F(20)···TYR452/HH/	Halogen(Fluorine)	2.15
F(2)···LYS190/O	Halogen(Fluorine)	2.81

Table. 4: Lipinski rule parameters of diflunisal molecule

Lipinski rule of 5 parameters	Values	Standard values
Log P	3.90	5
Molecular Weight	250.20g mol^{-1}	500g mol^{-1}
HBA	3	10
HBD	2	5
No-of rotatable bonds	2	10

Table. 5: Reactivity properties of diflunisal molecule

Molecular descriptor	Energy (a.u.)
Electron affinity $A=[-E_{LUMO}]$	0.067
Ionization potential I=[-EHOMO]	0.231
Global hardness $\eta=(I-A)/2$	0.082
softness $S=1/\eta$	12.203
Electronegativity $\chi=(I+A)/2$	0.149
Electrophilicity index $\omega=\mu^2/2\eta$	0.135

shows the ball and stick model of optimized structure of diflunisal molecule in the gas phase and the one lifted from the active site of HSA. The structure of diflunisal molecule in the gas phase appears to be linear, but transformed substantially in the active site. Comparison of geometrical parameters and confirmation of two different forms would help in understanding the drug-enzyme interaction. The average C-C bond distance of aromatic rings in the gas phase and active site was 1.396 Å and 1.388 Å, respectively. The bond length (gas phase/active site) of C=O was 1.225/1.213 Å. The length of the C−O (carboxyl) bond of diflunisal molecule was 1.346/1.319 Å and that of the C-O (phenyl) bond was 1.339/1.324Å. In general, the majority of bonds in the gas phase molecule were longer than their counterparts in the other form lifted from the active site due to which the molecule can form interactions with the amino acids present in the active site. However, the C-F bonds had a bond length of 1.352/1.324 Å for F(2)−C(10) and 1.348 /1.337 Å for F(20)−C(12).The C−H bond

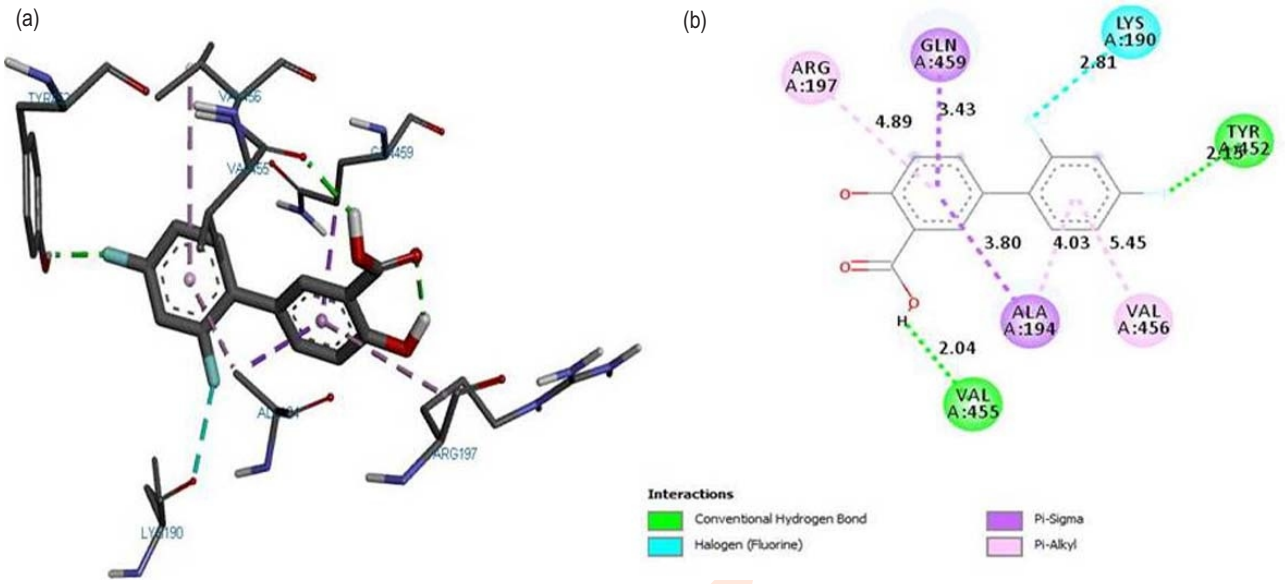

Fig .1: (a) HSA- diflunisal complex (b) Intermolecular interactions between diflunisal and HSA obtained by Biovia Discovery Studio 2017R2.

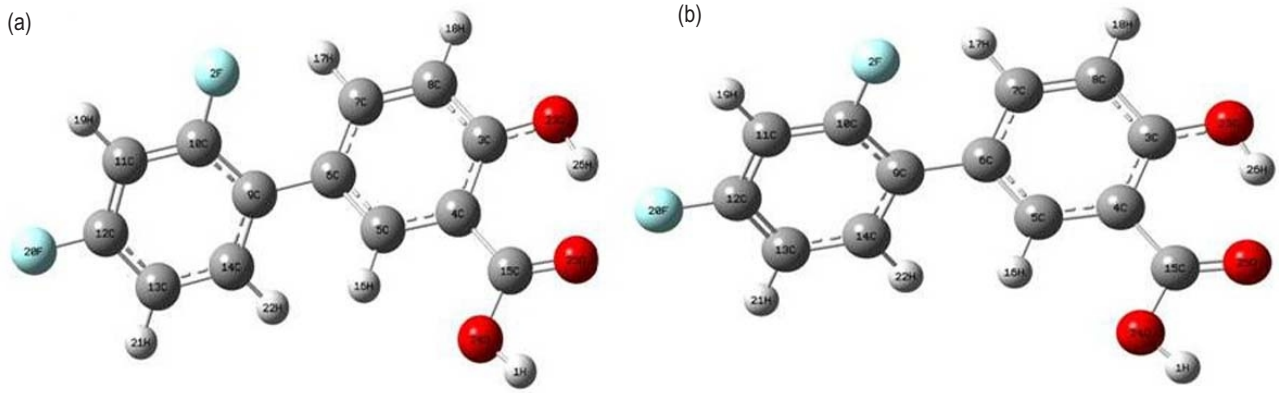

Fig . 2: Molecular structure of diflunisal in gas phase (A) and active site (B).

distances did not vary much between the two forms (Williams *et al.,* 1973). Significant changes in bond angles were observed. In both the phases, the aromatic C−C−C bond angles in both the rings slightly deviated from the ideal angle (120°) for sp2 hybridized carbon indicating slight deformation. Particularly, a slight decrement was noted in the C−C−O angle (124.39/120.19°). A remarkable difference in the majority of torsion angles between the two forms suggest that diflunisal molecule has conformational flexibility, which leads to protein-ligand interactions.

Different types of interactions between the diflunisal and HSA can be characterized from the bond topological properties of ligand in the gas phase and active site. Bader's quantum theory of atoms in molecules (QTAIM) was employed for charge density analysis. Data about electron density and Laplacian of electron density [$\nabla^2\rho$®] at the bond critical points (*bcp*) of both the forms of ligand are given in Table. 3 The electron density values of both forms do not vary much, except for the C-H bonds because of the hydrophobic interactions with the amino acids. Fig. 3 shows deformation of electron density plots of two rings present in diflunisal molecule lifted from the active site.

The maps help to visualize the lone pair positions of atoms in the molecule. The $\rho_{bcp}(r)$ values of aromatic C-C bond ranged from 2.024eA-3 to 2.142 eA^{-3}. The $\rho_{bcp}(r)$ of carboxyl C=O bond, C-O bond (carboxyl) and C-O bond (phenyl) were found to be 2.719e$Å^{-3}$ (C15-O25), 2.045e$Å^{-3}$ (C15-O24) and 2.064e$Å^{-3}$ (C3-O23), respectively. The electron density of C=O bond was intact

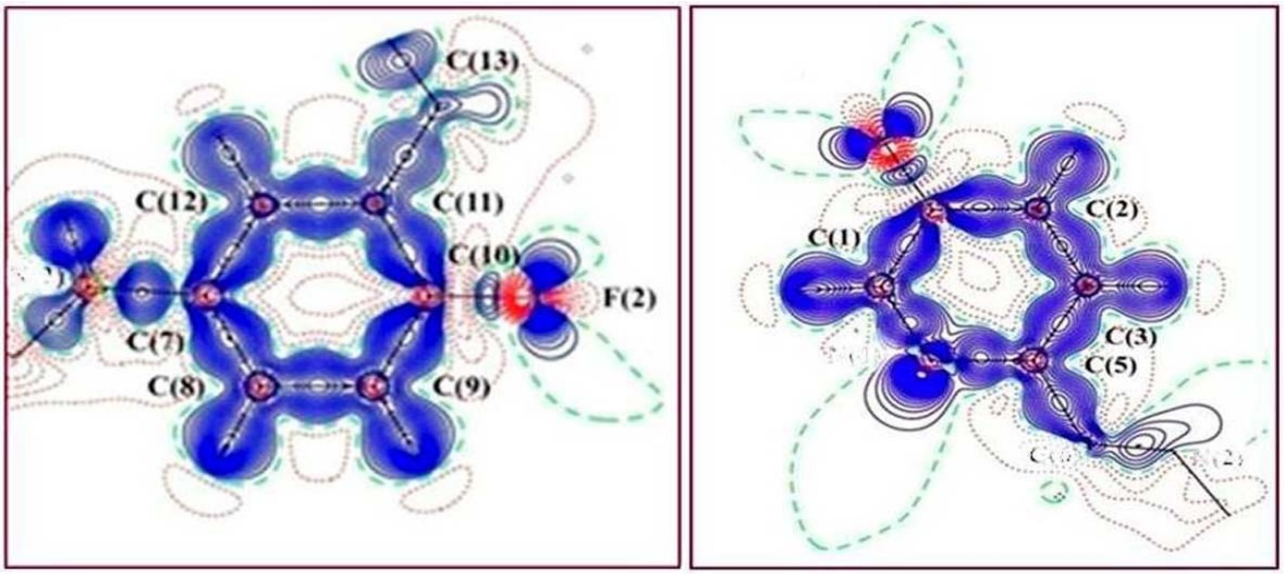

Fig . 3: Deformation electron density map of diflunisal molecule lifted from the active site of human serum albumin receptor.

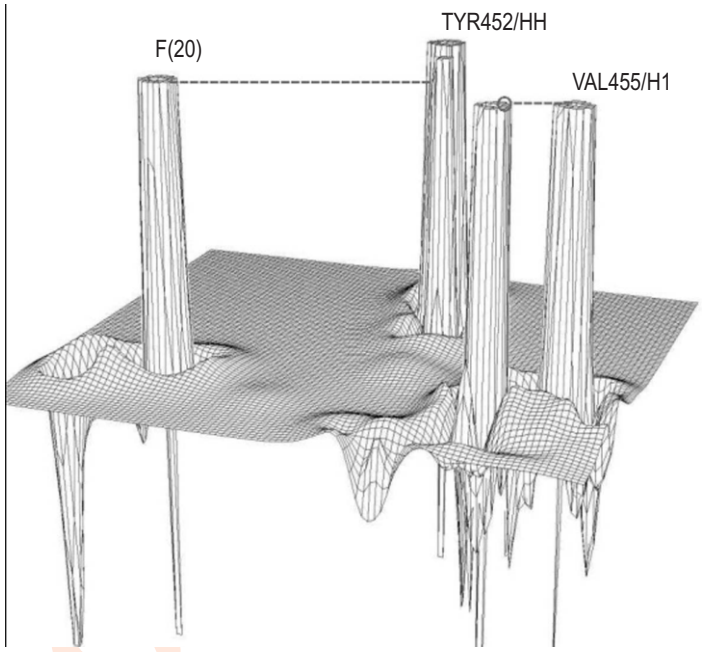

Fig . 4: Relief map of negative Laplacian of electron density, $\nabla^2\rho(r)$ (range −200 to +200 e Å^{-5}) for the O(24)H1···VAL455/OandF(20)···TYR452/OH hydrogen bonded dimer of diflunisalmolecule.

after it was placed at the active site. Similarly, in both the forms, the electron density of C-F bonds did not vary significantly. Concentration or depletion of charges at *bcp* can be studied by analyzing the Laplacian of electron density$\nabla^2\rho(r)$]. The $\nabla^2\rho(r)$ values at the *bcp* of both the forms conform well to each other, except at C-H bonds, which attribute the hydrophobic contacts between ligand and protein. The $\nabla^2\rho(r)$ values of aromatic C–C bonds ranged from -19.513 to -21.972 eÅ^{-5}. The $\nabla^2\rho(r)$ of carboxyl C=O bond (C15-O25), C-O bonds (C3-O23, phenyl and C15-O24, carboxyl) were found to be 7.531, -10.147, and -12.151 eÅ^{-5}, respectively. The closed-shell interaction (Chopra *et al.,* 2006) was noticed for C-F bonds (F(2)-C(10): 3.201/3.209 eÅ^{-5}; F(20)-

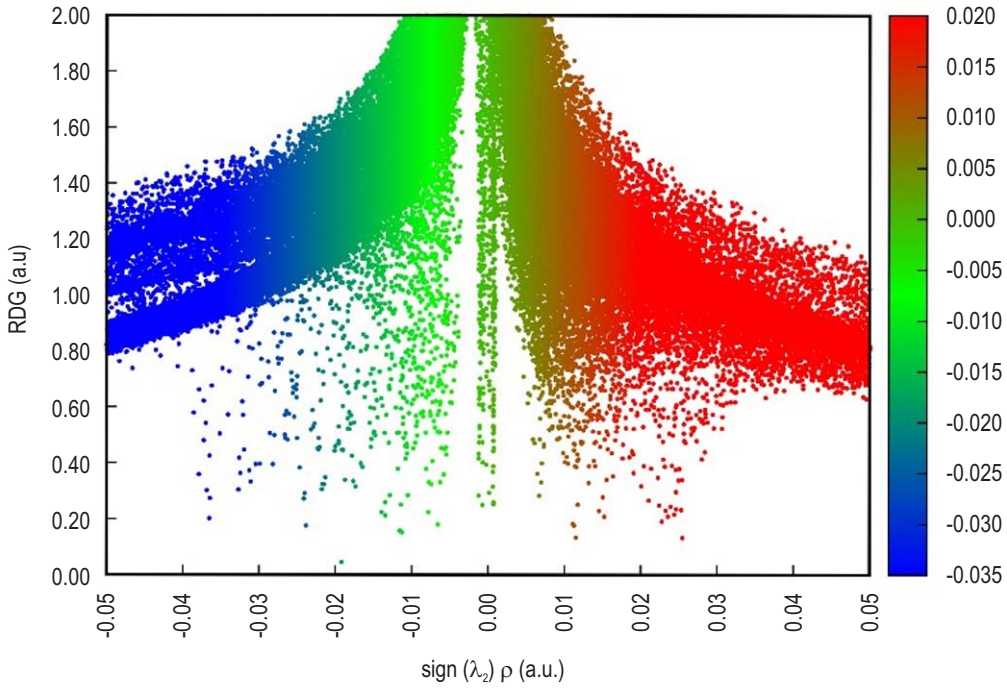

Fig . 5: RDG scatter plot showing weak and strong interactions between diflunisal and HSA enzyme. The surfaces are colored on a blue-green-red scale according to values of sign(λ_2/ρ), ranging from -0.06 to +0.05 au. Blue indicates strong attractive interactions, and red indicates strong nonbonded overlap.

C(12):3.288/3.234 eÅ^{-5}), as their values of Laplacian of electron density is positive, and the results are in agreement with the reported values (Hoffmann *et al.,* 2021). This electron delocalization in O-H bonding region attributed to the limited flexibility of radial functions used in the multipole model as well as the basis set and the electron correlation effect in theory. Similar positive $\nabla^2\rho(r)$ values [3.215/3.213 eÅ^{-5}] was calculated for intramolecular bond O (25)…H (26) bond. The above data agree well with the previously reported experimental and theoretical data (Ahmed *et al.,* 2013; Lai *et al.,* 2016). Moreover, the $\rho(r)$ and $\nabla^2\rho(r)$ of the new bonds formed between diflunisal and amino acids of HSA have been analyzed (Fig. 4). The $\rho_{bcp}(r)$ of the O(24) H1···VAL455/O bond was 1.60 eÅ^{-3} and its $\nabla^2\rho(r)$ was 2.41 eÅ^{-5}. Similarly, the $\rho_{bcp}(r)$ of F(20)···TYR452/OH bond was 0.72eÅ^{-3} and the $\nabla^2\rho(r)$ was 1.08 eÅ^{-5}. The values of $\rho(r)$ and $\nabla^2\rho(r)$ were found to decrease for the longer donor-acceptor distances and these values were compared well with the literature values (Kalaiarasi *et al.,* 2016; Aray *et al.,* 2019).

Apart from the H-bond interactions, the stability of HSA-Diflunisal complex was further empathized by five hyrdrophobic interactions framed between hydroxyl benzoic acid ring with ALA194, GLN459 and ARG197amino acid residue and difluorobenzene ring with ALA194 and VAL456. The existence of similar interactions was found in the earlier reports (Ghuman *et al.,* 2005) and the values were found to be comparable. The interaction of Diflunisal with HSA was well established from Non-covalent interaction energy (NCI). The NCI regions were identified from the reduced density gradient (RDG) (Boto *et al.,* 2016). The Multiwfn software (Lu, 2014) was used to obtain the RDG scatter plot as shown in Fig. 5. The hydrogen bonding, van der Waals and steric effect interaction in HSA-Diflunisal complex were represented by blue, green and red regions in the RDG scatter plot. The atomic charges of both the forms of diflunisal molecule were calculated by Mulliken Population Analysis (MPA) (Carbó-Dorca and Bultinck, 2004), Natural Population Analysis (NPA) (Reed *et al.,* 1985), and atoms in molecules (AIM) analysis. Due to the active site effect, charge distribution of gas phase molecule altered in the active site of HSA. In all the three models considered in the present study, all other carbons, except C(3), C(15) bonded to oxygen atoms and C(10), C(12) bonded to fluorine atoms carry a positive charge. Similarly, all the oxygen atoms of diflunisal molecule were found to bear a high negative charge. The dipole moment values of both the forms were calculated from the quantum chemical methods.

The dipole moment of diflunisal molecule in the gas phase was 2.15 D whereas the dipole moment of a molecule at active was 2.01 D. The effect of dipole moment variation in both the phases was well established from the calculation of charge balance (v). Fig. 6 represents the molecular electrostatic potential of Diflunisal molecules in active site environment. Also, the positive/ negative variance of ESP were calculated for both the

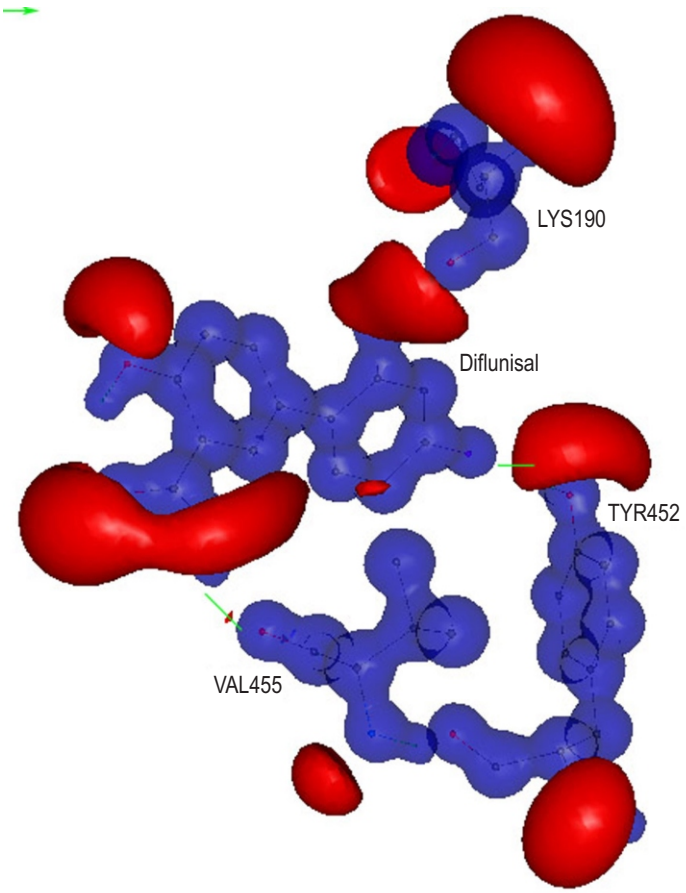

Fig. 6: Isosurface representation of ESP of diflunisal molecule active site of HSA enzyme. Blue, positive potential (+0.3 e Å^{-1}); red, negative potential (-0.15 e Å^{-1}).

gas and active site phases, and the corresponding values were 90.11Kcal-mol^{-1}/35. 41Kcal-mol^{-1} and 173.55 Kcal-mol^{-1}/21.57 Kcal-mol^{-1} respectively. These values reveals that the ESP distribution of molecule fluctuates remarkably in active site as their v value is calculated as 0.098 whereas the v value in gas phase was 0.202. Some molecular descriptors such as log P (partition coefficient), molecular weight, hydrogen bond acceptors, and donor count in a molecule are correlated with some of the molecular properties like membrane permeability and bioavailability. Lipinski's rule of 5 parameters (Chattaraj *et al.,* 2003) has been listed in (Table. 4).

The molecule has Log P value 3.90 which shows its good absorption, distribution and potency. Its molecular weight (250.2g mol^{-1}) is less than the reference value which shows its overall potency to qualify as a drug. The less number of HBA (hydrogen bond acceptor), HBD (hydrogen bond donor) and rotatable bonds than the standard values show the measure of molecular flexibility. Thus, the selected molecule fulfills the conditions and establishes itself as a potent drug and physiologically active. To get a clear picture of the nature and reactivity of the bio-molecules, the global reactivity descriptors such as electron affinity, ionization potential, chemical hardness, softness, electronegativity, and electrophilicity serve as the most important tools. Small ionization energy illustrates high reactivity and high ionization energy shows high stability and chemical inertness of the atoms and molecules (LoPachin *et al.,* 2012).

According to HSAB theory (Chattaraj *et al.,* 2003) the chemical hardness and softness are the key factors used to estimate the reactivity of the molecules. The calculated values of the descriptors of diflunisal molecule are presented in Table. 5. As the softness value of the molecule was high (12.203a.u.), the molecule is high reactive. The less toxicity of the molecule can be explained by low electrophilicity index value (0.135a.u.). In the viewpoints of reactivity descriptors, the molecule diflunisal has more polarizability, less toxicity, low kinetic stability, and high chemical reactivity. The present theoretical study shows that diflunisal molecule is capable of forming highly stable intermolecular interactions such as O(24)H1···VAL455/O, F(20)···TYR452/ OH with HSA, substantiated by topological and dipole moment studies. The flexible conformation of diflunisal

molecule in the active site of HSA proves the drug to be an inhibitor of HSA to ameliorate the conditions caused by osteoarthritis. The positive Laplacian of electron density values evidences the closed-shell interaction of C−F bonds. Further, the results of the drug-likeness study show that diflunisal is highly reactive and less toxic.

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Add-on Information

Authors' contribution: S.M. Shankar: Conceptualization, writing original draft, data collection; **A.D. Stephen:** Conceptualization and methodology, analysis and interpretation of results, Data interpretation, writing original draft, review and editing; **R.N. Devi:** Data interpretation, reviewing and editing; **S. Maruthamuthu:** Review and editing; **A.M. Musthafa, M. Pannipara, A.G.Al-Sehemi:** Addidtional studies carried out at revision stage.

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