Research Article

Synthesis, Bio-evaluation and Molecular Modeling Studies of (2S)-1-[({[1-substituted cyclohexyl] methyl} amino) acetyl] pyrrolidine-2-carbonitriles for their DPP-4 Inhibiting Activity

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ABSTRACT

Synthesis of certain vildagliptin analogues namely (2S)-1-[({[1-substituted cyclohexyl] methyl} amino) acetyl] pyrrolidine-2-carbonitriles (*1a-d*) was performed, biologically evaluated and molecular docking studied for their DPP-4 inhibiting activity. Compounds *1b* and *1c* at dose levels of 0.28mmol/kg and 0.24 mmol/kg, respectively, equivalent to 100 mg/kg exhibited greater DPP-4 inhibitory activity as well as reducing the effect on serum glucose level after oral administration to type 2 diabetic mice as compared to vildagliptin (0.33 mmol/kg) as reference drug. Molecular modeling studies resulted in good binding affinity of compounds *1b* and *1c* at the DPP-4 active site and were in favor of their observed anti-diabetic activity as compared to vildagliptin.

Keywords: Synthesis, pyrrolidine-2-carbonitrile, DPP-4 inhibitors, molecular docking.

INTRODUCTION

Diabetes mellitus is a metabolic disease which affects populations all over the world. WHO reported that almost 3 million deaths worldwide per year are due to diabetes and the rate is doubled over the past 15 years[1].

There are many types of marketed drugs to control and treat type 2 diabetes such as α-glucosidase inhibitors[2], thiazolidinediones[3-4], biguanides[5-6], meglitinides[7] and sulfonylureas[8].

Despite all these conventional drug types, there is still an importunate demand for safer and more effective antidiabetic agents[9-10]. One of the main causes that induce type 2 diabetes consists of the reduced potential of GLP-1 by DPP-4 enzyme. Inhibition of DPP-4 enzyme elevates endogenous GLP-1 and insulin level and thereby improves glucose secretion.

Thus, inhibition of DPP-4 enzyme could be considered as a positive therapeutic goal for the management of type 2 diabetes mellitus[11-15].

Clinical studies have proven that a number of such inhibitors could be used as antidiabetics[16-19], of which the most well-known are sitagliptin (Januvia®)[20-21], alogliptin[22] and the 2-cyanopyrrolidine derivatives, NVP-DPP728[23], denagliptin[24], saxagliptin[25] and vildagliptin (Galvus®)[26-27] (Figure 1).

The latter four which incorporate the active-site serine trapcyano- group in their structure are potent and reversible covalent inhibitors.

Hence, the aim of this work is the design and synthesis of certain vildagliptin analogues: namely (2S)-1-[({[1-substituted cyclohexyl] methyl} amino) acetyl] pyrrolidine-2-carbonitriles, (Figure1) having the general structure *1a-d* to be bioevaluated for their potential DPP-4 inhibition activity. Further, molecular docking study will be performed.

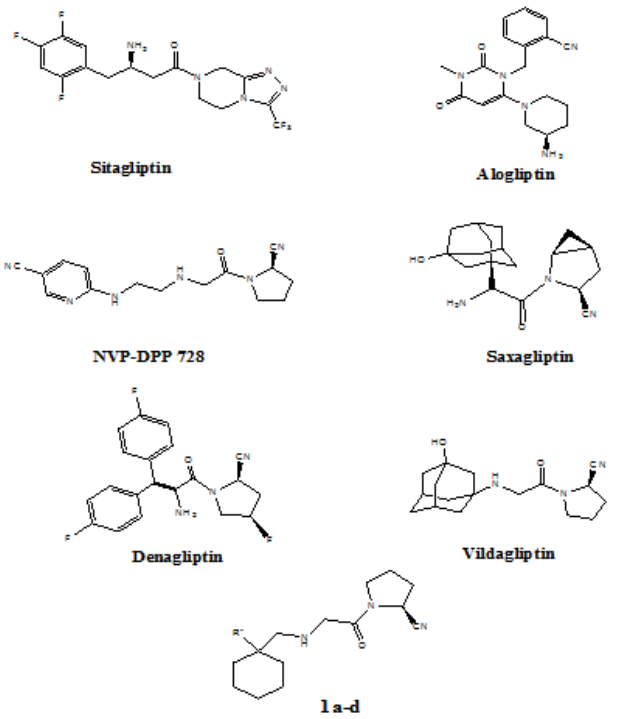


Figure 1: Structures of certain marketed DPP-4 inhibitors and the target compounds *1a-d*

MATERIALS AND METHODS

Chemistry

All melting points are uncorrected and measured with Electrothermal Capillary melting point apparatus. Infrared (IR) spectra were done neat for oils and as KBr pellets (for

solids) using a JASCO FT/IR-6100 Spectrometer and the values are represented in cm^{-1}. Jeol ECA 500 MHz Spectrometer was used to record ^{1}H NMR and ^{13}C NMR spectra using TMS as internal standard and the values of chemical shift were given in ppm on δ scale. The mass spectra were run on Finnigan Mat SSQ-7000 Spectrophotometer and Jeol JMS-AX 500. Elemental analyses were performed at the Microanalytical Laboratory, National Research Centre-Cairo-Egypt. Thin layer chromatography (TLC) was performed on Silica gel plates (Merck, 60F254). Illumination with UV light source (254nm) was used for spot visualization. Purification were achieved using Column chromatography on silica gel 60 (0.063-0.200) purchased from Merck.

Preparation of (S) 1-(2-chloroacetyl) pyrrolidine-2-carboxylic acid (*6*).

To a suspension of S-proline (10g, 0.087mol) in THF (100mL) was added chloroacetyl chloride (9.85 mL, 0.13mol) at room temperature and refluxed for 2 hours then cooled; diluted with water (10 mL) and stirred for 20min. Saturated brine solution was added and extracted with ethyl acetate (3×25mL). The combined organic layers were dried ($MgSO_4$) and evaporated. The resulting thick oil was stirred with diisopropyl ether (50mL) at room temperature for half an hour then cooled to 0°C for 1 hour, where the formed solid was filtered, washed and dried to afford white solid of the acid *6* (12.9g, 80%); mp 109-110°C[28].

Preparation of (2S)-1-(chloroacetyl)pyrrolidine-2-carboxamide *(5)*.

To a solution of the acid *6* (5.0g, 0.026mol) in dichloromethane (100mL) was added slowly a solution of dicyclohexylcarbodiimide (DCCDI) (5.4g, 0.026mol) in dichloromethane (25mL) at 10-15°C and stirred at room temperature for 1hour. Ammonium bicarbonate (20.6g, 0.26mol) was added to the mixture and stirred at room temperature for 48hours. The reaction mixture was cooled, filtered and evaporated. The residual oil was dissolved in THF and diisopropyl ether, stirred for 15min, cooled to 0°C and left to precipitate for 1hour. The formed solid was filtered, washed with diisopropyl ether, dried and purified on column chromatography using a mixture of petroleum ether: ethyl acetate (7:3) as an eluent, to yield white solid of compound *5* (2.73g, 55%); mp135-136°C[28].

Preparation of (2S)-1-(chloroacetyl) pyrrolidine-2-carbonitrile *(3)*

Trifluoroacetic anhydride (4.4mL, 0.032mol) was added to a suspension of the amide *5* (4.0g, 0.021mol) in THF (40mL) at 0-5°C. The mixture was stirred for 2hours at room temperature. Ammonium bicarbonate (12.4g, 0.157mol) was added portion wise to the reaction mixture at 5-10°C under stirring for 45min, followed by concentration under reduced pressure at 40°C. The residue was stirred for 1hour with toluene (60mL) at room temperature and filtered. The filtrate was evaporated under vacuum to give an oily substance which was stirred at room temperature with n-hexane (20mL) for 30min, cooled to 0-5°C and stand to crystallize for 30min, filtered and washed with cold n-hexane to afford 3.0g (83%) of compound *3* as white crystals, mp 52-54°C. Its spectral data were in agreement with the reported ones[28].

1-(Piperidin-1-yl)cyclohexanecarbonitrile *(4a)* was prepared as cited[29] to give a yellowish white solid (80% yield), mp 65°C.

General procedure for the preparation of 1-(4-ethyl and/or aralkyl piperazin-1-yl)cyclohexane carbonitriles *(4b-d)*

A solution of the appropriate substituted piperazine (0.01mol) in 5mL water was mixed carefully with concentrated hydrochloric acid (0.96 mL, 0.026mol) where pH was adjusted to 3-4. Cyclohexanone (0.81 mL, 0.01mol) was added to the resulting solution, then potassium cyanide (0.65g, 0.01mol) in water (1.7mL) was added, stirred at room temperature overnight, basified (10% NaOH) and the formed precipitate was filtered off and washed with water to afford the corresponding carbonitrile derivatives *4b-d* in 50-80 % yields.

The crude solids *4b-d* were pure enough to be used in the next step without further purification.

1-(4-Ethylpiperazin-1-yl) cyclohexane carbonitrile *(4b)*[30]

Yield 1.10g (50 %); white solid, mp 77-79°C.

1-(4-Benzylpiperazin-1-yl) cyclohexane carbonitrile *(4c)*[30]

Yield 2.26g (80 %); white solid, mp 94-96°`C.

1-(4-[4-Methoxybenzyl] piperazin-1-yl) cyclohexane carbonitrile *(4d)*

Yield 1.88g (60 %); yellowish white solid, mp 94-95°C. IR (KBr, cm^{-1}) showed bands at 2221 (CN), 2931, 2826, 1004. ^{1}H-NMR ($CDCl_3$) δppm: 1.50-2.12 (m, 10H, 5× CH_2, cyclohexyl protons), 2.47-2.66 (m, 8H, 4× CH_2, piperazinyl protons), 3.43 (s, 2H, N-CH_2-phenyl), 3.79 (s, 3H, O-CH_3), 6.83-6.85 (d, J= 8.6Hz, 2CH_{ar}), 7.21-7.23 (d, J= 8.6Hz, 2CH_{ar}).^{13}C-NMR ($CDCl_3$): 22.30, 25.02, 33.99 (5×CH_2-cyclohexyl), 46.68, 53.10 (4× CH_2-piperazinyl), 55.35 (OCH_3), 61.13, 62.20 (*C*-cyclohexyl, N-CH_2-phenyl), 113.66 (2×$CH_{ar.}$), 119.37(*C*≡N), 130.08($CH_{ar.}$), 130.34(2×$CH_{ar.}$), 158.80 ($CH_{ar.}$).Ms (EI) *m/z* (%) : 313 (100) (M^+), 287 (20),121 (90). Anal. Calcd. for $C_{19}H_{27}N_3O$: C, 72.81; H, 8.68; N, 13.41; O, 5.10. Found: C, 72.77; H, 8.79; N, 13.69; O, 5.06.

Synthesis of 1-[1-(4-ethyl and/or aralkyl piperazin-1-yl) cyclohexyl]methanamine *(2b-d)*

To a cold suspension of lithium aluminum hydride powder (1.9g, 0.049mol) in dry THF (100mL) was added dropwise under stirring a suspension of anhydrous aluminum chloride (2.1g, 0.016mol) in dry THF (5mL). A solution of the appropriate carbonitriles *4b-d* (0.011mol) in dry THF (15mL) was added dropwise to the cooled (0°C) reaction

mixture and stirring was continued for 24hours at room temperature, then quenched by slow addition of saturated solution of Na_2SO_4 at 0-5°C. The formed precipitate of lithium hydroxide and aluminum hydroxide was filtered off and washed with THF (10mL) and ethyl acetate (25mL). The combined filtrate and washings were dried and evaporated under vacuum to afford *2b-d* as pale yellow oils which were pure enough to be used in the next step without further purification. The spectral data of compounds *2b* and *2c* were in agreement with the reported ones.

1-[1-(4-Ethyl piperazin-1-yl) cyclohexyl] methanamine *(2b)*[30]

Yield 2.15g (87 %); pale yellow viscous oil.

1-[1-(4-Benzyl piperazin-1-yl) cyclohexyl] methanamine *(2c)*[30]

Yield 2.70g (85 %); pale yellow viscous oil.

1-[1-(4-[4-Methoxybenzyl] piperazin-1-yl) cyclohexyl] methanamine *(2d)*

Pale yellow viscous oil purified by column chromatography using chloroform: methanol (9:1) as a mobile phase, Yield 1.74g (50%). IR (KBr, cm^{-1}) showed bands at 3410, 3260 (NH_2), 1611,822; ^{1}H-NMR ($CDCl_3$) δppm 1.04-2.21 (m, 12H, 5× CH_2, cyclohexyl protons, NH_2), 2.39-2.67 (m, 10H, 4× CH_2, piperazinyl protons, CH_2-NH_2), 3.41 (s, 2H, N- CH_2-phenyl), 3.72 (s, 3H, O-CH_3), 6.77-6.79 (d, J= 8.6Hz, 2CH_{ar}), 7.14-7.16 (d, J= 8.6Hz, 2CH_{ar}); ^{13}C-NMR ($CDCl_3$): 22.28, 26.08, 34.30, (5×CH_2-cyclohexyl), 47.00, 48.30, 52.23 (4× CH_2-piperazinyl,CH_2-NH_2), 55.25 (OCH_3), 62.13, 63.62 (*C*-cyclohexyl, N-CH_2-phenyl), 113.81 (2×$CH_{ar.}$), 125.56($CH_{ar.}$), 130.64(2×$CH_{ar.}$), 158.88 ($CH_{ar.}$); Ms (EI) *m/z* (%) : 317 (5)(M^+), 287 (15), 121(100); Anal. Calcd. for $C_{19}H_{31}N_3O$: C, 71.88; H, 9.84; N, 13.24; O, 5.04. Found: C, 71.76; H, 9.75; N, 13.39; O, 5.11.

Synthesis of 1-[1-(piperidin-1-yl) cyclohexyl] methanamine *(2a)*[31]

The reaction was performed by adopting the same procedure for the synthesis of compounds *2b-d* using lithium aluminum hydride only without addition of aluminum chloride. The spectral data of *2a* was in agreement with the reported one. Yield 75 %; the dihydrochloride salt mp 265-267°C.

General procedure for the preparation of (2S)-1-[({[1-substituted cyclohexyl] methyl} amino) acetyl] pyrrolidine-2-carbonitriles *(1a-d)*

A solution of (2S)-1-(chloroacetyl) pyrrolidine-2-carbonitrile *(3)* (1.0g, 0.0054mol) in THF (10mL) was added dropwise to an ice-cooled stirred suspension of the appropriate cyclohexyl methanamine *2a-d* (0.0054mol), potassium iodide (0.9g, 0.0054mol) and anhydrous potassium carbonate (1.5g, 0.011mol) in THF (20mL). The reaction mixture was stirred at room temperature overnight. The resulting mixture was filtered, dried and evaporated under reduced pressure to give an oily residue, which was purified on column chromatography using chloroform: methanol (97:3) as a mobile phase to afford yellowish white solids of the target compounds *1a-d*.

(2S)-1-[({[1-Piperidinocyclohexyl] methyl} amino) acetyl] pyrrolidine-2-carbonitrile *(1a)*.

Yield 25 %; yellowish white solid, mp 120-122°C.IR (KBr, cm^{-1}): 3423(NH), 2245 (CN), 1655 (C=O); ^{1}H-NMR ($CDCl_3$)δppm: 1.23-2.16 (m, 21H,10× CH_2, cyclohexyl, piperidinyl protons and 1H, N*H*),2.71-2.87 (m, 2H, CH_2, pyrrolidinyl), 3.03-3.12 (m, 2H, CH_2, pyrrolidinyl) 3.41 (s, 2H, CH_2-NH), 4.08(s, 2H, NH-CH_2-CO), 4.24-4.30 (t, J=7.0Hz, 2H, CH_2-N-pyrrolidinyl), 4.60-4.68 (t, J=7.5Hz, 1H, *CH*-C≡N). ^{13}C-NMR ($CDCl_3$): 22.18, 22.72, 25.06, 25.30, 26.83, 29.70, 29.82 (5 × CH_2-cyclohexyl, 3× CH_2-piperidinyl and 2× CH_2-pyrrolidinyl), 44.89, 46.69, 49.92 (*C*H- and CH_2-pyrrolidinyl, 2×CH_2-piperidinyl), 60.85, 62.27, 69.33 (NH- CH_2CO, *C*-cyclohexyl and CH_2-NH), 118.04 (*C*≡N), 171.13 (*C*=O). MS (EI) m/z (%): 333 (3) (M^++1), 167(10), 70 (100). Anal. Calcd. for $C_{19}H_{32}N_4O$: C, 68.64; H, 9.70; N, 16.85; O, 4.81. Found: C, 68.76; H, 9.79; N, 16.79; O, 4.66.

(2S)-1-[({[1-(4-Ethylpiperazin-1-yl)cyclohexyl] methyl}amino) acetyl] pyrrolidine-2-carbonitrile *(1b)*

Yield 50 %; yellowish white solid, mp 218-221°C. IR (KBr, cm^{-1}): 3441(NH), 2243 (CN), 1659 (C=O); ^{1}H-NMR ($CDCl_3$) δppm: 1.16-1.93 (m, 14H, 1×CH_3 and 5×CH_2, cyclohexyl and 1H, N*H*), 2.15 -3.00 (m, 14H, CH_3-CH_2-, 2× CH_2, pyrrolidinyl and 4× CH_2, piperazinyl), 3.47 (s, 2H, CH_2-NH), 4.10 (s, 2H, NH- CH_2-CO), 4.31-4.37 (t, J=7.0Hz, 2H, CH_2-N-pyrrolidinyl),4.63-4.67 (t, J=7.5Hz, 1H, *CH*-C≡N). ^{13}C-NMR ($CDCl_3$) δppm: 12.82 (CH_3-CH_2), 22.65, 25.43, 25.99, 29.02, 29.87 (5×CH_2-cyclohexyl and 2×CH_2-pyrrolidinyl), 42.68, 47.18, 47.94, 48.17, 52.28, 53.89 (*C*H-and CH_2-pyrrolidinyl, 2×CH_2-piperazinyl, CH_2-CH_3, NH-CH_2-CO, *C*-cyclohexyl), 60.11, 62.76 (2×CH_2-piperazinyl and CH_2-NH), 117.78 (*C*≡N), 162.59 (*C*=O). Ms (EI) *m/z* (%): 362 (2) (M^++1), 196 (47), 82 (75). Anal. Calcd. for $C_{20}H_{35}N_5O$: C, 66.44; H, 9.76; N, 19.37; O, 4.43. Found: C, 66.56; H, 9.79; N, 19.41; O, 4.50.

(2S)-1-[({[1-(4-Benzyl piperazin-1-yl)cyclohexyl] methyl}amino) acetyl] pyrrolidine-2-carbonitrile *(1c)*

Yield 30 %; yellowish white solid, mp 152-154°C. IR (KBr, cm^{-1}): 3431(NH), 2242 (CN), 1656 (C=O); ^{1}H-NMR ($CDCl_3$) δppm: 1.32-2.16 (m, 11H, 5× CH_2, cyclohexyl protons and 1H, *NH*), 2.40-3.09 (m, 12H, 2× CH_2, pyrrolidinyl and 4× CH_2, piperazinyl protons), 3.46 (s, 2H, CH_2-NH), 3.55-3.81(m, 6H, NH-CH_2-CO, CH_2-N-pyrrolidinyl,N-CH_2-phenyl), 4.65-4.70 (t, J=7.5Hz, 1H, *CH*-C≡N), 7.23-7.24 (m, 5H, $H_{ar.}$).^{13}C-NMR ($CDCl_3$) δppm: 22.54, 25.31, 25.51, 27.68, 29.87 (5×CH_2-cyclohexyl and 2×CH_2-pyrrolidinyl), 46.23, 48.20, 51.26, 52.69, 53.20 (*C*H- and CH_2-pyrrolidinyl, 2×CH_2-piperazinyl, NH-CH_2-CO, *C*-cyclohexyl), 62.47, 62.85, 63.81 (2×CH_2-piperazinyl, CH_2-NH, N-CH_2-phenyl), 118.52(*C*≡N), 128.31 ($CH_{ar.}$), 128.41 (2×$CH_{ar.}$), 129.39 (2×$CH_{ar.}$), 137.14 (C_{ar}), 168.72 (*C*=O). MS (EI) m/z (%): 424 (5) (M^++1), 257 (70), 166 (27), 91 (100). Anal.

Calcd. for $C_{25}H_{37}N_5O$: C, 70.89; H, 8.80; N, 16.53; O, 3.78. Found: C, 70.75; H, 8.69; N, 16.69; O, 3.62.

(2S)-1-[({[1-(4-(4-Methoxybenzyl)piperazin-1-yl)cyclohexyl] methyl}amino) acetyl] pyrrolidine-2-carbonitrile *(1d)*

Yield 50 %; yellowish white solid, mp 188-190°C. IR (KBr, cm^{-1}): 3429 (NH), 2242 (CN), 1655 (C=O); ^{1}H-NMR ($CDCl_3$) δppm: 1.31-2.10 (m, 11H, 5× CH_2, cyclohexyl protons and 1H, *NH*), 2.51 - 2.99 (m, 12H,2× CH_2, pyrrolidinyl and 4× CH_2, piperazinyl protons), 3.42 (s, 2H, CH_2-NH), 3.51-3.70 (m, 6H, NH -CH_2-CO, CH_2-N-pyrrolidinyl, N-CH_2-phenyl), 3.79 (s, 3H, OCH_3), 4.77- 4.90 (t, J=7.5Hz, 1H, *CH*-C≡N), 6.85-6.94 (d, J=8.6, 2H, H_{ar}), 7.21-7.25(d, J= 8.6,2H, H_{ar}). ^{13}C-NMR ($CDCl_3$) δppm: 22.88, 25.53, 25.70, 28.00, 28.96 (5×CH_2-cyclohexyl and 2×CH_2-pyrrolidinyl), 42.61, 48.73,51.33, 52.18, 53.98 (*C*H-and CH_2-pyrrolidinyl, 2×CH_2-piperazinyl, NH-CH_2-CO, *C*-cyclohexyl), 55.36 (OCH_3), 60.58, 61.93, 64.68 (2×CH_2-piperazinyl,CH_2-NH,N-CH_2-phenyl), 114.79(*C*≡N), 129.21 ($CH_{ar.}$), 130.33 (2×CH_{ar}), 135.23(2×CH_{ar}), 139.27 (CH_{ar}), 161.32 (C=O). MS (EI) m/z (%): 454(3) (M^++1), 288(10), 121 (100). Anal. Calcd. for $C_{26}H_{39}N_5O_2$: C, 68.84; H, 8.67; N, 15.44; O, 7.05. Found: C, 68.78; H, 8.59; N, 15.59; O, 7.12.

Pharmacology

Animals

The dipeptidyl peptidase-4 (DPP-4) inhibition properties and the antidiabetic activity of the target compounds (2S)-1-[({1-substituted cyclohexyl]methyl}amino)acetyl}pyrrolidine-2-carbonitriles (*1a-d*) were evaluated using adult Swiss albino mice weighing 20-25 g. Animals were obtained from the Animals House of the National Research Centre, Cairo, Egypt. Animals were housed in polypropylene cages under the standard conditions of light (12 hours light/dark cycle), temperature (23 ± 2 °C) and were allowed free access to water and standard laboratory diet. All procedures were carried out according to the Ethics Committee of the National Research Centre and the recommendations for the appropriate care and use of laboratory animals, "Canadian Council on Animal Care Guidelines, 1984".

Drugs and Chemicals

Streptozotocin, Tween 80 and GLP-1 enzyme immunoassay (EIA) kit (Sigma Chemicals Co., St. Louis, MO, USA). Gly-Pro-Aminomethylcoumarin (AMC) (Cayman Chemical Company, Ann Arbor, MI, USA). Glucose Enzymatic colorimetric method kit (Biodiagnostic, Egypt). C-peptide ELISA kit (DRG International, Inc. USA). Vildagliptin (Galvus, Novartis, Egypt).

Induction of Diabetes

Acclimatization of the animals to the laboratory environment was for one week before starting the experiment. Diabetes was induced chemically according to the method of[32]. Mice were fasted for 18 hours before diabetes was induced by intraperitoneal injection (*i.p.*) of STZ (75 mg/kg) dissolved in cold sodium citrate buffer (0.01 M, pH 4.5, freshly prepared for immediate use) on three successive days. The mice were screened for diabetes starting 3 days after administering the first dose of STZ by testing for the presence of glucose in urine using urine strips. After one week from the first STZ dosing, the diabetic state of the animals was confirmed by measuring glucose level with a glucometer (Bionime, GmbH, Heerbrugg, Switzerland). The mice were considered diabetic when their fasting blood glucose levels was higher than 250 mg/dL.

Experimental Groups and Protocol

After fasting for at least 18 hours, diabetic animals were divided into six experimental groups. Each group consisted of 8 mice. Group I served as diabetic control. Animals in group II received orally vildagliptin (100 mg/kg=0.33 mmol/kg) as a reference drug[33]. Mice in groups III, IV, V and VI received orally one of the tested compounds *1a-d* (100 mg/kg corresponding to 0.3, 0.28, 0.24, and 0.23 mmol/kg), respectively in 7% tween 80. Non diabetic mice were used as normal control. After the indicated experimental period of 3 hours, mice were anesthetized and blood was collected directly from the heart. Serum were separated by centrifugation at 3000 rpm for 20 min at 4°C using cooling centrifuge (Sigma Laborzentrifugen GmbH, Germany) and stored in aliquots at -80 °C until analysis.

In-vivo Assessment of DPP-4 Inhibition

After fasting for at least 18 hours, mice were orally administered one of the test compounds or the reference drug suspended in 7 % tween 80 as a single dose of 100 mg/kg. Blood samples were collected 3 hours after dosing and immediately centrifuged to obtain serum for estimation of the DPP-4 activity. 50 µl of serum was added to each well of the 96 well flat bottomed microplate, followed by the addition of 50 µl of 60µM substrate (Gly-Pro-AMC). The rate of DPP-4 activity was measured after 30 min using the method described by Kondo[34] and the percent inhibition relative to initial DPP-4 activity was calculated.

Assessment of Glucagon-like Peptide 1 (GLP-1)

GLP-1 was estimated in mice serum according to the method described by[35] using GLP-1 competitive enzyme immunoassay (EIA) kit (sigma-Aldrich, St. Louis, MO, USA). The kit's microplate was pre-coated with secondary antibody. Following a blocking step, the plate was incubated with anti-GLP-1 antibody. The peptide standard or targeted peptide in the samples and the biotinylated GLP-1 peptide interact competitively with the GLP-1 antibody. The uncompeted biotinylated GLP-1 peptide interacts with streptavidin-horseradish peptidase (SA-HRP), catalyzing reaction which develops a color measured at 450 nm using ELISA plate reader AsysExpert Plus microplate reader (AsysHitechGmBH, Austria). The

intensity of the developed color is directly proportional to the amount of biotinylated GLP-1 peptide-SA-HRP complex and inversely proportional to the amount of GLP-1 peptide in the standard and the samples. A standard curve of known concentrations of GPL-1 peptide in the samples was established and the concentration of GLP-1 peptide in the samples was calculated accordingly.

C-peptide Immunoassay

The immunoassay of C-peptide is a useful parameter for the quantitation of insulin secretion as C-peptide and insulin are secreted in equimolar amounts. Moreover, the half-life of C-peptide in the circulation is 2-5 times longer than that of insulin. C-peptide level is considered a more stable indicator of insulin secretion than insulin due to its half-life that lasts 2-5 times longer in the circulation than insulin[36]. The C-peptide kit (DRG International, Inc. USA) is a competitive binding ELISA kit. The microplate wells are coated with anti-mouse antibody, which binds a monoclonal antibody directed towards a unique antigenic site on the C-peptide molecule. Endogenous C-peptide of a sample competes with a C-peptide horseradish peroxidase conjugate for binding to the coated antibody. The unbound conjugate is washed off after incubation. The amount of bound peroxidase conjugate is inversely proportional to the concentration of C-peptide in the sample. the intensity of color developed after addition of the substrate solution is inversely proportional to the concentration of C-peptide in the sample.

Assessment of Blood Glucose Level

Blood samples from the tail tip of normal control, diabetic control, and diabetic treated mice were collected at zero and 1 hour post-treatment for estimation of blood glucose level using the glucometer. Three hours from vildagliptin and test compounds oral administration, mice were anesthetized and blood was collected directly from the heart and deproteinized. The obtained supernatant was used for the determination of blood glucose by glucose oxidase/peroxidase method (Glucose Enzymatic colorimetric method kit, Biodiagnostic, Egypt) spectro-photometrically (Cary 100 UV-Vis, Agilent Technologies, VIC, USA)[37].

Oral Glucose Tolerance Test

Oral glucose tolerance test was assessed in male diabetic mice (n=6). The mice were fasted for 18 hours before the beginning of the study and then dosed orally with the vehicle (7%, tween-80) or vildagliptin or one of the tested compounds (100 mg/kg). After 30 minutes, glucose solution was orally administered at 2 g/kg[38] body weight. Blood samples were collected from the tail tip at zero time and directly from the heart 3 hours post-treatment. Serum samples were prepared for estimation of glucose levels by colorimetric assay using glucose oxidation/peroxidase method (Glucose Enzymatic colorimetric kit, Biodiagnostic, Egypt).

Statistical Analysis

Data are expressed as mean ± s.e.m., and statistical significance were assessed by one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test. Statistical significance was considered at P value <0.05.

Molecular Modeling Studies

Molecular modeling studies were performed using Accelrys Discovery Studio 2.5 operating system (Accelrys Inc., San Diego, CA, USA).

Molecules were built within DS, and conformational models for each compound were generated automatically.

This emphasizes representative coverage over a 20 Kcal/mole energy range above the estimated global energy minimum, and the best quality generation technique was chosen.

Docking study involved the following steps: - the docking analysis was carried out on the DPP-4 enzyme,- the 3D protein structure of DPP-4 enzyme co-crystallized with vildagliptin (code; 3W2T) with resolution 2.36 Å was downloaded from the Protein Data Bank of the Research Collaboration for Structural Bioinformatics (RCSB) Web site [www.rcsb.org], - the binding pocket of the DPP-4 was docked with the lead vildagliptin and the test compounds *1b* and *1c* after cleaning the protein, adding the missing hydrogen atoms and side chains as well as energy minimization according to DS protocol, - the binding pocket of vildagliptin with the connected amino acid molecules at sphere of radius= 6.3Å was identified and then docked with the target compounds *1b* and *1c* using Libdock-hotspots based technique.

Thereafter the docking scores of the best-fitted conformation of each of the docked molecules as well as the total number of hydrogen bonds and π-interactions with the amino acids at the binding pocket were recorded.

RESULTS AND DISCUSSION

Chemistry

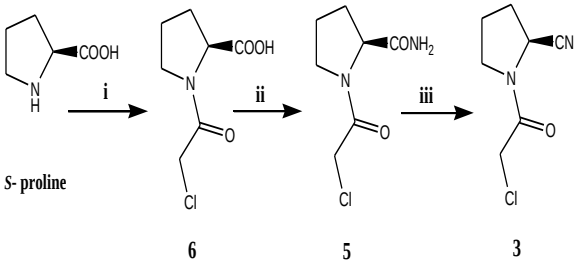
Syntheses of the beseeched compounds *1a-d* and their intermediates were illustrated in schemes 1 and 2. Compound (2S)-1-(chloroacetyl) pyrrolidine-2-carbonitrile (*3*) was the starting synthon to achieve (2S)-1-[({[1-substituted cyclohexyl] methyl} amino) acetyl] pyrrolidine-2-carbonitriles *1a-d*, through chloroacetylation of S-proline with chloroacetyl chloride followed by amidation of the carboxylic group of 6 to afford (2S)-1-(chloroacetyl) pyrrolidine-2-carboxamide (*5*), which through subsequent dehydration using trifluoroacetic anhydride the chloro-acetylated carbonitrile *3* was obtained (scheme 1).

Compound 1-[1-(piperidin-1-yl) cyclohexyl] methanamine (*2a*) was synthesized starting with the reaction of

cyclohexanone and piperidine with sodium cyanide and sodium bisulphite in water[29] to afford *4a*, while, the preparation of 1-[1-(4-ethyl piperazin-1-yl and/or aralkyl piperazin-1-yl)cyclohexyl] methanamine (*2b-d*) was achieved by the reaction of cyclohexanone with the appropriate substituted piperazine and potassium cyanide under Strecker synthesis conditions[39] to achieve the carbonitriles *4b-d*.

Subsequent reduction of the carbonitrile group using lithium aluminum hydride in THF in case of *4a* or in the presence of aluminum chloride in case of *4b-d* led to the desired amines *2a-d*.

Condensation of the chloroacetylated carbonitrile *3* with the appropriate methanamine *2a-d* in THF and in the presence of anhydrous potassium carbonate and potassium iodide resulted in the desired (2S)-1-[({[1-substituted cyclohexyl] methyl}amino) acetyl] pyrrolidine-2-carbonitriles (*1a-d*)**,** scheme 2.



Scheme 1: Synthesis of (2S)-1-(chloroacetyl) pyrrolidine-2-carbonitrile (*3*)

Reagents and Conditions:

i: $ClCH_2COCl$/ THF;
ii: DCCDI/NH_4HCO_3/ DCM;
iii: TFAA /NH_4HCO_3/THF

Pharmacology

The incretins are among the peptide hormones that are secreted by the L-cells of the GIT in response to the digestion of food.

They stimulate secretion of insulin from beta-cells in a glucose dependant manner.

Sustained insulin secretion is a result of enhancement of incretin activity leading to normalization of an elevated blood glucose level.

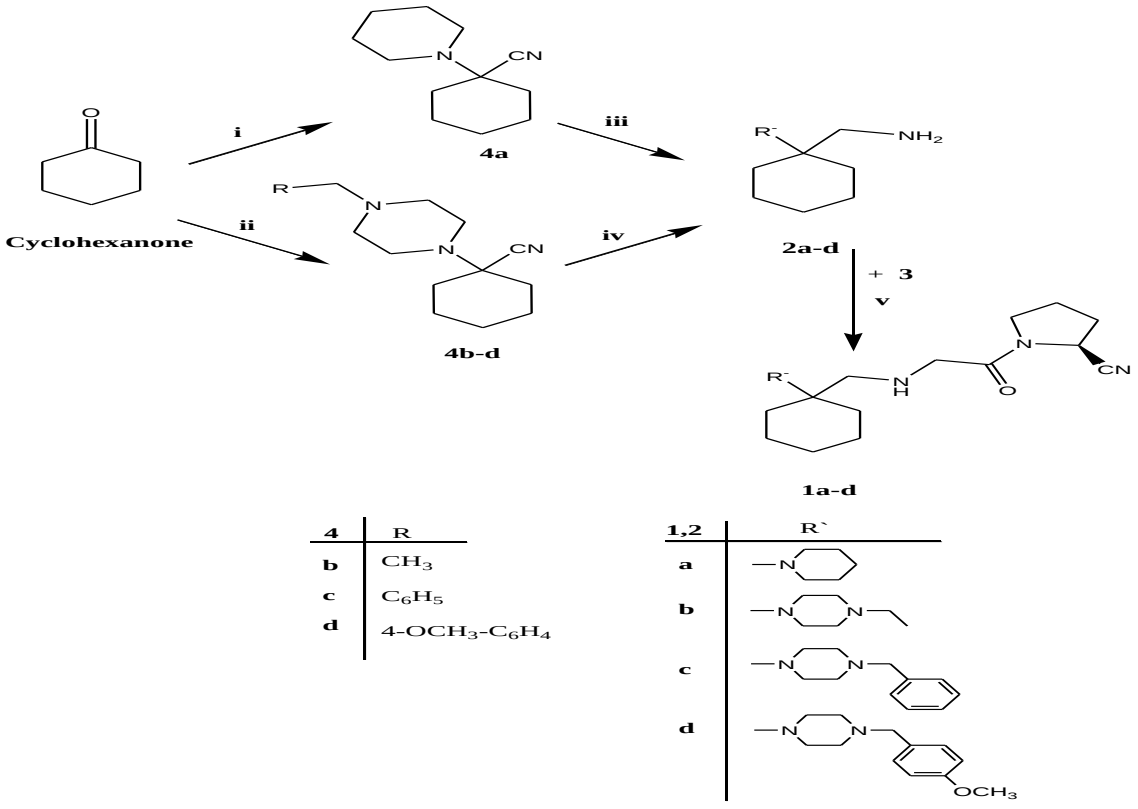
Thus, the incretin GLP-1 is considered as an important target for the treatment of diabetes mellitus type 2 (DMT2).

GLP-1 is rapidly degraded to the inactive GLP-1 form by the dipeptidyl peptidase-4 (DPP-4) enzyme, therefore is not a suitable oral medication.

Consequently, inhibition of DPP-4 is considered as an indirect approach to elevate the level of GLP-1 and looks promising as effective therapeutic strategy for the management of type 2 DM[40-42].

Data in Table 1 demonstrated that the percentage inhibition of serum DPP-4 activity in diabetic mice was 47% compared to normal level.

Regarding compounds *1a* (0.3 mmol/kg) and *1d* (0.23mm /kg) the percentage inhibition of DPP-4 activity was 85% and 106%, respectively, three hours after dosing.



Scheme 2: Synthesis of compounds *1a-d*

Reagents and Conditions: i: Piperidine/ NaCN/ $NaHSO_3/H_2O$; ii:1-(4-Substituted) piperazine, / KCN/ HCl conc (pH 3-4)/ H_2O; iii: $LiAlH_4$/THF; iv: $LiAlH_4$/ $AlCl_3$/THF; v: K_2CO_3 (anh.)/ KI/THF

Table 1: *In-vivo* serum DPP-4 inhibition of vildagliptin and test compounds **(***1a-d***)** in diabetic mice

Groups	Dose		DPP-4 inhibition (%)*
	mg/kg	mmol/kg	
Diabetic mice[#]	-	-	47.24
Vildagliptin	100	0.33	114.58
1a	100	0.3	85.01
1b	100	0.28	153.09
1c	100	0.24	138.43
1d	100	0.23	105.87

**In-vivo* serum DPP-4 inhibition (%) 3hours after dosing.

[#]Diabetes was induced by i.p. injection of STZ (75 mg/kg) for 3 consecutive days.

Table 2: H-bonds, π-interaction and LibDock scores of compounds *1b* and *1c*

Compound number	H-bonds (distance in Å)	π-interaction	LibDock score Kcal/mole
1b	Try 666 (2.45) Tyr 547 (2.30) Tyr 547 (2.01) Glu 205 (2.60) Glu 205 (2.80) Glu 206 (2.45) Ser 630 (4.40)	Tyr 666	-106.32
1c	Try 666 (2.46) Tyr 547 (2.01) Glu 205 (2.10) Glu 206 (1.70)	Phe 357	-115.72
Vildagliptin	Tyr 666 (2.80) Tyr 547 (2.20) Glu 205 (2.20) Ser 630 (3.14)	-	-109

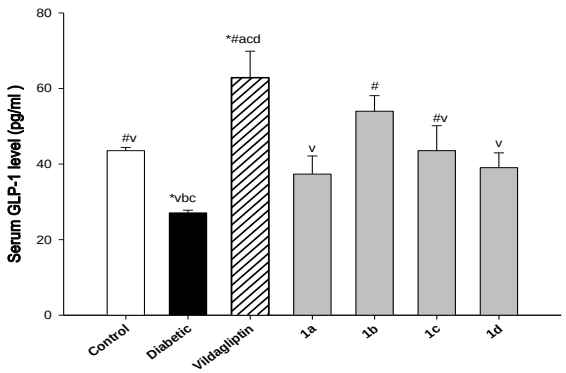


Figure 2: Effect of oral administration of vildagliptin and the compounds *1a-d* on serum GLP-1 level in diabetic mice.

Data are represented as the mean GLP-1 (pg/mL) ± s.e.m. of the number of animals in each group (n=6). Statistical analysis was carried out by one way ANOVA followed by Student Newman-Keuls test. * significantly different from normal control value at P< 0.05, [#] significantly different from diabetic value at P< 0.05, [v] significantly different from vildagliptin (0.33 mmol/kg) value at P< 0.05, [a] significantly different from compound *1a* (0.3 mmol/kg) value at P< 0.05, [b] significantly different from compound *1b* (0.28 mmol/kg) value at P< 0.05, [c] significantly different from compound *1c* (0.24 mmol/kg) value at P< 0.05, [d] significantly different from compound *1d* (0.23 mmol/kg) value at P< 0.05.

The introduction of the ethyl or benzyl piperazinyl moiety to the 4- position as in compounds *1b* (0.28 mmol/kg) and *1c* (0.24 mmol/kg), respectively, augmented the

inhibitory potency at 30 min as it reached 153% and 138%, respectively 3 hours after dosing (Table 1).

Consistent with the inhibitory effect on DPP-4 activity, treatment with compound *1b* (0.28 mmol/kg) or *1c* (0.24 mmol/kg) significantly increased the serum GLP-1 level by 2- and 1.6-folds, respectively as compared to that of diabetic untreated mice (Figure 2). Furthermore, treatment with compound *1b* or *1c* induced insignificant changes in GLP-1 level as compared to control and vildagliptin values.

Oral treatment of diabetic mice with vildagliptin (0.33 mmol/kg), compound *1b* (0.28 mmol/kg) or *1c* (0.24 mmol/kg) normalized the C-peptide level as it induced a significant increase in its level by 96%, 113% and 119%, respectively as compared to diabetic value (Figure 3). Meanwhile, treatment with compound *1d* (0.23mmol/kg) exerted significant increase in serum C-peptide by 79%. On the other hand, administration of compound *1a* (0.3 mmol/kg) showed insignificant change in C-peptide level as compared to diabetic value (Figure 3).

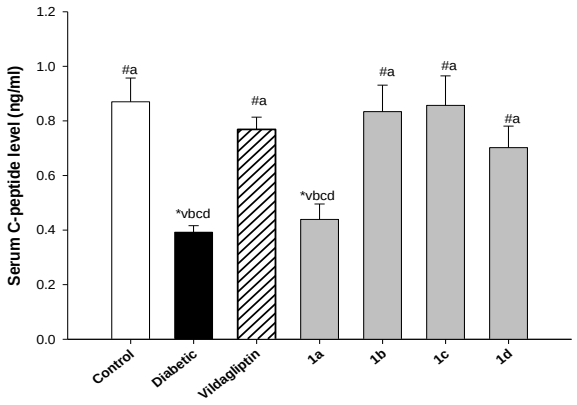


Figure 3: Effect of oral administration of vildagliptin and the compounds *1a-d* on serum C-peptide level in diabetic mice.

Data are represented as the mean C-peptide (ng/mL) ± s.e.m. of the number of animals in each group (n=6). Statistical analysis was carried out by one way ANOVA followed by Student Newman-Keuls test. * significantly different from normal control value at P< 0.05, $^{\#}$ significantly different from diabetic value at P< 0.05, $^{\#}$ significantly different from diabetic value at P< 0.05, ^{v} significantly different from vildagliptin (0.33 mmol/kg) value at P< 0.05, ^{a} significantly different from compound *1a* (0.3 mmol/kg) value at P< 0.05, ^{b} significantly different from compound *1b* (0.28 mmol/kg)value at P< 0.05, ^{c} significantly different from compound *1c* (0.24 mmol/kg) value at P< 0.05, ^{d} significantly different from compound *1d* (0.23 mmol/kg) value at P< 0.05.

To examine whether the DPP-4 inhibition by the compounds under investigation result in lowering serum glucose level in type 2 diabetes, the effect of compounds *1a-d* was tested on serum glucose level in type 2 diabetic mice using vildagliptin (0.33 mmol/kg) as reference drug. As shown in Figure 4, oral treatment with vildagliptin (0.33 mmol/kg), *1b* (0.28 mmol/kg) or *1c* (0.24 mmol/kg) normalize the serum glucose level in diabetic mice as they significantly reduce the serum glucose level by 67%, 57.5% and 57.8%, respectively, one hour post-administration and by 71%, 69% and 58%, respectively, three hours post-administration as compared to diabetic value. Meanwhile, treatment with compound *1a* (0.3 mmol/kg) or *1d* (0.23 mmol/kg) ameliorate the elevated blood glucose level of diabetic mice (Figure 4).

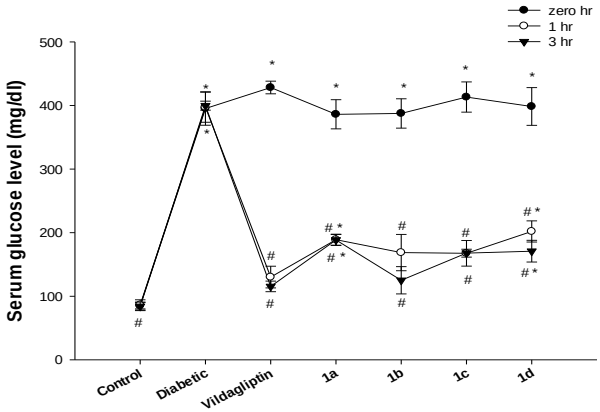


Figure 4: Effect of oral administration of vildagliptin and the compounds *1a-d* on serum glucose level in diabetic mice.

Each point represents the mean glucose (mg/dL) ± s.e.m. of the number of animals in each group (n=6). Statistical analysis was carried out by one way ANOVA followed by Student Newman-Keuls test. * significantly different from normal control value at P< 0.05.; $^{\#}$ significantly different from diabetic value at P< 0.05.

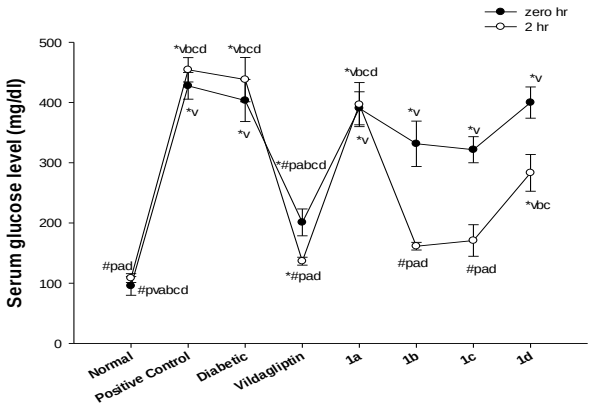


Figure 5: Effect of vildagliptin and the compounds *1a-d* on oral glucose tolerance in diabetic mice.

Indicated dose (100 mg/kg) of the test compounds were orally administered to diabetic mice 30 min before oral glucose challenge (2 g/kg). Blood samples were withdrawn 0 and 2 hours post treatment for estimation of glucose level. Data are represented as the mean glucose (mg/dL) ± s.e.m. of the number of animals in each group (n=8). Statistical analysis was carried out by one way ANOVA followed by Student Newman-Keuls test. * significantly different from normal value at P< 0.05, ^{P}significantly different from positive control value at P< 0.05, $^{\#}$ significantly different from diabetic value at P< 0.05, ^{v} significantly different from vildagliptin (0.33 mmol/kg) value at P< 0.05, ^{a} significantly different from compound *1a* (0.3 mmol/kg) value at P< 0.05, ^{b} significantly different from

compound *1b* (0.28 mmol/kg) value at P< 0.05, [c] significantly different from compound *1c* (0.24 mmol/kg) value at P< 0.05., [d] significantly different from compound *1d* (0.23 mmol/kg) value at P< 0.05.

The effect of compounds *1a-d* on oral glucose tolerance was examined in male diabetic mice. The results presented in Figure 5 revealed that oral administration of vildagliptin (0.33 mmol/kg), compound *1b* (0.28 mmol/kg) or *1c* (0.24 mmol/kg) normalized serum glucose level 2 hours after glucose loading, as they reduced the elevated glucose level by 69%, 63% and 61%, respectively, as compared to diabetic value. This inhibitory effect might be through a mechanism that involves DPP-4 inhibition. Meanwhile, administration of compound *1d* (0.23mmol/kg) ameliorated the rise in serum glucose level. On the other hand, compound *1a* (0.3 mmol/kg) showed insignificant change in serum glucose level as compared to that of diabetic value.

Molecular Modeling Studies

Molecular modeling study was initiated in order to support the assumed mode of action for the most active tested compounds and optimize a reliable model for predicating novel effective anti-diabetic hits. Docking study was carried out for compounds *1b* and *1c* into DPP-4 enzyme using Discovery Studio 2.5 software (Accelrys Inc., San Diego, CA, USA). X-ray crystal structure of DPP-4 enzyme with vildagliptin as a ligand molecule (3W2T) was obtained from protein data bank PDB[43].

The prepared protein was used in determination of the important amino acids in binding pocket. Interactive docking using Libdock protocol was carried out for poses of compounds *1b* and *1c* to the selected active site, after energy minimization using prepare ligand protocol. Re-docking vildagliptin with the same binding site showed docking energy = -109 kcal/mole with small root mean square deviation (RMSD) (0.709Å) deviation in comparison to its crystal structure. The small RMSD values proved the validity of the used docking processes[44].

Each docked compound was assigned a score according to its binding mode onto the binding site[45] that predicted binding energies and the corresponding experimental values as outlined in Table 2.

The molecular docking simulation study revealed that the binding mode of *1b* and *1c* is similar to vildagliptin in the DPP-4 active site (Figure 6, Table 2).

The pyrrolidine carbonitrile moiety occupies the S1 subsite where the cyano group forms two hydrogen bond acceptors with Tyr 666 and Tyr 547. Meanwhile the remaining part of the compounds *1b* and *1c* occupies the S2 and S2 extensive pockets where the piperazine basic nitrogens form hydrogen bond acceptors with Glu 205 and Glu 206. Regarding the π- interactions the ethyl derivative *1b* interacts with the hydrophobic residue Tyr 666 while the benzyl derivative *1c* interacts with the hydrophobic residue Phe 357 in the S2 extensive subsite. These decisive interactions with Tyr 666, Tyr 547, Glu 205, Glu 206 and Phe-357 are desirable for the expressed inhibitory activity of these compounds against DPP-4[46]. Moreover, it was found that libdock scores of compounds *1b* and *1c* were comparable and have no significant difference with that of vildagliptin.

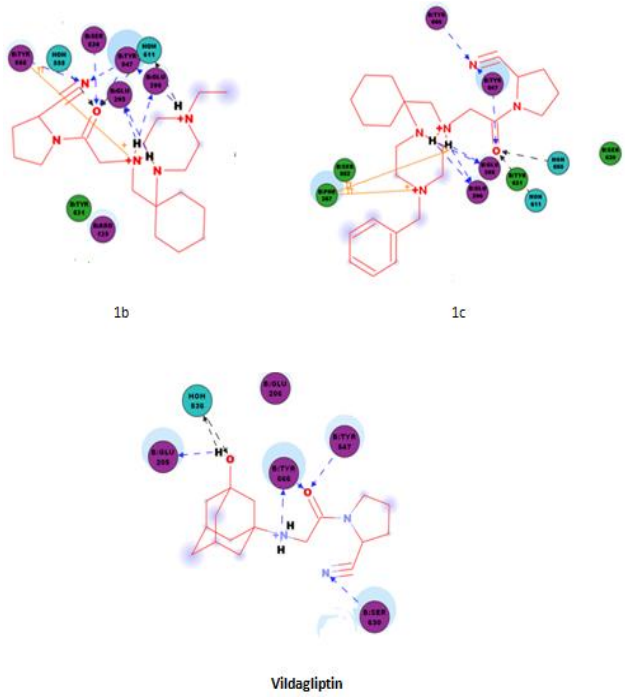


Figure 6: Key binding interactions of compounds *1b*, *1c* and *Vildagliptin* with the active site of DPP-4 enzyme. The figure was prepared using Accelrys Discovery Studio 2.5 operating system (Accelrys Inc., San Diego, CA, USA). The hydrogen bondings are depicted by blue dashed lines.

CONCUSION

In conclusion, according to the achieved appraisals, this study has revealed that, the introduction of the ethyl- or benzyl piperazinyl moiety to the 4-position as in compounds *1b* (0.28 mmol/kg) and *1c* (0.24 mmol/kg), equivalent to 100mg/kg exhibited greater DPP-4 inhibitory activity as well as reducing the effect on serum glucose level after oral administration to type 2 diabetic mice than the introduction of the piperidinyl moiety in *1a* (0.3 mmol/kg) or the p-methoxybenzylpiperazinyl moiety in *1d* (0.23 mmol/kg) to the cyclohexyl ring. These effects were accompanied by augmentation of GLP-1 and C-peptide levels. Thus, it is likely that the newly synthesized compounds increased the endogenous GLP-1 level by DPP-4 inhibition; consequently increased active GLP-1 and C-peptide levels may stimulate insulin secretion resulting in reducing serum glucose level in type 2 diabetic mice. Also, molecular docking studies on compounds *1b* and *1c* revealed that they exhibited good binding mode at the active site of DPP-4 enzyme complementing their biological activity. The potent DPP-4 inhibitors *1b* and *1c* are expected to have usefulness in development as therapeutic candidates for impaired glucose tolerance and type 2 diabetes.

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