Specific and Irreversible Cyclopeptide Inhibitors of Dipeptidyl Peptidase IV Activity of the T-Cell Activation Antigen CD26

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The dipeptidyl peptidase IV (DPP IV) activity of CD26 is characterized by its post-proline-cleaving capacity that plays an important but not yet understood role in biological processes. Here we describe a new family of specific and irreversible inhibitors of this enzyme. Taking into account the substrate specificity of DPP IV for P_2-P_1>-<P_1' cleavage, we have designed and synthesized cyclopeptides c[($^{\alpha}H_2N^+$)-Lys-Pro-Aba-(6-CH_2-S^+R_2)-Gly_n] $2TFA^-$ (Aba = 3-aminobenzoic acid, R = alkyl) possessing a proline at the P_1 position and a lysine in the P_2 position, which allows the closing of the cycle on its side chain. These molecules show a free N-terminus, necessary for binding to the CD26 catalytic site, and a latent quinoniminium methide electrophile, responsible for inactivation. Treatment of c[$^{\alpha}$Z-Lys-Pro-Aba-(6-CH_2-OC_6H_5)-Gly_n], obtained by peptide synthesis in solution, with R_2S/TFA simutaneously cleaved the Z protecting group and the phenyl ether function and led to a series of cyclopeptide sulfonium salts. These cyclopeptides inhibited rapidly and irreversibly the DPP IV activity of CD26, with IC_{50} values in the nanomolar range. Further studies were carried out to investigate the effect of the modification of the ring size (n = 2 or 4) and the nature of the sulfur substituents (R = Me, Bu, Oct). Cycle enlargement improved the inhibitory activity of the methylsulfonio cyclopeptide, whereas the increase of the alkyl chain length on the sulfur atom had no apparent effect. Other aminopeptidases were not inhibited, and a much weaker activity was observed on a novel isoform of DPP IV referred to as DPP IV-β. Thus, this new family of irreversible inhibitors of DPP IV is highly specific to the peptidase activity of CD26.

Introduction

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5), a membrane-bound exopeptidase, has been classically associated to the T-cell activation antigen CD26, a multifunctional sialoglycoprotein expressed on a variety of different epithelia and also by different hematopoietic cell types (for some recent reviews, see Yaron and Naider.[1] and Fleischer et al.[2]). Dipeptidyl peptidase IV (CD26) is a serine protease which has the unique specificity to cleave dipeptides from the N-terminus of polypeptides provided that proline is the penultimate residue.[1] In HIV infection, CD26 has been implicated in the viral entry process and its cytopathic effect.[3] Furthermore, DPP IV activity inhibition by HIV-1 Tat protein has been proposed as the mechanism of the lack of response to recall antigens observed in early stages of HIV infection;[4] however, the relevance of this inhibition in physiological conditions is unclear.[5] Whatever is the case, the addition of soluble CD26 can restore response to recall antigens of HIV-infected individuals in vitro.[6]

Irrespective of its peptidase activity, CD26 is associated with other molecules on the cell surface. It has been shown to be the main receptor of adenosine deaminase,[7] and on T-lymphocytes, CD26 is associated with CD45,[8] a cell-surface-expressed phosphotyrosine phosphatase involved in signal transduction. Both features of CD26, its signaling capacity and its peptidase activity, contribute to the costimulatory function of CD26 in T-cell activation events. However, the role of DPP IV activity of CD26 in these events is unclear. Some authors have described that small synthetic inhibitors of DPP IV impair mitogen and antigen stimulation of PBMC and other lymphocytic cell types.[9] In contrast, others have found no effect of DPP IV inhibitors on stimulated T-cells.[10] Similarly, contradictory results on the function of the DPP IV activity of CD26 have been reported by using cell lines expressing a mutated, catalytically inactive form of CD26.[11,12] The failure to understand the role of DPP IV activity is, in part, due to the fact that no physiological substrates have been identified. However, a broad spectrum of bioactive peptides, including some interleukines, chemokines, neuropeptides, and growth factors, can be potentially cleaved by DPP IV.

The availability of stable specific irreversible inhibitors or highly potent reversible inhibitors of DPP IV should be useful in studies for the determination of the physiological and pathological role(s) of this enzyme. Several competitive, tight-binding, or irreversible inhibitors of the enzyme are already known: oligopeptides with the N-terminal X-Pro sequence (X = various amino acid residues) such as the diprotins A and B (Ile-Pro-Ile or Val-Pro-Leu),[13,14] X-pyrrolidides and X-thiazolidides,[14−16] X-cyanoPro and X-cyanoThia,[17−19] X-phosphonoPro or Pip aryl esters,[20,21] X-boroPro,[22−25] X-ProCH_2N^+-

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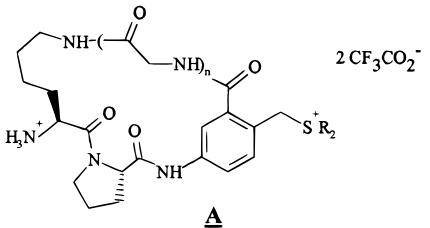
Chart 1. General Structure of the Cyclopeptides **A** Designed for Irreversible Inhibition of the DPP IV Activity of CD26



A

Me$_3$,[26] X-azaPro derivatives,[27] X-Pro-*N*-(arylcarbonyloxy)-amides,[28,29] and one of its ψ(CF=C) fluoro olefin isosteres.[30,31] Owing to the presence of a free amino N-terminus and the flexibility of the imino peptide bond, several of these inhibitors easily cyclize and are not very stable in solution.[32] The diacylated hydroxylamines are mechanism-based irreversible inhibitors of the protease: a demasked acylnitrene can react directly with an active site nucleophile or can lead, through a Lossen rearrangement, to an electrophilic isocyanate.[33]

We have previously designed and studied functionalized cyclopeptides containing a latent quinoniminium methide electrophile as suicide substrates for serine proteases.[34] Taking into account the substrate specificity of the DPP IV enzyme for P$_2$-Pro><-P′$_1$ cleavages (P$_1$ = Pro; Schechter and Berger notation[35]), we have now designed, synthesized, and studied cyclopeptides **A** (Chart 1): c[($^{\alpha}$H$_2$N$^+$)-Lys-Pro-Aba-(6-CH$_2$-S$^+$R$_2$)-Gly$_n$] 2TFA$^-$ (Aba = 3-aminobenzoic acid, R = alkyl), possessing the same latent electrophile, as selective suicide substrates for this exoprotease. These cyclopeptides were able to induce the complete, rapid, and irreversible inhibition of the DPP IV activity of CD26 with IC$_{50}$ in the nanomolar range. Their specificity was demonstrated by the lack of its effect on the activity of other peptidases, including the cell-surface-expressed DPP IV-β, the recently described protein with typical DPP IV activity.[36]

Results

From the relative weak importance of the nature of the P$_2$ side chain on the rate of hydrolysis of the enzyme substrates (vide infra), we hypothesized that the cyclization to the ϵ-amino function of a P$_2$ lysine residue would result in new molecules able to bind the catalytic site of the DPP IV activity in CD26. Moreover, this cyclization would leave the N-terminal α-amino group free and protonated, a necessary condition for the recognition by the enzyme (Chart 1). In macrocycles **A**, the substituted P′$_1$ aminobenzoic acid residue Aba-(6-CH$_2$-S$^+$R$_2$) is a precursor of the latent electrophilic quinoniminium methide cation. The presence of different numbers of glycine residues (*n*) in the cyclopeptides **A** allows the variation of the ring size, whereas the nature of the sulfur substituents (R) will modify the bulk and the lipophilicity in this part of the molecule. The influence of the leaving group ability on the inhibition efficiency and selectivity has been also examined by replacing the benzylic sulfonium substituent by an acetate group.

Peptide synthesis in solution, using DCC/HOBt for the formation of the amide bonds, was applied for the synthesis of the linear precursors of these macrocycles. The strategy involved the use of a nitro substituent as a latent amino group.[37] Coupling of the 2-(phenoxymethyl)-5-nitrobenzoic acid (Nba-[CH$_2$OPh], **5**[38]) with either ethyl diglycinate or ethyl tetraglycinate gave the substituted ethyl nitrobenzoyl polyglycinates **6a**,**b**. Selective catalytic hydrogenation of the nitro substituent, without hydrogenolysis of the benzyl ether function, occurred in the presence of platinium oxide in MeOH/DMF. The unstable aminobenzoyl polyglycine derivatives were rapidly acylated with *N*-Boc-L-proline to generate compounds Boc-Pro-Aba-[CH$_2$OPh]-Gly$_n$-OEt **7a**,**b**. Cleavage of the N-protecting group occurred in trifluoroacetic acid. In the following coupling step, the orthogonally diprotected Z-L-Lys(Boc)-OH derivative was used. The linear peptides **8a**,**b** were obtained in good yields. Cyclization of these precursors was achieved by the azide method: hydrazinolysis of the ester function leading to protected hydrazides **9a**,**b**, selective cleavage of the N$^{\epsilon}$-Boc group of the lysine residue in the presence of the N$^{\alpha}$-Z protecting group, treatment of the resulting hydrazides with an alkyl nitrite, and dilution in DMF in the presence of a tertiary amine. The cyclization yields were 45% and 44% for **10a**,**b**, respectively.

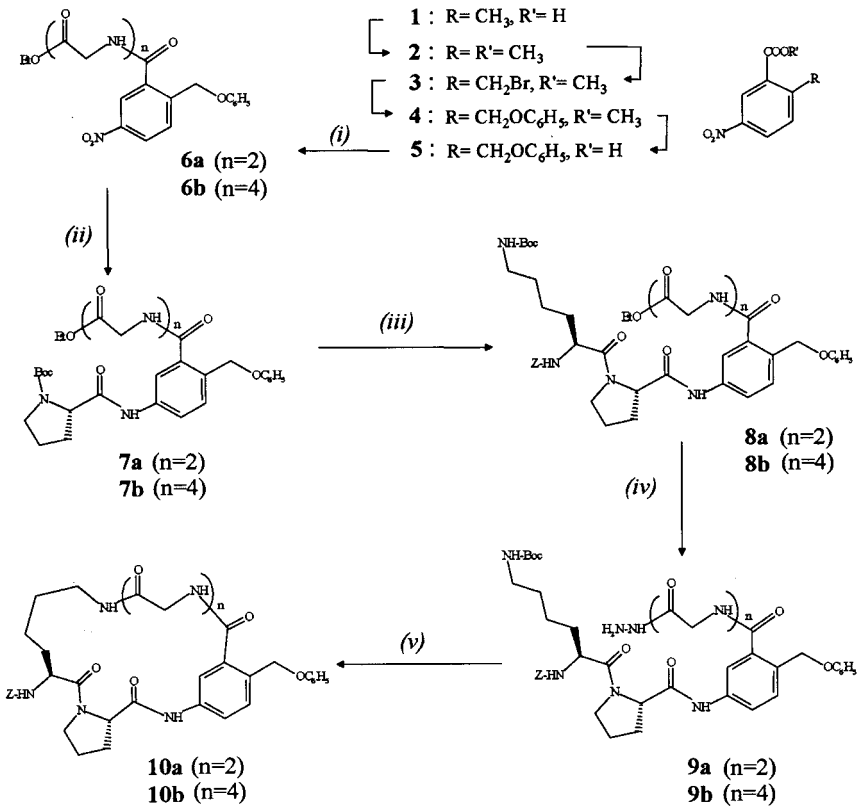
Organic sulfides, such as thioanisole, in trifluoroacetic acid are known to deprotect *O*-benzyltyrosine without the formation of O-to-C rearrangement products[39] and also to cleave the *N*-benzyloxycarbonyl protecting group.[40] The reactions occur by a "push−pull mechanism": nucleophilic attack of the sulfide lone pair on the benzylic carbon of a protonated ether or carbamate function. The byproducts in these deprotection reactions are sulfonium salts. We reasoned that treatment of the c[$^{\alpha}$Z-Lys-Pro-Aba-(6-CH$_2$-OC$_6$H$_5$)-Gly$_n$] cyclopeptides with various dialkyl sulfides in trifluoroacetic acid should cleave both the Z protecting group and the phenyl ether function (Scheme 2), thus leading to sulfonium salts having a protonated N-terminus. Effectively, such a treatment gave the bis trifluoroacetate salts **A**: **11a** (*n* = 2, R = Me, 62% yield), **11b** (*n* = 4, R = Me, 60% yield), **12a** (*n* = 2, R = Bu, 74.5% yield), **13a** (*n* = 2, R = Oct, 71% yield).

For the preparation of the cyclopeptide having a benzylic acetoxy substituent, the corresponding dimethylsulfonium salt **11a** was treated with potassium acetate in dry DMF. The resulting acetate was not very stable and decomposed during purification. The crude product was therefore reacted with di-*tert*-butyl dicarbonate to give the stable N-protected derivative which was easily purified by chromatography. Treatment with trifluoroacetic acid cleaved the Boc protecting group and led to the expected acetate salt **15a** in 76% yield.

Finally, selective hydrogenolysis of the Z protecting group of the cyclopeptide in the presence of the benzylic phenyl ether function was achieved by using a palladium on carbon catalyst in aqueous methanol and gave the cyclopeptide **16a** possessing a phenoxy substituent (Scheme 2).

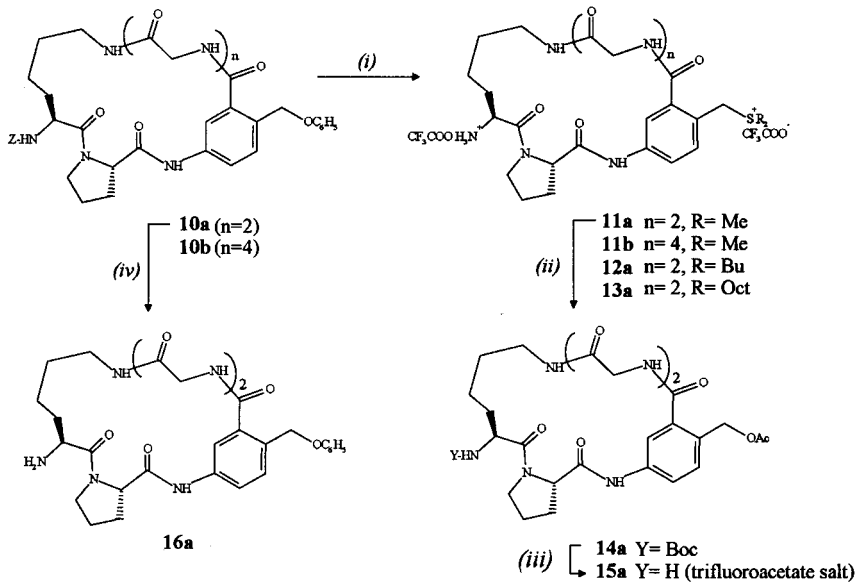
For comparison, simplified linear analogues of the

Scheme 1. Synthesis of the Cyclopeptides **10a**,**b** Having a Benzylic Phenoxy Substituent and a Terminal N-Z Protecting Group[a]



[a] (i) Gly_nOEt, DCC/HOBt; (ii) 1. H_2/PtO_2, MeOH−DMF, 2. Boc-Pro-OH, DCC/HOBt; (iii) 1. CF_3CO_2H or HCl/CH_2Cl_2, 2. Z-Lys(Boc)-OH, DCC/HOBt; (iv) NH_2NH_2/MeOH; (v) 1. CF_3CO_2H/CH_2Cl_2, 2. HCl/DMF−THF, 3. *i*-$PrNEt_2$/DMF.

Scheme 2. Last Steps of the Synthesis of Cyclopeptides **11a**−**16a**[a]



[a] (i) R_2S/CF_3CO_2H; (ii) 1. KOAc/DMF, 2. Boc_2O; (iii) CF_3CO_2H; (*iv*) H_2/Pd/C, MeOH.

Pro-Aba(6-CH_2-S^+Me_2)-OMe (**18a**,**b**) were prepared as above from the corresponding benzylic ethers H_2^+-X-Pro-Aba(6-CH_2-OC_6H_5)-OMe (**17a**,**b**).

Biological Studies. In all the studies described here, the dipeptidyl peptidase activity of CD26 and DPP IV-β was investigated using the enzymes in their natural habitat, i.e., by using either crude cell extracts the cell surface, and thus their enzymatic activity could be assayed by using intact cells.[36] As a source of CD26 and DPP IV-β, we used either human CEM cells overexpressing CD26 by transfection[49] or human C8166 cells which express only DPP IV-β.[36] In some experiments partially purified enzyme preparations were also used.[51] In the case of CD26, we also used immunoaf-

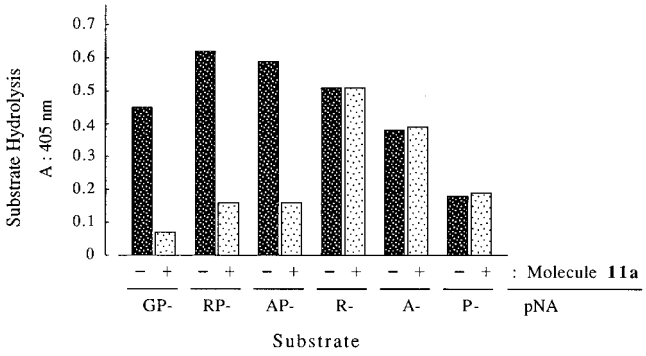


Figure 1. Specificity of molecule **11a**. Effect of inhibitor **11a** on different types of peptidase activities. Crude MOLT4 cell extracts[36] were assayed for the effect of 10 μM compound **11a** on different aminopeptidase activities. The peptidases tested were as follows: DPP IV by the cleavage of GP-pNA, RP-pNA, and AP-pNA; Arg-peptidase by the cleavage of R-pNA; Ala-peptidase by the cleavage of A-pNA; and Pro-peptidase by the cleavage of P-pNA. At 10 μM **11a**, the DPP IV activity against different substrates was inhibited by more than 75%, while no apparent effect on the other peptidases was observed. Abbreviations of the amino acids: G = glycine, P = proline, A = alanine, and R = arginine.

activity of different peptidases, extracts from human MOLT4 cells were used.[36]

Preliminary results pointed out that molecule **11a** was a potent inhibitor of DPP IV activity of CD26. Indeed, the IC_{50} value for the inhibition of the DPP IV activity of a purified preparation of CD26[36] was found to be 0.01 μM. For this reason, we first studied the specificity of this inhibitor by determining its effect on different aminopeptidases found in crude cell extracts. The peptidases tested were as follows: arginine-peptidase (EC 3.4.11.6) by the cleavage of Arg-pNA, alanine-peptidase (EC 3.4.11.2) by the cleavage of Ala-pNA, proline-peptidase (EC 3.4.11.5) by the cleavage of Pro-pNA, and DPP IV by the cleavage of different substrates (Gly-Pro-, Arg-Pro-, and Ala-Pro-pNA).[36] At 10 μM molecule **11a**, which is 3 orders of magnitude higher than its IC_{50}, the DPP IV activity monitored with the different substrates was inhibited by more than 80% (Figure 1). The remaining residual 20% activity was probably due to the background (i.e., a nonspecific and CD26-independent cleavage of the substrates) since it was observed even at 100 μM molecule **11a** (not shown). In contrast, molecule **11a** exerted no inhibitory effect on the activity of arginine-, alanine-, and proline-aminopeptidase (Figure 1). These results therefore demonstrated the specific nature of the DPP IV inhibition by molecule **11a**.

To investigate the irreversible nature of the inhibitory molecule **11a**, its effect on the DPP IV activity of CD26 expressed on the cell surface was studied. For this purpose, we established by transfection of CD26 cDNA, human CEM cells which express high levels of CD26, i.e., cells which express high levels of DPP IV activity on the cell surface. Consequently, intact cells (clone H01) could be assayed for DPP IV activity by incubation with an appropriate substrate, such as Gly-Pro-pNA.[36] Under these experimental conditions, molecule **11a** was found to be a potent inhibitor of cell-surface DPP IV activity, with maximum inhibition occurring at 2 μM.

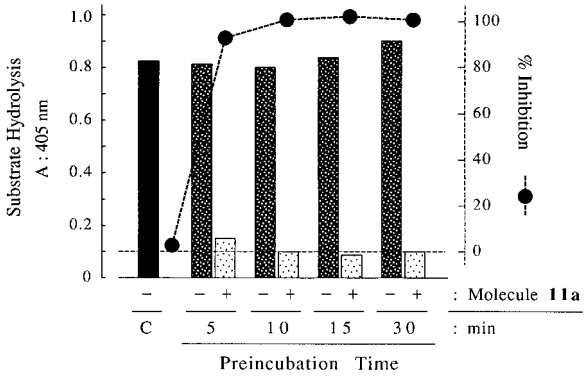


Figure 2. Kinetics of molecule **11a**-mediated inhibition of the cell-surface-expressed DPP IV activity of CD26. Intact CEM cells expressing high levels of CD26 (clone H01)[49] were incubated for 5, 10, 15, and 30 min in PBS with or without 5 μM compound **11a** at 37 °C and then washed twice with PBS. DPP IV activity of CD26 was then monitored by the cleavage of GP-pNA as described in the Experimental Section. The binding of the inhibitor **11a** to DPP IV active site is very rapid since 5 min is sufficient to obtain more than 75% inhibition.

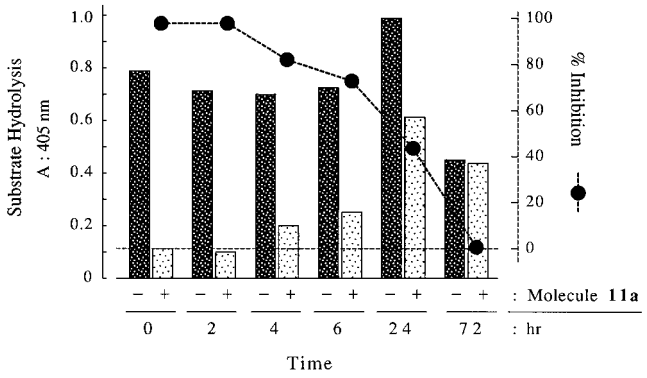


Figure 3. Irreversibility of DPP IV inhibition by molecule **11a** demonstrated by the cell-surface-expressed CD26. Intact CEM cells overexpressing CD26 (clone H01) were preincubated for 15 min in PBS in the absence or presence of 5 μM **11a** at 37 °C, then washed twice in PBS, and cultured in RPMI supplemented medium. Aliquots of cells were taken at the indicated times, washed twice with PBS, and then assayed for DPP IV activity by incubating cells with the substrate GP-pNA for 1 h at 37 °C. The samples at time 0 represent the DPP IV activity just after the 15 min of preincubation.

with 5 μM molecule **11a**. At times of 5, 10, 15, and 30 min, cells were washed extensively to remove unbound molecule **11a** before assay of the DPP IV activity. As shown in Figure 2, inhibition was almost maximal after 5 min of incubation, since the degree of inhibition was only slightly increased at 30 min. These results demonstrated that molecule **11a** has the capacity to bind CD26 rapidly and thus inhibit irreversibly its DPP IV activity. Second, to confirm the irreversible nature of molecule **11a**, cells were incubated with 5 μM inhibitor for 15 min before extensive washing and further incubation in the culture medium for up to 3 days. At different times during this period, cells were monitored for cell-surface-expressed DPP IV activity (Figure 3). The maximum inhibition observed following the 15-min incubation of cells with molecule **11a** (time 0 h) was found to last for several hours. At 6 h, there was a very slight difference on the maximum inhibition, whereas at 24 h, there was about 50% inhibition. Interestingly,

Table 1. Inhibition of DPP IV Activity by the Cyclopeptide Compounds[a]

cyclopeptides **A**			inhibition of Gly-Pro-pNa hydrolysis (IC_{50}, μM)	
ref	glycine (*n*)	leaving group	CD26	DPP IV-β
11a	2	S^+Me_2	0.012	1
11b	4	S^+Me_2	0.003	0.61
12a	2	S^+Bu_2	0.02	0.57
13a	2	S^+Oct_2	0.03	0.63
15a	2	OAc	1.5	3.5
16a	2	OPh	10	25

[a] The DPP IV activity was monitored by the cleavage of Gly-Pro-pNA in purified preparations of CD26 and DPP IV-β (Experimental Section). CD26 was purified using extracts of CEM H01 cells expressing very high levels of CD26.[49] DPP IV-β was purified using extracts of CD26-negative C8166 cells.[36] Both purification procedures were as described elsewhere.[51] Such purified preparations of CD26 and DPP IV-β were free of any significant contamination by other peptidases. CD26 and DPP IV-β preparations were preincubated for 15 min with different concentrations of each inhibitor ranging from 1 nM to 10 μM before adding the substrate Gly-Pro-pNA as indicated.

production of cells that express newly synthesized CD26 molecules. At 3 days, no inhibition of cell-surface DPP IV activity was observed. Taken together, these data indicate that the inhibitory effect of molecule **11a** is irreversible, since the DPP IV activity could be resumed only by the newly synthesized CD26 (Figure 3). CD26 being a cell-surface-expressed protein, its expression on the cell surface could be modified with respect to the different phases of the cell cycle. Consequently at 24 and 72 h, the DPP IV activity was variable in control cells (without preincubation with the inhibitor) (Figure 3). It should also be noted that the cell-surface expression of CD26 was not affected by coupling with molecule **11a**. Indeed, cells in the absence or presence of preincubation with molecule **11a** manifested similar levels of cell-surface-expressed CD26, as monitored by FACS analysis using anti-CD26-specific monoclonal antibodies (data not shown).

The other cyclopeptides, possessing different leaving groups (**12a**, **13a**, **15a**, **16a**) or a larger ring (**11b**) were also assayed for their capacity to inhibit the DPP IV activity of CD26 using crude extracts from CEM cells (clone H01). The IC_{50} values for the inhibition of Gly-Pro-pNA hydrolysis are given in Table 1. All of these cyclopeptides showed different IC_{50} values in the nanomolar or micromolar range. However, no correlation was observed between these IC_{50} values and the length or lipophilicity of the leaving group. Molecules **15a** and **16a** with leaving groups as acetate and phenoxy, respectively, manifested significantly reduced IC_{50} values. In contrast, increasing the ring size in a given molecule generated a compound with an increased inhibitory activity (Table 1, compare molecules **11a**,**b**).

By use of the CD26-negative T-lymphoblastoid cell line C8166, we have recently described a CD26-like cell-surface protein with typical DPP IV activity.[36] This novel form of DPP IV, referred to as DPP IV-β, was found to be distinct from CD26. However the pH optimum and the profiles for substrate molecules were found to be indistinguishable for both CD26 and DPP IV-β. Similarly, several previously described inhibitors

Table 2. Characterization of the Inhibition Kinetics of CD26 and DPP IV-β by the Cyclopeptide **11a**

	inhibition constants[b]	
enzyme[a]	K_I (μM)	k_{inact} (s^{-1})
CD26	0.085	$11.\ 10^{-4}$
DPP IV-β	0.470	$22.\ 10^{-5}$

[a] The DPP IV activities of CD26 and DPP IV-β were assayed by the use of extracts from CEM H01 (cells expressing very high levels of recombinant CD26) and C8166 cells (cells expressing only DPP IV-β), respectively. The cleavage of the substrate Gly-Pro-pNA (0.5 mM) was monitored in the presence of increasing concentrations of inhibitor **11a** by measuring absorbance at 405 nm. Preparation of extracts and assay conditions were as described before[36] and as in the Experimental Section. [b] Calculations were done as indicated in the Experimental Section. K_I is the equilibrium constant of the inhibitor binding to the enzyme, whereas k_{inact} is the constant of the irreversible reaction that leads to the inactivation of the enzyme.

essential to assess the action of the cyclopeptide inhibitors on DPP IV-β. The results given in Table 1 show that the inhibitory effect of the different irreversible cyclopeptide inhibitors is much more pronounced for CD26 compared to DPP IV-β. For example, it is interesting to note that the inhibitory effect of molecules **11a**,**b** is 83- and 203-fold higher on the DPP IV activity of CD26 compared to that of DPP IV-β, respectively (Table 1). In contrast to such a significant selectivity, molecules **15a** and **16a** exerted only about 2-fold difference between the two enzymes. These data might suggest that the higher specificity of the inhibitors on the DPP IV activity of CD26 could be related to the reactivity of the cyclopeptide sulfonium salts.

To further investigate the significant differences in the effect of the mostly studied inhibitorory molecule**11a** on DPP IV activity of CD26 and DPP IV-β (Table 1), we studied the kinetics of inhibition of both enzymes, by using the approach previously described for irreversible inhibitors of trypsin-like proteases.[34] This approach considers two steps in the inhibition process: first, the reversible binding of the inhibitor to the enzyme and, second, the cleavage and subsequent irreversible covalent modification of the enzyme leading to the loss of catalytic activity. The inhibition kinetics of the DPP IV activities of CD26 and DPP IV-β fit well to this model, and the equilibrium constant of the first step (K_I) as well as the kinetic constant of the second step, was calculated. The results are summarized in Table 2 and show that the higher potency of inhibitor **11a** on the DPP IV activity of CD26 is the consequence of a higher affinity for this enzyme as demonstrated by the K_I values, along with a faster inactivation rate as pointed out by the k_{inact} values found for CD26.

The difference in the inhibitory effect of irreversible inhibitors was further emphasized by testing the cell-surface-expressed enzymes. For this purpose, we used CEM cells expressing high levels of CD26 (clone H01) as a source of CD26 and C8166 cells as a source of DPP IV-β. Figure 4 shows the effect of different concentrations of molecule **11a** and a previously described reversible inhibitor, Lys-[Z(NO_2)]-pyrrolidide. As we had reported previously,[36] Lys-[Z(NO_2)]-pyrrolidide inhibited to a similar extent the DPP IV activity of both CD26 and DPP IV-β, with comparable IC_{50} values. On the

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