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Catalytic properties and inhibition of proline-specific dipeptidyl peptidases II, IV and VII

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There is currently intense interest in the emerging group of proline-specific dipeptidases, and their roles in the regulation of biological processes. Dipeptidyl peptidase IV (DPP-IV) is involved in glucose metabolism by contributing to the regulation of glucagon family peptides and has emerged as a potential target for the treatment of metabolic diseases. Two other proline-specific dipeptidases, DPP-VII (also known as quiescent cell proline dipeptidase) and DPP-II, have unknown functions and have recently been suggested to be identical proteases based on a sequence comparison of human DPP-VII and rat DPP-II (78 % identity) [Araki, Li, Yamamoto, Haneda, Nishi, Kikkawa and Ohkubo (2001) *J. Biochem.* **129**, 279–288; Fukasawa, Fukasawa, Higaki, Shiina, Ohno, Ito, Otogoto and Ota (2001) *Biochem. J.* **353**, 283–290]. To facilitate the identification of selective sub-

strates and inhibitors for these enzymes, a complete biochemical profile of these enzymes was obtained. The pH profiles, substrate specificities as determined by positional scanning, Michaelis–Menten constants and inhibition profiles for DPP-VII and DPP-II were shown to be virtually identical, strongly supporting the hypothesis that they are the same protease. In addition, substrate specificities, catalytic constants and IC₅₀ values were shown to be markedly different from those of DPP-IV. Selective DPP-IV and DPP-VII substrates were identified and they can be used to design selective inhibitors and probe further into the biology of these enzymes.

Key words: dipeptidyl peptidase II, dipeptidyl peptidase IV, quiescent cell proline dipeptidase.

INTRODUCTION

A large number of proteins and peptides are synthesized with proline in the penultimate position, and there is increasing evidence that proline-specific serine dipeptidases may play an important role in the regulation of the functions of these molecules. One such enzyme that has received considerable attention is the cell-surface enzyme, dipeptidyl peptidase IV (DPP-IV), which has been implicated in the hydrolysis of a variety of substrates, including neuropeptides, chemokines and peptides of the glucagon family [1,2]. In particular, the incretins glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) are known endogenous substrates for DPP-IV, suggesting a role for this enzyme in glucose control [3]. Indeed, DPP-IV inhibitors are efficacious in animal models of diabetes, and are currently under clinical development [4].

Quiescent cell proline dipeptidase (QPP) is a recently studied member of the ‘DPP-IV activity and/or structure homologues’ (DASH) proteins. These proteins are enzymes that are unified by their common post-proline-cleaving serine dipeptidase mechanism [5]. QPP was initially named as such because of the observation that QPP inhibitors were found to induce apoptosis in quiescent lymphocytes [6,7]. As a result of its relationship to the family of DASH proteins, QPP was recently renamed as DPP-VII.

There is ample evidence that DPP-VII is very closely related or identical to another member of the DASH protein family, namely

DPP-II (EC 3.4.14.2). DPP-II was first identified in bovine anterior pituitary extracts [8] and later biochemically and enzymically characterized in different tissues from multiple species [9–14]. This enzyme has been identified to play a role in the degradation of collagen, substance P and other oligopeptides or their fragments [15]. Both DPP-II and DPP-VII are reported to be 58-kDa N-glycosylated proteins that contain disulphide bonds and occur in oligomeric states. Specifically, DPP-VII is functionally active as a leucine zipper-mediated homodimer [16]. Both proteins are reported to be localized primarily in vesicles; however, DPP-II is described as a lysosomal enzyme, whereas DPP-VII is located in vesicles that are distinct from lysosomes. Although lysosomes and DPP-VII-containing vesicles have similar densities, the latter do not contain lysosome-specific markers such as lysosome-associated membrane protein 1, and do not colocalize with lysosomes as determined by confocal microscopy [17]. It should be noted that the identification of vesicle type for DPP-II was primarily based on density gradients at a time when specific markers for lysosomes were not available.

The encoding gene for human DPP-II has never been assigned. Recently, rat DPP-II was purified, cloned and functionally expressed [9,14]. These investigators suggested that DPP-II and DPP-VII represent the same enzyme, based on a sequence identity of 78 % between rat DPP-II and human DPP-VII, and the fact that the N-terminal sequence of DPP-II-like activity from human placenta [18] matched residues Ala²⁶ to Leu⁵⁸ of the encoded sequence of the human DPP-VII gene.

Abbreviations used: AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; DPP, dipeptidyl peptidase; DASH, DPP-IV activity and/or structure homologues; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide 1; QPP, quiescent cell proline dipeptidase.

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To facilitate the identification of selective substrates and inhibitors for these proline-specific dipeptidases, and to obtain additional evidence that human DPP-II and DPP-VII are identical, we compared these enzymes with respect to pH profiles, substrate specificity, catalytic constants and inhibitor profile. The properties of DPP-II and DPP-VII are indeed virtually identical, and highly distinct from those of DPP-IV. In particular, profound differences in the primary specificities of these enzymes, as revealed by positional scanning, suggest the design of selective inhibitors that may have utility in studies of enzyme function.

EXPERIMENTAL

Cloning, expression and purification of recombinant human DPP-VII

The gene for human DPP-VII was PCR-amplified from a human leucocyte Marathon-Ready™ cDNA library (ClonTech, Palo Alto, CA, U.S.A.) using the primers 5'-CCGGCTCCAGGAG-CGTTCTCCAGCAG-3' and 5'-GGTCAGAGGCTGAGT-CTGGGCCCCCAGC-3'. The PCR product was cloned into the TA cloning vector pCR2.1 (Invitrogen) and confirmed by DNA sequencing. The gene was amplified in a subsequent PCR reaction using primers 5'-CCGGTACCGACTACAAG-GACGACGATGACAAGCATATGGGCTTCCAGGAGCG-CTTCTCCAGCAG-3' and 5'-GGAAGCTTTCAGAGGC-TGAGTCTGGGCCCCACG-3', which added the coding sequence for an N-terminal FLAG® tag and enterokinase site (DYKDDDDK), digested with *Asp*718 and *Hind*III and cloned into a derivative of the insect cell expression vector pFastBac™ 1 (Life Technologies, Gaithersburg, MD, U.S.A.). This vector had been previously engineered to contain an N-terminal honeybee mellitin leader signal sequence (KFLVNVALVFMVYIYA) for protein secretion. The final construct comprised a DPP-VII protein from residue Gly³⁰ to the end, fused to an N-terminal FLAG® tag. This was confirmed by N-terminal sequencing of the purified protein (results not shown). The DPP-VII gene encoding from Ala²⁶ to the end was obtained by PCR-amplifying a 5'-gene fragment of the Gly³⁰ expression construct using the primers 5'-CCGGTACCGACTACAAGGACGACGATGACAAGC-ATATGGCCCCGACCCCGGCTTCCAGGAGCGC-3' and 5'-TCGACGTCCCGGAAGAACTGGTTGGAG-3'.

The PCR fragment was cloned into the same vector digested with *Asp*718 and *Aat*II restriction sites. Recombinant bacmid DNA and transfected SF9 cells were obtained using the BAC-TO-BAC™ Baculovirus Expression System (Life Technologies) according to the manufacturer's instructions. Recombinant virus was amplified and plaque-purified. The expression rate was similar for individual clones of each construct. Viral stocks were generated in Grace's medium + 10% (v/v) foetal calf serum and typically had a titre of 10⁸ pfu (plaque-forming units)/ml. For large-scale protein expression, recombinant human DPP-VII baculovirus was used to infect High Five insect cells, at a MOI (multiplicity of infection) of 1.0 for 72 h using ESF-921 serum-free medium (Expression Systems).

For protein purification, 1000 ml of culture media was pH adjusted to 7–7.5 and loaded on to an anti-FLAG® M2-agarose affinity gel (2.5 cm × 7 cm; Sigma). The column was washed with 500 ml of 10 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl and eluted with 100 mM glycine buffer (pH 3.5). Elution fractions were immediately neutralized using Tris/HCl buffer. Probably due to the low expression rate of DPP-VII, we could not obtain a homogeneous protein using a single affinity step alone, even when stringent wash conditions were applied. A second anti-FLAG affinity step executed on a smaller amount of fresh resin (2 ml) yielded a homogeneous protein. Dipeptidase-

Table 1 Purification of DPPs

Protein	Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold purification
Ala ²⁶ DPP-VII	Medium	140	5588	40		
	Affin-1	0.19	22 000	113 402	100	2835
	Affin-2	0.15	18 945	126 300	86	3158
Gly ³⁰ DPP-VII	Medium	139	1230	9		
	Affin-1	0.05	3100	62 000	100	6889
	Affin-2	0.03	2437	81 233	79	9026
DPP-II	Extract	34 970	33 100	0.9	100	
	AS-cut	897	28 173	31	85	34
	DEAE	121	3756	31	11	34
	Butyl-650	21	2350	112	7	124
	Protein A	7	2150	307	6	341
DPP-IV	Medium	131	4.7 × 10 ⁸	3.6 × 10 ⁶	100	
	Q-Sepharose	6	9.4 × 10 ⁷	6.7 × 10 ⁷	20	19

containing fractions were eluted as before, pooled and concentrated (Millipore Ultrafree centrifugal filter device, cut-off molecular mass of 10 000 Da). Total DPP-VII activity in the medium was always lower than that achieved after the first purification step due to the presence of an unknown reversible inhibitor in the medium. Therefore the yield for the first affinity purification was defined to be 100%, taken as a reference for the next step (Table 1). Typically, approx. 30 µg/l of Gly³⁰ DPP-VII and 150 µg/l of Ala²⁶ DPP-VII were obtained.

Cloning, expression and purification of recombinant human DPP-IV

The gene for human DPP-IV was PCR-amplified from a human kidney Marathon-Ready™ cDNA library (ClonTech) using the primers 5'-CTGGTACCAACAAAGGCACAGATGATGC-3' and 5'-CGAAGCTTCTAAGGTAAGAGAAACA-3'. The PCR product was cloned into the same expression vector used for DPP-VII, which contained the honeybee mellitin leader sequence employing restriction sites *Asp*718 and *Hind*III. The resulting construct secreted soluble DPP-IV (Asp²⁹ to the end) without any fusion tag. The insect cell expression was performed as described above. For protein purification, 1000 ml of culture medium was filtered (0.45 µm), diluted with 2 vol. of 10 mM Tris/HCl buffer (pH 8.0) and loaded on to a Q-sepharose (5 cm × 25 cm, fast flow, Pharmacia). The column was washed intensively with the same buffer and developed with a 9 h linear gradient from 0 to 2 M NaCl. DPP-IV protein eluted before any other protein and the first few fractions were > 99% pure.

Purification of DPP-II from human placenta

In an attempt to purify human DPP-II from placenta, its separation from DPP-IV-like activity was carefully monitored using the substrate Lys-Ala-AMC (where AMC stands for 7-amino-4-methylcoumarin) at pH 5.5 (DPP-II-specific), in contrast with Gly-Pro-AMC-cleaving activity at pH 7.5. Human placenta (500 g) was homogenized in 500 ml of 10 mM Tris/HCl buffer (pH 8.0) using a homogenizer. Cell debris was removed by centrifugation for 15 min at 10 000 g. The 40–60% sediment of a subsequently fractionated ammonium sulphate precipitation was resolved and dialysed against 10 mM Tris/HCl buffer (pH 8.0). The sample was applied on to an anion-exchange column [Fractogel EMD DEAE-650(M) column, 2.5 cm × 20 cm], equilibrated with 10 mM Tris/HCl buffer (pH 8.0) and

eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. DPP-II-containing fractions were pooled, saturated with ammonium sulphate to 20% and applied to a hydrophobic interaction chromatography-column [EMD Butyl-650(M), 2.5 cm × 15 cm], equilibrated with 10 mM Tris/HCl buffer (pH 8.0) containing 20% ammonium sulphate. The protein was eluted using a linear reverse gradient of 20–0% ammonium sulphate. DPP-II-containing fractions were pooled and concentrated (Millipore Ultrafree centrifugal filter device; cut-off molecular mass of 10000 Da). To remove co-purifying immunoglobulins from the sample, the DPP-II-containing fractions were filtrated over Protein A–Sepharose (2 ml resin).

Enzyme assays

DPP-VII and DPP-II were assayed continuously in 100 mM cacodylate buffer (pH 5.5) containing 0.1 mg/ml of BSA in a total volume of 100 μ l for 30 min at 37 °C, and read using a Spectramax Gemini plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Substrates Lys-Ala-AMC, Lys-Pro-AMC, Gly-Ala-AMC, Gly-Pro-AMC, Ala-Pro-AFC (where AFC stands for 7-amino-4-trifluoromethylcoumarin) were obtained from Bachem (Torrance, CA, U.S.A.) and Nle-Pro-AMC and Nle-Nle-rhodamine derivatives were synthesized in-house. The excitation/emission wavelengths for different fluorogenic substrates were 360/460 nm (AMC), 485/535 nm (rhodamine) and 400/505 nm (AFC). One unit of DPP-VII activity cleaved 1 mol of Nle-Pro-AMC/min at pH 5.5 and at 37 °C.

DPP-IV activity was assayed in 100 mM Hepes buffer (pH 7.5) and 0.1 mg/ml of BSA using the same assay method as described above. The combinatorial positional scanning dipeptide library, provided as DMSO stock solutions at 50 μ M for each compound, was obtained from Harris et al. [19]. It was used at 0.5 μ M/compound and 1% total DMSO using the individual assay conditions as described above. Inhibitors were obtained as follows: diprotin A from Bachem, benzamidin from Sigma and Pefabloc SC from Roche Molecular Biochemicals. All other inhibitors were synthesized in-house. IC₅₀ values were obtained using the above buffer conditions for each enzyme and substrates at the K_m level of concentration: 4 μ M Nle-Pro-AMC for both DPP-II and DPP-VII and 50 μ M Gly-Pro-AMC for DPP-IV. All inhibitors tested were competitive reversible inhibitors and K_i values were calculated using the Michaelis–Menten equation for competitive inhibition:

$$IC_{50} = K_i(1 + [S]/K_m)$$

Enzyme kinetics analysis

All kinetic constants were computed by direct fits of the data to the appropriate non-linear equation, employing a non-linear regression procedure (NLIN; N.A. Thornberry, unpublished work) or commercially available curve-fitting programs such as SigmaPlot.

RESULTS

Expression and purification of human DPP-VII and DPP-IV from insect cells

In the initial description of DPP-VII, the correct post-translational processing sites and the native N-terminal sequence were not identified. Therefore two different polypeptide constructs were expressed in a Baculovirus Expression System yielding proteins with the N-terminus at Gly³⁰ and Ala²⁶ respectively. Both constructs contained an N-terminal FLAG[®] tag (DYKDDDDK) to facilitate purification to homogeneity from

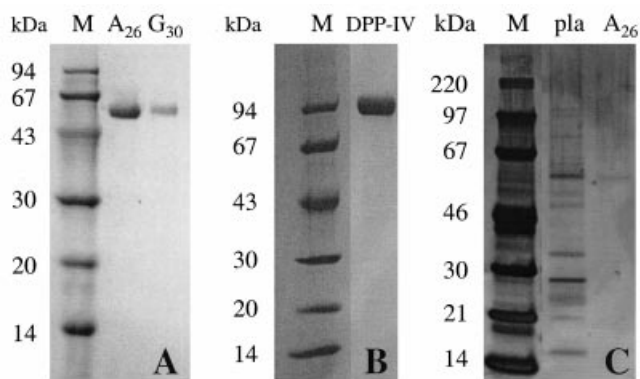


Figure 1 Purification of DPP

(A) Coomassie-stained SDS/PAGE (12% gel) of anti-FLAG-tag affinity-purified DPP-VII with an N-terminus at Gly³⁰ (G₃₀) or Ala²⁶ (A₂₆). (B) Coomassie-stained gradient SDS/PAGE (4–12% gel) of purified recombinant DPP-IV. (C) Silver-stained gradient SDS/PAGE (4–12% gel) of partially purified DPP-II (pla) from human placenta.

culture media using anti-FLAG[®] affinity purification (Figure 1). The purified proteins were thoroughly characterized with respect to their pH profiles, catalytic constants and inhibitor profiles and found to be enzymologically indistinguishable (Figures 2 and 4, Tables 1 and 3). Soluble recombinant DPP-IV was also secreted from insect cells analogous to DPP-VII, although without using a fusion tag to facilitate purification. The much higher expression level of DPP-IV compared with DPP-VII and its unique purification properties allowed for a rapid purification to homogeneity using a single anion-exchange column (Table 1 and Figure 1).

Purification of DPP-II from human placenta

DPP-II was partially purified from human placenta using sequential anion-exchange and hydrophobic interaction chromatography. The objective of the purification was to separate completely DPP-II activity from DPP-IV and other DASH family members that are present in this tissue. To facilitate this effort, Lys-Ala-AMC, previously reported to be a DPP-II-specific substrate at acidic pH, was employed to monitor DPP-II activity during the chromatography. Assays were conducted at pH 5.5. Conversely, assay of column fractions by Gly-Pro-AMC at pH 7.5 was used to determine the activity of DPP-IV and other DASH family proteins such as DPP8 [20] or fibroblast activation protein [21]. The results of the two major chromatography steps are shown in Figure 3. Anion-exchange chromatography only partially resolved the DPP-II activity from the activities of DPP-IV and related DASH enzymes, whereas the hydrophobic interaction column cleanly separated DPP-II from the other activities. The Gly-Pro-AMC cleaving activity at pH 7.5, which mirrors the DPP-II activity as determined by Lys-Ala-AMC activity at pH 5.5, indicates that this substrate can also be cleaved by this enzyme, albeit with reduced efficiency. The partially purified DPP-II sample used in the present study is shown in Figure 1. It is estimated that the purity of the enzyme is < 0.5% (Table 2).

Optimal pH for DPP-II, DPP-VII and DPP-IV

The optimal pH values for human DPP-II from placenta, recombinant human DPP-VII and recombinant human DPP-IV were determined across a broad pH range (3.5–8.5) using the

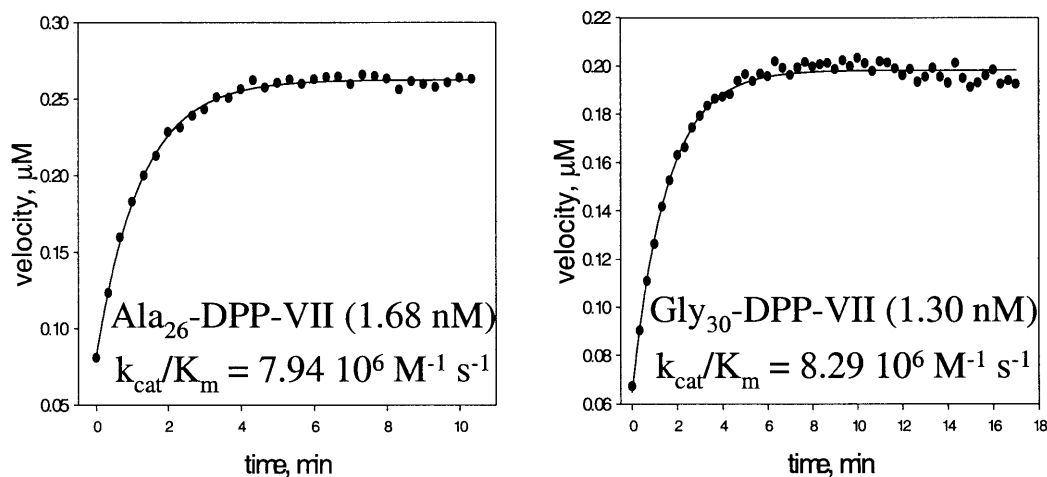


Figure 2 Determination of k_{cat}/K_m for Gly³⁰ and Ala²⁶ DPP-VII

k_{cat}/K_m values were determined in 100 mM cacodylate (pH 5.5), 0.1 mg/ml of BSA and 0.4 μ M Nle-Pro-AMC.

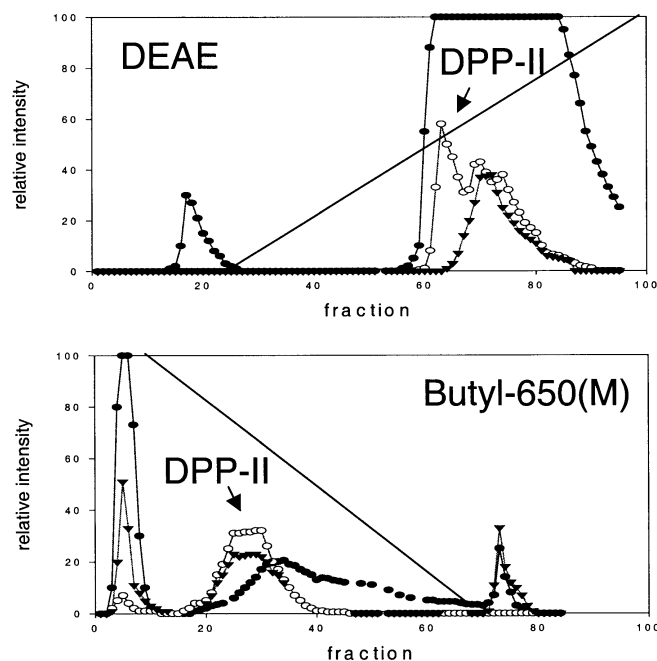


Figure 3 Partial purification of DPP-II from human placenta

The elution and activity profiles of (A) DEAE column and (B) Butyl-650: ●, total protein (A_{280}); ○, Lys-Ala-AMC-cleaving activity at pH 5.5; ▼, Gly-Pro-AMC-cleaving activity at pH 7.5. Straight lines indicate salt gradients. DPP-II-containing fractions were pooled: F61–66 (DEAE), F23–33 (Buty-650).

substrate Nle-Pro-AMC. As shown in Figure 4, the pH profiles for DPP-II and DPP-VII are virtually identical, with a peak activity at pH 5.5–6.0. The slight deviation of DPP-II from DPP-VII at pH 8 and higher is probably due to a minor contamination with another DASH protein family member (< 1%). In contrast with DPP-II and DPP-VII, DPP-IV is essentially inactive at acidic pH, with an optimal pH of 8.0–8.5. The pH profile of DPP-VII was also tested using two additional substrates, Gly-

Table 2 Comparison of substrate preferences

Assays were performed at 37 °C in 100 mM cacodylate buffer (pH 5.5) for DPP-VII and DPP-II and in 100 mM Hepes buffer (pH 7.5) for DPP-IV as average of two separate experiments. n.d., not determined.

Substrate	K_m (μ M)		
	DPP-VII	DP-II	DPP-IV
Lys-Ala-AMC	243 \pm 35	215 \pm 29	> 1000
Lys-Pro-AMC	14 \pm 1	11 \pm 1	35 \pm 4
Gly-Ala-AMC	1077 \pm 124	n.d.	> 1500
Gly-Pro-AMC	154 \pm 12	149 \pm 22	63 \pm 12
Nle-Pro-AMC	3 \pm 0.4	3 \pm 1	34 \pm 5
Ala-Pro-AFC	38 \pm 6	32 \pm 1	22 \pm 3
Nle-Nle-rhodamine	4 \pm 1	6 \pm 1	No cleavage
Nle-Pro-rhodamine	3 \pm 1	3 \pm 1	35 \pm 3

Pro-AMC and Nle-Nle-rhodamine; the profiles obtained differ insignificantly from that of Nle-Pro-AMC (results not shown).

Dipeptide substrate specificity

The substrate specificities of DPP-VII, DPP-II and DPP-IV were determined using a positional scanning synthetic combinatorial dipeptide substrate library, analogous to the one described by Backes et al. [22] (Figure 5). Positional scanning library analysis has previously been shown to be a powerful method for the determination of protease substrate specificity [23]. The positional scanning library used in this study has the general structure Xaa-Xaa-AMC. Amide bond hydrolysis occurs between the Xaa-Xaa dipeptide and the fluorogenic 7-amino-4-carbamoylmethylcoumarin, resulting in an increase in fluorescence. This library is composed of two sublibraries of 400 compounds each, utilizing the 20 naturally occurring amino acids, excluding cysteine and including norleucine. The P1 sublibrary is composed of 20 separate mixtures in which the P1 position is fixed, and the P2 position contains an equimolar mixture of all other amino acids. In the P2 sublibrary, the P2

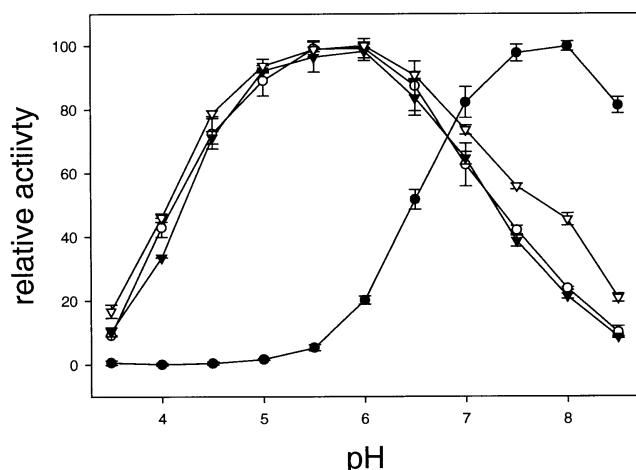


Figure 4 pH profiles of DPP-II, DPP-IV and DPP-VII

Relative activities of dipeptidases using 10 μ M substrate in 60 mM sodium citrate/phosphate buffer (pH range: 3.5–5.0) and 60 mM sodium phosphate buffer (pH range: 5.5–8.5) are shown: \circ , Gly³⁰ DPP-VII; \blacktriangledown , Ala²⁶ DPP-VII; ∇ , DPP-II; \bullet , DPP-IV. The results are averages of duplicate experiments.

residue is positionally defined, and the P1 position contains an equimolar mixture of all other amino acids. The results of assays with these libraries provide a complete understanding of the specificities of these dipeptidases in S₁ and S₂ subsites of the active sites.

Overall, the substrate specificities for DPP-VII and DPP-II are virtually identical and highly distinct from that of DPP-IV. All of the enzymes have a near-absolute specificity for proline in the P1 position. Nle is the next most preferred residue for DPP-VII and DPP-II, whereas DPP-IV prefers Ala, albeit with greatly reduced efficiency.

In the S₂ subsites, DPP-VII and DPP-II have a preference for Lys, Nle, Met and Ala, and do not tolerate aromatic and negatively charged residues. The subtle differences in the results obtained for DPP-II and DPP-VII are within the standard error of this experiment, owing to the low signal-to-noise ratio in these experiments. In contrast with the results obtained for DPP-VII and DPP-II, DPP-IV has a highly promiscuous S₂ subsite, with all residues well tolerated.

Catalytic constants for cleavage of optimal substrates

The K_m values for several substrates for DPP-VII, DPP-II and DPP-IV were determined, and evaluated in the context of the optimal dipeptide substrate specificities of these enzymes as determined by the positional scanning analysis. For all of the substrates tested, the kinetic constants for DPP-VII and DPP-II were identical within the error of the measurements. The lowest

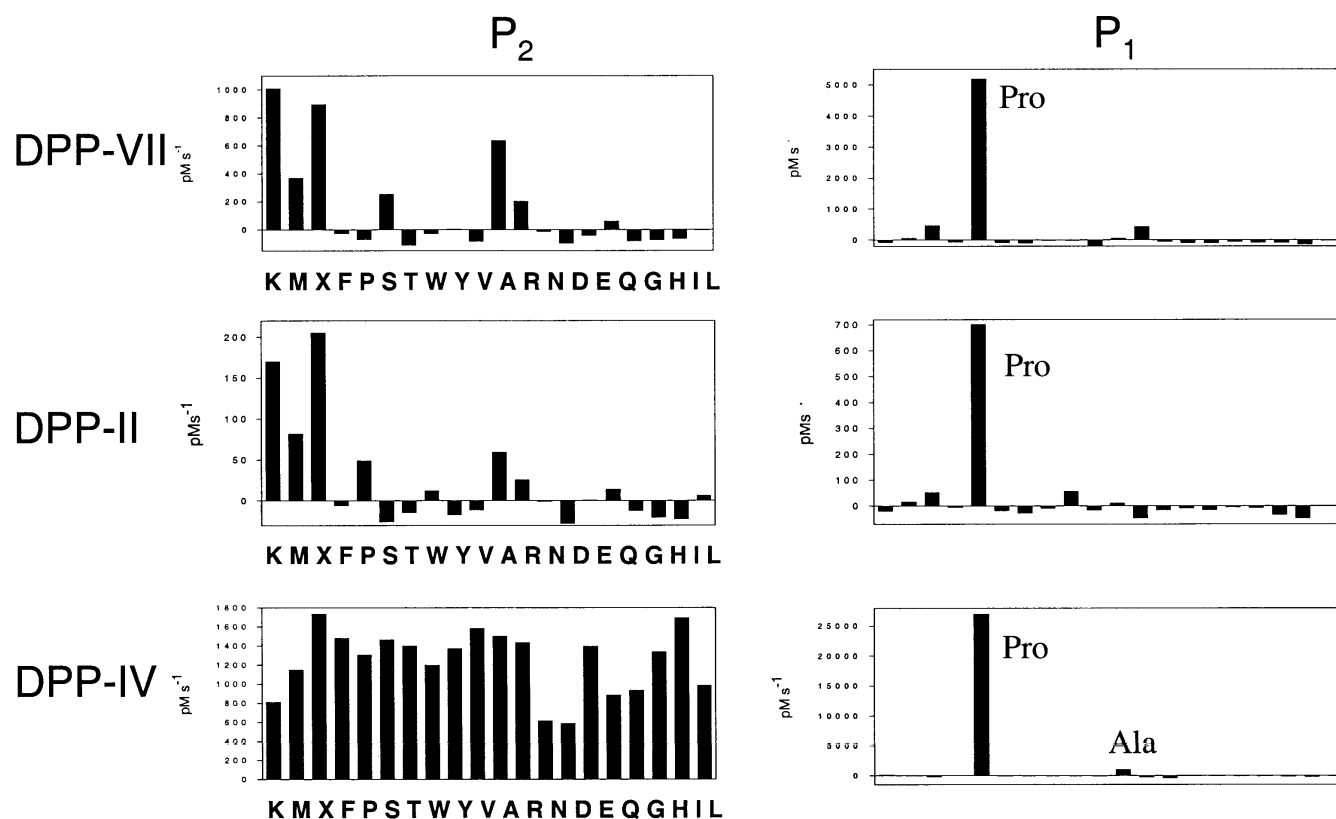


Figure 5 Positional scanning combinatorial dipeptide library profiles

Positional scanning combinatorial dipeptide library data for recombinant Gly³⁰ DPP-VII, human placenta DPP-II and human recombinant DPP-IV. Reactions contained 0.5 μ M of each library component per well. DPP-II and DPP-VII were measured in 100 mM cacodylate (pH 5.5), and DPP-IV in 100 mM Hepes (pH 7.5). Amino acid residues are shown in single letter code. X represents norleucine.

Table 3 Comparison of IC_{50} values

G_{30} -DPP-VII and A_{26} -DPP-VII denote DPP-VII proteins beginning at residues Ala²⁶ and Gly³⁰ respectively.

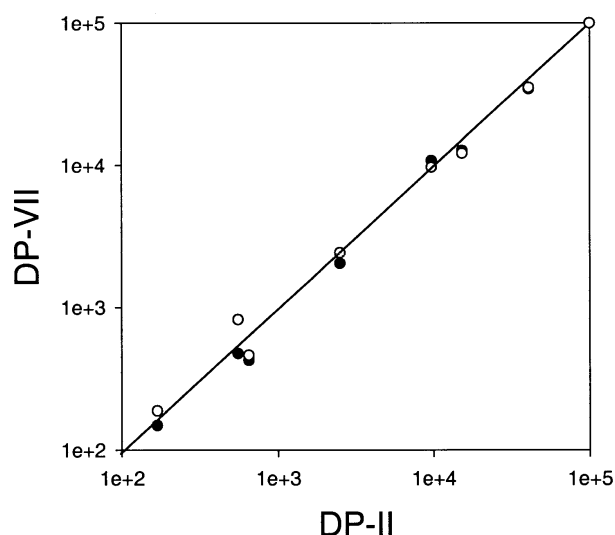
	K_i (nM)			
	G_{30} -DPP-VII	A_{26} -DPP-VII	DPP-II	DPP-IV
Lys thiazolidide	149	188	169	201
Ile cyanothiazolidide	475	820	556	< 1
Lys piperidide	425	461	651	671
Chg thiazolidide	2048	2425	2500	44
threo-Ile thiazolidide	10775	9680	9725	215
Val thiazolidide	12715	12130	15285	284
Diprotin A	34400	35200	40790	3500
Val pyrrolidide	> 100 000	> 100 000	> 100 000	891

K_m values were obtained for Lys-Pro-AMC, Nle-Pro-AMC and Ala-Pro-AFC, as anticipated from the results of the positional scanning analysis. The values obtained for DPP-IV were likewise consistent with the library results, in that the K_m values for all substrates containing Pro at P1 were highly similar, indicating a lack of specificity in the S_2 subsite.

The disparate specificity of DPP-II/DPP-VII and DPP-IV suggested that substrates highly selective for these enzymes could be identified. Exploiting the finding that Nle was tolerated in the P1 position of DPP-II and DPP-VII, a Nle-Nle-rhodamine derivative was synthesized and evaluated. This substrate was found to have $K_m = 6 \pm 1$ and $4 \pm 1 \mu\text{M}$ for DPP-II and DPP-VII respectively (Table 2). In striking contrast, absolutely no hydrolysis of Nle-Nle-rhodamine substrate by DPP-IV was observed, even when 500 times more enzyme (250 nM) was used than that used under standard assay conditions. Evidence that this lack of turnover was not due to the particular fluorescent group was provided in studies with an Nle-Pro-rhodamine derivative, which was hydrolysed by DPP-IV as efficiently as other Xaa-Pro-AMC substrates. Therefore Nle-Nle-rhodamine is a specific DPP-VII/DPP-II substrate, with a 50 000-fold selectivity over DPP-IV.

Inhibitor selectivity

Several classes of inhibitors have been described for proline-specific dipeptidases (see [1,2] for reviews). In particular, reversible inhibitors that have been identified for DPP-IV include diprotin A [2], cyanopyrrolidide [24], aminoacyl pyrrolidides and aminoacyl thiazolidides [1,2]. The selectivity of many of these inhibitors over other proline-specific enzymes has not been rigorously determined, although some inhibitors have been shown to inhibit DPP-VII with comparable affinity [7]. Dissociation constants (K_i) were determined for several of these compounds for DPP-VII, DPP-II and DPP-IV, with the results shown in Table 3. The IC_{50} values for DPP-VII and DPP-II are virtually identical, as illustrated in the correlation of IC_{50} on the double logarithmic plot shown in Figure 6 (correlation coefficient 0.998). The inhibitor selectivity of DPP-IV is distinct, and the values are similar to those described previously. Although the inhibitors shown in Table 3 do not reveal a DPP-II-selective inhibitor, Ile cyanothiazolidide and Val pyrrolidide have > 475- and > 112-fold selectivity respectively for DPP-IV over DPP-II. Lys thiazolidine and Lys piperidide were unselective, whereas the remaining four inhibitors showed 10–50-fold selectivity for DPP-IV.

**Figure 6 Double logarithmic plot of IC_{50} values from Table 3**

The K_i values obtained from eight inhibitors with either Gly³⁰ (●) or Ala²⁶ (○) DPP-VII correlate with the K_i values of DPP-II; $R = 0.998$. $1e+2$ denotes 1×10^2 (etc.)

DISCUSSION

A large number of peptides and proteins are synthesized with a proline residue in the penultimate position. Notable examples include proteins and peptides in the chemokine, cytokine and neuropeptide families. Although in many cases this probably serves simply to protect molecules from N-terminal degradation, in others it is believed to serve a regulatory function, since post-proline cleavage results in altered biological activity [25]. Several proline-specific dipeptidase (DASH) family members in addition to DPP-II, DPP-IV and DPP-VII have recently been identified, including DPP8 [20], fibroblast activation protein [21], attractin [26], DPP-IV- β [27] and others [5]. Although a role for DPP-IV in the regulation of metabolic peptides, such as the incretins GLP-1 and GIP, is well established, the functions of other DASH family members, and their relationship to each other, are unclear.

As described earlier, several lines of evidence have led to the hypothesis that the partially purified activity previously ascribed to DPP-II is, in fact, due to DPP-VII. The results presented in the present study strongly support this hypothesis. First, the enzymes have virtually identical substrate specificities as determined by a positional scanning library analysis. The subtle differences that were observed are well within the errors of the measurement and/or potentially due to minor impurities in the DPP-II preparation. Secondly, both enzymes have very similar kinetic constants for a number of substrates and inhibitors. Finally, their pH profiles are nearly superimposable. The only significant issue to be resolved relates to the reported subcellular localization of these enzymes. Although the results of density gradient studies are consistent with a lysosomal location for DPP-VII, the enzyme is clearly targeted to vesicles distinct from lysosomes [17]. DPP-VII and a typical lysosomal marker, lysosome-associated membrane protein 1, did not co-localize to the same vesicles, as determined by confocal microscopy [17]. In addition, DPP-VII-containing vesicles are neither clathrin-coated (adaptin- α -negative) nor *trans*-Golgi network-associated (Rab11-negative). The

conclusion that DPP-II is lysosomal is based primarily on studies employing density gradients for vesicle purification, and the observation that DPP-II has enzyme activity under acidic conditions [10,28,29]. These findings, however, are not inconsistent with the distinct vesicular subcellular localization described for DPP-VII. Further studies are required to determine the significance of these vesicles in the regulation of DPP-VII biology.

Understanding the function of DPP-VII, and other DASH family proteins, will require the identification of endogenous substrates and inhibitors for these enzymes. Previous studies [15] have suggested a role for the DPP-II activity in the lysosomal degradation of biologically important proline-containing oligopeptides such as collagen fragments, substance P, casomorphin and others. No macromolecular substrates of DPP-VII have been described. The results of the positional scanning library suggest that DPP-VII, like DPP-IV, has a strict preference for Pro in the P1 position, suggesting that substrates will have this feature. However, it is important to note that the best characterized endogenous substrates for DPP-IV are GLP-1 and GIP, both of which contain Ala in P1, indicating that primary sequence recognition is not the only important determinant of specificity for this enzyme [3]. It is interesting to note that Ala is the second most preferred residue in the S₁ subsite of DPP-IV by positional scanning analysis, which matches previously published substrate-specificity results [2].

The differences in primary substrate specificities of DPP-IV and DPP-VII, as revealed by positional scanning, indicate that there are profound differences in the S₁ and S₂ subsites of these enzymes, and suggest that selective inhibition can be achieved. Indeed, this was clearly demonstrated in a comparison of kinetic constants for a set of substrates and inhibitors. In general, the results are consistent with the primary specificity revealed by positional scanning. For example, lysine is well tolerated by both DPP-IV and DPP-VII, and Lys thiazolidide and Lys piperidide are equipotent against the two enzymes. Conversely, the other compounds tested were considerably less potent against DPP-VII, consistent with the more stringent S₂ specificity of this enzyme. Differences in S₁ specificity, notably the distinct ability of DPP-VII to tolerate Nle in the P1 position, should translate into selective inhibitors of this enzyme. Interestingly, recently reported DPP-II-selective inhibitors such as 2,4-diaminobutyrate piperidine showed considerably higher selectivity and potency for DPP-II over DPP-IV, confirming that the neutral bulkier piperidine group does not fit well in the more stringent S₁ pocket of DPP-IV [30]. Ile cyanothiazolidide and Val pyrrolidide were the most selective DPP-IV inhibitors identified in the present study, the latter of which has been used in various animal studies to study the effects of DPP-IV inhibitors [31]. Ile thiazolidide, which has only 50-fold selectivity over DPP-II, was also demonstrated to be efficacious in several animal studies [32]. A thorough biochemical characterization of these enzymes, such as that described in the present study for DPP-VII and DPP-IV, should facilitate the identification of selective substrates and inhibitors, which can be used as tools to elucidate their functions in the increasing number of biological processes that appear to be regulated by proline-specific peptidases.

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REFERENCES

- Demuth, H.-U. and Heins, J. (1995) Catalytic mechanism of dipeptidyl peptidase IV. In *Dipeptidyl Peptidase IV (CD26) in Metabolism and the Immune Response* (Fleischer, B., ed.), pp. 1–35. R. G. Landes Company, Austin, TX
- Augustyns, K., Bal, G., Thonus, G., Belyaev, A., Zhang, X. M., Bollaert, W., Lambey, A. M., Durinx, C., Goossens, F. and Haemers, A. (1999) The unique properties of dipeptidyl-peptidase IV (DPP IV/CD26) and the therapeutic potential of DPP IV inhibitors. *Curr. Med. Chem.* **6**, 311–327
- Meier, J. J., Nauck, M. A., Schmidt, W. E. and Gallwitz, B. (2002) Gastric inhibitory polypeptide: the neglected incretin revisited. *Regul. Pept.* **107**, 1–13
- Deacon, C. F. and Holst, J. J. (2002) Dipeptidyl peptidase IV inhibition as an approach to the treatment and prevention of type 2 diabetes: a historical perspective. *Biochem. Biophys. Res. Commun.* **294**, 1–4
- Šedo, A. and Malík, R. (2001) Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? *Biochim. Biophys. Acta* **1550**, 107–116
- Chiravuri, M., Schmitz, T., Yardley, K., Underwood, R., Dayal, Y. and Huber, B. T. (1999) A novel apoptotic pathway in quiescent lymphocytes identified by inhibition of a post-proline cleaving aminopeptidase: a candidate target protease, quiescent cell proline dipeptidase. *J. Immunol.* **163**, 3092–3099
- Underwood, R., Chiravuri, M., Lee, H., Schmitz, T., Kabacnel, A. K., Yardley, K. and Huber, B. T. (1999) Sequence, purification and cloning of an intracellular serine protease, quiescent cell proline dipeptidase. *J. Biol. Chem.* **274**, 34053–34058
- McDonald, J. K., Reilly, T. J., Zeitman, B. and Ellis, S. (1968) Dipeptidyl arylamidase II in the pituitary. Properties of lysylalanyl-β-naphthylamide hydrolysis: inhibition by cations, distribution in tissue, and subcellular localization. *J. Biol. Chem.* **243**, 2028–2037
- Araki, H., Li, Y.-H., Yamamoto, Y., Haneda, M., Nishi, K., Kikkawa, R. and Ohkubo, I. (2001) Purification, molecular cloning and immunohistochemical localization of dipeptidyl peptidase II from rat kidney and its identity with quiescent cell proline dipeptidase. *J. Biochem. (Tokyo)* **129**, 279–288
- Eisenhauer, D. A. and McDonald, J. K. (1986) A novel dipeptidyl peptidase II from porcine ovary. *J. Biol. Chem.* **261**, 8859–8865
- Sakai, T., Kojima, K. and Nagatsu, T. (1987) Rapid chromatographic purification of dipeptidyl peptidase II from human kidney. *J. Chromatogr.* **416**, 131–137
- Lynn, K. R. (1991) Isolation and some properties of dipeptidyl peptidase II and III from porcine spleen. *Int. J. Biol. Chem.* **23**, 47–50
- Huang, K., Takagaki, M., Kani, K. and Ohkubo, I. (1996) Dipeptidyl peptidase II from porcine seminal plasma: purification, characterization, and its homology to granzymes, cytotoxic cell proteinases (CCP 1–4). *Biochim. Biophys. Acta* **1290**, 149–156
- Fukasawa, K. M., Fukasawa, K., Higaki, K., Shiina, N., Ohno, M., Ito, S., Otogoto, J. and Ota, N. (2001) Cloning and functional expression of rat kidney dipeptidyl peptidase II. *Biochem. J.* **353**, 283–290
- Mentlein, R. and Struckhoff, G. (1989) Purification of two dipeptidyl peptidases II from rat brain and their action on proline-containing neuropeptides. *J. Neurochem.* **52**, 1284–1293
- Chiravuri, M., Lee, H., Mathieu, S. L. and Huber, B. T. (2000) Homodimerization via a leucine-zipper motif is required for enzymatic activity of quiescent cell proline dipeptidase. *J. Biol. Chem.* **275**, 26994–26999
- Chiravuri, M., Agarraberes, F., Mathieu, S. L., Lee, H. and Huber, B. T. (2000) Vesicular localization and characterization of a novel post-proline-cleaving aminodipeptidase, quiescent cell proline dipeptidase. *J. Immunol.* **165**, 5695–5702
- Ruecknagel, K. P., Rahfeld, J. U., Berger, R. and Fischer, G. PIR access code S77568
- Harris, J. L., Backes, B. J., Leonetti, F., Mahrus, S., Ellman, J. A. and Craik, C. S. (2000) Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 7754–7759
- Abbott, C. A., Yu, D. M. T., Woollatt, E., Sutherland, G. R., McCaughan, G. W. and Gorrell, M. D. (2000) Cloning, expression and chromosomal localization of a novel human dipeptidyl peptidase (DPP) IV homologue, DPP8. *Eur. J. Biochem.* **267**, 6140–6150
- Park, J. E., Lenter, M. C., Zimmermann, R. N., Garin-Chesa, P., Old, L. J. and Rettig, W. J. (1999) Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J. Biol. Chem.* **274**, 36505–36512
- Backes, B. J., Harris, J. L., Leonetti, F., Craik, C. S. and Ellman, J. A. (2000) Synthesis of positional-scanning libraries of fluorogenic peptide substrate libraries to define the extended substrate specificity of plasmin and thrombin. *Nat. Biotechnol.* **18**, 187–193
- Thornberry, N. A., Chapman, K. T. and Nicholson, D. W. (2000) Determination of caspase specificities using a peptide combinatorial library. *Methods Enzymol.* **322**, 100–110
- Hughes, T. E., Mone, M. D., Russell, M. E., Weldon, S. C. and Villhauer, E. B. (1999) NVP-DPP728 (1-[[[2-[(5-cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(S)-pyrrolidine], a slow-binding inhibitor of dipeptidyl peptidase IV. *Biochemistry* **38**, 11597–11603
- De Meester, I., Durinx, C., Bal, G., Proost, P., Struyf, S., Goossens, F., Augustyns, K. and Scharpe, S. (2000) Natural substrates of dipeptidyl peptidase IV. *Adv. Exp. Med. Biol.* **447**, 67–87

- 26 Duke-Cohan, J. S., Tang, W. and Schlossmann, S. F. (2000) Attractin: a cub-family protease involved in T cell monocyte/macrophage interactions. *Adv. Exp. Med. Biol.* **477**, 173–185
- 27 Jacotot, E., Callebaut, C., Blanco, J., Krus, B., Neubert, K., Barth, A. and Hovanessian, A. G. (1996) Dipeptidyl-peptidase IV- β , a novel form of cell-surface-expressed protein with dipeptidyl peptidase IV activity. *Eur. J. Biochem.* **239**, 248–258
- 28 Kugler, P. (1982) Quantitative histochemistry of the lysosomal dipeptidyl peptidase II in the proximal tubule of rat kidney. *Histochemistry* **76**, 557–566
- 29 Andersen, K. J. and McDonald, J. K. (1989) Lysosomal heterogeneity of dipeptidyl peptidase II active on collagen-related peptides. *Ren. Physiol. Biochem.* **12**, 32–40
- 30 Senten, K., Van der Veken, P., Bal, G., De Meester, I., Lambeir, A.-M., Scharpe, S., Bauvois, B., Haemers, A. and Augustyns, K. (2002) Development of potent and selective dipeptidyl peptidase II inhibitors. *Bioorg. Med. Chem. Lett.* **12**, 2825–2828
- 31 Deacon, C. F., Plamboeck, A., Moller, S. and Holst, J. J. (2002) GLP-1(9–36) amide reduces blood glucose in anesthetized pigs by a mechanism that does not involve insulin secretion. *Am. J. Physiol. Endocrinol. Metab.* **282**, E873–E879
- 32 Dimitrijevic, M., Stanojevic, S., Vujic, V., Kovacevic-Jovanovic, V., Beck-Sickinger, A., Demuth, H. and von Horsten, S. (2002) Effect of neuropeptide Y on inflammatory paw edema in the rat: involvement of peripheral NPY Y1 and Y5 receptors and interaction with dipeptidyl-peptidase IV (CD26). *J. Neuroimmunol.* **129**, 35–42

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