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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

6-Imino-2-thioxo-pyrimidinones as a new class of dipeptidyl peptidase IV inhibitors

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Abstract Dipeptidyl peptidase IV is a glycoprotein which removes N-terminal dipeptides from physiologically relevant polypeptides. An homologous series of 6-imino-2-thioxo-5-{[3,4,5-tris(methyloxy)phenyl]methyl}-2,5-dihydro-4(3*H*)-pyrimidinones has been tested for inhibition of DPP IV activity. The inhibitory effects at 0.1 mM were observed. Enzyme kinetic studies revealed that compounds inhibit DPP IV activity competitively. According to the molecular docking analysis, the inhibitors are anchored into the DPP IV hydrolytic site by interactions of the pyrimidinone core with Glu206, Tyr662, and Tyr547, with the alkyl chain entering the S1 pocket. We conclude that pyrimidinone-like compounds are a promising new scaffold for reversible inhibition of DPP IV.

Keywords DPP IV inhibitor · Pyrimidinone · Enzyme kinetics · Molecular docking · Cytotoxicity

Abbreviations

DPP IV	Dipeptidyl peptidase IV
GLP-1	Glucagon-like peptide-1
GIP	Glucose-dependent insulinotropic polypeptide
SAR	Structure–activity relationship
RMSD	Root mean square deviation
PDB	Protein Data Bank
GOLD	Genetic optimisation for ligand docking
MTS	3-(4,5-Dimethylthiazole-2-yl)-5-(3-carboximethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazole
ECACC	European Collection of Cell Cultures
THP-1	Human monocytic leukemia
HepG2	Human Caucasian hepatocyte carcinoma

Introduction

Dipeptidyl peptidase IV (CD26/DPP IV) is a 110-kDa glycoprotein which plays an important role in T cell activation, immune regulation, signal transduction, and apoptosis (Boonacker and Van Noorden Cornelis, 2003; Sato *et al.*, 2003). It interacts with antigen-presenting cell, and regulates cytokine and chemokine functions (Ohnuma *et al.*, 2008). DPP IV appears to play an important role in diabetes, obesity, anxiety, rheumatoid arthritis, multiple sclerosis, cancer, autoimmune diseases, and AIDS (Thompson *et al.*, 2007; Pro and Dang, 2004; Pratley and Salsali, 2007; Havre *et al.*, 2008). DPP IV acts to sequentially remove N-terminal dipeptides from proline-containing peptides such as incretins [glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)] (Wiedeman, 2007; Deacon, 2007), from

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some neuropeptides (Mentlein *et al.*, 1993; Mentlein, 1999), and from other growth factors, and vasoactive peptides. Incretin hormones are secreted in response to meal ingestion, thereby enhancing postprandial insulin and glucagons secretion, which modulate plasma glucose levels (Thongtang and Sriwijitkamol, 2008). In type 2 diabetic patients; the continuous infusion of GLP-1 decreases plasma glucose and haemoglobin A1c concentrations and improves beta-cell function (Madsbad *et al.*, 2008). In order to overcome its rapid inactivation, effective therapy with exogenously derived GLP-1 would require either continuous infusion or multiple daily injections. In order to conquer this barrier, two approaches have been pursued: (i) the creation of GLP-1 analogues (receptor agonists or incretin mimetics) with a low affinity for DPP IV and, resistance to enzymatic inactivation; and (ii) the development of compounds that inhibit the activity of DPP IV and, prevent inactivation of endogenous GLP-1 and GIP (Pratley and Salsali, 2007). The first two of such drugs, the incretin mimetic Exenatide® (Byetta-Amylin Pharmaceuticals) and the DPP IV inhibitor Sitagliptin® (Januvia-Merck & Co.) (Kesty *et al.*, 2008), were approved by the FDA in 2005 and 2006, respectively. Reported inhibitors of DPP IV have various heterocyclic structures and include pyrrolidines (Hughes *et al.*, 1999), piperazines (Brockunier *et al.*, 2004), amino-substituted pyrimidines (Peters *et al.*, 2004), etc. We propose 6-imino-2-thioxo-2,5-dihydro-4(3*H*)-pyrimidinones as a promising scaffold for design of new DPP IV competitive inhibitors, which differs from the other published ones. The homologous series of eight 6-imino-2-thioxo-pyrimidinones is defined by H, alkyl, or alkenyl chain substituents at the C_5 atom of the pyrimidinone ring (Fig. 1). This group of compounds has been extensively investigated by (i) DPP IV inhibition and enzyme kinetics, (ii) molecular docking and scoring approaches, and (iii) study of cytotoxicity in vitro.

Results and discussion

Synthesis, stability, and purity of the compounds

Compounds were synthesized in two steps (Fig. 1), in accordance with the procedure described previously (Glunčić *et al.*, 1986).

Compounds were synthesized originally in the early 1980s to be screened for anti-infective activity. Since significant anti-bacterial activity was not observed, compounds were stored in a corporative Compound Bank. After more than 25 years, the compounds were checked for their identity and purity and tested in a standard screening cascade. Remarkable stability and purity was observed after more than 25 years of the room temperature storage (all compounds were >95% pure, as determined by HPLC/MS/DAD analysis, data not shown, but available on request).

In-vitro inhibition of DPP IV activity and enzyme kinetic experiments

Inhibition of DPP IV activity by pyrimidinone-like compounds was tested using H-Gly-Pro-pNA tosylate as a substrate. Results are shown in Table 1 as percent inhibition at 0.1 mM compound concentration. Unsubstituted 6-imino-2-thioxo-5-(3,4,5-trimethoxy-benzyl)-2,5-dihydro-4(3*H*)-pyrimidinone (core scaffold) at position 5 (Compound **1**) did not inhibit DPP IV activity when tested at 0.1 mM. On the other hand, when methyl, ethyl, propyl, isopropyl, allyl, and butyl radicals were inserted at position 5 of the core scaffold, significant inhibition was obtained. The level of inhibition lay between 40 and 47%. Compound **5** was chosen for the determination of the type of DPP IV inhibition because of its good solubility in buffer. Time course experiments showed a linear increase in DPP IV activity during the first few minutes. Consequently, initial velocities were measured for 4 min and expressed as a change in absorbance at 405 nm with time (Fig. 2).

The calculated enzyme kinetic parameters in the absence of compound were $K_m = 0.7$ mM and $V_{max} = 5.6 \times 10^{-4}$. The K_m value is slightly higher than, but close to the previously published K_m values for this substrate (Abbott *et al.*, 2000). Values in the presence of 0.3 mM of Compound **5** were $K_m = 2.2$ mM and $V_{max} = 6.9 \times 10^{-4}$. A difference in K_m values, with and without compound, and almost no difference in V_{max} values are characteristics of competitive inhibition, for which $K_m^* = \alpha K_m$, where $\alpha = 1 + [I]/K_i$ (Marangoni, 2003). These results suggest that Compound **5** is a weak ($K_i = 160$ μM) competitive inhibitor of DPP IV. In order to confirm this and to gain a more detailed understanding of

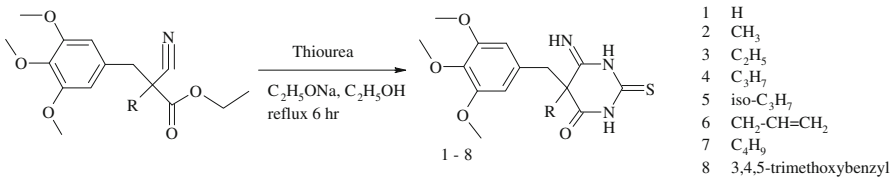

Fig. 1 Structures and synthesis of a series of 6-imino-2-thioxo-5-{[3,4,5-tris(methyloxy) phenyl]methyl}-2,5-dihydro-4(3*H*)-pyrimidinone compounds

Table 1 A series of pyrimidinone-like compounds with different side-chain-modifications

Compound	R	% of DPP IV inhibition
1	Hydrogen (H)	0
2	Methyl (CH_3)	40
3	Ethyl (C_2H_5)	40
4	Propyl (C_3H_7)	47
5	Isopropyl (iso-C_3H_7)	45
6	Allyl (CH_2–CH=CH_2)	47
7	Butyl (C_4H_9)	44
8	3,4,5-Trimethoxybenzyl	51

Percent inhibition of DPP IV is shown at 0.1 mM compound concentration

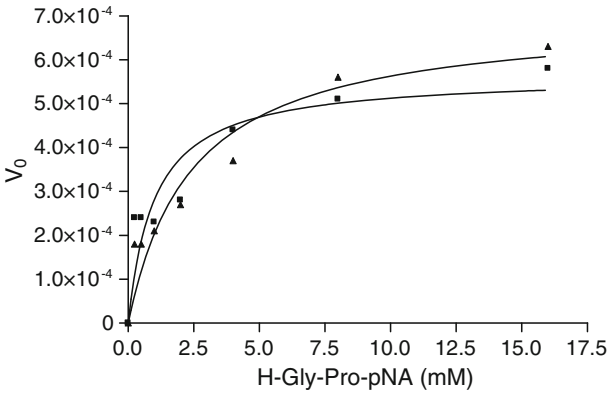

Fig. 2 Determination of the type of inhibition of DPP IV by Compound **5**. *Filled square*, without compound; *filled triangle*, 0.3 mM Compound **5**. Non-linear regression curve fitting was used for determination of kinetic parameters

compound-enzyme interactions, in silico studies were performed.

In silico studies

The catalytic site of DPP IV is buried in a small pocket within a large cavity placed at the interface of its two domains, the β-propeller and α/β-hydrolase domains. It has been found that residues surrounding the scissile bond P1–P1′ of a dipeptidyl-polypeptide mainly determine the substrate selectivity of DPP IV (Aertgeerts *et al.*, 2004). For substrate recognition, the substrate must enter the hydrophobic pocket S1, close to Ser630, and interact with glutamates Glu205 and/or Glu206 (Glu-Glu motif), that was shown to be essential for enzymatic activity (Abbott *et al.*, 1999). Interactions of ligands with side chains constituting the spacious S2 pocket, especially ones with hydrophobic, long or bulky side chains, generally showed an enhanced binding (Brandt, 2000; Peters *et al.*, 2004). We reproduced successfully the experimental poses of known inhibitors (RMSD <1.5 Å) with DPP IV crystal structures available from the PDB (Bernstein *et al.*, 1977; Berman *et al.*, 2003) using GOLD (Jones *et al.*, 1997; Hartshorn *et al.*, 2007, Thoma *et al.*, 2003; Peters *et al.*, 2004). Binding modes of pyrrolidine nitriles NVP-DPP728 (Hughes *et al.*, 1999), and FE999011 (Sudre *et al.*, 2002), reversible covalent DPP IV inhibitors, were satisfactorily predicted without applying covalent bond constraint. GOLD also successfully differentiated active from inactive compounds in reproducing the results of wet screening of an in-house compound library in the DPP IV enzymatic assay. The calculations were performed using the two different binding site conformations of the crystal structures with PDB code 1NU8 (Thoma *et al.*, 2003), and 1RWQ (Peters *et al.*, 2004). In these structures, DPP IV is complexed with competitive reversible non-covalent inhibitors of different chemical classes. In 1NU8, DPP IV is complexed with Ile-Pro-Ile (Diprotin), the pyrrolidine ring lying within the S1 pocket. In 1RWQ, it binds a 4-aminomethylpyrimidine inhibitor, and the dichlorophenyl ring enters the S1 pocket. In solution, the tested pyrimidinones may exist in an equilibrium complex of various ionic and tautomeric forms. Here, we considered only the most prevalent basic tautomer form [as attested by the software MN.TAUTOMER (Molecular Networks GmbH, 2004)] in its neutral and mono-protonated ionization states. According to ACD/pK_a (ACD/Labs, 2006), these molecules should be predominantly in the neutral ionization form (>80%) in neutral solutions. Since stereochemistry of the ring C_5 atom was not known, two possible enantiomers per molecular species were formed. For all the six C_5-substituted pyrimidinone derivatives, the predicted first GOLD/GoldScore ranked poses of both neutral and mono-protonated pyrimidinones in both 1NU8 and 1RWQ binding sites satisfy three recognition interactions: alkyl/alkenyl chain enters the S1 pocket, HN=C–NH fragment exhibits anchoring H-bond interactions with Glu206 and Tyr662, and the bulky trimethoxybenzyl group occupies the S2 pocket, providing favorable interactions with Arg125. The O=C–NH fragment of the pyrimidinone ring can interact with the Tyr547 amino acid residue, while the sulphur atom is orientated toward free space (Fig. 3).

In order for such a binding position to be achieved, the C_5 atom of the pyrimidinone ring must have the S configuration (i.e., R in the case of Compound **5**). The docking calculations were performed under the assumption of reversible non-covalent binding. The possibility of reversible covalent binding through interaction of Ser630 and 6-imino group of the compounds considered cannot be neglected (Kim *et al.*, 2006). However, the polar 6-imino 2-thioxo pyrimidinone heterocycle is quite unlikely to enter the S1 pocket, and,

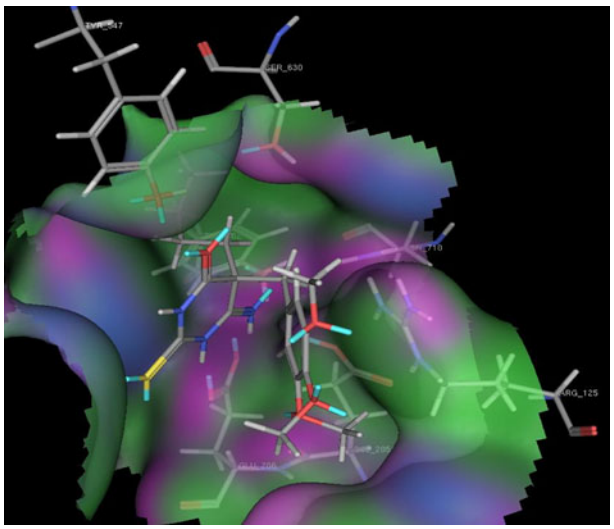

Fig. 3 Binding conformation of the pyrimidinones within the hydrolytic site of DPP IV. Interacting amino acid residues Glu206, Tyr662, Tyr547, and Arg125 are shown together with Compound **7**

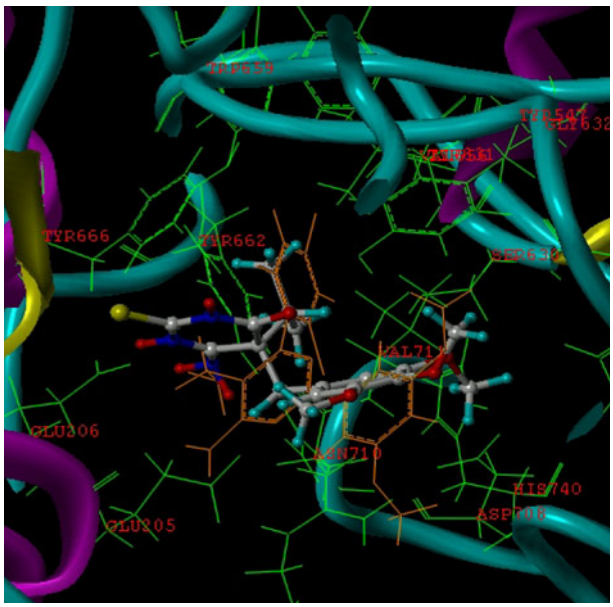

Fig. 4 Comparison of binding modes of the pyrimidinones, represented by Compound **5** and the 1RWQ ligand aminomethylpyrimidine, within the peptide-binding site of DPP IV

hence, such a binding mechanism is expected to be less probable. With regard to the energetics of the ligand–DPP IV interaction, the first-ranked poses at the GoldScore fitness scale also scored highly for the other scoring function considered, i.e., consensus score. In addition to the published DPP IV inhibitors, the best score (consensus score of 3) was obtained for pyrimidinones with the longest chains (Compounds **4** and **7**). In the compound set considered, the poorest score was obtained with the non-substituted Compound **1**. Its binding mode is analogous to the binding mode of its C_5-substituted derivatives (Compounds **2**–**7**). Although Compound **8** showed similar percentage of inhibition of DPP IV, its binding mode could not be determined unambiguously from docking analysis. Based on SAR for published DPP IV competitive inhibitors (Aertgeerts *et al*., 2004; Abbott *et al*., 1999; Brandt, 2000; Peters *et al.*, 2004), and molecular modeling results for the tested pyrimidinones (Fig. 4), it is expected that substitution of pyrimidinones at the C_5 ring position with pyrrolidine or phenyl rings should significantly increase their potency.

Cytotoxicity testing of the tested compounds

Dose–response curves for evaluation of cytotoxicity have been made for two human immortal cell lines—THP-1 and HepG2 cells, measured in terms of the mitochondrial succinate dehydrogenase activity of living cells, expressed as the percentage of the optical density (OD) values of non-treated cells. None of the compounds showed any cytotoxic effect or inhibition of cell growth of THP-1 or HepG2 cells at concentrations up to 0.1 mM.

Conclusion

The 6-imino-2-thioxo-pyrimidinone series of compounds showed inhibition of dipeptidyl peptidase IV activity at a concentration of 0.1 mM. Enzyme kinetic experiments revealed that the enzyme inhibition was competitive, indicating that the compounds bind to the peptide-binding site. The possible binding mode of the pyrimidinones to the hydrolytic site of DPP IV was proposed by combining knowledge of binding modes of known DPP IV ligands and molecular docking and scoring results for the homologous chemical series. The most likely binding mode for this group of compounds involves insertion of the alkyl/alkenyl chain into the S1 pocket, the HN=C–NH fragment making an H-bond network with Glu206 and Tyr662, the 6-carbonyl oxygen interacting with Tyr547, and the trimethoxybenzyl-group interacting with Arg125 in the S2 pocket. According to the proposed binding mode, S-enantiomeric forms (R in the case of Compound **5**) of the pyrimidinones are preferable, and it is expected that replacement of alkyl/alkenyl chains with a pyrrolidine or phenyl ring at the C_5 atom will significantly increase their potency for inhibition of DPP IV activity. Neither compound showed any cytotoxic effect or inhibition of cell growth of THP-1 and HepG2 cell lines at concentrations up to 100 μM. In conclusion, we propose 6-imino-2-thioxo-5-{[3,4,5-tris(methyloxy)phenyl]methyl}-2,5-dihydro-4(3*H*)-pyrimidinone as a new anchoring fragment in which substitution at C_5 position is likely to optimize inhibition of DPP IV.

Experimental protocols

Chemistry of pyrimidinone-like group of compounds

Compounds were synthesized in two steps, as described previously (Glunčić *et al.*, 1986). In brief, the ethyl ester of (3,4,5-trimethoxybenzyl)-cyanoacetic acid (Fig. 1, R=H) was used as the starting material, being first alkylated with corresponding halides in the presence of sodium alcoholate, to give ethyl esters of 2,2-disubstituted cyanoacetic acid. The reaction was performed in ethanol in the presence of sodium ethanolate, for 1 h at boiling temperature. In the second step, prepared esters were condensed with thiourea in ethanol with sodium ethanolate, for 6 h, at boiling temperature. Ethanol was then distilled off, the residue dissolved in water and acidified to pH 6 at 0°C with diluted hydrochloric acid to precipitate the compounds.

Inhibition of DPP IV and enzyme kinetics

DPP IV enzyme (R&D systems, USA, MN) kinetics and the activity of the compounds were tested using H-Gly-Pro-pNA tosylate (Bachem, Switzerland) as a substrate. Compound **5** (Table 1) was used for the determination of the enzyme inhibition type. The enzymatic reaction was performed in 100 µl of buffer containing 50 mM Tris, 150 mM NaCl at pH 7.9. Percent of inhibition and the type of inhibition were determined after 60-min reaction with 100 µM of each compound; 200 ng enzyme, 300 µM of Compound **5**, and 0.25–8 mM substrate. Initial velocities were determined by measuring absorbance at 405 nm during the first 4 min of the enzyme reaction. The non-linear regression (curve fit) curve was created using the Michaelis–Menten equation (1). Changes in kinetic parameters (K_m and V_{max}), after compound interaction with the enzyme, were used as parameters for the determination of the type of inhibition (Marangoni, 2003). Data analysis was performed using GraphPad Prism software version 3.02 (GraphPad software, San Diego, USA)

$$v = V_{max}[S]/(K_m + [S]), \quad (1)$$

where K_m is the concentration of substrate required to reach half-maximum velocity; V_{max} is the maximum reaction velocity; and $[S]$ is the substrate concentration.

In silico studies

The binding of the pyrimidinones to DPP IV was explored by molecular docking and a scoring method using the program GOLD, Version 2.1 (Jones *et al.*, 1995). It was assumed that the pyrimidinones bind in reversible non-covalent way. The binding site was defined as a sphere with an origin at the hydroxyl O-atom of the amino-acid residue Ser630 and a radius of 10 Å. Planar N-atom, amide bonds, and free ring corners of compounds were allowed to flip. Ten conformations were generated per compound and ranked according to GoldScore fitness function. Input conformations were pre-minimized by Tripos force field with Gasteiger–Hückel charges and distance-dependent dielectric constant $\varepsilon=\varepsilon(4r)$ (Tripos Inc., 2003). Geometrical optimizations were performed with at most 500 iterations, using a gradient of 0.05 kcal/molÅ as a terminating criterion. The three best-ranked positions were re-scored using ChemScore (Baxter *et al.*, 1998) and ASP (Astex Statistical Potential) (Mooij and Verdonk, 2005) fitness functions available in GOLD (Jones *et al.*, 1995). GoldScore, ChemScore, and ASP score were each converted to *Z*-score and binned by taking 0 as the cut-off value within SpotFire DecisionSite software, v. 8.2.1 (Spotfire 2005). The consensus score was calculated by summing the three binned scores and the best-ranked positions had the consensus score 3. Consensus scoring was done with seven known inhibitors within the scored compound set: Ile-Pro-Ile (Thoma *et al.*, 2003), NVP-DPP728 (Hughes *et al.*, 1999), FE999011 (Sudre *et al.*, 2002), (S)-isoleucine thiazolidide P32/98 (Sorbera *et al.*, 2001), cyclohexylglycine pyrrolidide, aminomethylpyrimidine representative (Peters *et al.*, 2004), and substituted piperazine representative (Brockunier *et al.*, 2004).

Cell lines and cytotoxicity assay

Cell lines were purchased from the ECACC—THP-1, monocyte, ECACC-88081201 (Tsuchiya *et al.*, 1980), and HepG2, epithelial, ECACC-85011430 (Aden *et al.*, 1979). Cells were maintained in complete RPMI 1640 medium (Institute of Immunology, Zagreb, Croatia) supplemented with 10% Fetal Bovine Serum (BioWest, S04382S1810) at 37°C in a 5% CO_2 atmosphere. Cytotoxicity assay was performed by using the MTS CellTiter 96® AQueous One Solution Cell Proliferation Assay (G358B, 18824201, Promega, USA). Each culture in the 96-well plate contained 50,000 (for HepG2) or 75,000 (for THP-1) cells. Cultures exposed to tested compounds were incubated for 24 h at 37°C in 5% CO_2. Thereafter, 15 µl of MTS reagent (Mosmann, 1983) was added directly to the cell lines. After an additional 2 h of incubation at 37°C in 5% CO_2, the absorbance was recorded at 490 nm using a spectrophotometric plate reader (Ultra, TECAN, USA). The method was programmed for the TECAN robotic system in GEMINI pipetting software (Verbanac *et al.*, 2005).

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