Biol. Chem., Vol. 382, pp. 1197 – 1205, August 2001 ·

Cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ Is a Potent Competitive Antagonist of the Interaction of Urokinase-Type Plasminogen Activator with Its Receptor (CD87)

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Urokinase-type plasminogen activator (uPA) represents a central molecule in pericellular proteolysis and is implicated in a variety of physiological and pathophysiological processes such as tissue remodelling, wound healing, tumor invasion, and metastasis. uPA binds with high affinity to a specific cell surface receptor, uPAR (CD87), *via* a well defined sequence within the N-terminal region of uPA (uPA$_{19-31}$). This interaction directs the proteolytic activity of uPA to the cell surface which represents an important step in tumor cell proliferation, invasion, and metastasis. Due to its fundamental role in these processes, the uPA/uPAR-system has emerged as a novel target for tumor therapy. Previously, we have identified a synthetic, cyclic, uPA-derived peptide, cyclo^{19,31}uPA$_{19-31}$, as a lead structure for the development of low molecular weight uPA-analogues, capable of blocking uPA/uPAR-interaction [Bürgle *et al.*, Biol. Chem. *378* (1997), 231 – 237]. We now searched for peptide variants of cyclo^{19,31}uPA$_{19-31}$ with elevated affinities for uPAR binding. Among other tasks, we performed a systematic D-amino acid scan of uPA$_{19-31}$, in which each of the 13 L-amino acids was individually substituted by the corresponding D-amino acid. This led to the identification of cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ as a potent inhibitor of uPA/uPAR-interaction, displaying only a 20 to 40-fold lower binding capacity as compared to the naturally occurring uPAR-ligands uPA and its amino-terminal fragment. Cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ not only blocks binding of uPA to uPAR but is also capable of efficiently displacing uPAR-bound uPA from the cell surface and to inhibit uPA-mediated, tumor cell-associated plasminogen activation and fibrin degradation. Thus, cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ represents a promising therapeutic agent to significantly affect the tumor-associated uPA/uPAR-system.

Key words: D-amino acid / Peptides / Protease / Receptor / Tumor invasion / Urokinase.

Introduction

The serine proteases urokinase-type plasminogen activator (uPA) and plasmin, in concert with other proteolytic enzymes (*e. g.* matrix metalloproteases, cysteine proteases), are involved in tumor-associated processes such as cell invasion and regulation of cell/cell and cell/matrix contacts (Reuning *et al.*, 1998; Schmitt *et al.*, 2000; Andreasen *et al.*, 2000). (pro-)uPA binds to a specific, high-affinity cell surface receptor, uPAR (CD87), which is composed of three structurally homologous, independently folded domains (Dear and Medcalf, 1998). In addition, there are also cellular binding sites for plasmin(ogen) (Félez, 1998). In consequence, binding of (pro-) uPA to cell membrane-anchored uPAR significantly increases plasmin generation due to a distinct increase of reciprocal activation of pro-uPA and plasminogen (Ellis *et al.*, 1991). Plasmin not only degrades a variety of components of the extracellular matrix (*e. g.* fibrin and laminin), but also activates certain matrix metalloproteases (in addition to pro-uPA) which break down additional structural proteins of the extracellular matrix such as various collagens. Thus, cell surface-triggered plasminogen activation plays a fundamental role in tissue remodeling, which is a prerequisite for tumor cell invasion and metastasis (Reuning *et al.*, 1998; Schmitt *et al.*, 2000; Andreasen *et al.*, 2000).

The interaction of pro-uPA or its activated form high molecular weight (HMW-)uPA with uPAR has been characterised in detail. Although the N-terminal domain I of uPAR contains major determinants for uPA-binding, high affinity interaction with uPA is dependent on the multidomain structure of the receptor, indicating that all three protein domains are involved in the formation of a composite ligand binding site (Ploug, 1998; Gårdsvoll *et al.*, 1999; Bdeir *et al.*, 2000). In contrast, uPA binds to uPAR *via* a defined continuous peptide sequence

(uPA_{19-31}) within the N-terminal region of uPA. Originally, the uPAR-binding epitope of uPA was identified by applying a linear peptide spanning amino acids 12–32 of uPA (in which, for technical reasons, Cys19 was substituted by Ala) in competition experiments (Appella *et al.*, 1987). This peptide was found to inhibit binding of the amino-terminal fragment (ATF) of uPA (which binds to uPAR with the same affinity as uPA but lacks the uPA protease domain) to cell surface uPAR. Later, several linear peptides spanning uPA_{14-32} were synthesised in which the naturally occurring amino acids were individually replaced by alanine (Ala scan) in order to identify the amino acids critical for binding to uPAR (Magdolen *et al.*, 1996). The exchange of Cys19, Lys23, Tyr24, Phe25, Ile28, Trp30, and Cys31, respectively, by Ala resulted in peptides with strongly impaired uPAR-binding capacities, whereas replacement of the other amino acids had no or little effect on uPAR binding. Finally, the minimal uPAR-binding region of uPA was located to uPA_{19-31} using synthetic peptides which were successively shortened from the amino- and/or carboxy-terminus starting with uPA_{10-32} (Bürgle *et al.*, 1997).

The region between amino acids Thr18 and Asn32 of uPA consists of a flexible, seven-residue Ω-loop (Asn22 to Ile28) which by means of a double stranded, antiparallel ß-sheet (between Thr18 to Ser21 and His29 to Asn32) is forced into a ring-like structure (Hansen *et al.*, 1994a, b; Magdolen *et al.*, 1996; Schmitt *et al.*, 2000). In uPA, Cys19 and Cys31, although in close proximity, form disulfide bonds with distinct cysteines (Cys11/Cys19 and Cys13/Cys31, respectively; Hansen *et al.*, 1994a, b). The short distance between Cys19 and Cys31 in the native molecule may explain why peptide $cyclo^{19,31}uPA_{19-31}$ with its 'illegitime' disulfide bond retains or displays an even increased uPAR-binding activity (Bürgle *et al.*, 1997). Recently, we successfully generated double-headed inhibitors directed at different tumor-associated proteolytic systems by exchanging a disulfide-linked loop in cystatin, which is non-essential for cysteine protease inhibition, with uPA_{19-31}. The results obtained indicate that cystatins with a uPAR-binding site are efficient inhibitors of cysteine proteases and uPA/uPAR-interaction at the same time (Muehlenweg *et al.*, 2000).

Other types of peptide antagonists of uPA/uPAR-interaction, which show no obvious similarity to uPA_{19-31}, have been identified by a bacteriophage peptide display approach (Goodson *et al.*, 1994). All of the reactive peptides (with the clone 20 peptide being most active) harbor relatively short common motifs, such as LWXXY and FXXYLW, which may represent a variation of the motif ^{24}YFXXIXW^{30} within the uPA molecule (only the critical hydrophobic amino acids for uPAR-binding are depicted) (Magdolen *et al.*, 1996; Bürgle *et al.*, 1997). Recently, a non-competitive, allosteric antagonist of pro-uPA/uPAR-interaction (Å6-peptide) derived from a non-receptor binding region of uPA ($uPA_{136-143}$) has been identified (Guo *et al.*, 2000). As demonstrated in various experimental *in vivo* model systems for competitive inhibitors of uPA/uPAR-interaction (Crowley *et al.*, 1993; Min *et al.*, 1996; Kobayashi *et al.*, 1998; Li *et al.*, 1998; Tressler *et al.*, 1999; Krüger *et al.*, 2000; Lutz *et al.*, 2001), administration of this non-competitive antagonist to tumor-bearing animals did also inhibited tumor growth and metastasis.

In the present study, the synthetic peptide $cyclo^{19,31}uPA_{19-31}$ was used as a lead structure to identify more potent small molecule inhibitors of uPA/uPAR-interaction. Initially, we substituted each of the 13 L-amino acids of peptide uPA_{19-31} individually by the corresponding D-amino acid. Subsequently, cyclic counterparts of those D-amino acid-containing peptides still displaying distinct uPAR-binding capacities were generated. In addition, we also synthesised the retro-inverso variant of $cyclo^{19,31}uPA_{19-31}$, substituted the cystin moiety in $cyclo^{19,31}uPA_{19-31}$ by lanthionine, and deleted the N- and C-terminal cysteines, resulting in peptide $cyclo^{20,30}uPA_{20-30}$. Finally, the most potent peptide, $cyclo^{19,31}$[D-Cys^{19}]-uPA_{19-31}, displaying an IC_{50} value of about 200 nM in flow cytofluorometric analyses, was tested for its capacity to displace uPAR-bound uPA from the cell surface of human cells and to inhibit uPA-mediated, tumor cell-associated plasminogen activation and fibrin degradation.

Results and Discussion

Stereochemical Modifications of uPA_{19-31}

Substitution of L-amino acids by corresponding D-amino acids in peptide analoga may yield additional information with respect to the stereochemical basis for the generation of specific secondary structures and their input on the interaction of the peptide ligand with its receptor. Furthermore, since most proteases are enantio-specific, and therefore do not cleave peptide bonds which are built up by D-/L- or L-/D-amino acids, the use of D-amino acids may also significantly improve the *in vivo* stability of such peptide analoga. D-amino acids in peptide ligands may also induce so-called ß-turns and, therefore, increase the rigidity of structural motifs which are important for the binding conformation.

We replaced each of the 13 L-amino acids of the linear peptide uPA_{19-31} individually by the corresponding D-amino acid. After purification by HPLC, the ability of these peptides to inhibit binding of fluorescently labeled uPA to uPAR-expressing human U937 cells was tested by flow cytofluorometry. The substitution of some L-amino acids by corresponding D-amino acids in uPA_{19-31} (Asn22, Lys23, Tyr24, Phe25, Ser26, Ile28, and Cys31) significantly reduced the binding capacities of these peptide variants as compared to the parental peptide uPA_{19-31} (Table 1). This points to the fact that the stereochemical orientation of these amino acids may be important for binding or that certain D-amino acids induce a structural change in the uPA_{19-31}-variant, thereby leading to a reduced interaction with uPAR. Interestingly, these

Table 1 uPAR-Binding Characteristics of uPA$_{19-31}$-Related Synthetic Peptides.

Peptide	Inhibition	
	Linear	Cyclic
uPA$_{19-31}$	+++	+++
[D-Cys^{19}]-uPA$_{19-31}$	+++	++++
[D-Val^{20}]-uPA$_{19-31}$	++	(+/–)
[D-Ser^{21}]-uPA$_{19-31}$	++	++
[D-Asn^{22}]-uPA$_{19-31}$	–	n.t.
[D-Lys^{23}]-uPA$_{19-31}$	–	n.t.
[D-Tyr^{24}]-uPA$_{19-31}$	–	n.t.
[D-Phe^{25}]-uPA$_{19-31}$	–	n.t.
[D-Ser^{26}]-uPA$_{19-31}$	–	n.t.
[D-Asn^{27}]-uPA$_{19-31}$	++	++
[D-Ile^{28}]-uPA$_{19-31}$	(+/–)	n.t.
[D-His^{29}]-uPA$_{19-31}$	++	(+/–)
[D-Trp^{30}]-uPA$_{19-31}$	++	(+/–)
[D-Cys^{31}]-uPA$_{19-31}$	(+/–)	n.t.
Retro-inverso-uPA$_{19-31}$	–	–
[Ala^{19}-S-Ala^{31}]-uPA$_{19-31}$	n.t.	(+/–)
[D-Cys^{21},Hph^{25},Cys^{29}]-uPA$_{21-30}$	n.t.	–
[Cys^{21},Ala^{24},Cys^{29}]-uPA$_{21-30}$	n.t.	–
uPA$_{20-30}$	–	–

The uPAR-binding capacities of linear and cyclic uPA-derived variants were tested by flow cytofluorometry. For this, PMA-stimulated human U937 cells (2.5 × 10^5 cells per 250 µl) were co-incubated with 16 ng FITC-pro-uPA and varying amounts of the uPA-peptides, and the cell-associated fluorescence was determined. Key: ++++, efficient competition of FITC-pro-uPA binding with < 1 µg peptide; +++, efficient competition with < 2.5 µg; ++, about 50% competition with 2.5 µg; (+/–), weak inhibition of FITC-pro-uPA binding with ≥ 10 µg peptide; –, no inhibition; n.t., not tested; Hph, homophenyl-alanine.

amino acids –with the exception of Cys31 – are located in the flexible Ω-loop of the uPAR-binding region of uPA. Substitution of the L-amino acids Val20, Ser21, Asn27, His29, Trp30, and, especially, Cys19, by the corresponding D-enantiomers yielded peptide variants with distinct uPAR-binding capacities. The linear peptide [D-Cys[19]]-uPA$_{19-31}$ inhibits the uPA/uPAR-interaction close in order to that of the cyclic lead structure cyclo[19,31]uPA$_{19-31}$ (Table 1).

Cyclic D-Amino Acid-Containing Variants of uPA$_{19-31}$

Cyclic counterparts of the linear uPA$_{19-31}$-derived peptides that display an inhibitory activity towards uPA/uPAR-interaction in the FACS analyses were generated, HPLC-purified and again tested by FACS analysis. Substitution of L-Cys by D-Cys at position 19 led to a distinct increase of biological activity (Figure 1). The IC_{50} value of peptide cyclo[19,31][D-Cys[19]]-uPA$_{19-31}$ to inhibit the interaction of FITC-labeled uPA with uPAR was approx. 200 nM, that of the lead peptide cyclo[19,31]-uPA$_{19-31}$ about 700 nM. The IC_{50} value of both unlabelled human uPA and ATF was 5 – 10 nM. Thus, the binding affinity of the D-Cys19-modified uPA-peptide is only 20- to 40-fold lower than that of the naturally occurring uPAR-ligands uPA and ATF.

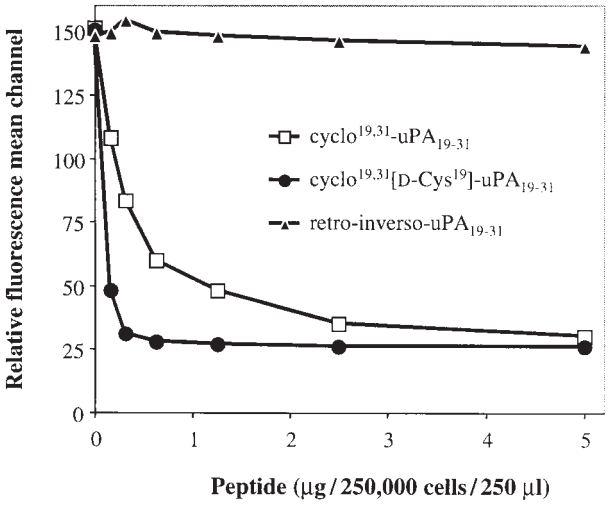

Fig. 1 Dose-Dependent Inhibition of Binding of Fluorescently Labelled pro-uPA to Cell Surface-Associated uPAR by Cyclo[19,31]-uPA$_{19-31}$ and Cyclo[19,31][D-Cys[19]]-uPA$_{19-31}$.
PMA-stimulated human U937 cells (2.5 × 10^5 cells per 250 µl) were coincubated with 16 ng FITC-pro-uPA and increasing concentrations of synthetic cyclic uPA-derived peptides at room temperature, and the cell-associated fluorescence determined. Whereas cyclo[19,31]-uPA$_{19-31}$ (IC_{50}: approx. 700 nM) and cyclo[19,31][D-Cys[19]]-uPA$_{19-31}$ (IC_{50}: approx. 200 nM) efficiently block uPA/uPAR-interaction, the retro-inverso peptide of uPA$_{19-31}$ (for the primary structure see Figure 2) exerts no effect. The result is representative of three independently performed experiments.

Other Cyclic uPA-Derived Peptide Variants

In addition to the peptides with single D-amino acid substitutions, we generated a cyclic peptide in which all L-amino acids were substituted by their corresponding D-enantiomers and in which all of the peptide bonds were inverted. This approach resulted in the so-called retro-inverso, protease-insensitive peptide of cyclo[19,31]-uPA$_{19-31}$ (Figure 2).

As initially proposed by Prelog and Gerlach (1964) and Shemyakin *et al.* (1969), the complete inversion of all chirality centers, together with the inversion of the amino acid sequence should lead to a similar topochemical orientation of the side chains than the parent peptide, provided the topology of the intramolecular hydrogen bonds is neglected. Indeed, several groups have observed that retroenantiomeric peptides may display comparable biological activities as the parent peptides (Chorev and Goodman, 1993). In line with this, the structural evaluation of a cyclic Fcε RI α-chain-derived peptide and its retro-inverso motif by NMR showed similarity of the surface topology of both compounds (McDonnell *et al.*, 1997). However, as demonstrated by Wermuth *et al.* (1997), the topology of the $\alpha_v\beta_3$ integrin inhibitor cyclo (–RGDfV–) and its retro-inverso peptide can differ, resulting in different activity profiles of the derivative or even complete loss of biological activity.

The retro-inverso peptide of uPA$_{19-31}$ did not show any uPAR-binding activity (Figure 1). This may due to the fact

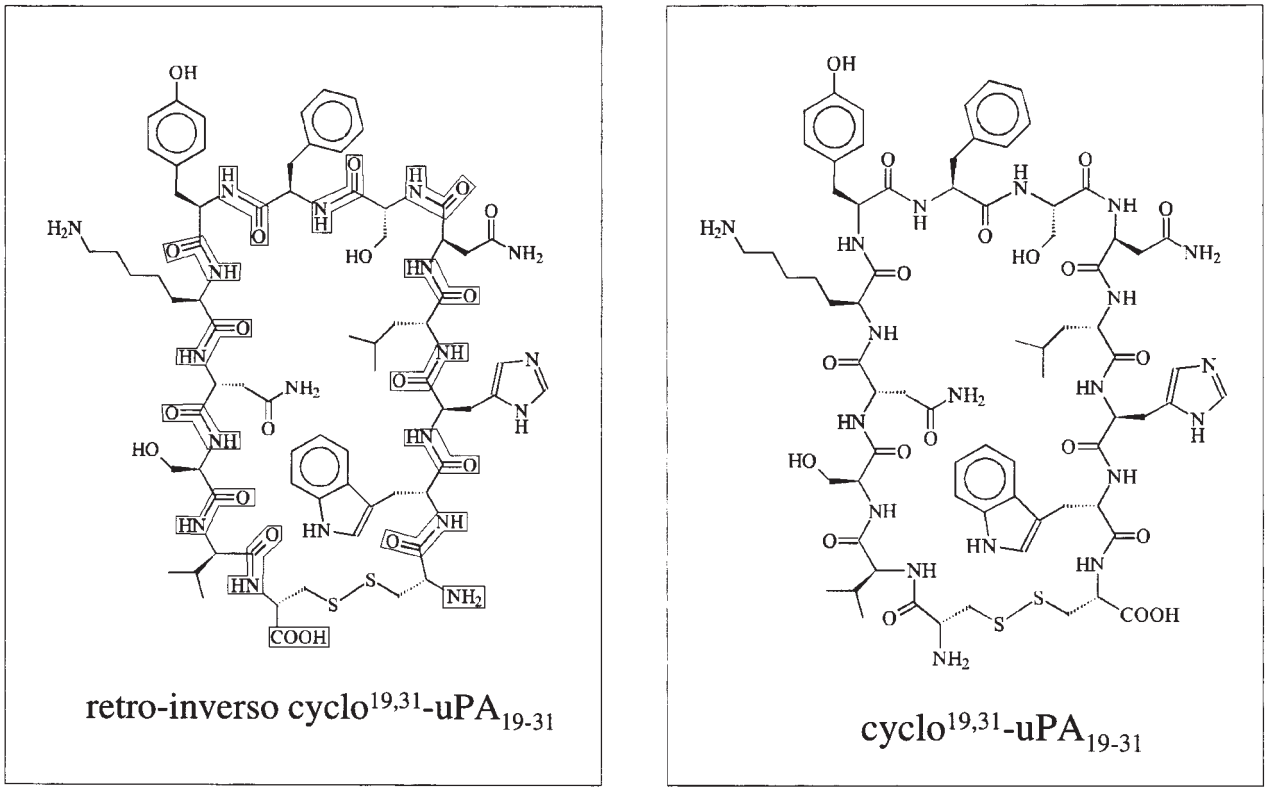

Fig. 2 Comparison of the Primary Structure of Retro-Inverso Cyclo^{19,31}-uPA$_{19-31}$ with Its Parental Peptide.
The differences between the two peptides are highlighted in the retro-inverso form. The sequence of cyclo^{19,31}-uPA$_{19-31}$ (with the disulfide bridge formed by Cys19 and Cys31) is: ^{19}Cys-Val-Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp-Cys^{31}.

that retro-D-peptides harbor exchanged N- and C-termini (Figure 2) and that these terminal groups in the peptide cyclo^{19,31}uPA$_{19-31}$ are important for the interaction with uPAR. Alternatively, the amide bonds in the backbone of the peptide (which are reversed in the retro-inverso peptide) may be necessary for the binding of the uPA-derived peptide to uPAR. One also has to take into account that the hydrogen bond pattern in retro-inverso peptides is different as compared to the parental peptides.

To further elucidate the importance of Cys19 and Cys31 in cyclic uPA-peptides for binding to uPAR, we generated two other peptide variants: (i) cyclo^{20,30}uPA$_{20-30}$ was generated by the formation of a peptide bond of the terminal -NH_2 group of Val20 and the terminal -COOH group of Trp30; (ii) cyclo^{19,31}[Ala^{19}-S-Ala^{31}]-uPA$_{19-31}$ corresponds to a peptide variant of cyclo^{19,31}-uPA$_{19-31}$, in which the cystine (Cys-Cys, which formally corresponds to Ala^{19}-S-S-Ala^{31}) is substituted by lanthionine (Ala^{19}-S-Ala^{31}).

This thioether analogue of the cystine is found in a variety of natural products, mainly in 'lantibiotics' such as nisin, subtilin, and cinnamycin (Jung, 1991; Sahl *et al.*, 1995). The biosynthesis of the lanthionine peptides occurs ribosomally by posttranslational stereoselective *Michael*-like addition of cysteine to dehydroalanine and always results in a D-configuration at the generated asymmetric center (Sahl *et al.*, 1995). Due to the increased interest in lantibiotics in pharmaceutical research, several routes to synthesise the mentioned thioether moiety were examined. Among these are the *Michael*-type addition to dehydroalanines (Miller, 1980; Probert *et al.*, 1996), nucleophilic ring-opening of serine-ß-lactones (Shao *et al.*, 1995; Arnold *et al.*, 1998), sulfur extrusions of protected peptides (Wakamiya *et al.*, 1983), *in situ* alkylations of immobilised peptidyl cysteine residues (Mayer *et al.*, 1998), and asymmetric alkylations of cysteines with ß-iodo-alanine derivatives (Dugave and Ménez, 1997). In our hand, the last mentioned method worked best regarding practical and chemical considerations.

In native human uPA, the distance between the Cα-atoms of Cys19 and Cys31 (which are not connected by a disulfide bond) is 6.1 Å (Hansen *et al.*, 1994a, b), whereas the average distance of cysteines engaged in disulfide bridges (as in peptide cyclo^{19,3}-uPA$_{19-31}$) is about 5.2 Å. The distance between the Cα-atoms at amino acid positions 19 and 31 is further narrowed down in cyclo^{19,31}[Ala^{19}-**S**-Ala^{31}]-uPA$_{19-31}$ to about 4.8 Å. Cyclo^{19,31}[Ala^{19}-**S**-Ala^{31}]-uPA$_{19-31}$ displayed a distinct – but compared to the parental peptide cyclo^{19,3}-uPA$_{19-31}$ significantly lower – inhibitory activity with respect to interference with the uPA/uPAR-interaction (Table 1). This indicates that the cystine-bridge in the lead uPA-peptide structure could be modified by peptido-mimetica to some extent.

In previous studies (Bürgle *et al.*, 1997), we observed that linear uPA$_{19-31}$-derived peptides harboring Cys to Ser substitutions ([Ser^{19}]-uPA$_{19-31}$; [Ser^{31}]-uPA$_{19-31}$; [Ser^{19},Ser^{31}]-uPA$_{19-31}$) or even shorter peptides such as uPA$_{20-30}$ did not interact with uPAR. We now have extended these analyses by synthesising and testing cyclo^{20,30}uPA$_{20-30}$ and observed that this peptide – similar to the above mentioned peptides – does not display any uPAR binding capacity (Table 1).

Cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ Effectively Displaces uPAR-Bound uPA from the Cell Surface

As shown in Figure 1, cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ efficiently blocks binding of uPA to uPAR, when both the peptide and the protease are added simultaneously to uPAR-exposing human tumor cells. We also wanted to know whether cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ will displace uPA from cell surface-uPAR. Therefore, we at first incubated promyeloid U937 cells (which were pre-treated under mild acid conditions to dissociate endogenous receptor-bound uPA) with FITC-conjugated HMW-uPA to allow binding to uPAR and, subsequently, added increasing amounts of cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ to the cells. As shown in Figure 3, cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ – as well as unlabeled HMW-uPA – efficiently reduced cell-associated fluorescence in a dose-dependent manner. A cyclic control uPA-peptide, which does not interact with uPAR (cyclo^{21,29}[D-Cys^{21}, Hph^{25},Cys^{29}]-uPA$_{21-30}$; Table 1) had no effect on dissociation.

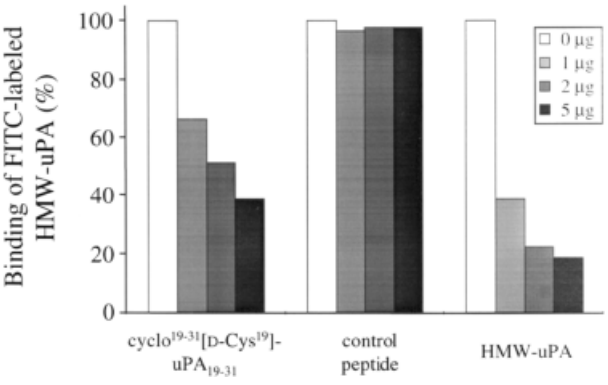

Fig. 3 Displacement of uPAR-Bound, Fluorescently Labelled HMW-uPA by Cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$.
PMA-stimulated human U937 cells (2.5 × 10^5 cells per 250 µl) were pre-incubated with 25 ng FITC-HMW-uPA, then increasing concentrations of synthetic cyclic uPA-derived peptides or unlabeled HMW-uPA were added (1 h, 37 °C) and, subsequently, the cell-associated fluorescence was determined. Whereas cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ and HMW-uPA efficiently displace FITC-HMW-uPA from the cell surface, a uPA-related peptide with no uPAR-binding activity (cyclo^{21,29} [D-Cys^{21},Hph^{25},Cys^{29}]-uPA$_{21-30}$) has no effect. The result is representative of three independently performed experiments.

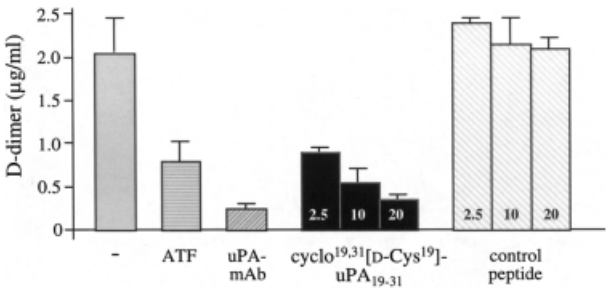

Fig. 4 Inhibition of Cell Surface-Associated uPA-Mediated Plasminogen Activation and Fibrin Degradation by Cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$.
uPA-producing ovarian cancer cells (OV-MZ-6) were seeded on fibrin gels in the presence of plasminogen (2 µg/ml medium) together with increasing amounts of synthetic peptides (2.5, 10 and 20 µM, respectively). As controls, either ATF (2 µg/ml medium) or mAb # 394 directed against human uPA (10 µg/ml medium) were added instead of the synthetic peptides. After 4 h, cell culture supernatants were analysed for the presence of D-dimer as the result of fibrin degradation by plasmin. Whereas cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ inhibits fibrin degradation in a dose-dependent manner, a uPA-related peptide with no uPAR-binding activity (cyclo^{21,29}[Cys^{21},Ala^{24},Cys^{29}]-uPA$_{21-30}$) has no effect. Both ATF and mAb # 394 also significantly inhibit D-dimer formation. Mean values (± SD) are given for measurements performed in duplicate.

Cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ Inhibits uPA-Mediated Plasminogen Activation and Fibrin Degradation

To test whether cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ does inhibit uPA-mediated plasminogen activation, we seeded uPA-producing ovarian cancer cells (OV-MZ-6) in the presence of plasminogen onto fibrin clots. After 4 h of incubation, supernatants containing fibrin degradation products (including D-dimer), produced *via* proteolytic action by plasmin, were collected. As can be seen from Figure 4, cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$ efficiently reduces the generation of D-dimer in a dose-dependent manner. A cyclic uPA-related control peptide, which does not interact with uPAR (cyclo^{21,29}[Cys^{21},Ala^{24},Cys^{29}]-uPA$_{21-30}$; Table 1) had no effect. As controls, the amino-terminal fragment of uPA (ATF), which also blocks binding of endogenously produced uPA to the cell surface, or a monoclonal antibody directed against human uPA, which inhibits the proteolytic activity of uPA, was employed. In both cases, a significant reduction of uPA-mediated plasminogen activation and, in consequence, fibrin degradation was observed (Figure 4).

Conclusions

Due to its important role in tumor-associated processes the urokinase-type plasminogen activation system emerged as an attractive target for anti-invasive and anti-metastatic therapy. Over the past years, a variety of substances known to interfere with the expression or reactiv-

ity of uPA or uPAR at the gene or protein level were developed, including antisense oligodeoxynucleotides or RNA (Wilhelm *et al.*, 1995; Mohan *et al.*, 1999), protease inhibitors (Towle *et al.*, 1993; Sperl *et al.*, 2000), recombinant soluble uPAR (Krüger *et al.*, 2000; Lutz *et al.*, 2001), or uPA-derived antagonists of uPA/uPAR-interaction. In the latter case, mostly large therapeutic molecules were generated and tested *in vivo*. For example, it was demonstrated that a uPA_{1-137}/IgG-chimera suppressed the metastatic capacity of human PC3 prostate carcinoma cells in nude mice (Crowley *et al.*, 1993). Similarly, reduction of growth of primary human breast carcinoma in immunodeficient mice was observed by administration of a fusion protein consisting of human or murine uPA_{1-48} fused to the Fc portion of human IgG (Tressler *et al.*, 1999). A bifunctional inhibitor, consisting of uPA_{1-134} fused to domain II of the urinary trypsin inhibitor (UTI), suppressed experimental tumor invasion and metastasis by blocking uPA/uPAR-interaction *via* the amino-terminal fragment of uPA within this chimeric molecule and by inhibition of plasmin activity *via* UTI (Kobayashi *et al.*, 1998). The application of large molecules to treat cancer patients appears rather difficult and depends on sophisticated, *e. g.* viral, delivery systems. Consequently, our aim was to develop synthetic, small peptide inhibitors derived from the continuous uPAR-binding sequence of uPA (Magdolen *et al.*, 1996; Bürgle *et al.*, 1997). Using the cyclic disulfide-bridged form of the minimal uPAR-binding region as lead structure ($cyclo^{19,31}uPA_{19-31}$), we have identified $cyclo^{19,31}[D\text{-}Cys^{19}]\text{-}uPA_{19-31}$ as – to our knowledge – the most potent competitive uPA-antagonist presently known that displays only a 20- to 40-fold lower uPAR-binding capacity as compared to the natural uPAR ligands uPA or ATF. $Cyclo^{19,31}[D\text{-}Cys^{19}]\text{-}uPA_{19-31}$ inhibits binding of uPA to uPAR on human tumor cells with more than 3-fold higher efficiency compared to the lead structure and at least with 10-fold higher efficiency compared to a high affinity uPAR ligand (clone 20 peptide) derived from bacteriophage display (Goodson *et al.*, 1994; Bürgle *et al.*, 1997). Furthermore, $cyclo^{19,31}[D\text{-}Cys^{19}]\text{-}uPA_{19-31}$ efficiently displaces uPAR-bound uPA from the cell surface of human cells and inhibits uPA-mediated, cell-associated plasminogen activation and fibrin degradation. We are currently synthesising a series of $cyclo^{19,31}[D\text{-}Cys^{19}]\text{-}uPA_{19-31}$-variants, in which particular amino acids are substituted by amino acids not occuring in natural proteins (*e. g.* substitution of lysine by ornithine or norleucine; tryptophan by 1-naphthyl- or 2-naphthyl-alanine, *etc*.). This strategy may lead to high affinity uPA-antagonists with increased biological availability due to higher resistance against proteolytic degradation. Recently, a synthetic non-competitive peptidic antagonist of the pro-uPA/uPAR-interaction, the Å6-peptide, has been successfully tested *in vivo*. The administration of Å6 – in a rather high concentration of 75 mg per kg per day – resulted in a significant inhibition of tumor growth and also suppressed the development of lymph node metastases in both syngeneic (rat breast cancer) and xenogeneic (human breast cancer in nude mice) tumor model systems (Guo *et al.*, 2000). Thus, high affinity competitive antagonists of uPA/uPAR-interaction, such as $cyclo^{19,31}[D\text{-}Cys^{19}]\text{-}uPA_{19-31}$ or variants thereof, may constitute potent therapeutic agents for inhibition of tumor growth and metastasis.

Materials and Methods

Solid-Phase Peptide Synthesis

Linear peptides were synthesised on a trityl polystyrene resin (PepChem, Tübingen, Germany) using an Applied Biosystems model 431A peptide synthesiser or a multiple peptide synthesiser, model SyRo II (MultiSynTech, Witten, Germany). Applying an orthogonal Fmoc-strategy (Carpino and Han, 1972; Fields and Noble, 1990), the amino acid side chains were protected with trityl- (Asn, Cys, Gln, and His), *tert*-butyloxycarbonyl- (Lys and Trp), *tert*-butyl (Asp, Glu, Ser, Thr, and Tyr), and 2,2,5,5,7,8-pentamethylchroman-6-sulfonyl- or 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg) groups. Couplings were performed twice for 1 h each at room temperature (RT) in NMP using a 3-fold excess of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBt)/Fmoc-amino acid with 2.8 equivalents of N,N-diisopropylethylamine (DIEA) in NMP. The Fmoc-group was removed by sequential treatment of the resins with an excess of 20% piperidine in DMF for 5 and 15 min, respectively. Cleavage of the peptides from the resins as well as removal of the side chain protecting groups, and work up was performed as follows (a and b, respectively):

(a) treatment with 82.5% trifluoroacetic acid (TFA)/5% phenol/2.5% ethanedithiol/5% thioanisol/5% H_2O (0 °C, 1 h; followed by RT, 1 h). In the case of 2,2,5,5,7,8-pentamethylchroman-6-sulfonyl protected Arg, peptides were treated for additional 12 h at RT. The crude peptides were precipitated in diethylether at – 30 °C, dissolved in methanol, precipitated as before, redissolved in tBuOH and then lyophilised. Tryptophan-containing peptides were treated additionally with 5% AcOH for 2 h prior to lyophilisation (White, 1992). Peptides were purified by HPLC using a Beckman Instruments System Gold or a Pharmacia Biotech Series 900 with a reversed phase C-18 column (Nucleosil 100 5-C18) or a YMC-Pack ODS/A column with H_2O and ACN plus 0.1% TFA as eluents applying an optimised gradient. Peptides cyclised *via* disulfide bridges were synthesised from the linear precursors through oxidation as described under (b).

(b) Treatment with 90% TFA/7% triisopropylsilane (TIPS)/3% H_2O (RT, 3 × 15 min). The solution was evaporated, the resulting white solid powder suspended in H_2O (0.1 – 0.3 mg/ml of the crude peptide), cooled with ice and diluted with DMSO (end concentration: 20% v/v). After 10 – 24 h the solvent was removed under reduced pressure, the resulting white solid powder dissolved in DMSO, diluted with an equal aliquot of H_2O, filtered, and purified by HPLC as described above.

Backbone cyclisation of uPA_{20-30} was performed as follows: the N-terminally deprotected peptidyl-resin was treated with 75% DCM/12.5% trifluoroethanol (TFE)/12.5% AcOH (RT, 2 h) and filtered. The resin was washed twice with DCM. The pooled solutions were evaporated in the presence of toluene and the resulting oil lyophilised from dioxane to yield a white powder. The linear peptide was dissolved in dry DCM/DMF (7:3 v/v, 0.4 mM). 1,3-diisopropylcarbodiimide (DIC), N-methylmorpholine (NMM), and 1-hydroxy-7-azabenzotriazole (HOAt) (1, 4, 2 equivalents,

respectively) were added at 0 °C under vigorous stirring. The solution was kept unstirred at 4 °C for 24 h when the completion of the reaction was indicated by HPLC analysis. The solvents were evaporated, the resulting oil dissolved in DCM, washed with H_2O, dried over Na_2SO_4 and the solvent then removed under reduced pressure. After lyophilisation from dioxane, side-chain deprotection was achieved as described above (method b), followed by HPLC purification.

Synthesis of cyclo[19,31][Ala[19]-S-Ala[31]]-uPA$_{19-31}$: the lanthionine building block (*R*)-benzyl-N-triphenylmethylalanyl-(*R*)-methyl-N-benzyloxycarbonylalanylsulfide was prepared from N-benzyloxycarbonyl-L-cysteinemethylester and N-triphenylmethyl-3-iodo-L-alaninebenzyl-ester according to a procedure published by Dugave and Ménez (1997). The suitable orthogonally protected (*R*)-benzyl-N-fluorenylmethyloxycarbonylalanyl-(*R*)-methyl-N-benzyloxycarbonyl-alanylsulfide was prepared by deprotection (1.2 eq. LiOH in H_2O/dioxane at pH 8–9, followed by treatment with 50% TFA/DCM) and reprotection with 1.1 eq. N-(9-fluorenylmethyloxycarbonyl)oxysuccinimide (Fmoc-ONSu)/DIEA, followed by flash chromatography over silica gel with an optimised hexane/ethyl acetate gradient including AcOH concentrations ranging from 0 and 10%. This lanthionine derivative was coupled to H-Val-Ser(tBu)-Asn(Trt)-Lys(Boc)-Tyr(tBu)-TCP as described above, followed by Fmoc deprotection, coupling of Fmoc-Phe-Ser(tBu)-Asn(Trt)-Ile-His(Trt)-Trp(Boc)-OH with 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/HOAt/*sym*-collidine (1, 1, 10 eq., respectively) and Fmoc deprotection. The resulting peptide was cleaved from the resin, cyclised and side-chain deprotected as described for the cyclo[20,30]uPA$_{20-30}$. The resulting peptide cyclo[19,31][L-Ala19(Z)-*S*-L-Ala31(OBn)]-uPA$_{19-31}$ was purified by HPLC and completely deprotected by treatment with 80% TFA, 10% trifluoromethanesulfonic acid (TFMS), 5% TIPS and 5% *m*-cresole (RT, 30 min). Preparative HPLC followed by lyophilisation from tBuOH yielded the desired peptide as a white solid.

Mass spectrometric and amino acid analysis for characterisation and quantitation of the peptide concentration was performed as described previously (Bürgle *et al.*, 1997).

Flow Cytofluorometry

The capacity of synthetic peptides to inhibit uPA/uPAR-interaction was assessed by FACS analysis with the FACScan flow cytofluorometer (Becton-Dickinson, Heidelberg, Germany; low power argon laser excitation at 488 nm) as described previously (Chucholowski *et al.*, 1992; Magdolen *et al.*, 1996; Bürgle *et al.*, 1997). Briefly, human U937 cells - stimulated with phorbol-12-myristate-13-acetate (PMA, 1 mM) for 48 h - were treated with 50 mM glycine-HCl, 0.1 M NaCl, pH 3.0, to dissociate endogenous receptor-bound uPA (1 min, RT). Subsequently, the acidic buffer was neutralised by an appropriate volume of 0.5 M HEPES/100 mM NaCl, pH 7.5. The cells were washed, centrifuged and resuspended in PBS/0.1% BSA (adjusted to 10^6 cells/ml). Subsequently, the cells were incubated simultaneously with 16 ng FITC-conjugated pro-uPA and varying amounts of the respective synthetic peptides in question (45 min, RT). Alternatively, cells were pre-incubated with 25 ng FITC-labeled HMW-uPA (15 min), before varying amounts of the respective synthetic peptides or unlabeled HMW-uPA were added. In these displacement studies, the fluorescence mean channel was determined after 1 h incubation at 37 °C.

Fibrin Matrix Degradation Assay

Cell-associated plasminogen activation was assessed using a fibrin degradation test as described previously (Reuning *et al.*, 1995). Briefly, fibrin gels were prepared in 24-well cell culture plates by incubating 200 µl of 50 mg/ml plasminogen-depleted fibrinogen (Calbiochem, Frankfurt, Germany), 50 µl of 150 mM $CaCl_2$, and 50 µl of 60 U/ml thrombin (Novagen, Schwalbach, Germany) for 1 h at 37°C. uPA-producing ovarian cancer OV-MZ-6 cells (Möbus *et al.*, 1992; Fischer *et al.*, 1998) were seeded on top of the fibrin gel (4×10^4 cells/well) in the presence of plasminogen (2 µg/ml medium) and varying amounts of the respective synthetic uPA-peptides. As controls, the inhibitory monoclonal antibody against human uPA B-chain, mAb # 394 (10 µg/ml), or the amino-terminal fragment of uPA, ATF (2 µg/ml) (both from American Diagnostica, Pfungstadt, Germany) were used. After 4 h of incubation at 37°C, cell culture supernatants were collected and analysed for the generation of fibrin degradation products (including D-dimer) by ELISA (D-dimer test Gold EIA kit #802, American Diagnostica).

Acknowledgements

The excellent technical assistance of C. Schnelldorfer, S. Creutzburg, and D. Helmecke is gratefully acknowledged. Part of this work was supported by grants of the Sonderforschungsbereich 469 (A4, B3), the Sonderforschungsbereich 456 (B9), the Graduiertenkolleg 333 of the Deutsche Forschungsgemeinschaft (DFG), and Roche Diagnostics, Penzberg. Christoph Riemer gratefully acknowledges a graduate fellowship of the Hanns-Seidel-Stiftung e.V.

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Received March 22, 2001; accepted May 7, 2001