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## Abstract

A known side-activity of the oral potassium-sparing diuretic drug amiloride is inhibition of the enzyme urokinase-type plasminogen activator (uPA, $K_i$ = 7 mu M), a promising anticancer target. Several studies have demonstrated significant antitumor/metastasis properties for amiloride in animal cancer models and it would appear that these arise, at least in part, through inhibition of uPA. Selective optimization of amiloride's structure for more potent inhibition of uPA and loss of diuretic effects would thus appear as an attractive strategy towards novel anticancer agents. The following report is a preliminary structure-activity exploration of amiloride analogs as inhibitors of uPA. A key finding was that the well-studied 5-substituted analogs ethylisopropyl amiloride (EIPA) and hexamethylene amiloride (HMA) are approximately 2-fold more potent than amiloride as uPA inhibitors.

## Keywords

Synthesis, preliminary, evaluation, amiloride, analogs, inhibitors, urokinase, type, plasminogen, activator, uPA, CMMB

## Disciplines

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## Publication Details

# Synthesis and Preliminary Evaluation of Amiloride Analogs as Inhibitors of the Urokinase-Type Plasminogen Activator (uPA)

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Keywords: urokinase-type plasminogen activator; uPA; inhibitor; amiloride; structure-activity relationships

Abbreviations: EIPA, ethylisopropyl amiloride; GPI, glycosylphosphatidylinositol; HMA, hexamethylene amiloride; HMW-uPA, high molecular weight urokinase-type plasminogen activator; NBS, *N*-bromosuccinimide; NCS, *N*-chlorosuccinimide; NHE1, sodium hydrogen exchanger 1; NIS, *N*-iodosuccinimide; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2; PAS, plasminogen activation system; rp-HPLC, reverse phase high performance liquid chromatography; SOSA, selective optimisation of side-activity; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

**Abstract**

A known side-activity of the oral potassium-sparing diuretic drug amiloride is inhibition of the enzyme urokinase-type plasminogen activator (uPA, $K_i$ = 7 μM), a promising anticancer target. Several studies have demonstrated significant antitumor/metastasis properties for amiloride in animal cancer models and it would appear that these arise, at least in part, through inhibition of uPA. Selective optimization of amiloride's structure for more potent inhibition of uPA and loss of diuretic effects would thus appear as an attractive strategy towards novel anticancer agents. The following report is a preliminary structure-activity exploration of amiloride analogs as inhibitors of uPA. A key finding was that the well-studied 5-substituted analogs ethylisopropyl amiloride (EIPA) and hexamethylene amiloride (HMA) are approximately 2-fold more potent than amiloride as uPA inhibitors.

Orally active non-cytotoxic agents capable of inhibiting the progression of primary tumors to metastasis have long been a goal in anticancer drug discovery.[1] Upregulation of the plasminogen activation system (PAS) is known to play a critical role in tumor invasion and metastasis[2,3] and several components of the PAS offer attractive drug targets in this context.[4-6] In its simplest form, the PAS comprises the serine protease urokinase-type plasminogen activator (uPA), its cognate glycosylphosphatidylinositol (GPI)-anchored cell surface-bound receptor (uPAR) and two endogenous serpins; plasminogen activator inhibitor 1 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2). Receptor-bound uPA efficiently cleaves and thus activates co-localised plasminogen at the cell surface, which reveals the broad spectrum serine protease plasmin. Plasmin activates downstream extracellular proteases (e.g. matrix metalloproteases) and latent growth factors and together these lead to pericellular proteolysis and remodelling of the local extracellular environment – key events required for metastasis (reviewed in Refs 7 and 8). To highlight the importance of the PAS in metastasis, upregulated uPA and PAI-1 have been shown to be the strongest known prognostic biomarkers of shortened disease-free survival and overall survival in breast cancer and the most accurate predictors of metastasis in lymph-node-negative tumours.[9]

One approach towards dampening PAS activity and potentially inhibiting metastasis is to target the serine protease activity of uPA. Several classes of uPA inhibitors have been reported and selected analogs have undergone preclinical evaluation as non-cytotoxic antitumor/metastasis agents.[10] An orally active uPA inhibitor prodrug MESUPRON® (Wilex AG, Germany) is currently undergoing two phase II trials for the treatment of breast and pancreatic tumors.[11] A general problem with uPA inhibitors, however, is that many contain highly basic amidines or guanidines which often confer poor drug-like properties.[12] These positively charged groups are necessary for making a key salt-bridge contact with Asp189 located at the base of uPA's S1-pocket. Scaffolds which could employ less basic groups to make this crucial interaction are thus sought after for elaboration into uPA inhibitors with improved properties.

Amiloride.HCl **1** is an orally administered potassium-sparing diuretic that has been used clinically for several decades, most commonly in combination with thiazide (e.g. hydrochlorothiazide) or loop diuretics (e.g. frusemide) as an antikaliuretic.[13] Studies have shown that amiloride is also a moderately potent competitive inhibitor of uPA ($K_i$ = 7 μM)[14] and that uPA inhibition correlates with significant antitumor/metastasis effects of the compound *in vivo*.[15] The published X-ray co-crystal structure of amiloride bound to uPA[16] (Figure 1) reveals that the drug positions its acylguanidine unit deep within the S1 pocket to make the key salt-bridge interaction with Asp189. The low μM uPA inhibitory potency of amiloride combined with its demonstrated antitumor/metastasis effects in animal models, the reduced basicity of its acylguanidine ($pK_a$ = 8.8),[17] its longstanding use as a safe oral $K^+$-sparing diuretic and its known selectivity for uPA over closely related off-target serine proteases (i.e. tissue-type plasminogen activator (tPA), plasmin, thrombin and kallikrein)[14] suggest it is an excellent starting point for a selective optimisation of side-activity (SOSA)[18] study aimed at producing a more potent, orally active uPA inhibitor for use in the cancer setting. The following report is a preliminary structure-activity exploration of amiloride analogs as inhibitors of uPA.

The reported amiloride:uPA X-ray structure showed that amiloride occupies the S1 and S1β sites of uPA by making a network of hydrogen bonds and van der Waals contacts in addition to the salt-bridge interaction with Asp189 (Figure 1).[16] The Ser190 hydroxyl group forms a hydrogen bond to a terminal nitrogen atom of the acylguanidine, which in turn forms a hydrogen bond with a water molecule bound deep within the S1 pocket. The Ser190 hydroxyl also forms a hydrogen bond to this water molecule, as does the acylguanidine carbonyl oxygen. The Gly219 carbonyl oxygen forms a weak hydrogen bond (O---N distance 3.3 Å) to the acylguanidine amide NH. The Ser195 hydroxyl group is hydrogen bonded to the amino group at C3, while the amino group at C5 forms a hydrogen bond to an

adventitious sulfate ion bound in the oxyanion hole. The sulfate ion is further held in place by hydrogen

bonds to the Ser195 hydroxyl, His57 imidazole nitrogen and Gly193 amide NH. The chlorine atom at

C6 partially occupies the S1β subsite and participates in interactions with residues Gln192, Gly216,

Gly219 and the Cys191-Cys220 disulfide bond.
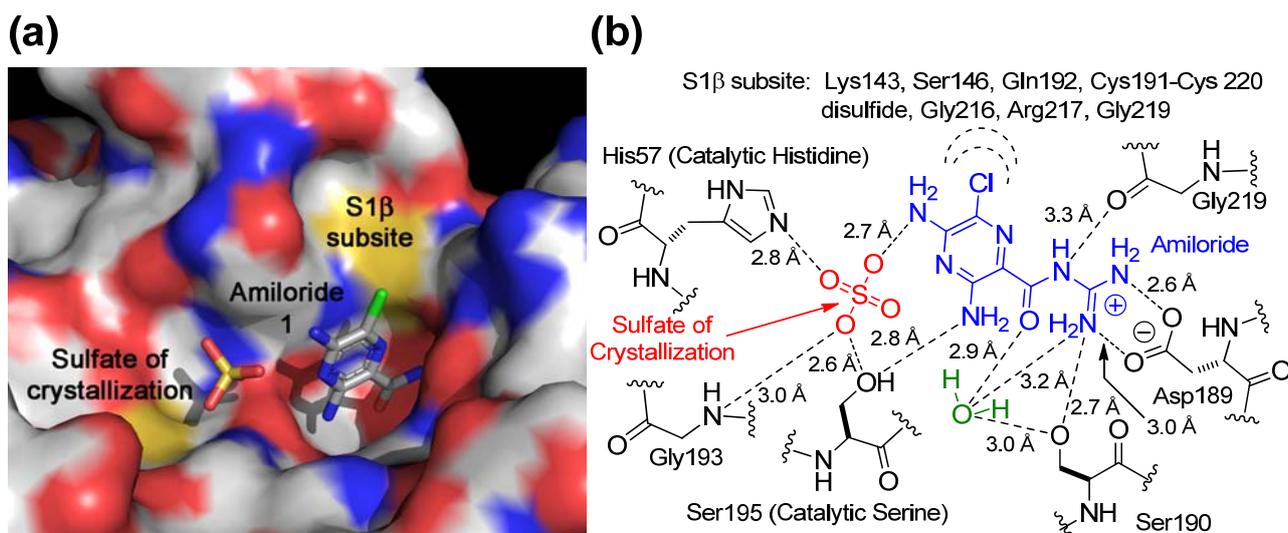


**Figure 1.** (a) X-ray crystal structure of the amiloride **1**:uPA complex (binding site only); PDB 1f5l.[16]

(b) Summary of the binding interactions between amiloride (blue), uPA (black), an adventitious sulfate

of crystallization (red) bound in the oxyanion hole and a water molecule (green) bound deep within the

S1 pocket.

The structural features of amiloride that were examined in this study are summarised in Figure 2. All

compounds were purified by preparative rp-HPLC to greater than 95% purity (analytical rp-HPLC) and

evaluated for uPA inhibitory potency using a 96-well plate *in vitro* enzyme assay. The commercially

available colorimetric uPA substrate Spectrozyme UK (American Diagnostica Inc.) was used with

active high molecular weight uPA (HMW-uPA) in all assays. Briefly, compounds were dissolved in

DMSO to create 20 mM stock solutions which were diluted with buffer in series in the plates to give

final DMSO concentrations of less than 2%. Two compounds were assayed per plate with triplicate

measurements taken for each inhibitor concentration. Assay blanks (no enzyme) were included to account for the colour of some inhibitors. Amiloride was included in all assays as a positive control ($IC_{50}$ = 11 μM under the assay conditions). Plates were incubated at 37 °C in a plate reader and absorbances read at 405 nm over time. Absorbance values were recorded for a time point taken from the linear region of plots of absorbance vs time and $IC_{50}$ values were calculated from sigmoidal dose response curves of absorbance vs log[inhibitor] using GraphPad Prism V. 5.01 software.
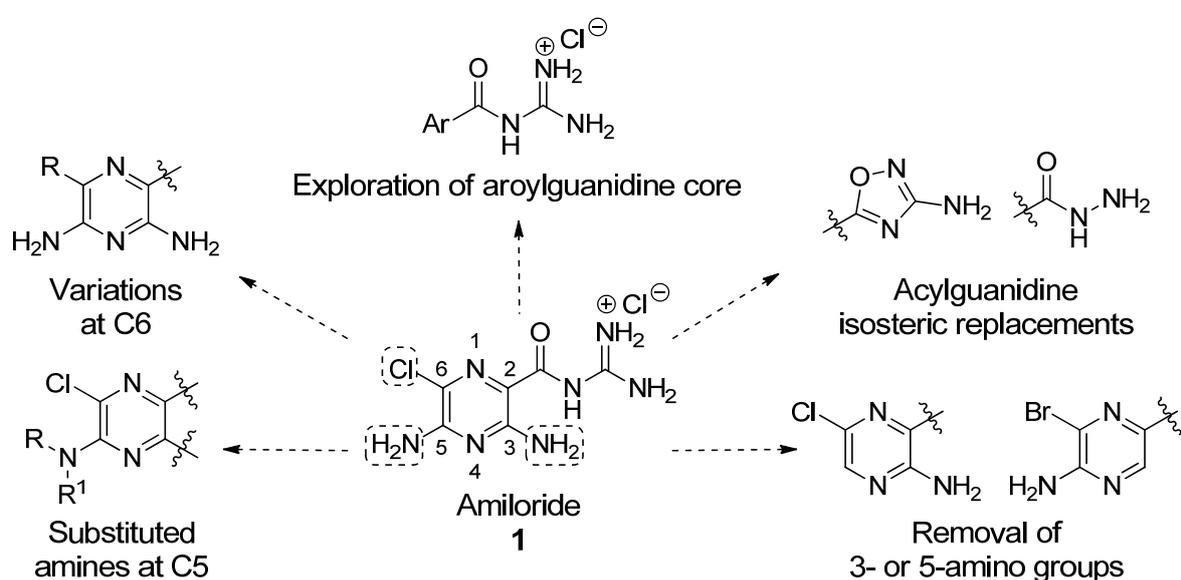


**Figure 2.** Summary of the amiloride structural features examined.

At the outset we were interested in establishing the importance of the acylguanidine:Asp189 interaction and, specifically, whether this interaction might be providing the bulk of amiloride's uPA affinity with other contacts perhaps playing only a minor role. A series of aroylguanidines **2-8** with varied aryl cores were thus synthesized by refluxing their commercially available methyl ester precursors with guanidine.HCl (pre-neutralised with Na in $^i$PrOH) in isopropanol. Methyl esters which were not commercially available were synthesised by methylation of their available carboxylic acids by using $CH_3I/Cs_2CO_3$ in *N,N*-dimethylformamide (DMF). As shown in Table 1, the aroylguanidines were all

essentially inactive with $IC_{50}$ values above 1 mM (except **7**, $IC_{50}$ = 222 μM). It was significant that 2-

pyrazinoyl guanidine **5** was inactive as this structure forms the core of amiloride. These findings are

evidence that the 2-pyrazinoyl guanidine core of amiloride is not on its own responsible for uPA

affinity and that the 3- and 5-amino and 6-chloro groups, in combination with the acylguanidine,

contribute significantly to binding. The improved potency of the substituted benzoylguanidine **7** (which

bears an aryl ring substitution pattern similar to amiloride) relative to benzoylguanidine **2** supports this

conclusion.

**Table 1.** Aroylguanidines with varied aryl cores.



| Compound | R | Yield % | $IC_{50}$, μM |
|----------|---|---------|----------------|
| **2** | | 89 | >1000 |
| **3** | | 42 | >1000 |
| **4** | | 27 | >1000 |
| **5** | | 56 | >1000 |
| **6** | | 27 | >1000 |
| **7** | | 62 | 222 |
| **8** | | 93 | >1000 |

The importance of the acylguanidine unit was further explored with analogs **9** and **10**, which incorporate 3-amino-1,2,4-oxadiazole and acylhydrazide isosteric replacements for the acylguanidine, respectively. Compound **9** was accessed in 39% yield by refluxing hydroxyguanidine hemisulfate[19] and sodium in ethanol with the commercially available methyl ester **11**.[20] Compound **10** was prepared in 81% yield by refluxing **11** with hydrazine hydrate in ethanol. Analogs **9** and **10** were both found to be inactive (IC$_{50}$ >1000 μM) (Scheme 1).



**Scheme 1.** Analogs incorporating acylguanidine isosteres.

The importance of the amino groups at the 5- and 3-positions of amiloride were investigated next with the deaminated analogs **12** and **13** (Scheme 2). Chlorination of the commercially available pyrazine methyl ester **14** by heating with *N*-chlorosuccinimide (NCS) in DMF at 80 °C gave **15** in 72% yield. Guanidinylation of **15** using the aforementioned conditions provided **12** in 87% yield. Guanidinylation of the commercially available methyl ester **16** afforded compound **13** (28%). The chloro analogue of **16** was not commercially available so the direct 3-deamino analogue of amiloride (i.e. 6-chloro-**13**) was

not pursued; however it was later noted that substitution of the 6-chloro group of amiloride **1** with bromide (i.e. 6-Br-amiloride **58**, Table 3) resulted in a 2-fold increase in activity. This suggests that 6-chloro **13** would most likely show very similar or only slightly increased activity relative to **13**.

It was found that removal of either 3- or 5-amino groups resulted in a total loss of activity ($IC_{50}$ >1000 $\mu$M). Loss of activity upon removing the amino group at C3 (i.e. **13**) may (partially) result from losing the hydrogen bond between this amine and the OH group of the catalytic Ser195 residue (Figure 1 (b)). Explaining the total loss of potency after removing the 5-amino group (i.e. **12**) is difficult since this group appears not to form any direct contacts with the enzyme, although it does form a hydrogen bond with the adventitious sulfate of crystallization bound in the oxyanion hole (Figure 1(b)).
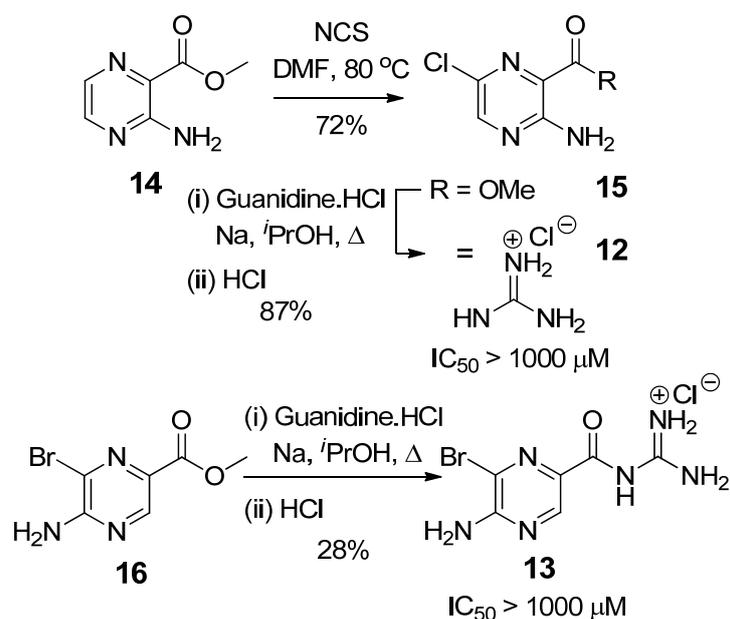


**Scheme 2.** Analogs with 3- and 5-amino groups removed.

A major part of the study focused on amiloride analogs carrying substituents at C5. This was due to their ease of synthesis and because many 5-substituted analogs are known which show reduced diuretic

effects relative to amiloride.[21] Application of the SOSA approach to identify amiloride analogs with high uPA inhibitory potency for use as antitumor/metastasis agents would (ideally) require that the evolved compounds show reduced diuretic activity and minimal effects on $K^+$-levels. While many amiloride analogs bearing hydroxyl, alkoxy, mercapto, alkylmercapto, alkyl, aryl and other substituents at the 5-position are reported,[21, 22] we chose to examine those carrying substituted amines at this position after the 5-deaminated derivative **12** was found to be inactive.

The amiloride:uPA X-ray co-crystal structure suggests that substituted amines at C5 should extend away from the pyrazine ring towards the oxyanion hole being occupied in the structure by the adventitious sulfate ion (Figure 1).[16] Analogs incorporating negatively charged groups attached to the amine via 1- or 2-carbon linkers (i.e. carboxylates **18** and **20**, sulfonate **21**, phosphonates **23**, **25**, **26** and tetrazoles **29** and **31**) were thus explored under the hypothesis that these groups could potentially mimic the sulfate ion and pick up favourable contacts within the oxyanion hole. Uncharged synthetic precursors of these inhibitors (carboxylate esters **17** and **19**, diethylphosphonates **22** and **24** and nitriles **28** and **30**, respectively) were also examined. Other 5-substituted analogs (**27**, **32**-**47**) were chosen in order to explore a cross-section of inhibitors containing aromatic rings, basic (or quaternary) amines capable of carrying positive charges, and compounds containing sulfur.

Nucleophilic aromatic substitution reactions of the 5,6-dichloropyrazinoyl guanidine **49** with requisite amines were used to prepare all 5-substituted analogs (Table 2). **49** was obtained in 84% yield from the commercially available methyl-5,6-dichloropyrazine carboxylate **48** by guanidinylation using the conditions described above.

**Table 2.** Analogs with substituted amines at C5.



| Compound | R | Yield % | IC$_{50}$ (μM) | Compound | R | Yield, % | IC$_{50}$ (μM) |
|---|---|---|---|---|---|---|---|
| **1** | NH$_2$ | a | 11 | **32** | (benzyl)NH | 97 | 8 |
| **17** | MeOC(=O)CH$_2$NH | 82 | 26 | **33** | (pyridin-2-ylmethyl)NH | 63 | 38 |
| **18** | HOC(=O)CH$_2$NH | 77[b] | 110 | **34** | (pyridin-3-ylmethyl)NH | 77 | 17 |
| **19** | EtOC(=O)CH$_2$CH$_2$NH | 69 | 25 | **35** | (pyridin-4-ylmethyl)NH | 81 | 6 |
| **20** | HOC(=O)CH$_2$CH$_2$NH | 83[c] | 102 | **36** | (4-F-benzyl)NH | 78 | 6 |
| **21** | HOS(=O)$_2$CH$_2$CH$_2$NH | 48 | > 1000 | **37** | (phenethyl)NH | 84 | 16 |
| **22** | (EtO)$_2$P(=O)CH$_2$NH | 45 | 37 | **38** | Et$_2$NCH$_2$CH$_2$NH | 88 | 14 |
| **23** | (HO)$_2$P(=O)CH$_2$NH | 75[d] | > 1000 | **39** | MeNHCH$_2$CH$_2$N(Me) | 83[f] | 3 |
| **24** | (EtO)$_2$P(=O)CH$_2$CH$_2$NH | 53 | 44 | **40** | (benzyl)N(Me)CH$_2$CH$_2$NH | 78 | 15 |
| **25** | (HO)$_2$P(=O)CH$_2$CH$_2$NH | 22[e] | > 1000 | **41** | (benzyl)NHCH$_2$CH$_2$NH | 52 | 28 |
| **26** | (EtO)(HO)P(=O)CH$_2$CH$_2$NH | 32[e] | > 1000 | **42** | (pyridinium)CH$_2$CH$_2$NH | 69 | 30 |
| **27** | (EtO)$_2$P(=O)N(H)NH | 24 | 10 | **43** | (benzyl)SCH$_2$CH$_2$NH | 92 | 21 |
| **28** | N≡CCH$_2$NH | 58 | 50 | **44** | (benzyl)S(=O)$_2$CH$_2$CH$_2$NH | 90[g] | 15 |
| **29** | (tetrazol-5-yl)CH$_2$NH | 63 | 177 | **45** | (tosyl)N(H)CH$_2$CH$_2$NH | 89 | 35 |
| **30** | N≡CCH$_2$CH$_2$NH | 61 | 16 | **46** | (N-ethyl,N-isopropyl)N | 78 | 6 |
| **31** | (tetrazol-5-yl)CH$_2$CH$_2$NH | 74 | 61 | **47** | (azepan-1-yl) | 26 | 6 |

[a]Amiloride.HCl **1** was purchased from Sigma-Aldrich. [b]Yield of **18** following hydrolysis (K$_2$CO$_3$, MeOH, H$_2$O) of **17**. [c]Yield of **20** following hydrolysis (K$_2$CO$_3$, MeOH, H$_2$O) of **19**. [d]Yield of **23** following hydrolysis (TMSCl) of **22**. [e]Yield of **25/26** following hydrolysis (TMSCl) of **24**. [f]Yield and IC$_{50}$ correspond to the unexpected bicycle **39** formed from the 2° amine cyclizing onto C6 and displacing the Cl. [g]Yield of **44** following oxidation (Oxone) of **43**.

Amines used in the synthesis of **17**, **19**, **21**, **22**, **24**, **28**, **30**, **32**-**39**, **41**, **43**, **45**-**47** were all commercially available while others required synthesis. Diethyl phosphorohydrazidate **50**, 2-(benzyl-methylamino) ethylamine **51** and 1-(2-aminoethyl)pyridinium bromide **52** (used in the synthesis of **27**, **40** and **42**, respectively) were prepared by reported methods (Scheme 3).[23-25] Tetrazolylalkylamines **53** and **54** (used in the synthesis of **29** and **31**, respectively) were prepared from commercially available alkyl chlorides by substitution with ammonia (i.e. **53**) or by conversion to the azide followed by Staudinger reduction with $PPh_3$ (i.e. **54**) (Scheme 3).
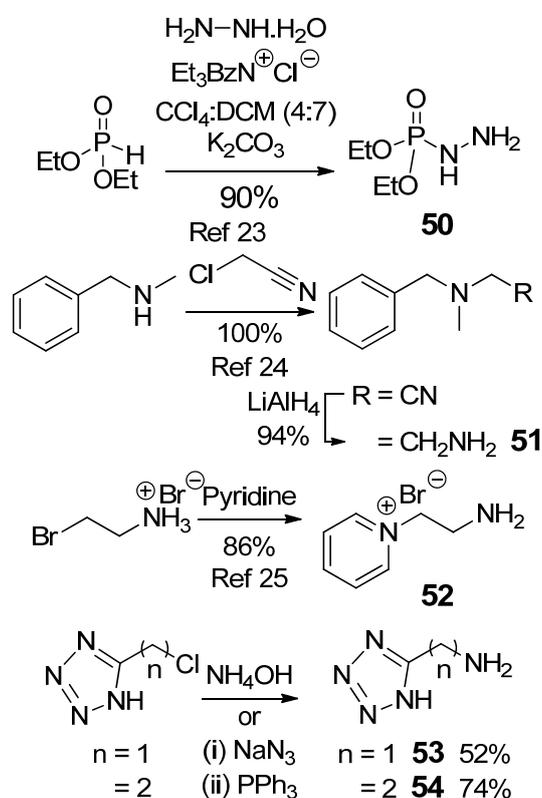


**Scheme 3.** Synthesis of non-commercially available amines **50**-**54**.[23-25]

Esters **17** and **19** were hydrolysed under basic conditions ($K_2CO_3$/MeOH/$H_2O$) to yield the carboxylic acids **18** and **20**, respectively. Phosphonate esters **22** and **24** were fully or partially hydrolysed using trimethylsilyl chloride to afford **23**, **25** and **26**. Oxidation of thioether **43** with Oxone yielded sulfone

**44**. An attempt to produce 5-(*N,N'*-dimethylethane-1,2-diamino)-amiloride by reaction of **49** with *N,N'*-dimethylethylenediamine instead resulted in formation of a novel bicycle **39**, where the free secondary amine of the initial 5-sustituted product had cyclised onto the 6-position by displacing the chloride.

The hypothesis that negatively charged groups appended at C5 might increase potency by acting as sulfate mimetics able to make favourable contacts in the oxyanion hole was found to be incorrect. All inhibitors bearing C5-substituents capable of carrying negative charges (i.e. **18**, **20**, **21**, **23**, **25**, **26**, **29**, **31**) showed dramatically reduced or total loss of activity relative to amiloride, while uncharged synthetic precursors of these inhibitors (i.e. **17**, **19**, **22**, **24**, **28** and **30**) universally showed less dramatic losses in potency ($IC_{50}$ = 25-50 µM). Only diethylphosphorohydrazidate **27** ($IC_{50}$ = 10 µM) retained the potency of amiloride. Benzylic extensions (compounds **32**-**36**) were well tolerated and produced slight (up to 2-fold) increases in potency (*p*-fluorobenzyl **36**, $IC_{50}$ = 6 µM). Of the three analogs substituted with benzyl pyridines (compounds **33**-**35**) only the *para*-substituted pyridine **35** ($IC_{50}$ = 6 µM) showed increased potency relative to amiloride. Extension of the benzylamine substituent to phenylethylamine caused a 2-fold drop in potency (**37**, $IC_{50}$ = 16 µM vs **32**, $IC_{50}$ = 8 µM).
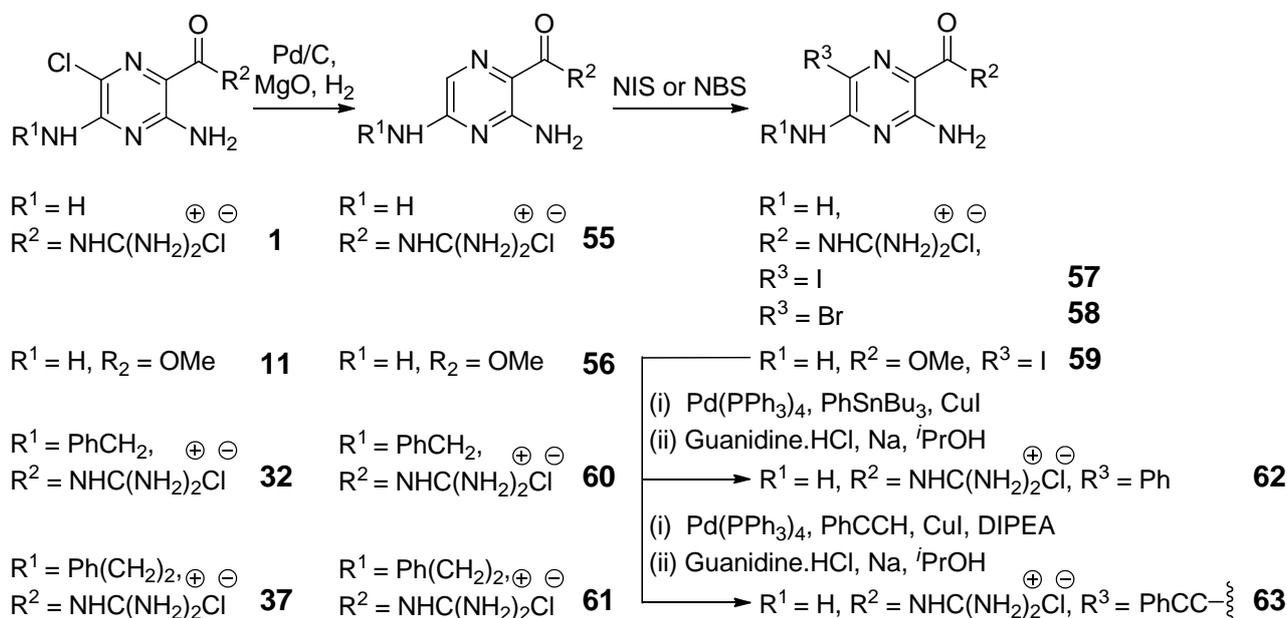
5-substituted analogs containing $2^o$, $3^o$ or $4^o$ nitrogens were investigated to examine the effects of positively charged groups in this region. Analogs **26** ($IC_{50}$ = 14 µM) and **40** ($IC_{50}$ = 15 µM) bearing $3^o$ amino groups showed only slightly reduced potency, while **42** (pyridinium, $IC_{50}$ = 30 µM) and **41** ($2^o$ amine, $IC_{50}$ = 28 µM) were ~3-fold less potent than amiloride. It was concluded that positive charges in C5 substituents do not confer increased potency but they are largely tolerated or produce only minor losses in potency.

Analogs **43**, **44** and **45**, which included sulfur in the C5-substituent, were examined because some potent uPA inhibitors have been shown to position sulfonamides in the oxanion hole.[26, 27] The sulfone **44** ($IC_{50}$ = 15 μM) was found to be slightly more potent than its corresponding sulfide **43** ($IC_{50}$ = 21 μM), while both compounds were slightly less potent than amiloride. Sulfonamide **45** ($IC_{50}$ = 35 μM) was more than 3-fold less potent than amiloride.

The overall trend with this series indicated that analogs bearing shorter amines at C5 tended to slightly improve inhibitor potency while the more extended amines produced no improvement or a reduction in potency. Inspection of the amiloride:uPA X-ray co-crystal structure (Figure 1) suggests that C5-substituted analogs should place the substituent near the oxanion hole, with longer substituents extending out further, probably towards solvent. The finding that only slight increases (up to 2-fold) and relatively minor reductions (except for the negatively charged analogs) in potency were observed with this diverse set of analogs suggests that only non-specific contacts are being made between the C5-substituents and the enzyme. The relatively flat SAR trend would be compatible with these substituents significantly interacting with the surrounding solvent.

Compounds **46** (commonly known as ethylisopropyl amiloride, EIPA) and **47** (hexamethylene amiloride, HMA) were of considerable interest in this study because they are two of the most potent known inhibitors of the sodium hydrogen exchanger 1 (NHE1), another emerging antitumor/metastasis drug target.[28] Amiloride is also a moderately potent NHE1 inhibitor ($K_{0.5}$ = 7 μM) but EIPA **46** and HMA **47** are 140 x and 524 x more potent, respectively.[17, 29] The likelihood that the antitumor/metastasis effects of amiloride (and analogs) *in vivo* arises through dual inhibition of both NHE1 and uPA was recently reviewed,[15] and it was noted there that the inhibitory potencies of EIPA and HMA against uPA had not yet been

reported. Compounds **46** (IC$_{50}$ = 6 μM) and **47** (IC$_{50}$ = 6 μM) are now shown to be equipotent uPA inhibitors and ~2-fold more potent than amiloride.
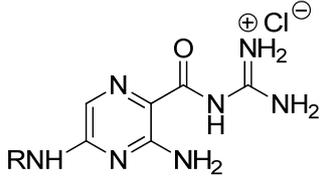
R$^1$ = H
R$^2$ = NHC(NH$_2$)$_2$Cl  **1**

R$^1$ = H, R$_2$ = OMe  **11**

R$^1$ = PhCH$_2$,
R$^2$ = NHC(NH$_2$)$_2$Cl  **32**

R$^1$ = Ph(CH$_2$)$_2$,
R$^2$ = NHC(NH$_2$)$_2$Cl  **37**

Pd/C, MgO, H$_2$

R$^1$ = H
R$^2$ = NHC(NH$_2$)$_2$Cl  **55**

R$^1$ = H, R$_2$ = OMe  **56**

R$^1$ = PhCH$_2$,
R$^2$ = NHC(NH$_2$)$_2$Cl  **60**

R$^1$ = Ph(CH$_2$)$_2$,
R$^2$ = NHC(NH$_2$)$_2$Cl  **61**

NIS or NBS

R$^1$ = H,
R$^2$ = NHC(NH$_2$)$_2$Cl,
R$^3$ = I  **57**
R$^3$ = Br  **58**

R$^1$ = H, R$^2$ = OMe, R$^3$ = I  **59**

(i) Pd(PPh$_3$)$_4$, PhSnBu$_3$, CuI
(ii) Guanidine.HCl, Na, $^i$PrOH
R$^1$ = H, R$^2$ = NHC(NH$_2$)$_2$Cl, R$^3$ = Ph  **62**

(i) Pd(PPh$_3$)$_4$, PhCCH, CuI, DIPEA
(ii) Guanidine.HCl, Na, $^i$PrOH
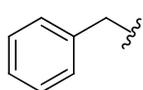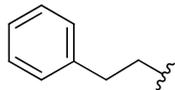R$^1$ = H, R$^2$ = NHC(NH$_2$)$_2$Cl, R$^3$ = PhCC–  **63**

**Scheme 4.** Synthesis of analogs with variations at C6.
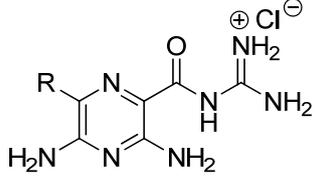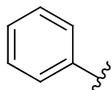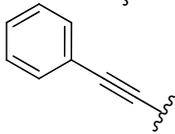
The amiloride:uPA X-ray structure (Figure 1) indicates that the C6 chloro group of amiloride projects toward and partially occupies the S1β site.[16] A selection of 6-substitued amiloride analogs which either remove or replace the chloro group were thus prepared to explore structure-activity relationships about the S1β site. Hydrodehalogenation (H$_2$, Pd/C,MgO)[21] of amiloride **1** and methyl ester **11** provided dechlorinated compounds **55** (59%) and **56** (98%), respectively. Reaction of **55** with *N*-iodosuccinimide (NIS) and *N*-bromosuccinimide (NBS) yielded aryl halides **57** (72%) and **58** (81%), and iodination of **56** provided the aryl iodide **59** in 98% yield (Scheme 4). Compounds **60** and **61** were produced by hydrodehalogenation (H$_2$, Pd/C,MgO) of **32** and **37** in 78% and 81% yields, respectively. Stille and Sonogashira couplings were carried out on iodide **59** with phenyltributylstannane and

phenylacetylene. Guanidinylation of the resultant esters afforded the 6-substituted analogs **62** and **63** (71% and 46% yields over two steps, respectively).

**Table 3.** IC$_{50}$ values of analogs with variations at C6.



| Compound | R | IC$_{50}$, μM |
|---|---|---|
| **55** | H | 19 |
| **60** | (benzyl) | 43 |
| **61** | (phenethyl) | 120 |



| Compound | R | IC$_{50}$, μM |
|---|---|---|
| **57** | I | 2 |
| **58** | Br | 5 |
| **62** | (phenyl) | 10 |
| **63** | (phenylethynyl) | 2 |

Compounds **55**, **60** and **61** (i.e. dechloro analogs of **1**, **32** and **37**, respectively) were used to explore the contribution of the chloro group to potency. The three dechloro compounds all showed reduced potency (~2-7.5 fold) compared to their 6-chloro variants (Table 3), confirming the favourable interaction of the chloro group with the S1β site. The effect of larger halogens at the 6-position was

also investigated. Relative to amiloride, iodide **57** and bromide **58** produced 5- and 2-fold improvements in potency, respectively, suggesting that the larger halogens interact more favourably with the S1β site. Previous uPA:inhibitor co-crystal structures showing that the S1β site can accommodate an aryl ring[12] led us to explore amiloride analogs **62** and **63** carrying phenyl and phenylacetylenyl substituents at C6. The two compounds were found to have equal (**62** $IC_{50}$ = 10 μM) or higher potency (**63** $IC_{50}$ = 2 μM) relative to amiloride.

Amiloride shows significant antitumor/metastasis effects in *in vivo* animal models and it would appear that that these probably arise from dual inhibition of uPA and NHE1.[15] The *in vitro* potency of amiloride against these two targets is relatively modest, however, so it is conceivable that an analog optimised for higher potency against both targets might yield a superior anticancer compound. Deliberately optimising compounds to potently interact with two or more independent targets (i.e. polypharmacology) is increasing being recognised as a viable drug development strategy.[30] The NHE1 inhibitory potency of many amiloride analogs has been reported and some show greatly increased potency relative to amiloride, including the well studied compounds EIPA **46** and HMA **47**. In order to advance our long-term goal of identifying potent dual NHE1/uPA inhibitors it is necessary to characterise the potency of amiloride analogs as uPA inhibitors.

This study initially explored simplified aroylguanidines and analogs containing acylguanidine isosteres. It was found that simple aroylguanidines are poor uPA inhibitors and should not be pursued further as alternative uPA inhibitor scaffolds. Analogs bearing acylguanidine isosteres were found to be totally inactive indicating that the acylguanidine group should be retained. Analogs lacking either the 3- or 5-amino substituent were used to establish the importance of these groups for inhibitor binding. Both compounds were found to be inactive.

It was postulated that analogs incorporating a negatively charged sulfate mimetic at C5 could pick up favourable contacts in the oxyanion hole of uPA. Surprisingly, analogs bearing negatively-charged groups all showed greatly reduced potency relative to amiloride. Ironically, the uncharged synthetic precursors of these compounds all showed much lower reductions in potency. Investigation of alkylaryl extensions at C5 were more fruitful, with several analogs showing slightly improved potency. Overall, substituted amines at C5 were generally well tolerated with inhibitors being as potent or only slightly less potent than amiloride. While exploration of these analogs did not yield an inhibitor with nanomolar potency as hoped, it succeeded in showing that substituted amines at the 5-position are at least well tolerated. Amiloride analogs substituted at the 5-position are known to show reduced diuretic effects.[21] If a high potency uPA inhibitor can eventually be evolved from amiloride it will be useful to know that the 5-position can be varied to reduce diuretic effects and to modulate physiochemical properties without dramatically altering uPA affinity.

Removal of the 6-chloro group was investigated with dechloro analogs; **55**, **60** and **61**. When compared with their 6-chloro counterparts, the three dechloro compounds each showed significantly reduced potency indicating that placing a group into the S1β pocket is important. Other analogs substituted at the 6-position with larger halogens (i.e. **57**, **58**) and aryl groups (i.e. **62**, **63**) showed improved potency, with **57** and **63** being the most potent compounds identified in the study (~6-fold increase in potency relative to amiloride).

Determining the uPA inhibitory potency of **46** and **47** was of particular interest since these compounds are two of the most potent NHE1 inhibitors known.[17, 29] The two compounds were shown in the current work to provide 2-fold improvements in potency relative to amiloride. While not large increases in potency the result confirms that 5-substituted amiloride analogs with very high potency against NHE1

are able to maintain potency against uPA (relative to the unsubstituted amiloride) – an important finding in the search for dual uPA/NHE1 inhibitors. It also supports that the demonstrated antitumor/metastasis effects of **46** and **47** most likely arise through dual inhibition of uPA and NHE1.

Analogs of **46** and **47** that retain the respective ethylisopropyl and hexamethylene amines at C5 while replacing the 6-chloro group with substituents that are able to make more favourable contacts in the S1β site may lead to more potent uPA inhibitors that retain potency against NHE1. It is worth noting that 6-Iodo-**46** and 6-Bromo-**47** are 313- and 566-fold more potent NHE1 inhibitors than amiloride, respectively, and 1.40- and 1.08-fold higher in potency than their 6-chloro counterparts **46** and **47**.[17] The current study has shown that converting amiloride to the 6-Bromo or 6-Iodo derivatives enhances uPA inhibition. The compounds 6-Iodo-**46**, 6-Bromo-**47** and derivatives of **46** and **47** that carry other C6-substituents are currently being examined in these laboratories in pursuit of dual uPA/NHE1 inhibitors.

**References**

[1]     Duffy, M. J. *Biotherapy* **1992**, *4*, 45.

[2]     Dass, K.; Ahmad, A.; Azmi, A. S.; Sarkar, S. H.; Sarkar, F. H. *Cancer Treatment Rev*. **2008**, *34*, 122.

[3]     Duffy, M. J. *Curr. Pharm. Des.* **2004**, *10*, 39.

[4]     Rømer, J.; Nielsen, B. S.; Ploug, M. *Curr. Pharm. Des.* **2004**, *10*, 2359.

[5]     Nozaki, S.; Endo, Y.; Nakahara, H.; Yoshizawa, K.; Ohara, T.; Yamamoto, E. *Anticancer Drugs* **2006**, *17*, 1109.

[6]     Tyndall, J. D. A.; Kelso, M. J.; Clingan, P.; Ranson, M. *Recent Pat. Anti-Canc.* **2008**, *3*, 1.

[7]     Ploug, M. *Curr. Pharm. Des.* **2003**, *9*, 1499

[8]     Croucher, D, R.; Saunders, D. N.; Lobov, S.; Ranson, M. *Nat. Rev. Cancer* **2008**, *8*, 535.

[9]     Look, M. P.; van Putten, W. L. J.; Duffy, M. J.; Harbeck, N.; Christensen, I, J.; Thomssen, C.; Kates, R.; Spyratos, F.; Fernö, M.; Eppenberger-Castori, S.; Sweep, C. G. J. F.; Ulm, K.; Peyrat, J-P.; Martin, P-M.; Magdelenat, H.; Brünner, N. et al. *J. Natl. Cancer Inst.* **2002**, *94*, 116.

[10]    Rockway, T, W.; Giranda, V, L. *Curr. Pharm. Des.* **2003**, *9*, 1483.

[11]    http://www.wilex.de/R&D/Mesupron.htm. (Accessed on July 7, 2011).

[12]    Rockway, T. W.; Nienaber, V.; Giranda, V. L. *Curr. Pharm. Des.* **2002**, *8*, 2541.

[13]    Sweetman, S. C. (ed.) Martindale: the complete drug reference [online]. London: Pharmaceutical Press <http://www.medicinescomplete.com/> (Accessed on 07 July 2011).

[14]    Vassalli, J-D.; Belin, D. *FEBS Lett.* **1987**, *214*, 187.

[15]    Matthews, H.; Ranson, M.; Kelso, M. J. *Int. J. Cancer* **2011**, *129*, 2051.

[16]    Zeslawska, E.; Schweinitz, A.; Karcher, A.; Sondermann, P.; Sperl, S.; Stürzebecher, J.; Jacob, U. *J. Mol. Biol.* **2000**, *301*, 465.

[17]    Kleyman, T. R.; Cragoe Jr., E. J. *J. Membr. Biol.* **1988**, *105*, 1.

[18]    Wermuth, C. G. *J. Med. Chem.* **2004**, *47*, 1303.

[19]    Walker, J. B.; Walker, M. S. *J. Biol. Chem.* **1959**, *234*, 1481.

[20]    Showell, G. A.; Gibbons, T. L.; Kneen, C. O.; MacLeod, A. M.; Merchant, K.; Saunders, J.; Freedman, S. B.; Patel, S.; Baker, R. *J. Med. Chem.* **1991**, *34*, 1086.

[21]    Cragoe Jr., E.; Woltersdorf, O. W.; Bicking, J. B.; Kwong, S. F.; Jones, J. H. *J. Med. Chem.* **1969**, *10*, 66.

[22]    Bicking, J. B.; Robb, C. M.; Kwong, S. F.; Cragoe Jr., E. J. *J. Med. Chem.* **1967**, *10*, 598.

[23]    Chen, G. S.; Wilbur, J. K.; Barnes, C. L.; Glaser, R. *J. Chem. Soc., Perkin Trans. 2* **1995**, 2311.

[24]    Lei M.; Tao, X.-L.; Wang, Y.-G. *Helv. Chim. Acta* **2006**, *89*, 532.

[25]    Contreras, J.-M.; Rival, Y. M.; Chayer, S.; Bourguignon, J.-J.; Wermuth, C. G. *J. Med. Chem.* **1999**, *42*, 730.

[26]    Fish, P. V.; Barber, C. G.; Brown, D. G.; Butt, R.; Collis, M. G.; Dickinson, R. P.; Henry, B. T.; Horne, V. A.; Huggins, J. P.; King, E.; O'Gara, M.; McCleverty, D.; McIntosh, F.; Phillips, C.; Webster, R. *J. Med. Chem.* **2007**, *50*, 2341.

[27]    Renatus, M.; Bode, W.; Huber, R.; Stürzebecher, J.; Stubbs, M. T. *J. Med. Chem.* **1998**, *41*, 5445.

[28]    Harguindey, S.; Arranz, J. L.; Wahl, M. L.; Orive, G.; Reshkin, S. J. *Anticancer Res.* **2009**, *29*, 2127.

[29]    Vigne, P.; Frelin, C.; Cragoe Jr., E. J.; Lazdunski, M. *Mol. Pharmacol.* **1984**, *25*, 131.

[30]    Arnaud, C. H. *Chem. Eng. News* **2011**, Jan 24, 32.