Targeting Tumor Cell Invasion and Dissemination In Vivo by an Aptamer That Inhibits Urokinase-type Plasminogen Activator through a Novel Multifunctional Mechanism

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Abstract

Data accumulated over the latest two decades have established that the serine protease urokinase-type plasminogen activator (uPA) is a potential therapeutic target in cancer. When designing inhibitors of the proteolytic activity of serine proteases, obtaining sufficient specificity is problematic, because the topology of the proteases’ active sites are highly similar. In an effort to generate highly specific uPA inhibitors with new inhibitory modalities, we isolated uPA-binding RNA aptamers by screening a library of 35 nucleotides long 2'-fluoro-pyrimidine RNA molecules using a version of human pro-uPA lacking the epidermal growth factor-like and kringle domains as bait. One pro-uPA–binding aptamer sequence, referred to as upanap-126, proved to be highly specific for human uPA. Upanap-126 delayed the proteolytic conversion of human pro-uPA to active uPA, but did not inhibit plasminogen activation catalyzed by two-chain uPA. The aptamer also inhibited the binding of pro-uPA to uPAR and the binding of vitronectin to the preformed pro-uPA/uPAR complex, both in cell-free systems and on cell surfaces. Furthermore, upanap-126 inhibited human tumor cell invasion in vitro in the Matrigel assay and in vivo in the chick embryo assay of cell escape from microtumors. Finally, upanap-126 significantly reduced the levels of tumor cell intravasation and dissemination in the chick embryo model of spontaneous metastasis. Together, our findings show that usage of upanap-126 represents a novel multifunctional mechanistic modality for inhibition of uPA-dependent processes involved in tumor cell spread. Mol Cancer Res; 1–12. © 2012 AACR.

Introduction

The urokinase-type plasminogen activator (uPA) is an extracellular serine protease playing a central role in tissue remodeling events in normal physiology and in pathophysiology, including cancer invasion and metastasis. uPA catalyzes the conversion of the extracellular zymogen plasminogen to the active matrix-degrading protease plasmin. uPA itself is secreted as a zymogen, pro-uPA, and is activated by the cleavage of a single peptide-bond between residues Lys158 and Ile159. The cleavage can be catalyzed by several proteases, including plasmin (1). uPA consists of an EGF domain (residues 5–46) and a kringle domain (residues 50–131), which together comprise the amino-terminal fragment (ATF), followed by an interdomain linker (residues 132–147), and the catalytic domain (residues 148–411). uPA is recruited to cell surfaces via high affinity interaction of the EGF domain with the glycosylphosphatidylinositol-anchored uPA receptor, uPAR. As plasminogen also accumulates on cell surfaces, via lysine binding sites in its kringle domains, the cell membranes provide an efficient template for plasminogen activation. Furthermore, binding of uPA to uPAR creates a proadhesive effect by increasing the binding efficiency of uPAR to the extracellular matrix protein vitronectin (2). Ligation of uPAR with vitronectin has in turn been found to initiate cytoskeletal rearrangements (3).

Several lines of experimental evidence link uPA with the invasive capacity of malignant tumors: uPA levels positively correlate with the invasive potential of different cell lines; prevention of uPA from binding to uPAR decreases invasion; antibodies and antisense oligonucleotides to uPA reduce metastasis of tumor cells in both mice and chick embryo models; mice lacking either the uPA or the plasminogen gene show lower incidence of metastasis than wild-type mice (ref. 4; for reviews, see refs. 1, 5–7). The basic hypothesis is, that uPA secreted by cancer cells or stromal cells catalyses generation of plasmin, which in turn facilitates cancer cell invasion into surrounding tissues directly, by degrading the basement membrane and ECM proteins or indirectly, by activating other proenzymes such as promatrix metalloproteases. However, uPA-promoted tumor spread also involves other activities, including adhesive interactions of the uPA/uPAR complex with vitronectin (8), cellular signaling...
mediated by uPA binding to uPAR (9, 10), and uPA-induced generation of plasmin, which then mediates activation of growth factors (11) or cleaves cell surface survival molecules (12, 13).

Almost 25 years ago, it was shown that breast cancer patients with high levels of uPA in their tumors have a significantly shorter disease-free interval and shortened overall survival than patients having low levels of tumor uPA (14, 15). In the case of breast cancer, so-called level I evidence studies supported the independent prognostic value of combined measurements of uPA and PAI-1 (16, 17). On the basis of such studies, the American Society of Clinical Oncology and the German Breast Cancer Society recommend the use of uPA and PAI-1 as prognostic markers in breast cancer patients (18, 19). High levels of uPA also correlate with aggressive disease in most other types of cancers (20). Measurements of uPAR levels are also of prognostic value (21).

As uPA represents a potential therapeutic target in cancer, a number of small molecule uPA inhibitors have been developed (5, 22). However, most of these inhibitors lack sufficiently documented specificity. To generate highly specific uPA inhibitors, we have turned to nucleic acid aptamers, which consist of RNA or single-stranded DNA. They are selected during an iterative in vitro process named "Systematic Evolution of Ligands by Exponential Enrichment" (SELEX; refs. 23, 24). The selection of oligonucleotides, which bind specifically and with high affinity to a target, is based on the ability of these molecules to fold into specific 3-dimensional structures. In the SELEX procedure, a library of 10^14 to 10^15 different oligonucleotide sequences are subjected to a target and binding sequences enriched over 6 to 18 iterative cycles. Several properties of aptamers make them suitable as drug candidates. They display dissociation constants (K_d) in the low to subnanomolar range. They are reported to be virtually nontoxic and nonimmunogenic. They can be modified chemically to obtain desired blood clearance and biodistribution patterns. One aptamer against VEGF has been approved by U.S. Food and Drug Administration for treatment of age-related macular degeneration and several others are in clinical trials primarily for use as anticoagulants (25–27).

Here we describe an RNA aptamer, upanap-126 (uPA nucleic acid aptamer-126), that has been selected with a truncated version of human pro-uPA (residues 134–411) as a bait. The aptamer inhibits plasmin-mediated pro-uPA activation, competes the binding of pro-uPA to uPAR and prevents the binding of pro-uPA/uPAR complex to vitronectin. Upanap-126 was tested in several cell function assays in vitro and the potency of the aptamer was shown in vitro in tumor invasion and intravasation model systems.

Materials and Methods

Proteins and reagents

Human soluble uPAR lacking the glycolipid anchor (residues 1–283) and a human pro-uPA variant (residues 134–411, S356A) lacking the EGF and kringle domains were produced in Drosophila cells as described for uPA lacking the EGF domain (residues 45–411; ref. 28). The ATF (residues 1–135) of uPA was prepared by proteolytic cleavage of active uPA (29). The EGF domain of human uPA (residues 1–48) was a kind gift from Dr. Steven Rosenberg (Chiron Corporation, Emeryville, CA; ref. 30). Recombinant human pro-uPA was generously provided by Abbott laboratories (Abbott Park). Glu-plasminogen purified from human plasma was a kind gift from Lars Sottrop-Jensen (Aarhus University, Aarhus, Denmark). Plasmid DNA encompassing the coding sequence for mutant T7 polymerase Y639F was generously donated by Dr. Rui Sousa (University of Texas Health Science Center, San Antonio, Texas). T7 polymerase Y639F was expressed in BL21 Escherichia coli cells and purified by ammonium sulphate precipitation followed by SP-sepharose chromatography. The following reagents were purchased from the indicated sources: Human uPA (Wakamoto); mouse uPA and human α2-antiplasmin (Molecular Innovations); human tPA (Genentech); H-D-Val-Leu-Lys-p-nitroanilide (S-2251) and H-D-Glu-Gly-Arg-p-nitroanilide (S-2444); Chromogenix); H-D-Val-Leu-Lys-7-amido-4-methylcoumarine (VLK-AMC, I-1390; Bachem Bioscience); aprotinin from bovine lung (Sigma-Aldrich); multimeric human vitronectin (BD Biosciences); and monoclonal antibody to mouse uPA clone H77A10 (Molecular Innovations). The following in-house produced antibodies and peptide were used: Monoclonal anti-uPA mAb-6 (31) and mAb-112 (32); monoclonal anti-uPAR mAb-R2 (33); polyclonal anti-human uPA F1609 (34) affinity purified using a human uPA-coupled Sepharose column; and mupain-1 peptide (35).

Selection of aptamers binding to the catalytic domain of uPA

SELEX was carried out as described (36) with minor modifications. The RNA library was constructed with a 35-nucleotide degenerate sequence and with 2'-fluoropyrimidines as described (37) using the primers 5'-CGGGGATCCTGAATACGACTCATAAGGGCCCAACAGACATT-3' containing the T7 promoter sequence and 5'-GATCCATGGGCACTATTTATATCAAC(N35)AATGTCGTTTGGTGCCC-3' containing the degenerate sequence. Enrichment of RNA aptamers with binding affinity toward the truncated version of human pro-uPA (residues 134–411), lacking the EGF and kringle domains and harboring the mutation S356A, was carried out by capturing the protein target on protein A-coupled Sepharose beads using the rabbit polyclonal anti-uPA antibody F1609 (34) essentially as described (37) with 12 rounds of enrichments. To obtain 2'-fluoropyrimidine–modified RNA molecules, RNA transcriptions were carried out using 2.5 mmol/L each of ATP and GTP (GE Healthcare), and 2.5 mmol/L each of 2'-F-dCTP and 2'-F-dUTP (2'-F-Y, Tri-Link Biotechnologies), and 50 μg/mL mutant T7 RNA polymerase Y639F. Selected RNA aptamer clones were transcribed from dsDNA generated by Klenow extension of primers constructed from the individual sequences. RNA
aptamers were purified on 8% denaturing polyacrylamide gels followed by micro gel filtration columns (micro Bio-spin 30 column, BioRad) exchanging the buffer to 10 mmol/L HEPES, 140 mmol/L NaCl, 2 mmol/L MgCl₂, and pH 7.4. 2′-F-Y RNA concentrations were determined by UV spectroscopy at 260 nm (1 A260 unit = 40 mg/mL).

Surface plasmon resonance analysis of aptamer binding

For surface plasmon resonance analysis, a Biacore T200 instrument (GE Healthcare) was used. Pro-uPA or ATF was captured on a CM5 chip coupled with the same polycyonal anti-uPA antibody F1609 (34), which was used for aptamer selections. Then, the selected aptamer clones were passed over the chip at 200 mmol/L for 60 seconds at 30 μL/min. Running buffer was 10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl (HEPES-buffered saline), containing 2 mmol/L MgCl₂, 0.05% tween-20, and 0.1% bovine serum albumin (BSA). Regeneration of the surface between each run was carried out using 10 mmol/L acetic acid, pH 2.7, 0.5 M NaCl for 25 seconds, 30 μL/min followed by 10 mmol/L glycine, pH 2.1 for 25 seconds, 30 μL/min. The capture level of pro-uPA and ATF did not vary significantly between running cycles indicating that the surface remained stable to the regeneration conditions. The previously described monoclonal antibodies mAb-112, binding the uPA catalytic domain (32), and mAb-6, binding the uPA ATF (38), were used as positive controls for binding.

Alternatively, a monoclonal antibody to mouse uPA, clone H77/A10, that binds in the ATF, was captured on a chip with anti-mouse-IgG, after which mouse uPA was injected to a final level of approximately 1,500 RU. Then, aptamer clones were injected over the surface at 500 mmol/L for 60 seconds at 30 μL/min, and with regeneration between each run using 10 mmol/L glycine, pH 1.7 for 180 seconds at 10 μL/min. The previously described mupain-1 peptide binding the catalytic domain of mouse uPA was used as positive control for binding (35).

In vitro enzyme assays

Coupled plasminogen activation assays with and without cells, direct pro-uPA activation assays, and pro-uPA cleavage assays analyzed by SDS-PAGE and immunoblotting were carried out essentially as described (39) in HEPES-buffered saline containing 0.1% BSA and 2 mmol/L MgCl₂. For pro-uPA activation assays, data were fit to a 4-parameter logistic nonlinear regression model yielding the IC₅₀ values for inhibition of pro-uPA activation (Eq. 1):

\[ y = \min + \frac{\max - \min}{1 + \left(\frac{x}{x_{50}}\right)^{n}} \]  

(equation 1)

For analyzing the relative amounts of single-chain pro-uPA and activated two-chain uPA generated in PC-hi/diss cell cultures, cells were grown in presence of 0.5 μmol/L upanap-126 or control RNA sequence. Following 48 hours, conditioned medium samples were collected and analyzed by reducing SDS-PAGE and immunoblotting with rabbit polyclonal anti-uPA antibody F1609 (34).

Surface plasmon resonance analysis of uPA-uPAR binding

Anti-uPAR monoclonal antibody mAb-R2 was immobilized onto a CM5 sensor chip and soluble uPAR was captured to approximately 130 RU by injecting 5 mmol/L uPAR over the surface for 120 seconds at a flow rate of 10 μL/min. Pro-uPAR, active uPA or the isolated ATF (10 mmol/L) was run in the absence or presence of aptamers (0–200 nmol/L) over the surface at a flow rate of 10 μL/min for 30 seconds. The sensor chip was regenerated with 0.1 M acetic acid, 0.5 M NaCl, pH 2.7 for 15 seconds at 30 μL/min. Data were fit to a 4-parameter logistic nonlinear regression model (Eq. 1) as described above. To ensure that the binding capacity of the sensor chip was not reduced between each cycle because of the repetitive regeneration, the binding of pro-uPA, active uPA, or ATF without aptamers was repeated in the end of each experiment. This analysis was found to give rise to the same binding levels as before the competition experiment was started.

Solid-phase assay for binding of pro-uPA-uPAR complex to vitronectin

Human multimeric vitronectin (0.5 μg/mL) was coated onto Maxisorp microtitre plates (Nunc). Upanap-126, the control RNA sequence or PAI-1 were individually added at a final concentration of 1 to 0.1 μmol/L preformed pro-uPA-uPAR complex in PBS containing 0.01% Tween 20 and 2 mmol/L MgCl₂. The mixtures were incubated for 30 minutes at room temperature, before being added to the vitronectin coated wells. The relative amounts of pro-uPA-uPAR bound to vitronectin were determined with anti-uPAR antibody mAb-R2, followed by Horseradish-peroxidase–conjugated rabbit–anti-mouse IgG (Dako).

Lamellipodia formation assay

The assay was done as described previously (28). Briefly, HEK293 cells were seeded in 24-well culture plates onto vitronectin-coated glass coverslips. The stable clone of HEK293 cells expressing a single-site mutant of human uPAR, uPARW32A, was used and cultured as described (28). At the day of experiment, new culture medium containing 20 mmol/L human pro-uPA or the isolated EGF-domain in the presence or absence of various concentrations of upanap-126 or control RNA sequence was added to the cells. After 24 hours, evaluation of lamellipodia-positive cells was carried out as described (28).

Matrigel invasion assay

Cell invasion assays in vitro and in vivo were conducted with PC-hi/diss cells (40). In Matrigel invasion assays, the upper side of membranes (8 μm pore Transwell, Fisher Scientific) was precoated with 2 μg Matrigel (BD Biosciences). Conditioned medium from chicken embryonic fibroblasts was used as a chemoattractant in the lower chamber. 1 × 10⁵ PC-hi/diss cells were plated in 100 μL of serum-free Dulbecco’s Modified Eagle’s Media atop Matrigel in the upper chamber. Upanap-126 or control RNA sequence was added to both the upper and lower chambers at a final...
concentration of 1 μmol/L, along with additionally supplemented 2 mmol/L MgCl₂. Following 48-hour incubation, the invaded PC-hi/diss cells were detached with trypsin/EDTA from the underside of the inserts, combined with nonadherent cells from the lower chamber, and counted.

**Intumesodermal microtumor model**

Escape from microtumors and invasion of PC-hi/diss cells in vivo was carried out in live chick embryos as described (41). Briefly, PC-hi/diss cells were labeled with CellTracker Green CMFDA (Molecular Probes, Invitrogen) and injected into the mesoderm layer of the chorioallantoic membrane (CAM) of 9-day-old chicken embryos. On day 2 after cell injections, developing microtumors were treated topically with 25 μL Dulbecco’s PBS (DPBS), supplemented with 5 μmol/L of upanap-126 or control RNA sequence, 5% dimethyl sulfoxide (DMSO) and 2 mmol/L MgCl₂. On day 6, embryos were injected intravenously with rhodamine-conjugated *Lens culinaris* lectin (LCA; VectorLabs) to highlight the vasculature, and portions of the CAM containing microtumors were excised and immediately imaged in the fluorescence microscope. Quantification of invasion distances was carried out as described (41). A total of 15 to 20 microtumors from 3 to 5 embryos were analyzed for each variable.

**The chick embryo model for tumor cell dissemination**

Analysis of spontaneous intravasation and dissemination of PC-hi/diss cells carried out in chick embryos was as described (40). Briefly, SPAFAS White Leghorn embryos (Charles River) were allowed to develop in a humidified incubator. After 10 days of incubation, 2.5 × 10⁶ PC-hi/diss cells were grafted through the window in the egg shell onto the CAM of each embryo. On days 2 and 4 after cell grafting, developing microtumors were treated topically with 100 μL DPBS supplemented with 2.5 μmol/L upanap-126 or control RNA sequence, 5% DMSO and 2 mmol/L MgCl₂. On day 7, primary tumors were removed and weighed, and portions of the CAM distal to the site of primary tumor development were excised and analyzed by *Alu*-qPCR to determine actual numbers of human cells in the chicken tissue, essentially as described (40).

**Table 1. Overview of enriched RNA sequences after selection against human pro-uPA.**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sequence of variable region</th>
<th>Abundance among sequenced clones, %</th>
<th>Inhibitory in activity assay⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>UUAUCGUACCUGUGUCUGUUACGAAUCUACUC</td>
<td>1.8</td>
<td>No</td>
</tr>
<tr>
<td>108</td>
<td>GGUUCUGGACCGCUGUGUCGCGAAGGAUCUAACAAU</td>
<td>1.8</td>
<td>No</td>
</tr>
<tr>
<td>126</td>
<td>CAUUGCGACGGUGUCGGGGAUAGUGCCGGAUUGU</td>
<td>50.9</td>
<td>Yes</td>
</tr>
<tr>
<td>130</td>
<td>UGCAGAUCGGCGAUCUGGCUGUUAGAUUUGACG</td>
<td>1.8</td>
<td>No</td>
</tr>
<tr>
<td>201</td>
<td>GGGCUGACCGCGUGUGAGUCGCACAUAGCAAGAC</td>
<td>3.5</td>
<td>No</td>
</tr>
<tr>
<td>226</td>
<td>UAUGAAUUGAUACCAUGCGAGGAGCGAAGUC</td>
<td>24.6</td>
<td>No</td>
</tr>
<tr>
<td>231</td>
<td>CAUGAUGCGGCGGACCGUGACCGCUCAGGACUCA</td>
<td>3.5</td>
<td>Yes</td>
</tr>
<tr>
<td>246</td>
<td>GUCUGACCGCGGCGUGUGGGAUGGCAUUGAUC</td>
<td>8.8</td>
<td>No</td>
</tr>
<tr>
<td>250</td>
<td>ACUUGCGAAGGCCAGUGUGUCGUGAAGGCAUA</td>
<td>3.5</td>
<td>No</td>
</tr>
</tbody>
</table>

⁴Unique sequences were screened for inhibitory function in a coupled plasminogen activation assay (see Fig. 1).

**Results**

**Selection of RNA aptamers targeting the serine protease domain of pro-uPA**

SELEX was conducted with the uPA catalytic domain containing part of the interdomain linker region (residues 134–411) in the inactive single-chainzymogen form and with the active site Ser356 mutated to alanine. Eighty-four clones from the pool of enriched aptamers obtained by 12 selection rounds were sequenced and found to contain 9 different RNA sequences (Table 1).

**Identification of aptamers that inhibit the plasminogen activation activity initiated by pro-uPA**

Effects of the selected RNA aptamers on plasminogen activation were assessed in an assay in which pro-uPA or active uPA was incubated with plasminogen and the amount of generated plasmin was evaluated by cleavage of a chromogenic substrate S-2251. Of the 9 sequences analyzed, 2 aptamers, namely, upanap-126 and upanap-231, were found to be inhibitory in the setting requiring pro-uPA activation for generation of plasmin (Fig. 1A). None of the aptamers inhibited in the plasminogen activation assay when active uPA was incubated together with plasminogen, indicating that the effect of the upanap-126 and upanap-231 aptamers was on pro-uPA activation rather than directly on uPA-mediated plasminogen activation (Fig. 1B). A non–uPA-binding RNA sequence had no inhibitory effects on plasmin generation and was used as a negative control (Fig. 1A and B).

In surface plasmon resonance analysis, the 2 inhibitory aptamers were found to bind full length human pro-uPA and active uPA with half maximal binding occurring at approximately 50 to 100 nmol/L for both aptamers (data not shown). Accurate Kᵦ determinations could not be made...

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because of biphasic dissociation kinetics. In the same surface plasmon resonance (SPR) format, no measurable binding of the aptamers to isolated human ATF could be shown, confirming that the binding site for the aptamers resides in the serine protease domain (SPD; data not shown). As the inhibition and binding levels of upanap-126 and upanap-231 were highly similar, upanap-126 was selected as a representative for further characterization, as this clone was the most abundant among sequenced clones after 12 rounds of selection (Table 1). As shown in Fig. 1C, the inhibitory effects of upanap-126 were dose dependent with a substantial reduction of generated plasmin activity in the range of 1 to 100 nmol/L aptamer. Importantly, upanap-126 displayed no measurable binding to mouse uPA or inhibition of plasminogen activation when catalyzed by human tissue-type plasminogen activator, tPA (data not shown).

Mechanism of inhibition of the plasminogen activation activity of uPA by upanap-126

We next evaluated the inhibitory mechanisms of upanap-126 in a more direct manner. First, its ability to inhibit pro-uPA activation was assessed. To this end, pro-uPA was incubated with plasmin for a period of time, after which further plasmamin activity was quenched by the addition of aprotinin. The amount of active uPA generated was estimated from the rate of hydrolysis of the small chromogenic uPA peptide substrate S-2444. The addition of upanap-126 inhibited pro-uPA activation with an IC50 value of 7.3 ± 0.3 nmol/L, whereas the control RNA sequence had no effect (Fig. 2A). Importantly, no effect of upanap-126 on uPA-mediated hydrolysis of S-2444 was observed (data not shown).

To directly analyze if the aptamers influenced the rate of cleavage of single-chain pro-uPA, plasmin was incubated with pro-uPA in the presence or absence of upanap-126 or control RNA sequence for various time periods, after which reaction products were visualized by reducing SDS-PAGE and immunoblotting. Upanap-126 significantly delayed the rate of plasmin-catalyzed cleavage of pro-uPA, whereas a control RNA sequence showed no effect (Fig. 2B).

Effects of upanap-126 on the binding of uPA to uPAR

We analyzed if upanap-126 could interfere with the binding of the uPA-uPAR complex to vitronectin, using an...
ELISA, in which vitronectin was coated onto the solid phase and the binding of the preformed pro-uPA-uPAR complex in the presence or absence of aptamers was analyzed. Upanap-126 decreased the binding between pro-uPA-uPAR complex and vitronectin (Fig. 3B). PAI-1 was used as a positive control for inhibition of the interaction between pro-uPA-uPAR and vitronectin, as PAI-1 binds the somatomedin B domain of vitronectin in the same position as uPAR (42). Passing upanap-126 over a SPR sensor surface with pro-uPA captured on immobilized uPAR showed that...
uponap-126 was unable to induce dissociation of the uPA-uPAR complex. In contrast, uponap-126 was observed to bind the pro-uPA-uPAR complex although with a clearly reduced affinity as compared with binding to pro-uPA captured by the polyclonal antibody F1609 (data not shown). These observations confirm that uponap-126 binds the preformed pro-uPA-uPAR complex directly and prevents the complex from binding to vitronectin, rather than inducing dissociation of uPA from uPAR. The reduced affinity for uPAR-bound pro-uPA also explains the higher IC_{50} value of uponap-126 for the pro-uPA/uPAR-vitronectin interaction compared with the values observed for the effects toward pro-uPA activation and uPA-uPAR association.

**Upnap-126 is an effective inhibitor of uPA-dependent processes on the cell surface**

To evaluate the effect of uponap-126 on cell-surface associated, uPA-catalyzed plasminogen activation, we monitored plasmin activity after addition of plasminogen and pro-uPA to U937 cells, which express high levels of uPAR. To score generation of plasmin exclusively at the cell surface, the assay was conducted in the presence of EGF domain. The control RNA sequence showed no inhibition (Fig. 4A, open circles). We also found that uponap-126 was able to inhibit plasminogen activation initiated by active two-chain uPA on the surface of U937 cells (data not shown), whereas no inhibition was observed in the cell-free assay (Fig. 1B). This observation shows the uPA-uPAR binding inhibitory activity of uponap-126 at the cellular level.

To evaluate a direct effect of aptamers on the proadhesive uPA-uPAR interaction with vitronectin in a cellular context, a newly adapted protrusion assay was used (28). In this assay, lamellipodia formation induced by uPAR-vitronectin binding is scored. In HEK293 cells expressing wild type uPAR, lamellipodia formation occurs uPA independently. In contrast, in cells expressing the single-site mutant uPAR_{W32A} (HEK-uPAR_{W32A}), lamellipodia formation is uPA dependent because the mutation weakens the interaction between uPAR and vitronectin. Lamellipodia formation by HEK-uPAR_{W32A} cells can, therefore, be rescued by addition of catalytically inactive pro-uPA_{S356A} or even the isolated EGF domain of uPA (28). Thus, this assay is suitable for evaluating whether uPA antagonists, which are able to disrupt the binding of uPA to uPAR or uPA-uPAR complex to vitronectin, would affect lamellipodia formation. The presence of uponap-126 caused inhibition of lamellipodia formation induced by pro-uPA_{S356A} in HEK-uPAR_{W32A} cells (Fig. 4B). However, in apparent agreement with its binding to the serine protease domain of pro-uPA, 1 μmol/L uponap-126 (i.e., a concentration that caused a substantial decrease in pro-uPA_{S356A}-induced lamellipodia formation) did not inhibit lamellipodia formation rescued by the isolated uPA EGF domain. The control RNA sequence showed no inhibition of lamellipodia formation in uPAR_{W32A}-expressing cells induced by either pro-uPA or uPA EGF domain (Fig. 4B).

**Human tumor cell invasion in vitro and in vivo is inhibited by uponap-126**

Next, we investigated the ability of uponap-126 to interfere with human tumor cell invasion. We used a highly disseminating variant of human PC-3 prostate carcinoma, that is, PC-hi/diss cells, generated by in vivo selection (40). Dissemination of this cell variant in chick embryo and mouse model systems has previously been shown to depend on the uPA system (40, 41). When PC-hi/diss cells were cultured in the presence of 500 nmol/L uponap-126, the amount of active uPA generated was substantially decreased (Fig. 4C). Correspondingly, the addition of 1 μmol/L uponap-126 resulted in a significant reduction of tumor cell invasion in the Matrigel invasion assay (Fig. 4D). The control RNA sequence showed no effect in these assays (Fig. 4C and D).

To evaluate the inhibitory effects of uponap-126 on tumor cell invasion in vivo, we used a newly developed model, in which fluorescently labeled tumor cells are inoculated into the CAM mesoderm of chick embryos developing ex ovo. Cell escape from intramesodermal microtumors and invasion into surrounding stroma is then visualized microscopically 5 to 6 days after cell injections (41). In the present study, treatment of developing PC-hi/diss microtumors with 25 μL of a 5 μmol/L solution of uponap-126 caused a 50% reduction in stromal invasion of tumor cells as compared with treatment with control RNA sequence (Fig. 5).

Finally, we investigated whether uponap-126 would inhibit spontaneous dissemination of PC-hi/diss cells in the chick embryo model for tumor cell metastasis. In this model, human tumor cells are grafted atop the CAM, a highly vascularized tissue that supports growth of primary tumors and also provides vascular conduits for tumor cell intravasation and dissemination (40, 41). The developing PC-hi/diss tumors were treated topically with 100 μL of a 2.5 μmol/L solution of aptamers and 7 days after grafting, the actual numbers of human cells that disseminated to the distal CAM were quantified by Alu-qPCR. Uponap-126 dramatically inhibited PC-hi/diss cell intravasation by more than 80%, but did not affect significantly the growth of the primary tumors (Fig. 6).

**Discussion**

The results presented in this study show that targeting functional exosites of uPA provides a conceptual and practical alternative to standard inhibition of proteolytic activity by targeting the enzyme’s active site. In this regard, uponap-126 inhibits plasminogen activation at the very apex of the activation cascade, that is, by delaying the proteolytic activation of pro-uPA. Moreover, uponap-126 inhibits other molecular interactions of pro-uPA, namely the association of
Figure 4. Effects of upanap-126 on uPA-dependent events in cell cultures. A, cell-surface-dependent plasminogen activation was assayed using acid-washed U937 cells distributed into 96-well plates. Plasminogen (200 nmol/L) and α2-antiplasmin (400 nmol/L) were added to cells before addition of 1 nmol/L pro-uPA, which had been preincubated alone or with aptamers (500 nmol/L) as indicated. The fluorogenic plasmin substrate VLK-AMC was added at 200 μmol/L and fluorescence monitored once a minute. B, Pro-uPA–dependent lamellipodia formation in HEK293 cells expressing human uPARW32A was analyzed by plating the cells onto vitronectin-coated glass coverslips. On the day of experiment, 20 nmol/L pro-uPA or EGF domain along with upanap-126 or control RNA sequence, at final concentrations indicated under the bars, was added to the cells. After a 24-hour incubation, evaluation of lamellipodia formation was carried out by assigning the number 0 (no lamellipodia-positive cells present) or 1 (at least 1 lamellipodia-positive cell in field) to 6 randomly chosen fields for each experimental condition. The protrusion was defined as the combined score obtained from 4 independent researchers for all fields of the samples in question (range 0–24). C and D, effects of upanap-126 on the amount of active uPA generated in cell culture and on cell invasion were assessed using the PC-hi/diss cell variant expressing high amounts of pro-uPA. C, PC-hi/diss cells were grown in the presence of 0.5 mmol/L upanap-126 or control RNA sequence. After 48 hours, samples of conditioned medium were collected and analyzed by reducing SDS-PAGE and immunoblotting using anti-uPA polyclonal antibody F1609. Single-chain pro-uPA runs as a 54,000 Da band, whereas two-chain active uPA runs as 2 bands at 34,000 Da (SPD) and 20,000 Da (ATF). D, in Matrigel invasion assays, PC-hi/diss cells were allowed to invade through the matrix barrier in the absence or presence of upanap-126 or control RNA (1 μmol/L) toward conditioned medium from chicken embryonic fibroblasts. The data are presented as percentage of invasion in the presence of control RNA sequence and are means ± SEM. *, *P < 0.05; 2-tailed Student t test. ***, *P < 0.001; 2-tailed Student t test.
pro-uPA to uPAR and the subsequent binding of the pro-

uPA-uPAR complex to vitronectin. These inhibitory effects
were observed in both cell-free model systems and cell
functional assays. In contrast, upanap-126 does not inhibit
plasminogen activation catalyzed by active uPA, and shows
no measurable effect on uPA-mediated hydrolysis of a small
chromogenic peptide substrate. Furthermore, upanap-126
efficiently inhibits uPA-dependent cellular invasion in both
cell culture and live animal settings.

Upanap-126 appears to be highly specific for human uPA
over other serine proteases. First of all, upanap-126 only
inhibited plasminogen activation initiated using human pro-

uPA and not when using the other plasminogen activator,
human tissue-type plasminogen activator. Secondly, upa-

nap-126 does not bind measurably to mouse uPA, which is
more similar to human uPA than any human homologous
protease. Considering that upanap-126 must bind to the
SPD, it is not surprising that the aptamer inhibits pro-uPA
activation. However, it is unexpected that upanap-126
inhibits interactions distant from the SPD, that is, uPAR
binding to the EGF domain of uPA, as well as vitronectin
binding to uPAR, an interaction induced by uPA-uPAR
complex formation. These observations can be explained by
the fact that although RNA aptamers have relatively low
molecular masses, they actually have relatively large dimen-
sions compared with their target proteins, as evident from
published X-ray crystal structures of proteins in complex
with RNA aptamers. Thus, a thrombin-binding RNA apta-
mer with molecular mass of 8 kDa had maximum length of
about 45 Å. Comparably, human thrombin has a molecular
mass of 36 kDa and a maximum length of approximately 55
Å (43). Similarly, an aptamer against an IgG1 Fc-fragment
with molecular mass of 8 kDa had maximum length of about
45 Å, whereas the IgG1 Fc fragment has a molecular weight
of 50 kDa and a maximum length of approximately 70 Å
(44). With a molecular mass for upanap-126 of about 26
kDa, its length may well approach 100 Å, which should be
sufficient to cover a region from the primary binding site in
the uPA SPD up to its EGF domain (45). The observation
that upanap-126 inhibits the binding of pro-uPA to uPAR
with a lower IC50 than the binding of active two-chain uPA

![Figure 5](image-url) Upanap-126 inhibits the invasion of PC-hi/diss cells in the intramesodermal chicken embryo assay. A, CellTracker labeled PC-hi/diss cells (green) were injected into the CAM mesoderm of chick embryos. Developing microtumors were treated topically with control RNA sequence or upanap-126 (25 μL of 5 μmol/L solution). Rhodamine-conjugated LCA was injected to highlight the CAM vasculature (red). Portions of the CAM containing microtumors were excised and images were digitally acquired at original ×100 magnification. Microtumor borders and invasion distances are indicated by white dashed lines. B, quantification of invasion was conducted in digitally captured images by averaging the 5 longest distances of cell invasion for each microtumor. Data are presented as percentage of PBS control (100%) determined for 15 to 18 individual microtumors per treatment variable and are means ± SEM. ***, P < 0.001; 2-tailed Student t test.

![Figure 6](image-url) Upanap-126 inhibits PC-hi/diss cell intravasation. PC-hi/diss cells (2 × 10^6) were grafted onto the CAM of day 10 chicken embryos. On day 2 and 4 following cell grafting, the developing primary tumors were treated topically with 100 μL of 2.5 μmol/L control RNA sequence, upanap-126, or vehicle (DPBS supplemented with 5% DMSO). A, on day 7, the primary tumors were excised and weighed. B, intravasation of PC-hi/diss cells in the presence of inhibitors was quantified in portions of the distal CAM by qPCR. Data are presented as number of human cells identified within 10^6 chicken cells in the CAM tissue and are means ± SEM. **, P < 0.01; 2-tailed Student t test.
to uPAR (Fig. 3A) could be caused by a slightly differential affinity of upanap-126 for pro-uPA versus active uPA or differences in the interaction of pro-uPA and active uPA with uPAR. Previously, we showed that uPA acquire an increased overall interdomain flexibility upon conversion of pro-uPA to active uPA (45).

By using full-length human uPA as a target, we previously selected RNA aptamers, which were all directed against the ATF of uPA (37). Similar to upanap-126, several of the ATF-binding aptamers were found to inhibit the uPA-uPAR interaction (37). In addition, we also generated and characterized a murine monoclonal antibody, mAb-112, capable of inhibiting pro-uPA activation (32). Similar to upanap-126, mAb-112 delays the proteolytic activation of pro-uPA. By interfering with zymogen activation and receptor binding, upanap-126 combines the two inhibitory mechanisms displayed individually by the ATF-binding aptamers (37) and the pro-uPA activation-blocking mAb-112 (32). In addition, upanap-126 inhibits the binding of the preformed pro-uPA-uPAR complex to vitronectin, a modality that to the best of our knowledge has not been observed with any other compound.

Both in the in vitro and in vivo assays, the inhibitory activities displayed by upanap-126 led to efficient inhibition of uPA-mediated tumor cell invasion and dissemination. For invasion experiments, we used a variant of the human prostate carcinoma PC-3 cell line, PC-hi/diss, selected in vitro for high disseminating potential. The uPA activity was shown to be central for the increased dissemination of this cell variant and the inhibition of pro-uPA activation by mAb-112 significantly decreased intravasation levels of PC-hi/diss cells in the CAM and mouse xenograft models (40). Recently, it was elucidated that it was the initial escape of PC-hi/diss cells from the primary tumor and tumor cell invasion into the surrounding stroma that were facilitated by active uPA during tumor cell spontaneous dissemination (41). Herein, upanap-126 was found to reduce PC-hi/diss invasion in a Matrigel assay in vitro. Importantly, upanap-126 was also inhibitory for PC-hi/diss cell escape and stromal invasion in vivo. Furthermore, upanap-126 substantially reduced intravasation and vascular dissemination of PC-hi/diss cells to secondary sites in the chick embryo model, thereby validating the antimetastatic potential of this novel pro-uPA binding aptamer in live animals.

The inhibition of pro-uPA activation, preventing plasminogen conversion to active plasmin, is likely to be the primary cause for the reduction of human tumor cell invasion displayed by upanap-126 in the chick embryo, similarly to what has been found for mAb-112 (40). However, it has been shown that the interaction between uPA and its cell-surface receptor uPAR is necessary for an increased invasive potential of the human cell lines HeLa and Wish in the CAM model (46). Therefore, the role of upanap-126 in vivo might be dual and involve inhibition of two mechanisms, which both facilitate tumor dissemination, namely uPA-uPAR binding to vitronectin and plasminogen activation. Further experiments would be needed to unravel this point. By stable silencing uPA in the PC-hi/diss cells combined with the expression of mutant uPA’s lacking the uPAR binding site or the activation site, the mechanism of upanap-126 in vivo could possibly be clarified. In addition, upanap-126’s ability to inhibit the interaction of uPA-complexed uPAR to vitronectin in the chick embryo models remains a subject for future verification. When uPAR interacts with vitronectin, it initiates actin reorganization and increases cell motility (3). Blocking this interaction could, therefore, lead to reduced cell migration. Importantly, the binding site for uPAR in the somatomedin B domain of vitronectin is very well conserved between human and chick vitronectins and human uPAR on the surface of PC-hi/diss cells is likely able to bind to chicken vitronectin in the live embryo model systems.

Aptamers targeting functional exosites of proteases have previously been described (reviewed in ref. 25). Thus, aptamers inhibiting the binding of cofactors tissue factor and FVa to coagulation factors FVIIa and FXa, respectively, were shown to interfere with coagulation (47, 48). Aptamers inhibiting more than 1 functional characteristic of their targets have also been described. Thus, a thrombin binding aptamer, named HD-1, inhibited prothrombin activation (49), thrombin-catalyzed fibrin clot formation, and thrombin-catalyzed cleavage of PAR-1 (50). However, these functional events of thrombin were all related to binding of substrates and cofactors to the same exosite I, whereas binding of upanap-126 affects multiple different functional sites of pro-uPA.

In conclusion, we have generated a novel uPA inhibiting RNA aptamer, upanap-126, selected against the serine protease domain and part of the linker region. Functional characterization of upanap-126 showed that this aptamer inhibits uPA-mediated activities through multiple molecular mechanisms. The multifaceted mode of functional inhibition was shown to be efficient in reducing uPA-mediated tumor cell invasion and dissemination in several in vitro and in vivo model systems, thereby highlighting the utility of simultaneously targeting several functional exosites of the pro-uPA molecule.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Targeting Tumor Cell Invasion and Dissemination \textit{In Vivo} by an Aptamer That Inhibits Urokinase-type Plasminogen Activator through a Novel Multifunctional Mechanism

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