IJPSR (2018), Volume 9, Issue 10 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 23 June, 2018; received in revised form, 12 September, 2018; accepted, 18 September, 2018; published 01 October, 2018

ANALYSIS OF CRYSTAL STRUCTURES OF DIPEPTIDYL PEPTIDASE 4 (DPP 4) CO-CRYSTALLIZED WITH DIVERSE INHIBITORS

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Keywords:

DPP 4, GLP-1, Gliptins, Interaction, Binding pose, Drug discovery

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ABSTRACT: Crystal structures of target are an indispensible tool in modern methods of rational drug design and discovery. Hence, judicious selection of crystal structures for virtual screening and ligand docking is essential. In recent times, a number of new chemical entities (NCEs) have been introduced into the drug discovery pipeline with these methods in treatment of type 2 diabetes. Dipeptidyl peptidase 4 (DPP 4) enzyme splits an incretin based glucoregulatory hormone glucagon like peptide -1(GLP-1) from N-terminal of peptide, where penultimate amino acid is either alanine or proline. Several DPP 4 inhibitors, "gliptins", are approved for management of type 2 diabetes or are under clinical trials. Crystal structures of DPP 4 have been released by various research groups that may assist rapid discovery of new DPP 4 inhibitors. 18 crystal structures of DPP 4 bound to various inhibitors are analyzed in the present work to gain insight into interactions between the protein and ligands. Chemically all DPP 4 inhibitors are diverse in nature but occupy same binding site. Key amino acid residues essential for optimum interaction between protein and ligands are discussed with emphasis on orientation of ligand in active site of DPP 4.

INTRODUCTION: Type 2 diabetes (T2D) is one of the major health concerns around the world as the prevalence of diabetes is rising in all corners of the world. In 2014, 422 million of the world's population had diabetes. The numbers are expected to increase up to 642 million by 2040. The global prevalence of diabetes among adults over 18 years of age has almost doubled from 4.7% in 1980 to 8.5% in 2014. The prevalence of diabetes has been increasing since last 3 decades and is growing most rapidly in low- and middle-income countries [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(10).4460-71

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(10).4460-71

Diabetes is a major risk factor for cardiovascular disease (CVD), *i.e.*, Myocardial Infarction, angina, atherosclerosis, stroke, hypertension, *etc*. T2D is characterized by insulin resistance and insufficiency. Main aim of management of T2D is to control hyperglycaemia [2]. Existing therapeutics for T2D include biguanides, sulphonylureas, thia-zolidinediones, meglitinides and α-glucosidase inhibitors. Poor glycaemic control in spite of aggressive therapy is a demoralizing factor in the management of T2D.

Newer agents are being developed that increase insulin secretion. Glucagon-like peptide-1 (GLP-1) and gastric inhibitor peptide (GIP) are naturally occurring incretin hormones that are released in the gut after meals. They stimulate the release of insulin. Naturally produced GLP-1 is degraded by enzyme dipeptidyl peptidase 4 (DPP 4) in gut and

hence has a very short half-life of less than two minutes. Incretin function is impaired in patients with T2D and thus their blood sugar levels are not properly regulated, leading to adverse clinical events. DPP 4 inhibition is an established approach for the management of T2D. DPP 4 inhibitors extend the action of insulin by delaying the degradation of GLP-1 and suppress the release of glucagon; ultimately reducing elevated blood glucose, a feature of T2D patients. DPP 4 inhibitors have advantage of minimal hypoglycemia because they stimulate insulin secretion in a glucose dependent fashion. Another advantage is that treatment with DPP 4 inhibitors does not lead to weight gain in diabetic patients; the vast majority of whom are obese. The intense focus directed towards the discovery and development of DPP 4 inhibitors by various research groups and pharmaceutical industries has increased, reflecting their therapeutic potential for management of T2D.

Incretin Hormones and Dipeptidyl Peptidase 4 (DPP 4, EC 3.4.14.5): Incretins hormones, mainly GLP-1 and GIP are involved in maintaining insulin level in glucose-dependent manner. GLP-1 is released from intestinal-L cells after meals. GLP-1 binds to receptors located on pancreatic β- cells and stimulates biosynthesis, leading to release of insulin. Another beneficial effect of GLP-1 is the reduction in the secretion of glucagon from pancreas. Glucagon stimulates the liver to convert glycogen to glucose, thus increasing blood sugar levels. GLP-1 has many other beneficial effects. It induces feeling of satiety, prolongs gastric emptying and reduction in body weight. GLP-1 and GIP are thought to have beneficial effects on the pancreas such as increase in β-cell mass and their survival. GIP is released from K cells and is profoundly involved in maintenance of glucose level by triggering insulin release. Both these peptides have very short half-lives (2 min and 4 min respectively) and are inactivated by DPP 4 [3, 4, 5, 6].

DPP 4 is a large transmembrane serine protease containing 766 amino acids, which selectively cleaves XAA-Ala and XAA-Pro dipeptides from the N-terminus of peptides and proteins, (XAA are other amino acids); **Fig. 1**. Other substrates for DPP 4 include Neuropeptide Y, circulating peptide hormones like peptide YY, GLP- 1 and gastric inhibitory peptides. It has very wide tissue distribution with expression in exocrine pancreas, kidney, gastrointestinal tract, biliary tract, thymus, spleen, lymph nodes, uterus, placenta, prostate, adrenal, and mammary glands.

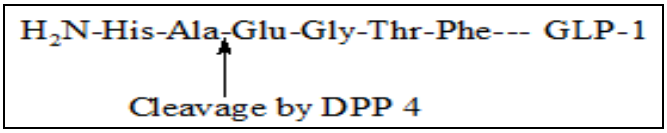

FIG. 1: DPP 4 CLEAVAGE POINT IN GLP-1 AT THE PENULTIMATE POSITION FROM N-TERMINAL

Administration of synthetic inhibitor of DPP 4 prevents cleavage of GLP-1 and GIP from N-terminal, and leads to elevated plasma levels of incretin hormones, increased insulin secretion and reduced glucose levels with lowered risk of hypoglycemia in diabetic patients. Apart from reducing blood glucose level by enhancement of GLP-1 level, DPP 4 inhibition also provides protection against cardiovascular risks and diabetic nephropathy.

The modern approach towards development of an inhibitor requires crystal structures of the target enzyme for use in docking simulations. About 80 crystal structures of human DPP 4 have been released, in apo form as well as with a variety of inhibitors bound to the active site. Chemically all DPP 4 inhibitors are diverse in nature and occupy the same binding site [7, 8, 9, 10]. In the present work binding modes of some important DPP 4 inhibitors bound to the crystal structure are analyzed. Brief overview of the complete structure of DPP 4 is discussed before elaborating the substrate binding sites and highlighting differences among them. Essential amino acid residues involved in interactions between ligands and DPP 4 enzyme binding site also are summarized.

Structure of DPP 4: X-ray crystal structure of DPP 4 has been solved in apo form as well as in bound form to various inhibitors. More than 80 crystal structures of DPP 4 have been solved till date and deposited to Protein Data Bank by different research groups. Human DPP 4 exists as a homodimer. It is a 110 kda transmembrane glycoprotein. Cytoplasmic tail (1-6 amino acid residues), transmembrane region (7-28 amino acid residues) and extracellular domain (29-766 amino acid residues) make up the large DPP 4 enzyme.

Each subunit is composed of C-terminal *α* / *β*-hydrolase domain and N-terminal 8 bladed *β* – propeller domain. Inhibitors bind to a small pocket in the cavity located between the two domains making up a volume of 30-45 Å approximately. Key residues from both domains constitute the binding site of DPP 4. Large cavity is formed at the interface of an *α* / *β*- hydrolase domain and an eight-bladed *β*- propeller domain. Active site is also positioned in this large cavity. Subsites in the active site of a protease are generally outlined by the binding site occupied by the substrate peptide.

The amino acids in the substrate peptide are numbered from the point of cleavage (P2, P1, P'1, P'2 . . .), and the protein subsites occupied by the respective amino acids are also numbered in a similar manner (S2, S1, S'1, S'2..), **Fig. 2**. X-ray analysis has confirmed that the S1 pocket is composed of a catalytic triad having SER630, ASP708, and HIS740; an oxyanion hole having TYR547, TYR631; and highly hydrophobic amino acid residues *i.e*. VAL656, TRP659, TYR662, TYR666, ASN710, and VAL711. The S2 pocket also consists of various hydrophobic residues in which GLU205 and GLU206 dyad and ARG125 are present. A large cavity is defined beyond S2 pocket known as S2 extended pocket, which is made up of VAL207, SER209, PHE357 and ARG358 [11, 12].

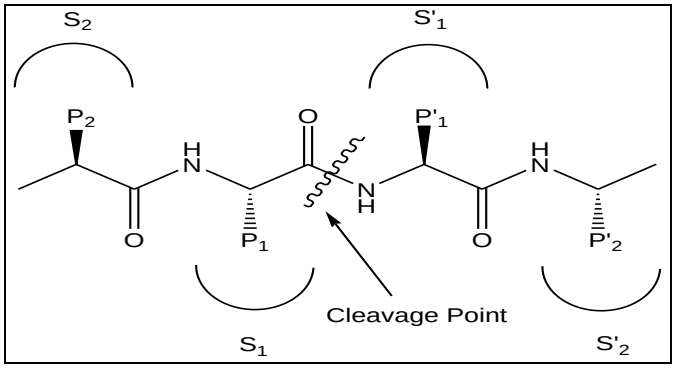

FIG. 2: NOMENCLATURE OF SUBSTRATE INHIBITOR RESIDUES AND THEIR CORRESPONDING BINDING SITES IN THE ACTIVE SITE OF ENZYME

Early DPP 4 Inhibitors: DPP 4 was validated as druggable target as its function is effectively inhibited by synthetic small molecules. As a result of considerable interest and intense research work from different researchers and pharmaceutical industries, a large number of different DPP 4 inhibitors have been developed for management of T2D (non-insulin dependent) till date. There are more than 100 patents filed worldwide of diverse chemical compounds as DPP 4 inhibitors. Chemically all inhibitors are heterocyclic (pyrrolidine, pyrimidine, xanthine, thiazolidine), amino acid amide, alkyl amines and carbocyclic. Chemically diverse small molecule inhibitors of DPP 4 have been granted regulatory approval for management of T2D in various countries while some inhibitors are under various phases of development. All the DPP 4 inhibitors reduce glycated haemoglobin (HbA1c) in range of 0.5 to 1.

Compounds 1 and 2 **Fig. 3** are valinepyrolidide (Novartis) and isoleucinethiazolidide (Probiodrug/ Merck) respectively. They inhibited DPP 4 at Ki of 255 nm and 130 nM respectively. They were the initially designed DPP 4 inhibitors based on the structure of DPP 4 substrate peptide. They are known as peptidomimetic DPP 4 inhibitors. Peptidomimetics are also termed as substrate like, *i.e.* mimic penultimate cleavage sequence of DPP 4 substrates. Small 5-membered heterocycles like pyrrolidine, thiazolidine substituted with amino acid at penultimate P_1 position are proline mimetics of DPP 4 substrate and hence are termed as peptidomimetics. They were not developed further since they suffered from lack of selectivity and poor physicochemical properties.

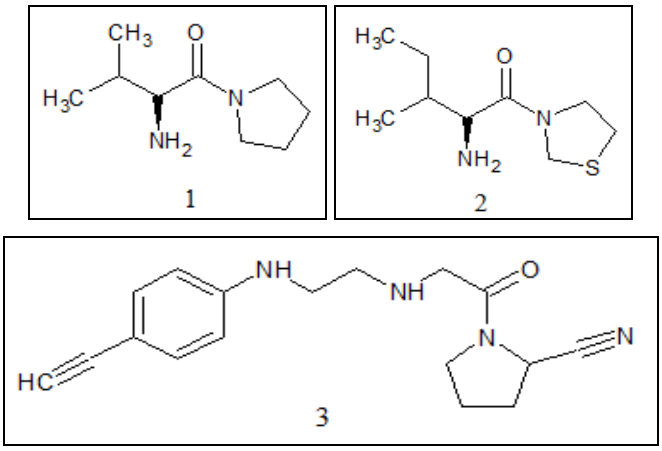

FIG. 3: SOME EARLY DPP 4 INHIBITORS

Peptidomimetic inhibitors containing electrophile at 2nd position of five membered rings interact with S_1 subsite by forming a covalent interaction with –OH of SER630 in catalytic triad; thereby forming an immediate adduct. They were initially reported as potent inhibitor of DPP 4. Small molecules devoid of electrophile were designed later but were

found to be less potent. Therefore, small molecules containing electrophile which enable them to interact with SER630 in catalytic site were designed and evaluated. 2-cyanopyrrolidine containing inhibitors like compound 3 (Novartis) showed potent enzyme inhibition with IC_{50} = 22 nM, and they were further explored for lead optimization [13], **Fig. 3**.

MATERIAL AND METHODS:

Comparison of X-Ray Co-Crystal Structures: The co-crystal structures of DPP 4 co-crystallized with 18 inhibitors were downloaded from the Protein Data Bank in .pdb file format [14, 15, 16, 17, 18, 19]. They were superimposed on each other for analysis of their binding mode in active site of DPP 4. Details of PDB files and DPP 4 inhibitors analyzed in the present work are outlined in **Table 1**. Chemical structures of DPP 4 inhibitors are shown in **Fig. 4**.

Analysis of Binding Modes: Analysis of binding modes of various bound DPP 4 inhibitors was performed using Discovery Studio Visualizer 4.1, Accelrys Inc., USA [20].

TABLE 1: PDB CODE, BOUND LIGAND AND OTHER DETAILS OF CO-CRYSTAL STRUCTURE OF DPP 4 STUDIED

S. no.	PDB	Ligand	Resolution (A°)	IC_{50} (nM)	Innovator
1	3BJM	Saxagliptin	2.36	3.37	Bristol-Myers Squibb
2	3W2T	Vidlagliptin	2.35	3.50	Novartis
3	1X70	Sitagliptin	2.10	18.00	Merck & Co
4	4PNZ	Fluoro-omarigliptin	1.90	2.20	
5	3VJK	Teneligliptin	2.49	0.37	Mitsubishi Tanabe Pharma
6	3VJL	9	2.39	5.60	
7	3VJM	10	2.10	0.40	
8	3F8S	Gosogliptin	2.43	13.00	Pfizer Inc
9	3KWF	Carmegliptin	2.40	6.80	Hoffman La Roche
10	3WQH	Anagliptin	2.85	3.30	Sanwa Kagaku Kenkyushu/Kowa
11	2ONC	14	2.55	13.00	Takeda / Furiex
12	5KBY	Trelagliptin	2.24	4.00	
13	2RGU	Linagliptin	2.60	1.00	Boehringer Ingelheim
14	4A5S	17	1.62	17.00	Argenta Discovery
15	2FJP	18	2.40	4.30	Merck & Co
16	2QT9	19	2.10	2.30	
17	2QTB	20	2.25	4.80	
18	3OPM	21	2.72	47.00	Takeda Pharmaceuticals

RESULTS AND DISCUSSION:

Analysis of PDB: Interaction between DPP 4 enzyme and ligands are represented as 2D ligand interaction diagram. These were generated by defining enzyme and inhibitor in active site using Receptor-Ligand Interaction tool available in Discovery Studio Visualizer 4.1. Details of binding modes of various DPP 4 inhibitors in active site are discussed in this section.

3W2T and 3BJM: Both Gliptins are chemically similar containing 2-cyanopyrrolidine moiety. The binding modes of these are almost similar. Pioneering DPP 4 inhibitors containing 2-cyanopyrrolidine were developed successfully. Vildagliptin and saxagliptin, respectively are bound to PDB id 3W2T and 3BJM. Both these compounds are peptidomimetic inhibitors of DPP 4. Vildagliptin is a potent and selective, DPP 4 inhibitor with antihyperglycemic properties (IC_{50}= 3.50 nM), discovered by Novartis. Saxagliptin was found to be more potent (IC_{50}= 3.37 nM) than vildagliptin with better pharmacokinetic profile. It was approved by FDA in 2009. It can be given once daily and is well tolerated in diabetic patients. Cyanopyrrolidine moieties in their structure bind to S_1 pocket and nitrile group interacts covalently with SER630 in catalytic site *via* formation of immmediate adduct. Nitrogen of the immediate makes connects with the side-chain hydroxyl of TYR547 by hydrogen bond interaction. Primary amino group of saxagliptin and secondary amino group of vildagliptin form salt bridge interaction with GLU205 and GLU206 respectively. Admantane moiety is oriented towards the S_2 subsite and the carbonyl group interacts with ASN710 *via* hydrogen bond. The hydroxyl group present on the admantyl group in vildaglipitn form hydrogen bonds with HIS126 and SER209.

Cyclopropanated cyanopyrrolidine moiety in saxagliptin enhances the chemical stability of saxagliptin towards intramolecular cyclization. Cyclopropane moiety additionally makes hydrophobic interaction with TYR666 in S_1 pocket. There is also a direct hydrogen bond interaction between hydroxyl group on admantyl group on saxagliptin with side chain of TYR547, **Fig. 5**. These two additional interactions of saxagliptin with DPP 4 active site may contribute to its higher potency.

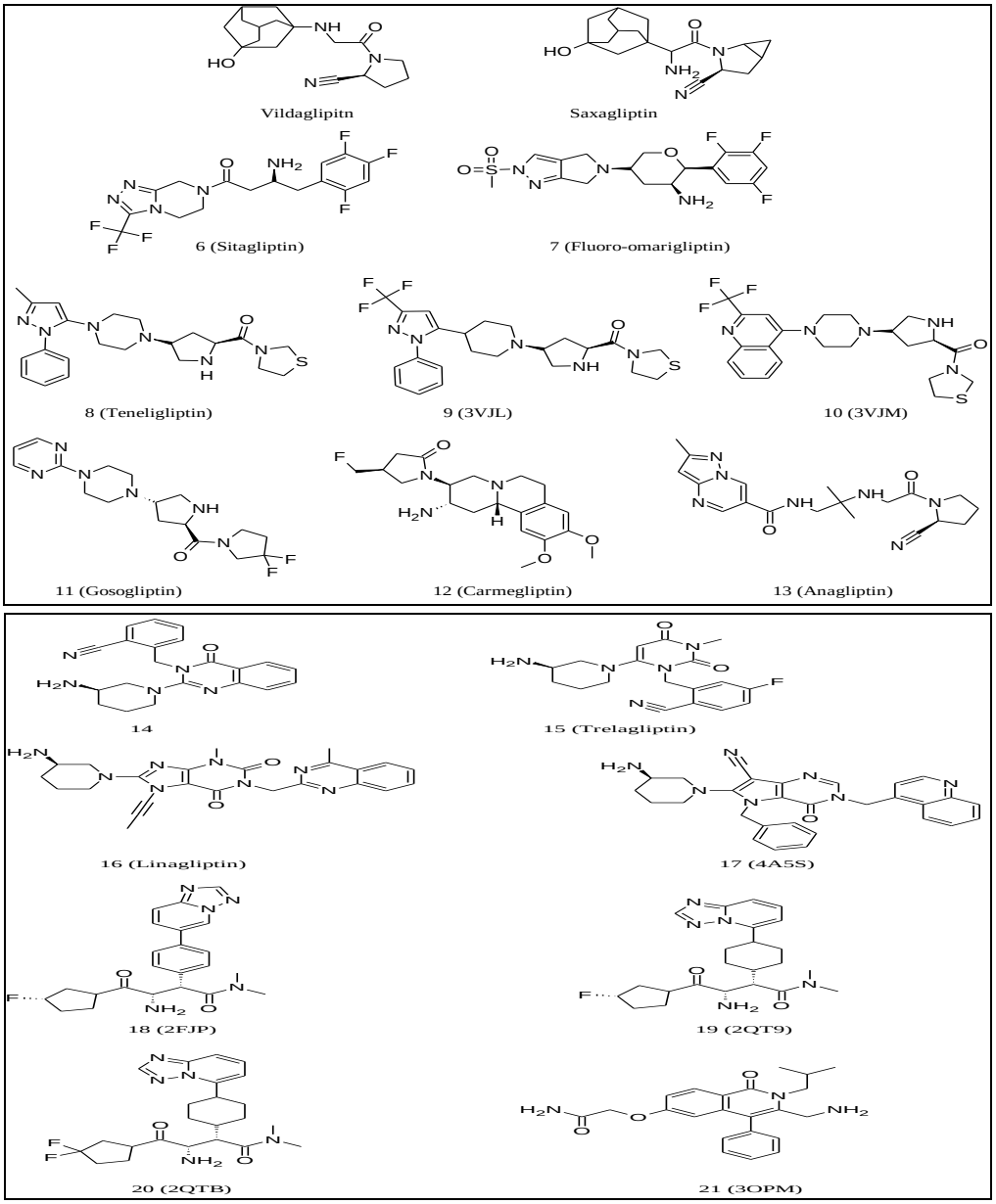

FIG. 4: VARIOUS CO-CRYSTALLIZED LIGANDS OF DPP 4

1X70 and 4PNZ: Sitagliptin and fluoro-omarigliptin are respectively bound to crystal structure 1X70 and 4PNZ. Sitagliptin was invented by Merck Inc and it contains triazolopiperazine ring system fused with *β*- amino acid amide. It is a potent, orally active DPP 4 inhibitor (IC_{50} = 18 nM), **Fig. 4**. Fluoro-omarigliptin is a long acting DPP 4 inhibitor, which is also developed by Merck Inc. It is a competitive, reversible inhibitor of DPP 4 (IC_{50} = 1.6 nM) and is more potent than sitagliptin. Triazolopiperazine moiety in sitagliptin is replaced by pyrrolopyrazole moiety in fluoroomarigliptin. An additional tetrahydropyran ring incorporated in fluoroomarigliptin renders it rigid as compared to sitagliptin. Both ligands make similar contacts with active site of DPP 4. Fluorinated phenyl ring present in both inhibitors interacts with S_1 hydrophobic pocket and it is

stacked between tyrosine residues (TYR662 and TYR666) and histidine (HIS740). Primary amino group in both ligands interact with GLU205 via salt bridge interaction and hydrogen bonding interaction with oxygen atom of hydroxyl group of TYR662. The fused triazolopiperazine and pyrrolopyrazole moieties of both ligands bind to S_2 and S_2 extensive site, making π–π stacking interaction with the side chain of PHE357. The trifluoromethyl group situated on triazole ring in sitagliptin interacts with the side chains of ARG358 and SER209, **Fig. 6.** Analogs of sitagliptin which lack trifluoromethyl group showed reduced potency as compared to sitagliptin, highlighting the importance of ARG358 and SER209 in binding of DPP 4 inhibitors. The pocket where the trifluoromethyl moiety binds is quite tight. Potency of analogs was reduced when trifluoromethyl moiety was replaced by larger groups. *2R, 3S, 5R* stereoisomer of fluoro-omarigliptin is the most potent of all 8 possible stereoisomer due to presence of three chiral centers. 2, 3, 5-trifluorophenyl and 3- amino group should be trans with respect to each-other for optimal binding with DPP 4. It has half-life of 120 h making it suitable for once a week administration for management of T2D.

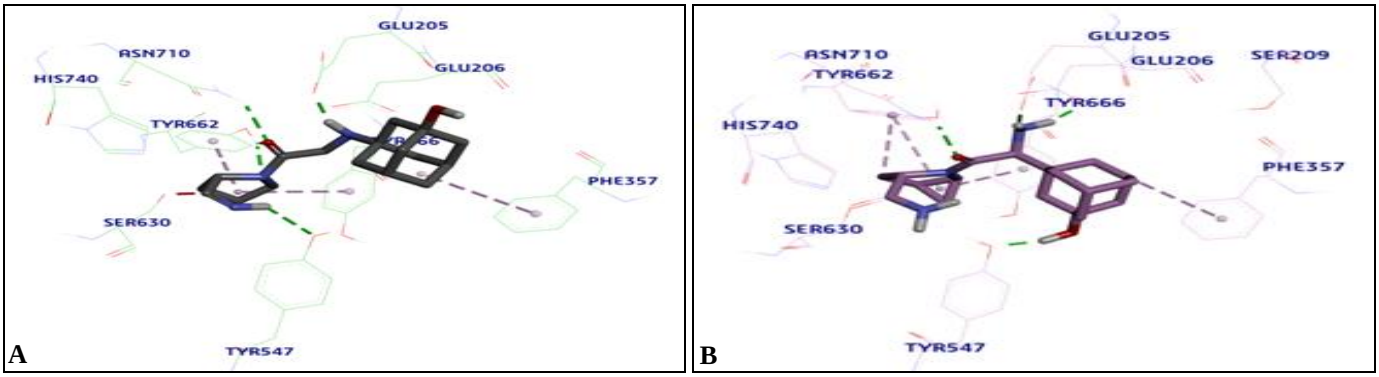

FIG. 5: BINDING MODE OF (A) SAXAGLIPTIN AND (B) VILDAGLIPTIN IN DPP 4 ACTIVE SITE

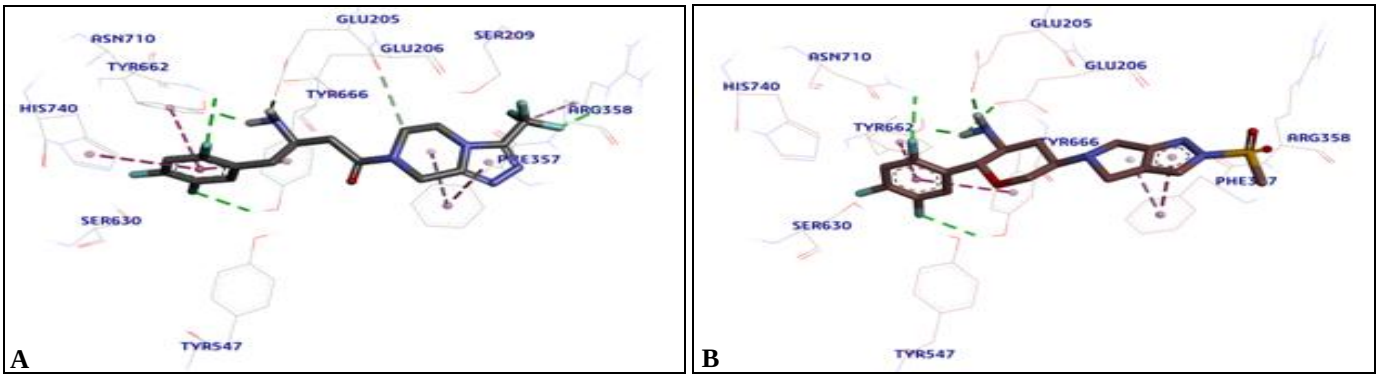

FIG. 6: BINDING MODE OF (A) SITAGLIPTIN AND (B) FLUOROOMARIGLIPTIN

3VJK, 3VJM, and 3VJL: Teneligliptin and compounds 9 and 10 **Fig. 4** of Mitsubishi Tanabe Pharma are bound to crystal structures 3VJK, 3VJL and 3VJM respectively. They all belong to the same chemical class of L-prolylthiazolidines substituted with bicyclic heteroarylpiperazines at *γ* –position. Teneligliptin (MP513) is an orally active, highly potent, selective, long acting DPP 4 inhibitor. An X-ray co-crystal structure of teneligliptin revels that five rings present in structure are accommodated nicely and fit well into the active site of DPP 4 and assume "J-shape". S_1 hydrophobic pocket is occupied by thiazolidine moiety. The salt bridge to GLU206 is retained by the secondary amino group of the proline moiety; while, the carbonyl oxygen interacts with ASN710 via hydrogen bond. The pyrazolyl ring extends towards S_2 extensive site and stacked against PHE357 making π-π interactions and piperazinyl ring also interacts with PHE357 *via* CH–π interaction, **Fig. 7A**. In the S_2 extensive subsite, the carbon at 4-position of the phenyl ring makes a weak hydrogen bond. It is very close to the carbonyl oxygen of the main chain of VAL207. S_2 extensive subsite that accommodates the phenyl ring is very specific because any substitution on

para position of ring and replacement of phenyl by cyclohexyl ring does not result in favorable interaction. This could be explained by reduced activity of such analogs. The hydrophobic interaction of phenyl ring of teneligliptin in S_2 extensive site is stronger than that of the trifluoromethyl substituent of sitagliptin, this possibly explains the enhanced potency of teneligliptin (IC_{50} = 0.37 nm). Compound 9, and 10 bind in similar manner as teneligliptin to DPP 4 active site whereby pyrzole and quinoline scaffold of compound 9 and 10 occupy S_2 extensive sites **Fig.7B** and **7C**. Compound 9, contains central piperidine ring in place of piperazine ring in teneligliptin.

It is bound to crystal structure 3VJL forms similar types of interactions with the S_1 hydrophobic subsite and proline moiety makes hydrogen bonding interactions *via* the secondary amino group. However, 1-phenylpyrazol-2-yl part of the compound is orientated in completely different way from that of the piperazinyl ring of teneligliptin. The phenyl group of teneligliptin forces side chain of ARG358 to be slightly away, making S_2 extensive subsite assessable. Teneligliptin was found to inhibit increase of plasma glucose levels after an oral glucose load in Zucker fatty rats at 0.3 mg/kg dose. Teneligliptin has been approved for T2D in Japan. In India, it was approved for marketing in 2015.

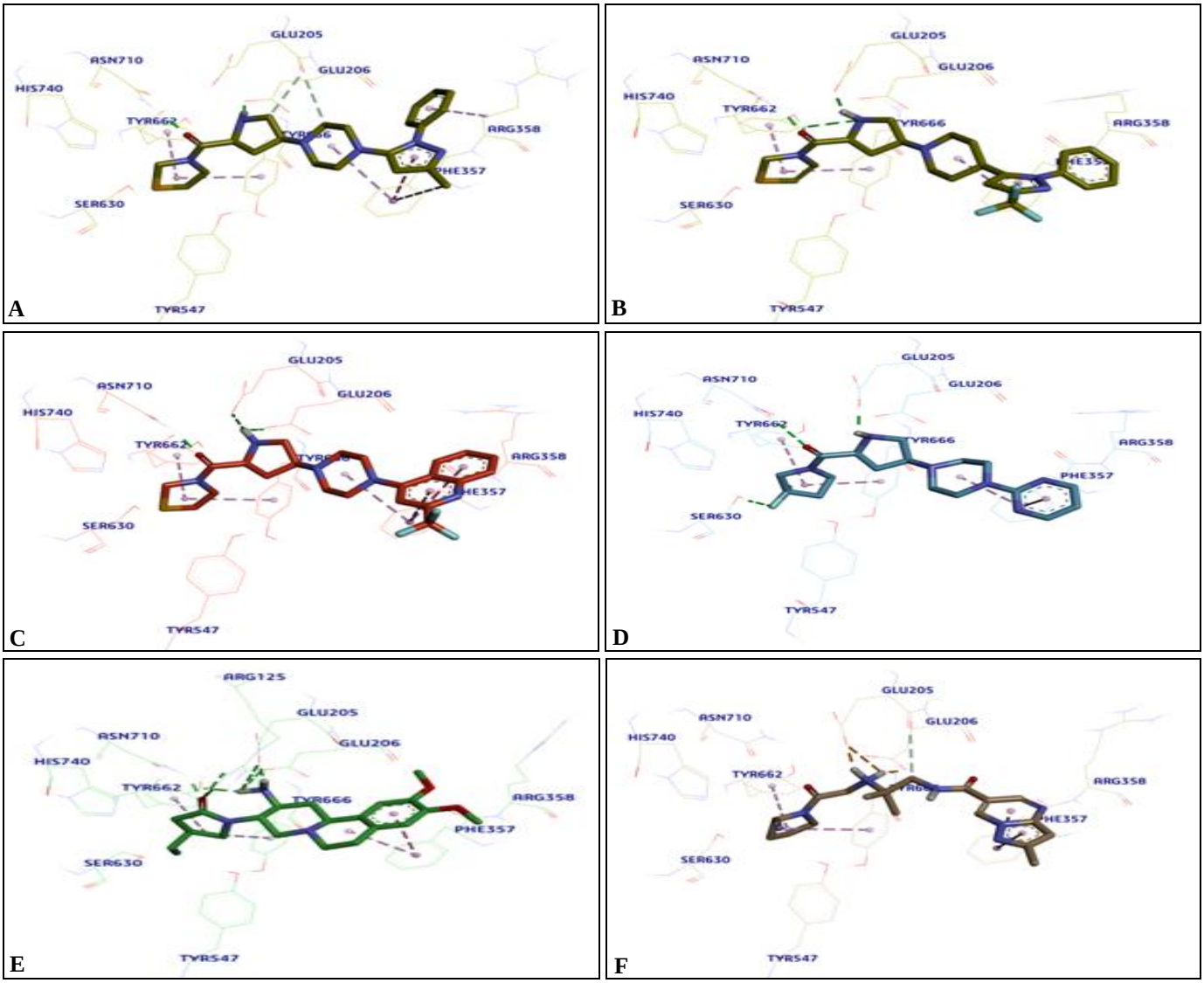

FIG. 7: BINDING MODE OF (A) TENELIGLIPTIN AND (B) LIGAND 9, BINDING MODE OF (C) LIGAND 10 AND (D) GOSOGLIPTIN, BINDING MODE OF (E) CARMEGLIPTIN AND (F) ANAGLIPTIN

3F8S: Gosogliptin is bound to crystal structure 3F8S of DPP 4. It is a derivative of 4-substituted proline amide. Discovered and developed by Pfizer Inc., it is having IC_{50} value of 13 nM. It showed

good pharmacokinetic profile and oral bio-availability in preclinical studies including less plasma protein binding. The co-crystal structure of gosogliptin with human DPP 4 reveals that difluoropyrrolidide moiety exclusively forms hydrophobic interactions with TYR631, SER630, VAL 656, TYR666 and TYR662 in S_1 subsite. The secondary amine of the pyrrolidine ring makes a salt bridge with GLU205 and hydrogen bond with ARG125 amino acid residues. The pyrimidine ring makes π- π stacking interaction with PHE357. One of the fluorine atoms on the pyrrolidines ring is close to SER630 and TYR632, which permit a hydrogen bond formation with either the side chain or the main chain of residues, **Fig. 7D**.

3KWF: Carmegliptin is bound to crystal structure 3KWF of DPP 4. It is chemically aminobenzo [a] quinolizine derivative with non-aromatic substituents in S_1 binding pocket. It has an IC_{50} value of 6.8 nM. It was discovered by F. Hoffmann-La Roche Ltd. It was the first DPP 4 inhibitor to contain tricyclic scaffold and pyrrolidone scaffold as S_1 recognition moiety. Crystal structure analysis reveals that the *p*-fluoromethyl substituent is oriented towards the lipophilic S_1 subsite which is essential for affinity. Carbonyl oxygen of lactam forms hydrogen bond with amido -NH of ASN710, while the guanidine moietyof ARG125, it interacts through a cation-dipole type of interaction, which would mimic the amide carbonyl present in substrates and derivatives of substrate. Primary amino group interacts with GLU205 and GLU206 dyad through formation of salt bridge and through hydrogen bonds to TYR662, **Fig. 7E**. Tetrahydroisoquioline moiety fits into S_2 and S_2 extensive pocket and makes π-π stacking interaction with PHE357. Carmegliptin has very good pharmacokinetic properties, with no or minimal metabolism and balanced excretion *via* renal and hepatic routes. It was found to be a safe oral anti-diabetic agent with once daily administration in clinical phase 1 and 2 studies.

3WQH: Anagliptin belongs to the pyrazolo-pyrimidine chemical class and was discovered by Sanwa Kagaku Kenkyushu. It is another potent DPP 4 inhibitor with IC_{50} of 3.3 nM. It has 2-cyanopyrrolidine moiety in its structure that binds to S_1 pocket and makes hydrophobic interactions with TYR631, TRP659, TYR662, and TYR666. Secondary amino group makes salt bridge interaction GLU205 and GLU206. The fused pyrazolopyrimidine ring system binds to S_2 and S_2 extensive site, making $\pi-\pi$ stacking with the side chain of PHE357, **Fig. 7F.**

2ONC and 5KBY: Compound 14 and Trelagliptin are bound to crystal structures 2ONC and 5KBY respectively. Both gliptins are selective DPP 4 inhibitor having IC_{50} of 13 nM and 4 nM respectively. Trelagliptin is a fluorinated analogue of aloglptin. Aminopiperidine motif of both ligands forms salt bridges to GLU205 and GLU206. Cyanobenzyl group at N-3fills the S_1 hydrophobic pocket and is involved in $\pi-\pi$ stacking interaction with TYR662. Additionally nitrogen atom of –CN group makes H-bond with ARG125, **Fig. 8A and B**. The carbonyl group at C-4 interacts with the backbone NH of TYR631 *via* hydrogen bonding interaction. Quinazolinone and uracil ring of compound 14 and trelagliptin respectively interact with TYR547 *via* π- π stacking. The presence of a fluorine atom at position 5 of the cyanobenzyl group in trelagliptin brings it in close proximity to TRP659 and TYR631 for and provides additional attraction between fluorine and hydrogen atoms of TRP659 and TYR631, which explains increased activity of trelagliptin as compared to compound 14. Trelagliptin was launched in Japan in 2015.

2RGU and 4A5S: Linagliptin and compound 17 are bound to crystal structures 2RGU and 4A5S respectively. Both ligands occupy DPP 4 active site in similar fashion. Linagliptin is derivative of xanthine moiety and compound 17 is a deazahypoxanthine derivative. IC_{50} values of linagliptin and compound 17 are 1 nM and 17 nM respectively. Their co-crystal structure reveals that amine group on the piperidine ring forms hydrogen bonds with GLU205 and TYR662. Xanthine ring system and deazahypoxanthine ring systems were found to be involved in $\pi-\pi$ interaction of aromatic ring of TYR547. TYR547 moves from its position in the apo structure for interaction to take place when bound to ligands. It is a very good example of conformational change induced by inhibitor binding to DPP 4. The carbonyl oxygen in both ligands interacts with NH of TYR631 through hydrogen bond.

Both rings in quinazoline of linagliptin are found to make *π-π* stacking with indole ring of TRP629, while only pyridine portion of quinoline in ligand 17 makes π-π stacking with pyrrole ring of indole in TRP629 in S'$_2$ subsite, **Fig. 8C** and **D**.

Higher potency of linagliptin as compared to other DPP 4 inhibitors can be attributed to this unique interaction in S'$_2$ subsite. It is suitable for once a day administration for the management of T2D owing to its longer duration of action.

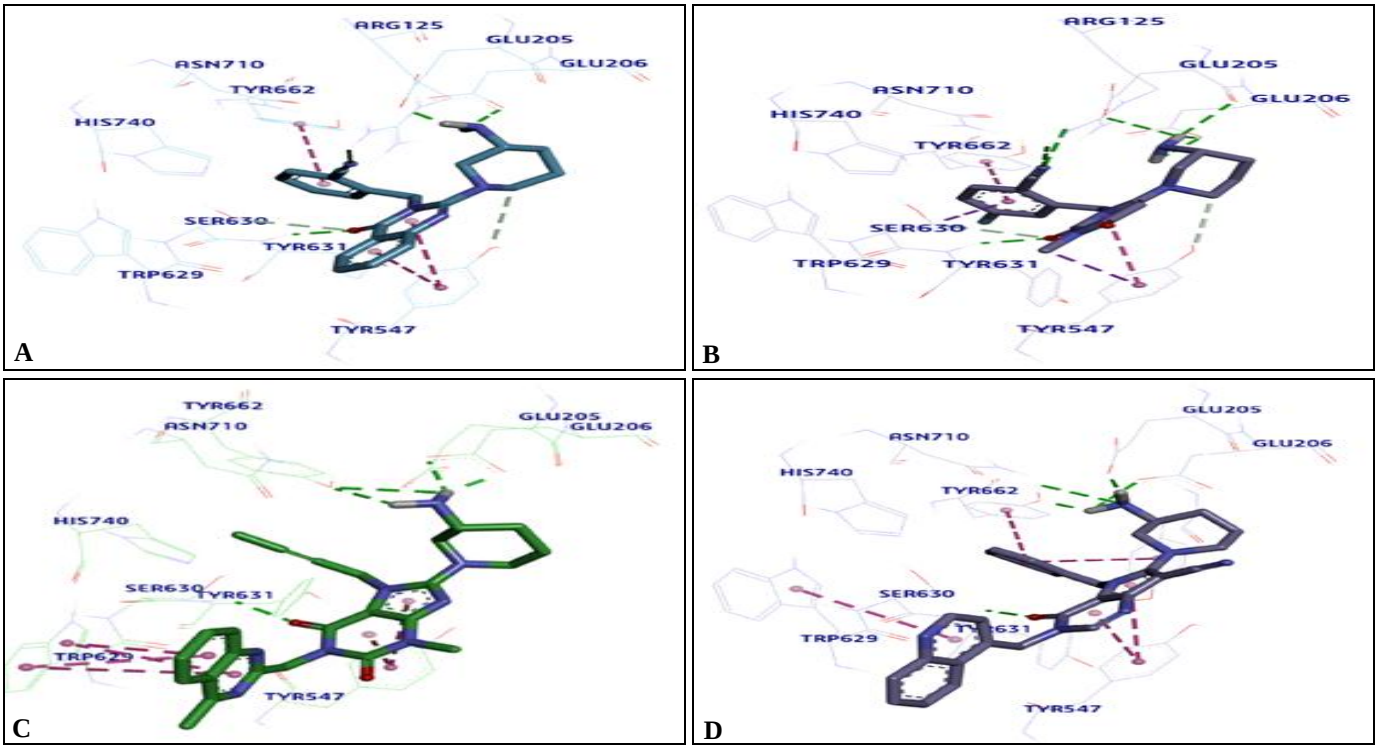

FIG. 8: BINDING MODES OF (A) LIGAND 14, (B) TRELAGLIPTIN, (C) LINAGLIPTIN AND (D) LIGAND 17

2FJP, 2QT9 and 2QTB: The inhibitors in the 2FJP, 2QT9 and 2QTB crystal structures are 18, 19 and 20 respectively. They have triazolopyridine fused heterocyclic ring system that binds to S_2 and S_2 extensive site while fluoropyrrolidine moiety is placed optimally in S_1 subsite. 2FJP is *β*-substituted biarylphenylalanine amide derivative while 2QT9 and 2QTB are *β*- substituted phenycyclo- hexylalanine amide derivatives. The pyrrolidine moiety present in inhibitors occupies the S_1 hydrophobic pocket and interacts with TYR662 and TYR666. Carbonyl groups attached to the pyrrolidines are involved in hydrogen bonding with side chain of ASN710. The primary amino groups of inhibitors interact with the side chain of TYR662 *via* hydrogen bond and with GLU205 through salt bridge interaction. Carbonyl group of *N*, *N*-dimethyl amide makes a hydrogen bond with side chain of TYR547. Central phenyl and cyclohexyl ring in inhibitors 18 and 19 and 20 respectively extends triazolopyridine heterocycles close to ARG358. The fused triazole ring interacts with PHE357 by forming π-π stacking interaction. Triazole nitrogen accepts hydrogen bond from side chain of ARG358, **Fig. 9A, B** and **C**. The pyrrolidine moieties in inhibitors in the structures 2FJP, 18 (IC_{50} = 4.3 nm) and 2QT9, 19 (IC_{50} = 2.3 nm) have monofluoro substitution and in structure 2QTB, 20 (IC_{50} = 4.8 nm) have difluoro substitution.

3OPM: Ligand bound to crystal structure 3OPM is compound 21, an isoquinolone derivative. It is an inhibitor having IC_{50} value of 47 nM. 3-aminomethyl group interacts with the GLU205 and TYR662. Bulky portion of 2-isobutyl group forms CH-π interaction with aromatic ring of PHE357. 4-phenyl ring is oriented towards hydrophobic S_1 pocket. 6-carbamoylmethoxy group extends Van der Waals interactions with TYR547, and NH_2 group of amide makes weak contact with TRP629. 6-carbamoylmethoxy is placed deep in S_1 pocket. Hydrophobic residues VAL546, TYR547, TRP629, and hydrophilic residues LYS554, ASP545 make up the pocket, **Fig. 9D**. Isoquinolone nucleus present in this ligand mimics a P_2–P_1 dipeptidyl substrate for DPP 4 inhibition.

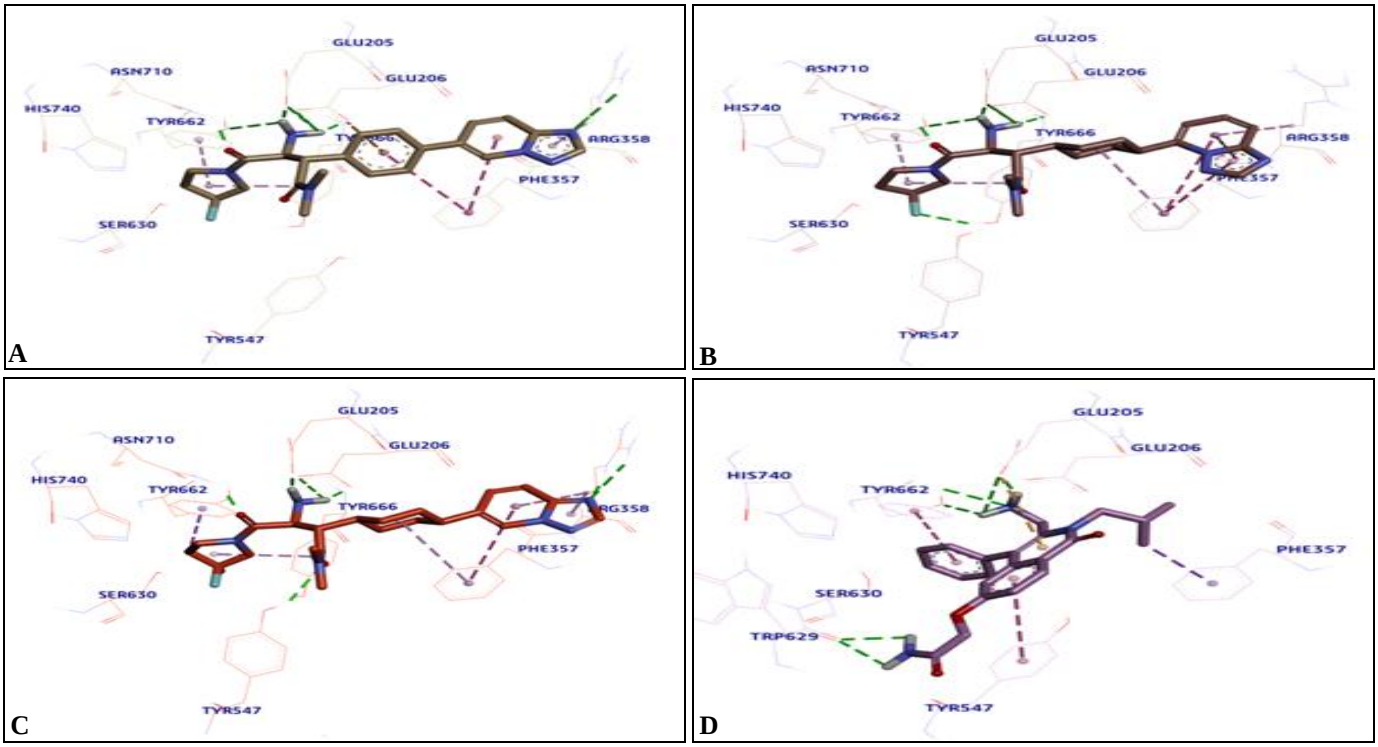

FIG. 9: BINDING MODE OF LIGANDS BOUND TO CRYSTAL STRUCTURE (A) LIGAND 18, (B) LIGAND 19, (C) LIGAND 20 AND (D) LIGAND 21

CONCLUSION: Different inhibitors may interact with different subsites of enzyme active site but key features for binding of all DPP 4 inhibitors are hydrophobic interactions with TYR662, TYR666, hydrogen bond interaction with TYR547, and ASN710 in S_1 subsite. Salt bridge interaction with N-terminal recognition site of enzyme, *i.e.*, GLU205 and/or GLU206 is essential for enzyme inhibition, which is found in all co-crystal structures. In S_2 site hydrophobic interaction with PHE357, electrostatic interaction with ARG358 and hydrogen bond interaction also takes place between DPP 4 and some inhibitors.

Some inhibitors bind to S_2 extensive region contributing to their potency as well as higher selectivity over other isoforms of DPP and related enzymes. This is due to the fact that the hydrophobic S_2 extensive site is not clearly differentiated in other members of the family including DPP 8, DPP 9 and fibroblast activation protein. Designing of DPP 4 inhibitors which bind to S_2 extensive site can increase potency and selectivity over other related prolyl peptidases. Common generalized binding mode for gliptins may be proposed based on analysis of crystal pharmacophoric features should be considered while designing new DPP 4 inhibitors, *i.e.*, an aliphatic primary or secondary amine for salt bridge or hydrogen bonding interaction with GLU205 and/ or GLU206, hydrophobic or aromatic group occupying S_1 subsite and bulky aromatic group that can orient towards S_2 subsite for optimum binding, **Fig. 10**.

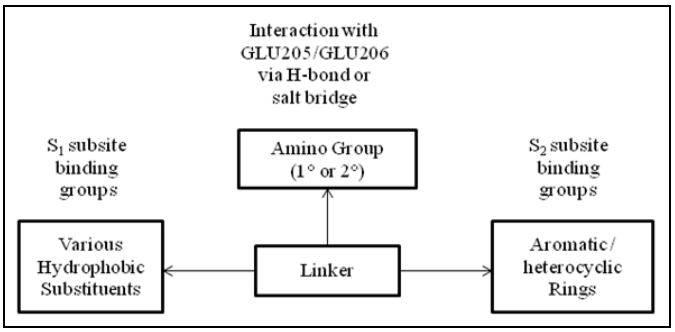

FIG. 10: PROPOSED PHARMACOPHORIC REQUIREMENTS FOR DESIGN OF NEW DPP 4 INHIBITORS

Some simple rules can be advised for identification and design of potent DPP 4 inhibitors in different virtual screening experiments in light of analysis of binding modes of co-crystal structures of DPP 4 with its differing synthetic inhibitors. Fragment, heterocyclic or other cyclic rings in inhibitors can involve S_1 subsite and make the most number of π-π association or potentially hydrophobic contacts, **Fig. 10**. In the N-terminal recognition region, a positively charged aliphatic 1° or 2° amino group

would act as a hydrogen bond donor and it can make salt-bridge contact with the GLU205 and the GLU206 dyad and a hydrogen bond with the TYR662 hydroxyl functionality. Aromatic and/ or heterocyclic scaffolds are critical for π-π stacking interaction with PHE358 in S_2 extensive subsite. There might be 2-3 carbon atoms interface between essential amino groups associating with heterocyclic scaffolds. Substitution with an electronegative atom or group on aromatic and/ or heterocyclic scaffolds occupying S_2 extensive site is likely to orient the molecule towards ARG358 for making electrostatic interaction in S_2 extensive site. Interaction of a compound with S_2 extensive region contributes to potency for DPP 4 inhibition. For compounds binding to S'_1 and S'_2 subsites, placement of moieties that can stack against aromatic rings of TYR547 and TRP629 (π-π stacking) would enhance strength of inhibitors.

A detailed study of the existing molecules interacting at the same target can guide the researcher identify key features required for successful discovery of active and potent molecules for the treatment of any disease.

ACKNOWLEDGEMENT: The authors are also thankful to Accelrys Inc., USA for providing Discovery Studio Visualizer 4.1 for performing computational analysis for the present research work.

CONFLICT OF INTEREST: Nil

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How to cite this article:
Upadhyay J and Gajjar A: Analysis of crystal structures of dipeptidyl peptidase 4 (DPP 4) co-crystallized with diverse inhibitors. Int J Pharm Sci & Res 2018; 9(10): 4460-71. doi: 10.13040/IJPSR.0975-8232.9(10).4460-71.