Selective Urokinase-Type Plasminogen Activator Inhibitors. 4. 1-(7-Sulfonamidoisoquinolinyl)guanidines†

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1-Isoquinolinylguanidines were previously disclosed as potent and selective inhibitors of urokinase-type plasminogen activator (uPA). Further investigation of this template has revealed that incorporation of a 7-sulfonamide group furnishes a new series of potent and highly selective uPA inhibitors. Potency and selectivity can be achieved with sulfonamides derived from a variety of amines and is further enhanced by the incorporation of sulfonamides derived from amino acids. The binding mode of these 1-isoquinolinylguanidines has been investigated by X-ray cocrystallization studies. uPA inhibitor **26** was selected for further evaluation based on its excellent enzyme potency (K_i 10 nM) and selectivity profile (4000-fold versus tPA and 2700-fold versus plasmin). *In vitro*, compound **26** is able to inhibit exogenous uPA in human chronic wound fluid ($IC_{50} = 0.89\ \mu M$). *In vivo*, in a porcine acute excisional wound model, following topical delivery, compound **26** is able to penetrate into pig wounds and inhibit exogenous uPA activity with no adverse effect on wound healing parameters. On the basis of this profile, compound **26** (UK-371,804) was selected as a candidate for further preclinical evaluation for the treatment of chronic dermal ulcers.

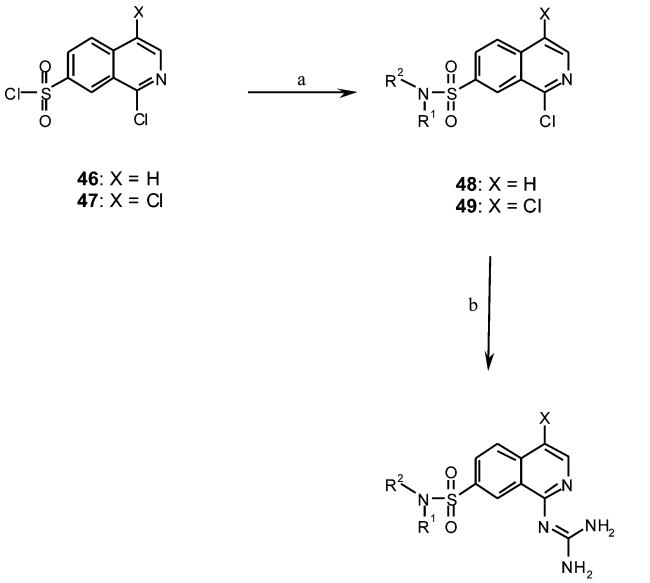
Introduction

Urokinase-type plasminogen activator (uPA)[a] (urokinase or urinary-type plasminogen activator; International Union of Biochemistry classification number EC.3.4.21.31) is a serine protease of the trypsin family produced by a large variety of cell types (smooth muscle cells, fibroblasts, endothelial cells, macrophages, and tumor cells). It has been implicated in cellular invasion and tissue remodeling. A principal substrate for uPA is plasminogen, which is converted by cell surface-bound uPA to the serine protease plasmin. Locally produced, high plasmin concentrations mediate cell invasion by breaking down the extracellular matrix. Important processes involving cellular invasion and tissue remodeling include wound repair, bone remodeling, angiogenesis, tumor invasiveness, and spread of metastases.[1,2]

Conditions of particular interest to us include chronic dermal ulcers which are a major cause of morbidity in the aging population. Chronic dermal ulcers are characterized by excessive uncontrolled proteolytic degradation resulting in ulcer extension, loss of functional matrix molecules (e.g., fibronectin), and retardation of epithelialization and ulcer healing. A number of groups have investigated the enzymes responsible for the excessive degradation in the wound environment, and the role of plasminogen activators has been highlighted.[3−7] Normal human skin demonstrates low levels of plasminogen activators which are localized to blood vessels and identified as tissue-type plasminogen activator (tPA). In marked contrast, chronic ulcers demonstrate high levels of uPA localized diffusely throughout the ulcer periphery and the lesion and readily detectable in wound fluids.

Thus, overexpression of uPA in the wound environment has the potential to promote uncontrolled matrix degradation and inhibition of tissue repair. Inhibitors of the enzyme thus have the potential to promote healing of chronic wounds. Several

Scheme 1. General Synthesis of 1-Guanidino-7-isoquinolinesulfonamides[a]



46: X = H
47: X = Cl

48: X = H
49: X = Cl

X = H: **4**, **5**

X = Cl: **6-10**, **36**, **40**, **41**, **44**, **45**

[a] (a) R^1R^2NH, NEt_3, CH_2Cl_2; (b) $HN{=}C(NH_2)_2{\cdot}HCl$, NaH or *t*-BuOK, DMSO or DME, 90 °C.

† PDB ID: 2jde and 2uwk.

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[a] Abbreviations: urokinase-type plasminogen activator, uPA; tissue-type plasminogen activator, tPA.

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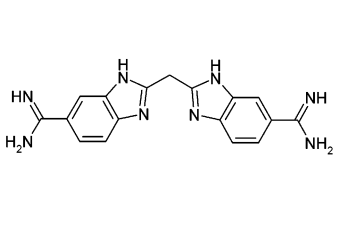
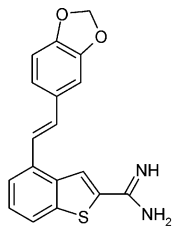
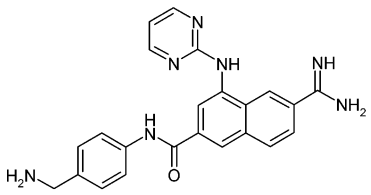
Chart 1. Nonpeptidic uPA Inhibitors



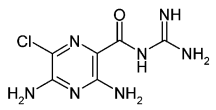
BABIM
Ki = 2,300 nM



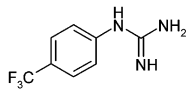
B-623
IC_{50} = 70 nM



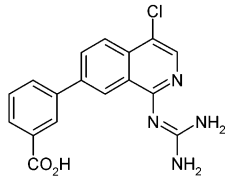
1
IC_{50} = 0.68 nM



amiloride
Ki = 7,000 nM



2
Ki = 6,000 nM



3
Ki = 17 nM

related enzymes such as tPA, which also acts *via* production of plasmin, play a key role in the fibrinolytic cascade. Hence, it is important that an inhibitor has adequate potency and selectivity for uPA relative to both tPA and plasmin to avoid the possibility of antifibrinolytic side effects.

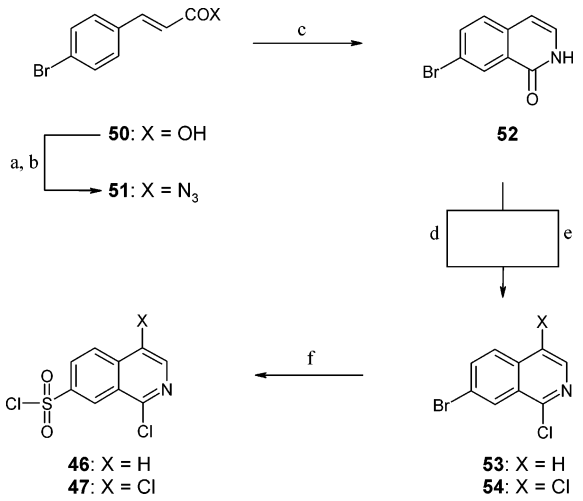
Two types of nonpeptidic, low molecular weight inhibitors of uPA are emerging (Chart 1).[8] Various aromatic amidines, e.g., BABIM, have been reported to inhibit uPA.[9] These compounds are generally weak and/or nonselective for uPA relative to related serine proteases. Bridges disclosed a series of benzothiophene amidines (e.g., B-623) with significantly greater potency and selectivity with respect to tPA and plasmin,[10] and Wendt reported a series of potent naphthyl amidines (e.g., **1**).[11] There are few reports of guanidine derivatives as uPA inhibitors. Amiloride is a weak but selective inhibitor of uPA,[12] and various substituted phenylguanidines are reported to have a similar level of potency (e.g., **2**).[13] Our own efforts identified 1-isoquinolinylguanidine **3** (UK-356,202) as a potent and selective inhibitor of uPA.[14]

The compounds described herein are potent reversibly competitive inhibitors of uPA enzymatic activity, with selectivity for uPA relative to the fibrinolytic enzymes tPA and plasmin.

Chemistry. The methods for the synthesis of inhibitors disclosed in Tables 1−5 (compounds **4**−**45**) are described in Schemes 1−5. The general method for the synthesis of 1-guanidino-7-isoquinolinylsulfonamides is outlined in Scheme 1. Sulfuryl chlorides **46** and **47** were reacted with a number of amines to give the corresponding sulfonamides **48** and **49** respectively, and the guanidine group was then introduced by reaction with guanidine free base to give compounds **4**−**10**, **36**, **40**, **41**, **44**, and **45**.

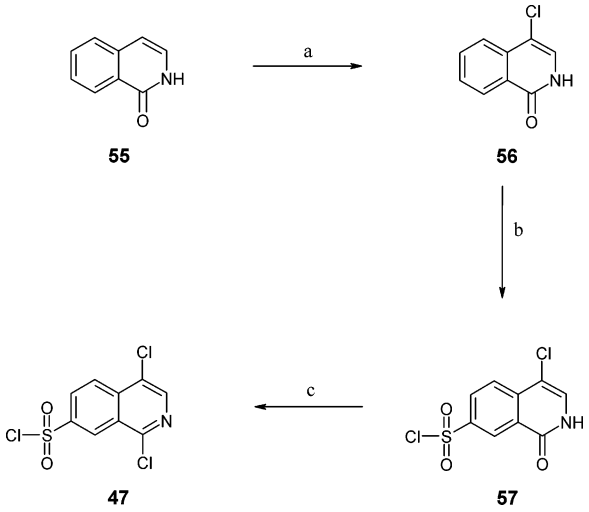
7-Isoquinolinylsulfonyl chlorides **46** and **47** were prepared by two methods (Schemes 2 and 3). The first method employed

Scheme 2. Synthesis of 7-Isoquinolinesulfuryl Chlorides **46** and **47**. Method 1[a]



[a] (a) $SOCl_2$, 80 °C; (b) NaN_3, acetone−H_2O, 0 °C; (c) 270 °C, Ph_2O [CAUTION]; (d) for **53**: $POCl_3$, 110 °C; (e) for **54**: PCl_5, 140 °C; (f), i. *n*-BuLi, THF−Et_2O, −78 °C, ii. SO_2Cl_2, hexane.

Scheme 3. Synthesis of 1,4-Dichloro-7-isoquinolinesulfuryl Chloride (**47**). Method 2[a]

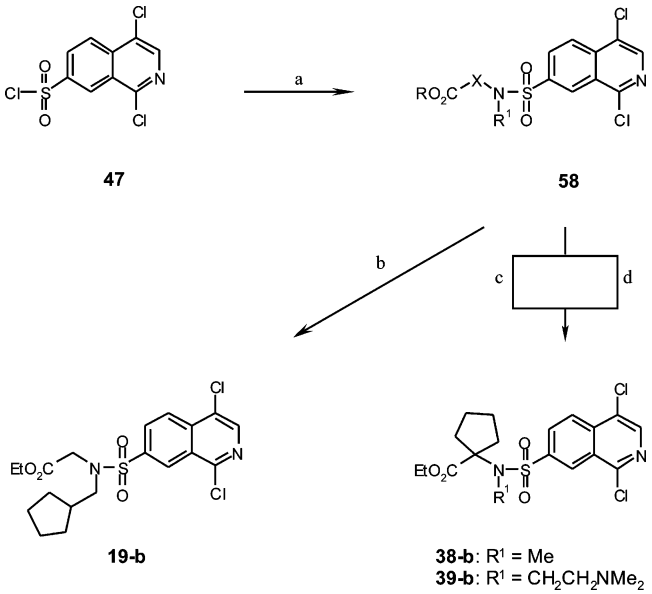


[a] (a) NCS, MeCN; (b) $ClSO_3H$, 100 °C; (c) $POCl_3$, MeCN.

a Curtius-like rearrangement[15] of the cinnamyl azide **51** to give the isoquinolone **52** which was then converted to the 1-chloroisoquinoline **53** by treatment with $POCl_3$, or to the 1,4-dichloroisoquinoline **54** by reaction with PCl_5. 7-Bromoisoquinolines **53** and **54** were then converted to the 7-sulfonyl chlorides **46** and **47** respectively by low-temperature lithiation (Br→Li exchange) with *n*-BuLi and then reaction with sulfuryl chloride. Sulfuryl chlorides **46** and **47** prepared by this method were used *in situ* without isolation. An improved synthesis of **47** is shown in Scheme 3. It had been shown that 1(2*H*)-isoquinolinone (**55**) undergoes electrophilic nitration at the 5 and 7 positions[16] and so it was proposed that introduction of the 4-Cl atom prior to electrophilic sulfonylchlorination should promote attack at the 7-position by sterically blocking the 5-position; this strategy proved successful. Thus, chlorination of **55** with *N*-chlorosuccinimide (NCS) gave **56**, reaction with neat $ClSO_3H$ then furnished **57**, and finally treatment of **57** with $POCl_3$ gave **47**.

The preparation of sulfonamides derived from amino acids is shown in Scheme 4. A few *sec*-sulfonamides (**58**: R1 = H) were further functionalized at this stage by N-alkylation to give

Scheme 4. Synthesis of 7-Isoquinolinesulfonamides Derived from Amino Acids[a]



[a] (a) RO_2C-X-NH-R^1, NEt_3, CH_2Cl_2; (b) cyclopentylmethanol, DEAD, PPh_3, THF; (c) for **38b**: K_2CO_3, MeI; (d) for **39b**: K_2CO_3, $Me_2NCH_2CH_2Cl$, DMF.

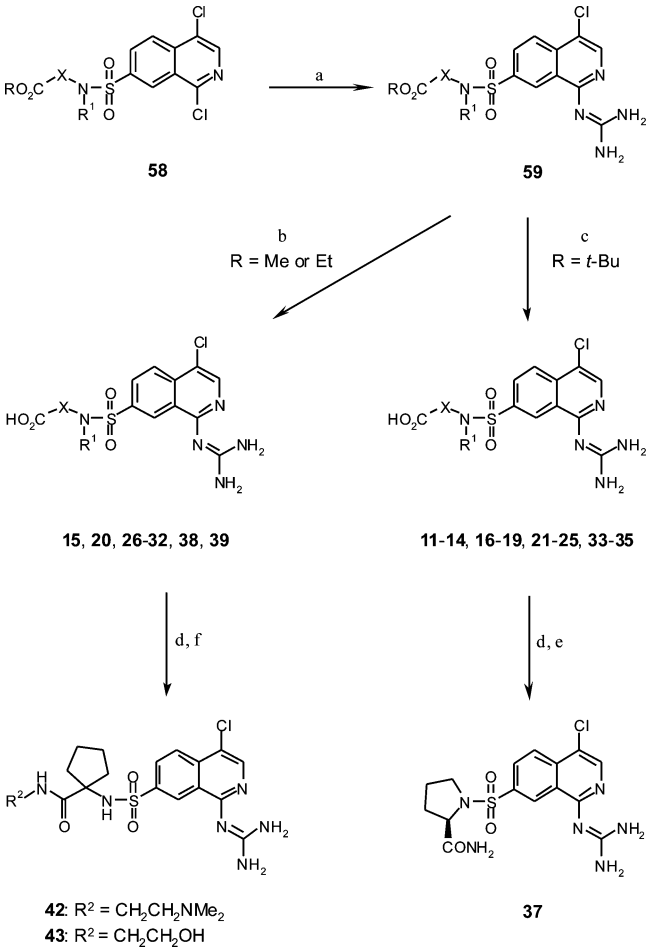
tert-sulfonamides. Glycine derivative **13b** (**58**: X = CH_2, R = Et, R1 = H) was reacted with cyclopentylmethanol under Mitsunobu conditions to give **19b**, and cycloleucine derivative **28a** (**58**: **X** = $C(CH_2)_4$, R = Et, R1 = H) was converted to **38b** by treatment with K_2CO_3-MeI, or to **39b** by reaction with $K_2CO_3-Me_2NCH_2CH_2Cl$. The guanidine group was then introduced into the template by reaction of **58** with guanidine free base to give 1-guanidinoisoquinolines **59** (Scheme 5). For the inhibitors which incorporate a protected carboxy group, the acid was unmasked at this stage. Methyl and ethyl esters **59** (R = Me or Et) were hydrolyzed with dilute NaOH and deprotection of *tert*-butyl esters **59** (R = *t*-Bu) was performed with HCl or TFA. Acids **26** and **33** were converted to the amides **42**, **43**, and **37** respectively by initial treatment with oxalyl chloride to give the corresponding acid chloride and then reaction with the appropriate amine.

Compound Design

In our earlier studies with heterocyclic guanidines as uPA inhibitors,[17,18] we showed that 1-isoquinolinylguanidine had moderate affinity for inhibition of uPA (1900 nM). This template was then further developed by the incorporation of a *m*-benzoic acid at the 7-position of the isoquinoline ring to yield compound **3** (17 nM) which is a potent and selective inhibitor of uPA.[14] We believe the 100-fold increase in potency is due to a binding interaction between the benzoic acid group and solvent-exposed Arg-217 of the enzyme active site in a similar manner to the binding between the glutamic acid of H-Glu-Gly-Arg-CH_2Cl (EGR-cmk) and Arg-217 as described in the first crystal structure of uPA.[19] This hypothesis may also explain the high degree of selectivity demonstrated by **3** for uPA compared to tPA and plasmin, as the corresponding residue in both these enzymes is Leu-217, and no similar interaction is possible.

With compound **3** selected for clinical development for the treatment of chronic dermal ulcers, our objective was to discover an agent that would have improved potency, high selectivity over tPA and plasmin, and physicochemical properties suitable for the *topical* delivery to an open chronic wound. There was

Scheme 5. Synthesis of 1-Guanidino-7-isoquinolinesulfonamides Derived from Amino Acids[a]

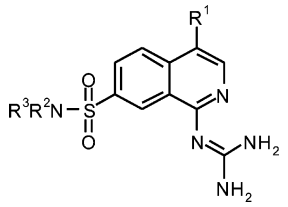


[a] (a) HN=$C(NH_2)_2$·HCl, NaH or *t*-BuOK, DMSO or DME or DMA, 90 °C; (b) NaOH, MeOH; (c) HCl or TFA; (d) $(COCl)_2$, DMF, CH_2Cl_2; (e) NH_3, CH_2Cl_2; (f), amine, CH_2Cl_2.

ample scope to further explore the 7-position of the 1-isoquinolinyl guanidine template, and we decided to investigate 7-sulfonamides. We reasoned that an appropriately positioned sulfonamide group would promote an opportunity to gain additional affinity through a backbone H-bonding interaction with Gly-216 of uPA in a similar manner to those interactions observed with other small, noncovalent serine protease inhibitors.[20] In addition, we hypothesized that a polar sulfonamide group would also confer improved solubility and lower lipophilicity for this series of uPA inhibitors, and these combined properties may aid delivery of compound into the wound environment.

The synthesis of 7-isoquinolinylsulfonyl chlorides **46** and **47**, and precedented chemistry for the introduction of the guanidine group, allowed the rapid exploration of SAR in this series. The first analogue, primary sulfonamide **4**, demonstrated encouraging potency for uPA (K_i 280 nM) and selectivity over tPA and plasmin, and both potency and selectivity were further enhanced by the incorporation of a 4-chloro atom in the isoquinoline template **6** (K_i 140 nM) (Table 1). This observation is consistent with all previous examples in the heterocycle guanidine series, and so the 4-Cl atom was then retained for all further analogues. Secondary sulfonamides **5** and **7** and tertiary sulfonamide **8**, all derived from simple lipophilic amines, also gave an enhancement in uPA inhibitory potency compared to **4** and **6**,

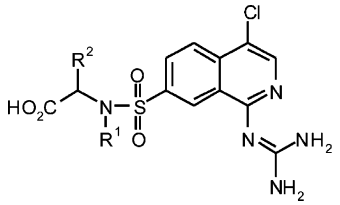
Table 1. 1-Guanidino-7-sulfonamidoisoquinolines



compound	R^1	NR^2R^3	K_i (nM)[a] uPA	tPA	plasmin
4	H	NH_2	280	24000	13000
5	H	NHPh	160	15000	3200
6	Cl	NH_2	140	>100 μM[b]	>100 μM
7	Cl	NH-cyclopentyl	71	>30 μM[c]	>100 μM
8	Cl	pyrrolidinyl	130	>30 μM	>30 μM
9	Cl	morpholinyl	330	ND	ND
10	Cl	4-methyl-1-piperazinyl	550	ND	ND
11	Cl	2-$NHC_6H_4CO_2H$	86	>100 μM	33000
12	Cl	3-$NHC_6H_4CO_2H$	59	>100 μM	9800
13	Cl	$NHCH_2CO_2H$	48	>30 μM	>30 μM
14	Cl	$NHCH_2CH_2CO_2H$	58	>100 μM	>100 μM
15	Cl	$NHCH_2C(Me)_2CO_2H$	43	ND	ND

[a] Calculated K_i (see Experimental Section). [b] <50% inhibition @ 100 μM. [c] <50% inhibition @ 30 μM. [d] ND = not determined.

Table 2. Substituted Glycine Derivatives



compound	R^1	R^2	K_i (nM)[a] uPA	tPA	plasmin
16	Me	H	54	>100 μM[b]	>100 μM
17	Ph	H	29	>100 μM	>100 μM
18	CH_2Ph	H	35	>100 μM	>100 μM
19	CH_2cyclopentyl	H	31	>100 μM	23000
20	H	*R*-Me	28	ND[c]	ND
21	H	*S*-Me	21	ND	15000
22	H	*R*-$CHMe_2$	19	38000	16000
23	H	*S*- $CHMe_2$	66	>100 μM	45000
24	H	*R*-CMe_3	22	>100 μM	>100 μM
25	CH_2Ph	*S*-Me	27	>100 μM	ND

[a] Calculated K_i. [b] <50% inhibition @ 100μM. [c] ND = not determined.

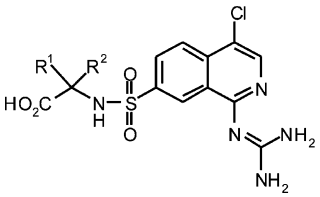
whereas sulfonamides **9** and **10**, derived from polar amines, were inferior to **6** but still better than having no substituent at the 7-position.

These preliminary results gave encouragement that our objective should be achievable in this series. We then prepared a number of 7-sulfonamides derived from amino acids in an attempt to further improve affinity for uPA through (i) an interaction between the carboxylate with Arg-217, (ii) a sulfonamide H-bond interaction with Gly-216, and (iii) additional nonspecific lipophilic binding between the amino acid carbon framework and the lipophilic residues of the active site of uPA.

The first examples derived from aminobenzoic acids, glycine, and β-alanines, compounds **11**−**15** respectively (Table 1), once again gave a modest enhancement in affinity (K_i 40−80 nM) while retaining selectivity for tPA and plasmin. Of these, glycine derivative **13** (K_i 48 nM) was selected for further modification, and the effects of substitution on the sulfonamide N atom, on the amino acid α-carbon, and on both of these positions simultaneously were investigated (Table 2). The incorporation of a small selection of *N*-alkyl and *N*-aryl sulfonamides, compounds **16**−**19**, gave at best less than a 2-fold increase in affinity with the larger groups. Substitution on the amino acid α-carbon proved to be a more successful with sulfonamides derived from alanine, valine, and *tert*-leucine **21**, **22**, **24** (K_i 20−30 nM) having potency approaching that of compound **3**. Although inhibitors derived from the two enantiomers of alanine (**20** and **21**) could be accommodated equally, when amino acids incorporating larger alkyl α-groups were introduced there was a preference for the *R*-isomer (**22** versus **23**). Compound **25** incorporated both *N*-alkyl and α-carbon groups (i.e., a hybrid of **18** and **21**) and again demonstrated that the *N*-alkyl group contributed little to the binding of the inhibitor (compare **21** and **25**).

The results of the alanine isomers, **20** and **21**, prompted us to explore α,α-disubstituted glycine derivatives (Table 3). The geminal dimethyl compound **26** proved to be our most potent (K_i 10 nM) and selective inhibitor to date, and potency could be further enhanced by incorporation of a small alkyl ring **27**−**29** (K_i 6−8 nM). Comparison of cycloleucine derivative **28** (K_i 5.8 nM) with cyclopentyl sulfonamide **7** (K_i 71 nM) shows that the acid group bestows a 10 fold increase in potency. Further modification of the cyclohexyl spiro-fused ring system (**29**) demonstrated that both polar groups, such as tetrahydropyran

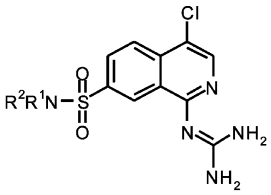
Table 3. α,α-Disubstituted Glycine Derivatives

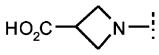
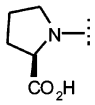
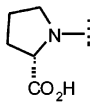
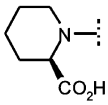
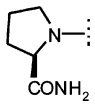


compound	R^1; R^2	K_i (nM)[a] uPA	tPA	plasmin
26	Me, Me	10	40000	27000
27	$CH_2CH_2CH_2$	7.4	>100 μM[b]	16000
28	$CH_2CH_2CH_2CH_2$	5.8	38000	14000
29	$CH_2CH_2CH_2CH_2CH_2$	8	>100 μM	39000
30	$CH_2CH_2OCH_2CH_2$	7.4	>100 μM	41000
31	$CH_2CH_2N(Me)CH_2CH_2$	11	ND[c]	71000

[a] Calculated K_i. [b] <50% inhibition @ 100μM. [c] ND = not determined.

Table 4. Cyclic Derivatives



Compound	NR^1R^2	K_i (nM)[a] uPA	tPA	plasmin
32		330	ND[c]	ND
33		9.9	>100μM[b]	>100μM
34		111	>100μM	>100μM
35		23	>100μM	>100μM
36		160	ND	ND
37		54	ND	ND

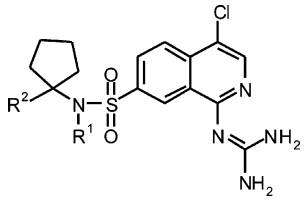
[a] calculated K_i. [b] <50% inhibition @ 100μM. [c] ND = not determined.

30, and basic groups, such as *N*-methyl piperidine **31**, could be accommodated at this position.

A number of sulfonamides derived from cyclic amino acids were prepared (Table 4). The analogues **32**−**35** demonstrate that the position of the acid group is important for gaining good affinity and the sulfonamides derived from the two enantiomers of proline, **33** and **34**, again demonstrated a preference for the *R*-isomer (cf. **22** and **23**). The proline derivative **33** was then modified to see if the carboxylic acid was essential for good potency and selectivity, or if an alternative group could be accommodated at the α-carbon. Alcohol **36** and carboxamide **37** were prepared as the preferred *R*-enantiomers. Comparison of **36** (K_i 160 nM) and **37** (K_i 54 nM) with proline isomer **33** (K_i 9.9 nM) and the unsubstituted pyrrolidinyl sulfonamide **8** (K_i 130 nM) confirmed the importance of the acid for affinity but also highlighted that appropriately functionalized carboxamides may also be potent inhibitors of uPA.

The final set of sulfonamides were derived from the cycloleucine derivative **28** (K_i 5.8 nM), our most potent uPA inhibitor prepared so far (Table 5). Substitution of the sulfonamide N

Table 5. Cycloleucine Derivatives



compound	R^1	R^2	K_i (nM)[a] uPA	tPA	plasmin
38	Me	CO_2H	11	ND[c]	ND
39	$CH_2CH_2NMe_2$	CO_2H	2.6	23000	32000
40	H	CO_2Et	14	23000	>100 μM[b]
41	H	CH_2OH	67	ND	ND
42	H	$CONHCH_2CH_2NMe_2$	30	ND	>100 μM
43	H	$CONHCH_2CH_2OH$	10	108000	>100 μM
44	H	$CON(CH_2CH_2)_2O$	1.6	34400	>30 μM
45	H	$CON(CH_2CH_2)_2NMe$	1.4	34700	65000

[a] Calculated K_i. [b] <50% inhibition @ 100μM. [c] ND = not determined.

Table 6. Comparison of Physicochemical Properties of **3** and **26**

physicochemical property[a]	**3**	**26**
MW	340.8	385.8
Log $D_{7.4}$	2.3	0.0
pK_a	2.35, 8.4	3.4, 7.7
solubility: human plasma	60−120 μM	1500 μM
PBS (pH 6.5)	<1.0 μg/mL	2.3 μg/mL
PPB: human	99.8	96.7

[a] PBS: phosphate buffered saline. PPB: plasma protein binding.

atom was revisited in this series (cf. Table 2), and, as before, the introduction of a Me group (**38**) had no beneficial effect. However, the incorporation of a pendent amino group **39** gave a very potent (K_i 2.6 nM) and selective inhibitor, once again showing an additional basic center could be accommodated at an appropriate position. The search for a nonacid group was also more extensively explored in this series with compounds **40**−**45**. Ethyl ester **40** had good affinity (K_i 14 nM), but once again compounds that incorporated an alcohol (**41**, K_i 67 nM) contributed little to binding (cf. **7**, K_i 71 nM). Following the encouraging result with proline carboxamide **37**, a small series of carboxamides were prepared derived from **28**. Secondary carboxamides with a pendent amine **42** or, more preferably, a pendent alcohol **43** were tolerated; however, our most potent and selective uPA inhibitors were derived from tertiary carboxamides. Morpholine amide **44** and *N*-methylpiperazine amide **45** are both potent inhibitors of uPA (K_i ca. 1−2 nM) and demonstrate >20000-fold selectivity over tPA and plasmin.

Throughout the course of analogue preparation, compounds were evaluated for their physicochemical properties, and selected results for **26**, along with **3**, are shown in Table 6. Sulfonamide **26** is of an acceptable size (as measured by MW) and reasonable lipophilicity (Log $D_{7.4}$) and is within our target range for topical delivery. The ionization constant of the guanidine group in **26** (pK_a 7.7) is somewhat lower than usual and warrants additional comment. There are a number of factors that contribute to the weak basicity of the guanidine: (i) the electron-deficient nature of the isoquinoline ring, (ii) a putative internal H-bond between an NH of the guanidine and the N atom of the isoquinoline ring; this interaction serves to hold the guanidine essentially in the plane of the isoquinoline,[17] and (iii) the electron-withdrawing effects of the 7-sulfonamide substituent.

The solubility of the inhibitors was routinely measured in phosphate-buffered saline (PBS), and then the solubility of preferred compounds was measured in human plasma as a surrogate for human chronic wound fluid. The increased solubility of **26** compared to **3** (>10-fold) is likely to aid delivery of the compound from a suitable formulation into the wound environment.[21,22] The lower lipophilicity of **26**, compared to **3**, translated to a lowering of plasma protein binding (PPB) and a higher free drug fraction (16-fold).

Table 7. Selectivity vs Enzymes of the Coagulation Cascade for **3** and **26**

enzyme[a]	K_i /nM or % inhib **3**	**26**
thrombin (b)	2% inhib @ 10 μM	ND
Factor VIIa (h)	3% inhib @ 10 μM	ND
Factor IXa (h)	1240 nM	13% inhib @ 12.5 μM
Factor Xa (h)	0% inhib @ 10 μM	0% inhib @ 10 μM

[a] Human (h), bovine (b). [b] ND = not determined.

Table 8. Comparison of *in Vitro* Potency of **3** and **26**

	3	**26**
in vitro primary potency, measured K_i/μM	0.017 ± 0.0007	0.012 ± 0.002
in vitro potency in porcine wound homogenate, IC_{50}/μM	8.35 ± 2.5	0.39 ± 0.05
in vitro potency in human wound fluid, IC_{50}/μM	6.91 ± 1.18	0.89 ± 0.26

The selectivity of **3** and **26** for uPA over several serine proteases which have important functions in the coagulation cascade was assessed (Table 7).[23] No significant inhibition of these enzymes was observed as measured by chromogenic enzyme assays.[24] Furthermore, the effects of **3** upon thrombin time (TT), activated partial thromboplastin time (APTT), and prothrombin (PT) times of human plasma were evaluated as functional measures of the potential effects upon the coagulation cascade. Compound **3** did not significantly affect TT, APTT, or PT over the concentration range 0.3−100 μM where clinically relevant changes in clotting times are regarded as >2-fold increase in normal values.

On the basis of the enhanced potency and selectivity profile, and attractive physicochemical properties, **26** was selected for further evaluation using two *in vitro* models for uPA inhibition in physiological wound samples (Table 8). The ability of **26** to inhibit exogenous uPA added to porcine acute wound tissue homogenate, and exogenous uPA added to human chronic wound fluid, was measured. Compound **26** demonstrated an ability to inhibit uPA in both these systems to a greater extent than compound **3**. This enhanced ability of **26** to inhibit uPA is explained by the improved affinity for uPA in conjunction with the significantly greater free fraction. The drop off in

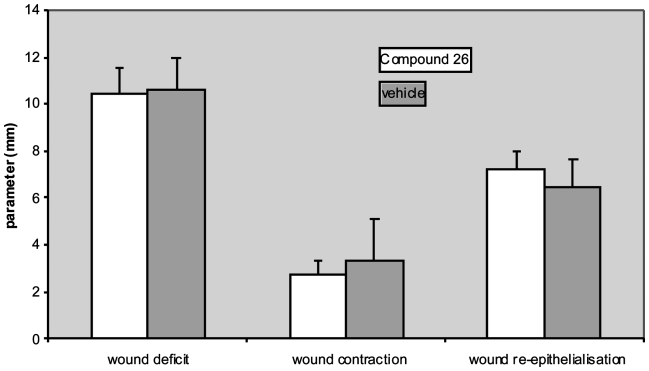


Figure 1. Effect of **26** on wound healing parameters. Compound **26**: white bars, $n = 7$ (mean ± SEM); vehicle: gray bars, $n = 8$ (mean ± SEM).

potency of **3** and **26** in these assays compared to the uPA primary assay is simply a function of the reduction of the free drug levels by nonspecific binding to tissue homogenate or plasma proteins.

It should be noted that there is no animal model of chronic wound healing that demonstrates uPA upregulation which is predictive of efficacy in human chronic dermal ulcers. Therefore, **26** was evaluated in an acute (normal) wound model.[25,26] It was not expected that a uPA inhibitor would have a beneficial effect on wound healing parameters in this model as uPA levels are not elevated. However, as uPA is present in acute wounds, it was important to demonstrate that uPA inhibition by **26** is not detrimental to normal processes of dermal repair. Thus, the aim of this *in vivo* study was to determine the ability of topically applied **26** to inhibit exogenous uPA, to determine topical and systemic pharmacokinetics, and to demonstrate safety with respect to wound closure parameters. A porcine acute excisional wound model was employed as pig is a 'tight skinned' mammal like human and has similar acute healing processes. An excisional wound model would allow quantification of healing parameters over a period of several days.

Two female pigs were subjected to eight excisional wounds. The wounds were dressed and treated daily for 10 days with either 1 mL of a 10 mg/mL formulation of **26** in hydrogel vehicle, or hydrogel vehicle alone (control). On day 11 the animals were sacrificed, terminal blood samples were taken to assess any systemic exposure of the compounds, and the wounds were excised from the surrounding normal skin. The central portion of each wound was fixed and mounted for histological analysis of wound re-epithelialization. The remaining wound tissue was homogenized and analyzed for its ability to inhibit uPA activity.

Compound **26** demonstrated no adverse effects on acute wound closure in this model. Comparison of results from wounds treated with **26** and vehicle, or with vehicle alone, as measured by wound deficit, wound contraction, and wound re-epithelialization showed no significant difference (Figure 1). Compound **26** was shown to penetrate into the pig wounds following topical delivery. Concentrations of **26** in the dermis were 41.8 ± 9.0 μM. This result was supported by the *ex vivo* assay results which demonstrated 82.3 ± 7.6% inhibition of exogenous uPA. Hence, compound **26** has the correct physicochemical properties to penetrate wounds and is able to inhibit uPA activity. In addition, topical dosing of **26** caused no detected systemic exposure (limit of detection = 20 nM).

Enzyme−Inhibitor Binding

As inhibitor synthesis was underway and our understanding of SAR was evolving, contemporaneous studies were undertaken to cocrystallize one of these inhibitors in the active site domain of uPA. Compound **33** was cocrystallized with uPA, and X-ray data to 2.3 Å resolution was collected for the uPA−**33** complex (Figure 2). Compound **33** was unambiguously modeled into the initial difference maps, and the binding mode for **33** in uPA was defined. The structure shows the guanidine group making the previously defined interactions in the S1 pocket. The 4-chlorine atom packs against the side chain of Ser-195, which has rotated to accommodate the inhibitor perturbing the catalytic triad. Further direct interactions between uPA and inhibitor **33** are limited to the proline acid moiety which is in good hydrogen-bonding distance to the Nϵ of Arg-217 and the main-chain NH of Gly-218. Also of note, **33** does not gain any affinity through an interaction with Gly-216, and the excellent selectivity is clearly not gained through an interaction with the 97 insertion loop (Thr-97A-Leu-97B) (which is unique to uPA and nonexistent in tPA or plasmin), as **33** does not contact that region of the uPA active site.

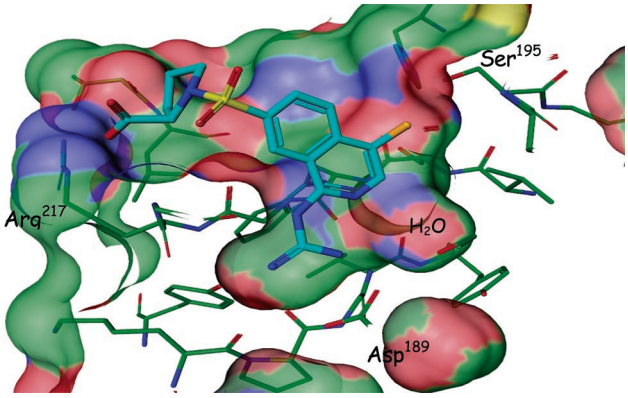


Figure 2. Inhibitor **33** (blue) in uPA X-ray showing enzyme surface and key residues.

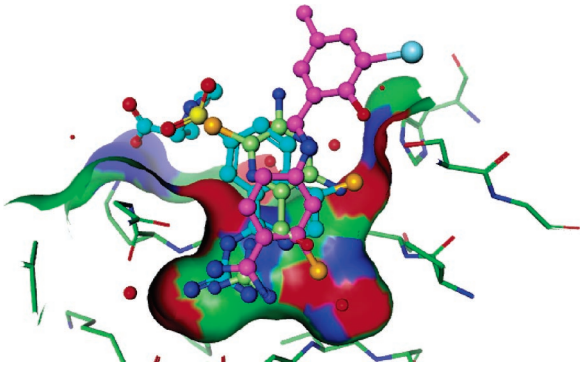


Figure 3. Overlay of the crystal structures of **33** (blue), amiloride (green), and APC-11421 (magenta). The trajectory of exit from P1 is maintained.

Following the initial crystal structure of uPA in 1995,[19] a number of high resolution, noncovalent inhibitor−uPA complex crystal structures have been determined and are now available in the protein data bank (PDB).[27−30] The complex with amiloride,[28] which maintains the guanidine group interactions, shows the same trajectory out of P1 as **33** (Figure 3). Additional conserved interactions within P1 include the position of a H-bond acceptor: the nitrogen of the isoquinoline ring of **33** overlaps with the carbonyl moiety of amiloride, accepting a good H-bond from the conserved water within P1; this water in turn forms an H-bond to the side chain of Ser190. The significance of this H-bond network in selectivity of inhibitors over serine protease members which have an Ala at position 190 (e.g., tPA

and factor Xa) has been established by a series of selective inhibitors from Axys[30] in which a halogen atom displaces the P1 water. Selectivity of **33** and amiloride may be due to enhancing this hydrogen-bonding network to the serine, an interaction not available when an alanine is present.

Conclusion

We have shown that introduction of a 7-sulfonamide substituent into the 1-isoquinolinyl guanidine template has furnished a new series of potent and highly selective uPA inhibitors. Potency and selectivity can be achieved with sulfonamides derived from amines and is further enhanced by the incorporation of sulfonamides derived from amino acids (e.g., **26**−**31** and **33**). The most potent and selective examples are sulfonamides derived from cycloleucine which has either the free carboxylate (**28** and **39**) or a carboxamide (**44** and **45**) as part of the amino acid moiety. The binding mode of these 1-isoquinolinylguanidines in uPA was confirmed by X-ray cocrystallization studies. Sulfonamide ligand **33** adopts a similar trajectory of exit from the P1 pocket as has been seen previously although this binding mode could not have been predicted from the uPA-EGR-cmk structure.

Finally, on the basis of the excellent enzyme potency and selectivity profile, ability to inhibit exogenous uPA in human chronic wound fluid, physicochemical properties suitable for topical application, and safety profile in the porcine excisional wound model, **26** (UK-371,804) was selected as a candidate for further preclinical evaluation for the treatment of chronic dermal ulcers.

Experimental Section

Chemistry. General Details. Melting points (mp) were determined using open glass capillary tubes and either Gallenkamp or Electrothermal melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) data were obtained using a Varian Unity 300 or a Varian Inova 400. Low-resolution mass spectral (LRMS) data were recorded on a Fisons Instruments Trio 1000 (thermaspray) or a Finnigan Mat. TSQ 7000 (APCI). The calculated and observed ions quoted refer to the isotopic composition of lowest mass. Elemental combustion analyses (Anal.) were determined by Exeter Analytical UK. Ltd. Column chromatography was performed using Merck silica gel 60 (0.040−0.063 mm). Log $D_{7.4}$ determinations were similar to those described by Stopher and McClean.[31] pK_a were determined by Sirius Analytical Instruments Ltd (UK). The following abbreviations are used: *N*-chlorosuccinimide, NCS; 1,2-dimethoxyethane, DME; dimethyl sulfoxide, DMSO; trifluoroacetic acid, TFA.

General Methods for the Synthesis of 1-Guanidino-7-isoquinolinesulfonamides. 4-Chloro-1(2*H*)-isoquinolone (56). A solution of NCS (9.66 g, 72 mmol) in MeCN (80 mL) was added dropwise to a stirred solution of 1-(2*H*)-isoquinolone (**55**) (10 g, 69 mmol) in MeCN (250 mL) which was being heated under reflux. The mixture was heated under reflux for an additional 1.5 h and then cooled to room temperature. The resulting precipitate was collected by filtration, with MeCN rinsing, and then dried *in vacuo* to give **56** (11.3 g, 62.9 mmol) as a pale pink solid. ^{1}H (DMSO-d_6, 300 MHz) δ 7.5 (1H, s), 7.6 (1H, dd), 7.8−7.9 (2H, m), 8.25 (1H, d), 11.5 (1H, br s), ppm; LRMS 180, 182 (MH^+), 359, 361, 363 (M_2H^+).

4-Chloro-1-oxo-1,2-dihydro-7-isoquinolinesulfonyl Chloride (57). 56 (20.62 g, 115 mmol) was added portionwise to stirred chlorosulfonic acid (61 mL, 918 mmol) at 0 °C. The mixture was heated at 100 °C for 3.5 d and then cooled to room temperature. The reaction mixture was added in small portions onto ice−water [CAUTION], and the resulting precipitate was collected by filtration. The solid was washed with water, triturated with MeCN, and then dried *in vacuo* to give **57** (18.75 g, 67.4 mmol) as a cream solid. ^{1}H (DMSO-d_6, 400 MHz) δ 7.45 (1H, s), 7.8 (1H, d), 8.0 (1H, d), 8.5 (1H, s), 11.5 (1H, br s) ppm.

1,4-Dichloro-7-isoquinolinesulfonyl Chloride (47). $POCl_3$ (9.65 mL, 103.5 mmol) was added to a stirred suspension of **57** (22.1 g, 79.6 mmol) in MeCN (500 mL) at room temperature, and the mixture was then heated at reflux for 15 h. On cooling, the MeCN solution was decanted from the insoluble sludge and evaporated *in vacuo*. The residue was extracted with hot EtOAc and evaporated to leave a solid which was stirred with Et_2O (1.2 L) at room temperature overnight. The ethereal solution was decanted from the insoluble material and evaporated *in vacuo* to give **47** (20 g, 67 mmol) as a pale yellow solid. ^{1}H (DMSO-d_6, 400 MHz) δ 8.2 (2H, s), 8.5 (1H, s), 8.55 (1H, s) ppm.

Synthesis of 7-Sulfonamides (49). General Method. A solution of **47** (2.0 mmol) in CH_2Cl_2 (3 mL) was added to a stirred solution of the amine (2.4 mmol) and NEt_3 (0.70 mL, 5.0 mmol) in CH_2Cl_2 (3 mL), and the mixture was stirred at room temperature for 24 h. The mixture was diluted with CH_2Cl_2 (50 mL), washed with dilute HCl (2 M), saturated aqueous $NaHCO_3$, and brine, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by column chromatography upon silica gel.

Synthesis of 1-Guanidino-7-isoquinolinesulfonamides. General Method. NaH (30 mg, 80% dispersion by weight in mineral oil, 1.01 mmol) was added in one portion to a stirred suspension of guanidine hydrochloride (154 mg, 1.61 mmol) in DME (5.0 mL), and the mixture was heated at 60 °C under N_2 for 45 min. A solution of the 1-chloroisoquinoline (**49**) (0.40 mmol) in DME (2.0 mL) was added and the mixture heated at 95 °C for 4 h. The solvents were evaporated *in vacuo*, and the residue was purified by column chromatography upon silica gel.

Complete experimental procedures, along with spectroscopic and analytical data for the preparation of all intermediates and target compounds for the uPA inhibitors **4**−**45** can be found in the Supporting Information.

Preparation of uPA Inhibitors 3, 26, and 33. (4-Chloro-7-(3-carboxyphenyl)isoquinolin-1-yl)guandine Monohydrate (3). The preparation of **3** has been reported previously.[14] off-white solid. mp >300 °C; ^{1}H (DMSO-d_6, 300 MHz) δ 7.3 (3H, br s), 7.63 (1H, t), 7.98 (4H, m), 8.11 (1H, d), 8.27 (1H, s), 8.96 (1H, s) ppm; LRMS 341, 343 (MH^+); Anal. ($C_{17}H_{13}ClN_4O_2S\cdot H_2O$) C, H, N.

2-{[(4-Chloro-1-guanidino-7-isoquinolinyl)sulfonyl]amino}-isobutyric Acid Hydrochloride (26). Methyl 2-{[(1,4-Dichloro-7-isoquinolinyl)sulfonyl]amino}isobutyrate (26b). A mixture of methyl 2-aminoisobutyrate (1.24 g, 8.07 mmol), NEt_3 (2.34 mL, 16.9 mmol), and **47** (2.00 g, 6.74 mmol) in CH_2Cl_2 (120 mL) was stirred at 23 °C for 15 h. The mixture was diluted with CH_2Cl_2 (100 mL), washed with dilute HCl (2 M), saturated aqueous $NaHCO_3$, and brine, dried (Na_2SO_4), and evaporated *in vacuo* The residue was purified by column chromatography upon silica gel using hexane−EtOAc (70:30) as eluant to give **26b** (1.13 g, 3.00 mmol) as a white solid. mp 159.5−161 °C (EtOAc); ^{1}H ($CDCl_3$, 400 MHz) δ 1.5 (6H, s), 3.7 (3H, s), 5.55 (1H, s), 8.25 (1H, d), 8.35 (1H, d), 8.5 (1H, s), 8.9 (1H, s) ppm; LRMS 377 (MH^+).

1-{[(4-Chloro-1-guanidino-7-isoquinolinyl)sulfonyl]amino}-isobutyric Acid Methyl Ester (26a). NaH (199 mg, 80% dispersion by weight in mineral oil, 6.63 mmol) was added in one portion to a stirred solution of guanidine hydrochloride (1.05 g, 10.6 mmol) in DMSO (20 mL), and the mixture was heated at 50 °C under N_2 for 20 min. **26b** (1.00 g, 2.65 mmol) was added in one portion and the mixture heated at 80 °C for 6.5 h. The cooled mixture was poured into water (200 mL) and extracted with EtOAc (3 × 75 mL), and the combined organic extracts were washed with water and brine, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by column chromatography upon silica gel using CH_2Cl_2−MeOH−0.880NH_3 (95:5:0.5 then 90:10:01) as eluant to give the product. Recrystallization with EtOAc gave **26a** (270 mg, 0.675 mmol) as yellow solid. mp >170 °C (dec); ^{1}H (CD_3OD, 300 MHz) δ 1.4 (6H, s), 3.5 (3H, s), 8.15−8.25 (3H, m), 9.1 (1H, s) ppm; LRMS 400, 402 (MH^+).

2-{[(4-Chloro-1-guanidino-7-isoquinolinyl)sulfonyl]amino}-isobutyric Acid (26). A solution of NaOH (1 mL, 2 M, 2 mmol)

was added to a solution of **26a** (204 mg, 0.51 mmol) in MeOH (8 mL), and the mixture was heated at 40−50 °C for 16 h. The cooled mixture was neutrilized with dilute HCl (1 mL, 2 M) to give a precipitate. The solid was collected by filtration, with copious water washing, and then dissolved in concd HCl. The solvents were evaporated *in vacuo*, azeptroping with PhMe, and then dried under high vacuum to give **26**·HCl (68 mg, 0.16 mmol) as a pale cream solid. mp 258 °C (dec); ^{1}H (CD_3OD, 400 MHz) δ 1.45 (6H, s), 8.4 (1H, d), 8.4 (1H, s), 8.45 (1H, d), 8.9 (1H, s) ppm; LRMS 386, 388 (MH^+); Anal. ($C_{14}H_{16}ClN_5O_4S$·HCl·0.8H_2O) C, H, N.

***N*-[(4-Chloro-1-guanidino-7-isoquinolinyl)sulfonyl]-D-proline Hydrochloride (33). *N*-[(1,4-Dichloro-7-isoquinolinyl)sulfonyl]-D-proline *tert*-Butyl Ester (33b).** A mixture of d-proline *tert*-butyl ester hydrochloride (340 mg, 1.64 mmol), NEt_3 (0.50 mL, 3.6 mmol), and **47** (400 mg, 1.35 mmol) in CH_2Cl_2 (30 mL) was stirred at 23 °C for 20 h. The mixture was diluted with CH_2Cl_2 (50 mL), washed with dilute HCl (2 M), saturated aqueous $NaHCO_3$, and brine, dried ($MgSO_4$), and evaporated *in vacuo* to give **33b** (550 mg, 1.28 mmol) as a white solid. mp 80−82 °C; ^{1}H ($CDCl_3$, 400 MHz) δ 1.4 (9H, s), 1.9−2.0 (3H, m), 2.2 (1H, m), 3.4−3.6 (2H, m), 4.4 (1H, m), 8.3 (1H, d), 8.4 (1H, d), 8.5 (1H, s), 8.9 (1H, s) ppm; LRMS 431 (MH^+), 448 (MNH_4^+).

***N*-[(4-Chloro-1-guanidino-7-isoquinolinyl)sulfonyl]-D-proline *tert*-Butyl Ester (33a).** Guanidine hydrochloride (220 mg, 2.3 mmol) was added in one portion to a stirred suspension of NaH (55 mg, 80% dispersion by weight in mineral oil, 1.83 mmol) in DME (8 mL) and the mixture was heated at 60 °C under N_2 for 30 min. **33b** (250 mg, 0.58 mmol) was added and the mixture heated at reflux for 5 h. The cooled mixture was diluted with EtOAc, washed with water and brine, and dried ($MgSO_4$), and the solvents were evaporated *in vacuo*. The residue was purified by column chromatography upon silica gel using CH_2Cl_2−MeOH−0.880NH_3 (97:3:0.3) as eluant to give **33a** (200 mg, 0.44 mmol) as a yellow solid. mp >170 °C (dec); ^{1}H ($CDCl_3$, 400 MHz) δ 1.45 (9H, s), 1.7−1.8 (1H, m), 1.8−2.05 (3H, m), 3.3−3.45 (1H, m), 3.5−3.6 (1H, m), 4.3 (1H, dd), 6.3−6.6 (4H, br), 8.05 (1H, d), 8.1 (1H, d), 8.1 (1H, s), 9.2 (1H, s) ppm; LRMS 454, 456 (MH^+).

***N*-[(4-Chloro-1-guanidino-7-isoquinolinyl)sulfonyl]-D-proline (33). 33a** (50 mg, 0.11 mmol) was dissolved in a solution of EtOAc saturated with HCl (10 mL) and the mixture stirred at room temperature for 2.5 h. The mixture was concentrated *in vacuo*, azeotroping with CH_2Cl_2, to give **33**·HCl (40 mg, 0.092 mmol) as a white powder. mp >200 °C (dec); ^{1}H (CD_3OD, 400 MHz) δ 1.7−1.85 (1H, m), 1.9−2.2 (3H, m), 3.4−3.5 (1H, m), 3.5−3.6 (1H, m), 4.4 (1H, dd), 8.4 (1H, d), 8.45 (1H, s), 8.5 (1H, d), 8.9 (1H, s) ppm; LRMS 397, 399 (MH^+). Anal. ($C_{15}H_{16}ClN_5O_4S$·1.0HCl·0.2H_2O·0.25CH_2Cl_2) C, H, N.

Biology. Determination of Inhibitor Potency and Selectivity. High molecular weight human uPA from urine, 3000 IU/vial (Calbiochem, 672081), was reconstituted in H_2O to give 30000 IU/mL stock and stored frozen (−18 °C). Chromogenic urokinase substrate pyro-Glu-Gly-Arg-*p*-nitroanilide (S-2444), 25 mg/vial (Quadratech, 820357), was reconstituted in H_2O to give 3 mM stock and stored at 4 °C. Human tPA stimulator (Chromogenix 822130−63/9) was reconstituted to 1 mg/mL in buffer and used fresh. Human tPA (one chain), 10 μg/vial (Chromogenix, 821157-039/0), was reconstituted to 4 μg/mL in buffer and used fresh. S-2288, chromogenic substrate for serine proteases, 25 mg/vial (Chromogenix, 820852−39), was reconstituted in H_2O to give 10 mM stock and stored at 4 °C. Human plasmin, 2 mg/vial (Quadratech, 810665), was reconstituted to 1 mg/mL in buffer and stored frozen (−18 °C). Chromozym-PL (Boehringer Mannheim, 378 461), 1 mM stock in buffer, was prepared fresh.

IC_{50} and K_i values for compounds were calculated by incubation of 33 IU/mL uPA with 0.18 mM S-2444 (substrate) and various compound concentrations, all diluted in uPA assay buffer (75 mM Tris, pH 8.1, 50 mM NaCl). A preincubation of compound with enzyme was carried out for 15 min at 37 °C, followed by substrate addition and further incubation for 30 min at the same temperature. The final assay volume was 200 μL. Absorbance was read at 405 nM following preincubation (background, time zero measurement) and following the 30 min incubation with substrate using a SPECTRAMax microplate reader (Molecular Devices Corporation. Background values were subtracted from the final absorbance values. Percentage inhibition was calculated and plotted against compound concentration to generate IC_{50} values. The enzymatic K_i was calculated from the known K_m of the substrate, 90 μM using the equation

$$K_i = IC_{50}/((1 + ([S]/K_m))$$

The method for analysis of tPA inhibition was similar to that for uPA inhibition. The assay utilized final concentrations of tPA at 0.4 μg/mL with 0.1 mg/mL tPA stimulator, 0.4 mM H-D-Ile-Pro-Arg-*p*-nitroanilide dihydrochloride substrate (S-2288), and various concentrations of inhibitors made up in uPA assay buffer. Preincubation was carried out with compound, enzyme, and enzyme stimulator, as for uPA, prior to the incubation with substrate. Incubation time was 60 min at performed at 37 °C. Data analysis was identical to that described above for uPA, using a known K_m for tPA of 250 μM.

Plasmin inhibition was assayed by incubating human plasmin at 0.7 μg/mL with 0.2 mM Chromozym-PL (substrate) and various concentrations of inhibitors in uPA assay buffer. Preincubation was carried out as for uPA, and the incubation was performed at 37 °C for 30 min. Data manipulation and percentage inhibition was calculated as for uPA, using a known K_m for plasmin of 200 μM.

The measured K_i of compounds for uPA was determined using a number of different substrate (30−300μM) and inhibitor (0−150 nM) concentrations. Compounds were diluted from a 10 mM stock in DMSO to the required concentration in uPA assay buffer. The assay format was identical to that carried out for IC_{50} determination. Following substrate addition the cleavage rate was measured at 30 s intervals for 30 min at 37 °C at 405 nm). The rates of substrate cleavage were used to determine K_i for the compound:enzyme complex.

Determination of Potency in Porcine Wound Homogenate and Human Wound Fluid. The uPA bioassay used a peptide probe to quantify the amount of active uPA inhibitor compound in a particular biological sample. The probe biotin−glutamate-glycine-arginine−chloromethyl ketone (PFBL-1) molecule is directed to the active site of uPA (*via* glutamate-glycine-arginine) and binds irreversibly to the enzyme (*via* the chloromethyl ketone moiety). The degree of probe:uPA enzyme complex was then detected and quantified by colorimetric analysis (*via* biotin−streptavidin label). Our studies have demonstrated that the probe:uPA enzyme interaction, although irreversible, could be blocked in a concentration-dependent fashion by the presence of competitive uPA inhibitors. Therefore, the concentration of compound in wound sample governs the amount of probe−uPA enzyme complex, which is formed at any particular time. As the glutamate-glycine-arginine moiety was not exclusively specific for uPA, the amount of specific probe−uPA enzyme complex formed was separated from other probe−enzyme complexes by protein electrophoresis.

Assay of uPA Inhibitor Activity in Human Wound Fluid. Human chronic wound fluids 9001, 9003, and 9004 were obtained from Dr. M. Stacey. Inhibition of exogenous uPA added to wound fluid samples was assayed by incubating 0.5 μL of 375 ng/μL human uPA with 0.5 μL of various concentrations (0.01−100 mL) of compound dissolved in DMSO, with 2 μL 100% human chronic wound fluid, at 37 °C for 15 min. This was followed by incubation with 0.5 μL of 7 mM biotin−Glu-Gly-Arg−chloromethyl ketone (PLFB-1) in PBS at 37 °C for 10 min. Samples were prepared for gel electrophoresis by the addition of 15 μL of sample buffer containing 5% 2-mercaptoethanol and boiling for 5 min.

Assay of uPA Inhibitor Activity in Pig Granulation Tissue Homogenate. Twelve full thickness 15 mm punch biopsies were taken from the back of a pig. Granulation tissue was allowed to accumulate for 3 days, followed by excision of the wound. Pig wounds were stored at −70 °C and then kept on ice. An 8 mm punch biopsy was made through the wound bed, and the dermis was homogenized (30% w/v) in uPA assay buffer (75 mM Tris,

pH 8.1, 50 mM NaCl) with a hand-held glass homogenizer. Homogenates were stored at −20 °C.

Inhibition of exogenous uPA added to pig wound homogenate supernatants was assayed by incubating 10 mL of granulation tissue homogenate supernatant with 1 μL of 375 ng/mL human uPa and 1 μL of PFBL-1 (final concentration of 1μM). This assay mixture was incubated at 37 °C for 10 min, shaking throughout. Samples were prepared for gel electrophoresis by the addition of 12 μL of sample buffer containing 5% 2-mercaptoethanol and boiling for 5 min.

Electrophoresis and Western Blotting. Human wound fluid samples were analyzed with a gel loading of 5.5 μL/lane and pig granulation tissue homogenates with a gel loading of 15 μL/lane. The separation was carried out at 125 V for ∼120 min using an XCellII Mini-Cell). The gel was removed to transfer buffer (48 mM Tris base, 39 mM glycine, 10% (v/v) methanol) for 15 min before blotting onto a nitrocellulose membrane using a Trans Blot SD cell 'semi-dry' system (BioRad).

Detection of uPA Inhibition. The nitrocellulose membrane was blocked with 3% bovine serum albumin (BSA) in water at room temperature for 30 min and washed twice for 5 min in 2 mM Tris-HCl, 50 mM NaCl (TBS)/0.05% Tween (TTBS). The membrane was then incubated with Streptavidin-AP conjugate (1 U/mL in TBS for 30 min) and washed twice in TTBS for 7.5 min before the addition of BCIP/NBT (one tablet in 10 mL water) for 5 min. The color development was halted by washing with water. The nitrocellulose was dried, and the intensity of staining of the uPA bands was assessed using the imaging densitometer (BioRad Model GS-700). The % inhibitions and 50% inhibition values of the added uPA were calculated.

Topical Application of compound 26 to Porcine Acute Excisional Wounds. Compound **26** was assessed in a model of porcine acute excisional wound healing to ensure no adverse effects or inhibition of any measureable aspect of wound healing and a measure of topical and systemic pharmacokinetics.

Formulation. The vehicle gel for this study was made from the following reagents: Glycerol (5%), Lutrol F127 (2%), Blanose Carboxy Methyl Cellulose (CMC) Hydrogel (7HF) (3%), and water (to 100%). Lutrol F127 was dissolved and the glycerol dispersed in water at 4 °C. Once at room temperature, CMC was added during vortex mixing until fully dissolved. For production of gel containing 26, solid compound was passed through a 180 μM pore sieve and added to the glycerol/water/F127 mixture, prior to addition of CMC. The final 26 concentration in the suspension was 10 mg/mL. Vehicle or compound containing gel was then loaded into 2 mL sterile syringes and stored at 4 °C prior to use. Dose levels: 26; 1 mL of 10 mg/mL (3.18 mg/cm^2, 10 mg/day) applied topically at daily intervals.

Animal Study. Female pigs (crossbreed of Danish country, Duroc, and Yorkshire) were subjected to eight full thickness excisional skin wounds of 20 mm diameter (four on each side of the spine), using a circular knife. Wounds were dressed and treated daily for 10 days with 1 mL of a 10 mg/mL formulation of compound formulated in hydrogel or hydrogel alone (control). Each wound was assessed daily for inflammation, hemorrhage exudation, necrosis, hypergranulation, granulation tissue deposition, and wound area by planimetry. On day 11 the animals were sacrificed and terminal blood samples were taken to assess any systemic exposure of the compounds. Following treatment the wounds were excised from the surrounding normal skin. The central portion of each wound was fixed and mounted for histological analysis of wound re-epithelialization. The remaining wound tissue was homogenized as described above and analyzed for its ability to inhibit uPA activity using the assay described above.

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Supporting Information Available: Complete experimental details, along with spectroscopic and analytical data, for the preparartion of all intermediates and target compounds for the uPA inhibitors **4**−**45**. A reaction scheme for a synthesis of amino acid derivatives which are not readily available. A detailed account of the enzyme−inhibitor modeling and X-ray structure determination of both uPA and trypsin. Experimental details for the uPA and trypsin X-ray structure determinations. Materials and methods for solubility measurements in PBS and human plasma. This material is available free of charge via the Internet at http://pubs.acs.org.

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