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Design, synthesis and biological evaluation of novel pyrazolo-pyrimidinones as DPP-IV inhibitors in diabetes ☆

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ABSTRACT

We report the design, synthesis, biological activity and docking studies of series of novel pyrazolo[3,4-*d*] pyrimidinones as DPP-IV inhibitors in diabetes. Molecules were synthesized and evaluated for their DPP-IV inhibition activity. Compounds **5e**, **5k**, **5o** and **6a** were found to be potent inhibitors of DPP-IV enzyme. Amongst all the synthesized compounds, 6-methyl-5-(4-methylpyridin-2-yl)-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (**5k**) was found to be the most active based on in vitro DPP-IV studies and also exhibited promising in vivo blood glucose lowering activity in male *Wistar* rats.

Diabetes mellitus is recognized as world's major health problem affecting millions of people. It is characterized by abnormally high levels of plasma glucose that occurs due to the variable degrees of insulin resistance produced by the pancreatic β cells and dysfunction of β cells.[1]

Insulin secretion enhancement by pancreatic islet β-cells has been a major goal for the treatment of Type 2 Diabetes[2] but these treatments have side effects viz. severe hypoglycemia, weight gain, edema and nausea. In the area of diabetes, researchers have found various novel targets. One such target that has been found very effective in modulating the disease physiology is Dipeptidyl Peptidase-IV (DPP-IV) enzyme. DPP-IV is the major enzyme involved in the regulation of incretin hormone. It is a serine protease that cleaves the N-terminal dipeptide with a preference for L-proline or L-alanine at the penultimate position.[3] DPP-IV inhibitors stabilize endogenous GLP-1 and induce insulin secretion in a glucose-dependent manner in contrast to insulin tropic agents which release insulin in glucose independent manner and manifest hypoglycemia as a side effect.[2] The inhibition of DPP-IV results in higher levels of circulating active GLP-1 and improves glucose tolerance.[4]

DPP IV belongs to a family of enzyme that contains several closely related members like DPP-II, DPP-VIII, DPP-IX and FAP-α.[5] In order to inhibit DPP-IV enzyme, selectivity is of prime importance. Currently available 'Gliptins' namely, Sitagliptin, Vildagliptin, Saxagliptin, Alogliptin, Linagliptin, and Gemigliptin[5–11] are associated with several side effects including nasopharyngitis, headache, nausea, hypersensitivity, skin reactions and pancreatitis. Pancreatitis is the most severe side effect of DPP-IV inhibitors.[5] The side-effects observed with the 'Gliptins' are specific to the molecules which inhibit other substrates rather than DPP-IV per se. To avoid the side effects associated with the inhibition of other members of DPP family like DPP-II, DPP-VIII, and DPP-IX it becomes necessary to design selective inhibitors of DPP-IV enzyme.

Large numbers of structurally diverse molecules were designed against DPP-IV enzyme inhibition. We report here new chemical classes of DPP-IV inhibitors containing fused pyrazolo-pyrimidinones. As mentioned earlier, DPP-IV cleaves at proline residue of N-terminal site. Incorporation of a small electron rich heterocycle at the proline site has ability to provide reactive center to accept DPP-IV's catalytic activity for its inhibition, thus it can impart the potency of molecule.[12] Pyrazole scaffold has electron rich 'N' containing center in its structure. Thus it has ability to serve as proline mimics and expected to cleave DPP-IV enzyme.[13] In addition, several reported molecules containing pyrazole moiety showed potent inhibition of DPP-IV action.[5,14,15] Whereas pyrimidine is a privileged structure and important for its biological activity.[16] Ring 'N'

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of pyrimidinone has ability to form H-boding interaction with Arg125 present in S2 pocket of DPP-IV enzyme.[17] Pyrimidinone has 10,000 fold selectivity for DPP-IV over DPP-VIII and DPP-IX as '—C=O' forms H-bonding interaction with Tyr631.[2,12,18] This special chemical feature imparts its selectivity towards other DPP's. Two pharmacophores, pyrazoles[13] and pyrimidinones[17] both independently act on DPP-IV enzyme so combining these two pharmacophores in a single molecule may lead to a potent and selective inhibitor of DPP-IV enzyme (see Fig. 1).

As depicted in Scheme 1, synthesis of pyrazolo[3,4-*d*]pyrimidinone based DPP-IV inhibitors (**5a**–**5t**) and (**6a**–**6e**) were synthesized. Pyrazole synthesis was carried out by reaction of

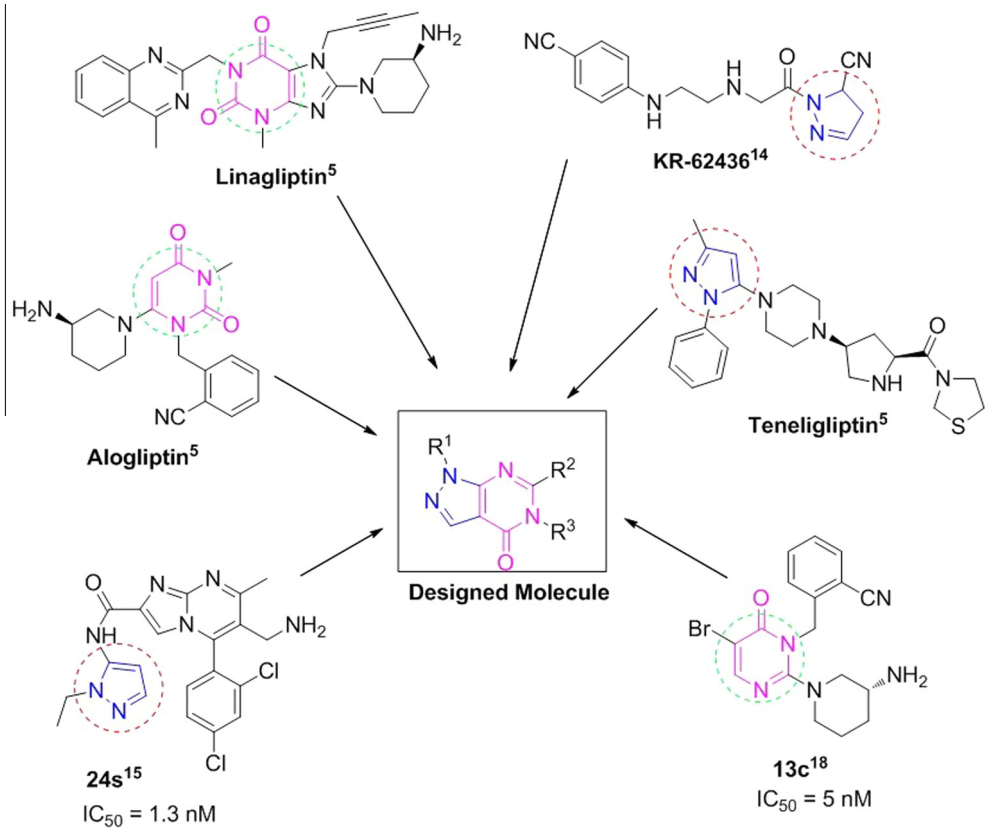
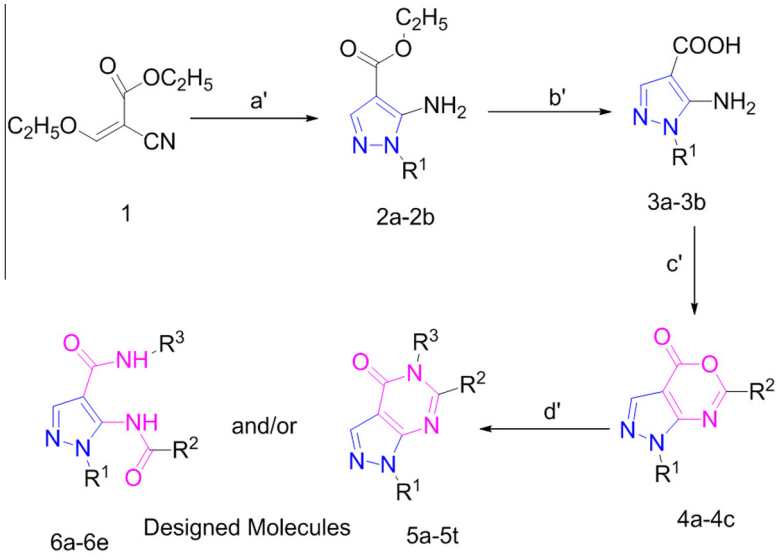


Figure 1. Designed pyrazolo-pyrimidinones. (Refer Table 1 for specific substitutions R^1, R^2 and R^3).



Scheme 1. Synthesis of pyrazolo-pyrimidinones. R^1 = -phenyl, -4-methoxyphenyl. R^2 = -methyl, -trifluoromethyl. R^3 = -phenyl, -4-fluoromethyl, -4-ethylphenyl, -3-chlorophenyl, -4-methyl-pyridine-2-yl, -5-bromo-pyridine-2-yl-5-nitro-pyridine-2-yl, -4-(4-chlorophenyl)thiazol-2-yl, -3-methyl-thiophene-2,4-dicarboxylate-5-yl, etc. Reagents and conditions: (a′) Hydrazines (1.0 equiv), ethanol, 80 °C, 6 h, (b′) NaOH (3.0 equiv), ethanol, 80 °C, 7 h, (c′) anhydrides (6.0 equiv), tetrahydrofuran, 70 °C, 10 h, (d′) amine derivatives (2.0 equiv), dimethylformamide, 150 °C, 15 h.

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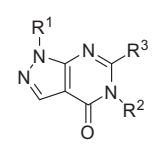
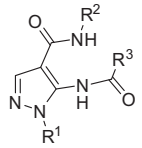
Table 1
List of synthesized compounds (compounds **5a** to **5t** and **6a** to **6e**) and its DPP-IV inhibitory activity



Compounds 5a to 5t



Compounds 6a to 6e

Code	R^1	R^2	R^3	% yield[a]	IC_{50}[b] (μM)
5a	Phenyl	Phenyl	Methyl	70	ND
5b	Phenyl	4-Fluorophenyl	Methyl	60	ND
5c	Phenyl	4-Ethylphenyl	Methyl	70	ND
5d	Phenyl	3-Chlorophenyl	Methyl	85	ND
5e	**Phenyl**	**2,4,6-Trimethylphenyl**	**Methyl**	**60**	**1.32 ± 0.09**
5f	4-Methoxyphenyl	4-Fluorophenyl	Methyl	60	ND
5g	Phenyl	Benzyl	Methyl	55	ND
5h	Phenyl	2,4-Difluorophenyl	Methyl	50	ND
5i	**Phenyl**	**Pyridine-2-yl**	**Methyl**	**54**	**4.0 ± 0.02**
5j	**Phenyl**	**Pyrimidine-2-yl**	**Methyl**	**60**	**24 ± 0.13**
5k	**Phenyl**	**4-Methylpyridine-2-yl**	**Methyl**	**49**	**1.06 ± 0.09**
5l	**Phenyl**	**5-Bromopyridine-2-yl**	**Methyl**	**48**	**11 ± 0.1**
5m	Phenyl	5-Nitropyridine-2-yl	Methyl	41	ND
5n	4-Methoxyphenyl	Pyridine-2-yl	Methyl	46	ND
5o	**4-Methoxyphenyl**	**4-Methylpyridine-2-yl**	**Methyl**	**52**	**2.23 ± 0.02**
5p	4-Methoxyphenyl	5-Bromopyridine-2-yl	Methyl	38	ND
5q	4-Methoxyphenyl	5-Nitropyridine-2-yl	Methyl	55	ND
5r	4-Methoxyphenyl	4-(4-Chlorophenyl)thiazol-2-yl	Methyl	51	100 ± 0.03
5s	Phenyl	3-Methylthiophene-2,4-dicarboxylate-5-yl	Methyl	58	ND
5t	4-Methoxyphenyl	3-Methylthiophene-2,4-dicarboxylate-5-yl	Methyl	42	ND
6a	**Phenyl**	**4-Fluorophenyl**	**Trifluoromethyl**	**70**	**1.25 ± 0.04**
6b	Phenyl	4-Chlorophenyl	Trifluoromethyl	75	ND
6c	Phenyl	5-Bromopyridine-2-yl	Methyl	35	ND
6d	Phenyl	4-(4-Chlorophenyl)thiazol-2-yl	Methyl	45	ND
6e	Phenyl	Pyridine-2-yl	Methyl	25	ND
Sitagliptin					**0.018 ± 0.001**[20]

ND not determined because of their low inhibitory activities.
Potent DPP-IV inhibitors are given in bold.
[a] Isolated yield.
[b] IC_{50} value represents the concentration of each compound resulting in 50% inhibition.

hydrazine derivatives with ethylethoxymethylene cyanoacetate (**1**) to obtain substituted ethyl 5-amino-1*H*-pyrazole-4-carboxylate (**2**).[19] Then compounds (**2**) were hydrolyzed to get substituted 5-amino-1*H*-pyrazole-4-carboxylic acids (**3**). Acids then reacted with anhydride derivatives to obtain substituted pyrazolo[3,4-*d*][1,3] oxazin-4(1*H*)-ones (**4**) which on condensation with different amines produced a series of various 1*H*-pyrazolo[3,4-*d*]pyrimidin-4 (5*H*)-one derivatives (**5**). Uncyclized product of this reaction was isolated (**6**) and also checked for its DPP-IV inhibition. All the compounds were purified by column chromatography and characterized by various spectroscopic techniques (^{1}H NMR and ^{13}C NMR). Purity of the compounds was checked by HPLC chromatogram (see Supplementary data for spectral data).

The in vitro DPP-IV inhibition was determined in compounds (**5a** to **5t**) and (**6a** to **6e**). All the molecules were screened for their DPP-IV enzyme inhibition activity using colorimetric assay. Compounds were incubated with human recombinant DPP-IV enzyme and Gly-Pro-*p*-nitroanilide substrate.[20] Cleavage of Gly-Pro-*p*-nitroanilide and formation of *p*-nitroaniline was measured by using micro-plate reader (Biotek, India). Compounds **5e**, **5i**, **5j**, **5k**, **5l** and **5o** and **6a** showed DPP-IV inhibition activity in micro molar range. DPP-IV inhibition varied across all the tested compounds depending on their substitution. Trimethyl phenyl group in compound **5e**, 4-methylpyridine group in compound **5k** and compound **5o** showed potent inhibition of DPP-IV enzyme (Table 1). The activity was quantified in terms of percentage inhibition of DPP-IV enzyme and was found to be 82%, 81%, 75% and 72% for compounds **5k**, **5o**, **5e** and **6a**, respectively, at 100 μM concentration. IC_{50} of these compounds were found to be 1.06 μM for **5k**, 2.23 μM for **5o** and 1.32 μM for **5e** and 1.25 μM for **6a**.

Molecules which showed promising activity from in vitro studies were tested for their cytotoxicity on normal cells using isolated rat macrophages.[21] None of them showed cytotoxicity towards normal cells. Further compounds were evaluated for their in vivo efficacy in male *Wistar* rats. All experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee prior to initiation of the experiment (Registration No. PERD/IAEC/2013/0011).

A combination of high-fat-diet and low dose of streptozotocin (dose: 40 mg/kg) was administered to 6–8 weeks old male *Wistar* rats (200–250 g body weight) for induction of hyperglycemia.[22] The animals were divided in 6 groups ($n = 6$), normal control group, disease control group, positive control group (Sitagliptin), compound **5e** test group, compound **5k** test group and compound **6a** test group. The dose of sitagliptin was 10 mg/kg whereas the test compounds were dosed at 50 mg/kg bodyweight. All the compounds were administered orally, as a suspension in 0.2% agar. Blood glucose level (as shown in Fig. 2) was measured by using automated biochemical analyser (Em360, Transasia, Germany). Blood glucose lowering was observed in all animals treated with the test molecules compared to disease control animals. Blood glucose level decreased in disease control group from 515.0 mg/dl to 136.4 mg/dl for sitagliptin, 162.6 mg/dl for **5e**, 198.5 mg/dl for **5k** and 193.2 mg/dl for **6a** treated animals after 15 days of treatment.

To see the possible interactions of the newly synthesized ligands with the DPP-IV enzyme, docking study was carried out

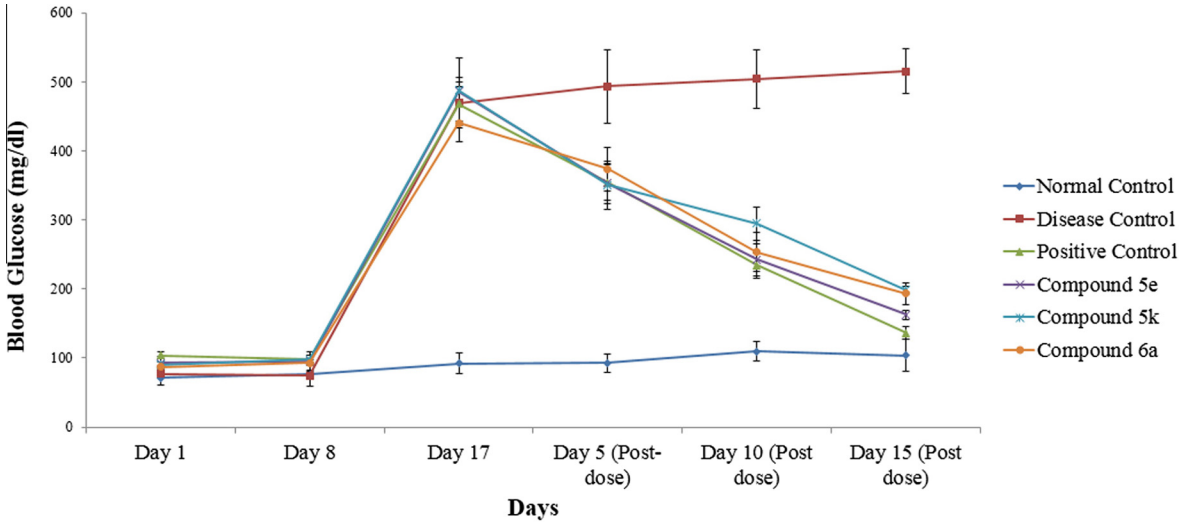


Figure 2. In vivo results of blood glucose level. Statistical analysis was done by One-way ANOVA. Data shown are mean ± SEM ($n = 6$) $^{*}p < 0.05$. Normal control: vehicle. disease control: streptozotocin (40 mg/kg) + high-fat-diet. Positive control: sitagliptin (10 mg/kg). Compound **5e**: dose (50 mg/kg), compound **5k**: dose (50 mg/kg), compound **6a**: dose (50 mg/kg).

using surflex dock module of SYBYL X-2.0. The X-ray structure of the DPP-IV enzyme (PDB ID: 2RGU) was obtained from the protein databank and the protein structure was prepared. For docking study, the ligands were geometrically optimized and Gasteiger–Huckel charges were assigned to the structure. The pyrazolo-pyrimidinone group of the derivatives docked into the binding site pocket and compared with the standard drug sitagliptin (Fig. 3).

Figure 3A illustrates an overlay of the docking stimulated 3D structural model of compound **5k** and sitagliptin with DPP-IV enzyme. H-bonding interactions were shown in yellow dotted line. Compound **5k** shows important interactions with DPP-IV enzyme similar to that of sitagliptin. 'N' of pyrimidinone moiety shows H-bonding with Arg125. This site is also responsible for π–π interaction in its S2 pocket. '—C═O' group fits well into the S1 pocket of enzyme having important residues Ser630 and Tyr631. Free 'N' of pyrazole moiety interacted with His740. Whereas phenyl moiety of pyrazole accommodate well in hydrophobic S1 pocket of enzyme. 4-Methylpyridine moiety of compound **5k** is responsible for H-bonding interaction with Asn710 in catalytic region of enzyme. The inhibitory potency of compound **5k** is higher since the docking results show that compound **5k** fits very well into the binding pocket of DPP-IV enzyme. Total Score and Consensus Score (CScore) of compound **5k** was 7.69 and 5, respectively. Sitagliptin shows additional binding with Glu205 and Ser209 in S2 pocket and Val207 in S1 pocket (see Table 2).

Figure 3B illustrates an overlay of the docking simulated 3D structural model of low potent compound **5b** and sitagliptin binding to DPP-IV enzyme. From the docking result of this compound it is clear that overlay of compound **5b** did not match with sitagliptin. Compound **5b** did not show interaction with important residues. Compound **5b** shows H-bonding interaction with Ser630 and Tyr631 in S1 pocket but not showing any interaction with Arg125 in S2 pocket. Additional H-bonding interactions observed with residues like Gly632, Gly633, Trp629, and Lys554. Total Score and CScore of compound **5b** was 3.6 and 2, respectively (see Table 2).

Docking results of some of the representative molecules were depicted in Table 2. Total Score value of docked compound implies binding capacity of ligand. CScore represents ranking of the affinity of ligands bound to the active site of a receptor. CScore is generated by combination of G_Score, D_Score, PMF_Score and ChemScore.[23] Compound **5e** does not have 'N' containing heterocycle in its catalytic region so, H-bonding with Asn710 is not present. Though compound **5e** has other important residues for interaction like Ser630, Tyr631, Gly632, Gly633, Trp629, Lys554, His740 and Arg125. Total Score of compound **5e** was 6.5 which is less compared to compound **5k** but it is showing promising activity in in vitro studies. Compounds **5i**, **5j** and **5l** incorporate well into biding site having pyridine, pyrimidine and 5-bromo-pyridine moiety. Compound **5o** shows additional binding interactions with Gly632,

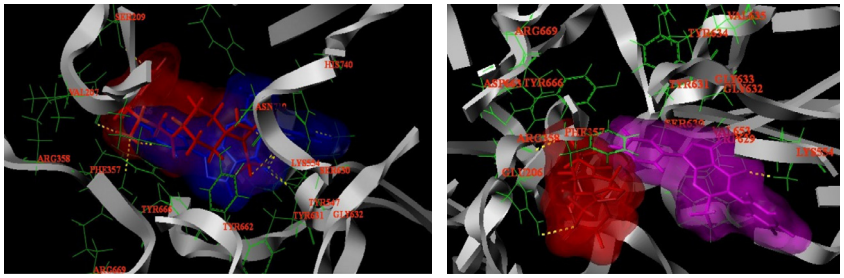


Figure 3. Docking study results (PDB ID: 2RGU). (H-bonding interactions were shown in yellow dotted line and amino acid residues presented in green color). (A) Overlay of compound **5k** (blue sticks) with Sitagliptin (red sticks): H-bonding interactions of compound **5k**: Ser630, Tyr631, His740, Tyr662, Asn710, and Arg125. H-bonding interactions of Sitagliptin: Ser630, Tyr631, Tyr662, Glu205, Val207, Asn710, Ser209. (B) Overlay of compound **5b** (pink sticks) with Sitagliptin (red sticks): H-bonding interactions of compound **5b**: Ser630, Tyr631, Gly632, Gly633, Trp629, and Lys554. H-bonding interactions of Sitagliptin (red sticks): Ser630, Tyr631, Tyr662, Glu205, Val207, Asn710, Ser209.

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Table 2
Docking results of compounds with interacting binding residues

Ligand	Docking score		Interacting residues with docked ligand
	Total score	CScore	
Compound **5e**	6.5	5	Ser630, Tyr631, Gly632, Gly633, Trp629, Lys554, His740, Arg125
Compound **5i**	7.56	5	Ser630, Tyr631, Tyr662, Asn710, His740, Arg125
Compound **5j**	7.41	4	Ser630, Tyr631, Tyr662, Asn710, Gly632, His740, Arg125
Compound **5k**	7.69	5	Ser630, Tyr631, Tyr662, Asn710, His740, Arg125
Compound **5l**	7.43	4	Ser630, Tyr631, Gly632, His 740, Arg125
Compound **5o**	7.57	4	Ser630, Tyr631, Gly632, Gly633, Trp629, Lys554, Asn710, Arg125
Compound **6a**	7.0	4	Ser630, Tyr631, Arg358, Asn710, Glu205, Tyr547, Arg125
Compound **5b**	3.6	2	Ser630, Tyr631, Gly632, Gly633, Trp629, Lys554
Compound **5n**	4.0	3	Ser630, Tyr631, Gly632, Gly633, Trp629, Lys554
Compound **5t**	6.0	4	Ser630, Tyr631, Tyr662, Asn710, Arg125
Compound **6e**	3.9	3	Ser630, Tyr631, Gly632, Gly633, Trp629, Lys554
Sitagliptin	9.01	5	Ser630, Tyr631, Tyr662, Glu205, Val207, Asn710, Ser209

Total score: Binding capacity of ligand with receptor.
CScore: ranges from 0 to 5 (higher the score higher is the affinity of ligand with receptor).

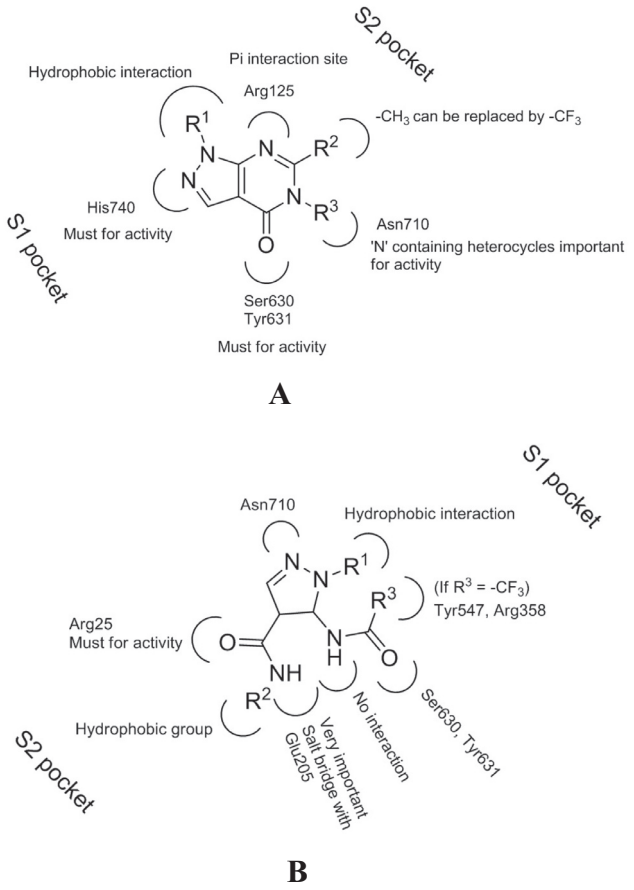

Figure 4. Structure activity relationship (SAR) study of synthesized molecules. (A) Structure activity relationship (SAR) study of compounds **5a**–**5t**. (B) Structure activity relationship (SAR) study of compounds **6a**–**6e**.

Gly633, Trp629 and Lys554 because of its methoxyphenyl group in S1 pocket. Methoxyphenyl does not show promising in vitro activity in other compounds like compounds **5f**, **5n**, **5p**, **5q**, **5r** and **5t** except compound **5o**. In compound **6a** '—NH' group forms salt bridge with Glu205 which is very important binding interaction observed in sitagliptin. Compound **6a** has '—CF_3' group that enhances its interaction with Tyr547 and Arg358 in its S2 pocket. Several other compound are inactive because they could not fit into the pocket of DPP-IV active site like compounds **5a**, **5c**, **5d**, **5f**, **5g** and **5h**. Though there are some exceptional compounds which has shown good binding affinity but they did not show good in vitro inhibitory activity as observed in compounds **5s** and **5t**.

Based on in vitro and docking studies, we could derive a structure activity relationship (SAR) of synthesized molecules but some of the molecules did not fit into this SAR. Compound **5e** does not have 'N' containing heterocycle but shows potent inhibition of DPP-IV action. Whereas in some molecules like **5m**, **5n**, **5p** and **5q** 'N' containing heterocycle did not show beneficial effect on inhibition of enzyme. Based on SAR we could come to a conclusion that in order to enhance potency of compound we should modify substitutions on S2 pocket of enzyme. '—NH' and '—NH_2' has ability to form salt bridge with Glu205 and Glu206 and H-bonding interaction with Arg356 and Phe357. This study will be helpful for further developing of these molecules with better activity (see Fig. 4).

In summary, a novel scaffold of pyrazolo[3,4-*d*]pyrimidinones was designed using a dual pharmacophore approach and a number of structurally diverse molecules containing the aforementioned scaffold were synthesized. Most of the synthesized compounds were found active from in vitro studies and a few from in vivo experiments. The most potent molecule of this series will be taken as a lead to optimize it further as an anti-diabetic drug acting through DPP-IV inhibition.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.09.015.

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