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Dipeptidyl Peptidase IV in the Immune System

Effects of Specific Enzyme Inhibitors on Activity of Dipeptidyl Peptidase IV and Proliferation of Human Lymphocytes

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Summary: Dipeptidyl peptidase IV (DP IV) is a membrane peptidase playing a significant role in the process of activation and proliferation of human thymus-derived lymphocytes. This conclusion is drawn from

(1) the induction of this enzyme on mitogen-activated T lymphocytes (cf. Schön, E. & Ansorge, S. (1990) *Biol. Chem. Hoppe-Seyler* **371,** 699–705) and
(2) the impairment of different functions of activated T cells in the presence of specific inhibitors and antibodies against DP IV (Schön, E. & al. (1987) *Eur. J. Immunol* **17,** 1821–1826).

This paper is aimed at testing new active site-specific peptide inhibitors for their efficiency as inhibitors of lymphocyte DP IV and DNA synthesis of mitogen-stimulated lymphocytes.

These inhibitors comprise (i) diacylhydroxylamine derivatives of Xaa-Pro or Xaa-Ala peptides, (ii) different oligopeptides with N-terminal Xaa-Pro-sequences, and (iii) amino-acid amides of the pyrrolidide and the thiazolidide type.

The thiazolidides of ε-(4-nitrobenzyloxycarbonyl)-L-lysine and of L-isoleucine as well as Ala-Pro-nitrobenzoylhydroxylamine are the most effective inhibitors in both test systems, yielding half-maximal inhibitory concentrations in the micromolar range. Cell viability was not impaired in this effective concentration range.

Other inhibitors of DP IV are one to two orders of magnitude less efficient in the suppression of lymphocyte proliferation.

Dipeptidylpeptidase IV im Immunsystem: Einfluß von spezifischen Inhibitoren auf die Aktivität der Dipeptidylpeptidase IV und die Proliferation humaner Lymphozyten

Zusammenfassung: Das Membranenzym Dipeptidylpeptidase IV (DPIV) stellt ein wichtiges regulatorisches Element der Thymus-abhängigen Lymphozyten dar. Das läßt sich ableiten aus der Induzierbarkeit des Enzyms nach mitogener Stimulation und aus der Hemmbarkeit einer Reihe von funktionellen Aktivitäten der aktivierten T-Zellen in Gegenwart spezifischer DP IV-Inhibitoren.

Neuentwickelte active-site-spezifische Inhibitoren wurden hinsichtlich ihrer Fähigkeit getestet, die lymphozytäre DPIV-Aktivität und die Proliferation Mitogen-stimulierter Lymphozyten zu hemmen. Dabei kamen folgende Gruppen von Inhibitoren zum Einsatz: (i) Diacylhydroxylamin-Derivate von Xaa-Pro- und Xaa-Ala-Peptiden, (ii) verschiedene Oligopeptide mit einer N-terminalen Xaa-Pro-Sequenz und

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Enzyme:
Dipeptidyl peptidase IV, dipeptidyl-peptide hydrolase (EC 3.4.14.5)

Abbreviations:
ACD, anticoagulant citrate dextrose; Cap, hexanoic acid or hexanoyl, DIFP, diisopropyl fluorophosphate; DP IV, Dipeptidyl peptidase IV, CD 26; [^{3}H]dThd, tritiated thymidine; IL, interleukin; MNC, mononuclear cell(s) from human blood; PHA, phytohemagglutinin A, lectin from *Phaseolus vulgaris;* pNA, 4-nitroanilide; T cell, thymus-derived lymphocyte; Z, benzyloxycarbonyl

(iii) Aminosäureamide vom Pyrrolidid- und vom Thiazolidid-Typ.

Die Thiazolidide von ε-(4-Nitrobenzyloxycarbonyl)-L-Lysin und von L-Isoleucin sowie Ala-Pro-Nitrobenzoylhydroxylamin erwiesen sich als stärkste Inhibitoren in bezug auf die Hemmung der DP IV-Aktivität und der Proliferation humaner Lymphozyten mit halbmaximalen Hemmkonzentrationen im mikromolaren Bereich. Unspezifische Effekte auf die Vitalität der Lymphozyten konnten dabei ausgeschlossen werden.

Die wirksamen Hemmkonzentrationen der Inhibitoren unterscheiden sich zum Teil um Größenordnungen in den beiden Testsystemen.

Key terms: Dipeptidyl peptidase IV, T lymphocyte, lymphocyte proliferation, active site inhibitor, peptidase.

Dipeptidyl peptidase IV (EC 3.4.14.5) was described for the first time by Hopsu-Havu et al. in 1966[1]. This membrane peptidase has been found to occur in a variety of human organs and tissues[2,3]. As a typical ectoenzyme of the plasma membrane[4], DP IV is expressed in high density in membrane regions organized as microvilli, such as brush border regions of the small intestinum, the kidney tubulus and the liver sinusoidal membrane[5]. In the human immune system, the enzyme is expressed almost exclusively in the thymus-derived lymphocytes[6,7].

Despite many investigations on the significance of this enzyme as a marker in lymphoproliferative diseases and in patients with disturbed immune functions[8–12], the role of this enzyme in the physiology of the immune system is not yet understood. In 1985 we were the first to show that in the presence of specific inhibitors of DP IV the proliferation of human lymphocytes stimulated by mitogenic lectins and alloantigens is suppressed in a dose-dependent manner[13].

Moreover, DP IV activity was found to be raised after mitogenic stimulation in vitro[14,15]. This induction of enzyme activity with a peak in the G1 phase is paralleled by an increasing density of epitopes reacting with antibodies against this enzyme[16]. In 1988, DP IV was classified as CD 26 under the entry *lymphocyte activation markers* at the 4th Workshop on Leukocyte Differentiation Antigens[17].

Recently, we found that various functions of T lymphocytes, such as production of cytokines, interleukin-2-augmented proliferation and as helpers of B lymphocytes, are dependent on the activity of this membrane peptidase, either directly or indirectly[18,19].

These investigations on the role of DP IV in the physiology of lymphocytes were backed by the development of specific inhibitors serving as key tools in our studies. These active site inhibitors are potential immunomodulatory drugs. To be selected for application in animal models, these compounds have to meet several criteria in vitro. The present paper describes their efficiency at low doses, sufficient stability under cell-culture conditions and lack of cytotoxic side effects in vitro. A series of diacyl hydroxylamines of Xaa-Pro- and Xaa-Ala-peptides, oligopeptides with N-terminal Xaa-Pro as well as amino acyl pyrrolidides and thiazolidides have been tested for their ability to inhibit lymphocyte DP IV activity and DNA synthesis of mitogen-stimulated lymphocytes. Aminoacyl amides with heterocyclic amide structures were found to be the most efficient inhibitors of lymphocyte proliferation in vitro.

Materials and Methods

1) Cell preparation

Mononuclear cells of human venous blood drawn from healthy volunteers were separated using Boyum's method of density gradient centrifugation with minor modifications as described earlier[18]. For cell cultures, only heparinized blood samples were used as a lymphocyte source. For biochemical tests, ACD-treated blood donations obtained as buffy coats from the blood bank served as a lymphocyte source.

2) Test system DP IV activity

Enzyme source: MNC at a cell density of approximately 5×10^7/m*l* were agitated for 30 min at 4 °C with 0.1% Triton X-100. The cell lysate was centrifuged at $1.7 \times 10^4 \times g$ for 30 min. The supernatant was used for determination of DP IV activity in lymphocyte lysates.

Substrate: Gly-Pro-pNA, final concentration 1mM.

Buffer: 0.1M KH_2PO_4/K_2HPO_4 buffer pH 7.6.

Assay: 0.025 m*l* enzyme source was preincubated with an equal volume of inhibitor for 60 min at room temperature. After adding 0.05 m*l* substrate solution, the reaction was allowed to proceed at 37 °C for 120 min and stopped by adding 0.4 m*l* of 1M sodium acetate buffer pH 4.5. The absorbance of liberated 4-nitroaniline was read in a spectrophotometer at 390 nm. Using this approach, less than 2% of Gly-Pro-pNA-degrading activity in lymphocytes can be attributed to enzymes other than DP IV[20].

3) Test system lymphocyte proliferation

The lymphocyte culture system used to evaluate the effect of DP IV inhibitors on mitogen-induced lymphocyte proliferation was described previously in detail[18] and is only summarized in the following.

Culture medium: based on RPMI 1640, supplemented as described[18], and with the addition of 10% pooled inactivated human

Culture vessels: polystyrene 96-well, flat-bottom microtiter plates; 0.2-m*l* cultures containing 2×10^5 MNC, were prepared in quadruplicate for each condition.

Mitogen: phythaemagglutinin A (Wellcome, Beckenham, Kent) at final concentrations of 0.3 and 0.03%.

Cell cultures were kept at 37 °C in a humidified 5% CO_2/air atmosphere. Total culture time was 96 h, including the incorporation of 37 kBq (1μCi) [3H]thymidine per well for the last 6 h. Cell suspension (0.1 m*l*) was harvested on filter paper and prepared for liquid scintillation counting in a beta counter. DP IV inhibitors were added at the beginning of the culture in concentrations ranging from 0.5μM to 0.5mM.

4) Test system cell viability

To exclude unspecific toxic side effects of the inhibitors on human lymphocytes, the viability of cells cultured in the presence and absence of mitogen under conditions as described above was tested after 0, 2, 24, 48, 72 and 96 h of culture. In brief, 0.01 m*l* cell suspension was put on glass slides and incubated with 0.01 m*l* 0.01% ethidium bromide for 30 s followed by addition of 0.01 m*l* 0.01% acridine orange solution. The cell viability was evaluated using a fluorescence microscope, where viable cells show a green nucleus and dead cells appear red.

5) Synthesis of amino-acid and peptide derivatives

The compounds listed in the table were synthesized by known standard methods for peptide synthesis. Phe-Pro-NHO-CO-C_6H_4-NO_2 and Ala-Pro-NHO-CO-C_6H_4-NO_2 were prepared according to the method described[21].

For the synthesis of the amino-acid pyrrolidides and thiazolidides[22] the corresponding Boc-amino-acid was coupled with pyrrolidine and thiazolidine, respectively, using the mixed anhydride procedure. After removal of the Boc-group with hydrogen chloride in acetic acid (in case of the Boc-amino-acid thiazolidides in presence of thioanisole under nitrogen atmosphere) the resulting amino-acid derivatives were isolated as hydrochlorides.

The dipeptides Ile-Pro, Lys [Z(NO_2)]-Pro and Lys(Cap)-Pro were obtained by coupling of the pentafluorophenyl or *N*-hydroxysuccinimide ester of the Boc-protected N-terminal amino-acids or amino-acid derivatives with L-proline followed by removal of the Boc-group with hydrogen chloride in acetic acid.

In the case of the tripeptides diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) the Boc-protected N-terminal dipeptides (synthesized as described above) were coupled with L-isoleucin-*tert*-butyl ester and L-leucin-*tert*-butyl ester, respectively, by the method of mixed anhydrides followed by simultaneous removal of the protecting groups with hydrogen chloride in acetic acid. Diprotin A and diprotin B were recrystallized from ethanol/ether.
β-Casomorphin-(1–5)-peptide (Tyr-Pro-Phe-Pro-Gly) and morphiceptin (Tyr-Pro-Phe-Pro-NH_2) were synthesized by stepwise elongation from the C-terminal end as described previously[23].

For the synthesis of the lymphotoxin-(1–5)-peptide (Leu-Pro-Gly-Val-Gly) a (2+3)-segment condensation was successful. Boc-Leu-Pro was coupled with Gly-Val-Gly-OMe using the mixed anhydride procedure. After alkaline hydrolysis and removal of the Boc-group with hydrogen chloride in acetic acid the pentapeptide was purified by crystallization from methanol/ether. The homogeneity of the amino-acid derivatives and the peptides, usually characterized as hydrochlorides, was checked by TLC and HPLC (purity >98%).

Results and Discussion

The starting point for the present study was the evidence that dipeptidyl peptidase IV is involved in the physiology of T lymphocytes. This was revealed by previous studies showing suppression of mitogen-or alloantigen-induced proliferation, lymphokine production and B cell differentiation in cultures of human MNC in the presence of specific inhibitors or antibodies to DP IV[13,18,19]. Thus, DP IV inhibitors are regarded as immunomodulating substances with potential therapeutical significance. Before any in vivo application, the following in vitro criteria should be met by these compounds: (i) high selectivity with regard to the enzyme, (ii) high efficiency at low concentrations, (iii) no cytotoxic action in the effective concentration range, and (iv) sufficient stability under cell culture conditions.

From these considerations a test hierarchy was developed to answer the following questions.

(1) Is the compound to be tested specific and effective against lymphocyte DP IV activity?
(2) Is the compound toxic for lymphocytes in vitro?
(3) How does the compound influence the mitogen-induced lymphocyte proliferation?

Three groups of DP IV inhibitors were screened with regard to the above questions:

(i) diacylhydroxylamine derivatives of Xaa-Pro- and Xaa-Ala-peptides;
(ii) oligopeptides comprising Xaa-Pro and substituted derivatives thereof, the microbiological tripeptides diprotin A and B, the N-terminal pentapeptides of lymphotoxin, β-casomorphine, morphiceptin;
(iii) amino acyl amides of the pyrrolidide and the thiazolidide type.

Effects of DP IV inhibitors on lymphocyte DP IV activity

The table summarizes the effect of inhibitors toward DP IV activity in lymphocyte extracts. The order of the compounds corresponds to their inhibitory efficiency in the biochemical test system. Fig. 1 shows a selection of three typical inhibition curves obtained in these studies.

Peptidyl-*N,O*-diacylhydroxylamines represent a group of inhibitors of serine and cysteine proteinases that act in an irreversible manner[21,24–27]. The Phe-Pro- and Ala-Pro-derivatives were equally effective against lymphocyte DP IV, whereas the Ala-Ala-derivative (not included in the table) expectedly showed only minor effects on DP IV with Gly-Pro-pNA as a substrate. The *N*-(Phe-Pro)-*O*-(nitrobenzoyl)-hydroxylamine, however, was strongly toxic for human lymphocytes at concentrations of 10μM and was therefore excluded from cell culture experiments.

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