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Dipeptidyl peptidase II and leukocyte cell death

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Abbreviations:
DPP, dipeptidyl peptidase
QPP, quiescent cell proline dipeptidase
CLL, chronic lymphocytic leukaemia
VbP, Val-boro-Pro
FAPα, fibroblast activation protein α
EBSS, Earle's balanced salt solution
CdA, 2-chloro-2′-deoxyadenosine
PI, propidium iodide
-pNA, -p-nitroanilide

ABSTRACT

Dipeptidyl peptidase (DPP) II (E.C. 3.4.14.2) is an intracellular protease that releases, preferably at acidic pH, N-terminal dipeptides from oligopeptides with Pro or Ala in the penultimate position. The natural substrates and the physiological role of DPPII remain unclear. The aim of the present study was to investigate the involvement of DPPII activity in different forms of cell death (apoptosis, necrosis and autophagy) in human leukocytes. We determined specific DPP activities in leukocytes. Compared to other subpopulations of peripheral blood mononuclear cells (PBMC), we observed relatively high DPPII specific activity in monocytic cells, opening new perspectives for further investigation of the DPPII functions. A second intriguing finding was that DPPII specific activity increased during necrosis, whereas induction of apoptosis or autophagy did not affect any of the dipeptidyl peptidase activities. Finally, we showed that inhibition of DPPII (>90%) using the in vitro applicable, highly potent (K_i of 0.082 ± 0.048 nM) and selective DPPII inhibitor UAMC00039, did not induce any form of cell death in leukocytes. These data are of importance for a more precise interpretation of the in vitro and in vivo effects of other dipeptidyl peptidase inhibitors.

1. Introduction

Dipeptidyl peptidase II (DPPII, E.C. 3.4.14.2) is an intracellular protease that localizes to the vesicular system. It releases, preferably at acidic pH, N-terminal dipeptides from oligopeptides with Pro or Ala in the penultimate position. According to cytochemical studies, DPPII is found in normal as well as in a number of malignant haematological cells. It is present in both T and B lymphocytes, the former usually showing a stronger cytochemical signal [1–3]. Assessment of the number of DPPII

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positive lymphocytes represents a simple and reliable prognostic criterion in patients with B type chronic lymphocytic leukaemia (B-CLL) [4,5]. Patients with B-CLL displaying a high number of DPPII positive cells had a worse prognosis [1]. Recently, susceptibility to the dipeptidyl peptidase inhibitor Val-boro-Pro (VbP)-induced apoptosis of CLL B cells was suggested to be a novel prognostic factor in CLL [6]. Moreover, the ratio of serum DPPII versus dipeptidyl peptidase IV (DPPIV) activity has been proposed as a biochemical index of cancer [7–9]. Despite recent investigations on possible peptide substrates and in vivo function of DPPII, the natural substrates and the physiological role remain undefined [10,11]. Among the dipeptidyl peptidases able to cleave post-proline bonds, dipeptidyl peptidase IV (DPPIV) has been studied most extensively [12]. The human fibroblast activation protein α (FAPα) shares the highest sequence homology with DPPIV and bears both DPPIV-like and collagenase/gelatinase activity but is not expressed in most normal adult tissues [13].

In 1999, Chiravuri et al. observed that inhibitors of post-proline cleaving dipeptidyl peptidases such as VbP caused apoptosis in quiescent lymphocytes in a process independent of DPPIV [14]. The effect was attributed to the enzyme human quiescent cell proline dipeptidase (QPP). Later QPP and DPPII were proven to be identical [15–17], suggesting a possible role for DPPII in cell death. However, the discovery of some new members of the DPPIV family, such as DPP8 and DPP9 made us look at the selectivity of DPP inhibitors from a different perspective. Previous studies using these compounds require reinterpretation. The DPP inhibitor VbP, also known as Talabostat or PT-100, has great therapeutic potential. Four phase II studies are ongoing in cancer patients, and Talabostat is also under development for the treatment of hematopoietic disordes, such as neutropenia, anemia and thrombocytopenia. The hematopoietic target in these studies was identified as fibroblast activation protein α (FAPα) [18,19].

Since the DPPs are similar with respect to their catalytic mechanism and substrate selectivity, the development of potent and selective DPPII-inhibitors is a challenging task. The highly potent and selective DPPII-inhibitor *N*-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(*S*)-butane-diamine dihydrochloride (UAMC00039) [20] proved to be a useful tool for in vivo investigations [11]. UAMC00039 demonstrated in vivo efficacy and oral availability without evidence for acute toxicity. The high selectivity of the inhibitor enabled us to differentiate between DPPII and DPPIV/8/9 activities in biological systems. Therefore, this compound seemed to be an excellent tool for the investigation of DPPII activity during different forms of cell death. In vitro and in vivo model systems support the hypothesis that a variety of cell death programs may be triggered in distinct circumstances. There is growing evidence that besides apoptosis, autophagic and necrotic forms of cell degeneration may be programmed [21]. The aim of the present study was to investigate the involvement of DPPII activity in cell death (apoptosis, necrosis and autophagy) of human leukocytes. First, the specific DPPII activity was determined in different subpopulations of peripheral blood mononuclear cells (PBMC). Secondly, DPPII specific activity was assessed during cell proliferation and after induction of different types of cell death. Thirdly, we studied whether UAMC00039 was able to inhibit intracellular DPPII in cell culture and whether inhibition of DPPII induced cell death. To the best of our knowledge, this is the first study addressing the influence of various forms of cell death on specific DPPII activity.

2. Materials and methods

2.1. Materials

The DPPII inhibitor *N*-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(*S*)-butane-diamine dihydrochloride (UAMC00039, Fig. 1) and the DPPIV/8/9 inhibitor Bis{4-[(ethoxycarbonyl)-methylaminocarbonyl]phenyl} 1-((*S*)-prolyl)pyrrolidine-2(*R*,*S*)-phosphonate (AB207) were synthesised as described [12,20,22]. DPPII and DPPIV were purified from human seminal plasma [17,23]. The DPP activity in cell homogenates after the removal of DPPII and DPPIV by affinity chromatography was considered to be DPP activity not caused by DPPII or DPPIV. DPP activity presumably caused by DPP8, DPP9 and DPPIV is termed 'non-DPPII' DPP activity (DPPIV/8/9). Buffy coats were obtained from the Antwerp blood transfusion center. All cell culture products including PBS, RPMI 1640 medium, Earle's balanced salt solution (EBSS), foetal bovine serum (FBS), penicillin and streptomycin were obtained from Invitrogen. Ficoll-Paque Plus was from Amersham Biosciences. MG-132, nigericine and 2-chloro-2′-deoxyadenosine (CdA) were obtained from Calbiochem. Annexin V-FITC and propidium iodide (PI) were purchased from BD Biosciences. Cell lines (Jurkat, HL60, U937) were obtained from the American Type Culture Collection. Lys-Ala-p-nitroanilide (Lys-Ala-pNA), Ala-Pro-pNA and Gly-Pro-pNA were obtained from Bachem. Phorbol 12-myristate 13-acetate (PMA), etoposide, bovine serum albumin (BSA) and cacodylic acid were from Sigma. All other chemicals were obtained from ICN Biomedicals.

2.2. Cells

The human monocytic cell line U937 was grown in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated FBS at 37 °C in 5% CO_2/95% air. To induce monocytic cell differentiation, U937 cells (0.25×10^6 ml^{-1}) were cultured in the presence of

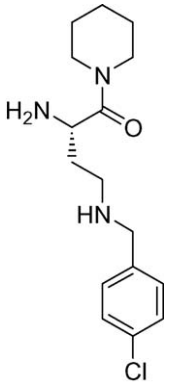

Fig. 1 – Structure of the DPPII inhibitor UAMC00039. The DPPII inhibitor *N*-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(*S*)-butane-diamine dihydrochloride.

10 ng/ml PMA for 72 and 96 h. Cells were scraped with a rubber policeman. Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient centrifugation of buffy coats, washed three times in RPMI and incubated overnight in complete medium at 37 °C in 5% CO_2/95% air before use. We used the non-adherent cells (predominantly lymphocytes) in our experiments, unless stated otherwise. For the isolation (positive selection) of peripheral $CD4^+$, $CD19^+$ and $CD14^+$ cells, the non-adherent PBMC were incubated with Dynabeads CD4 (T helper/inducer) and Dynabeads CD19 (panB) and the adherent cells with Dynabeads CD14 (monocytes/macrophages) according to the manufacturer's instructions (Dynal Biotech ASA, Oslo, Norway). Cells were lysed (vide infra) while attached to the beads. Assays were conducted in RPMI without FBS unless stated otherwise.

Enzyme activity and protein content were determined after lysing the cells. Washed cells were suspended (~1–10 × 10^6 cells/100 μl) in lysisbuffer (1% octylglucoside in 0.05 M cacodylic acid–NaOH buffer pH 5.5, 10 mM EDTA, 70 μg/ml aprotinin), incubated for 1 h at 4 °C and centrifuged for 10 min at 12,000 rpm (4 °C). The resulting supernatant was used as cell lysate.

Cell proliferation was assessed by cell counting (Neubauer-improved counting chamber) and protein measurements. Protein content was determined according to Bradford [24] or by using the bicinchoninic acid (BCA) protein assay kit (Pierce, USA) with BSA as a standard.

2.3. Enzyme assays

Enzyme activities were determined kinetically for 10 minutes at 37 °C by measuring the initial velocities of p-nitroaniline release (405 nm) from the substrate using a Spectramax plus microtiterplate reader (Molecular devices). One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol p-nitroaniline from the substrate per minute under assay conditions. DPPII enzymatic activity was determined using Lys-Ala-pNA (1 mM) in 0.05 M cacodylic acid–NaOH buffer pH 5.5 containing 10 mM EDTA and 14 μg/ml aprotinin [17]. Analoguous to the enzyme assay of Chiravuri et al. for QPP [14], we also used 1 mM Ala-Pro-pNA in 0.05 M HEPES buffer pH 7.0 containing 10 mM EDTA and 14 μg/ml aprotinin. DPPII, DPPIV, DPP8 and DPP9 can all cleave Ala-Pro-pNA under these conditions [25,26]. 'Non-DPPII' DPP activity (DPPIV/8/9) was determined using 0.5 mM Gly-Pro-pNA in 0.05 M Tris buffer pH 8.3 containing 10 mM EDTA and 14 μg/ml aprotinin.

2.4. Inhibitor characterization

Inhibition of DPPII activity was analysed spectrophotometrically as described above except that the DPPII inhibitor was preincubated with the enzyme sample for 15 min at 37 °C prior to the addition of substrate. IC_{50} values were obtained with substrate concentrations near the K_M value and at least 10 different inhibitor concentrations were used. IC_{50} values were calculated using Grafit software [27]. To investigate the selectivity of the inhibitor, IC_{50}-values for purified DPPIV and DPP8/9 were also measured.

The inhibition constant of the inhibitor for the active site (K_i) was determined using seven different substrate concentrations (10–1000 μM Ala-Pro-pNA) and at least ten different inhibitor concentrations (0–50 nM). Per substrate concentration, the data were fitted to the equation for tight-binding inhibition (Eq. (1)) [28] using GraFit version 5 [27] to calculate the $K_{i\text{-app}}$.

$$v = v_0\left(1 - \frac{\left(([E]_0 + [I] + K_{i\text{-app}}) - \sqrt{([E]_0 + [I] + K_{i\text{-app}})^2 - 4[E]_0[I]}\right)}{(2[E]_0)}\right) \quad (1)$$

where v is the measured velocity, v_0 the velocity in the absence of inhibitor, $[E]_0$ the total enzyme concentration, [I] the final inhibitor concentration, and $K_{i\text{-app}}$ is the apparent equilibrium inhibition constant. The different $K_{i\text{-app}}$ were plotted against their associated substrate concentration and the K_i was determined by calculating the intercept with the Y-axis using GraFit version 5 [27]. All experiments were performed in triplicate.

The reversibility of DPPII inhibition by UAMC00039 was investigated kinetically for 30 min under pseudo-first order conditions at 37 °C (5 nM UAMC00039 final concentration in 200 μl reaction volume). In a first experiment, the reaction was started by adding 190 μl of 1 mM Ala-Pro-pNA to 10 μl of a preincubated (15 min) mixture of 100 nM inhibitor and enzyme. In a second experiment, the reaction was started by adding 5 μl of enzyme to 195 μl of a mixture of inhibitor and 1 mM Ala-Pro-pNA. Both progress curves were compared.

2.5. Inhibition of intracellular DPPII

Stability of UAMC00039 in RPMI medium or assay buffer (50 mM cacodylate buffer pH5.5) was monitored at 37 °C. The inhibitors' capacity (IC_{50}) to inhibit DPPII was measured at different time points (up to 48 h).

U937 cells were incubated with various concentrations of inhibitor for 15 min at 37 °C in RPMI. Cells were then washed with PBS, lysed and assayed for DPPII activity; IC_{50} values were calculated as described above. Concentration–response and time–response curves were generated from incubations of PBMC with UAMC00039 (0.01 nM–1 μM) in RPMI at 37 °C for 1, 5, 15, 30 and 60 min. Washed cells were lysed overnight at 4 °C using 100 mM HEPES buffer pH 7.4, 10 mM EDTA, 70 μg/ml aprotinin and 1% octylglucoside.

2.6. Cell death induction and measurement

U937 cells or PBMC (0.5 × 10^6 cells/ml) were treated with the DPPII inhibitor UAMC00039 (1 and 100 μM), the DPPIV/8/9 inhibitor AB207 (5 μM) and with different triggers of cell death for 24 or 48 h at 37 °C in 5% CO_2/95% air. The topoisomerase II-inhibitor etoposide (50 μM) and the proteasome inhibitor MG132 (10 μM) were used as positive controls for apoptosis [29,30]. A 2-chloro-2′-deoxyadenosine (CdA, 10 μg/ml) was used as an apoptosis trigger in resting PBMC (after 24 h of incubation) [31,32]. The K^+ ionophore nigericine (10 μM) was used as a trigger for necrosis in the monocytic U937 cells [33]. Cells underwent amino acid deprivation in EBSS to induce autophagy [34].

2.6.1. *Viability measurements*

Viability was assessed microscopically by trypan blue exclusion (0.2% trypan blue in PBS) after culturing cells in the presence of 100 μM of inhibitor for up to 48 h. In addition, neutral red uptake was evaluated. Cells were incubated with fresh medium containing neutral red (100 μg/ml). After 2 h, cultures were washed twice with PBS and the neutral red in the cells was extracted with 0.05 M Na_2HPO_4 in 50% ethanol. Neutral red absorbance was detected at 540 nm. The viability was calculated with regard to the untreated cell control, which was set to 100%.

2.6.2. *Annexin V/propidium iodide (PI) staining*

Phosphatidylserine expression was determined by flow cytometric analysis using annexin V-FITC. Loss of membrane integrity (necrosis) was assessed by using PI. After 2, 6 and 24 h of incubation, cells were washed with PBS to remove non-incorporated inhibitor, resuspended in binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and subsequently labelled with annexin V-FITC. PI (final concentration 8 μg/ml) was added immediately prior to flow cytometric analysis using a FACSort analytical flow cytometer (BD Biosciences, San Diego, CA). Cells were classified into the following fractions: viable cells (annexin V^-/PI^-), apoptotic cells (annexin V^+/PI^-) and necrotic cells (annexin V^+/PI^+).

2.6.3. *Determination of apoptosis by DNA fragmentation assays*

DNA fragmentation into nucleosomal bands was detected using agarose gel electrophoresis as described previously [35]. Briefly, cells were lysed with hypotonic lysisbuffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100). Then 0.5 mg/ml proteinase K was added and lysates were incubated for 1 h at 50 °C. At the end of the incubation, 10 μl of DNase-free RNase (0.5 mg/ml) was added to the lysates and the tubes were incubated for 1 h at 37 °C. After precipitation overnight (−20 °C) with 1/10 volume of 3 M sodium acetate and 1 volume of isopropanol, the pellets were air dried and dissolved in water. After adding loading buffer (Fermentas), DNA samples were analysed by 1.8% agarose gel electrophoresis and stained with ethidium bromide.

2.6.4. *Electron microscopy*

Cells were harvested and fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde for 2 h, then rinsed (3 × 10 min) in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5% saccharose and postfixed in 1% OsO_4 solution for 1 h. After dehydration in an ethanol gradient (70% ethanol [20 min], 96% ethanol [20 min], 100% ethanol [2 × 20 min]), samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM 10 microscope at 80 kV.

2.6.5. *Effect of cell death on specific DPP activity*

After 2, 6 and 24 h, dipeptidyl peptidase activities were measured as described above. At the same time points viability/cell death was investigated by annexin V/PI staining.

2.7. *Statistical analysis*

Data are expressed as mean ± S.E.M. For the statistical analysis, the SPSS statistical package (SPSS for Windows, v. 12.0; SPSS, Chicago, IL) was used. Differences between groups

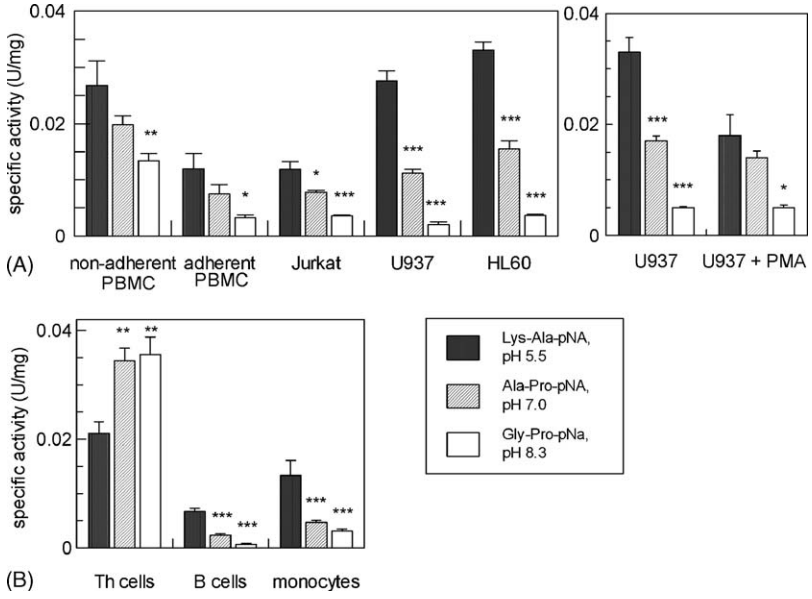

Fig. 2 – DPP specific activities in different leukocytes. (A) After lysing cells (U937 cells and PMA-stimulated U937 cells (72 h), HL60 cells, Jurkat cells, PBMC), enzyme activities were determined as described in Section 2: Lys-Ala-pNA at pH 5.5 (DPPII activity; solid); Ala-Pro-pNA at pH 7.0 (DPPII/IV/8/9 activity; hatched) and Gly-Pro-pNA at pH 8.3 ('non- DPPII' DPP activity; open). Specific activities are given ($n \geq 3$). (B) Non-adherent PBMC were used for the isolation of $CD4^+$ (T helper/inducer) and $CD19^+$ (panB) cells and adherent PBMC for the $CD14^+$ (monocytes/macrophages) cells using Dynabeads. Enzyme activities were determined after lysing cells while attached to the beads. Specific activities are given ($n = 6$). The data represent the mean ± S.E.M. The differences between the activity groups were assessed with one-way ANOVA, followed by the Dunnett test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. DPPII activity.

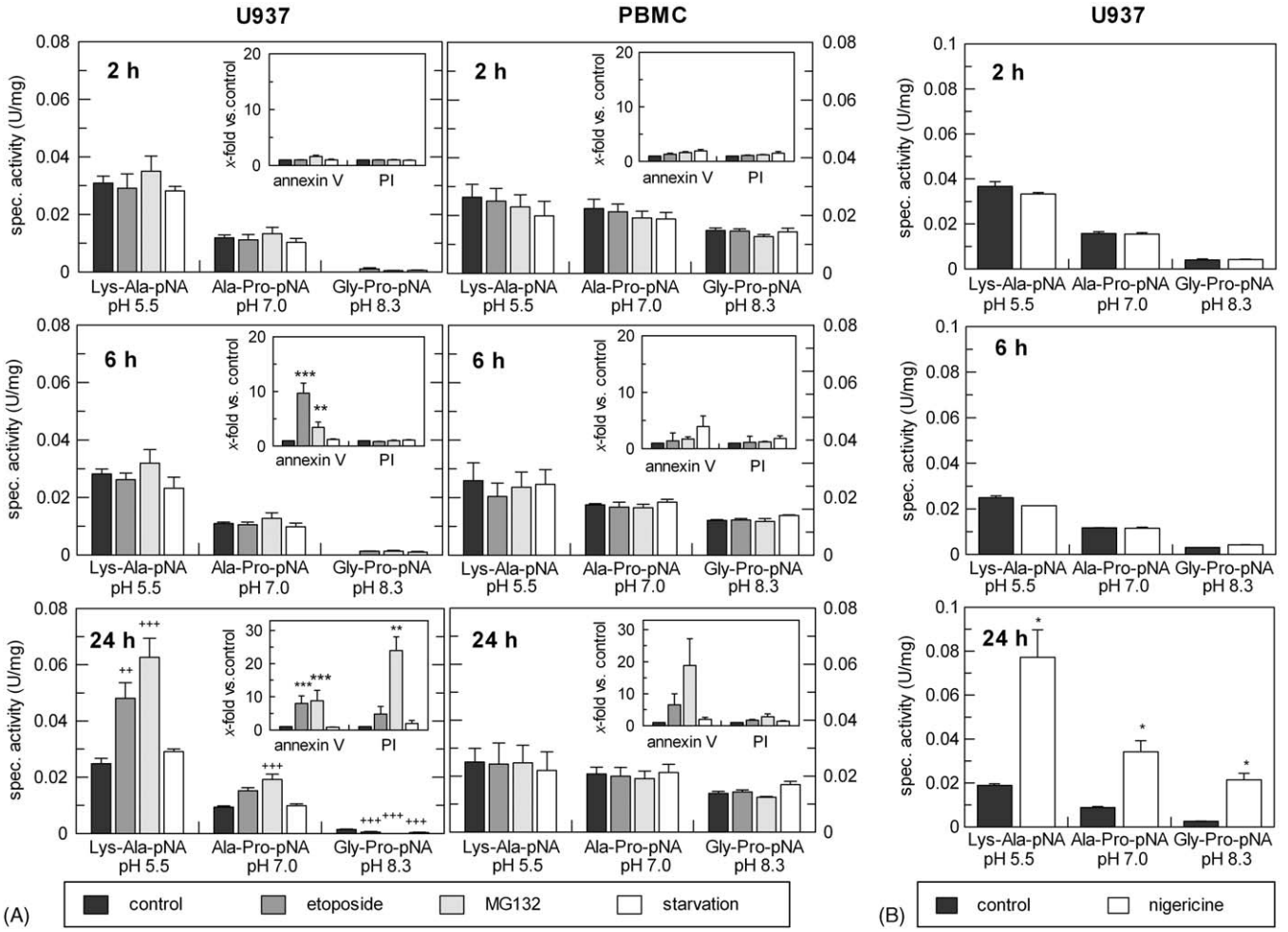

Fig. 3 – Influence of cell death on specific DPP activities in U937 cells and PBMC. The effect of different types of cell death on the specific DPP activities in U937 cells and PBMC was investigated. Cells were cultured in the presence of the indicated stimuli for 2, 6 and 24 h. After lysing the cells, enzyme activities were determined as described in Section 2: Lys-Ala-pNA at pH 5.5 (DPPII activity); Ala-Pro-pNA at pH 7.0 (DPPII/IV/8/9 activity) and Gly-Pro-pNA at pH 8.3 ('non-DPPII' DPP activity). (A) Specific DPP activities were investigated during apoptosis induced by 50 μM etoposide (grey) and 10 μM MG132 (hatched) and under starvation conditions (autophagy; open). Since the controls with and without DMSO were the same, only one control (black) is shown as a reference. The data represent the mean ± S.E.M. of 4 (PBMC) or 6 (U937) separate experiments. Specific activities are given. Treatment had no effect after 2 and 6 h of incubation in U937 cells and over the whole incubation time in PBMC. The annexin V/PI staining is presented in the inset ($n = 3$). The data were analysed using an univariate ANOVA, followed by the Dunnett test. $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. control specific activity; inset: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control annexinV/PI staining. (B) Specific DPP activities in U937 cells were investigated during nigericine-induced necrosis. Cells were cultured in the presence of 10 μM nigericine or control (EtOH) ($n = 2$). Unpaired two-tailed Student's t-test was used to analyze the specific activities. $^{*}p < 0.05$ vs. control.

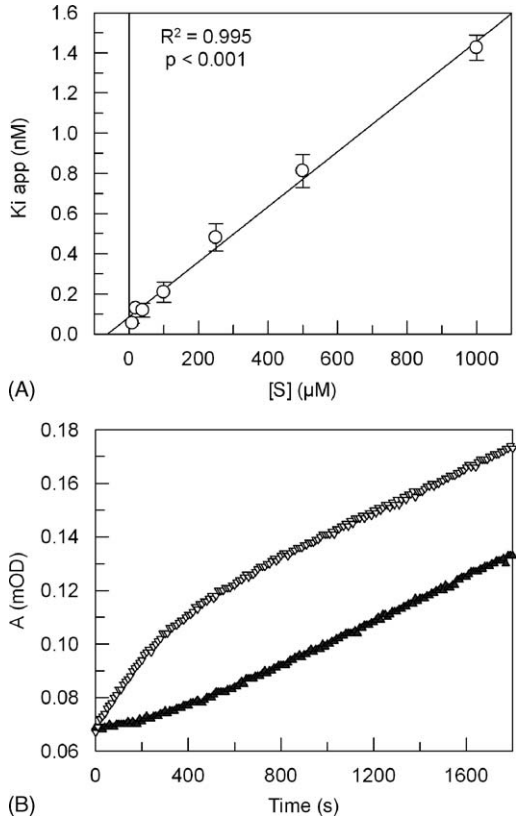

Fig. 4 – Inhibition characteristics of UAMC00039. (A) K_i of DPPII inhibition by UAMC00039. The different $K_{i\text{-app}}$, obtained using the equation for tight-binding inhibition (Eq. (1)) as described in Section 2 were plotted against their associated substrate concentration and the K_i was determined by calculating the intercept with the Y-axis (K_i of 0.082 ± 0.048 nM). The data represent the mean ± S.E.M. of three separate experiments. (B) Reversibility of DPPII inhibition by UAMC00039. The reversibility of DPPII inhibition by UAMC00039 was investigated as described in materials and methods using 5 nM inhibitor as final concentration. In a first experiment, the reaction was started by adding substrate (Ala-Pro-pNA) to a mixture of preincubated UAMC00039 and DPPII (▲, lower curve, dissociation curve of the inhibitor–enzyme complex). In a second experiment, the reaction was started by adding enzyme to a mixture of inhibitor and substrate (▽, curve of inhibitor binding to the enzyme). The slow recovery of the enzymatic activity, after dilution of the pre-formed enzyme–inhibitor complex, indicated that UAMC00039 was a reversible DPP II inhibitor.

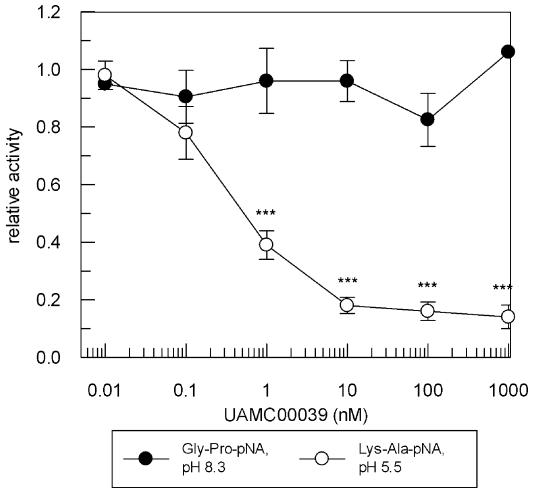

Fig. 5 – Applicability of UAMC00039 in cell culture experiments. A concentration–response curve was generated by incubation of PBMC with UAMC00039 ranging from 0.01 nM to 1 μM at 37 °C for 5 minutes. Lysates were prepared immediately after washing. DPPII (Lys-Ala-pNA pH 5.5) and 'non-DPPII' (Gly-Pro-pNA pH 8.3) DPP activities were measured. Relative enzyme activities are given (enzyme without inhibitor = 1). The data represent the mean ± S.E.M. of at least two separate experiments (n = 2–8). The differences between the activity groups were assessed with one-way ANOVA, followed by the Dunnett test. $^{***}p < 0.001$ vs. control (0 nM UAMC00039, not shown).

were assessed using one-way or univariate analysis of variance (ANOVA), followed by the Dunnett test. A value of $p < 0.05$ was considered significant. n represents the number of experiments. An unpaired two-tailed Student's t-test was used to analyze specific activities of Fig. 3B and D-PPII specific activities of Th cells and B cells in Fig. 2B. Linear regression analysis was carried out on the data shown in Fig. 4A.

3. Results

3.1. DPPII activity in leukocyte subpopulations

The DPP activities in resting primary mononuclear cells (adherent and non-adherent cells) and in several human leukemia cell lines (Jurkat, U937, HL60) were compared (Fig. 2A). Under our lysing conditions that were optimized for the study of DPPII, the monocytic U937 and HL60 cells showed the highest DPPII vs. 'non-DPPII' DPP activity. In U937 cells, 'non-DPPII' DPP activity balanced around the detection limit. DPPII activity in PMA-stimulated U937 cells was significantly lower ($p < 0.05$) than in the control cells both after 72 and 96 h of incubation (Fig. 2A right panel). Among primary lymphocytes, DPPII activity was higher in T helper cells ($CD4^+$) than in B cells ($CD19^+$) ($p = 0.001$, Fig. 2B). Again, monocytes ($CD14^+$) showed relatively high DPPII activity.

Specific DPP activities were measured in U937 cells in different cell culture conditions for 96 h. Specific DPPII activity did not change in experiments with medium containing serum and medium without serum. However, DPPII specific activity increased after 96 h of amino acid deprivation (supplementary data). At this moment necrosis was observed.

3.2. Effect of cell death on DPP activities

The effect of different types of cell death on the specific DPP activities in U937 cells and PBMC was investigated (Fig. 3A)

Table 1 – Selectivity of UAMC00039 and AB207

	DPPII inhibition IC_{50}	DPPIV inhibition IC_{50}	DPP8 inhibition IC_{50}
UAMC00039 [54]	0.48 ± 0.04 nM	165 ± 9 μM	142 ± 27 μM
AB207 [54]	>1000 μM	14 ± 1 nM	530 ± 1 nM

using the cell death inducers etoposide, MG132 and amino acid depletion (EBSS). Etoposide and MG132 caused apoptosis in U937 cells within 6 h of incubation as demonstrated by annexin V^+/PI^- staining. After 24 h, etoposide and MG132-induced apoptosis evolved to secondary necrosis as demonstrated by microscopy and annexin V^+/PI^+ staining. Apoptosis and amino acid depletion-induced autophagy did not affect DPPII or 'non-DPPII' DPP activities. However, secondary necrosis led to an increase of the DPPII specific activity. PBMC were quite resistant to the apoptosis triggers since apoptosis was only observed after 24 h of incubation with etoposide or MG132. Apoptosis of PBMC did not affect DPPII or 'non-DPPII' DPP specific activities.

The increase in DPPII specific activity during secondary necrosis prompted us to evaluate DPPII activity in U937 cells after triggering necrosis. Since 10 μM nigericine is known to elicit necrosis in monocytic cell lines [33], its effect on DPPII activity was investigated in U937 cells. Similar to secondary necrosis, DPPII specific activity increased 3.5-fold after 24 h nigericine treatment, (Fig. 3B).

3.3. *Inhibition characteristics of UAMC00039*

UAMC00039 is a potent reversible competitive tight binding DPPII inhibitor with a K_i of 0.082 ± 0.048 nM (Fig. 4). Because UAMC00039 has a subnanomolar IC_{50} towards DPPII compared to an IC_{50} of more than 100 μM for 'non-DPPII' DPP activity present in leukocytes, the inhibitor shows high selectivity towards DPPII (Table 1). The efficacy of a DPPII inhibitor in cell culture depends not only on the inhibitors' potency towards the enzyme but also on its stability in the medium and its ability to enter the cell. UAMC00039 was stable for at least 48 h at 37 °C in culture medium and in DPPII assay buffer. The compound was able to enter PBMC within 1 min resulting in a

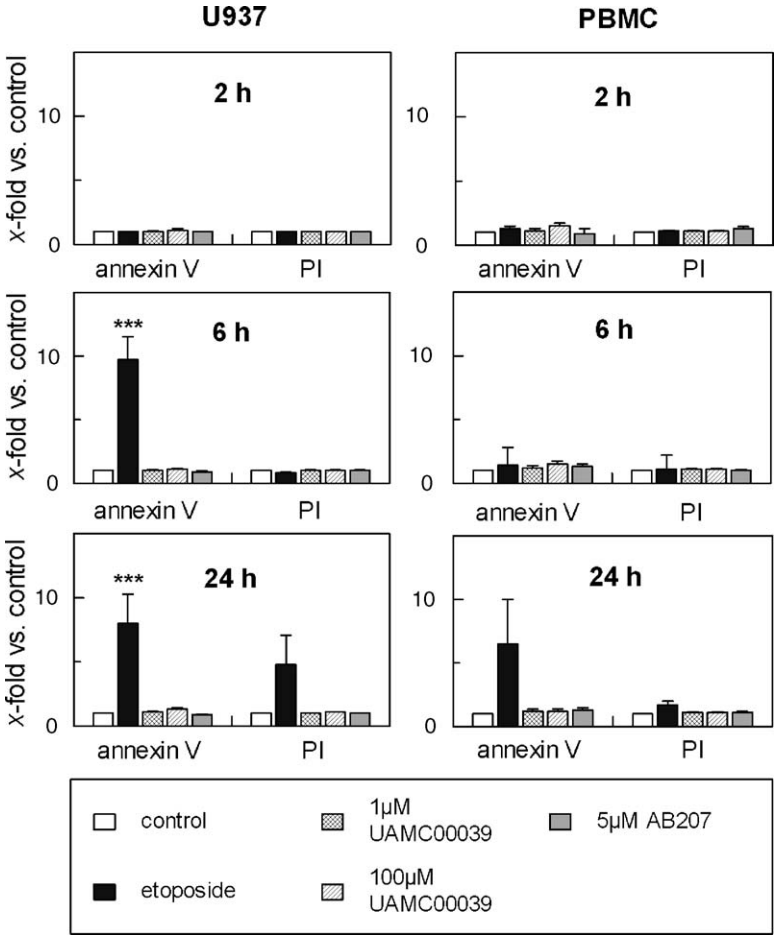

Fig. 6 – Effects of DPP inhibitors on cell death parameters in U937 cells and PBMC. U937 cells and PBMC were incubated for 2, 6 and 24 h with the specific DPPII inhibitor UAMC00039 and the DPPIV/8/9 inhibitor AB207. 50 μM etoposide was used as a positive control for apoptosis. Apoptosis and necrosis were evaluated by annexin V/PI staining ($n = 3$). The data were analysed using an univariate ANOVA, followed by the Dunnett test: $^{***}p < 0.001$ vs. control annexinV/PI staining.

concentration-dependent inhibition of intracellular DPPII activity without affecting the 'non-DPPII' DPP activity (Fig. 5). Maximal efficacy was reached at 100 nM. Upon incubation of intact U937 cells with UAMC00039, intracellular DPPII was inhibited with an IC_{50} in the same order of magnitude as that of both the purified enzyme and DPPII in U937 cell lysates (supplementary data).

3.4. *Effect of DPPII and 'non-DPPII' inhibition on cell death induction*

To ensure that sufficient inhibitor concentrations accumulated in the DPPII containing vesicles of U937 cells and PBMC, 1 and 100 μM UAMC00039 were used in the cell death assays. Both concentrations of UAMC00039 inhibited DPPII activity of PBMC and U937 cells more than 90%. Although UAMC00039 is a reversible inhibitor (Fig. 4B), DPPII was inhibited over the entire duration of the cell death assays due to the high concentration and stability of the compound in medium.

Trypan blue exclusion experiments suggested that UAMC00039 concentrations up to 100 μM were non-toxic for U937 cells for at least 48 h. Based on neutral red uptake by PBMC and U937 cells, inhibition of DPPII or DPPIV/8/9 did not affect viability of the cells whereas neutral red uptake by positive controls (treated with etoposide, MG132, CdA) was significantly decreased (data not shown).

Based on annexinV/PI staining (Fig. 6) and DNA laddering experiments (supplementary data), inhibition of DPPII (1 and 100 μM UAMC00039) or DPPIV/8/9 (5 μM AB207) did not result in apoptosis or necrosis of U937 ($n = 3$) and PBMC ($n = 3$) cells after 24 h of incubation. Concentrations up to 100 μM UAMC00039 did not affect MG132-triggered cell death.

Transmission electron microscopic analysis of U937 cells treated with the DPPII inhibitor UAMC00039 did not reveal vacuolization, which is a marker of autophagy [21]. Other morphological differences between control and treated cells could not be observed.

Based on total protein and cell count, cell proliferation was unaffected when DPPII activity was inhibited (>90% inhibition) for up to 96 h. We did not observe any upregulation of 'non-DPPII' DPP activity when DPPII was inhibited (data not shown).

4. Discussion

Cellular DPPIV-like enzymatic activity represents the sum of the hydrolytic activities of several DPPIV activity and/or structure homologues (DASH) including the plasma membrane localized DPPIV and FAPα as well as the intracellular proteases DPPII, DPP8 and DPP9 [36]. Experimental data on DPPII activity in different leukocytes and during the cell cycle are scarce and primarily based on non-quantitative cytochemical studies [1–3]. In this study, we focussed on the involvement of DPPII activity in leukocyte cell death. The PBMC showed considerable interindividual variability in enzyme activities and in addition resting lymphocytes were quite resistant to the induction of apoptosis. Therefore, in addition to PBMC, we chose the monocytic U937 cells. We could not observe any changes in the specific DPPII activity during proliferation of U937 cells which confirms the suggestion that DPPII plays a role in cell differentiation, rather than in cell proliferation [37–39]. However, DPPII activity decreased after PMA-stimulation in the U937 cells.

Based on intense cytochemical staining of DPPII in transitional ameloblasts from decalcified rat mandibles where apoptosis is thought to occur, DPPII has also been linked to cell death [40]. Furthermore, histochemical studies employing ovaries from cycling rats localized DPPII primarily to atretic follicles and during pregnancy, when all developing follicles are targeted for atresia, ovarian levels of DPPII are some three- to eight-fold higher [41]. However, the results of the present study demonstrate that induction of apoptosis in U937 cells had no effect on DPPII specific activity.

Autophagy, a nonapoptotic type of cell death, has been described as a means to resist starvation by degrading intracellular components for reuse. Since DPPII is known to cleave short peptides such as tripeptides [10,41,42], it may also be involved in the recycling of amino acids. Moreover, DPPII is localized to acidic vesicles [43] and based on cytochemical localization experiments in rat incisor tooth ameloblasts, reaction products of DPPII are present in phagosomes [40]. Autophagy is characterized by formation of numerous acidic vacuoles and therefore it is tempting to speculate that DPPII is involved in the breakdown of peptides in autophagosomes. ''Starvation'' conditions are routinely used to trigger autophagy. However, DPPII activity did not increase during starvation-induced autophagy of U937 cells. In the context of DPPII's catabolic function, we also investigated whether inhibition of the proteasome had an effect on DPPII activity and whether combined DPPII and proteasome inhibition affected cell death induction. Yet, we demonstrated that DPPII specific activity only increased when apoptosis evolved to secondary necrosis. In tissue culture, apoptotic cells can quickly proceed to secondary necrosis in the absence of uptake by phagocytic cells [44]. The increase in specific DPPII activity was confirmed using nigericine as a trigger for necrosis in U937 cells. A possible explanation for this observation may be that necrotic cells, due to leaky membranes, have minimal protein content [45].

According to Chiravuri et al. inhibition of DPPII activity by the boronic acid derivative VbP causes apoptosis in quiescent lymphocytes in a process independent of DPPIV [14]. Recent counterscreening efforts demonstrated that the boronic acid derivatives (e.g. VbP), initially designed as DPPIV inhibitors and also known to inhibit DPPII and FAP [19,46], in addition potently inhibit DPP8 and DPP9 [47]. Moreover, in another study the nonselective VbP as well as a selective DPP8/9 inhibitor inhibited proliferation and IL-2 release in T cells [48], properties that were previously attributed to DPPIV. DPPII and DPPIV selective compounds had no effect in these assays. The concentrations of VbP used by Chiravuri at al. [14], largely exceeded the nanomolar IC_{50}'s of all DPPs [47,48], which raises the possibility that leukocyte apoptosis elicited by VbP was not due to DPPII/QPP inhibition. The conditions used for measuring the QPP/DPPII activity (Ala-Pro-AFC pH 7.5) also did not exclude involvement of the new DPP members DPP8 and/or 9.

The availability of a highly potent and selective DPPII inhibitor UAMC00039 prompted us to further investigate the possible role of DPPII activity in different forms of cell death.

We showed that UAMC00039 proved to be suitable for cell culture experiments. The selectivity of this inhibitor enabled us to differentiate between DPPII and other related peptidases in the cell. The IC_{50} for intracellular DPPII in intact U937 cells seemed even lower than the IC_{50} for purified DPPII or DPPII in cell lysates. Possibly the basic inhibitor is enriched in the acidic vesicular compartment where DPPII is located.

Viability tests, DNA laddering assays and annexin V/PI-staining experiments showed that neither apoptosis nor necrosis occurred in U937-cells and PBMC after incubation with the DPPII inhibitor. Nonetheless, DPPII activity was inhibited for >90%. As reported in literature, resting lymphocytes showed a low sensitivity to the different apoptotic triggers [49–51].

At present, there is a lack of good markers for biochemical detection of autophagy. A growing body of evidence indicates that mammalian microtubule-associated protein 1 light chain 3 (LC3), in particular endogenous levels of the processed form LC3-II, is a biomarker for autophagy because it functions as a structural component during autophagosome formation. However, due to low expression levels of the protein, LC3 immunoblotting could not be used for U937 cells [52]. Transmission electron microscopy, currently the ''golden standard'' for monitoring autophagy both in tissue and cultured cells [53], did not reveal signs of autophagy in UAMC00039 treated U937 cells. In conclusion, our results clearly demonstrate (1) that inhibition of DPPII catalytic activity did not induce apoptosis, autophagy or necrosis in human leukocytes and (2) that DPPII specific activity increased during necrosis, but not during apoptosis or autophagy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2006.04.009.

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