Inhibitors of Urokinase Reduce Size of Prostate Cancer Xenografts in Severe Combined Immunodeficient Mice

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Advances in Brief

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Abstract

Proteolytic enzymes are required to mediate tumor cell invasion and metastasis. The urokinase plasminogen activator (uPA) is commonly overexpressed by many human cancers. Therefore, uPA is a logical target to inhibit cancer invasion and metastasis. However, uPA inhibitors also reduce tumor growth. We used a mutated form of plasminogen activator inhibitor type 1 to conform a correlation between the inactivation of uPA and tumor size; we have compared these results with the action of p-aminobenzamidine and amiloride, known inhibitors of uPA. Our results show that blocking uPA by uPA inhibitors reduces tumor size in experimental animals. Our molecular simulation of docking inhibitors to the urokinase reveals that all tested small molecule inhibitors bind in proximity of uPA’s specificity pocket, a critical site for future search of novel anticancer uPA inhibitors.

Introduction

Proteolytic enzymes are required to mediate tumor cell invasion of adjacent tissues, initiating the metastatic process. uPA2 is commonly overexpressed by many different human cancers (1). This enzyme activates plasminogen to plasmin, a protease that attacks a broad spectrum of proteins. It also activates a latent form of collagenases, creating an area of high proteolytic activity in the vicinity of primary site of origin (1–4).

Increased uPA activity has been noticed in human prostatic cancer cell lines with metastatic behavior (5). Moreover, animals injected with prostatic cancer cells containing high amounts of uPA developed metastatic lesions earlier and more frequently than animals injected with the same cell expressing lower amounts of uPA (6). Additionally, it has been reported that uPA activity is increased in metastatic sites when compared with primary prostate tumors in experimental animals (7).

Increased activity of uPA on the cancer cells has been traditionally associated with the ability of cancer cells to invade and to metastasize. Therefore, it is reasonable to consider inhibition of uPA as a method for limiting cancer invasion and metastasis. However, using competitive inhibitor of uPA, Billstrom et al. (8) showed that p-aminobenzamidine caused dose-dependent inhibition of uPA activity and a decreased rate of tumor growth in mice instead of reducing metastasis. Amiloride, another uPA inhibitor, reduces the rate of tumor growth of the tumor cells in the hepatomas and intestinal carcinomas (9–11).

The mechanism of action leading to the decrease in tumor growth rate is not clear. Proteolysis is responsible for degradation of proteins, invasion, or metastasis, but not for the proliferate properties of the cancer. It is difficult to envision that the diminishing size of tumor is due simply to blocking of uPA activity of cancer cells. Instead, the inhibitors may be interacting with the elements of the extracellular matrix, which also express uPA. For example, the neovascular bed surrounding tumors has been reported to contain high amounts of uPA and its receptor. Binding of proteolytically inactive ligand to uPA receptor reduces the amount of uPA on the surface of capillary endothelial cells and reduces tumor growth (12). It is also possible that p-aminobenzamidine and amiloride possess anticancer activity based on a mechanism that is not related to uPA inhibition. As an example, amiloride has been postulated to be an anticancer agent based on cellular acidification (13).

To clarify the mechanism of decreased tumor growth rate, we used human prostate cancer cells that either express (DU145) or do not express (LnCaP) uPA. We used a novel inhibitor of uPA with an extended half-life (14, 15) to determine whether these proteins cause a reduction of tumor growth in these tumor systems. Because PAI-1 has no other known activity than blocking uPA, a decrease in tumor growth would support the hypothesis that the previously described effect of amiloride and p-aminobenzamidine is mediated via the uPA inhibitory effects.

We hypothesize that amiloride, p-aminobenzamidine, and PAI-1 are anticancer agents and that they are acting on tumor indirectly by reduction of uPA activity. Our results showed that all inhibitors reduce size of tumors, regardless of whether they express or do not express uPA, and all of them are binding to or are in the proximity of the S1 position of uPA. Mutated PAI-1 with no anti-uPA activity showed no anticancer activity.

Materials and Methods

Expression and Purification of Mutated PAI-1. The mutants of PAI-1, BH14, P14Arg, and GATE were expressed in the Escherichia coli strain BL21 (DE3) pLys S (Fig. 1). The bacterial cells transformed with pSELPA1-1 BH14 plasmid for PAI-1 (half-life t1/2 = 160 h), GATE plasmid for PAI-1 (half-life t1/2 = 6 h), and pSELPA1-1 P14Arg plasmid for inactive PAI-1 were grown in LB broth to an absorbance at 650 nm of 0.5. PAI-1 production was induced by addition of 1 mm isopropyl β-d-thiogalactoside, and growth was continued for 2 h. All subsequent steps were done in a cold room. Cells were harvested by centrifugation, washed once, and resuspended in 5 ml of buffer [10 mm MgCl2, 1 mm EDTA, and 0.025 Tris-HCl (pH 7.5)]. Bacterial cells were disrupted by snap freezing in liquid nitrogen, and the cell debris was removed by centrifugation. The supernatant was passed over a Sephacryl S-200 column (2.5 × 100 cm) equilibrated with buffer [0.15 M NaCl and 0.025 M Tris-HCl (pH 7.5)]. Fractions from this and subsequent purifications were analyzed by electrophoresis to detect the Mr, 43,000 fraction that is characteristic for PAI-1. Fractions containing PAI-1 were pooled and applied to a hydroxylapatite column (2.5 cm × 2 cm). This column was washed and subsequently eluted with a 200-ml linear gradient in the range 0.13–0.25 M of sodium phosphate (pH 6.6). Fractions containing PAI-1 were pooled, diluted 1:1, and applied to...
heparin-agarose column (1.5 × 2 cm) equilibrated with 0.1 M sodium phosphate (pH 6.6). The column was washed and eluted with the same buffer.

**Amidolytic Assay of Anti-uPA Activity.** Tris (50 mM) with 0.01% Tween 80, 0.01% PEG 8000 (pH 8.8), and 10 KIU/ml sterile aprotinin (Sigma Chemical Co., St. Louis, MO) was incubated with 1 μg of uPA and decreasing amounts of inhibitor (initially 100 μg/ml) for 15 min; 100 μl of this mixture was incubated in 96-well microplates with 50 μl of Spectozyme UK 80, 0.01% PEG 8000 (pH 8.8), and 10 MU/ml sterile aprotinin (Sigma) with 0.1 ml of tumor cell suspension in Matrigel (approximately 5 × 10⁶ cells) and incubated at 37°C for 4 h. Absorbance is inversely proportional to the uPA inhibitory activity (17).

**Cell Line and Cell Culture Conditions.** The human prostate cancer cell lines DU145 and LnCaP were purchased from American Type Culture Collection of Ohio's IACUC committee. Retired breeders, SCID mice, were purchased from the National Cancer Institute. Animals were injected with 0.1 ml of tumor cell suspension in Matrigel (approximately 5 × 10⁶ cells) in both rear flanks.  p-Aminobenzamidine and amiloride (200 mg/kg body weight) were given to animals with established tumors in drinking water. The control group received water only. PAI-1(s) were injected by tail vein injections at 5-day intervals (2280 μg/kg of body weight). Tumor volume (height × length × width) was determined twice a week. Mice were euthanized at the end of the study. Comparison of tumor volume was performed by two-sample (control versus treated) unequal-variance t test (Welch-Satterthwaite approximation) for significance level P < 0.05 using Prophet 227 computer program (NIH/BBN Systems and Technologies, http://www.prophet.bbn.com).

**Search for uPA Inhibitors by Ludi Module.** The coordinates of the crystal structure of the low molecular weight, nonglycosylated human uPA were obtained from Dr. C. Phillips (Oxford Centre for Molecular Sciences, University of Oxford, Oxford, United Kingdom) (19). Selection of uPA inhibitors by Ludi module was done using InsightII Ludi program. Ludi predicts protein-ligand interactions through the use of interaction sites. For each atom or functional group of the protein that is capable of participating in a nonbonded contact, a set of interaction sites is generated. This set of interaction sites encompasses the range of suitable positions for a ligand atom or functional group involved in a putative interaction. Ludi distinguishes four types of interaction sites: H-donor, H-acceptor, lipophilic-aliphatic, and lipophylic aromatic. The Ludi program searches in a 12-Å radius from the center of active site (S1), with a maximum root mean square of 0.6 (root mean square value expressed as kcal mol⁻¹ Å⁻¹; Ref. 20).

**Docking Calculations.** To conform Ludi calculations in the S1 site and allow flexibility in the S1 position, the docking calculations were done for the selected inhibitors. The basis of the docking calculation was the calculation of the potential energy for a given configuration of two molecules. The evaluation of the nonbonded energy between two molecules is used as a guide for the preferred orientation of one molecule in relation to the other. The source of parameters used for this calculation is the force field file included in the commercial software package from Biosym and used in conjunction with a suitable program. We have used the consistent valence force field approach (20). Typical calculations usually include a search within a 12-Å radius from the S1 site, at least 50 different starting orientations, and up to 2000 iterations for each structure to allow convergence of the minimization process.

**Results and Discussion**

**Inhibition of uPA Activity by p-Aminobenzamidine, Amiloride, and PAI-1(s).** We have tested anti-uPA activity for the following uPA inhibitors: p-aminobenzamidine, amiloride, and PAI-1(s). Anti-

### Table 1 uPA inhibitory activity of selected substances

<table>
<thead>
<tr>
<th>Substance</th>
<th>IC₅₀</th>
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<tr>
<td>p-Aminobenzamidine</td>
<td>180 μM</td>
</tr>
<tr>
<td>Amiloride</td>
<td>150 μM</td>
</tr>
<tr>
<td>PAI-1 1B14 mutant</td>
<td>No activity</td>
</tr>
<tr>
<td>PAI-1 PI4Arg mutant</td>
<td>78 μM</td>
</tr>
<tr>
<td>PAI-1 GATE mutant</td>
<td>80 μM</td>
</tr>
</tbody>
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uPA activity was detected for p-aminobenzamidine, amiloride, and PAI-1 mutants 1B14 and GATE, but not for PAI-1 mutant P14Arg (Table 1).

Inhibition of Tumor Growth. We have found that all inhibitors of uPA reduced tumor size in experimental animals. The most prominent effect was observed in p-aminobenzamidine, in which we have observed complete remission of tumors in some animals (Fig. 2A). Complete regression of tumors using this agent has not been reported previously. It must be noted that the p-aminobenzamidine is light sensitive and degrades in water solution. In our experiments, it was prepared daily and kept in a lightproof container. Consequently, the effective concentration of the inhibitor in our experiments was probably higher than in others. Therefore, it is possible that the complete regression of tumors observed in our experiments could be attributed to this fact. Reduction of tumor size was observed when amiloride was used. However, the concentration was toxic to animals, and this inhibitor was eliminated from further study.

The mechanism of reduction of tumor size by uPA inhibitors remains somewhat puzzling. One possible explanation is that uPA associated with cancer cells binds inhibitors and is later internalized into the cancer cells. Both p-aminobenzamidine and amiloride could be accumulated in such a concentration that they are toxic to the cancer cells. PAI-1 should not lead to similar toxicity. Therefore, we use PAI-1 proteins with extended half-lives for assessment of these proteins on tumor growth. The only known function of PAI-1 is inhibition of uPA. However, due to its short half-life (1–2 h in 37°C), it has not been used yet in anticancer therapy. Recently, mutated forms of PAI-1 with an extended half-life (up to 160 h) have been developed, which opened the possibility of using them as anticancer agents.

We observed a reduction in tumor size for the following PAI-1 mutants: 1B14 mutant and GATE mutant, but not for P14Arg (Thr 333→Arg 333) mutant (Fig. 2B). This particular mutant (P14Arg), although structurally identical to the wild form of plasminogen activator inhibitor, does not inhibit uPA at all. These long-acting PAI-1s strongly support the hypothesis that all uPA inhibitors indeed diminish tumor growth by a common mechanism; i.e., by a decrease of uPA activity. Furthermore, growth of tumors not expressing uPA was also inhibited (Fig. 3). This supports the hypothesis that uPA inhibitors are interacting with the elements of the extracellular matrix that express uPA rather than directly with the cancer cells.

Small molecular uPA inhibitors are potent anticancer agents. Unfortunately, those we have used are toxic for humans in concentrations that inhibit uPA. Therefore, new inhibitors must be found. This can be achieved by computerized molecular simulation of uPA inhibition by small molecules. However, it must be determined whether a common binding site of uPA inhibitors exists on the uPA molecule.

Molecular Simulation of uPA Inhibition by Small Molecules. We have selected four known uPA inhibitors for a simulation: penicillin, p-aminobenzamidine, amiloride, and B623. Ampicillin was used. However, the concentration was toxic to animals, and this inhibitor was eliminated from further study.

The statistical differences are as follows: *, insignificant between groups for P < 0.05; **, significant between control, p-aminobenzamidine, and amiloride; and ***, significant between control, P14Arg, and GATE, 1B14 for P < 0.05.

Table 2. Calculated determinants of uPA inhibition

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calculated $K_i$</th>
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<tr>
<td>Ampicillin$^a$</td>
<td>74 μM</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>37 mM</td>
</tr>
<tr>
<td>p-Aminobenzamidine</td>
<td>257 μM</td>
</tr>
<tr>
<td>Amiloride</td>
<td>151 μM</td>
</tr>
<tr>
<td>B623</td>
<td>26 μM</td>
</tr>
</tbody>
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$^a$Ampicillin does not inhibit uPA; however, according to molecular simulation, it binds to uPA in a distant site from the S1 position and with a higher affinity than carbenicillin.
used as a control to determine whether a common binding site of uPA exists for all of these inhibitors. Ampicillin is closely related structurally to penicillin, but does not possess any anti-uPA activity. In general, we found good agreement between the published inhibitory capacity expressed by IC_{50} and by calculated determinant of inhibitory ability, K_{i} (Table 2; Refs. 21 and 22).

Docking calculations have shown that all of these inhibitors bond in close proximity to the S1 specificity pocket and catalytic triad (His 57, Asp 102, Ser 195) of uPA (Fig. 4). More precisely, we have found that with penicillin, p-aminobenzamide, and amiloride, these small molecules are located inside the specificity pocket, forming hydrogen bonds with amino acids of the catalytic triad and/or with one or more of the amino acids (Asp 189, Gly 218, Tyr 228) considered to be essential in the recognition of PAI-1 by uPA (23). This specificity pocket is responsible for binding the side chain of arginine from PAI-1 and plasminogen. Therefore, any inhibitor located in this position prevents the penetration of arginine into the specificity pocket, a step in which the substrate is recognized, and consequently prevents proteolytic cleavage of plasminogen and its activation to plasmin.

B623 forms hydrogen bonds with Thr 56, and instead of being inserted in the S1 specificity pocket, it is located outside of it. However, this location is in close proximity to the catalytic triad, and Asp 189, Gly 218, Tyr 228, and B623 create a space hindrance that prevents interaction between uPA and plasminogen. Carbenicillin, as the weakest uPA inhibitor, binds to uPA in peripheral regions of the S1 pocket and is expected to interfere weakly with uPA substrates. Ampicillin binds to uPA in a distant site from the S1 pocket and the catalytic triad, and therefore does not interfere with the binding of uPA to its substrates such as plasminogen or PAI-1.

There are no crystallographic data on the localization of uPA inhibitors within the uPA molecule. However, p-aminobenzamide, which inhibits other serine proteases, has been found near the S1 position in trypsin in the same orientation and close to a position found in uPA by computational molecular simulations (20). This fact and the biochemical data presented above support the conclusion that the S1 position in uPA is critical for control of uPA activity by small molecule inhibitors. Furthermore, ampicillin, which does not express anti-uPA activity, has been found in a distant site from the catalytic triad and S1 pocket. This fact confirms the suitability of molecular simulation in the prediction of inhibitory activity of urokinase. The inhibitors described above are probably unlikely candidates to be used in anticancer therapy in humans due to their weak inhibitory activity or toxicity. However, they provide a good model in the search for novel and high-affinity S1-directed uPA inhibitors.

All of the inhibitors tested are inhibitors of uPA. The mutants of PAI-1 inhibit uPA with high affinity and specificity. However, small molecule inhibitors could inhibit other proteases if they are structurally related to the active site of uPA. Therefore, we cannot exclude the possibility, even if slight, that other proteases were inhibited also.

We conclude that small molecule inhibitors and protein uPA inhibitors reduce tumor growth presumably by inhibiting uPA activity in the extracellular matrix. Reduction of uPA activity is achieved by blocking the S1 active site of uPA. This is a common binding site for all tested inhibitors including PAI-1, which in addition to this site interacts with others. Future searches for novel, high-affinity S1-directed uPA inhibitors should concentrate on this site.

Acknowledgments

uPA was a generous gift from Dr. R. Hart (American Diagnostica, Inc., Greenwich, CT). The PAI-1 mutants (B14, P14Arg, and GATE) were generous gifts from Dr. D. Ginsburg (University of Michigan, Ann Arbor, MI) and Dr. R. D. Gerard (University of Texas), respectively. We thank Dr. S. H. Selman (Medical College of Ohio) for helpful discussions and for reading this manuscript.

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_Cancer Res_ 1997;57:559-563.

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