The serine protease urokinase-type plasminogen activator (uPA) interacts with a specific receptor (uPAR) on the surface of various cell types, including tumor cells, and plays a crucial role in pericellular proteolysis. High levels of uPA and uPAR often correlate with poor prognosis of cancer patients. Therefore, the specific inhibition of uPA with small molecule active-site inhibitors is one strategy to decrease the invasive and metastatic activity of tumor cells. We have developed a series of highly potent and selective uPA inhibitors with a C-terminal 4-amidinobenzylamide residue. Optimization was directed toward reducing the fast elimination from circulation that was observed with initial analogues. The x-ray structures of three inhibitor/uPA complexes have been solved and were used to improve the inhibition efficacy. One of the most potent and selective derivatives, benzylsulfonyl-D-Ser-Ser-4-amidinobenzylamide (inhibitor 26), inhibits uPA with a $K_i$ of 20 nM. This inhibitor was used in a fibrosarcoma model in nude mice using $lacz$-tagged human HT1080 cells, to prevent experimental lung metastasis formation. Compared with control (100%), an inhibitor dose of 2 × 1.5 mg/kg/day reduced the number of experimental metastases to 4.6 ± 1%. Under these conditions inhibitor 26 also significantly prolonged survival. All mice from the control group died within 43 days after tumor cell inoculation, whereas 50% of mice from the inhibitor-treated group survived more than 117 days. This study demonstrates that the specific inhibition of uPA by these inhibitors may be a useful strategy for the treatment of cancer to prevent metastasis.

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The detachment of malignant cells from the primary tumor and their subsequent migration within the surrounding tissue, including intravasation and extravasation into blood and lymph vessels, leads to tumor dissemination and the formation of metastases at distant loci. Whereas a solid tumor can be removed by surgery or treated by radio-, chemo-, or hormone therapy, invasive tumor cells that have spread over the whole body can form secondary tumors leading to poor prognosis or death of cancer patients. Several proteases, such as matrix metalloproteases, the cysteine proteases cathepsin B and L, the aspartyl protease cathepsin D, and serine proteases, e.g. plasmin and uPA, are involved at multiple stages during growth, invasion and progression of tumors, including metastases formation (1). High levels of expression of these proteases often correlate with poor prognosis for cancer patients (2). However, in several clinical cancer trials with different types of non-specific matrix metalloprotease inhibitors, disappointing results with poor benefit and severe side effects were observed (3). This stimulated the search for alternative proteases with matrix degrading activity as new targets for anti-cancer drugs. An important role in metastasis has been recently ascribed to the plasmin-plasminogen activation system and especially to uPA.

Both, uPA and the second endogenous plasminogen activator tPA are trypsin-like serine proteases that can activate plasmin into enzymatically active plasmin, a broad spectrum serine protease. Whereas the main biological function of tPA seems to be associated with fibrinolysis, uPA is a central molecule in pericellular proteolysis (4). uPA is produced by a variety of cells as a single chain pro-uPA that binds to a specific receptor (uPAR) on the surface of tumor cells. This is in contrast to tPA for which no cell surface receptor is known. Pro-uPA bound to its receptor is converted into enzymatically active uPA mainly by plasmin. Some other proteases, such as cathepsins B and L, plasma kallikrein, and the transmembrane serine protease matriptase, may also be involved in uPA activation (5, 6). This cell surface focused active uPA, in turn, catalyzes plasminogen activation more efficiently than fluid-phase uPA (7). The generated plasmin can then activate pro-
forms of several matrix metalloproteases, as well as activate additional pro-uPA.

Several studies have established that uPA and uPAR levels are elevated in cancer patients making them diagnostic markers and attractive targets for anti-cancer drugs (8, 9). There are several potential ways to influence the uPA/uPAR system. In addition to interference with the expression of these proteins by antisense oligonucleotides, it is also possible to block the ligation of uPA to its receptor by treatment with antibodies or competitive analogues (5). A third strategy is the reduction of the proteolytic activity of the enzyme by treatment with synthetic, small molecule inhibitors. However, compared with other trypsin-like serine proteases, especially the clotting enzymes thrombin or factor Xa, only a few basic inhibitor structures are known, which selectively block uPA (10–12). Clinical trials were recently initiated using 2,4,6-(trisopropyl)phenylsulfonyl-3-amidinophenylalanine-N-(ethylxocarbonyl)-piperazine (13). However, results with regard to the efficacy of this nonspecific protease inhibitor (Ki for uPA 0.41 μM) in cancer patients have not yet been published.

Recently, we described the first analogues of a new series of tripeptide-derived uPA inhibitors containing an N-terminal Bzls-ω-Ser moiety, an amino acid in the L-configuration as a P2 residue, and a C-terminal 4-aminobenzylamide group in the P1 position (14). For one derivative from this series (Bzls-ω-Ser-Ala-4-Amba) an x-ray structure in complex with uPA was recently published together with the structure of related uPA inhibitors containing a P1-arginal or P1-4-guanidinobenzylamide group (15).

A pharmacokinetic analysis revealed that these first analogues were rapidly cleared from the blood of rats with a half-life shorter than 20 min after intravenous administration due to a relatively fast hepatobiliary elimination. Although this limited their use in animal studies, we recently demonstrated that moderate efficacy of these first generation analogues on inhibition of liver metastases formation is a murine T-cell lymphoma model (16).

In this report we describe the results obtained from a systematic optimization of this inhibitor type. By modification of the P4 and/or P2 residues using a strategy described for other types of benzamidine-derived serine protease inhibitors (17, 18) we demonstrate that incorporation of additional charged or polar groups result in an increase in half-life of the inhibitors. Among the newly synthesized analogues, we could identify highly potent (Ki value <20 nM) and selective uPA inhibitors with prolonged half-life in the circulation of rats, which were useful for animal studies to demonstrate their efficacy as inhibitors for experimental metastases formation. The structures of three analogues with Gly, Ser, and Ser(Bzl) in the P2 position in complex with a human uPA variant was solved by x-ray crystallography, which provides a basis for their high affinity and selectivity as uPA inhibitors. The design of these uPA inhibitors, their pharmacokinetic properties, and use in an experimental tumor model are presented in this article. An inhibitor dose of 2 × 1.5 mg/kg/day of compound 26 (benzylsulfonyl-ω-Ser-4-aminobenzylamide, Ki 20 nM) significantly reduced the formation of experimental lung metastases in mice. These results support the hypothesis that uPA may be a potential target for the development of new anti-metastatic agents.

MATERIALS AND METHODS

Synthesis

Some of the substituted benzyllsulfonylchlorides are commercially available (Maybridge, Cornwall, United Kingdom, or Array Biopharma, Boulder, CO), all other analogues were prepared from the appropriate sodium sulphonates by treatment with PCl₅ in phosphorous chloride at 80 °C for 4–5 h. The reaction mixture was poured into ice water and vigorously stirred. Finally, the solidified sulfonylchloride was filtered, washed with water, and dried in vacuum. All of the used sodium sulfonates were obtained from the corresponding benzylbromides by refluxing with 1.1 eq of Na₂SO₃ in water for 8 h, followed by crystallization from water (19). The inhibitors were synthesized by standard methods using side chain-protected P2 and P3 amino acids. The crude inhibitors were obtained after side chain deprotection and final hydrolysis followed by purification using a preparative reversed-phase HPLC.

In the case of inhibitor 26, which was used as a lead compound in animal studies, an optimized synthesis strategy without side chain protection of both serine residues was developed (Scheme 1). Briefly, Boc-4-(acetyloxamidino)benzylamide II was obtained in three steps from p-cyanobenzylamine (Showa Denko, Japan) using a procedure described previously (20). After deprotection, Boc-Ser was coupled to III by the mixed anhydride procedure and the Boc group was removed by trifluoroacetic acid to give intermediate V. Bzls-ω-Ser-OH, prepared by silylation of H-ω-Ser-OH and addition of benzylsulfonyl chloride, was attached to V using PyBop/DIEA as coupling reagent. The final hydrolysis resulted in crude inhibitor 26, which was purified by cation exchange chromatography using an ammonium acetate gradient. The analytical methods (MS, HPLC, and NMR) and a detailed synthesis procedure for inhibitor 26 are described in the Supplemental Materials.

Enzyme Kinetics and Preparation of Rat uPA

The kinetic measurements with human uPA, plasmin, TPA, trypsin, thrombin, and factor Xa were performed as reported previously (21). Rat uPA was cloned, expressed, and activated according to the following procedures.

Cloning—To generate a PCR template for rat uPA cloning, reverse transcription was performed using a first strand cDNA synthesis kit (Roche) and a rat liver poly(A)⁺ RNA (Clontech). In the subsequent PCR, 5‘-ACCATGAGAGTCCTGGCTGGCAGCC-3’ and 5‘-TAACTGATGATGATGAGCGAAGGCTAGGCCATTCTCTTCTCC-3’ were used as primers. The PCR was performed with 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. A mixture of Taq and Pfu polymerase was used. The resulting PCR product was cloned into pC DNS.1/V5-His/TOPO (Invitrogen) following the manufacturer’s instructions. The correct sequence of the resulting rat-uPApcDNA3 vector was confirmed by sequencing.

Expression and Activation—For expression of full-length rat pro-uPA, uPApcDNA3 was transformed into baby hamster kidney 21 cells.
The cells were cultured in RPMI1640 with 5% fetal calf serum (Invitrogen) at 37°C. Expressed protein was isolated from the cultured supernatant by nickel-nitrioltriacetic acid (Qiagen) chromatography under native conditions. Elution fractions that contained pro-uPA were pooled and dialyzed against Tris-buffered saline. Activation of pro-uPA was performed with biotinylated plasmin as described previously (22).

**Pharmacokinetic Measurements**

Anesthetized female Wistar rats, 240–320 g body weight (Charles River-Wiga, Sulzfeld, Germany), were used for the determination of the elimination half-life of selected inhibitors, as previously described (17, 21). The inhibitors were administrated in aqueous solution intravenously at a dose of 1 mg/kg. Blood samples were withdrawn into 3.8% sodium citrate solution (1/10, v/v) at different times after administration. The blood sample volume removed was replaced by injecting the corresponding volume of saline.

Citrated plasma was obtained by centrifugation at 1200 × g for 10 min, and the concentrations of the inhibitors were determined by a spectrophotometric assay. In preliminary assays it was demonstrated that these chromogenic measurements were in good agreement with results obtained with a reversed-phase HPLC system, which we have used previously for monitoring the inhibitor concentration in plasma (17). Therefore, 150 or 175 μl of 50 mM Tris-HCl buffer (pH 8.0, containing 0.154 mM NaCl) and 25 μl of substrate Pefachrome uPA (benzoyl-β-Ala-Gly-Arg-p-nitroanilide, stock 2 mM) were treated with 50 or 25 μl of a plasma sample at room temperature. The reaction was started by addition of 50 μl of uPA (Rhesothrom 500,000, Curocane AG, Kleinostheim, Germany), dissolved in 0.9% NaCl as a 1 mg/ml solution, and monitored at 405 nm over a period of 5 min. From the observed reaction rate the inhibitor concentration in plasma was calculated using a calibration curve prepared with inhibitor concentrations in the range of 0.05–10 μg/ml.

**Crytalization**

The crystallization of the enzyme-inhibitor complexes, data collection, and structure refinement were performed as described previously using a C122S mutant of the serine protease domain of uPA (βκ-uPA) (15, 23). The βκ-uPA-inhibitor complexes were prepared by soaking the inhibitors in crystals of the βκ-uPA-benzamidine complex. The refinement statistics for x-ray analysis of all βκ-uPA-inhibitor complexes is given in the Supplemental Materials.

**Experimental Metastasis Assay**

Pathogen-free, female CD1 nu/nu mice (9 weeks old; 25 g on average, Charles River) were inoculated with 1 × 10<sup>6</sup> lacZ-tagged human fibrosarcoma cells (HT1080) into the tail vein of each mouse at day 0. HT1080 cells were stably transfected with a lacZ-coding plasmid (PLZ 12) to allow single cell detection of metastases (24). The lacZ-tagged fibrosarcoma model offers a quantitative system after X-gal staining, allowing the study of experimental lung metastasis within 22 days after tumor cell inoculation (t.c.i.) as cells colonize the lung upon intravenous injection. At day 22, this results in the formation of macrometastatic foci (diameter >0.2 mm) in the lung. For the treatment, inhibitor 26 was freshly dissolved daily in sterile pyrogen-free H<sub>2</sub>O to a final concentration of 0.5% (v/v) ethanol.

For the experimental lung metastasis assay, starting from day −1 (1 day before t.c.i.), 200 μl of the inhibitor solution or vehicle control were administered intraperitoneally twice daily at 1.5 mg/kg (equivalent to 3 mg/kg/day) until day 1 (1 day after t.c.i. = short treatment) or until the day prior to organ extraction at day 22 after t.c.i. (long treatment). Additionally, the body weight of mice was measured before and after the experiment as an indicator of health condition. A second experiment with a long treatment of inhibitor 26, in this case subcutaneously administered, was performed with the same treatment regimen.

For the survival study, 200 μl of vehicle or inhibitor 26 solution were administered twice daily at a dose of 1.5 mg/kg (equivalent to 3 mg/kg/day) subcutaneously from day −1 until day 100. During the entire survival study the mice were monitored twice daily in terms of fitness and health status and sacrificed only when found to be moribund.

The first three treatments of all experiments were performed at 24, 12, and 1 h before t.c.i. to influence early colonization of the lung by tumor cells through the inhibitor presence. Mice of the control and treatment groups were sacrificed 22 days after t.c.i., when experimental macrometastases are clearly visible and well quantifiable in the lung in this model. Lungs were removed and stained with X-gal (Roche Diagnostics, Penzberg, Germany). Indigo blue macrometastatic foci on the surface of the organs were counted, allowing assessment of the metastatic pattern (24).

**Statistical Analysis of the in Vivo Experiments**

Data of the experimental metastasis assay were analyzed using the Mann-Whitney rank sum test. The Kaplan-Meier survival curves were compared using the log rank test. Data were considered significantly different when p < 0.05.

**Zymography**

Snap-frozen tissue (one lung lobe) was homogenized for 20 s in a minin beadbeater<sup>™</sup> in the presence of zirconium beads (1.0 mm diameter, Biospec Products Inc.) and 300 μl of extraction buffer (50 mM Tris/HCl, 5 mM CaCl<sub>2</sub>, 200 mM NaCl, 1% Triton X-100, pH 7.5). After centrifugation of the tissue homogenate (4°C, 12,000 × g, 10 min), the supernatant was collected, snap frozen in liquid nitrogen, and stored in aliquots at −80°C. Pellets of HT1080 cells were washed twice with phosphate-buffered saline and resuspended in Tris-buffered saline (pH 8.5), 0.1% (v/v) Triton X-100. Zymographic detection of uPA activity was performed with 7.5 μg of total protein per lane (10% SDS-PAGE with 1 mg/ml gelatin and 3.8 μg/ml plasminogen (Roche Applied Science), non-reducing conditions). Plasminogen served as substrate for the uPA present in cell supernatants or homogenates of tissue, generating plasmin, which then degraded gelatin. After electrophoresis, proteins were renatured and the gel was incubated at 37°C for 19 h in 100 mM Tris/HCl (pH 7.5) containing 200 mM EDTA to eliminate any gelatinase activity present in the samples. The gels were stained as described previously (25). Stained molecular weight markers (Kaleidoscope Prestained Standards, Bio-Rad) and human HMW-uPA (RiboPharm, Haan, Germany) served as standards.

**RESULTS**

**Enzyme Kinetics**—Besides uPA, five additional trypsin-like serine proteases were used to determine the specificity of the inhibitors. Trypsin was used as a standard enzyme for this protease class, whereas thrombin, factor Xa, tPA, and plasmin were selected to predict a possible influence on blood coagulation and fibrinolysis because of inhibitor treatment, which could lead to side effects during in vivo experiments.

The previously reported lead structure Bzls-D-Ser-Gly-4-Amba (inhibitor 1) consists of four segments abbreviated as P4-P3-P2-P1 according to the nomenclature from Schechter and Berger (26). Assuming that 4-Amba at P1 and the P3 amino acid D-Ser are essential residues in this inhibitor type, the P4-sulfonyl group and P2 residue were mainly optimized. Two additional inhibitors with modifications in the P3 position were synthesized to evaluate the contribution of the D-Ser side chain for uPA affinity, whereas thrombin, factor Xa, tPA, and plasmin were selected to predict a possible influence on blood coagulation and fibrinolysis because of inhibitor treatment, which could lead to side effects during in vivo experiments.

For variation of the P4 position, the benzylsulfonyl residue was maintained, because we demonstrated previously that analogues with the homologous phenylethanesulfonyl group and the shorter phenylsulfonyl residue were more than 10 times less potent uPA inhibitors (14). The inhibitors summarized in Table I are mainly modified at the para position of the P4 phenyl ring or contain an additional free or esterified carboxyl group. The carboxyl groups were introduced because several examples with structurally related carboxylated protease inhibitors for thrombin or factor Xa are known from literature, which were less prone to a rapid hepatobiliary elimination (17, 18, 27).

The substitution of the P4 position by a single halogen or methyl residue (inhibitors 2, 3, and 4) has little influence on uPA affinity, whereas a moderate improvement in potency toward all of the used proteases was obtained with compound 8 containing an amino group at the para position. All other inhibitors were less active but still inhibit uPA with K<sub>i</sub> values <0.5 μM. Most of the analogues were relatively selective, only some trypsin inhibition was observed. However, trypsin, which is released from the pancreas, appears only in the intestine and does not come into contact with the blood system or other tissues. Therefore, poor trypsin selectivity should not have any
impact on the efficacy of these inhibitors in animal studies, as long as they are only parenterally applied. Because of small differences in affinity the Bzl-D-Ser segment was maintained for the optimization of the P2 position (Table II).

Based on the inhibition constants determined for inhibitor 19, a D-amino acid residue is not accepted at the P2 position. Some of the analogues summarized in Table II are more potent uPA inhibitors compared with the lead compound 1. However, for 18 and 20 with Ala or Pro, the selectivity was strongly reduced. Specifically compound 20 is a potent thrombin inhibitor (Ki/H11005 12 nM) and could influence blood clotting. This was not surprising because Pro is the preferred P2 residue in many types of thrombin inhibitors (28). In contrast, inhibitor 26, containing a Ser at P2 was a potent uPA inhibitor and maintained good selectivity. A high uPA affinity (Ki/H11005 8.9 nM) was obtained also for Arg derivative 34. However, because of its plasmin affinity (Ki/H11005 0.2 nM) it might also affect the fibrinolytic system. During the course of the clinical development of the thrombin inhibitor melagatran it was determined that the inhibition constant for plasmin should be above 0.5 nM to avoid bleeding (29). To evaluate the importance of the D-Ser side chain two additional analogues were synthesized (Table III).

Both, derivatives 35 and 36, are significantly less potent uPA inhibitors than the reference 1, whereas only minor differences between these 3 compounds with respect to the other enzymes were observed. This was expected, because the x-ray structure indicates that the D-Ser hydroxy group acts as a hydrogen bond donor for the carbonyl oxygen of Leu<sup>97B</sup> and as an acceptor for the uPA residue His<sup>99</sup> (15). Surprisingly, only little differences in the uPA inhibition between the D-Ala and D-Dap derivatives were observed, because the free amino group of the D-Dap derivative should still allow the formation of a hydrogen bond to the carbonyl of Leu<sup>97B</sup>, which is not possible with the D-Ala compound.

The inhibition constants for inhibitors with dual modifications in positions P4 and P2 are summarized in Table IV. Because of their high uPA affinity the P2 residues Ala and Ser were combined with the carboxyl group containing P4 residues, which were found to have an impact on the elimination rate (see Fig. 1A). In addition, their methylester precursors and other inhibitors with Cl or methyl groups were investigated. Also in this series the inhibitors with Ala at P2 were gener-
ally more potent toward uPA than the corresponding Gly (see Table I) and Ser analogues. Remarkably, all compounds with a free carboxyl group in the para and meta position of the benzylsulfonyl group had a very high selectivity for uPA over plasmin, tPA, thrombin, and factor Xa. The results of the structure-activity relationship, which are summarized in Tables I–IV, revealed a series of potent and selective uPA inhibitors that were further characterized with respect to their elimination behavior.

Elimination Studies—For selected inhibitors the time courses of plasma elimination were analyzed after intravenous injection (1 mg/kg) in rats. The plasma concentration-time data were analyzed according to a biphasic two-compartment model (30). Typically, after a rapid distribution (α-phase) a more decelerated terminal elimination (β-phase) was observed for all compounds under study. Fig. 1A shows the terminal elimination half-life of the β-phase for these analogues. The given values represent the mean of 3 or 4 animal experiments. In some cases with fast elimination (t1/2 < 0.3 h) only two rats were investigated.

The incorporation of a carboxyl group at the para and meta positions of the benzyl ring in position P4 in combination with Gly as the P2 residue (inhibitors 9 and 11) significantly reduced the elimination rate in rats compared with the lead inhibitor 1. Surprisingly, ortho-substituted inhibitor 13 was cleared relatively fast. The effects were less pronounced with the P4 carboxylated inhibitors, which contain Ala as the P2 residue (inhibitors 40 and 42). This indicates the strong influence of small structural changes on the pharmacokinetic behavior within this type of inhibitors.

A prolongation of elimination was seen also with inhibitors containing polar or charged amino acids at P2 (inhibitors 24, 26, 32, and 34 containing Ser, Glu, Lys, and Arg, respectively), as well as with compound 8, which contains a 4-aminobenzylsulfonyl residue in the P4 position. However, because of its aniline-like structure, the amino group of inhibitor 8 should not be protonated at physiological pH.

In contrast, the analogues with a carboxylated benzyl ring at P4 in combination with Ser at position P2 (inhibitors 46 and 47) were eliminated significantly faster than the corresponding analogues with Gly. All of the more hydrophobic analogues were cleared as fast as the reference inhibitor 1. As an example, Fig. 1B shows the elimination curves obtained for inhibitor 26 as a function of administration type. The terminal elimination rates and plasma levels are similar after intravenous and intraperitoneal treatments, whereas slightly higher inhibitor concentrations were found after subcutaneous administration.

X-ray Crystallography—Because of its high affinity, selectivity, and prolonged elimination behavior we selected inhibitor 26 together with the reference compound 1 for structure analysis. Fig. 2, A and B, shows a stereo view on the active site region of βc-uPA in complex with analogue 1 (Protein Data Bank code 1SC8, resolution 2.4 Å) and compound 26 (Protein Data Bank code 1VJA, resolution 2.0 Å), determined by x-ray crystallography, respectively. Both inhibitors obey a similar overall binding mode and adopt a turn-like conformation, whereas the peptide backbone binds as a short anti-parallel β-sheet to uPA residues Ser214 and Gly216. This binding mode was also found for similar tripeptide-derived inhibitors in complex with other trypsin-like serine proteases, e.g. in case of thrombin or trypsin (31, 32).
The amidino group of the P1-Amba residue forms a symmetric salt bridge to Asp189 of uPA, whereas its phenyl ring fills the S1 pocket between uPA residues 215–219, 191–193, and Tyr228 at the back. At the opposite side, the S1-pocket is closed by the benzyl group of the P4 residue of the inhibitor. This group fills a shallow hydrophobic subpocket at the surface of the enzyme surrounded by uPA residues Gln192, Gly219, and the Cys220–Cys191 disulfide bridge. In addition, one oxygen atom of its sulfonamide is hydrogen bonded to the NH of Gly219.

The hydroxyl group of the P3-D-Ser in both inhibitors interacts with the carbonyl oxygen of Leu97B and one nitrogen of His99 from uPA. Together with Thr97A the residue Leu97B is part of an uPA-specific insertion loop, which restricts access of the more bulky P3 inhibitor residues in the D-configuration. Therefore, these hydrogen bonds to Leu97B and His99 are important for the affinity as well as for the selectivity of this inhibitor class, because among human trypsin-like serine proteases both residues specifically exist only in uPA. In contrast, the P2 side chain OH of inhibitor 26 is directed to an artificial sulfate present in the crystallization buffer. It is assumed that this hydrophilic group is normally exposed to the solvent.

Surprisingly, the more hydrophobic benzyl-protected analogue 27 is a relatively potent uPA inhibitor ($K_i = 28$ nM), although it was suggested previously that uPA accepts only small and sterically less demanding P2 residues. Therefore, to examine the binding mode of the P2 Ser(Bzl) side chain, we investigated the inhibitor 27:uPA complex (Fig. 3). The x-ray structure of this complex (Protein Data Bank code 1VJ9, resolution 2.3 Å) revealed that the benzyl ring at the P2 side chain of the inhibitor is located close to the imidazole rings of His99 and His57, and to the side chains of Tyr94 and Asp60A.

We took advantage of the proximity between the benzyl group of the P2 residue from inhibitor 27 and the carboxyl group of the uPA residue Asp60A and synthesized inhibitors 32 and 34 containing P2 amino acids with basic side chains. Arg containing compound 34 was revealed to be a very potent uPA inhibitor ($K_i = 8.9$ nM), which indicates that the guanidino group might interact with Asp60A. Fig. 4 shows the structure of the inhibitor 34:uPA complex obtained from molecular modeling using the software package Sybyl. The minimization procedure was performed with the MMFF94 force field (33) and MMFF94 charges over 1000 steps using the Powell algorithm (34). The dielectric constant was set to eight with a distance dependent function.

**Experimental Metastasis Studies**—Because of its favorable overall profile (high in vitro potency, high selectivity, and slow elimination rate) compound 26 was selected for in vivo studies to demonstrate its efficacy on the inhibition of experimental lung metastases formation of a human fibrosarcoma in nude mice. Although inhibitor 34 with the P2 Arg was a second candidate (see Table II), it was eliminated because of its possible influence on fibrinolysis ($K_i$ for plasmin 0.2 μM) and po-
tential hypotensive effects, which have often been described for strongly basic protease inhibitors (35, 36).

The lacZ-tagged HT1080 fibrosarcoma cells were analyzed for expression of uPA to justify that this is an appropriate model for the evaluation of uPA inhibitors. Enzymatically active uPA can exist in two different forms, as high molecular weight uPA (HMW-uPA) or low molecular weight uPA (LMW-uPA). LMW-uPA is produced from HMW-uPA by proteolytic cleavage between the protease domain and its N-terminal fragment, which contains the growth factor domain that is necessary for binding of uPA to uPAR (7). Therefore, only HMW-uPA can bind to uPAR and is able to focus its proteolytic activity on the surface of tumor cells.

By zymography we found that the HT1080 cells express significant amounts of HMW-uPA, (Fig. 5, lane 1), whereas in metastasis-free lungs only LMW-uPA could be detected (Fig. 5, lanes 2 and 3). The amount of HMW-uPA positively correlated with increasing numbers of metastases (Fig. 5, lanes 4–8).

We determined the efficacy of compound 26 to inhibit experimental lung metastases formation in two different experimental settings. On the one hand, we determined, whether a short treatment (between day −1 to day +1, when extravasation-associated proteolysis takes place) would be sufficient to inhibit experimental lung metastases formation. In a second group, we continued treatment of the mice until day 21 (end of study) to investigate possible additional efficacy on progression of metastasis. In both cases, treatment with uPA inhibitor 26 significantly reduced formation of macrometastases (>0.2 mm). Short and long term inhibitor treatment resulted in a remarkably high anti-metastatic efficacy of ~62 and ~95%, respectively (Fig. 6A). Second, long term treatment was significantly more efficient than short term treatment. Fig. 6B shows the X-gal-stained surface of three representative lungs (with metastasis numbers around the mean of each group) from the control and the inhibitor-treated groups. In addition, over the period of the whole experiment, treatment with inhibitor 26 resulted in a significant protection from body weight loss, as an indicator of good health condition (vehicle treated control group: −8.9 ± 2.7%; short treatment: +8.6 ± 2.9%; long treatment: +14.0 ± 1.8%).

Based on these data we postulated that inhibitor 26 may prolong the survival time of mice in the same tumor model. Therefore, the effect of a prolonged inhibitor treatment starting at day −1 until day 100 using the same dose regimen, followed by a treatment-free period, was investigated in a survival experiment (Fig. 7). All mice in the vehicle-treated control group died within 43 days after t.c.i. In contrast, we found a significantly increased survival of mice in the inhibitor-treated group. If at all, treated mice revealed only few but enlarged experimental pulmonal metastases. In survivors after day 117, no tumor mass was detectable upon sectioning. On day 420, the last inhibitor-

![Fig. 3. Structure of βc-uPA covered by a semitransparent surface colored by surface potential from positive (blue) to negative (red) in complex with inhibitor 27 (Protein Data Bank code 1VJ9), containing Ser(Bzl) at the P2 position. Selected amino acids are labeled.](Image 61x378 to 303x537)

![Fig. 4. Stereo view of the model of the inhibitor 34:human-uPA complex. The inhibitor is drawn as sticks with atom dependent colors. For simplification, only the hydrogens, which are attached to the terminal side chain nitrogens of the P2 arginine, are shown. The protein is visualized by a Connolly surface, blue and red surface areas showing hydrogen acceptors and donators, respectively. Gray areas have no hydrogen bonding properties. The guanidino group of the Arg side chain in the P2 position of the inhibitor probably forms a salt bridge (yellow lines with distances given in Å) to the carboxyl group of Arg102A, found specifically only in human uPA. This model was generated from the x-ray structure of the inhibitor 27-βc-uPA complex (see Fig. 3) by replacement of the oxygen and phenyl ring in the Ser(Bzl) side chain by a CH3- and guanidino group, respectively, followed by energy minimization of the enzyme-inhibitor complex using the software package Sybyl version 6.9.1. (Tripos).](Image 214x39 to 563x317)
treated mice died. These data demonstrate that treatment with inhibitor 26 is beneficial in preventing tumor progression in this fibrosarcoma model.

**DISCUSSION**

Recent findings indicate the importance of the plasmin-plasminogen activation system in tumor invasion and metastasis formation (37). Specifically, up-regulation of uPA, uPAR, and PAI-1 in tumor tissues often correlates with increased malignancy and poor prognosis for patients with breast cancer and several other solid malignant tumors (5, 38–40). However, at present only a few potent and selective uPA inhibitors have been developed, which could be used in animal tumor models to evaluate the influence of the proteolytic activity of uPA on metastasis and invasion.

Starting from our first lead 1, a selective and potent uPA inhibitor, we developed a new series of selective analogues with reduced elimination rates to improve their efficacy for *in vivo* studies. We have demonstrated that incorporation of charged or polar groups increased the half-life of the inhibitors in rats because of a reduced hepatobiliary clearance. The strongest effect was observed for those inhibitors that were substituted with a carboxyl group in *para* or *meta* positions of the P4 benzylsulfonyl residue (inhibitors 9 and 11 with t1/2 of 2 and 1.3 h, respectively). Such effects were observed previously with several other types of carboxyl-modified serine protease inhibitors (17, 18, 41). In the case of our lead 1 <20% of the inhibitor could be detected in bile, whereas this amount was reduced to less than 5% with inhibitors 9 and 11. For several of the inhibitors with an additional polar or charged group, <80–90% of the inhibitor dose could be detected in the urine of rats. Therefore renal clearance seems to be the dominant elimination route. In contrast, after administration of more hydrophobic derivatives relatively large amounts of the inhibitor were found in the bile: for example, 65% in inhibitor 27 with Ser(Bzl) at P2.

In comparison to inhibitor 1, the incorporation of a carboxyl group in the *para* or *meta* positions of the P4 benzyl group resulted in a similar 3–5-fold loss in inhibitory potency toward uPA, plasmin, trypsin, and factor Xa, whereas the affinity for thrombin was decreased more than 10-fold. Molecular modeling revealed that this might be because of some electrostatic repulsion induced by thrombin-specific residue Glu192, which comes in close contact to the P4 benzyl ring and is replaced by the non-charged Gln192 in all of the other used proteases.

The loss in uPA affinity found for the P4 carboxyl-modified inhibitors could be compensated by replacement of the P2 Gly with Ala. Both analogues 40 and 42 maintained sufficient selectivity as uPA inhibitors, but were more rapidly eliminated than inhibitors with Gly in P2. This indicates that small structural changes might strongly affect half-life.

A significant prolongation of elimination was also observed after incorporation of polar or charged P2 amino acids, like Ser, Gln, Lys, and Arg. The Arg-containing inhibitor 34 was also one of the most potent compounds, found within this series. Based on the x-ray structure of the inhibitor 27-uPA complex (Fig. 3) and the model of compound 34 in the active site of uPA (Fig. 4) we assume that the Arg-guanidino group interacts with the carboxyl side chain of uPA residue Asp^60A. This Asp^60A is a specific residue present only in human uPA, which is replaced by Gln and Asn in the mouse and rat enzymes, respectively. To prove our hypothesis, we determined the inhibition constants for 3 selected inhibitors, 1, 26, and 34, also for rat uPA. In the case of inhibitor 26, only marginal differences between the Ki values for human and rat uPA were observed (20 and 19 nM, respectively), whereas derivative 1 was a 4-fold more potent inhibitor of the rat enzyme (Ki, 7.9 nM). In contrast, compound 34 was less potent toward rat uPA (Ki, 20 nM). These kinetic results assist the modeled structure of the inhibitor 34-uPA complex shown in Fig. 4.

A similar interaction important for species selectivity was
The injection of HT1080 human fibrosarcoma cells into nude mice is a well established, widely used tumor model in preclinical assays, addressing the potential of protease inhibitors and other anti-tumor drugs to inhibit tumor growth and dissemination (44–47). However, because of the tail vein injection of tumor cells, these experimental metastasis assays are only suitable to test the effect of protease inhibitors on the late stages of metastasis, such as extravasation and growth of metastases in distal organs. These assays cannot reflect the influence of inhibitors on the initial intravasation steps of metastasis (48).

The results from the fibrosarcoma model reveal the efficacy of compound 26 on inhibition of experimental metastasis formation after intraperitoneal application. Nearly reproducible results were obtained also after subcutaneous treatment, which is a clinically more relevant method of application, with the same dose in a second experiment (experimental metastasis reduction to 15.1 ± 4.06% compared with control, n = 11). A reduced number of experimental metastases was also observed at a lower inhibitor concentration (subcutaneous application of 0.75 mg/kg/day, experimental metastasis reduction to 41.3 ± 6.3%, n = 6). However, further studies are necessary to establish a statistically significant dose/efficacy relationship over a broader range of inhibitor concentrations.

It should be noted that under the present conditions inhibitor 26 was well tolerated in mice and no relevant side effects have been observed because of inhibitor treatment. All clotting parameters remained unchanged and no hematomas were found at the site of inhibitor injections. In addition, no effect on blood pressure was detected.

It is likely that the high in vivo efficacy of inhibitor 26 in this fibrosarcoma model is mainly related to an effective and selective inhibition of the proteolytic activity of HMW-uPA in complex with uPAR on the surface of tumor cells and therefore, in the down-regulation of the plasmin/plasminogen activator system. Undoubtedly, the activation of plasmin and of additional downstream proteases, such as matrix metalloproteases, are critical steps for extracellular remodeling, angiogenesis, and metastasis, because pericellular proteolysis is thought to induce intra- and extravasation of tumor cells into lymph and blood vessels as a prerequisite for their dissemination (2, 5).

Primary tumor growth and metastasis also require rapid cell proliferation. It has been shown by others that a uPA-triggered intracellular signal transduction is involved in cell proliferation, cell adhesion, and migration. However, the mechanism of the mitogen-like function of uPA seems to be cell-type specific.

In a human epidermal tumor cell line CCL20.2 (49) and GUBSB melanoma cells (50), uPA-induced cell proliferation requires uPAR binding and enzymatic activity of uPA. Similar effects were also demonstrated with non-malignant vascular smooth muscle cells, in which only enzymatically intact uPA could induce a mitogenic response (51). However, in osteosarcoma cells and also in human ovarian cancer cells, the mitogen-like function of uPA was independent of its enzymatic activity. In addition the N-terminal fragment or uPA-derived peptides also exerted a mitogenic effect (52, 53).

An additional mechanism, which could be blocked by a synthetic act-site inhibitor, is the complex formation between the serpin-type inhibitor PAI-1 and uPA-uPAR, which requires a free active site of uPA. This ternary complex formation can result in uptake of the whole complex via a lipoprotein receptor-related protein, followed by an intracellular degradation of the PAI-uPA complex and recirculation of free uPA to the cell surface (54, 55). In this mechanism the free uPA can bind new uPA and therefore, the proteolytic activity is focused back to the cell surface and can facilitate invasion and metastasis. Although no experimental data are currently available we hypothesize that this recycling mechanism might be interrupted in the presence of a synthetic uPA inhibitor.

In summary, we have developed a series of highly potent and selective uPA inhibitors as promising agents in cancer treatment. For the lead compound inhibitor 26, we have demonstrated a strong antimetastatic efficacy in a preclinical tumor model resulting in significantly prolonged survival of mice. The potency of this and other inhibitors will be evaluated in further studies with other appropriate tumor types in which uPA and uPAR expression are up-regulated and are known to be correlated with tumor progression and metastasis.

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