High-affinity urokinase-derived cyclic peptides inhibiting urokinase/urokinase receptor-interaction: effects on tumor growth and spread

Sumito Sato[a], Charlotte Kopitz[b], Wolfgang A. Schmalix[c], Bernd Muehlenweg[c], Horst Kessler[d], Manfred Schmitt[a], Achim Krüger[b], Viktor Magdolen[a,*]

[a] *Klinische Forschergruppe der Frauenklinik, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, D-81675 München, Germany*
[b] *Institut für Experimentelle Onkologie und Therapieforschung der Technischen Universität München, München, Germany*
[c] *Wilex AG, München, Germany*
[d] *Institut für Organische Chemie und Biochemie, Technische Universität München, München, Germany*

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Abstract **Urokinase-type plasminogen activator (uPA) binds with high affinity to its specific cell surface receptor (uPAR) (CD87) via a well-defined sequence within the N-terminal region of uPA (uPA_{19-31}). Since this uPA/uPAR-interaction plays a significant role in tumor cell invasion and metastasis, it has become an attractive therapeutic target. Two small peptidic cyclic competitive antagonists of uPA/uPAR-interaction have been developed, based on the uPAR binding site in uPA: WX-360 (cyclo^{21,29}[D-Cys21]-uPA_{21-30}[S21C;H29C]) and its norleucine (Nle) derivative WX-360-Nle (cyclo^{21,29}[D-Cys21]-uPA_{21-30}[S21C;K23Nle;H29C]). These peptides display an only five to 10-fold lower affinity to uPAR as compared to the naturally occurring uPAR-ligand uPA. In this study, WX-360 and WX-360-Nle were tested in nude mice for their potency to inhibit tumor growth and intraperitoneal spread of *lacZ*-tagged human ovarian cancer cells. Intraperitoneal administration of either cyclic peptide (20 mg peptide/kg; 1× daily for 37 days) into the tumor-bearing nude mice resulted in a significant reduction of tumor weight and spread within the peritoneum as compared to the untreated control group. This is the first report demonstrating effective reduction of tumor growth and spread of human ovarian cancer cells in vivo by small synthetic uPA-derived cyclic peptides competitively interfering with uPA/uPAR-interaction. Thus, both WX-360 and WX-360-Nle are promising novel compounds to reduce dissemination of human ovarian carcinoma.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Urokinase-type plasminogen activator; Competitive uPA-derived peptide antagonists; Cancer

1. Introduction

The cellular plasminogen activation system consisting of the serine protease plasmin, urokinase-type plasminogen activator (uPA), its specific receptor (uPAR) (CD87) and the two inhibitors PAI-1 and PAI-2 plays a significant role in tumor-associated processes such as cell proliferation, migration, invasion and metastasis [1–5]. Binding of (pro)-uPA to its cell surface receptor not only generates a pericellular proteolytic system, furthermore, surface-associated feedback-activation of pro-uPA by plasmin results in potentiation of proteolytic activity compared to activation in solution [6]. In addition to this, pro-forms of other proteolytic enzymes such as matrix metalloproteinases are activated by plasmin as well, allowing tumor cells to degrade the surrounding extracellular matrix. This matrix degradation facilitates cell migration and enables tumor cells to detach from the primary tumor and to spread to distant loci in the body [1,2,5].

uPA binds with high affinity to its cell surface receptor uPAR (CD87), via a binding site within the N-terminal region of the molecule. Previously, we have determined the minimal binding region spanning amino acids 19–31 of uPA and designed a synthetic cyclic uPA-derived peptide, cyclo-$^{19,31}uPA_{19-31}$, serving as a lead structure for the development of small uPA-derived competitive peptide antagonists to interfere with uPA/uPAR-interaction [7]. Initially, we performed a systematic D-amino acid scan of uPA_{19-31}, in which each of the 13 L-amino acids of the molecule was individually substituted by the corresponding D-amino acid. This resulted in the selection of cyclo^{19,31}[D-Cys^{19}]-uPA_{19-31} (WX-307), a potent inhibitor of uPA/uPAR-interaction ($IC_{50} \approx 200$ nM), displaying only a 20–40-fold lower binding capacity as compared to the naturally occurring uPAR-ligand uPA [8]. Reduction of the ring-size and subsequent D-amino acid scanning led to cyclo^{21,29}[D-Cys21]-uPA_{21-30}[S21C;H29C] (WX-360), displaying a slightly improved binding to uPAR ($IC_{50} \approx 40$ nM) as compared to WX-307 [9,10]. In order to potentially increase its in vivo stability against proteolytic degradation, the lysine residue of WX-360 was replaced by norleucine, yielding WX-360-Nle (cyclo^{21,29}[D-Cys21]-uPA_{21-30}[S21C;K23Nle;H29C]). This cyclic peptide was found to exert similar binding affinities ($IC_{50} \approx 70$ nM) than WX-360 [11].

In previous experiments, we have targeted uPAR-expressing tumor cells in in vivo models by gene transfer of a recombinant soluble form of uPAR (suPAR) as a scavenger for uPA into human ovarian and breast cancer cells, resulting in high-level synthesis of this protein [12,13]. In both tumor models, we observed profound inhibitory effects of suPAR on primary tumor growth, tumor spread, or experimental metastasis. In the present study, we investigated whether inhibition of uPA/uPAR-interaction by small synthetic uPA-derived peptides represents an alternative strategy to affect primary tumor growth and metastasis. For this, we inoculated human ovar-

*Corresponding author. Fax: (49)-89-4140 7410.
E-mail address: viktor@magdolen.de (V. Magdolen).

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ian cancer cells OV-MZ-6#8-BAG into the peritoneum of nude mice, treated the mice with either of the two peptides WX-360 or WX-360-Nle and analyzed the effects of this treatment on primary tumor growth and spread in vivo.

2. Materials and methods

2.1. Peptides

Two synthetic cyclic peptides have been developed based on the lead structure WX-307 (cyclo^{19,31}[D-Cys^{19}]-uPA$_{19-31}$) [8], WX-360 (cyclo^{21,29}[D-Cys^{21}]-uPA$_{21-30}$[S21C;H29C]) and its derivative WX-360-Nle (cyclo^{21,29}[D-Cys^{21}]-uPA$_{21-30}$[S21C;K23Nle;H29C]), in which lysine 23 is substituted by norleucine [9–11].

2.2. Cell lines

OV-MZ-6#8 is a clonal cell line derived from the parental cell line OV-MZ-6, which was originally established from a patient with advanced serous cystadenocarcinoma of the ovary [14]. OV-MZ-6#8 cells were transfected with the bacterial *lacZ* gene which codes for β-galactosidase and, thus, the resulting OV-MZ-6#8-BAG cells can be stained with the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) in order to follow spreading of tumors in in vivo models [15]. As tested by ELISA, the *lacZ*-tagged cell line, as its parent cell line, expresses both uPAR and uPA. OV-MZ-6#8-BAG cells were cultured as previously described [16].

2.3. Tumor model

Pathogen-free female athymic (nu/nu) mice (4–6 weeks old), were obtained from Charles River Laboratories (Sulzfeld, Germany). 3×10^7 OV-MZ-6#8-BAG cells were suspended in 500 μl PBS and inoculated into the peritoneal cavity of nude mice. These mice were divided into three groups, which either received WX-360, WX-360-Nle (20 mg/kg/day, respectively), or vehicle only (5% mannitol, 0.6% DMSO) in a blinded manner. The peptides or the vehicle only were injected intraperitoneally once per day for 37 days (treatment started 1 day post-inoculation). At the end of the study, the mice were sacrificed, all intraperitoneal organs removed and stained with X-Gal [17] in order to facilitate identification of the spreading tumor cells. Tumor weight and total situs weight were measured for evaluation of the tumor mass as described previously [13]. Finally, the blinded code was uncovered and statistical analysis performed.

2.4. Statistical analysis

Significant differences in tumor weight between the groups were calculated using Student's *t*-test owing to normal distribution of the data. For data on tumor weight over total situs weight, the normal distribution test was not applicable; therefore, a Kruskal–Wallis one-way analysis and Dunn's multiple comparison test were performed to investigate for differences between the three groups. A significance level of ≤ 0.05 was considered statistically significant.

3. Results and discussion

Binding of uPA to uPAR supports tumor cell proliferation, invasion and metastasis. To inhibit this interaction, synthetic cyclic peptides, derived from the uPAR-binding sequences within the N-terminal region of uPA (uPA$_{19-31}$), were applied

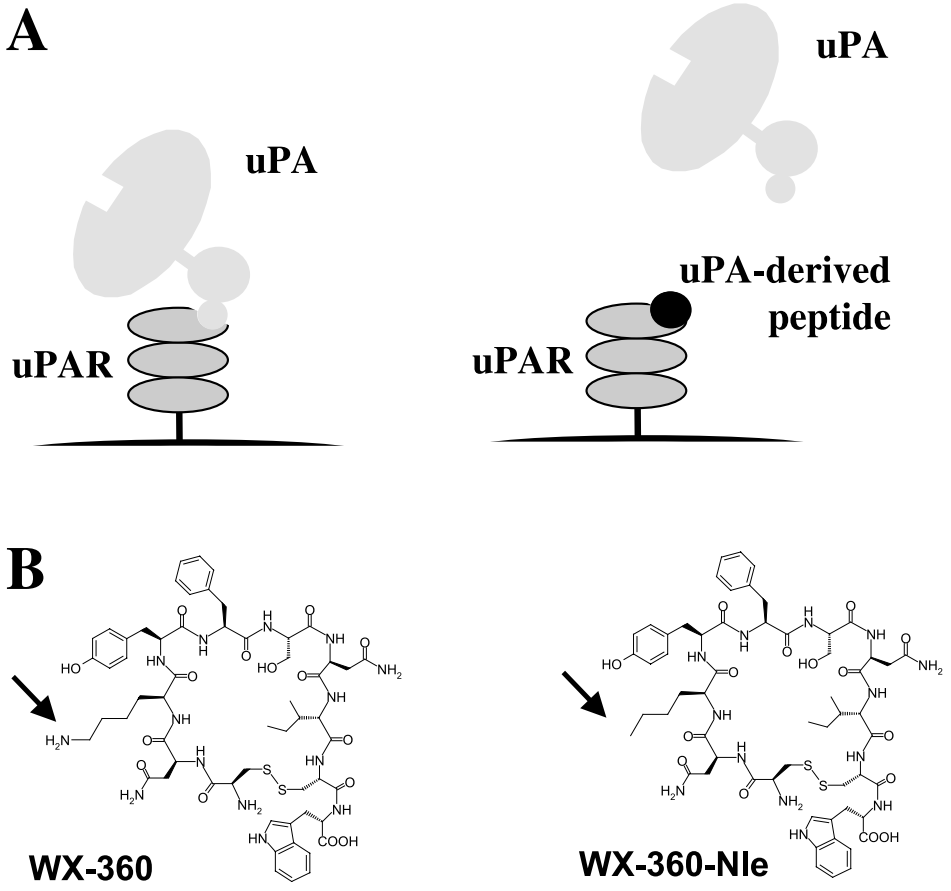

Fig. 1. Competitive antagonists of uPA/uPAR-interaction. A: Binding of uPA to the tumor cell surface via its cell membrane receptor uPAR facilitates tumor cell proliferation, invasion and metastasis [5,8]. uPA binds to uPAR by the continuous peptide sequence uPA$_{19-31}$. Based on this sequence, small cyclic peptidic competitive antagonists of uPA/uPAR-interaction such as WX-360 and WX-360-Nle have been developed which efficiently block binding of uPA to uPAR [8–11]. B: Structures of WX-360 (cyclo^{21,29}[D-Cys21]-uPA$_{21-30}$[S21C;H29C]) and its norleucine derivative WX-360-Nle (cyclo^{21,29}[D-Cys21]-uPA$_{21-30}$[S21C;K23Nle;H29C]). The arrows point to the side chain of lysine (K^{23}) in WX-360 and norleucine (Nle^{23}) in WX-360-Nle, respectively.

and tested whether such competitive antagonists of uPA/uPAR-interaction reduce tumor burden of a human ovarian carcinoma in nude mice (Fig. 1).

After intraperitoneal inoculation of *lacZ*-tagged OV-MZ-6#8-BAG cells, mice were treated once per day in a blinded manner either, with synthetic cyclic peptides (WX-360 or WX-360-Nle; 20 mg/kg/day) or vehicle until autopsy. After 37 days, mice were sacrificed and all intraperitoneal organs stained with X-Gal. To describe any therapeutic effect of the different peptides, tumor weight over total situs weight was assessed. In the control group, typically large tumor nodules on the left peritoneum and beneath the liver (near the site of tumor cell inoculation), as well as an abundant tumor spread across the peritoneal muscle layer, mesentery and the diaphragm was observed. By treating the mice with WX-360 and WX-360-Nle, fewer and smaller tumor nodules were present than in the control animals. Often (three of 15 mice in both the WX-360- and WX-360-Nle-treated groups versus one of 16 mice in the control group), a larger nodular tumor mass was not even detectable (Fig. 2). The statistical analysis of the ratios of tumor weight over total situs weight proved that treatment of mice carrying human ovarian cancer cells with synthetic uPA-derived cyclic peptides resulted in a significant reduction of tumor burden (WX-360 versus control, $P = 0.0002$; WX-360-Nle versus control, $P = 0.023$) if compared to the untreated control group (Fig. 3A). A trend towards a lower tumor weight in the WX-360 group was observed. Treatment of the nude mice by either peptide did not result in serious side effects during the time of treatment. However, after 1 week, in some mice treated with WX-360-Nle, an obvious excitability was seen. In addition to the tumor weight, we also measured organ weights of the mice to assess organotoxic side effects of the peptides. No significant differences were observed between all three groups (Fig. 3B), suggesting that treatment with the peptides did not affect organ growth of the mice.

A considerable number of studies have already shown that interference with the plasminogen activation system leads to reduction of tumor invasion and metastasis. Therefore, this system with its increased activity in tumor cells represents an attractive target to attack tumor invasion and metastasis [18]. Various strategies have been employed to reduce the expres-

Fig. 2. Tumor spread of OV-MZ-6#8-BAG cells within the peritoneum. X-Gal-stained organs of mice treated with intraperitoneal injection of vehicle only (5% mannitol, 0.6% DMSO) (A), WX-360 (B), or WX-360-Nle (C) (37 days; treatment with 20 mg peptide/day/kg; a single injection per day). At the end of the experiment, in the control group typically a large tumor nodule near the peritoneal inoculation site at the left, as well as an abundant tumor spread across the peritoneal muscle layer, the mesentery and the diaphragm was observed (A). In the groups of mice treated with the uPA antagonists, fewer and smaller tumor nodules and strong reduction in tumor cell dissemination was observed (B,C). Three representative examples of each group are depicted.

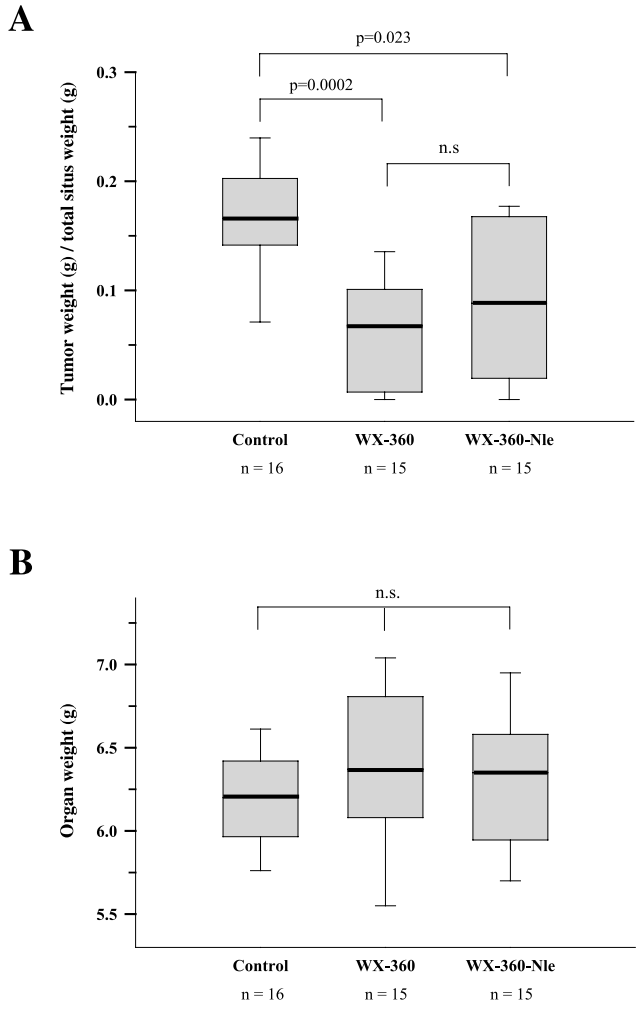

Fig. 3. Effect of synthetic cyclic competitive uPA-derived peptide antagonists on primary tumor growth and spread. After inoculation of OV-MZ-6#8-BAG cells, nude mice were treated with either WX-360, WX-360-Nle (in each case 20 mg/kg/day), or vehicle for 37 days (single intraperitoneal injection per day). The groups were compared to each other with respect to tumor burden (given as the ratio of tumor weight over total situs weight) (A) and organ weight (B). The box plot indicates the 25th and 75th percentiles, the vertical bars mark the 10th and 90th percentiles, respectively. The median value is indicated by a bold bar. n.s., not significant.

sion of uPA or uPAR or to impair the proteolytic activity, including the use of synthetic active site inhibitors of uPA as well as antisense oligodeoxynucleotides or RNA directed against uPA and uPAR mRNA [3,4,18]. Other concepts are based on competitive inhibitors of uPA/uPAR-interaction, which, in fact, also strongly affect experimental tumor invasion and metastasis [18,19]. For example, high-level synthesis of a suPAR in the vicinity of mammary or ovarian tumor cells significantly reduced tumor burden and spread or experimental metastasis in xenogenic mouse models due to inhibition of uPA-binding to tumor cell-associated uPAR [12,13]. uPA-derived therapeutic molecules, harboring the uPAR binding site have also been used frequently in animal experiments; two different uPA/IgG chimera (containing either uPA_{1-137} or uPA_{1-48}) suppressed the metastatic capacity of human PC3 prostate carcinoma cells and B16 melanoma growth and neovascularization, respectively, in in vivo experiments [20,21]. Furthermore, bifunctional inhibitors encompassing two functionally independent domains, both directed against the uPA/plasmin system, have been successfully applied in in vivo models. A hybrid protein, consisting of the receptor-binding amino-terminal fragment of uPA (ATF), linked to the bovine pancreas trypsin inhibitor (inhibits plasmin activity also at the cell surface), strongly inhibited neointima formation and restenosis [22]. In another set of experiments, a bifunctional inhibitor encompassing ATF and domain II of the serine protease inhibitor UTI reduced experimental tumor invasion and metastasis of ovarian carcinoma and choriocarcinoma cells [23]. All of these animal experiments provide 'proof of principle' for the use of antagonists of uPA/uPAR-interaction in tumor inhibition. However, the application of large recombinant proteins for treatment of patients appears rather difficult and depends on sophisticated, e.g. viral, delivery systems [18]. Therefore, others and we have concentrated on the development of small, synthetic competitive or non-competitive peptide antagonists [7–11,24–27].

Previously, we have demonstrated in in vitro assays that synthetic uPA-derived peptides such as $cyclo^{19,31}$-uPA_{19-31} or its [D-Cys19]-derivative WX-307 efficiently inhibit binding of uPA to cell surface-associated uPAR on human tumor cells [7]. These peptides not only block binding of uPA to uPAR but are also capable to displace uPAR-bound uPA from the cell surface and to inhibit uPA-mediated, tumor cell-associated plasminogen activation and fibrin degradation [7,8]. Reduction of the ring-size and D-amino acid scanning led to WX-360 ($cyclo^{21,29}$[D-Cys^{21}]-uPA_{21-30}[S21C;H29C]), displaying a five-fold higher affinity towards uPAR than WX-307 ($IC_{50} \approx 40$ nM versus ≈ 200 nM) [9,10]. Furthermore, as WX-360 harbors a lysine residue (K^{23}) and, thus, a target site for serine proteases such as plasmin, K^{23} was replaced by the non-protein amino acid norleucine (WX-360-Nle). This derivative still displays high binding affinity ($IC_{50} \approx 70$ nM). Stability testing showed that both WX-360 and WX-360-Nle, respectively, were highly resistant to proteolytic degradation in human and rodent plasma or serum whereas other uPA-derived peptides lacking a non-natural D-amino acid (such as $cyclo^{19,31}$-uPA_{16-32}) were significantly less stable: after 1 h incubation at 37°C, peptide $cyclo^{19,31}$-uPA_{16-32} was completely degraded, whereas WX-360 and WX-360-Nle, respectively, were stable over a period of 24 h. After incubation with high amounts of plasmin (5 μg of peptide were incubated with 0.01 U [≈4 μg] of plasmin at 37°C), however, WX-360 was degraded, whereas WX-360-Nle was completely resistant to proteolysis [11].

In the present study, we demonstrate that treatment of tumor-bearing mice with small, synthetic, cyclic, competitive uPAR-binding site-derived uPA antagonists results in a highly significant reduction of tumor burden and also dissemination in the peritoneal cavity in vivo. In the nude mouse model, we used human ovarian cancer cells, which typically induce a large primary tumor and abundant intraperitoneal metastases [13]. The results are in line with those of a previous study, in which administration of a synthetic non-competitive peptidic antagonist of uPA/uPAR interaction, the so-called Å6-peptide (75 mg per kg per day; two intraperitoneal injections per day), inhibited tumor growth significantly and suppressed the development of lymph node metastases in several breast cancer models [26].

Substitution of K^{23} in WX-360 with the non-protein amino acid norleucine did not significantly alter the biological effi-

cacy of the peptide, although WX-360-Nle displays a further increased proteolytic stability as compared to its parent peptide WX-360. Compared to WX-360, WX-360-Nle is distinctly less soluble in aqueous solutions, because the exchange of the positively charged lysine side chain by the aliphatic norleucine side chain results in a zero net charge of the peptide. Interestingly, some mice developed an excitable behavior during the treatment with WX-360-Nle, which was characterized by heavy struggle against injections and an aggressive behavior beginning after the first week of administration. It is tempting to speculate that the lower solubility of WX-360-Nle may give rise to intraperitoneal peptidic precipitates. The behavioral changes in the mice receiving WX-360-Nle may be related to a poor tolerability of such intraperitoneal precipitates.

In conclusion, we have demonstrated that small synthetic cyclic competitive uPA peptide-antagonists can effectively reduce tumor growth and spread of human ovarian cancer cells in a mouse tumor model. These results strongly suggest that the peptides WX-360 and WX-360-Nle are sufficiently stable within the peritoneal cavity to efficiently interfere with uPA/uPAR-interaction on the tumor cells. Since ovarian cancer is a disease, that spreads throughout the abdominal cavity, putative new uPAR-directed drugs could be administered intraperitoneally. Thus, WX-360 and WX-360-Nle represent promising new compounds to reduce tumor burden and dissemination of human ovarian carcinomas.

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