Selectivity Profiling of HIV Protease Inhibitors against Glut-4

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TH4 page

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Table of Content

List of Abbreviations

Sr. No:-	*Abbreviations*	
1.	Ac	Acetyl
2.	Arg	Arginine
3.	Asp	Aspartic acid
4.	CD4	Receptor on the surface of cells within the immune system
5.	CRF's	Circulating Recombinant Forms
6.	DNA	Deoxyribonucleic acid
7.	ED_{50}	50% inhibitory concentration in cell-assay
8.	EIAV	Equine infectious anemia virus
9.	FIV	Feline Immunodeficiency virus
10.	FDA	US Food and Drug administration
11.	Gag	Polyproteins containing structural proteins
12.	Gag-gene	Genome for Gag polyproteins
13.	Gln	Glutamine
14.	Gly	Glycine
15.	GLUT	Glucose transporter
16.	gp120,41	Glycoprotein 41 and 120
17.	HAART	Highly active antiretroviral therapy
18.	HMIT	H+/myo-inositol symporter
19.	ATV	Antiretroviral therapy
20.	IC_{50}	Concentration of inhibitor resulting in 50% inhibition
21.	Ile	Isoleucine
22.	IN	Integrase
23.	K_i	Inhibitory constant
24.	MVV	Maximum voluntary ventilation
25.	MFS	Major facilitative superfamily
26.	NNRTI	Non-nucleoside reverse transcriptase
27.	NRTI	Nucleoside reverse transcriptase
28.	PDB	Protein Data Bank
29.	P24	Capsid protein
30.	P17	Matrix protein
31.	Phe	Phenylalanine
32.	Pol	Polyproteins containing functional enzymes
33.	PI	Protease Inhibitors
34.	Pol-gene	Genome for polyproteins
35.	PR	HIV protease
36.	RT	Reverse transcriptase
37.	RNA	Ribonucleic acid
38.	SIV	Simian Immunodeficiency Virus
39.	Thr	Threonine
40.	Val	Valine

List of Figures

List of Tables

Abstract

Human Immunodeficiency Virus (HIV) protease is retro pepsin, essential in the life cycle of the HIV virus causing Acquired Immuno Deficiency Syndrome (AIDS). HIV protease plays an essential role in the maturation of infectious virions. HIV-1 protease inhibitors cause inhibition of this activity however, the use of these HIV protease inhibitors have been reported to cause hyperlipidemia, lipodystrophy, liver toxicities and hyperinsulinemia. The outcome of hyperinsulinemia is Type 2 Diabetes. Various studies revealed the increase risk of Diabetes Mellitus in HIV protease therapy patients. One of the major reasons of this outcome is the overlap in substrates/inhibitors profile of HIV protease and various glucose transporters. GLUT4 has been known as the major glucose transporter in cells therefore, selectivity of HIV-protease inhibitors against GLUT-4 is of prime importance while designing new drug candidates for HIV treatment. In this project various structure based *in silico* strategies have been utilized to design novel, potent and selective inhibitors of HIV proteases. Our results demonstrates the importance of Leu B23, Asp A25, Val A32, Asp B25, Ile A50, Asp B25, Gly A27 and Asp A29 for the selective interaction of HIV-protease-inhibitors . Final and selective pharmacophoric pattern of HIV-protease inhibitors consist of one hydrogen bond donor, one hydrogen bond acceptor, one hydrophobic and one aromatic feature. The present study could pave the way towards design of highly selective, potent inhibitors of HIV-protease with minimum risk of diabetes.

Chapter 1

Introduction

AIDS has been becoming one of the most prevailing diseases in world now a day but in Pakistan it is not yet a dominant epidemic [1]. However, it may become a major health issue in future. The treatment strategies available now-a-days uses combination therapy called Highly Active Antiretroviral therapy (HAART).

In the early 1980's the causative agent of Acquired Immuno Deficiency Virus (AIDS) was found to be Human Immunodeficiency Virus (HIV-1). At the onset of disease no drug therapy was available to act against it. An anticancer drug Azidothymidine (AZT) was the first drug that showed some counter effect against the virus [2]. However, after having the insights of structure and lifecycle of virus abundant development of other drugs targeting the viral proteins occurred. Among these viral proteins Proteases (PR) gained importance because inactivation of HIV-1 PR by either mutation or chemical inhibition leads to the production of immature and noninfectious viruses [2, 3].

HIV-1 protease inhibitors have been reported to be an important cause of Hyperinsulinemia [4]. Hyperinsulinemia is a condition when there is excess level of insulin in circulating blood stream. It is mostly associated with metabolic defects caused due to disturbances in glucose homeostasis [5]. In 1999 Carr *et al*, reported that the protease inhibitors used as anti-retro viral agents causes risk of cardiovascular disease and diabetic complications. They observed that 98% patients were at cardiovascular risk 16% developed glucose intolerance and about 7% developed diabetes mellitus [6].

Moreover, Dufer *et al*, reported that HIV-protease inhibitors such as Ritonavir, Nelfinavir and Indinavir effect directly on pancreatic β-cells and decrease hormone secretion

by interacting with β-cell [Ca^{+2}] and other ion channels. Ca^{+2} ions are increased due to increased influx of Ca^{+2} during glucose stimulated insulin secretion [7].

HIV belongs to the genus Lentivirus. As the name indicates "*Lenti*" is Latin word meaning "*slow"* and it has been named so because its incubation time period is very long. This genus has been further classified into five species [8] as shown in figure 1.

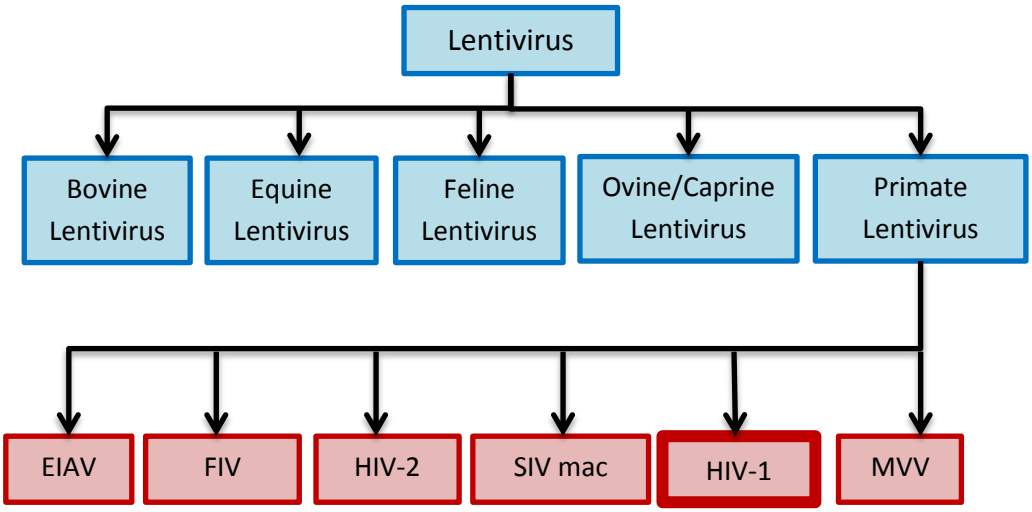

Figure 1:- Classification of Primate Lentivirus

Furthermore, HIV is divided into two species HIV-1 and HIV-2. HIV-2 is less easily transmitted and duration of infection and illness is longer. Whereas, HIV-1 is predominant virus involve in AIDS however, HIV-2 is concentrated in West Africa and is rarely found elsewhere. The HIV-1 has been classified into four groups based on strains and within group M (major) nine genetically distinct subtypes or clades have been identified as shown in figure 2 [9, 10].

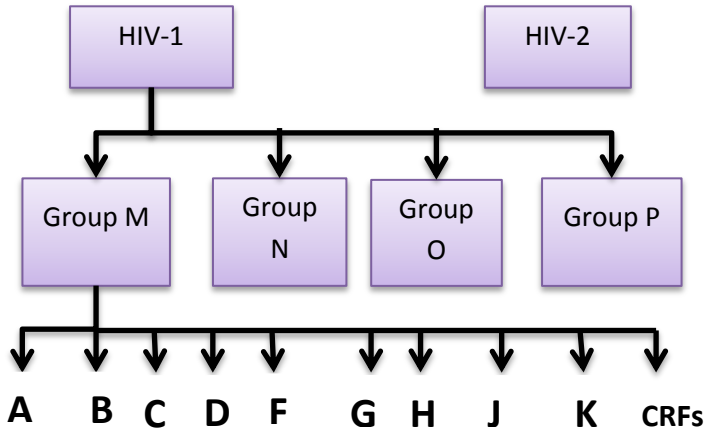

Figure 2:- HIV-1 subtypes

M (major), O (outlier), N (new), P (Cameroonian woman), CRF's (circulating recombinant forms)

1.1 HIV-1 Proteases:-

The major cause for AIDS is HIV-1 virus as it is predominant as compared to HIV-2 virus [11]. HIV structure consists of a viral capsid that contains:-

- A lipid bilayer
- A surface glycoprotein (gp120)
- A trans membrane protein (gp41)
- A shell of matrix protein (p17)
- A capsid protein (p24)
- A nucleocapsid protein (p7)
- Three viral enzymes protease (PR) , reverse transcriptase (RT) and integrase (IN) [3].

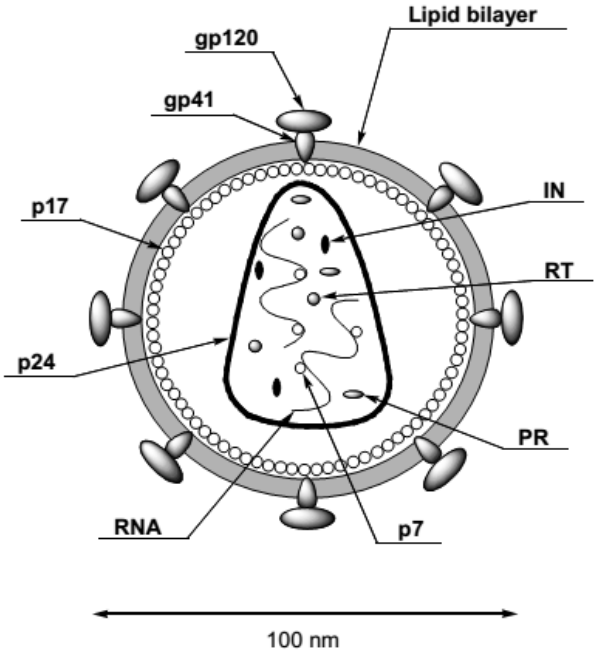

Figure 3:- Schematic diagram of the mature HIV-1 taken from Alterman *et al,* **[3]**

Viral proteases are responsible for the maturation of poly protein precursors into individual functional proteins [3]. Navia and coworker in 1989 successfully retrieved crystal structure of HIV protease. HIV protease consist of 99 amino acids that function as a homodimer[12]. Each monomer consists of extended ß-sheet region known as flap and this site plays an important role in substrate binding. The stability of dimeric structure is due to these β-sheets. Proteases cause cleavage of translated viral gag-pol polyproteins into mature proteins that can infect new cells [13]. Inhibition of HIV-1 Protease by using small molecules can aid value in the treatment of AIDS.

Figure 4:- HIV protease structure (PDB : 3TLH) , Taken from Navia *et al,* **[13]**

The lifecycle of HIV-1 consist of six steps:-

1) Adsorption and fusion of virion with the help of a CD4+ T-lymphocyte into the host cell
2) Reverse transcription where reverse transcriptase catalyzes the conversion of one copy of the genomic viral RNA into double stranded viral DNA
3) Integration in which integrase catalyzes the integration of virus into host chromosome to form a provirus DNA
4) Viral gene expression through making copies of HIV-1 genomic material and shorter strands of messenger RNA that in end form long chains of HIV-1 precursor proteins
5) Proteases cleave long chain poly peptide precursor proteins into mature virions
6) In budding each virion is separated and matured virion is ready to infect other cells [3].

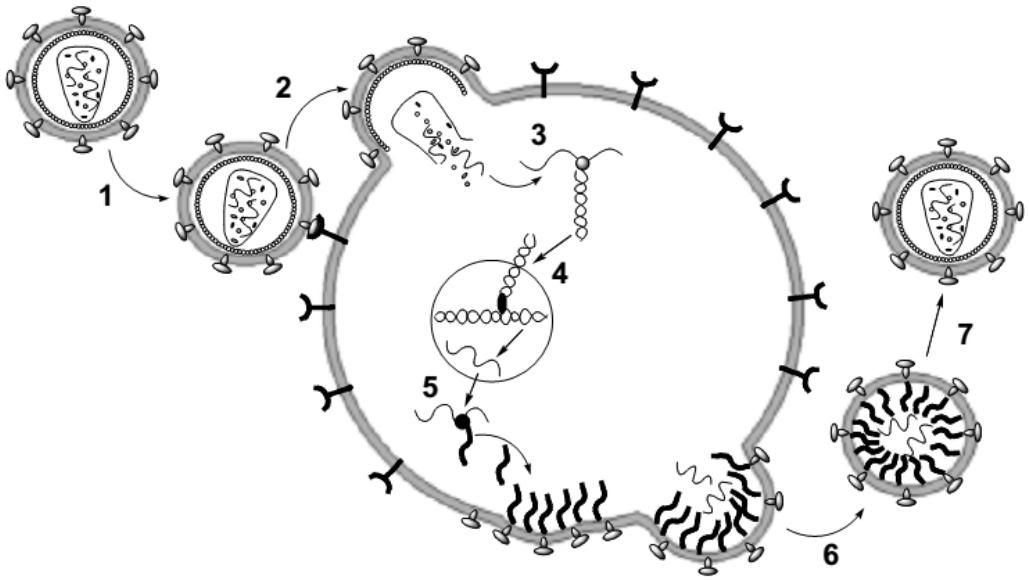

Figure 5:- Schematic drawing of the replicative cycle:

1. Adsorption 2.Attachment and fusion 3. Reverse transcription 4. Integration 5.Translation 6. Viral budding 7. Maturation via protease activities.

Taken from Alterman *et al*, **[3]**

Proteases play an important role in maturation of viral components. Core proteins are produced as a result of long polypeptides, which must be cuted into smaller fragments for being functional. The protease inhibitors block the site where protein cleavage occurs and mature core proteins are thus not produced. This results in immature virion that are further unable to infect other cells. There is basically an amino acid change that reduces the binding affinity of protease enzyme at active site [3].

Five classes of drugs has been identified that interrupt HIV life cycle. Enfuvirtide and Maraviroc that inhibit the fusion of viral envelop with host cell membrane. Nucleoside and Non-Nucleoside reverse transcriptase inhibitors. Nucleoside transcriptase inhibitors inhibit reverse transcription process. Integrase inhibitors for example Raltegravir that inhibit the integration of viral RNA into host DNA. Then the most important protease antagonists

(Saquinavir, Ritonavir etc.) that inhibit the maturation of newly formed virions to infect other healthy host cells [14].

Among all antiretroviral therapies, protease inhibitors have been considered to be more potent because they restrict the production of further mature virions thus inhibiting the viral load and preventing replication process. PI's have also been essential because they protect the host cell proteins from cleavage caused due to viral proteases. These include Saquinavir, Ritonavir, Indinavir, Nelfinavir, Amprenavir, Lopinavir, Fosamprenavir, Atanavir, Tipranavir and Darunavir[3].

The protease inhibitor therapy cause major metabolic side effects that mainly include peripheral lipodystrophy, hyperlipidemia, insulin resistance and type 2 diabetes. Of all these diabetes have been identified as a major side effect produced as a result of their use [4]. Many recent studies have identified that the cause of this insulin resistance is mainly due to the inhibition of glucose transporter (GLUT-4) that is essential for translocation of glucose in blood [15].

Therefore, we need to develop selective inhibitors of HIV proteases that do not interact with human proteases and thus, the side effect produced due to their use could be avoided.

1.2 Glucose transporter:-

Glucose transporters are the members of Major Facilitator Superfamily (MFS) that are basically the sugar transporters [16]. As the name indicates the GLUT transporters plays an important role in the transportation of all hexoses, monosaccharide's, polyols, urate and other sugar molecules across the membrane structures. These GLUT transporters have been classified into 14 GLUT proteins [17].

All Glucose transporter proteins consist of merely 500 amino acid residues and have been categorized on the basis of sequence similarity among them. Class 1 consists of Glucose transporters 1-4 and 14; Class 2 consists of transporter protein 5, 7, 9 and 11; and Class 3 comprises of transporter 6, 8, 10, 12 and HMIT [16].

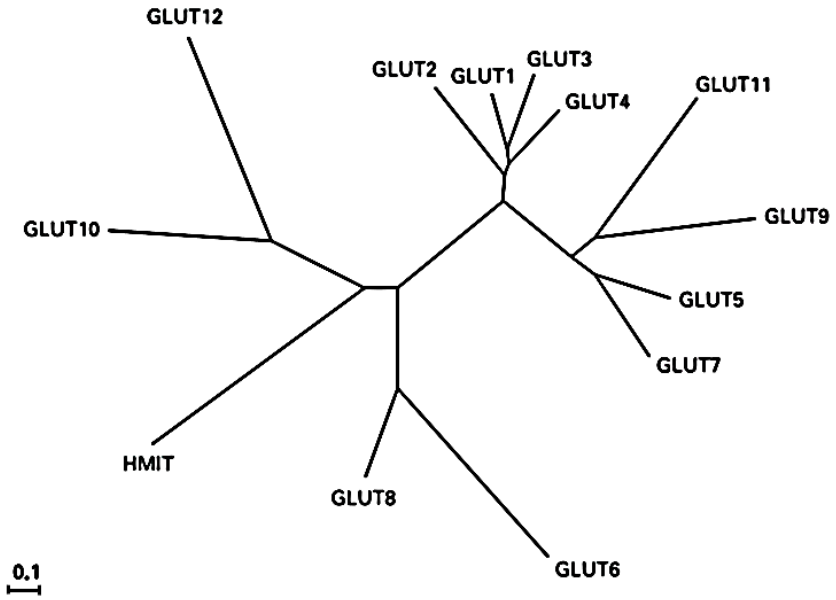

Figure 6:- Dendogram of GLUT family , Taken from Wood *et al,* **[16]**

All these transporter proteins have 12 Trans membrane structure with a single site of N-linked glycosylation and a central cytoplasmic linker domain along with both N and C termini facing the cytoplasm. All the transporters have different substrates and they are expressed at different tissue sites. The following table 1 represents the transporter protein with their substrates and cellular/subcellular expression sites along with the linked diseases [16].

Table 1:- GLUT transporters expression and link to disease

Taken from Wood *et al*, [16]

Protein Name	Predominant Substrates	Tissue distribution and cellular/subcellular expression	Link to disease
GLUT 1	Glucose, galactose, mannose, Glucosamine	Erythrocytes, brain, blood–brain barrier, blood-tissue barrier, many fetal tissues	Paroxysmal exertion-induced dyskinesia, dystonia-18, Glut1 deficiency syndrome
GLUT 2	Glucose, galactose, fructose, mannose, glucosamine	Liver, islet of Langerhans, intestine, kidney, brain	Fanconi-Bickel syndrome, (type2 diabetes)
GLUT 3	Glucose, Galactose, Mannose, Xylose,	Brain (neurons), testis	
GLUT 4	Glucose, Glucosamine	Adipose tissue, Skeletal and cardiac muscles	Type 2 Diabetes
GLUT 5	Fructose	Small intestine and Kidney	
GLUT 6	Glucose	Brain, Spleen, Leucocytes	
GLUT 7	Glucose, Fructose	Small intestine, colon, testis, prostate	
GLUT 8	Glucose, Fructose, Galactose	Testis, brain, adrenal gland, liver, spleen, brown adipose tissue, lung	
GLUT 9	Urate	Kidney, liver, small intestine, placenta, lung, leucocyte	Renal hypouricemia
GLUT 10	Glucose, Galactose	Heart, lung, brain, liver, skeletal, muscle, pancreas, placenta, kidney	Arterial tortuosity syndrome
GLUT 11	Glucose, fructose	Heart, muscle	
GLUT 12	Glucose	Heart, prostate, skeletal muscle, placenta	
GLUT 14		Testis	
HMIT	Myo-inositol	Brain, Adipose tissue	

In 1988, David James first reported that an insulin sensitive glucose transport protein is present at membrane surface [18]. Later on , Hresko *et al,* observed that GLUT-1 and GLUT-4 are predominantly inhibited by HIV inhibitors with different affinities [19].

Glucose transporters (GLUT'S) play an important role in the transportation of glucose across the membranes. The GLUT family consist of 13 members that are classified into further 3 classes. GLUT 4 belongs to Class I subfamily. GLUT 4 is mainly expressed in adipocytes and muscle cells. When insulin binds to the IR GLUT 4 translocation occurs [17]. The PI3-kinase and TC10 pathways are required for the complete activation of GLUT 4 translocation. Any obstruction in these pathways leads to insulin resistance that ultimately leads to Diabetes Mellitus type 2. Mueckler in the year 2001 shown that GLUT-4 is responsible for glucose transportation stimulated by insulin[20].

Glut 4 consists of 509 amino acids and 12 trans-membrane helices traversing through lipid bilayer [21]. N-terminal and C-terminal are located in cytoplasmic region and form an important region for transportation mechanism of GLUT4. Till now no specific study is available for structural understanding of GLUT4 but GLUT1 being close homolog to it reveals some structural features. It has been shown by the studies that TM segments 1, 2,4,5,7,8,10 and 11 form the glucose transport channel and this forms a permeation pore for the transport of glucose. The outer helices 3, 6, 9and 12 stabilize the whole channel [21].

Based on the above mentioned studies it is evident that GLUT-4 plays an important role in glucose translocation stimulated by insulin during HIV inhibitors use. The structural features that are important in insulin regulated GLUT-4 transport protein can be seen in figure 11 as follows

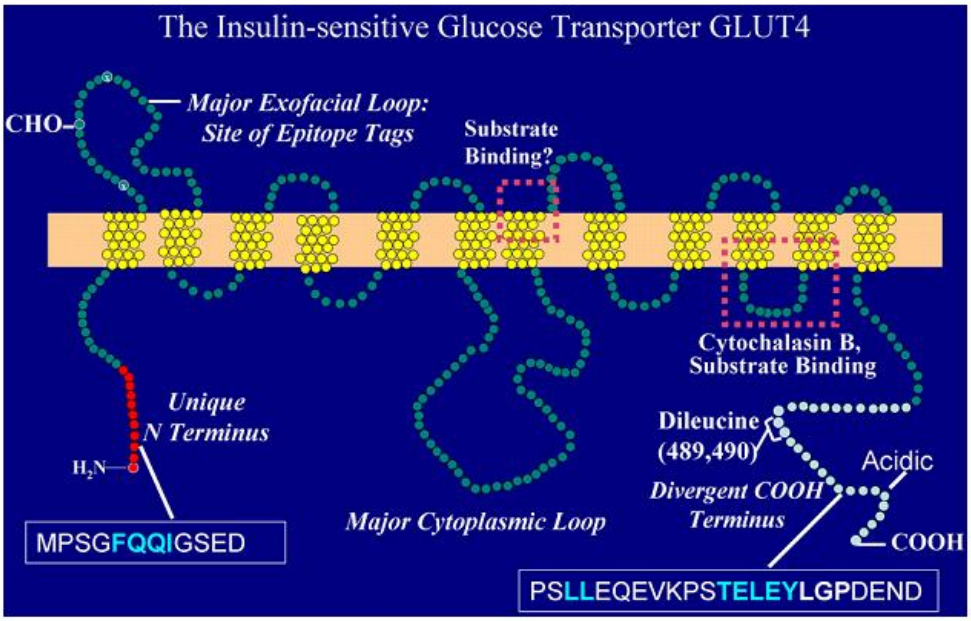

Figure 7:- Insulin stimulated glucose transport function taken from Huang *et al*, **[22]**

GLUT-4 is mainly expressed in skeletal and adipose tissues therefore, whenever the blood glucose level is disturbed the GLUT-4 transporter plays an important role in regulating it to normal [16]. GLUT-4 consist of 12 Trans membrane domains having a unique –N (phenyl alanine residue) & -COOH (dileucine & acidic motifs sequences in terminal cytoplasmic domains). Depletion of GLUT-4 at either level cause insulin resistance & diabetes to occur. It has been reported that RBP4 is released into serum when GLUT-4 is less in adipose tissues & insulin resistance is achieved [22].

Therefore, present project was designed to shed light on specific 3D structural features important for the inhibition of HIV proteases during ART therapy.

1.3 Aims of the project

- Identification of specific 3 D structural features, important for the inhibition of HIV-1 protease during maturation stage
- Identification of 3 D features that trigger Glut-4 inhibition
- To enhance potency and minimize toxicity of already developed HIV protease inhibitors

Chapter 2

Literature Review

Many investigation in the past few decades reported that HIV protease inhibitors cause hyperlipidemia, lipodystrophy and hyperinsulinemia [23] . In 2005, Brown *et_al*, conducted a study to determine the prevalence and incidence of Diabetes Mellitus [4]. The study analyzed 1278 patients that were further divided into three groups: (i) HIV seronegative (ii) HIV infected not using HAART (iii) HIV infected using HAART. They find the ratio of patients who developed Diabetes Mellitus was greater among HIV infected using HAART therapy [4]. In 2011, Hresko *et al*, reported that HIV protease inhibitors also act as competitive inhibitors of cytoplasmic glucose binding site of GLUT'S specifically for GLUT 1 and GLUT4 [19].

Since the discovery of first HIV inhibitor, many studies were performed to identify their 3D structural features that are important for ligand protein interaction. It includes various empirical approaches that include structure activity relationships (SAR), quantitative structural activity relationships (QSAR) and pharmacophore models. Hitherto no significant work has been done to identify the physiochemical features that are important in determining selectivity against the GLUT-4. In our study, structure based approaches have been used to identify important structural feature required for mounting selectivity in HIV protease inhibitors against GLUT 4.

Till now 456 structures for HIV-1 protease has been published along with co-crystallized antagonists. In 1989, Navia *et al*, for the first time reported X-ray crystallographic structure of HIV-1 protease. This study revealed the structural information that suggests that HIV-protease is a homo dimer enzyme consisting of two symmetrically related subunits whereas each unit consists of 99 amino acid residues [12]. The meeting point

of these two subunits form the active site of the enzyme. This active site is made of two Asp-Thr-Gly conserved sequence that make it differentiated aspartic protease family [12].

Saquinavir was the first protease inhibitor that was used for the treatment of HIV. It inhibits the multiplication of HIV virus by inhibiting the binding of polyproteins at the active site [24].

HIV-1 protease is functional only as a dimer, this dimer is formed from two identical subunits having twofold C2 symmetry. This dimeric face consist of eight interacting N- and C- terminal residues (residues 1-4 and 96-99) on each chain. These residues are located within four-stranded β-sheets having polar side chains consisting of Gln2, Thr4 and Asn98. This site is exposed towards the solvent whereas interior of enzyme consists of residues Pro1, IIe3, Leu97 and Phe99. To maintain the dimeric structure of protein, four C-terminal residues (96-99) are very important as they are involved in extensive inter-chain interactions along with 34 hydrogen bonds and 4 salt bridges [25].

In 1992, Sawyer *et al*, observed the structure-activity relationship of the inhibitor U-85548E with HIV-1 protease. They concluded that the fragments of the inhibitor P_4-P_2 Ser-Gln-Asn moiety showed 10^3 fold efficiency in binding with the receptor. Whereas, tripeptidomimetic analogs also showed good inhibitory potency against HIV-1 PR [26].

Thanki *et al*, provided a crystal structure of HIV-1 protease complexed with didydroxyethylene containing inhibitors [27]. The study revealed that the inhibitor U-75875 (Noa-His-Hch.k. [CH(OH)CH(OH)]Vam-Ile) is having a diol, that replaces the usual scissile bond. Therefore, there was only one hydroxyl group to interact with active site aspartates of the enzyme. This complex was studied to modify inhibitor side chains so that new drug molecules with optimal activity could be designed to overcome the resistance problem [27]. This inhibitor is a diastereomer having R configuration at both of the chiral carbon atoms and

its best interactions were seen with active site aspartates. This study assumes that the stereo chemical changes do not eliminate HIV-1 PR inhibitory activity [27]. Thaisrivongs *et al*, reported the change from R,R to R,S diol configuration causes a 20-fold drop in binding affinity towards the enzyme [28].

Thaisrivongs *et al*, in 1996 used structure based approach and identified the 5, 6-dihydro-4-hydroxy-2-pyrone (U-96988) as first clinical candidate for the potential treatment of HIV infection. The important residues identified for activity was aspartic acid [28].

In 2008 Zhang *et al*, analyzed the structure and function of HIV-1 protease, using Simplicial Neighborhood Analysis of Protein Packing Method (SNAPP) [29]. This method was used to predict the mutagenesis effect on enzymatic activity of the HIV-1 protease. In his study he identified that hydrophobic core residues and residues at the dimeric interface were very important for its activity [29].

Yadav *et al*, used both structure based and ligand based drug design approach to develop novel HIV-1 protease inhibitors [30]. He first generated a pharmacophore model using a series of 47 compounds belonging to cyanoguanidines and cyclic urea derivatives. The pharmacophore model generated, showed four important features i.e. two hydrogen bond acceptors (HBA) and two hydrophobic features (HY). Another model retrieved from above pharmacophore showed five important features including one hydrogen bond donor (HBD), two hydrogen bond acceptors (HBA) and two hydrophobic groups (HY). The comparison of both generated models by structure based and ligand based *in silico* strategies have four common points two hydrogen bond acceptors (HBA) and two hydrophobic groups (HY) [30].

In 2007, Ghosh *et al*, used structure based strategies and showed TMC-114 (Darunavir) as a potent inhibitor of HIV-protease enzyme [31]. They used structure based methods to overcome the resistance problem that provoked due to the mutation in HIV-

enzyme developed against the peptidic character that was causing reduction in binding of ligand into the active site. He found that the stability of hydrogen bond interaction will reveal the best binding of ligand with the receptor backbone [31].

Manikrao *et al*, 2012, performed a docking study with Darunavir as HIV-protease inhibitor utilizing the software MOE and concluded that carbonyl, sulfonyl and furan oxygen are playing the most important role in interaction with HIV-protease [32]. Furan oxygen of darunavir act as hydrogen bond donor whereas, carbonyl oxygen acts as hydrogen bond acceptor, and the interacting residues were AspB25 and AspA25 with interatomic distances of 2.8 and 2.59A˚. Other important residues were AspB30, AspA30, GlyA49, IleA50 and GlyB49.

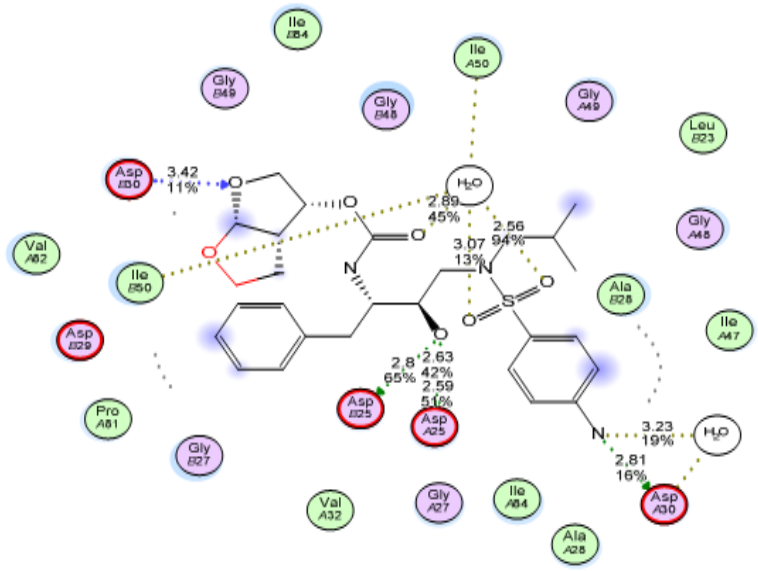

Figure 8:- Hydrogen bonding interaction of Darunavir and HIV-protease virus taken from Manikrao *et al,* **[32]**

They also measured the Vander Waal and surface interactions observed around Darunavir within 8A˚.

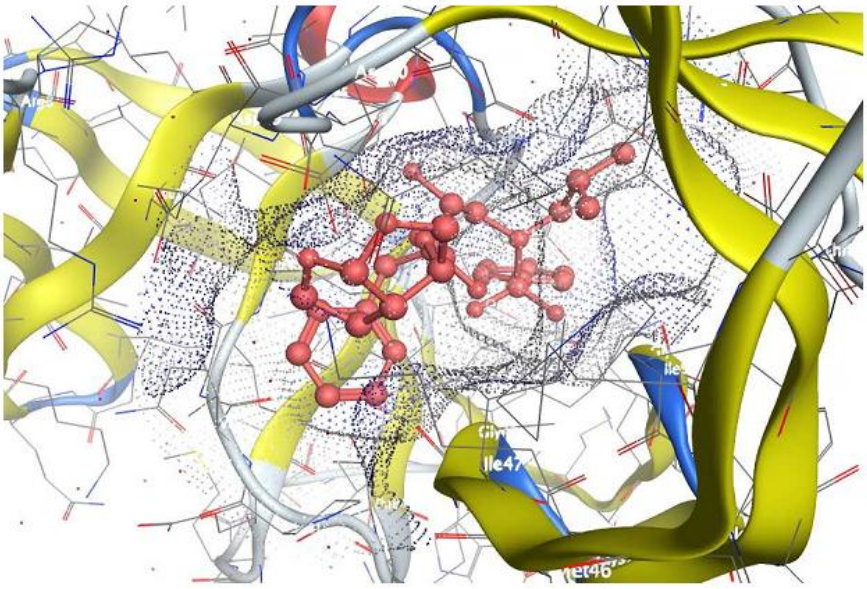

Figure 9:- Vander Waal surface around Darunavir taken from Manikrao *et al,* **[32]**

The structure selected for current study is PDB ID 4KB9 HIV-1 protease presented by Ghosh *et al,* in 2013 [33]. This model is selected because it is the latest structure available at highest resolution. In his study tricyclic P2 ligands were cocrystallized to get excellent antiviral activity along with maximum binding interactions with enzyme. He revealed some new molecular interactions that make this class of compounds potent inhibitors of HIV-1. This is a HIV-1 wild type protease cocrystallized at 1.29A˚ resolution and having an R factor of 0.14. The inhibitor in this case makes extensive interactions at active site of enzyme and notably displays favorable polar interactions including hydrogen bonds and weaker C - H….O and C H….π interactions. The hydroxyl group of inhibitor interacts with all the four carboxylate oxygen atoms of Asp 25 and Asp25´ with interatomic distances of 2.6-3.2A˚. Other important residues were Asp 30´, Gly 27, Ile47, Val32, Ile84, Leu76, Ile50´, Asp 30 and Asp 29 [33]. Other structures that were available with lowest resolution are listed below:-

Table 2:- X-ray Crystallographic models available for HIV-1 Protease

PDB Code	Exp. Method	Resolutions A°	Classification	Year	Reference
4KB9	X-ray diffraction	1.29	Hydrolase/hydrolase inhibitor	2013	Wang *et al*
3NU5	X-ray diffraction	1.29	Hydrolase /hydrolase inhibitor	2010	Wang *et al*
3OK9	X-ray diffraction	1.27	Hydrolase /hydrolase inhibitor	2010	Wang *et al*
2NNK	X ray diffraction	1.25	Hydrolase /hydrolase inhibitor	2007	Tie *et al*
3SPK	X ray diffraction	1.24	Hydrolase /hydrolase inhibitor	2011	Wang *et al*
3NU4	X ray diffraction	1.20	Hydrolase /hydrolase inhibitor	2010	Wang *et al*
3CYX	X ray diffraction	1.20	Hydrolase /hydrolase inhibitor	2008	Liu *et al*
2J9K	X ray diffraction	1.20	Hydrolase /hydrolase inhibitor	2007	Mailto *et al*
2NNP	X ray diffraction	1.20	Hydrolase /hydrolase inhibitor	2007	Tie *et al*
1K1T	X ray diffraction	1.20	Hydrolase /hydrolase inhibitor	2002	Mahalingam *et al*
3OXC	X ray	1.16	Hydrolase	2010	Kovalevesky

diffraction	/hydrolase inhibitor	*et al*

3D-QSAR COMFA and CoMSIA models were built by Cunha *et al*, in 2009 by using HOE/BAY-793 analogs. The results obtained showed steric, electrostatic and hydrogen bond donors are important for the enzyme-inhibitor interaction. They obtained a Multifit alignment (MFA) pose of compound 31 (HOE/BAY-793) showing the maximum hydrogen bonding interaction with an extended ligand conformation as shown in figure below. They used peptidomimetic derivatives having vicinal diol group as a central unit and three highly lipophilic side chains were used that were important for binding into the pocket and the residues involved in this interaction pattern were Asp 29, Ile 47 and Gly 48 as are shown in figure [34].

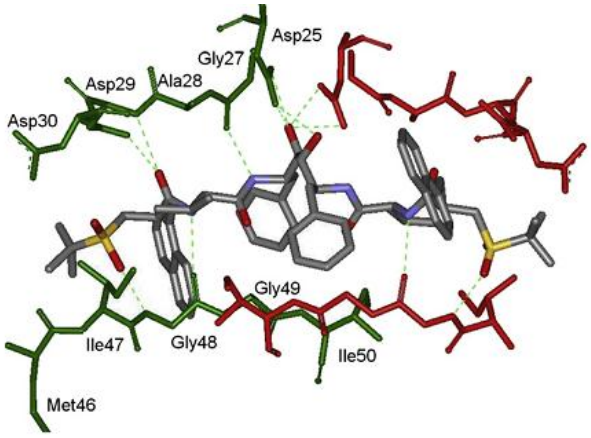

Figure 10:- HOE/BAY-793 showing inhibitor–protease hydrogen bonding interactions

Taken from da Cunha *et al*, **[30]**

Durdagi *et al*, in 2009 conducted a computational study using 3D QSAR, molecular docking and molecular dynamic simulation on fullerene based inhibitors of HIV-1 PR. They extract 3D QSAR models, performing docking analysis and molecular dynamic simulations [35]. The results revealed that HIV-1 protease attains a closed form with the flaps being

pulled towards the bottom of the active site Asp 25. The important features that were identified includes steric (42.6%), electrostatic (12.7%), H-bond donor (16.7%) and H-bond acceptor (28%) [35].

Marger *et-al*, in 1990 used various statistical methods to categorize many transport proteins of eukaryotes and bacteria for the first time [36]. The common feature was a structural motif of 12 transmembrane spanning α-helical segments among all. Five clusters were made that include drug resistance proteins, sugar facilitators, and facilitators for Krebs's cycle intermediates, phosphate ester-phosphate antiporters and oligosaccharide-H + symporters. Glucose transporters belongs to sugar facilitators cluster of these major facilitator superfamily (MFS)[36].

Mohan *et al*, in 2010 studied the intrinsic dynamic behavior of GLUT4 and the conformational changes mediated by substrate binding and ATP binding has been studied. In the apo state, form GLUT4 attains an open conformation towards extracellular region. This exofacial binding is stable occluded conformation for substrate. However, during ATP binding, GLUT 4 attains a compact interface for the two domains [21].

In 2012 Mohan *et al*, built first a homology model for GLUT 4 that was based on experimental data available from GLUT1. In this work the ATP, the substrate and the inhibitor binding site of GLUT4 were reconnoitered.

Table 3:- Structural features of GLUT1 and GLUT4

	GLUT 1	GLUT 4
Organism	Homo sapiens	Homo sapiens
Protein	Solute carrier family 2, facilitated glucose transporter member	Solute carrier family 2, facilitated glucose transporter member 4
Gene	SLC2A1	SLC2A4
Function	Transport wide range of aldoses, pentoses and hexoses, vitamin C metabolism, regulation of insulin secretion	Translocation of GLUT4 to plasma membrane, facilitative Na independent glucose transporter, transcriptional regulation of white adipocyte differentation
Amino acid	492	509
Helices	12	12
Involvement in disease	GLUT1 deficiency syndrome, Epilepsy, Dystonia	NIDDM

Chapter 3

Methodology

3.1 Databases:-

A series of 5 highly selective inhibitors of HIV-protease and GLUT-4 have been collected from literature. They include 2 Carbamate, 2 Sulfonamide and one Peptidomimetic derivatives selective for HIV-Protease [37-39]. GLUT-4 inhibitors include 3 Chrome-4-one, 1-Trioxy benzoate and one kaempiferol derivative [40, 41]. These inhibitors showed activity values in low micromolars as shown in table 3 and 4.

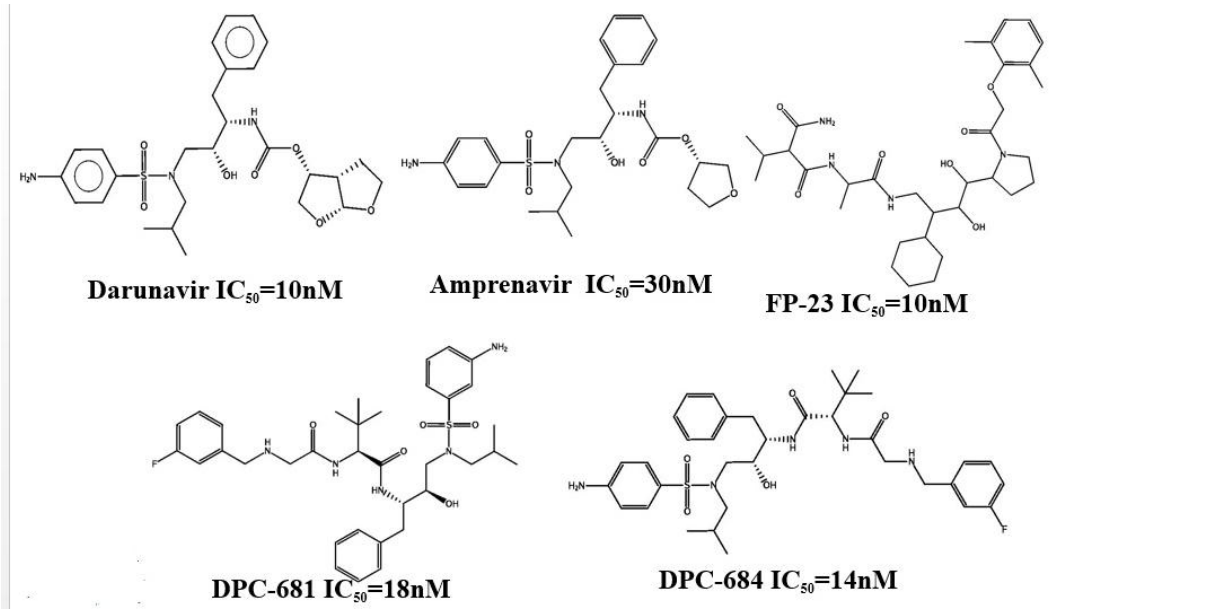

Figure 11:- HIV-1 Protease inhibitors

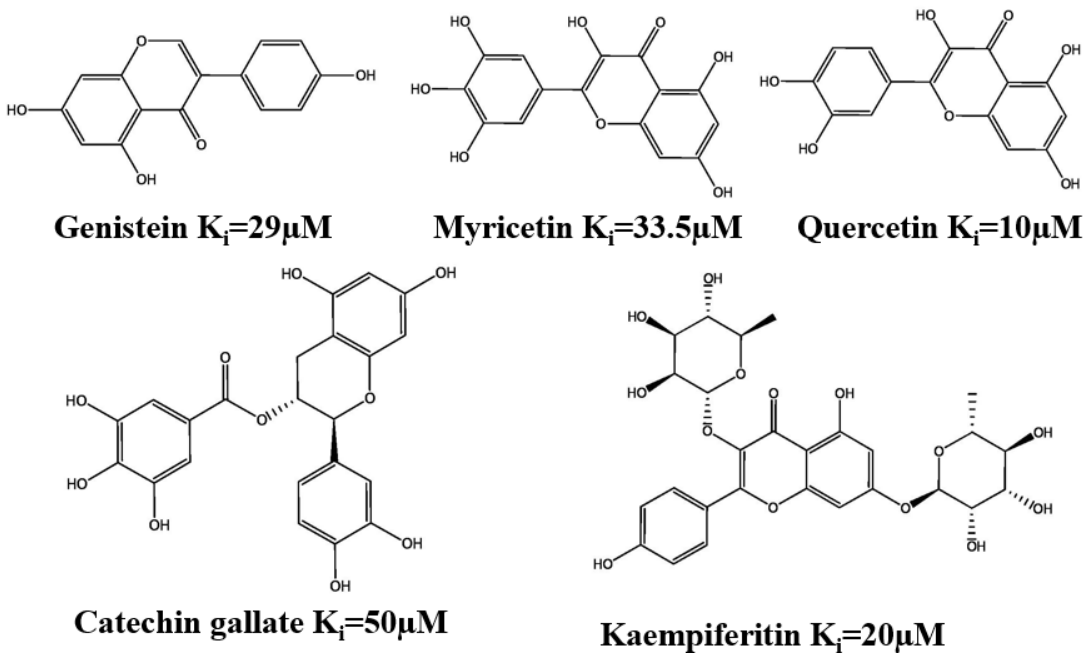

Figure 12:- Selective Inhibitors of GLUT-4

3.2 Homology Modeling:-

Homology modeling of GLUT 4 was performed as previously described by Mohan *et al*, [21] GLUT 1 model with PDB ID 4PYP was used as template to model human GLUT-4. Primary sequence of human GLUT 4 (P14672) was retrieved from uniprot KB databank [42]. Multiple sequence alignment of the target and the templates was performed using T-coffee [43] with default parameter. The aligned sequences were then manually edited in Swiss PDB viewer (spdbv) to match the topologies as previously described by Mohan *et al*, for GLUT 4. A total of 100 models have been generated and accessed on the bases their energy values. After that validation was done using PROCHECK [44] values and ERRAT [45] scores.

3.3 Docking and Pose Analysis:-

Docking was performed to identify the ligand-protein interactions pattern and to obtain most probable binding conformations of HIV proteases inhibitors. On the basis of structure activity relationships, dataset of 5 ligands was selected for docking analysis. Each

set of ligands was separately docked in HIV protease and GLUT 4 to elucidate selective ligand-protein interactions. Docking simulations were carried out using MOE suite v .09.2014. Both the ligands and the proteins were considered flexible and were allowed to flip the stereo-conformations around their chiral centers. Binding site was strictly restricted to the transmembrane domain. A total of 100 genetic runs for each ligand were performed to ensure reproducibility and optimization flexible binding of the ligands in the binding site. Slow (most accurate) protocol was used for balanced computational cost and accuracy.

The docking results were further analyzed to identify the final binding conformations. Initially common scaffold clustering on the basis of RMSD was performed to minimize the conformational space. Clusters with maximum number of docked ligands were considered for further ligand protein interactions. Additionally to supplement the pose analysis a consensus scoring was performed using scoring function London DG implemented in MOE version .09.2014. Cluster from RMSD analysis with conformations resembling with highest ranked poses were considered winners. Furthermore, to avoid errors in conformation selection, the final clusters were accredited with already available SAR data. Cluster with known interactions represented the most probable binding conformations and were used in flexible alignments and important interaction study.

3.4 Flexible Alignment:-

Flexible alignment was performed to analyze similar coordinates that will help us in analyzing the important and maximum number of amino acids involved in binding. The winning conformations along with the undocked molecules were imported in MOE version .09.2014. A total of 100 alignment conformations for each ligand protein were generated and

ranked according to their energy values. The minimum energy system with best alignment was further subjected to further analysis.

3.5 Pharmacophore Modelling:-

Pharmacophore modelling is an interactive approach for discovery of novel scaffold that is used in the screening of newer targets or new database. To identify the important features for biological activity a pharmacophore is encoding of chemical structural features into a 3 D query that is further matched with other molecular data.

The docked pose of FP-23 (401) was selected for building a pharmacophore model as it was the most active ligand among the whole dataset. The test set was formed using the inhibitors of HIV-1 protease and GLUT-4, 250 conformations of each ligand were generated using MOE version .09.2013. All the conformations were saved using packed data command in MOE. This packed data was then used as test set for validation of pharmacophore model.

Chapter 4

Results and Discussion

4.1 Datasets:-

Two datasets have been used in underlying study, one consists of selective inhibitors of HIV and second data set is of GLUT-4 specific inhibitors.

4.2 HIV-Protease dataset:-

Dataset of HIV inhibitors consists of Darunavir, Amprenavir [38] , DPC-681 , DPC-684 [37] and FP-23 [39]. The data set selected consist of compounds having IC_{50} ranging from 0.01µM (**FP-23**) to 18µM (**DPC-681**) and 14µM **(DPC-684**) whereas, **Darunavir** and **Amprenavir** having IC_{50} of 10µM. All these compounds were found to be selective inhibitors of HIV-protease causing no effects on glucose uptake [37-39].

Docking poses of selective inhibitors of Darunavir, Amprenavir, DPC-681 and DPC-684 analyzed common scaffold cluster analysis. 11 clusters were obtained containing all docked ligands, were selected for further ligand protein interaction pattern. Further best cluster was chosen using consensus scoring method. London DG, Alpha HB and Affinity dg scoring implemented in software MOE methods were used. Then, FP-23 best pose was selected after selecting the best cluster.

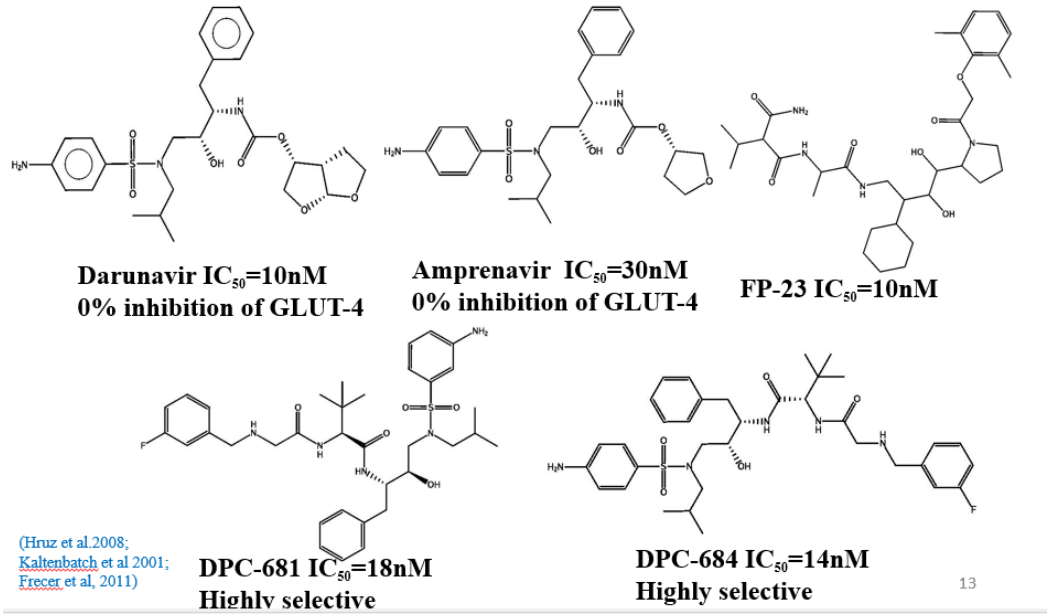

Figure 13:- Structure of HIV protease inhibitors

4.3 GLUT-4 Dataset:-

Compounds that showed highest selectivity towards inhibition of GLUT-4 include Genistein having K_i at 29µM[41], Quercetin, Myricetin and Catechin gallate having K_i at 16 , 33.5 and 90µM respectively [40] and Kaempiferitin was found to have 80% inhibition at 20µM [46].

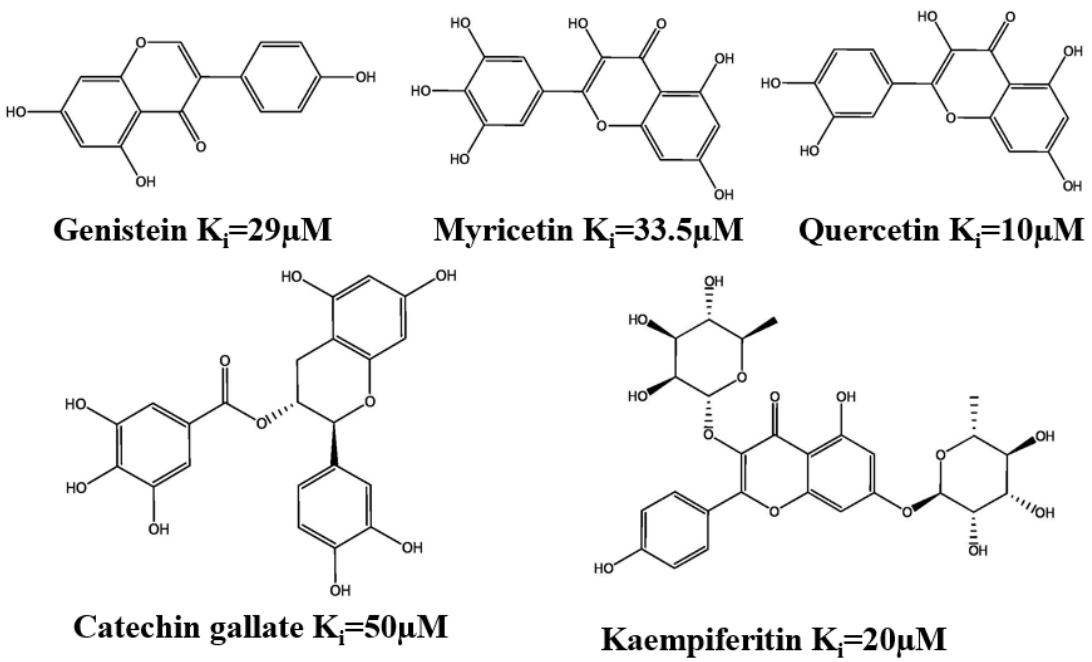

Figure 14:- GLUT-4 inhibitor dataset

4.4 SAR of GLUT-4 inhibitors:-

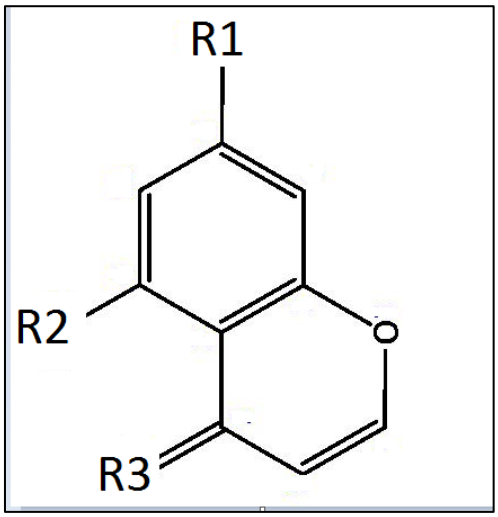

Figure 15:- Common Scaffold of GLUT 4 inhibitors

All compounds are polyphenols with different substitutions at R1, R2 and R3. Genistein showed maximum inhibitory effect due to presence of two aromatic rings that are substituted by a hydroxyl group at R3 and a phenol at R1 position. However, SAR study of

Quercetin indicate that less polar substituents at 3,4,5,7-tetrahydroxyflavonol skeleton produce stronger inhibitory effects[47].

From the literature and structural analysis it is evident that the activity against GLUT-4 among these compounds is due to phenol and the other is benzophenone substitutions. If the substituents at R1, R2 and R3 are more polar then it will reduce the activity whereas bulky groups at these positions also responsible for reduction in the biological activity.

4.5 Homology modeling of GLUT-4:-

Homology model for GLUT 4 were generated by using modeling program MODELLER based on the sequence alignment suggested by Mohan *et al,* [21]. Multiple sequence alignment was performed using CLUSTLAW 1.83 online tool. Figure 14 represents the alignment file

Figure 16:- Multiple sequence alignment of GLUT-1 sequence with GLUT-4

Using automated procedure, 100 models were generated and accessed on the bases of PROCHECK values and ERRAT scores as described in methodology. The Ramachandran plot of the final model showed 94.8% residues present in favored regions, 4.8% in additional allowed regions. Furthermore, only Ala 47 and Ala 251 were in disallowed regions (see figure 15).

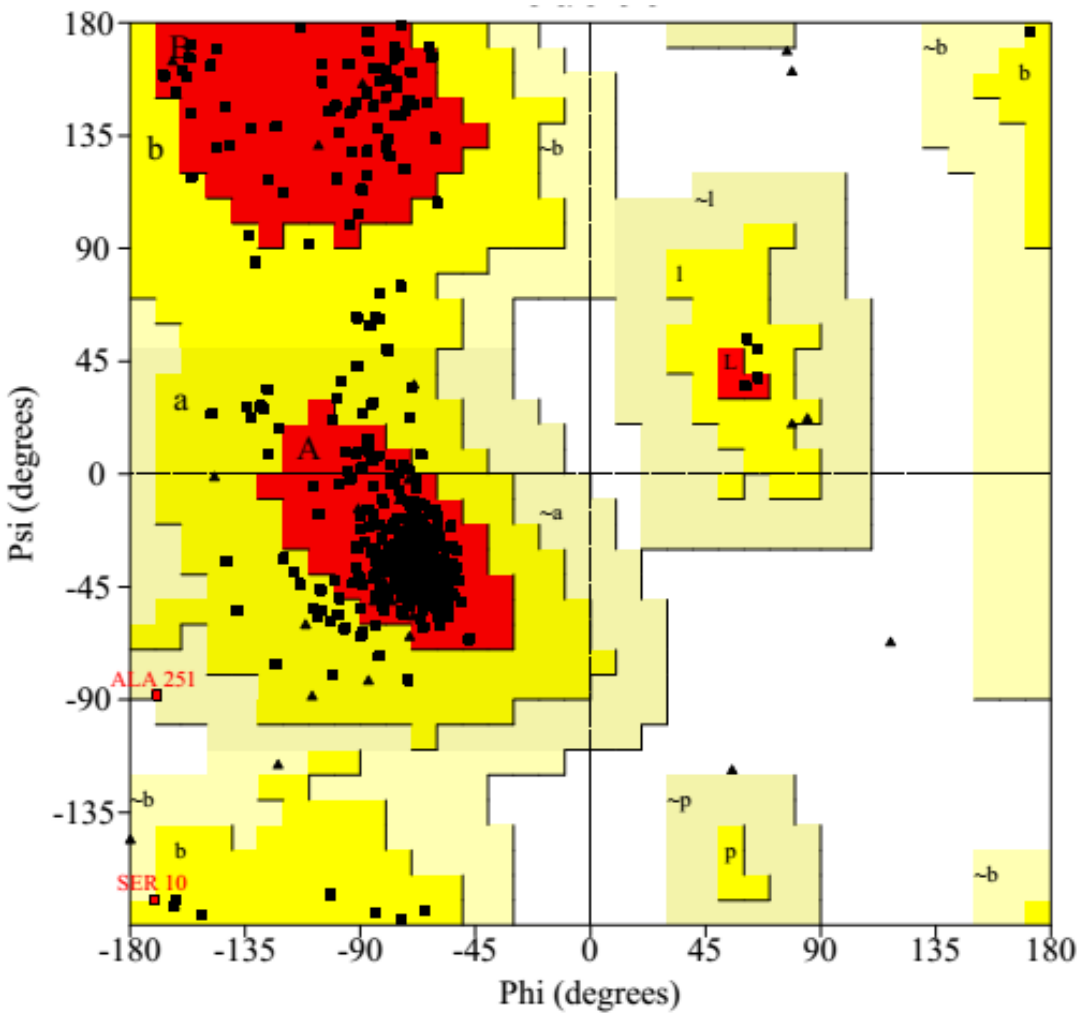

Figure 17:-Ramachandran plot of GLUT 4 model

4.6 GLUT-4 Model:-

The model obtained consist of 12 Trans membranes forming a channel having length 38.07A˚ and width 42.95A˚. The transmembrane structure can be clearly observed in the figure 16.

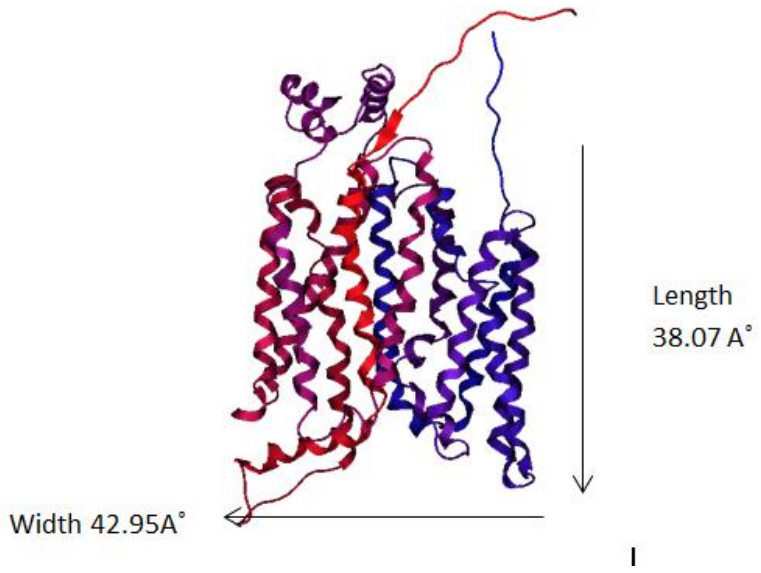

Figure 18:- Homology model of GLUT-4

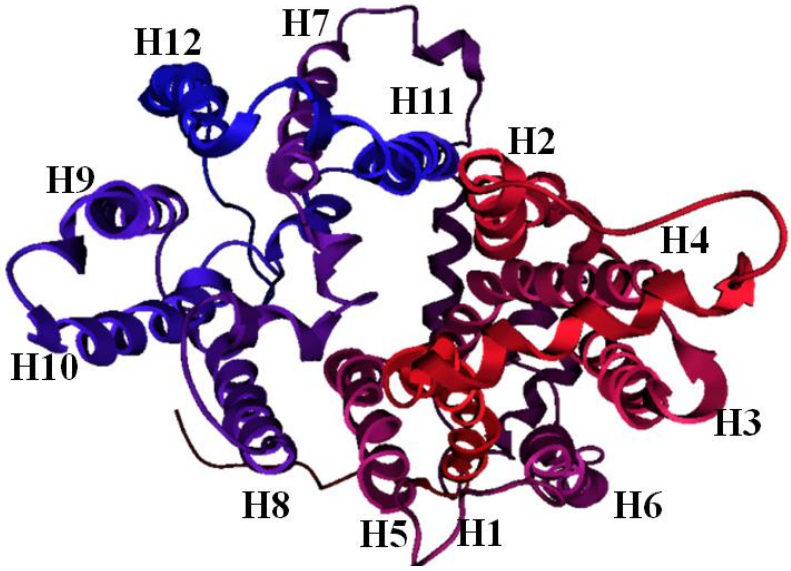

Figure 19:- Transmembrane view of GLUT-4

4.7 HIV-Protease structure:-

The HIV-1 protease structure was obtained from PDB: 4KB9. The binding pocket is already defined in case of HIV-1 protease model however for GLUT-4 pocket identification

mutagenesis data was used. Then by utilizing the available information the docking was performed.

4.8 Docking and Pose Analysis:-

Despite wide spread use of docking in ligand-protein interaction analysis, it has several limitations in predictions of native cellular conformations. Therefore, to avoid maximum uncertainty and algorithm biasness, a very exhaustive protocol for docking was optimized. Additionally, SAR guided Common Scaffold Cluster Analysis and Consensus Scoring Method was performed to identify the best ranked binding conformations of each ligand.

4.9 Docking of HIV protease inhibitors within HIV protease binding pocket:-

Ghosh *et-al,* designed and synthesize the new potent inhibitors of HIV-1 protease having tricyclic moiety common among all the new ligands. X-ray studies were performed to check the ligand-protein interactions. In our study we have used this structure with PDB Id 4KB9.

Specific inhibitors of HIV-protease were first docked using flexible docking method with Alpha Triangle as placement method and London DG as scoring method in MOE 2014.09. 100 poses were generated for each ligand in the docking process. In order to analyze most probable binding conformation of the ligands Common Scaffold Cluster analysis was performed. The common scaffold protocol used is shown in figure 18.

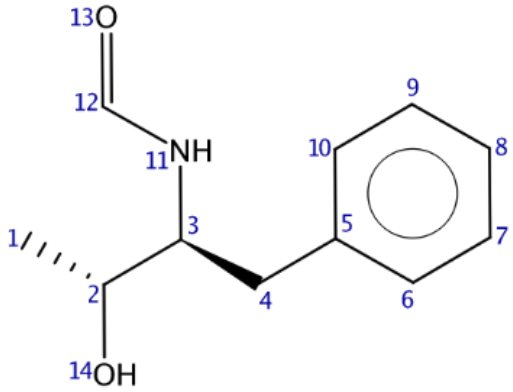

Figure 20:- Common Scaffold of Docking 1

Total 9 clusters were formed that were having all docked ligands. Final cluster was selected on the basis of Consensus Scoring. Cluster 3 was finalized having the best poses to interact at the active site.

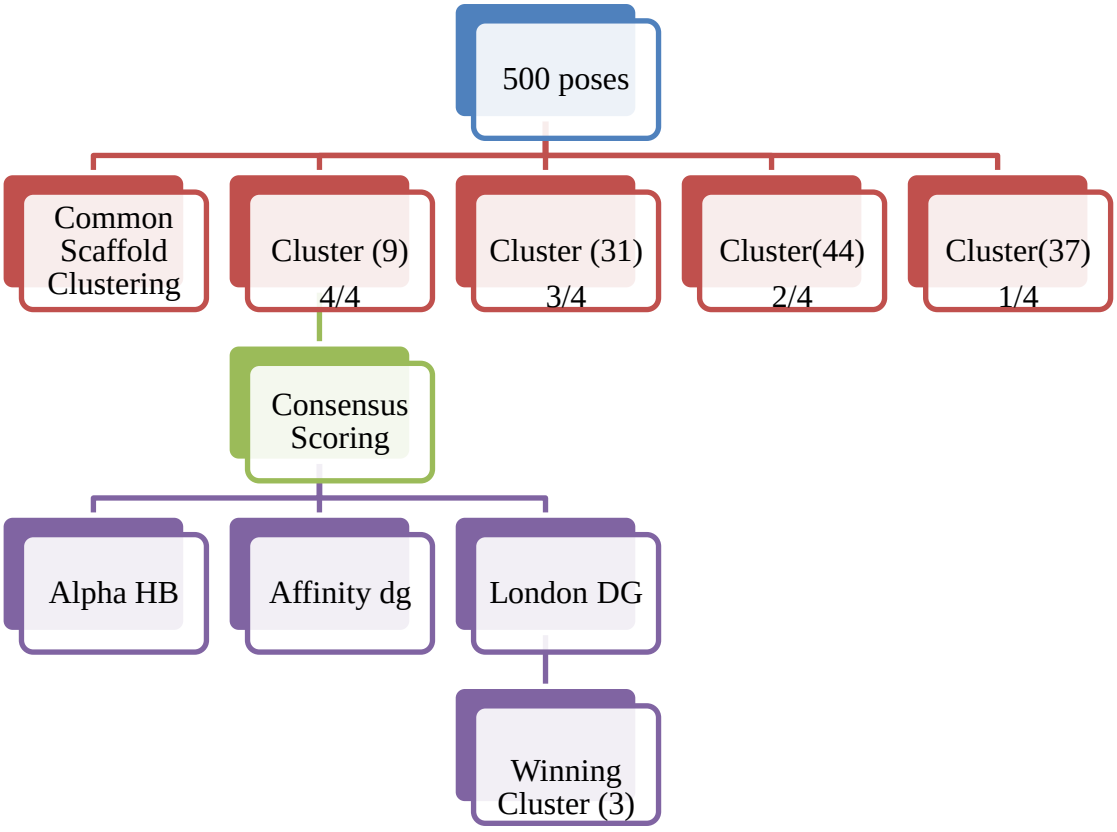

Figure 21:- Flow chart of cluster analysis and consensus scoring

The best pose selected is of ligand FP-23 determined using the flexible alignment method based on pharmacophoric features. Thus, the best selected pose of FP-23 showed interaction with Ile50, Gly48, Asp25 and Ala28 (see figure 20).

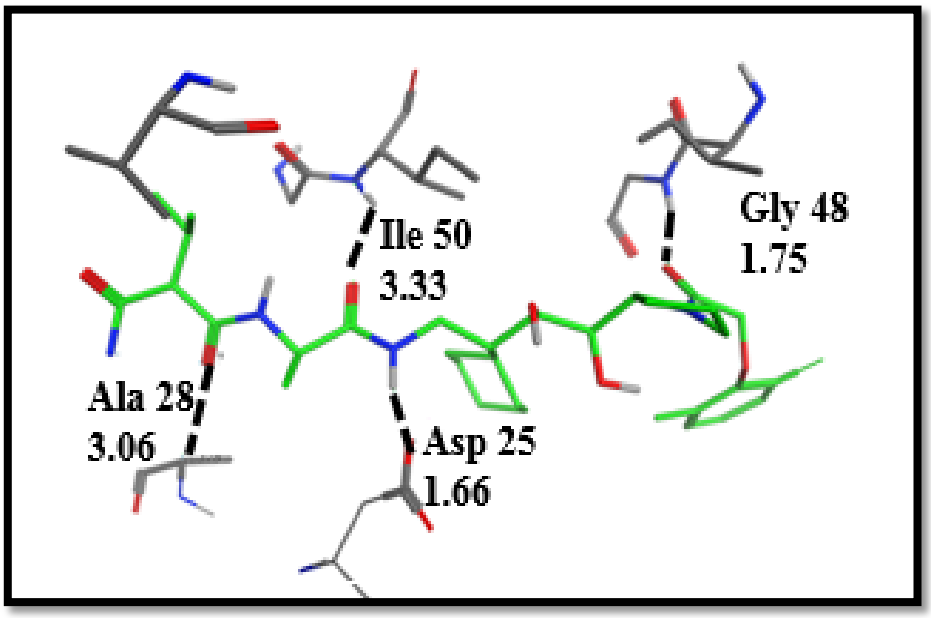

Figure 22:- Important interacting residues in HIV-HIV docking

4.10 Ligand – Protein interactions:-

Discussion:-

The residues common amongst all the drugs in interacting with HIV-1 protease pocket are Gly A48, Gly B48, Asp A25, Asp B25, Ile A50, Ile B50 and Ala A28. The other residues involved include Asp A29 showing donor interaction with –NH of the drug DPC-681, Arg B8 showed hydrophobic interaction with the benzene ring of DPC-684 and Ala B28 showed hydrogen bond acceptor linkage with drug FP-23. Aspartic acid residues are interacting due to lone pair present at the terminal of double bonded oxygen atoms. The hydrogen attached with the amide of drug binds with this lone pair and oxygen is having more electronegative effect so a positive electrostatic potential is created around the drug. Due to this reason the HIV-1 protease shows hydrogen bonding interactions mostly. Arg B8 is the only one residue

that is showing a hydrophobic interaction in the pocket in case of drug DPC-684(see figure 21).

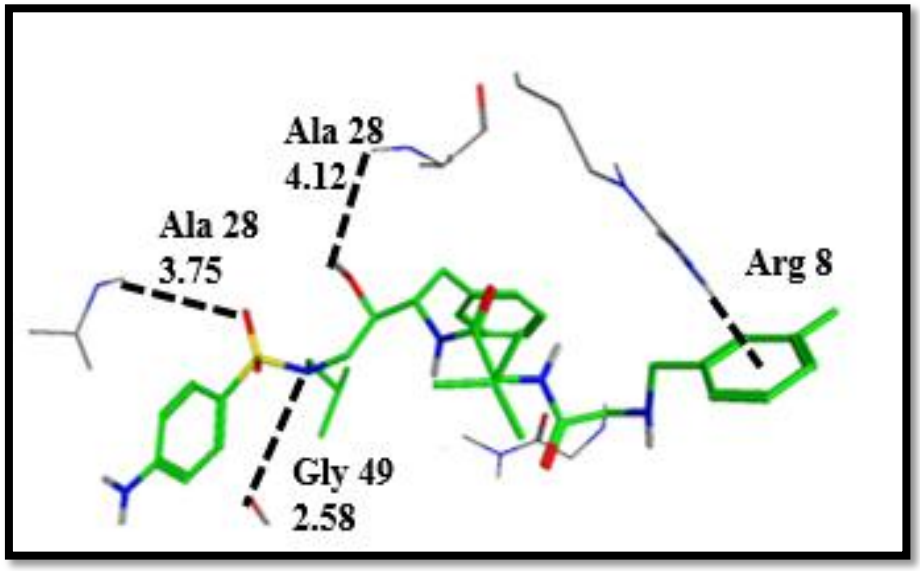

Figure 23:- Interaction of DPC-684 in HIV Protease binding pocket

4.11 <u>Docking of GLUT-4 inhibitors within GLUT-4 binding pocket</u>:-

Underlying study of GLUT-4 transporter posed a great challenge primarily because limited binding cavity information was available earlier by Mohan *et al*, [21] .

Molecular docking protocol was performed using GLUT-4 homology model as a receptor and GLUT-4 inhibitors were used as ligands. Same protocol was followed as using cluster analysis and consensus scoring. The best interacting residues were Asn 176, Asn 427, Trp 404, Met 420, Gly 424 and Ser 153(see figure 22), they all showed hydrogen bond donor interactions with the highly selective GLUT 4 inhibitor drugs. However, hydrophobic interactions were most prominent. The residues involved in hydrophobic interactions were Phe 38, Asn 431, Trp 404, Trp 428, Gly 154 and Ser 153. Trp 404 and Trp 428 were also involved in arene arene bonding interactions. According to our study we concluded that GLUT-4 binding pocket is most likely to have donor and hydrophobic interactions. Thus, to interact into GLUT-4 pocket the ligand should be of hydrophobic nature

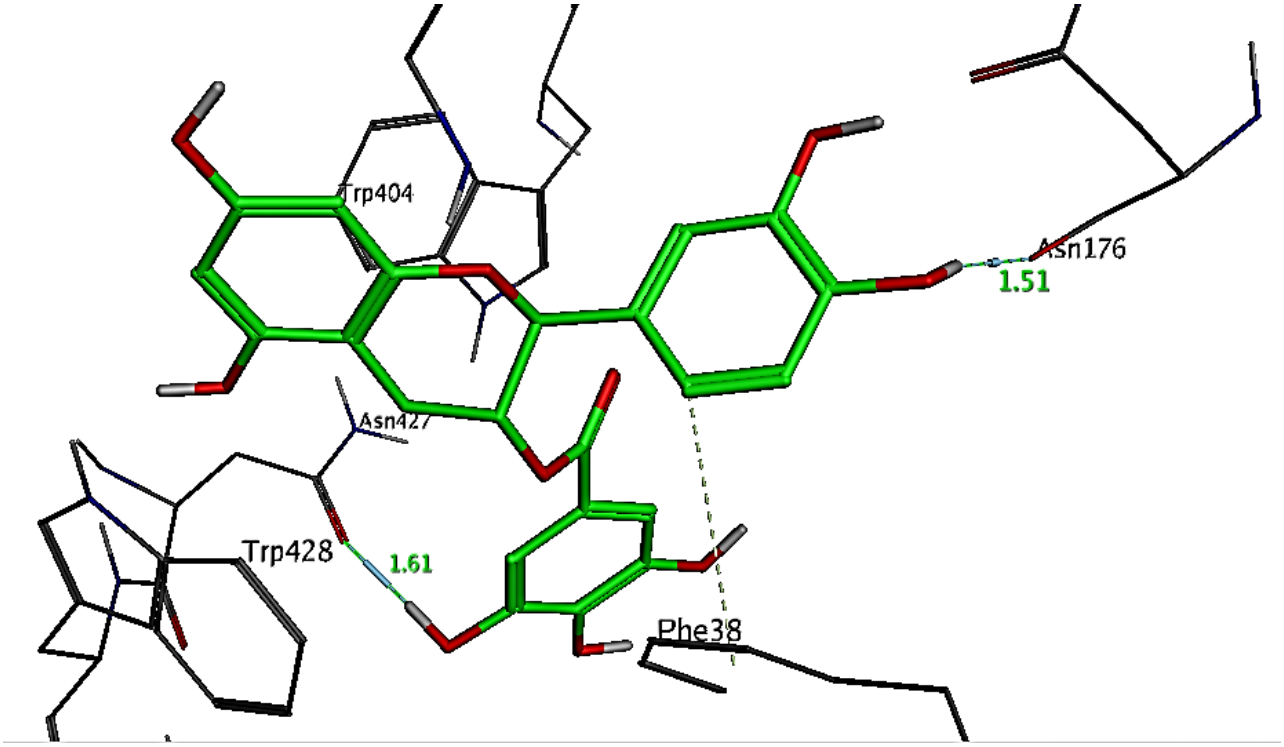

Figure 24:- Important residues involved in GLUT4-GLUT4 docking

Cross-docking

4.12 Docking of HIV-inhibitors into GLUT-4 pocket:-

Same protocol was followed as mentioned above. The best aligned poses of ligands were as shown in figure 23.

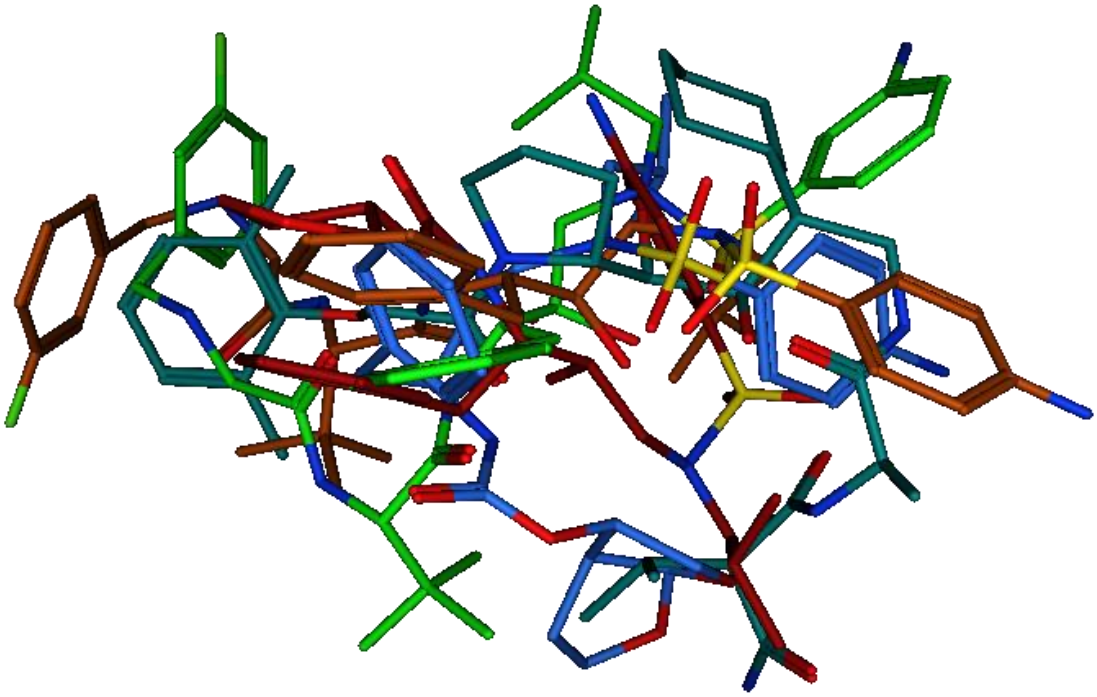

Figure 25:- Best aligned poses in Cross-docking

Ligand Protein interactions:-

Interaction of Amprenavir in GLUT 4 pocket:-

The interactions can be seen in following figures:-

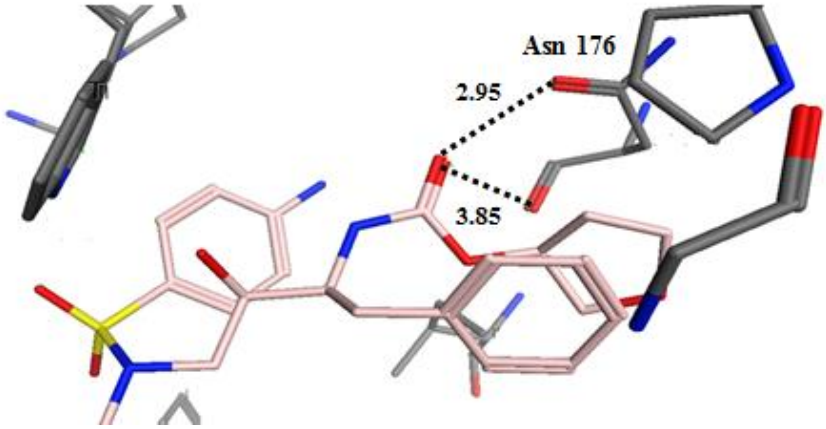

Figure 26:- Interactions of Amprenavir in GLUT-4 pocket

The residues that were involved in interaction were Trp 404 and Asn 176(see in figure 24). Trp 404 showed unfavorable hydrophobic interaction with Amprenavir and Asn 176 showed donor donor unfavorable interaction in the binding pocket. These interactions showed the specicificity of this drug towards HIV protease.

Interaction of Darunavir in GLUT 4 pocket:-

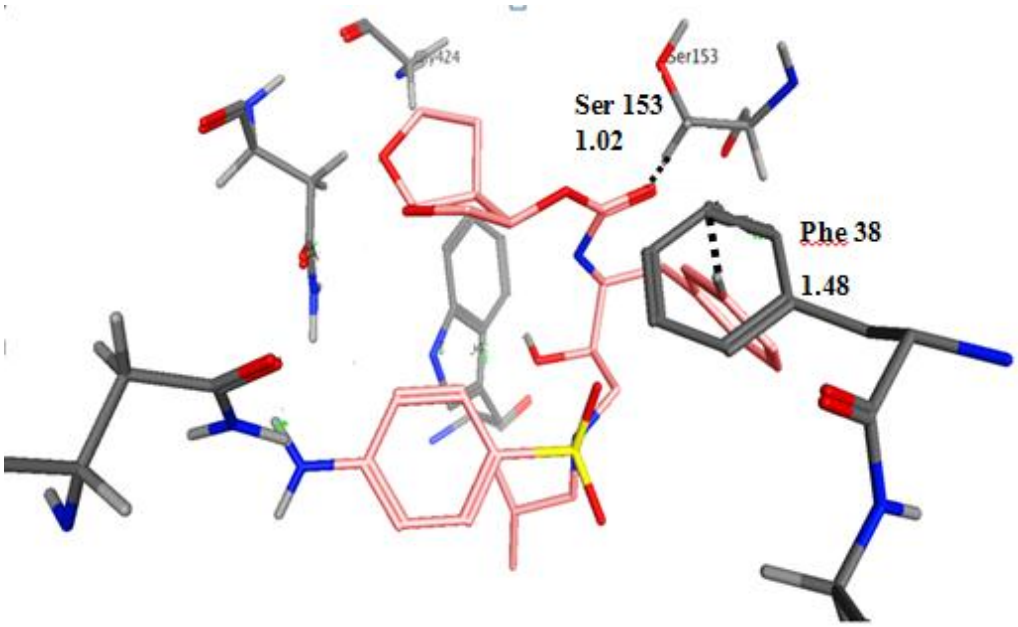

Figure 27:- Interactions of Darunavir in GLUT pocket

The unfavorable interaction pattern in figure 25 has been shown by Phe 38 and Ser 153 , the former showed hydrophobic imbalance that would result in increase in energy of the system that would be unfavorable and this shows the specificity of this drug.

Interaction pattern of DPC-681 and DPC-684:-

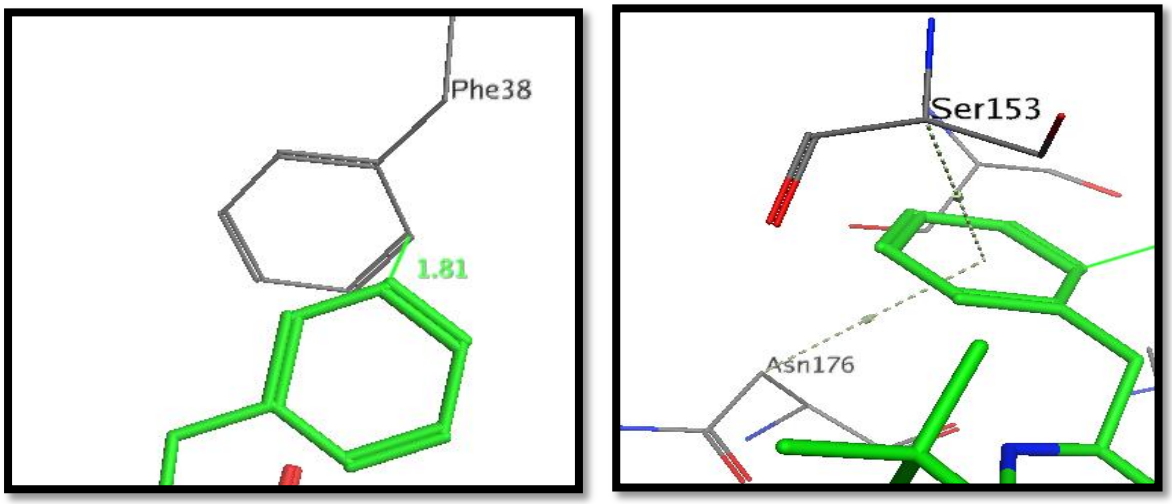

Figure 28:-Interaction of DPC-681and DPC-684 in GLUT-4 pocket

The residue Phe 38 caused instability in binding with GLUT 4 in case of DPC-681 and Ser 153 and Asn 176 showed steric clashes that are unfavorable for its binding in GLUT 4 pocket as shown in figure 26.

Interaction of FP-23 in GLUT 4 pocket:-

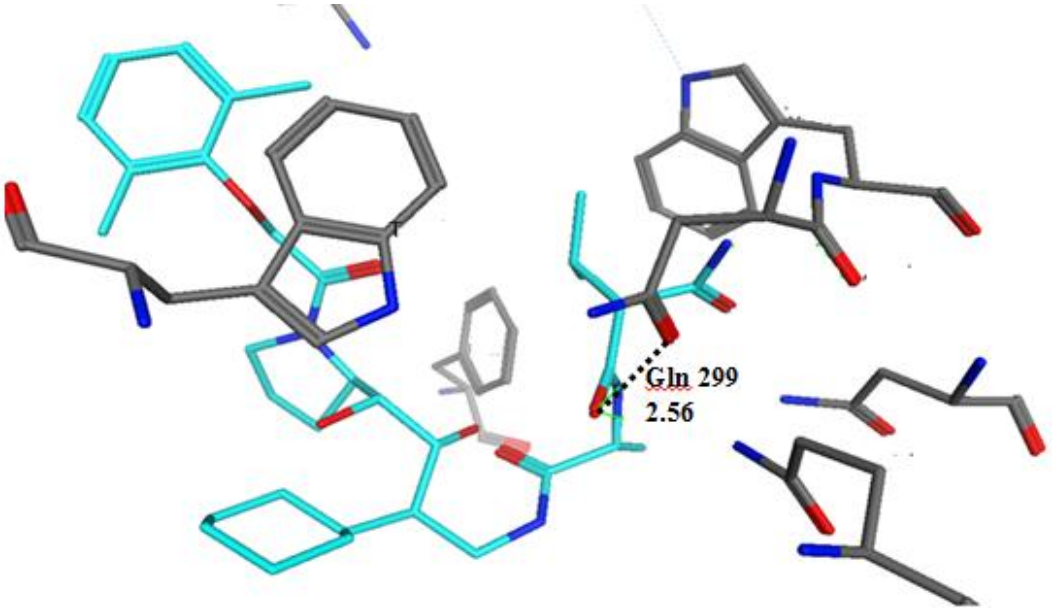

Figure 29:- Interactions of FP-23 in Glut-4 pocket

In case of FP-23 residue Gln 299 showed unfavorable interaction by donor-donor repulsion as shown in figure 27.

Discussion:-

The residues that were involved in the selectivity were Asn 431, Trp 404, Trp 428, Asn 427, Phe 38, Asn 304, Gln 299, Ser 153 and Asn 176. All these residues were specifically involved in fruitful binding of its own ligands into the pocket. But here in our studies these residues are clearly inhibiting the binding of HIV-1 protease inhibitors into GLUT-4 pocket so they are the selectivity determining residues of the pocket. Thus here we can hypothesize that the GLUT-4 pocket mainly farewells the hydrophobic ligands and H-bond acceptor ligands binding.

4.14 Pharmacophore Model:-

The pharmacophore generated from 3 D structure of FP-23 (401 pose) consist of four features: one hydrogen bond donor (HBD), one hydrogen bond acceptor (HBA), one hydrophobic group (HY) and one aromatic group (Aro). The pharmacophore model and inter-feature distances are shown in figure 28 and 29.

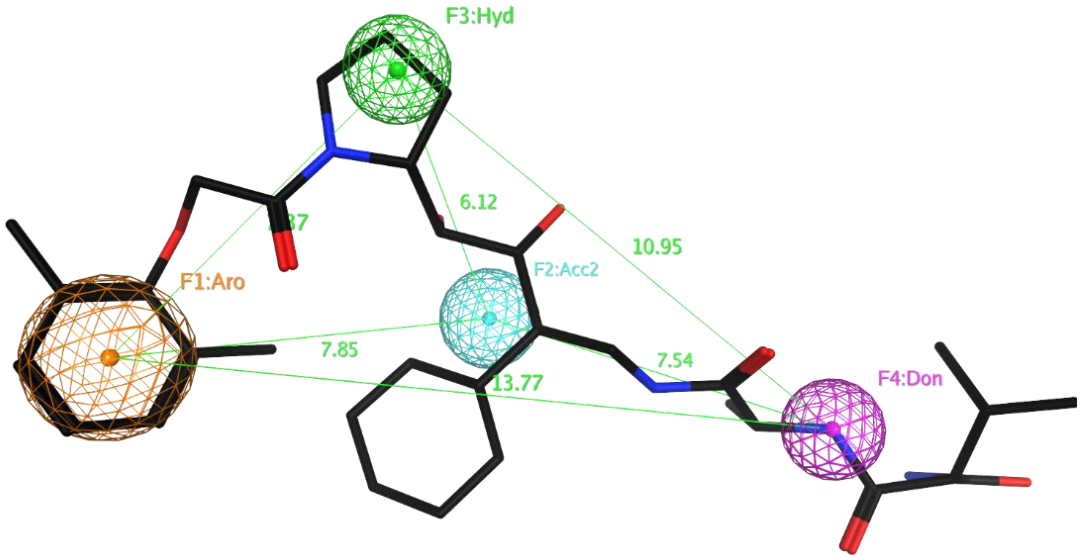

Figure 30:- Mapped pharmacophoric features of FP-23 compound

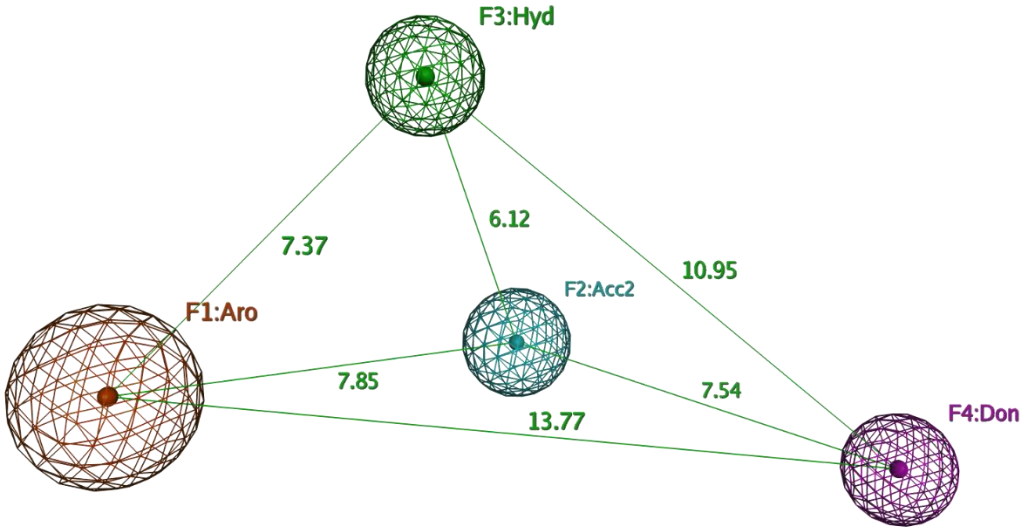

Figure 31:-Inter feature distances of pharmacophore model

Table 9:- Inter-feature distances of pharmacophore model

Features	Hydrophobic A˚	Aromatic A˚	Hydrogen bond Donor A˚	Hydrogen bond Acceptor A˚
Hydrophobic	0	7.37	10.95	6.12
Aromatic	7.37	0	13.77	7.85
Hydrogen bond Donor	10.95	13.77	0	7.54
Hydrogen bond Acceptor	6.12	7.85	7.54	0

Discussion:-

The present study was conducted to attain the selectivity among the HIV-1 protease inhibitors as they block the GLUT-4 transporter. Pharmacophore modelling is used here to identify the structural and orientational features important for HIV-1 protease inhibitors. The important features identified were HBD, HBA, HY and Aro for inhibitory action including the selectivity against GLUT-4. This model is significant because it does not select the GLUT-4 inhibitors from test set.

Same approach was used by Ataul Islam and his colleagues. They used a set of 30 compounds for building a pharmacophore model explaining the inhibitory effect of HIV-1 protease inhibitors but they did not identified the selectivity against GLUT-4. The identified features in their model were also hydrogen bond donor and acceptor, hydrophobic and aromatic ring [48]. However, Yadav *et al*, in 2012 used the both structure and ligand based approach the pharmacophore model obtained consist of five features namely two hydrogen

bond acceptors, one hydrophobic and one hydrogen bond donor. This model was identified as a significant because it complemented the features obtained from ligand based study too [30].

Conclusion:-

From the present study it is concluded that the four identified pharmacophoric features namely one hydrogen bond donor, one hydrogen bond acceptor, one hydrophobic and one aromatic ring are of great importance for HIV-1 protease inhibitors selectivity. The HIV-1 protease residues that are having the most determining behavior were Asp A25, Asp B25, Asp A29, Asp B29, Val B32, Ile A50 and Gly A27. Asp 25 and Asp A29 were already recognized as important for ligand protein interaction in all past studies. The other residues were also discussed earlier but in underlying study they are the key determinants for selectivity against GLUT-4 in humans.

Chapter 5

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