Inhibition of urokinase has been shown to slow tumor growth and metastasis. To utilize structure-based drug design, human urokinase was re-engineered to provide a more optimal crystal form. The redesigned protein consists of residues Ile^{16}-Lys^{243} (in the chymotrypsin numbering system; for the urokinase numbering system it is Ile^{159}-Lys^{404}) and two point mutations, C122A and C123A. This pocket could be utilized in future drug design of more potent nonpeptidic urokinase inhibitors.

Cancer cell invasion, the spread and growth of tumor metastases, is a primary cause of mortality and morbidity of malignancy (2), and this invasion requires the degradation of basement membranes and other extracellular protein structures. Urokinase has been shown to be strongly associated with tumor cells (3) and to play a role in basement membrane degradation via a cascade mechanism involving activation of plasminogen and the metalloproteases (4–6). Furthermore, inhibitors of urokinase have been reported to slow tumor metastasis as well as growth of the primary tumor (7–15). These inhibitors include the small molecules 4-iodo benzo(b)thiophene-2-carboxamidine and phenylguanidine at 2.0–2.2 Å resolution. All three inhibitors bind at the primary binding pocket of urokinase. The structures of amiloride and 4-iodo-benzo(b)thiophene-2-carboxamidine also reveal that each of their halogen atoms are bound at a novel structural subsite adjacent to the primary binding pocket. This site consists of residues Gly^{218}, Ser^{146}, and Cys^{191}–Cys^{220} and the side chain of Lys^{143}. This pocket could be utilized in future drug design efforts. Crystal structures of these three inhibitors in complex with urokinase reveal strategies for the design of more potent nonpeptidic urokinase inhibitors.

In this crystal form (if they were not blocked by the irreversible covalent inhibitor). Hence, although this system may be used for modeling of small molecule urokinase inhibitors, it may not provide an ideal system for structure-based drug design. A crystal system exists for urokinase, although it does not fully encompass the preferred properties outlined above.

Human low molecular weight (LMW) urokinase has been crystallized in complex with the peptidic inhibitor Glu-Gly-Arg-chloromethyl ketone (1). This structure reveals the geometry of the urokinase active site as well as the orientation of a peptide inhibitor in the substrate-binding groove. However, the LMW urokinase crystals diffract to lower resolution (2.5 Å resolution, synchrotron radiation; 3.0 Å resolution, rotating anode source) and utilize co-crystallization to achieve the target-ligand complex. In addition, the active site is in close contact with another molecule because of a noncrystallographic 2-fold axis near the active site. This interaction could limit minor ligand induced conformational shifts and perhaps distort the active site conformation. Furthermore, the noncrystallographic and crystallographic packing effectively blocks the active site such that it would be difficult to diffuse small molecules into the active site in this crystal form (if they were not blocked by the irreversible covalent inhibitor). Hence, although this system may be used for modeling of small molecule urokinase inhibitors, it may not provide an ideal system for structure-based drug design. Therefore, to design an anti-cancer therapeutic, a new crystal form of human urokinase was sought to facilitate the application of structure-based drug design. The strategy utilized protein engineering and information from the reported LMW urokinase structure to design an altered protein sequence to yield a new crystal form.
The new form of urokinase, micro-urokinase, crystallizes under conditions very similar to the low molecular weight form (1), although crystal packing and data quality are very different. This new crystal form contains a monomer in the asymmetric unit and diffracts to ultra-high resolution (d_{min} = 1.03 Å). In addition, this crystal form has an open active site permitting direct diffusion of compounds into the apo-crystals and is therefore ideal for providing precise structure determinations for urokinase ligand complexes by the soaking technique.

The re-engineered crystal system and soaking technique were utilized to determine the co-crystal structure of urokinase in complex with a series of small molecule inhibitors at 2.0 or 2.2 Å resolution. Two of these inhibitors, amiloride (24), and B428 (25, 26), have been shown to reduce tumor size and metastasis (8, 12–15), whereas the effect of the third, phenylguanidine (27) has not been reported to date. These complex structures were completed to determine the binding orientation of each compound to urokinase. This information in turn may be utilized to design molecules of increased potency toward discovery of an anti-cancer therapeutic compound.

**EXPERIMENTAL PROCEDURES**

**Recombinant Micro-urokinase**—Micro-urokinase was engineered by polymerase chain reaction manipulations using a human urokinase cDNA as a template (28). The C279A and N302Q mutations were made by the method of polymerase chain reaction based site-directed mutagenesis. Urokinase native leader sequence was fused directly to Bsu36I by polymerase chain reaction. This product was ligated to a baculovirus transfer vector pVJP10z (29). The final expression vector sequence was confirmed by DNA sequencing.

The pVJP10z-micro-urokinase vector was transfected into SF9 cells by the calcium phosphate precipitation method using the BaculGold kit from Pharmingen (San Diego, CA). Single recombinant virus expressing micro-urokinase was plaque purified by standard methods, and a large stock of the virus was prepared. Large scale expression of micro-urokinase was performed in suspension in High-Five cells, (Invitrogen, San Diego, CA) growing in Excel 405 serum free medium (JRH Biosciences, Lenexa, KS) at 27 °C. Urokinase activity in the supernatant was measured by amidolysis of the chromogenic substrate H-D-pyroglutamyl-Gly-L-Arg-p-nitroanilide (S2444; Helena Laboratories, 30). Briefly, 0–50 μM concentration of inhibitors were tested a/p 6 mg/ml urokinase or micro-urokinase toward the chromogenic substrate, S2444. The urokinase was eluted micro-urokinase protein was captured onto Sartorius membrane and amide (10 mM), benzamidine (5 mM), and EDTA (1 mM) were added to the starting material for purification. Protease inhibitors, iodoacetamide (30), benzamidine (5 mM), and EDTA (1 mM) were added. The final expression vector sequence was confirmed by 50 μM concentration of inhibitors were testedamiolysis of the chromogenic substrate H-D-pyroglutamyl-Gly-L-Arg-p-nitroanilide (S2444; Helena Laboratories, 30). Briefly, 0–50 μM concentration of inhibitors were tested a/p 6 mg/ml urokinase or micro-urokinase toward the chromogenic substrate, S2444. The urokinase was eluted micro-urokinase protein was captured onto Sartorius membrane and amide (10 mM), benzamidine (5 mM), and EDTA (1 mM) were added to the starting material for purification. Protease inhibitors, iodoacetamide (30), benzamidine (5 mM), and EDTA (1 mM) were added. The final expression vector sequence was confirmed by DNA sequencing.

The pVJP10z-micro-urokinase vector was transfected into SF9 cells by the calcium phosphate precipitation method using the BaculGold kit from Pharmingen (San Diego, CA). Single recombinant virus expressing micro-urokinase was plaque purified by standard methods, and a large stock of the virus was prepared. Large scale expression of micro-urokinase was performed in suspension in High-Five cells, (Invitrogen, San Diego, CA) growing in Excel 405 serum free medium (JRH Biosciences, Lenexa, KS) at 27 °C. Urokinase activity in the supernatant was measured by amidolysis of the chromogenic substrate H-D-pyroglutamyl-Gly-L-Arg-p-nitroanilide (S2444; Helena Laboratories, 30). Briefly, 0–50 μM concentration of inhibitors were tested a/p 6 mg/ml urokinase or micro-urokinase toward the chromogenic substrate, S2444. The urokinase was eluted micro-urokinase protein was captured onto Sartorius membrane and amide (10 mM), benzamidine (5 mM), and EDTA (1 mM) were added. The final expression vector sequence was confirmed by DNA sequencing.

The pVJP10z-micro-urokinase vector was transfected into SF9 cells by the calcium phosphate precipitation method using the BaculGold kit from Pharmingen (San Diego, CA). Single recombinant virus expressing micro-urokinase was plaque purified by standard methods, and a large stock of the virus was prepared. Large scale expression of micro-urokinase was performed in suspension in High-Five cells, (Invitrogen, San Diego, CA) growing in Excel 405 serum free medium (JRH Biosciences, Lenexa, KS) at 27 °C. Urokinase activity in the supernatant was measured by amidolysis of the chromogenic substrate H-D-pyroglutamyl-Gly-L-Arg-p-nitroanilide (S2444; Helena Laboratories, 30). Briefly, 0–50 μM concentration of inhibitors were tested a/p 6 mg/ml urokinase or micro-urokinase toward the chromogenic substrate, S2444. The urokinase was eluted micro-urokinase protein was captured onto Sartorius membrane and amide (10 mM), benzamidine (5 mM), and EDTA (1 mM) were added. The final expression vector sequence was confirmed by DNA sequencing.

The pVJP10z-micro-urokinase vector was transfected into SF9 cells by the calcium phosphate precipitation method using the BaculGold kit from Pharmingen (San Diego, CA). Single recombinant virus expressing micro-urokinase was plaque purified by standard methods, and a large stock of the virus was prepared. Large scale expression of micro-urokinase was performed in suspension in High-Five cells, (Invitrogen, San Diego, CA) growing in Excel 405 serum free medium (JRH Biosciences, Lenexa, KS) at 27 °C. Urokinase activity in the supernatant was measured by amidolysis of the chromogenic substrate H-D-pyroglutamyl-Gly-L-Arg-p-nitroanilide (S2444; Helena Laboratories, 30). Briefly, 0–50 μM concentration of inhibitors were tested a/p 6 mg/ml urokinase or micro-urokinase toward the chromogenic substrate, S2444. The urokinase was eluted micro-urokinase protein was captured onto Sartorius membrane and amide (10 mM), benzamidine (5 mM), and EDTA (1 mM) were added. The final expression vector sequence was confirmed by DNA sequencing.

The pVJP10z-micro-urokinase vector was transfected into SF9 cells by the calcium phosphate precipitation method using the BaculGold kit from Pharmingen (San Diego, CA). Single recombinant virus expressing micro-urokinase was plaque purified by standard methods, and a large stock of the virus was prepared. Large scale expression of micro-urokinase was performed in suspension in High-Five cells, (Invitrogen, San Diego, CA) growing in Excel 405 serum free medium (JRH Biosciences, Lenexa, KS) at 27 °C. Urokinase activity in the supernatant was measured by amidolysis of the chromogenic substrate H-D-pyroglutamyl-Gly-L-Arg-p-nitroanilide (S2444; Helena Laboratories, 30). Briefly, 0–50 μM concentration of inhibitors were tested a/p 6 mg/ml urokinase or micro-urokinase toward the chromogenic substrate, S2444. The urokinase was eluted micro-urokinase protein was captured onto Sartorius membrane and amide (10 mM), benzamidine (5 mM), and EDTA (1 mM) were added. The final expression vector sequence was confirmed by DNA sequencing.

The pVJP10z-micro-urokinase vector was transfected into SF9 cells by the calcium phosphate precipitation method using the BaculGold kit from Pharmingen (San Diego, CA). Single recombinant virus expressing micro-urokinase was plaque purified by standard methods, and a large stock of the virus was prepared. Large scale expression of micro-urokinase was performed in suspension in High-Five cells, (Invitrogen, San Diego, CA) growing in Excel 405 serum free medium (JRH Biosciences, Lenexa, KS) at 27 °C. Urokinase activity in the supernatant was measured by amidolysis of the chromogenic substrate H-D-pyroglutamyl-Gly-L-Arg-p-nitroanilide (S2444; Helena Laboratories, 30). Briefly, 0–50 μM concentration of inhibitors were tested a/p 6 mg/ml urokinase or micro-urokinase toward the chromogenic substrate, S2444. The urokinase was eluted micro-urokinase protein was captured onto Sartorius membrane and amide (10 mM), benzamidine (5 mM), and EDTA (1 mM) were added. The final expression vector sequence was confirmed by DNA sequencing.
contains three ordered sulfate ions, and two alternate side chain conformations located at the active site. All backbone atoms are well defined in the final model with atomic B-factors of 20 Å^2. The B428 model is refined to 2.0 Å resolution with a R_free of 20.9% and an R_factor of 22.1%. The amiloride model is refined to 2.2 Å resolution with a R_factor of 21.5% and a R_free of 29.1%. The phenylguanidine model is refined to 2.0 Å resolution with a R_factor of 18.9% and a R_free of 22.1%. Data for the complex structures were of quality comparable to that of native structures collected under the same conditions on a rotating anode source.

### RESULTS

**Redesign of LMW Urokinase**—To redesign the LMW urokinase sequence for the purpose of improving the crystal characteristics, the LMW urokinase coordinate file (Protein Data Bank entry 1LMW) was examined for sequences of excessively high B-factor, suggesting areas of disorder. The hypothesis is that areas of high disorder in the structure may contribute to the overall disorder of the crystals and/or may interfere with optimal crystal packing. The LMW urokinase structure consists of residues 136–158 of the A-chain and 159–411 of the B-chain connected by a disulfide bridge between Cys148 and Cys279 (urokinase numbering)^2. The B-chain corresponds to the serine protease domain, whereas the 21 residue A-chain lacks the kringle and epidermal growth factor domains present in full-length urokinase. The A-chain is reported to be an area of high disorder (1), and examination of the protein data bank entry 1LMW reveals that residues 148–155 of the A-chain have an average B-factor of 64 Å^2 ranging from 26 Å^2 for the disulfide-linked sulfur of residue Cys148 to 110 Å^2 for Pro155. The very high B-factors for the A-chain confirm this observation. Consequently, the A-chain was removed as a first step in the redesign. Furthermore, to remove the resultant free thiol on the B-chain, Cys148 was mutated to an alanine.

Further examination of the LMW urokinase coordinate file indicates a second area of disorder consisting of residues 405–411 of the C terminus where the average B-factor is 147 Å^2. Residues 407–411 represent a five residue extension in urokinase relative to other trypsin-like serine proteases. However, because residues 405–406 also have high atomic B-factors, the entire 405–411 segment was removed. The final potential site for disorder is the glycosylation site at residue 302. This glycosylation site was removed by an N302Q mutation to facilitate expression of the glycosylation-free protein in baculovirus. Hence, the re-engineered urokinase (micro-urokinase) consists of residues Ile159–Lys404 (Ile146–Lys243 chymotrypsin numbering system) with the two point mutations C279A (C122A) and N302Q (N145Q).

**Micro-urokinase Crystal Packing**—Micro-urokinase crystallizes with a monomer in the asymmetric unit (P2_12_12_1), whereas the LMW urokinase crystal form has a dimer in the asymmetric unit (R3) with intimate contacts at the substrate-binding site. Specifically, in LMW urokinase, residues 94–101 from each molecule (chymotrypsin numbering system as aligned by Ref. 1)^2 form a series of intermolecular main chain hydrogen bonds resulting in an extended four stranded β-sheet (1). From the LMW urokinase structure, it was seen that this loop decreases the size of the S1 pocket relative to that at the substrate-binding site of other serine proteases such as thrombin, Factor Xa and tissue plasminogen activator (1, 37–39). Hence, this loop provides a critical structural feature of the substrate-binding groove. However, because of the close crystal contact at this site in the LMW urokinase crystals, the possibility existed that the structure of the substrate-binding site may be distorted or conformationally restricted. The new crystal form of micro-urokinase lacks the close crystal contact present in LMW urokinase, and an overlay of the two structures indicates that the conformation of this loop is essentially identical in the two crystal forms. Consequently, it is unlikely that packing in either crystal system affects the conformation of this loop and the resultant shape of the S1 pocket, although the more open micro-urokinase packing may allow for inhibitor-induced conformational shifts.

Examination of crystal packing at the A-chain-binding cleft gives insight into why micro-urokinase yields different lattice packing and better diffracting crystals (a sample of the final model of residues 265–404 electron density map at 1.5 Å resolution is shown in Fig. 1A). In LMW urokinase, the A-chain binds in a cleft composed of residues 25–29, 116–122, and 201–208. In the crystal structure of micro-urokinase, there is no A-chain, and the A-chain-binding cleft is partially occupied by a symmetry related molecule. Specifically, a hydrophobic loop extending from 144 to 150 in the symmetry related molecule is directly bound at the A-chain site such that Tyr149–OH of the loop is involved in two hydrogen bonds at the A-chain cleft (Ser202-N and Ser135-O). In LMW urokinase, the A-chain blocks this set of interactions. Thus, in micro-urokinase, removal of the A-chain exposes a new “binding site” for the 144–150 loop of another micro-urokinase molecule permitting a new lattice to form. This interaction at the A-chain cleft probably contributes to the improved crystal quality by being both a site of nucleation as well as by facilitating close contact between adjacent molecules.

### Table I

| Data quality statistics | Complete | I/σ | R_sym (square) | R_factor | R_free |
|------------------------|----------|-----|----------------|----------|--------|
| %                      |          |     |                |          |        |
| Native                 | 96.6     | 15  | 0.075          | 19.1     | 21.8   |
| 1.53–1.50 Å            | 95.3     | 9   | 0.113          | 21.2 (1.57–1.50) | 25.8 (1.57–1.50) |
| B428                   | 89.9     | 16.8| 0.083          | 20.9     | 27.7   |
| 2.05–2.0 Å             | 88.4     | 5   | 0.203          | 20.0     | 29.4   |
| Amiloride              | 99.8     | 12.4| 0.108          | 21.5     | 29.1   |
| 2.3–2.2                | 99.8     | 4.3 | 0.358          | 19.1     | 26.9   |
| Phenyl guanidine       | 90.3     | 12.5| 0.086          | 18.9     | 22.1   |
| 2.06–2.00              | 94.2     | 4.5 | 0.254          | 24.3     | 24.8   |

^2 The urokinase numbering system is used for discussion of the sequence re-engineering work, whereas the chymotrypsin numbering system as aligned by Ref. 1 is used for discussion of the serine protease domain structure for micro-urokinase.
Micro-urokinase and LMW urokinase are nearly identical in structure (overall rms deviation for main chain atoms, 0.8 Å) with one significant structural change near a site of re-engineering. As discussed above, removal of the A-chain results in an empty cavity. One loop (201–210) forming this site undergoes a conformational shift relative to LMW urokinase with rms deviation (main chain) ranging from 1.1 to 1.8 Å with the largest shift being for Arg 206. However, although this loop is involved in a crystal packing interaction, the conformation of the 144–150 of the symmetry related molecule is the same for both micro-urokinase and LMW urokinase. Other sites of variation include the flexible loop at residues 37–37D (rms deviation main chain, 1.7–3.5 Å), residues 17–19 (rms deviation main chain, 1.1–2.1 Å) and residues 185B–186 (rms deviation main chain, 1.7 Å). All areas were of high b-factor in the LMW urokinase structure (b-factor > 60–90 Å²) but of significantly lower b-factor in the micro-urokinase structure (b-factor < 20 Å²) with the exception of residues 17–19, which were of low b-factors in both structures. The 17–19 segment was clearly defined in the final 2F₀ − Fc electron density maps of micro-urokinase and is not near any re-engineered sites. Residues 185B–186 were remodeled in the higher-resolution structure. In the lower resolution LMW urokinase structure, Trp 186 was exposed to solvent and Gln 185B was buried. The higher resolution data clearly placed Trp 186 in the protein core with Gln 185B exposed to solvent.

Active Site of Native Micro-urokinase—Like the overall molecular fold, the active sites of LMW urokinase and micro-urokinase are nearly identical (rms deviation, <0.8 Å). The higher resolution data did not depict any large side chain movements relative to LMW urokinase but did show an alternate side chain conformation for two residues (Fig. 1B and C) in addition to a bound sulfate ion (see Fig. 3C). The sulfate ion is bound near the oxyanion hole (40), where O1 is accepting hydrogen bonds from Gly 193-NH (2.8 Å) and Ser 195-OH (2.8 Å), whereas O2 is accepting a hydrogen bond from His 145-Nε2 (2.8 Å). Hence, the higher resolution data revealed more structural details at the active site.

In Fig. 1B, native 1.5 Å 2F₀ − Fc (contoured at 1 σ) and F₀ − Fc (contoured at 3 σ) electron density maps depict that the side chain of His 99 is in multiple conformations. These maps were calculated before the alternate conformation had been included in the model. As presented in Fig. 1B, one His 99 conformation is identical to that observed with LMW urokinase. In this conformation, His 99-Nε1 accepts a hydrogen bond from Tyr 206-OH (2.9 Å). In the alternate conformation (modeled into the green positive peak; Fig. 1B), the His 99 imidazole is rotated approximately 90° about the Cβ-Cγ bond resulting in a different hydrogen bonding pattern. Here, His 99-Nε1 can donate a hydrogen bond to Asp 102-Oε1 (3.2 Å). The His 99 side chain forms part of both the S2 and S3 pockets. Hence, a change in the conformation of His 99 results in a change in the overall shape of S2 and S3, suggesting that the side chain movement would effect a drug design strategy directed toward the substrate-binding groove.

The side chain of Cys 42 is also observed in two side chain conformations and is near the active site (Fig. 1C). In what is likely the major conformation, the Cys 42-Cys 58 disulfide bridge is intact. However, in the alternate conformation, the disulfide is broken and the Cys 42 thiol group lies in a small hydrophobic pocket formed by the side chains of Phe 59, Ile 29, and Val 41. This side chain shift is unexpected as the Cys 42-Cys 58 disulfide bridge is present all trypsin-like serine protease structures, and its proximity to the catalytic triad suggests that it may structurally stabilize the active site. Hence, one might expect the catalytic activity to be affected when this disulfide bridge is broken. On the other hand, one must note that this observation occurs in the solid state and that further solution work would be necessary to determine its physiological significance.

### Table II

| Inhibitor                  | Ki (μM) |
|----------------------------|---------|
| LMW-urokinase              |         |
| B428                       | 0.490 ± 0.018 | 0.512 ± 0.022 |
| micro-urokinase            |         |
| Ring numbering is shown in conjunction with the chemical structure for each inhibitor. |

**Phenylguanidine**

20.6 ± 1.0 17.4 ± 1.1
S2444 substrate concentrations were 0.8, 1.0, 1.3, 2.0, and 4.0 mM. B428 says with S2444 as described under “Experimental Procedures.” Amidolytic chromogenic assays with S2444 as described under “Experimental Procedures.” S2444 substrate concentrations were 0.8, 1.0, 1.3, 2.0, and 4.0 mM. B428 concentrations were 0 nM (V), 250 nM (A), 500 nM (O), and 1000 nM (K). Data represent the means of triplicate determinations. $K_i$ values were determined by replots of slope versus inhibitor concentration (inset) and are represented in Table II.

Examination of crystal packing at the active site reveals that the micro-urokinase molecules pack forming a solvent channel that leads to the active site groove. Therefore, small molecule inhibitors may diffuse into the crystal and bind at the active site. This is important from a structure-based drug design perspective because it facilitates soaking as a method of forming protein-compound complex crystals. The soaking method was used to obtain crystal structures with the three known urokinase inhibitors, B428, amiloride, and phenylguanidine. These structures were obtained at high resolution and provide a starting point for structure-based drug design of a nonpeptidic urokinase inhibitor.

B428—B428 has been reported to inhibit human urokinase with an $I_{50}$ value of 0.320 μM (Refs. 25 and 26 and Table II). B428 inhibition was tested versus LMW urokinase and micro-urokinase, and Fig. 2 presents the Lineweaver-Burke analysis for the effect of B428 on the activity of micro-urokinase. The results show that B428 competitively inhibits micro-urokinase as observed for the native enzyme (25, 26). As listed in Table II, B428 inhibits LMW urokinase with a $K_i$ of 0.490 μM while inhibiting micro-urokinase with a $K_i$ of 0.512 μM. Hence, $K_i$ values for the native and re-engineered forms of the protein are essentially identical and are consistent with reported $I_{50}$ values (25, 26).

The B428-micro-urokinase co-crystal structure was completed to 2.0 Å resolution. In the complex structure, the $2F_o - F_c$ and $F_o - F_c$ maps indicate that His$^99$ is in two conformations as observed in the native structure although Cys$^{42}$ is observed only in the conformation in which the Cys$^{42}$-Cys$^{58}$ disulfide bridge is intact. It is unclear why only one conformation is present in the complex structure although Cys$^{42}$ is observed essentially identical and are consistent with reported $I_{50}$ values (25, 26).

In addition to interactions at S$_1$, the 4-iodo group is pointing out of the S$_1$ pocket away from the substrate-binding groove and is making van der Waals’ interactions with the side chain of Cys$^{220}$ and the main chain atoms of Gly$^{218}$. These residues are making hydrogen bonding interactions with the side chain of Lys$^{143}$. This interaction is consistent with the B428-urokinase crystal structure where the 4-iodo group partially accesses the S$_1$ pocket.
thermore, B623 inhibits urokinase with an IC50 of 0.07 μM (25, 26). Based upon the crystal structure of B428-micro-urokinase, it is possible that this larger 4-substituent is occupying more of the S1b pocket and consequently binds more tightly to urokinase. Hence, access to this novel pocket has been shown to confer an increase in binding potency and may serve as a site for further substitution in structure-based drug design.

Examination of the crystal structure of B428-urokinase shows that the 5 and 6 positions of the benzo(b)thiophene-2-carboxamidine are also open for substitution, whereas the 3 and 7 positions are buried within the S1 pocket and therefore less likely to accommodate a substituent. Of these, the 5 position does not directly point toward any pockets of the urokinase molecule because it points toward Gln192 and out toward bulk solvent. Hence, substitution at this position is less likely to confer a large increase in binding potency. On the other hand, the 6 position points toward the urokinase catalytic site although the position appears partially blocked by the side chain of the active site Ser195. The distance from Ser195-OH to the 6 position carbon is 3.2 Å; therefore incorporation of a substitution at this position may require a shifting of the benzothiophene scaffold away from Ser195. Additionally, substitutions at the 6 position would not orient toward the substrate-binding groove accessed by Glu-Gly-Arg-chloromethyl ketone. Substitutions at the 6 position would have to bend back toward the substrate-binding site or access other subsites. Nevertheless, the 4 and 6 positions appear to be the best substitution sites toward increasing the binding potency of B428, and both sets of substitutions will likely occupy sites apart from the substrate-binding groove.

Amiloride—Amiloride has been reported to inhibit human urokinase with a K_i of 30 μM (24) or IC50 of 7 μM (25, 26). As observed with B428, amiloride also competitively inhibits LMW uroki-
nase and micro-urokinase with similar values (\(K_i = 7.2 \, \mu M\) for LMW urokinase, and \(K_i = 6.9 \, \mu M\) for micro-urokinase). Amiloride is a weaker urokinase inhibitor than B428 (Table II) but may have more favorable pharmacological properties because the compound is an orally active commercial drug (46). To compare the binding modes of amiloride and B428 and to establish strategies for development of a more potent amiloride-based urokinase inhibitor, the co-crystal structure of amiloride micro-urokinase was completed at 2.2 Å resolution.

Examination of the \(2F_o - F_c\) (contoured at 1 \(\sigma\)) and \(F_o - F_c\) (contoured at 3 \(\sigma\)) electron density maps at the active site shows that all atoms of the inhibitor are clearly defined in both maps (Fig. 4A). In addition, the maps show His\(^{99}\) in two conformations and the Cys\(^{42-58}\) disulfide bridge intact as observed in the B428 complex. The data also indicate that amiloride binds at the S\(_1\) pocket as observed with B428 (Fig. 4C).

The crystal structure of amiloride-micro-urokinase indicates that amiloride is making more hydrogen bonding interactions at the S\(_1\) site than B428 while maintaining some of the van der Waals’ interactions within the pocket. The size of the amiloride pyrazine scaffold is smaller than the B428 benzothiazepine such that even though the pyrazine ring is in contact with the rim of the S\(_1\) pocket as observed for B428, the extent of the packing interactions is smaller. In place of the thiophene ring, the 3-amino and 2-acylguanidine groups of amiloride are making hydrogen bonding interactions. Specifically, the 3-amino group is packed underneath the side chain of Ser\(^{195}\) as shown in Fig. 4B where it is donating a hydrogen bond to Ser\(^{195-\text{O\gamma}}\) (3.1 Å). The carbonyl of the acyl guanidine group is accepting a hydrogen bond (2.9 Å) from a buried solvent molecule bound directly above Tyr\(^{228}\). The guanidine-NH is donating a hydrogen bond to Gly\(^{218-\text{O\gamma}}\) (3.1 Å). As observed with B428, the amide-like nitrogens are donating hydrogen bonds to Gly\(^{218-\text{O\gamma}}\) (2.7 Å) and Asp\(^{199-\text{O\delta1}}\) (3.0 Å) or to Asp\(^{199-\text{O\delta2}}\) (3.0 Å), and Ser\(^{190-\text{O\gamma}}\) (2.7 Å). The hydrogen bonding geometry of the guanidinium group is also very similar to that observed for Arg\(^{P_1}\) in the Glu-Gly-Arg-chloromethyl ketone-LMW urokinase structure (1). Hence, although the core scaffolds of both B428 and amiloride are bound at the S\(_1\) pocket, the nature of the interactions within the pocket are different.

The crystal structure of amiloride-micro-urokinase reveals strategies for structure-based drug design of a more potent small molecule inhibitor. One potential site of substitution is the 6 position. The 6-chloro group of amiloride is accessing the strategies for structure-based drug design of a more potent actions within the pocket are different.

Phenylguanidine—Phenylguanidine inhibits urokinase with a \(K_i\) of 20.6 \(\mu M\) (27) and is therefore a weaker inhibitor of urokinase than either amiloride or B428 (Table II). This inhibitor also competitively inhibits micro-urokinase with a \(K_i\) consistent with the LMW form (\(K_i = 20.6 \, \mu M\) LMW for urokinase, and \(K_i = 17.4 \, \mu M\) for micro-urokinase). To compare the binding mode of this inhibitor to amiloride and B428 and to determine potential sites of substitution, the co-crystal structure of phenylguanidine-micro-urokinase was completed at 2.0 Å resolution.

The phenylguanidine-micro-urokinase active site structure is very similar to that in the presence of B428 and amiloride. His\(^{99}\) is observed in multiple conformations while the Cys\(^{42-58}\) disulfide bridge is intact. Additionally, the \(2F_o - F_c\) (contoured at 1 \(\sigma\)) and \(F_o - F_c\) (contoured at 3 \(\sigma\)) electron density maps (Fig. 5A) obtained using the urokinase model in the absence of inhibitor and before any refinement cycles shows that all atoms of the inhibitor are clearly defined in both maps. The inhibitor was found to bind at the S\(_1\) pocket (Fig. 5B).

Even though both amiloride and phenylguanidine have scaffolds of the same size, the phenyl ring of phenylguanidine binds very differently from the pyrazine ring of amiloride (Fig. 5, B and C). Specifically, the phenylguanidine ring packs underneath Ser\(^{195}\) and is interacting with the main chain atoms of Val\(^{213-\text{Trp}^{215}}\) as well as the side chain of Val\(^{213}\). The ring also interacts with the main chain atoms of Ser\(^{190-\text{Cys}^{58}}\) as well as the side chain of Ser\(^{190}\). The differential ring packing is most likely due to amiloride possessing one additional linker atom between the guanidine and aromatic groups relative to phenylguanidine (Table II) because the guanidine groups are oriented very similarly. Specifically, the guanidine-NH is donating a hydrogen bond to Gly\(^{218-\text{O\gamma}}\) (3.9 Å), whereas the amide-like nitrogens are donating hydrogen bonds to Asp\(^{199-\text{O\delta1}}\) (2.9 Å) or to Asp\(^{199-\text{O\delta2}}\) (3.0 Å) and Ser\(^{190-\text{O\gamma}}\) (3.3 Å). Thus, it is likely that the core scaffold of amiloride (pyrazine ring) orients differently than the phenyl group of phenylguanidine because the binding is being driven by the hydrogen bonding geometry of the guanidinium groups rather than the van der Waals’hydrogen bonding interactions of the core groups even though interactions of the core groups most certainly contribute to the compound binding.

The phenyl guanidine urokinase structure also shows that Gln\(^{192}\) has changed conformation and is in hydrophobic contact with the inhibitor (Fig. 5B) such that it is blocking the entrance to the S\(_1\beta\) pocket. In the native and the B428 or amiloride complex structures, the S\(_1\beta\) pocket is open where Gln\(^{192}\) is accepting a hydrogen bond from Lys\(^{243}\) (3.3 Å) and donating a hydrogen bond to Tyr\(^{153}\) (3.1 Å). Thus, a conformational shift of this side chain requires breaking two hydrogen bonds. This is not the case for other serine proteases such as thrombin where there is no hydrogen bonding partner for Glu\(^{192}\) and the side chain is free to move in both conformations (49, 50). For urokinase, it appears that the binding of certain inhibitors such as phenyl guanidine does break the two Gln\(^{192}\) hydrogen bonds and conformationally shift Gln\(^{192}\) to maximize hydrophobic desolvation of the compound. Hence, Gln\(^{192}\) may be induced to shift conformation and because Gln\(^{192}\) may act as a switch to the entrance to S\(_1\beta\) from S\(_1\), noting the orientation of this side chain is important in a drug design strategy.

The crystal structure of phenylguanidine-urokinase suggests a structure-based drug design strategy different from that with B428 or amiloride. Both B428 and amiloride are capable of directly accessing the S\(_1\beta\) pocket, whereas the binding orientation of phenylguanidine is such that a similar interaction cannot be achieved by direct substitution of the phenyl ring (Fig. 5C) even with movement of Gln192 to the S\(_1\beta\) open position. Specifically, as shown in Fig. 5 (B and C), the 2 and 3
positions could point toward the S$_{1\beta}$ pocket but are too far away to support direct interaction with S$_{1\beta}$. In fact, substitution of the phenyl ring with halogens at both the 2 and 3 positions did not result in any increase in inhibitory potency (27). On the other hand, substitution at position 4 with a chloro- or trifluromethyl-group resulted in an increase in inhibition to $K_i$ values of 6.8 and 6.5 $\mu$M, respectively (27). This 4 substitution is expected to orient toward the side chain of Ser$^{195}$ and may obtain binding energy from a favorable van der Waals' packing interaction with Ser$^{195}$ and the S$_{1}$ pocket. The 5 and 6 positions are within the S$_{1}$ pocket and therefore less open for substitution. Because interactions with the S$_{1\beta}$ pocket are expected to confer an increase in binding potency and because phenylguanidine may not directly access this site, modification of the scaffold may be a promising drug design strategy for this series.

Further examination of an overlay of the crystal structures of phenyl guanidine and amiloride micro-urokinase (Fig. 5C) shows that the binding of the two scaffolds is complementary. The lack of overlap between the two groups suggests that the phenyl and pyrazine rings could be fused to form a 1-naphthylguanidine system. The naphthyl ring would be expected to occupy the sites of both core scaffolds and could therefore maintain the positive characteristics of both the phenylguanidine and amiloride series. This would include utilization of the 4-chloro or 4-trifluromethyl substitutions in the phenylguanidine series as well as access to the S$_{1\beta}$ pocket exploited by amiloride and B428. Hence, a merging of the amiloride and phenylguanidine scaffolds would be predicted to benefit from the additivity of both sites and create a more potent and easily optimized urokinase inhibitor.

**DISCUSSION**

Urokinase inhibitors have been shown to affect tumor metastasis and growth in vivo making urokinase an attractive anti-cancer target. However, these existing compounds lack all of the properties necessary for a therapeutic agent and require optimization. Crystallography driven structure-based drug design based on a series of ligand-protein crystal structures can be utilized to optimize urokinase inhibition. The properties of the protein crystals can affect the efficiency of structure-based drug design because a larger number of more accurate struc-

---

**Fig. 5.** A, initial $2F_o - F$ (purple) and $F_o - F$ (green) maps contoured at 1 and 3 $\sigma$, respectively, for the binding site of phenyl guanidine before refinement. B, molecular surface micro-urokinase as calculated by the program package QUANTA (Molecular Simulations Inc.) depicting interactions between B428 and micro-urokinase. The inhibitor and inhibitor surface are shown in orange, whereas the protein and protein surface are shown in cyan. C, overlay of the crystal structures of amiloride (purple) and phenyl guanidine (black) micro-urokinase, showing that the two scaffolds occupy different areas of the S$_{1}$ pocket.
Crystal Structures of Urokinase at High Resolution

7247

tures provides a better description of the relationship between binding interactions and binding energy. Fortunately, advances in molecular biology can be used to engineer the protein to obtain crystal systems that facilitate faster and more exact structure determinations and enhance the drug design cycle (47). Such a method has been used to design a crystal system for human urokinase for optimization of a urokinase inhibitor.

The sequence of LMW urokinase was redesigned to produce a new crystal form that would permit a more ideal system for structure-based drug design. Specifically, LMW urokinase was re-engineered to minimize the areas of disorder that may likely cause suboptimal crystal packing. This recombinant protein, micro-urokinase, produces crystals with close packing interactions at the A-chain cleft, which would be blocked in LMW urokinase. This close molecular packing results in crystals that diffract to high resolution on a rotating anode source (1.6–2.0 Å). However, even though the micro-urokinase molecules are closely packed, the active site is both unoccupied and open to solvent channels in the crystal. This property readily allows compounds to be diffused into the crystal and has facilitated the determination of crystal structures in the presence of three reported urokinase inhibitors toward design of an anti-cancer agent.

The micro-urokinase crystal system and soaking method was used to determine the co-crystal structures of micro-urokinase complexed with the inhibitors B428 (25, 26), amiloride (24), and phenylguanidine (27). Each of the co-crystal structures gives insight into favorable compound-protein interactions that contribute to the binding of these inhibitors to urokinase. The primary binding force is likely the hydrogen bonds between each inhibitor’s amidine or guanidine group and Asp189. This salt bridge interaction is common to many guanidine or amiloride complexes with trypsin or trypsin-like serine proteases such as thrombin, factor Xa, or tissue plasminogen activator complexes with trypsin or trypsin-like serine proteases. Hence, both hydrophilic and hydrophobic interactions at the A-chain cleft, which would be blocked in LMW urokinase, produces crystals with close packing interactions at the S1 subpocket, is composed of the disulfide bridge at Cys191–Cys220 residues Ser146 and Gly218 and the side chain of Lys214. The S1β subpocket is also present in the LMW urokinase structure (Protein Data Bank entry 1LMW) and is away from any re-engineered sites. The crystal structure of phenyl guanidine urokinase reveals that Gln192 may act as a switch for the closing and opening of S1β. In the native and B428 or amiloride complex structures, the S1β pocket is open, and Gln192 is involved in two hydrogen bonds (Lys343 and Tyr151). However, in the presence of other inhibitors such as phenyl guanidine or Glu-Gly-Arg-chloromethyl ketone (1), the hydrogen bonds are broken, and the conformation of Gln192 shifted such that its side chain is in van der Waals’ contact with the inhibitor. In this conformation, the entrance to S1β is blocked, and the shift is most likely induced to maximize interactions with the inhibitor. Hence, although the S1β pocket may be blocked by the induced movement of Gln192, its proximity to S1 makes it an attractive substrate for structure-based drug design.

The halogen atoms of B428 and amiloride are interacting with the entrance to the S1β subsite (Gly218–Cys220). Interactions at this site have been shown to confer a significant increase in inhibitory potency for the benzo(b)thiophene-2-carbuxamide series where the 4-iodo group (IC50 = 0.32 μM) or 4-benzodioxolanylketone (IC50 = 0.07 μM) inhibit more strongly than the 4-hydro compound (IC50 = 3.7 μM) (25, 26). The increase in potency observed for both substitutions is most likely due to packing interactions at the S1β pocket. Phenyl
guanidine lacks a halogen atom to access the S1β pocket, and examination of the structure reveals that the pocket cannot be easily accessed by a direct substitution of the phenylguanidine ring. However, an overlay of the phenylguanidine crystal structure with that of amiloride reveals that the two scaffolds could be merged to form a 1-guanadyl naphthalene. This compound could, in turn, access the S1β pocket. Hence, urokinase co-crystal structures with B428, amiloride, and phenylguanidine indicate that all three scaffolds may provide either direct or indirect access to the S1β pocket. Furthermore, this newly described subsite has great potential for the future design of more potent urokinase inhibitors for the treatment of cancer.

Acknowledgments—We thank Dr. Bruce Littlefield of the Eisai Company for initial supplies of B428 and Dr. Todd Rockway for synthesis of ε-amino caproic acid p-carboxyphenyl ester chloride. We also thank Dr. Stephen Betz for critical examination of the manuscript and Dr. Jonathan Greer for many helpful discussions and critical examination of the manuscript.

REFERENCES

1. Spraggon, G., Phillips, C., Nowak, U. K., Ponting, C. P., Saunders, D., and Dobson, C. M. (1995) Structure 3, 681–691.
2. Kohn, E. C. (1991) Pharmacol. Ther. 52, 235–244.
3. Quax, P. H., van L. R. T., Verspaget, H. W., and Verheijen, J. H. (1990) Cancer Res. 50, 1488–1494.
4. Behrendt, N., Ronne, E., Ploug, M., Petri, T., Lober, D., Nielsen, L. S., Schleuning, W. D., Blasi, F., Appella, E., and Dano, K. (1990) J. Biol. Chem. 265, 6453–6460.
5. Schmitt, M., Janicke, F., Moniwa, N., Chucholowski, N., and Pache (1992) Biochem. Hoppe-Seyler 373, 611–622.
6. Duffy, M. J. (1990) Blood Coagul. Fibrinolysis 1, 681–687.
7. Astedt, B., Billstrom, A., and Lecerand, I. (1995) Fibrinolysis 9, 175–177.
8. Evans, D., Sloan-Stakleff, K., Arvan, M., and Guyton, D. (1988) Clin. Exp. Metastasis 16, 355–357.
9. Banerji, A., Fernandes, A., Bane, S., and Ahire, S. (1998) Cancer Lett. 129, 15–20.
10. Kobayashi, H., Gotoh, J., Shinohara, H., Moniwa, N., and Terao, T. (1994) Thromb. Haemostasis 71, 474–480.
11. Xiao, G., Liu, Y., Gentz, R., Sang, Q., Goldberg, I., and Shi, Y. (1999) Proc. Nat. Acad. U. S. A. 96, 3700–3705.
12. Rablani, S., Harakakis, P., Davidson, D., Henkin, J., and Mazar, A. (1995) Int. J. Cancer 63, 840–845.
13. Alonso, D., Tejera, A., Farias, E., Jeffe, O., and Bomez, D. (1998) Anticancer Res. 18, 4499–4504.
14. Alonso, D., Farias, E., Lameda, D., Davel, L., Puricelli, L., and Joffer, K. (1996) Breast Cancer Res. Treat. 40, 229–233.
15. Jakun, J., Keck, R., Krzyzanek-Jakan, E., and Swierz, R. (1997) Cancer Res. 57, 559–563.
16. Browner, M. F., Smith, W. W., and Castelano, A. L. (1995) Biochemistry 34, 6602–6610.
17. Chand, P., Bahu, Y. S., Banita, S., Chu, N., Cole, L. E., and Kotian, P. L. (1997) J. Med. Chem. 40, 4030–4052.
18. Erickson, J., Neidhart, D. J., VanDrie, J., Kempf, D. J., and Wang, X. C. (1990) Science 249, 527–530.
19. Lam, P. Y., Jadhav, P. K., Eyermann, C. J., Hodge, C. N., and Ru, Y. (1994) Science 263, 380–384.
20. Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., and Pak, J. Y. (1996) Nature 384, 644–648.
21. Luong, C., Miller, A., Barnett, J., Chow, J., and Ramesha, C. (1996) FEBS Lett. 577–587.
22. Verlinde, C. L., and Hol, W. G. (1994) Structure 5, 33, 474–480.
23. Luzatti, P. V. (1952) Acta Crystallogr. 5, 403–410.
24. Vassalli, J. D., and Belin, D. (1987) J. Exp. Med. 164, 825–835.
25. Bridges, A. J., Lee, A., Schwartz, C. E., Towle, M. J., and Littlefield, B. A. (1993) Bioorg. Med. Chem. Hoppe-Seyler 214, 489–495.
26. Schleuning, W. D., Blasi, F., Appella, E., and Dano, K. (1990) J. Biol. Chem. 265, 6453–6460.
27. Yang, H., Henkin, J., Kim, K. H., and Greer, J. (1999) J. Med. Chem. 42, 2956–2961.
28. Lo, K.-M., and Gillies, S. D. (1991) Biochi. Biophys. Acta 1088, 217–224.
29. Wang, J., Birlar, B., and Reich, E. (1995) Protein Sci. 4, 1758–1767.
Crystal Structures of Urokinase at High Resolution

30. Barlow, G. H. (1976) Methods Enzymol. 45, 239–244
31. Segel, I. H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, John Wiley & Sons, New York
32. Menegatti, E., Guarneri, M., Bolognesi, M., Ascenzi, P., and G. A. (1989) J. Enzyme Inhibition 2, 249–259
33. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
34. Navaza, J. (1994) Acta Crystallogr. A 50, 157–163
35. Brumberger, A. T. (1993) X-PLOR, version 3.1, Yale University Press, New Haven, CT
36. Sheldrick, G. M. (1990) SHELX97, Gottingen University, Gottingen, Germany
37. Bode, W., Mayer, I., Baumann, U., Huber, R., and Stone, S. R. (1989) EMBO J. 8, 3467–3475
38. Lembke, D., Bauer, M., Huber, R., Fischer, S., Rudolph, R., Kohnert, U., and Bode, W. (1986) J. Mol. Biol. 258, 117–135
39. Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blankenbol, D. T., Cardin, A. D., and Kistel, W. (1993) J. Mol. Biol. 232, 947–966
40. Henderson, R. (1970) J. Mol. Biol. 54, 341–354
41. Bode, Q., Turk, D., and Sturzbecher, J. (1990) Eur. J. Biochem. 193, 175–182
42. Bode, W., and Schwager, P. (1975) J. Mol. Biol. 98, 693–717
43. Banner, D. W., and Hadvory, P. (1991) J. Biol. Chem. 266, 20085–20093
44. Renatus, M., Bode, W., Huber, R., Sturzbecher, J., Prasa, D., Fischer, S., Kohnert, U., andStubbs, M. (1997) J. Biol. Chem. 272, 21713–21719
45. Brandstetter, H., Kuhne, A., Bode, W., Huber, R., von der Saal, W., Wirthenboum, K., and Engl, R. (1996) J. Biol. Chem. 271, 29988–29992
46. Baba, W. I., Lant, A. F., Smith, A. J., Townshend, M. M., and Wilson, G. M. (1968) Clin. Pharmacol. Ther. 9, 318–327
47. Price, S., and Nagai, K. (1995) Curr. Opin. Biotechnol. 6, 425–430
48. Steitz, T., Henderson, R., and Blow, D. (1969) J. Mol. Biol. 46, 337–348
49. Blow, D. (1976) Acc. Chem. Res. 9, 145–152
50. Sigler, P., Jeffery, B., Matthews, B., and Blow, D. (1966) J. Mol. Biol. 15, 175–192
51. Birkenstock, J., and Blow, D. (1972) J. Mol. Biol. 68, 187–240
52. Malikayil, J. A., Burkhart, J. P., Schreuder, H. A., Broersma, R. J., Tardif, C., Kutcher, L. W., Mehdi, S., Schatzman, G. L., Neises, B., and Peet, N. P. (1997) Biochemistry 36, 1034–1040
53. Das, J., and Kimball, S. D. (1995) Bioorg. Med. Chem. 3, 999–1007