Identification of a novel inhibitor of urokinase-type plasminogen activator

Ming Zhu,1 Vijay M. Gokhale,2 Lajos Szabo,2 Ruben M. Munoz,1 Hyounggee Baek,2 Sridevi Bashyam,2 Laurence H. Hurley,2 Daniel D. Von Hoff,1 and Haiyong Han1
1Division of Clinical Translational Research, Translational Genomics Research Institute, Phoenix, Arizona and 2College of Pharmacy, Arizona Cancer Center and BIO5 Institute, The University of Arizona, Tucson, Arizona

Abstract
Urokinase-type plasminogen activator (uPA), a highly restricted serine protease, plays an important role in the regulation of diverse physiologic and pathologic processes. Strong clinical and experimental evidence has shown that elevated uPA expression is associated with cancer progression, metastasis, and shortened survival in patients. uPA has been considered as a promising molecular target for development of anticancer drugs. Here, we report the identification of several new uPA inhibitors using a high-throughput screen from a chemical library. From these uPA inhibitors, molecular modeling and docking studies identified 4-oxazolidinone as a novel lead pharmacophore. Optimization of the 4-oxazolidinone pharmacophore resulted in a series of structurally modified compounds with improved potency and selectivity. One of the 4-oxazolidinone analogues, UK122, showed the highest inhibition of uPA activity. The IC50 of UK122 in a cell-free indirect uPA assay is 0.2 μmol/L. This compound also showed no or little inhibition of other serine proteases such as thrombin, trypsin, plasmin, and the tissue-type plasminogen activator, indicating its high specificity against uPA. Moreover, UK122 showed little cytoxicity against CFPAC-1 cells (IC50 > 100 μmol/L) but significantly inhibited the migration and invasion of this pancreatic cancer cell line. Our data show that UK122 could potentially be developed as a new anticancer agent that prevents the invasion and metastasis of pancreatic cancer.


Introduction
Urokinase-type plasminogen activator (uPA) is a serine protease that functions in the conversion of the circulating zymogen plasminogen to the active, broad-spectrum serine protease plasmin. Plasmin, in turn, mediates the pericellular proteolysis of extracellular matrix components and activates other proteases such as matrix metalloproteinases and collagenases that lead to the further degradation and remodeling of the extracellular matrix. uPA is secreted as an inactive single-chain proenzyme by many different cell types and exists in a soluble or a cell-associated form by binding to a specific membrane uPA receptor (uPAR; refs. 1, 2). Structurally, active uPA is a two-chain polypeptide linked by a single disulfide bond. uPA has three major domains: an amino-terminal growth factor domain that contains the binding site for uPAR, a kringle domain that is involved in the non-uPAR-mediated signaling transduction and the stabilization of the binding of uPA to uPAR, and a carboxy-terminal sequence that contains the catalytic site (3–7). Besides the proteolytic function, upon binding to uPAR, uPA is involved in initiating versatile intracellular signal pathways that regulate cell proliferation, adhesion, and migration through its interaction with various integrins and vitronectin (8).

Studies have found that uPA and uPAR levels are elevated in various malignancies, including breast, pancreatic, gastric, lung, and colorectal carcinoma (9, 10). Overexpression of uPA enhances or confers the metastatic phenotype in animal models (11, 12). Importantly, high levels of uPA and uPAR have been clinically correlated with cancer progression and poor patient prognosis in several tumor types, including breast and gastrointestinal cancers (13, 14). Furthermore, suppression of uPA expression by antisense or short interference RNA can inhibit cancer cell migration and invasion and decrease the metastatic phenotype (15, 16). Compared with wild-type mice, mice lacking a functional uPA gene failed to develop invasive tumors (17). Preclinical studies have shown that either inhibition of uPA activity or prevention of uPA binding to its receptor reduces tumor growth, angiogenesis, and metastasis (18–20). These studies collectively suggested that inhibition of uPA activity might be an effective strategy to inhibit tumor development and progression. Hence, uPA has become a major target for development of small-molecule inhibitors as potential anticancer drugs. To date, several nonpeptidic small-molecular weight inhibitors of uPA have been reported. These inhibitors can generally be classified into two types: aromatic amidines and derivatives of guanidines (21). Aromatic amidine–derived uPA inhibitors include benzimidazole (22), benzothiophene amidines (23), quinoline amidines (24), isocoumarin amidines (25), and naphthamidines (20). Guanidine-based inhibitors of uPA include amiloride (18, 26), pyridinylguanidines...
(27, 28), and phenylguanidines (29, 30). Many of these inhibitors have been evaluated in animal models and results from some of the inhibitors have been very promising (18, 20, 21, 31, 32). One compound, WX-UK1, which showed potent antitumor growth and antimetastasis activity in a rat breast cancer model, is currently in phase I clinical trials (20).

Here, we report the identification of a new class of uPA inhibitors that are potent inhibitors of uPA enzymatic activity, with high selectivity for uPA relative to other serine proteases such as tissue-type plasminogen activator (tPA), trypsin, plasmin, and thrombin.

Materials and Methods

Reagents

Starting materials and solvents for chemical synthesis were purchased from common commercial suppliers and used as received or distilled from the appropriate drying agent. The chemical compound library consisting of ~16,000 small-molecule compounds was purchased from NanoSyn, Inc. (Menlo Park, CA). Amloride and DMSO were from Sigma (St. Louis, MO). Live/Dead kit was purchased from Molecular Probes, Inc. (Eugene, OR).

Chemical Synthesis

Reactions requiring anhydrous conditions were done under an atmosphere of argon. 1H nuclear magnetic resonance spectra were obtained using a Bruker AMX instrument at 300 MHz. The coupling constant, J, is reported in Hertz and referred to as apparent peak multiplicity rather than coupling constant. Low-resolution electrospray ionization mass spectra were recorded on a Finnigan LCQ classic ion trap instrument by using direct infusion of 50 to 100 μmol/L of the analytes in a mixture of methanol and H2O (methanol/H2O, 1:1). High-resolution and accurate mass measurements were carried out on an Ionspec 4.7 T Fourier transform ion cyclotron resonance instrument using electrospray ionization of the same analyte solutions. All compounds were assessed as being >95% pure using high-performance liquid chromatography using water/acetonitrile gradient containing 0.1% trifluoroacetic acid.

4-AMIDINOBENZALDEHYDE HYDROCHLORIDE

To a solution of 4-cyanobenzaldehyde (1.15 g, 0.076 mole) in dioxane (4.5 mL), Et2O (1.7 mL) and methanol (1.1 mL) were added with dry HCl gas (3.18 g, passed through two H2SO4 towers) at ice-bath temperature (4). The solution was left at 5°C for 92 h, then poured into 50 mL of Et2O and stirred for 1 h. The iminoether hydrochloride was collected, washed with ether, and redissolved in 10 mL H2O. It was then converted to alkaline with 10% NaOH and extracted with ether. The extracts were washed with saturated brine, dried with MgSO4, and concentrated under reduced pressure to give a semisolid iminoether free base. The iminoether was dissolved in 20 mL methanol and treated with 6 mL of 10% aqueous NH4Cl and heated at 90°C (oil bath) for 2 h. The mixture was cooled down to room temperature and poured into 75 mL acetone. The precipitate formed was removed by filtration and the filtrate was diluted with 200 mL acetone, whereupon the product precipitated. The filtrate was then concentrated in vacuum to remove methanol and H2O and was treated again with acetone. The combined product was stirred with ethereal HCl for 1 h, then filtered and washed with fresh ether to give compound 4 (ref. 33; 0.6 g, 28% yield). Mp 208 to 210°C; 1H nuclear magnetic resonance (DMSO-d6): 6: 8.01 (d, J = 8.1 Hz, 2H, CH), 8.12 (d, J = 8.0 Hz, 2H, CH, 9.29 (br s, 2H, NH2), 9.55 (br s, 2H, NH2), 10.14 (s, 1H, CHO). MS (ES), [M+H]+ 149.1 (calculated for C8H8N2O, 148.06).

4-[(5-Oxo-2-Phenyl-4(5H)-Oxazolylidine)-Methyl]-Benzenecarboxilimidamide (UK122)

To a sample of polyphosphoric acid (1.0 g), 4-amidinobenzaldehyde hydrochloride (4; 0.1 g, 0.54 mmol) and hippuric acid (3; 0.097 g, 0.54 mmol) were added. The mixture was heated on an oil bath (90°C) for 4 h followed by pouring water into the reaction mixture. The yellow precipitate formed from the mixture was then washed several times with water. This crude material was purified by high-performance liquid chromatography (gradient 70-5/20 min, H2O-acetonitrile, 5.2 mL/min flow rate) to give 0.044 g (23% yield) solid product (UK122). The high-performance liquid chromatography purity of the compound is 96.2% (at 254 nm, Rt 9.3 min, gradient 95-5/10 min, H2O-acetonitrile with 0.1% trifluoroacetic acid, flow rate 1.3 mL/min) and 95.8% (at 254 nm, Rt 12.6 min, gradient 95.5%-10 min, 5%-5%/5 min, H2O-methanol with 0.1% trifluoroacetic acid, flow rate 1.0 mL/min). 1H nuclear magnetic resonance (DMSO-d6): 6: 7.46 (s, 1H, CH), 7.68 (m, 2H, CH), 7.78 (m, 1H, CH), 7.93 (d, 2H, J = 8.4 Hz, CH), 8.18 (d, 2H, J = 7.2 Hz, CH), 8.50 (d, 2H, J = 8.5 Hz, CH), 9.05 (br s, 2H, NH2), 9.41 (br s, 2H, NH2). HRMS (electrospray ionization) [M+H]+ 292.1085 (calculated for C17H13N3O2, 291.10078).

Molecular Modeling

Molecular modeling calculations were done using the Sybyl 6.9 package (Tripos, Inc., St. Louis, MO). A crystal structure of uPA bound with an inhibitor (benzothiophene-2-carboxamide) was obtained from the Protein Data Bank (PDB ID: 1C5X) for docking calculations (34). The protein structure was first separated from the inhibitor molecule and refined using molecular minimization with added hydrogens. All molecular minimizations were carried out using the Tripos forcefield, which is the default forcefield in Sybyl 6.9. This refined protein structure was used in the subsequent docking studies. All docking calculations were done using FlexX suite as implemented in Sybyl package. Docking calculations were carried out using standard default variables for the FlexX program. FlexX scores were used for analysis of docking orientations.

Cell Culture and Drug Treatment

CFPAC-1, a pancreatic ductal adenocarcinoma cell line, was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were incubated in a humidified (37°C, 5% CO2) incubator and passaged upon reaching 90% confluence.
Amiloride and test compounds were dissolved in DMSO, and diluted to working concentrations in growth medium right before use. Cells were treated with the drugs or DMSO (0.1%) controls at concentrations and time periods indicated in the figures.

**uPA Enzymatic Activity Assay**

uPA activity was measured using a plasmin-coupled indirect enzymatic assay in flat-bottomed 96-well microtiter plates. Briefly, 5 units of purified human uPA (high molecular weight, American Diagnostica, Inc., Stamford, CT) and 2 μL of test compounds were added to a reaction buffer [50 mmol/L Tris (pH 7.4), 0.1% PEG 8000 and 6 mmol/L aminohexanoic acid] containing 0.1 mg/mL bovine plasminogen and incubated at room temperature for 10 min. Ten microliters of a plasmin-specific chromogenic substrate, H-D-Nle-HHT-Lys-pNA.2AcOH (Chemicon, Temecula, CA), were then added to the above reaction system to a final concentration as 0.25 mmol/L (final volume 100 μL). For the direct uPA assay, 10 units of purified human uPA (American Diagnostica) and 2 μL of test compounds (final concentration at 10 μmol/L) for chemical library screening were added to a reaction buffer [50 mmol/L Tris (pH 7.4), 0.1% PEG 8000] and incubated at room temperature for 10 min. Ten microliters of a specific urokinase substrate, pyroGLU-GLY-ARG-pNA-HCl (Chemicon, Temecula, CA), with a K_m of 42 μmol/L was then added to the above reaction system to a final concentration of 0.25 mmol/L (final volume 100 μL). The final concentration of DMSO in both reaction systems was 1%. Upon the addition of substrates, the assay plates were immediately transferred to a Bio-Tek KC4 Signature Bioassay reader (Winooski, VT) and read at 405 nmol/L being washed twice with PBS, cells were incubated with calcein/AM (2 μmol/L) in PBS for 30 min at 37°C. Calcein/AM is absorbed by living cells and subsequently converted by cytosolic esterases into a green fluorescent product (excitation/emission 495 nm/530 nm). At the end of incubation, the number of viable cells was determined by measuring the florescence signal intensity using the Prism 4 software (GraphPad Software, San Diego, CA).

**Cell Cytotoxicity Assay**

The cytotoxicity of the compounds in the CFPAC-1 pancreatic cancer cell line was determined using the Live/Dead Viability/Cytotoxicity Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, CFPAC-1 cells were seeded in 96-well culture plates at a density of 2 × 10^4 well and grown in the continuous presence of test compounds for 48 h. After being washed twice with PBS, cells were incubated with calcein/AM (2 μmol/L) in PBS for 30 min at 37°C. Calcein/AM is absorbed by living cells and subsequently converted by cytosolic esterases into a green fluorescent product (excitation/emission 495 nm/530 nm). At the end of incubation, the number of viable cells was determined by measuring the florescence signal intensity using the Bio-Tek KC4 Signature Bioassay reader.

**Cell Motility Assay**

The microliter-scale radial monolayer motility assay were done as previously described (35). Briefly, 10-well slides (Erie Scientific, Portland, NH) were coated with 0.1% bovine serum albumin and 10 μg/mL fibronectin. Cells were seeded through a cell sedimentation manifold (CSM, Inc., Phoenix, AZ) at 2,500 cells per well to establish a circular 1- to 2-mm diameter confluent monolayer at the center of the substrate-coated well. Twenty-four hours postseeding, a circle circumscribing the cells was measured. The cells were then treated with DMSO (0.1%) as solvent control or test compounds and were allowed to further migrate out for 24 h. The circular cell images were then captured by a Spot camera (Diagnostic Instruments, Sterling Heights, MI) under an inverted microscope (Axiovert, Carl Zeiss, Thornwood, NY). The images were analyzed using the Scion Image software (Scion Corporation, Frederick, MD) to determine the diameters of the circles circumscribing the cell populations. The assay results were reported as the changes in the diameter of the cell circles over the 24-h period (μm/h).
Cell Invasion Assay
Cell invasion assay was carried out using the BD BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA) following the protocol provided by the manufacturer. Briefly, CFPAC-1 cells (2.5 x 10^4 per well) in serum-free DMEM medium were plated into the upper wells of the invasion chambers and treated with vehicle or test compounds at indicated concentrations. In the lower chambers, 5% fetal bovine serum was added as a chemo-attractant. After 24-h incubation at 37°C, the cells that were associated with the top surfaces of the chamber membranes were removed using cotton swabs. The cells that penetrated through the Matrigel-coated membrane to the underside surface of the membrane were stained with Accustain crystal violet solution (Sigma) for 1 min at room temperature. The inside and outside of the chamber were then thoroughly washed with PBS. The membrane was removed and put into a tube containing 100 μL of 30% acetic acid to dissolve the crystal violet in the cells. The number of cells that had invaded through the filter was determined by measuring the absorbance of crystal violet at 490 nm using the Bio-Tek KC4 Signature Bioassay reader.

Results
Identification of Novel uPA Inhibitors Using Chemical Library Screening
We first used a cell-free uPA enzymatic assay to screen a chemical library consisting of 16,000 compounds. This was a direct uPA assay based on the cleavage of a chromogenic peptide substrate by uPA and done in a 96-well plate format. A final concentration of 10 μmol/L in 1% DMSO was used in the initial screen of chemical library compounds. Compounds with 50% or more inhibition on uPA activity compared with DMSO control (final concentration 1%) were selected for evaluation at three concentrations (10, 1, and 0.1 μmol/L) in triplicates. Compounds that showed concentration-dependent inhibitory activity and >50% inhibition at 10 μmol/L were subjected to further evaluation at six concentrations to determine the IC50 values. Interestingly, four of these positive hits share very similar chemical structures (NS47847, NS47844, NS47751, and NS47731 in Fig. 1A). As shown in Table 1, the IC50 values of these compounds against uPA range from 0.2 to 1.3 μmol/L, which is significantly more potent than the known uPA inhibitor, amiloride (Fig. 1B; Table 1: IC50 15 μmol/L). A structure similarity search of our chemical library found a fifth compound (NS47851 in Fig. 1A) that had a similar structure but was not active against uPA in our assays (IC50 >20 μmol/L; Table 1).

To further evaluate the specificity of the positive hits, we tested the compounds in assays for three additional serine proteases that are structurally closely related to uPA. They were tPA, plasmin, and trypsin. As shown in Table 1, these positive hits also strongly inhibited all three proteases with IC50 values in the low-micromolar range. This result indicates that these positive hits are not very specific to uPA and could be merely general protease inhibitors. It has been reported that uPA inhibitors with high specificity generally do not inhibit cancer cell growth (26, 36). However, all four compounds except NS47844 had cytoxicity against the CFPAC-1 pancreatic cancer cells (Table 1), indicating that these compounds might also target cellular proteins other than uPA.

Lead Optimization Using Molecular Modeling
As shown in Table 1, four confirmed actives were identified from library screening and shared similar structural features. Structures of these molecules, NS47751, NS47731, NS47847, and NS47844, are shown in Fig. 1. These molecules showed moderate inhibitory activity against uPA but were not highly specific to uPA. Compound NS47851 identified by a structure similarity search was not active against uPA. To design new uPA inhibitors with improved potency and selectivity, we did docking and molecular modeling studies on the confirmed actives. The compounds were first docked into the active site of the uPA enzyme using the FlexX docking program. As can be seen from Table 1, the FlexX docking scores of the compounds correlate fairly well with their IC50 values against uPA. Compound NS47751 (IC50 0.4 μmol/L) showed the best FlexX score of −20.3. The docking model of NS47751 is shown in Fig. 2A. The oxazolidinone ring of NS47751 shows extensive hydrogen bonding interactions with the active site residues of uPA. As can be seen from Table 1, the FlexX docking scores of the uPA active site is critical for the selectivity of uPA inhibitors (37). We therefore compared the docking models revealed that although the thiophene ring of NS47751 and NS47731 interacts favorably with the active site, extensive hydrogen bonding interactions in the S1 pocket were not present. It has been shown that interactions in the S1 pocket of the uPA active site is critical for the selectivity of uPA inhibitors (37). We therefore compared the docking models of NS47751 and amiloride, an inhibitor of uPA known to have high specificity toward uPA. As shown in Fig. 2B, the docking model of amiloride (FlexX score −33.0) exhibits extensive hydrogen bonding interactions with S1 pocket residues through amidine functional group. Based on these observations, we designed a new molecule (UK122; Fig. 1B) that retains the oxazolidinone pharmacophore but with a phenylamidine substitution to increase the interactions in the S1 pocket. The docking model of UK122 within the active site of uPA is shown in Fig. 2C. The presence of amidino group significantly improves the interactions in the S1 pocket and, therefore, could potentially increase the selectivity against uPA. As can be seen from the docking model, besides the interactions in the S1 pocket, the central oxazolidinone ring system exhibits extensive hydrogen bonding interactions with active site residues His199, Asp194, Ser195, and Gly198. The FlexX docking score for UK122 is −40.1, which is significantly higher than those of the confirmed actives as well as amiloride (Table 1). The docking results were also confirmed using a second crystal
structure of uPA/inhibitor complex (PDB ID 1OWE; ref. 38). The UK122 docking models were very similar between the structures. The FlexX docking score for UK122 in the 1OWE structure is −38.6. We therefore synthesized UK122 and evaluated its biological activity in various assays (see below).

Chemical Synthesis of UK122

The synthetic scheme for UK122 is shown in Fig. 3. The amidino benzaldehyde oxazolidinone derivative (UK122) was prepared from the amidino benzaldehyde salt (4, 39). This intermediate was synthesized from 4-cyanobenzaldehyde (1) via the iminoether (2) with the earlier published procedure (33). The resulted crude azlactone was purified by high-performance liquid chromatography and the benzene-carboximidamide product was isolated as previously described (33). The chemical information of UK122 and other intermediates is given in the Materials and Methods section.

Biological Activity of UK122

UK122 Is a Potent and Selective Inhibitor of uPA. We first evaluated the inhibitory activity of UK122 in cell-free enzymatic assays for uPA, tPA, plasmin, thrombin, and trypsin. The results are presented in Table 1. UK122 exhibited IC₅₀ values of 0.2, 1.0, 2.0, and 2.0 µmol/L for uPA, tPA, plasmin, and trypsin, respectively. UK122 also showed high selectivity towards uPA, with IC₅₀ values for tPA and plasmin being 10-fold higher than for uPA. In addition, UK122 displayed negligible cytotoxicity, with an IC₅₀ value of 120 µmol/L.

Table 1. Protease-inhibitory activities and FlexX docking scores of the positive hits identified from library screen

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ (µmol/L) or % inhibition (concentration, µmol/L)</th>
<th>FlexX scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uPA</td>
<td>tPA</td>
</tr>
<tr>
<td>NS47847</td>
<td>1.3</td>
<td>3.5</td>
</tr>
<tr>
<td>NS47844</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>NS47751</td>
<td>0.4</td>
<td>2.5</td>
</tr>
<tr>
<td>NS47731</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>NS47851</td>
<td>45 (20)</td>
<td>42 (10)</td>
</tr>
<tr>
<td>Amiloride</td>
<td>15.0</td>
<td>9 (10)</td>
</tr>
</tbody>
</table>

Figure 1. Chemical structures of novel uPA inhibitors. A, four positive and one negative (NS47851) hits identified from the Nanosyn chemical library using a cell-free uPA assay. B, UK122 is a new structure derived from NS47751 and amiloride.
The IC$_{50}$ of UK122 in the uPA assay is 0.2 µmol/L, whereas in tPA, plasmin, thrombin, and trypsin, its IC$_{50}$ values are >100 µmol/L. This indicates that UK122 has at least 500-fold selectivity against uPA. The order of potency of UK122 against the enzymes is uPA > trypsin > thrombin, tPA > plasmin. At 100 µmol/L, UK122 inhibited trypsin activity by 38%, thrombin by 8%, and tPA by 5%. UK122 showed no inhibitory activity against plasmin at 100 µmol/L. These results showed that UK122 not only retained the potency of the parent compounds against uPA but also had a significant increase in selectivity.

UK122 Inhibits Cancer Cell Migration and Invasion but not Proliferation. To evaluate the effect of UK122 on the proliferation of pancreatic cancer cells, cell viability was carried out in CFPAC-1 cells in the presence of a serial dilution of the test compounds (starting from 10 to 100 µmol/L). The CFPAC-1 cell line was chosen in this study because it has been shown to express high levels of uPA at both RNA and protein levels and are highly invasive (40–42). At 100 µmol/L of UK122, we did not observe any cell growth or cell morphology change 48 h after treatment. As mentioned above, uPA-specific inhibitors generally do not inhibit cancer cell growth. This result therefore further supports the conclusion that UK122 is a specific uPA inhibitor.

Cell migration and invasion are important processes in many pathologic situations and is a fundamental aspect of the metastatic process. The proteolytic cascade system of plasminogen activation, directed by the uPA and its receptor uPAR, has long been recognized as performing a central role in these processes. We therefore examined the effect of UK122 on the cell migration and invasion of the
pancreatic cancer cells. We first used a microliter-scale radial monolayer motility assay to measure the effects of UK122 on CFPAC-1 cell migration. As shown in Fig. 4, in a 24-h period, 100 μmol/L of UK122 significantly suppressed the cell migration on fibronectin-coated slides by 80% compared with the negative controls. The inhibitory effect of UK122 compounds on cell migration was also dose dependent (Fig. 4). Consistent with the cell-free uPA inhibition results, UK122 showed significantly higher inhibitory activity against CFPAC-1 cell migration than amiloride in all three concentrations tested ($P = 0.005$ between the two treatment groups based on the two-factor with replication ANOVA test). In addition, UK122 significantly reduced the invasiveness of CFPAC-1 cells in a Matrigel-coated Boyden chamber assay. As shown in Fig. 5, at 100 μmol/L, UK122 inhibited CFPAC-1 invasion by 68% in the invasion assay, which is 30% more potent than amiloride at the same concentration ($P = 0.017$).

In summary, using structure-activity information obtained from chemical library screen and molecular modeling studies, we designed and synthesized a novel uPA inhibitor that is highly potent and specific against uPA in cell-free assays and exhibits potent antimigration and invasion activities that are consistent with the cellular function of uPA in cell-based assays.

Discussion

tPA plays a significant role in tumor cell invasion and metastasis. It has become an attractive therapeutic target in a variety of tumor types in recent years (1, 9). One of strategies to suppress tPA activity is to use the synthetic small-molecule inhibitors. In this study, we identified four confirmed actives for the inhibition of tPA by screening a chemical library. On the basis of initial structure-activity analysis of the lead compounds and molecular docking studies, we designed and synthesized UK122, a molecule that has better interactions within the active site of tPA. UK122 as a novel uPA inhibitor contains a pharmacophore (the oxazolidinone ring) that, to our best knowledge, has not been reported previously for uPA inhibitors. This oxazolidinone group forms extensive hydrogen bonding interactions with residues His$^{99}$, Asp$^{194}$, Ser$^{195}$, and Gly$^{198}$ in the active site of uPA. UK122 also contains a phenylamidine group that interacts with residues Asp$^{189}$ and Ser$^{190}$ in the S$_1$ pocket. The Ser$^{190}$ in the S$_1$ pocket has been considered to be one of the important specificity factors because it is present in uPA, plasmin, and trypsin, but is replaced by Ala$^{190}$ in tPA and thrombin (34, 43).

To be therapeutically amenable, it is important that an uPA inhibitor does not inhibit other closely related serine proteases such as tPA, trypsin, thrombin, and potentially plasmin. Both uPA and tPA can activate plasminogen into enzymatically active plasmin, whereas the main biological function of tPA seems to be associated with fibrinolysis of which inhibition can result in serious cardiac problems (44). Therefore, it is especially important that uPA inhibitors do not inhibit enzymes of the fibrinolytic system to avoid the possibility of antifibrinolytic effects. Trypsin is a broad-spectrum serine protease and is involved in many important biological processes such as digestion, defense, development, and blood coagulation (45). It has been shown that trypsin is expressed in various carcinomas and tumor-derived cell lines. Yamashita et al. (46) recently
reported that trypsin has a tumor-suppressive role in cancer progression. Being a ubiquitous protein, plasmin has a physiologic role throughout the body. Collectively, inhibiting these serine proteases would mean serious side effects in vivo. With little inhibitory activity against tPA, trypsin, thrombin, and plasmin, UK122 presents an ideal lead for further development. UK122 structure can be further modified to improve its pharmacokinetic properties. For instance, the highly basic phenylamidine group can be replaced by an aminopyridine group to potentially improve its oral bioavailability. This aminopyridine group would act as a bio-isosteric replacement for the phenylamidine group, thus preserving its interactions with the S1 pocket residues. Similar replacement has been reported to improve the oral bioavailability of the naphthyiamidine-derived uPA inhibitors (47). Our preliminary FlexX docking studies showed that such replacement approach could result in molecules with uPA binding affinities similar to that of UK122 (data not shown).

The cancer metastatic process involves a series of tightly coupled events. These include the detachment of tumor cells from the primary site, migration of the cells accompanied by regulated proteolysis of the extracellular matrix/basement membrane, dissemination of the tumor cells through the vasculature, and, finally, adhesion and proliferation of the cells at a secondary site. Evidence is mounting to show that inhibition of uPA activity can have a dramatic effect on these processes. Invasive pancreatic cancers show increased expression levels of uPA and uPAR, compared with benign pancreatic tumors and normal pancreas (48, 49). Increased expression of uPA and uPAR correlates with advanced-stage pancreatic cancer, and high levels of uPA and uPAR are considered poor prognostic factors for patients with pancreatic cancer (50). In this study, we showed that UK122 could significantly inhibit the migratory and invasive capacity of pancreatic cancer cells in vitro. We are currently in the process of further optimizing UK122 to generate a potential clinical candidate.

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Molecular Cancer Therapeutics

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