Design, Synthesis, and Structure−Activity Relationships of Novel 2-Substituted Pyrazinoylguanidine Epithelial Sodium Channel Blockers: Drugs for Cystic Fibrosis and Chronic Bronchitis

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Amiloride (**1**), the prototypical epithelial sodium channel (ENaC) blocker, has been administered with limited success as aerosol therapy for improving pulmonary function in patients with the genetic disorder cystic fibrosis. This study was conducted to synthesize and identify more potent, less reversible ENaC blockers, targeted for aerosol therapy and possessing minimal systemic renal activity. A series of novel 2-substituted acylguanidine analogues of amiloride were synthesized and evaluated for potency and reversibility on bronchial ENaC. All compounds tested were more potent and less reversible at blocking sodium-dependent short-circuit current than amiloride. Compounds **30**−**34** showed the greatest potency on ENaC with IC_{50} values below 10 nM. A regioselective difference in potency was found (compounds **30**, **39**, and **40**), whereas no stereospecific (compounds **33**, **34**) difference in potency on ENaC was displayed. Lead compound **32** was 102-fold more potent and 5-fold less reversible than amiloride and displayed the lowest IC_{50} value ever reported for an ENaC blocker.

Introduction

Sodium channels are ubiquitous in nature and are classified either as neuronal voltage-gated sodium channels, which are selectively blocked with neurotoxins (tetrodotoxin or saxitoxin) or the antiepileptic drug phenytoin,[1] or as epithelial/degenerin sodium channels (ENaC), which are selectively blocked by a substituted acylguanidine known as amiloride (**1**).[2] Amiloride is a potassium-sparing diuretic that was developed by Cragoe and his colleagues at Merck Pharmaceuticals in the early 1960s and has been widely used clinically as an adjunctive treatment with the earlier developed loop diuretics to help minimize the hypokalemia commonly experienced with the sulfonamide agents.[3,4]

In the respiratory epithelium, the apical sodium channel helps regulate the thin layer of airway surface liquid (ASL) that lines airway surfaces. The ASL provides the necessary milieu for cilia function and cephalad mucociliary clearance (MC), which comprises the primary innate defense mechanism of the respiratory tract.[5−7] Because cystic fibrosis (CF) pathogenisis was thought to involve defective mucus clearance, amiloride was tested as an inhalation therapy for enhancing MC and cough clearance in the respiratory tract of CF patients in 1986.[8] The rationale for amiloride inhalation as a therapy for CF has been supported by direct evidence that increasing ASL volume enhances MC,[9−11] which in turn would provide relief from mucus plugging in CF airways. Although the therapeutic utility of amiloride in CF patients was inconclusive,[8,12−17] the rationale for an aerosol pharmacotherapy that delivers an active compound directly to a protein that controls ASL volume and, hence, mucus clearance, warranted the development of novel more efficacious ENaC blockers tailored for inhalation therapy.

Amiloride, which was designed for oral administration, failed as a form of inhalation therapy because of (1) the restricted drug delivery associated with inhalation therapy, that is, its limited solubility restricted the mass that could be delivered by a conventional nebulizer; (2) limited potency; (3) rapid absorption by airway epithelia; and (4) rapid wash-off (rapid reversibility) from ENaC.[16,18−21]

The proposed ENaC subunit stoichiometry is a heteromeric protein complex comprised of three subunits (α, β, and γ) with at least two possible configurations: (1) the 2:1:1 configuration, where the channel is in an $\alpha\beta\alpha\gamma$ complex,[22] and (2) the 3:3:3 (α:β:γ) configuration or eight to nine subunits of which a minimum of two are the γ subunit. [23,24] Amiloride is proposed to block ENaC at concentrations ranging from approximately 0.2−1 μM via a direct exofacial block, (the invasion hypothesis) proposed by Cuthbert in 1976[25] and supported by Li et al.[26] The model, consists of two steps: first, the positively charged acylguanidinium side chain of amiloride invades the channel pore and interacts with an anionic site (a fixed negatively charged residue, e.g., aspartic or glutamic acid) forming an encounter complex, and second, the negative charge from the chlorine atom on the pyrazine ring binds to an electropositive site (positively charged residue, e.g., arginine, histidine, or lysine) on the channel to form a stable blocking complex.[26] Li et al. further added that the differences in ENaC blocker potency were due to changes in the on and off rate constants of the compound to the binding site on the channel.[26,27] Adding to this model, Venanzi et al., using molecular electrostatic potential analysis, found that the distance between the stable blocking complex formed by the charged acylguanidinium moiety and the chlorine atom on the pyrazine ring defined an important spatial requirement that allowed for the stable amiloride block.[28]

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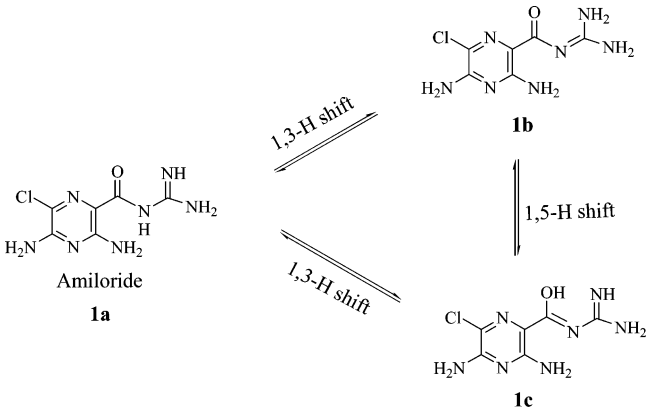


Figure 1. Tautomeric interconversions of amiloride free base in solution; **1a** acylamino, **1b** acylimino, and **1c** isoimino.

The potential binding site of amiloride to ENaC was investigated using site-directed mutagenesis studies. Two separate groups identified the serine 583 residue located on the α subunit to be an important site that maintains ENaC amiloride activity,[29,30] and glycine residues 525 and 537 from the β and γ subunits, respectively, in an area known as the the pre-M2 segment.[31] Furthermore, residues 278−283 within the α-subunit ectodomain have been suggested to participate in amiloride binding (specifically the pyrazine ring moiety).[29,32]

The objective of this study was to identify more potent, less reversible ENaC blockers than amiloride that would be suitable for a once or twice daily aerosol therapy in patients suffering from chronic obstructive pulmonary diseases (COPD) such as CF. To this end, we synthesized a novel focused library of 2-acylguanidine ENaC blocker analogues and report the intrinsic activity (potency) and reversibility on airway ENaC. On the basis of this study, we propose adding three new auxiliary binding sites to the invasion hypothesis, which rationalizes the increased potency of the new ENaC analogues.

Chemistry. Amiloride (**1a**) in solution exists as a mixture of the three tautomers represented in Figure 1. Previous studies by Smith et al.[33] have shown that the free base exists primarily as acylimino tautomer **1b**, whereas the physiologically active species exists as the protonated form of the acylamino tautomer **1a**[2] (Figure 1). These structural representations (**1a** and **b**) have been used to represent amiloride and its analogues in both the patent and scientific literature. We use the acylamino representation (**1a**) for convenience throughout this article with the understanding that the structures are, in reality, a hybrid of the three forms with the actual amount of each dependent on the pH and the nature of the substituents.

New compounds **2**−**4** and **7**−**42** were all made by coupling amine precursors with **43a** or **b**. Thus, the synthesis of amiloride derivatives **2**−**4** and **7**−**12** were achieved by coupling guanidines made in situ from commercial amines with 3,5-diamino-6-chloropyrazine-2-carboxylic acid methyl ester (**43a**) (method A) or by coupling amines made in multisteps with methylthio pseudourea (**43b**) which was prepared according to the reported procedure (Scheme 1).[34] These methods served as general procedures for the preparation of all of the novel amiloride analogues reported in this study.

Syntheses of para-substituted analogues with varied lengths of linear chain started from the requisite 4′-substituted phenyl alcohols **44a**−**e** (Scheme 2). The hydroxyl group in compounds **44a**−**e** was activated to the mesylate ester and subsequently displaced by sodium azide. The resulting azides **45a**−**e** were reduced to the amines **46a**−**e** by triphenylphosphine and water in THF. The coupling of amines **46b**−**e** with **43b** directly afforded compounds **15**, **21**, **22,** and **27**. Compound **22** was further converted to its corresponding acid analogue **25** by saponification with lithium hydroxide. The methylcarboxylate in compound **46d** was reduced by $LiAlH_4$ to its corresponding alcohol **46e**, which was subsequently converted to compound **27** by coupling with **43b**. To study the effect of the chain length of para-hydroxy-substituted analogues on blocking ENaC, the demethylation of compounds **46a**−**c** and **46f** by treatment with a 48% hydrobromic acid produced phenols **47a**−**d**, which were similarly converted to target compounds **13**, **14**, **16**, and **42**. Compound **16** was further converted to its corresponding sulfuric acid derivative **26** by the treatment of **16** with pyridine sulfur trioxide complex.

An alternative synthesis for compound **47c** is illustrated in Scheme 3. The carboxylic acid group of the commercially available **48** was activated by treatment with iso-butylchloroformate, the product of which was then treated in situ with methanolic ammonia to afford amide **49**. Amide **49** was reduced to its corresponding amine by a borane−THF complex. The reduction product, without purification, was subsequently subjected to demethylation by hydrobromic acid to afford **47c** as an HBr salt. Compared to a procedure in the literature for the synthesis of compound **47c**,[35] the synthetic procedure described herein for the synthesis of **47c** is shorter and easier to scale-up (see Experimental Section).

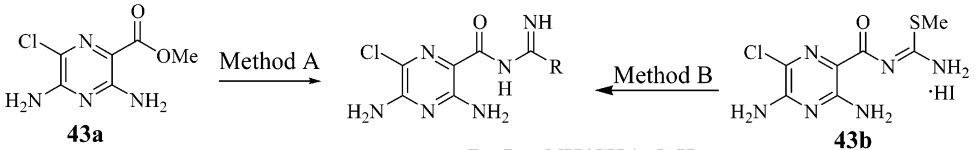
To study the effect of the location of the hydroxyl group on the phenyl ring, compounds **17**−**19** were synthesized by employing the Sonogashira reaction[36] for the preparation of key intermediates **52a**−**e** (Scheme 4). Compounds **20**, **23**, and **30** were also synthesized by this method. Thus, the substituted phenylhalides **50a**−**e** were treated with *N*-Boc-protected 3-butynylamine **51** ($n = 2$), which was prepared from 3-butynyl alcohol according to the reported procedure.[37] The triple bond in the protected amines **52a**−**e** was saturated by hydrogenation to afford products **53a**−**c** and **e**. The 4-nitro group in compound **52d** was concomitantly reduced to give **53d**. The treatment of **53a**−**d** with 48% aqueous hydrogen bromide affected the removal of both the Boc and methyl protecting groups present in these compounds. The resulting amines **54a**−**d** were coupled with **43b** to give desired compounds **17**−**20**. The Boc-protecting group in **53a** and **e** was separately cleaved by TFA, affording compounds **55a** and **e**, which were subsequently converted to target compounds **23** and **30**.

Syntheses of the analogues containing an oxygen atom in the linear chain linker are depicted in Scheme 5. The alkylation of **56a** with **57a** and **56b** with **57b**, mediated by sodium hydride, afforded **58a** and **b**, which were then treated with methylamine to remove the phthalimide protecting group. Free amines **59a** and **b** were then converted to target compounds **24** and **29**. Compound **24** was converted to its phenolic derivative **28** by treating with aqueous 48% hydrogen bromide.

The synthesis of compound **31** started from intermediate **47c** (Scheme 6). The protection of the terminal amine in **47c** by a carbobenzyloxy protecting group afforded **60a**; the allylation of **60a** with allyl bromide provided advanced intermediate **61**. Hydroboration−oxidation of the terminal alkene in **61** afforded the desired product **62** with the concomitant production of a 10−15% yield of byproduct **63**, in which the hydroxy group was at the 2′- position. Cleavage of the carbobenzyloxy protecting group in **62** by hydrogenolysis afforded amine **64**, which was converted to target compound **31** by coupling with **43b**.

Syntheses of compounds **32**−**36** are shown in Scheme 7. The diol side chain of these structures was established by a reaction

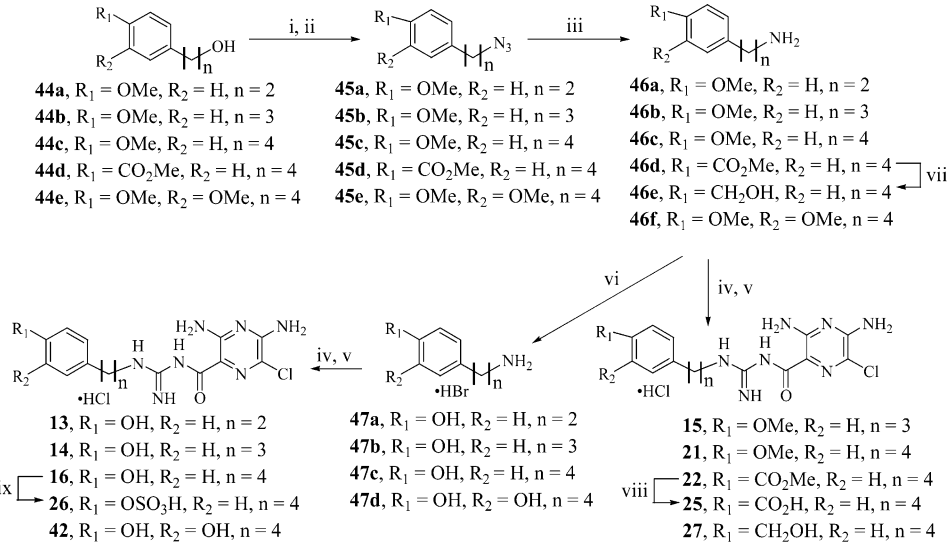
Scheme 1. General Procedure (Method A or B)[a]



2, R = $NH(CH_2)_5CH_3$ **7**, R = $NH(CH_2)_2C_6H_5$
3, R = $NH(CH_2)_7CH_3$ **8**, R = $NH(CH_2)_3C_6H_5$
4, R = $NH(CH_2)_2OH$ **9**, R = $NH(CH_2)_4C_6H_5$
10, R = $NH(CH_2)_5C_6H_5$
11, R = $NH(CH_2)_6C_6H_5$
12, R = $NH(CH_2)_8C_6H_5$

[a] Reagents: Method A: (i) RNH_2, 1*H*-pyrazole-1-carboxamidine hydrochloride, *i*-Pr_2EtN, DMF; (ii) **43a**, 25% NaOMe, MeOH. Method B: **43b**, RNH_2, *i*-Pr_2EtN, EtOH (or THF).

Scheme 2[a]



44a, R_1 = OMe, R_2 = H, n = 2
44b, R_1 = OMe, R_2 = H, n = 3
44c, R_1 = OMe, R_2 = H, n = 4
44d, R_1 = CO_2Me, R_2 = H, n = 4
44e, R_1 = OMe, R_2 = OMe, n = 4

45a, R_1 = OMe, R_2 = H, n = 2
45b, R_1 = OMe, R_2 = H, n = 3
45c, R_1 = OMe, R_2 = H, n = 4
45d, R_1 = CO_2Me, R_2 = H, n = 4
45e, R_1 = OMe, R_2 = OMe, n = 4

46a, R_1 = OMe, R_2 = H, n = 2
46b, R_1 = OMe, R_2 = H, n = 3
46c, R_1 = OMe, R_2 = H, n = 4
46d, R_1 = CO_2Me, R_2 = H, n = 4
46e, R_1 = CH_2OH, R_2 = H, n = 4
46f, R_1 = OMe, R_2 = OMe, n = 4

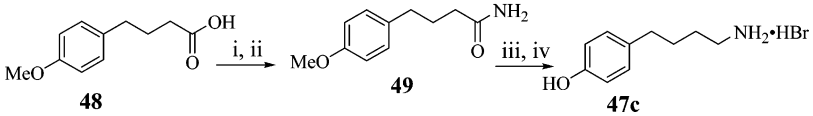
13, R_1 = OH, R_2 = H, n = 2
14, R_1 = OH, R_2 = H, n = 3
16, R_1 = OH, R_2 = H, n = 4
26, R_1 = OSO_3H, R_2 = H, n = 4
42, R_1 = OH, R_2 = OH, n = 4

47a, R_1 = OH, R_2 = H, n = 2
47b, R_1 = OH, R_2 = H, n = 3
47c, R_1 = OH, R_2 = H, n = 4
47d, R_1 = OH, R_2 = OH, n = 4

15, R_1 = OMe, R_2 = H, n = 3
21, R_1 = OMe, R_2 = H, n = 4
22, R_1 = CO_2Me, R_2 = H, n = 4
25, R_1 = CO_2H, R_2 = H, n = 4
27, R_1 = CH_2OH, R_2 = H, n = 4

[a] Reagents: (i) MsCl, pyr., THF; (ii) NaN_3, DMF; (iii) Ph_3P, THF, H_2O; (iv) Method B (Scheme 1); (v) Method C: concd HCl, MeOH; (vi) 48% HBr, H_2O; (vii) $LiAlH_4$, THF; (viii) LiOH, THF. (ix) Pyridine sulfur trioxide complex, pyridine.

Scheme 3[a]



[a] Reagents: (i) iso-butylchloroformate, NMM, THF; (ii) NH_3, MeOH; (iii) BH_3/THF; (iv) 48% HBr.

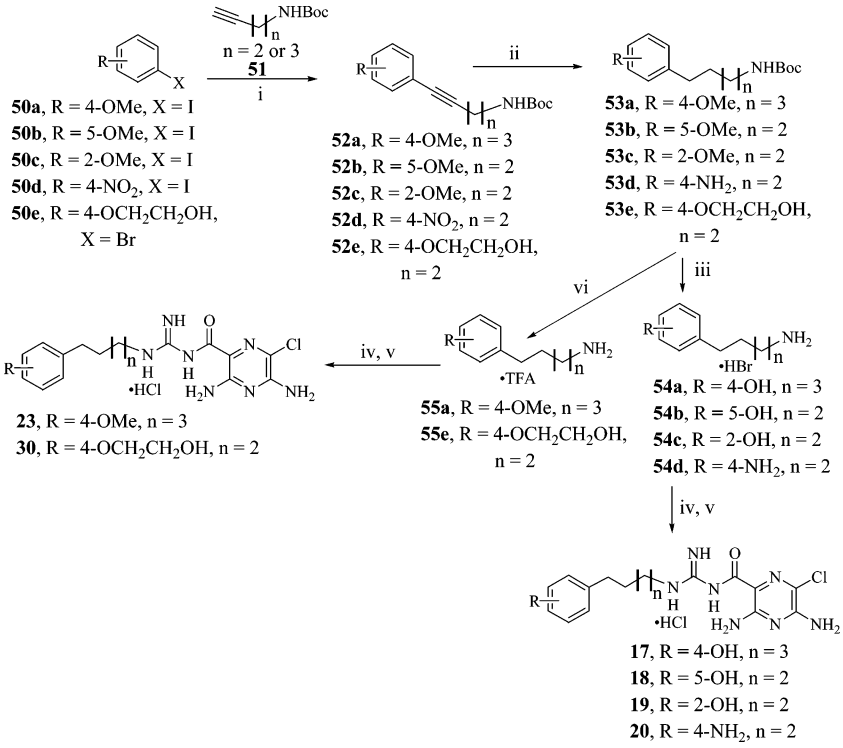
of the phenol hydroxy group with glycidol in the presence of a catalytic amount of triethylamine (0.005−0.1 equiv). For the syntheses of the enantiomerically pure targets **33** (*R*) and **34** (*S*), the corresponding enantiomerically pure glycidols *R* and *S*, respectively, were used. The enantiomers of the racemic compound **32** were separated by chiral HPLC, and their ratio was found to be 1:1. To ensure stereochemical integrity, the enantiomeric purity for compounds **33** and **34** was determined using the same method as that above, and the compounds were found to possess 100% ee. Compounds **65a**−**e** were subject to catalytic hydrogenolysis to remove the Cbz protecting group. The deprotected amines **66a**−**e** were then coupled with compound **43b** to afford target compounds **32**−**36**, which were characterized as either a methanesulfonic acid salt or a hydrochloride salt.

The synthesis of compounds **39** and **40** is illustrated in Scheme 8. The Sonogashira coupling of the appropriately substituted iodophenol **67a** and **b** with *N*-Boc-protected butynylamine **51** followed by the reduction of the triple bond afforded compounds **69a** and **b**, which underwent alkylation with THP-protected 2-bromoethanol to give compounds **70a** and **b**. The deprotection in an acidic medium (TFA) simultaneously removed both the Boc and THP protecting groups, giving key intermediates **71a** and **b** (TFA salt). The coupling of **71** with **43b** afforded desired products **39** and **40**, respectively.

The synthesis of the carboxylic acid derivatives **37** and **38** is illustrated in Scheme 9. The alkylation of the phenolic hydroxyl group in **60a** with the appropriate bromide afforded compounds **72a** and **b**. The cleavage of the carbobenzyloxy protecting groups in **72** followed by the coupling of the resulting deprotected compounds **73a** and **b** with **43b** from Scheme 1 using the conventional conditions for this type of reactions completed the synthesis of compounds **37** and **38**.

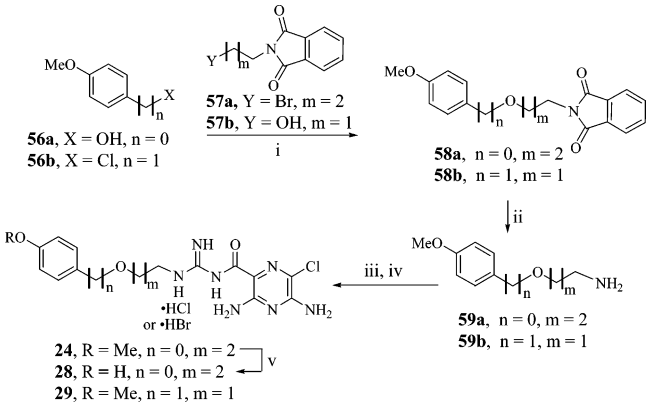
The synthesis of compound **41** is described in Scheme 10. The coupling of **60a** with the fully protected sugar **75** (D-glucuronic acid) mediated by trifluoroborane etherate afforded

Scheme 4[a]



[a] Reagents: (i) **51**, $Ph_3P/PdCl_2$, CuI, Et_2NH, THF; (ii) H_2, Pd/C, MeOH; (iii) 48% HBr; (iv) Method B (Scheme 1); (v) Method C (Scheme 2); (vi) TFA, CH_2Cl_2.

Scheme 5[a]



[a] Reagents: (i) NaH, THF; (ii) $MeNH_2$, MeOH; (iii) Method B (Scheme 1); (iv) Method C (Scheme 2); (v) 48% Hydrobromic acid.

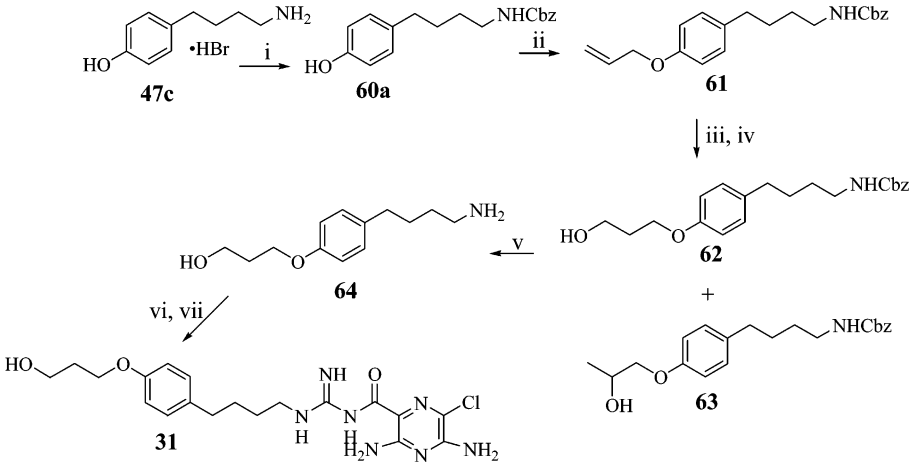
76, which underwent hydrogenolysis to remove the carbobenzyloxy protecting group, giving the amine **77**. The coupling of **77** with compound **43b** followed by a global saponification reaction with sodium hydroxide to cleave all acetyl protecting groups as well as the methyl ester afforded the final compound **41** as the sodium salt.

Biological Results. Pharmacology and SAR in Vitro. All ENaC blockers listed in Table 1 were evaluated using an electrophysiological assay utilizing bronchial airway epithelia grown at an air/liquid interface. The short-circuit current (I_{sc}) and transepithelial resistance (R_t) were recorded from primary canine bronchial epithelial cultures mounted in modified Ussing chambers. The IC_{50} values were calculated from a 12-point concentration−effect curve, where the IC_{50} value represents the mucosal concentration required to inhibit 50% of the sodium-dependent current. Recovery or reversibility is a qualitative index of drug wash-off and was measured after maximal inhibition (the channel is in the fully blocked state) followed by three consecutive mucosal washes. Recovery is reported as the percent of starting (basal) I_{sc} after the third and final apical wash. In general, amiloride and other commercially available ENaC blockers tested in this study were less active compared to previously published data, which were derived from multiple methods and different tissues.[2] In our in vitro study, (1) all compounds were tested using the procedure described herein (i.e., utilizing one method and only one tissue type); (2) the rank order in potency between amiloride **1a** and benzamil **6** was similar (11.8 compared to 9.7-fold) to that in previous reports; (3) the canine bronchial epithelial cells that were utilized had bioelectric properties similar to that of human bronchial epithelia;[38] and (4) the canine bronchial epithelial cells provided a robust dynamic range (approximately 65 $\mu A/cm^2$) with a dominant sodium-dependent current (>90% of total I_{sc}), facilitating the detection of small shifts in intrinsic blocking activity.

Using this model, the IC_{50} value for **1a** (amiloride) was 776 nM (Table 1). After the full-block (final concentration of the concentration−effect response) with amiloride, the sodium-dependent current returned to its basal value (96% recovered) after three consecutive apical washes.

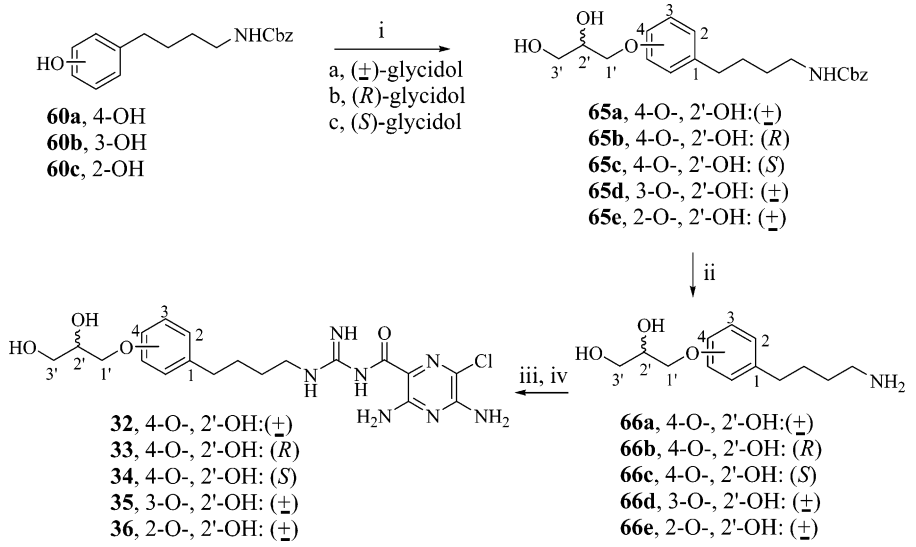
The acylguanidinium cation is essential for ENaC activity[2] but lacks the potency necessary to achieve our objectives. Our early SAR focused on the 2-position because it appeared to us to hold the most promise for increasing potency. Substituting one of the terminal amino groups by hydrophobic alkyl groups **2**−**3** or the more hydrophilic ethanol **4** produced compounds with an approximately 7.5-fold increase in potency and decreased reversibility compared to those of amiloride. Substituting a phenyl group (phenamil **5**) or a benzyl group (benzamil **6**) increased potency 1.9- and 11.8-fold respectively and also decreased reversibility. Discussion on the differences in the phenamil IC_{50} value reported in this study compared to values reported in the literature[2,39] have been previously addressed.[40]

Scheme 6[a]



[a] Reagents: (i) CbzCl, $NaHCO_3$, H_2O; (ii) allyl bromide, NaOH, DMF; (iii) BH_3/THF; (iv) H_2O_2, NaOH; (v) Method D: H_2, Pd/C, EtOH or MeOH; (vi) Method B (Scheme 1); (vii) Method C (Scheme 2).

Scheme 7[a]



[a] Reagents: (i) glycidol, Et_3N, EtOH, reflux; (ii) Method D (Scheme 6); (iii) Method B (Scheme 1); (iv) Method C (Scheme 2) or CH_3SO_3H, EtOH.

Having established in our hands that benzamil was more potent than phenamil, we synthesized a homologous series of aralkyl derivatives, all of which (with the exception of the phenoctyl analogue **12**) significantly increased potency compared to that of phenamil (Figure 2). Increasing the number of carbon atoms between the terminal nitrogen atom of the guanidine moiety and the aromatic ring thus produced compounds **7**−**11**, which were of greater potency (to a maximum of 27-fold) and were less reversible than amiloride. Although not statistically significant, it appeared that phenylbutyl derivative **9** was the most active compound of this series.

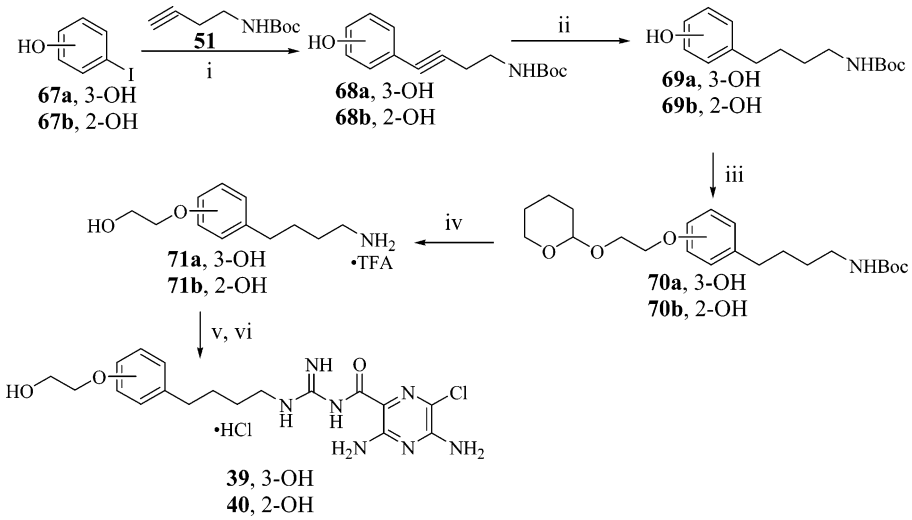
We, therefore, focused additional SAR studies on phenylbutyl derivatives. The addition of the 4′-hydroxyl group to the optimal four-carbon linker (**9**) increased potency approximately 2-fold, making the phenol (**16**) 46-fold more potent than amiloride. Incorporating the four-carbon linker with the 4′-methoxy group (**21**) produced a slight increase in potency with no differences in reversibility compared to that of **16**.

Before we continued our SAR around the 4′ position, we wanted to confirm our original assumption that the 4-phenylbutyl chain was optimal for potency. We probed the effect of chain length in the phenol (**13**, **14**, **16**, and **17**) and anisole (**15**, **21**, and **23**) series. As predicted, the 4-phenylbutyl derivatives **16** and **21** were significantly more active than their lower and higher homologues, thus fully establishing the 4-phenyl series as optimal.

We also confirmed that the lipophilic nature of the four carbon linker side chain was important. Incorporating an oxygen atom at position 2 or position 3 in the alkyl chain of the 4′-methoxy analogues (**24** and **29**, respectively) decreased potency compared to that of compound **21**. The loss of potency was also found in phenol **28** compared to that in **16**. Reversibility also increased by substituting oxygen for methylene in the side chain. Thus, replacing a lipophilic methylene group in the linker with an oxygen atom was deleterious to our objective.

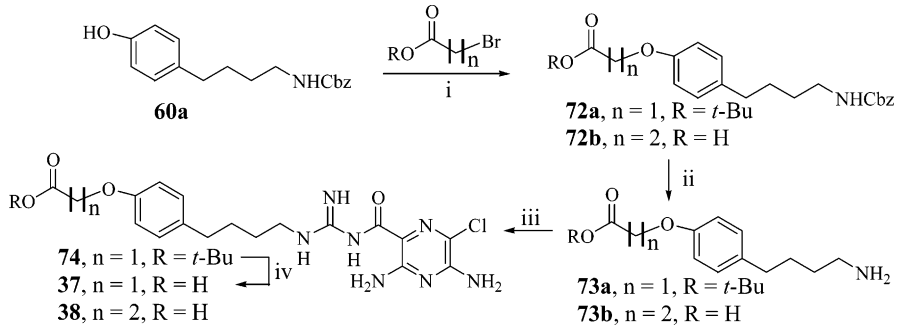
At this point, we had satisfied our first two design criteria of increasing potency and making the compounds less reversible. We approached our third criterion by focusing on incorporating groups that could become inactive once they traversed the epithelial lining of the lung and entered the systemic circulation. Of particular note is compound **16**, a phenol that was designed on the basis of a soft drug/antedrug approach.[41−43] The phenol can be viewed as a metabolite of compound **9** and could also serve as a substrate for conjugation and/or potential oxidation. We found that two of its potential metabolites **41**(conjugation) and **42** (oxidation) were indeed less potent and more reversible

Scheme 8[a]



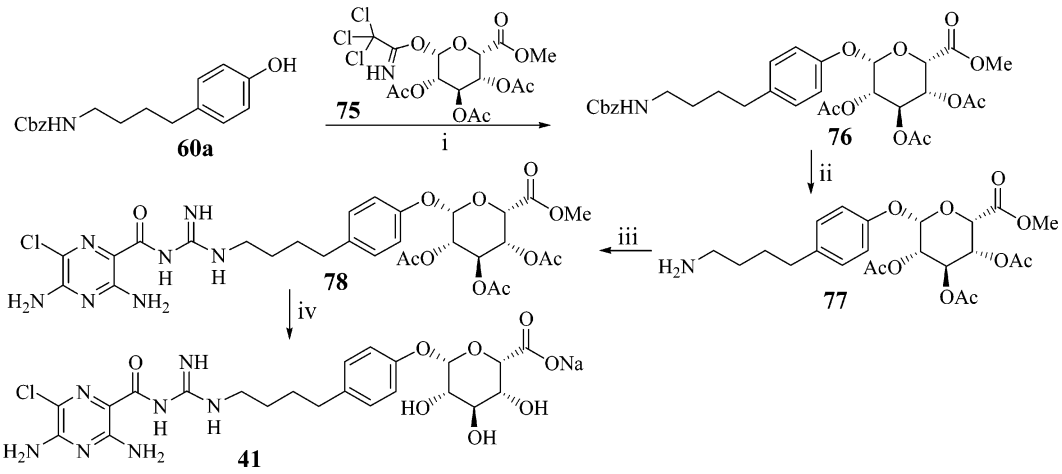
[a] Reagents: (i) Et_2NH, CuI, $PdCl_2$, Ph_3P, THF; (ii) H_2, Pd/C, EtOH; (iii) 2-(2-bromoethoxy)tetrahydro-pyran, K_2CO_3, acetone; (iv) TFA, CH_2Cl_2; (v) Method B (Scheme 1); (vi) Method C (Scheme 2).

Scheme 9[a]



[a] Reagents: (i) NaH, THF; (ii) Method D (Scheme 6); (iii) Method B (Scheme 1); (iv) TFA, MeOH.

Scheme 10[a]



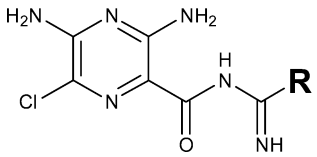
[a] Reagents: (i) $BF_3{\cdot}OEt_2$, CH_2Cl_2; (ii) Method D (Scheme 6); (iii) Method B (Scheme 1); (iv) NaOH, H_2O, THF.

than parent **16**. On the basis of these results with compound **16**, we then turned our attention to substitutions that could provide other metabolic pathways toward inactivation. In addition to being more potent than amiloride by at least an order of magnitude, we found that these compounds were all significantly more active than potential metabolites **25** and **41**.

Ester **22** and benzyl alcohol **27** did not change potency and reversibility compared to those of phenol **16**. However, the potential metabolite of **22** and **27**, carboxylic acid **25**, decreased in both potency and reversibility. Other groups such as sulfonic acid **26** and aniline **20** retained good potency. We did not pursue these analogues further in this study because we had moved on to other compounds that showed more promise.

We then continued investigating the 4′-substituted position. We speculated that the potency of primary alcohols **30** and **31** could be decreased by conjugation in plasma or by hepatic oxidation to the carboxylic acid. The reduced potency of the respective carboxylic acids **37** and **38** compared to that of the primary alcohols (**30** and **31**) supported this hypothesis. Glycol **32** combines the features of both **30** and **31** and was prepared

Table 1. Intrinsic Blocking Activity and Recovery of ENaC by Substituted 2-Acylguanidine Derivative Using Primary Canine Bronchial Epithelial Cells



compd	R	$IC_{50} \pm SD$ (nM)[a]	% recovery ± SD[a]
1[b] amiloride	NH_2	776 ± 326 (36)	96 ± 22 (35)
2	$NH(CH_2)_5CH_3$	87 (1)	24 (1)
3	$NH(CH_2)_7CH_3$	108 ± 61 (2)	7 ± 3 (2)
4	$NH(CH_2)_2)OH$	107 (1)	77 (1)
5[b] Phenamil	NHC_6H_5	401 ± 150 (4)	15 ± 6 (4)
6[b] Benzamil	$NHCH_2C_6H_5$	66 ± 33 (56)	47 ± 24 (52)
7	$NH(CH_2)_2C_6H_5$	66 ± 21 (5)	21 ± 15 (5)
8	$NH(CH_2)_3C_6H_5$	50 ± 21 (5)	23 ± 11 (4)
9	$NH(CH_2)_4C_6H_5$	29 ± 5 (6)	16 ± 10 (5)
10	$NH(CH_2)_5C_6H_5$	68 ± 22 (3)	6 ± 4 (2)
11	$NH(CH_2)_6C_6H_5$	58 ± 41 (2)	12 ± 2 (2)
12	$NH(CH_2)_8C_6H_5$	477 ± 365 (4)	12 ± 10 (4)
13	$NH(CH_2)_2C_6H_4$4-OH	62 ± 48 (10)	65 ± 25 (10)
14	$NH(CH_2)_3C_6H_4$4-OH	88 ± 24 (6)	65 ± 17 (6)
15	$NH(CH_2)_3C_6H_4$4-OCH_3	124 ± 64 (2)	57 ± 29 (2)
16	$NH(CH_2)_4C_6H_4$4-OH	17 ± 9 (32)	24 ± 15 (31)
17	$NH(CH_2)_5C_6H_4$4-OH	64 ± 56 (8)	29 ± 11 (8)
18	$NH(CH_2)_4C_6H_4$3-OH	20 ± 8 (3)	14 ± 0.2 (2)
19	$NH(CH_2)_4C_6H_4$2-OH	30 ± 17 (2)	11 ± 1 (2)
20	$NH(CH_2)_4C_6H_4$4-NH_2	21 ± 1 (3)	36 ± 21 (3)
21	$NH(CH_2)_4C_6H_4$4-OCH_3	15 ± 12 (7)	19 ± 15 (6)
22	$NH(CH_2)_4C_6H_4$4-CO_2CH_3	22 ± 11 (6)	13 ± 2 (5)
23	$NH(CH_2)_5C_6H_4$4-OCH_3	77 ± 9 (3)	5.5 ± 2 (3)
24	$NH(CH_2)_3OC_6H_4$4-OCH_3	25 (1)	25 (1)
25	$NH(CH_2)_4C_6H_4$4-CO_2H	51 ± 27 (6)	65 ± 18 (6)
26	$NH(CH_2)_4C_6H_4$4-SO_3H	21 ± 5 (2)	53 ± 7 (2)
27	$NH(CH_2)_4C_6H_4$4-CH_2OH	14 ± 7 (4)	20 ± 10 (4)
28	$NH(CH_2)_3OC_6H_4$4-OH	32 (1)	73 (1)
29	$NH(CH_2)_2OCH_2C_6H_4$4-OCH_3	41 (1)	72 (1)
30	$NH(CH_2)_4C_6H_4$4-$O(CH_2)_2OH$	9 ± 4 (10)	29 ± 17 (8)
31	$NH(CH_2)_4C_6H_4$4-$O(CH_2)_3OH$	7 ± 3 (7)	17 ± 13 (7)
32	$NH(CH_2)_4C_6H_4$4-$OCH_2CHOHCH_2OH$	8 ± 3 (368)	20 ± 11 (345)
33	$NH(CH_2)_4C_6H_4$4-$OCH_2CHOHCH_2OH$[c]	9 ± 3 (12)	25 ± 15 (12)
34	$NH(CH_2)_4C_6H_4$4-$OCH_2CHOHCH_2OH$[d]	7 ± 2 (10)	18 ± 16 (10)
35	$NH(CH_2)_4C_6H_4$3-$OCH_2CHOHCH_2OH$	26 ± 13 (2)	37 ± 8 (2)
36	$NH(CH_2)_4C_6H_4$2-$OCH_2CHOHCH_2OH$	28 ± 0.4 (2)	63 ± 9 (2)
37	$NH(CH_2)_4C_6H_4$4-OCH_2CO_2H	48 ± 13 (8)	79 ± 25 (8)
38	$NH(CH_2)_4C_6H_4$4-$O(CH_2)_2CO_2H$	20 ± 4 (4)	65 ± 43 (4)
39	$NH(CH_2)_4C_6H_4$3-$O(CH_2)_2OH$	17 ± 3 (2)	30 ± 18 (2)
40	$NH(CH_2)_4C_6H_4$2-$O(CH_2)_2OH$	31 ± 7 (2)	21 ± 4 (2)
41	$NH(CH_2)_4C_6H_4$4-O-D-glucuronic acid	73 ± 50 (2)	86 ± 40 (2)
42	$NH(CH_2)_4C_6H_3$3,4-di OH	42 ± 6 (5)	45 ± 22 (5)

[a] All values are the mean ± SD. The number in parenthesis is the number of individual observations. [b] Commercially available epithelial sodium channel blockers. [c] The *R*-enantiomer of compd **32**. [d] The *S*-enantiomer of compd **32**.

because first, it has a highly oxidized side chain, which may resemble an already metabolized form, and second, we hypothesized that it would stay on the apical side longer than compounds **30** and **31**. In fact, by placing an oxygen atom at the 4′ position and incorporating ethanol **30**, propanol **31**, or glycol **32** generated the lowest IC_{50} values ever reported (9, 7, and 8 nM, respectively) for blocking ENaC and were 3.3-, 5.6-, and 4.9-fold, respectively, less reversible than amiloride.

Having now found a series of compounds that were 2 orders of magnitude more potent than amiloride, less reversible, and conceptually antedrugs or soft drugs, we looked at the details of the regio and stereospecificities of glycol **32**. The ortho (**36**) and meta (**35**) analogues were significantly less active than the para analogue by 3- to 5-fold. The para-position regioselectivity was similar to that found with compounds **30**, **39**, and **40**. Furthermore, the regiochemical para preferences for ENaC block increased with size in the following order: *p*-$OCH_2CHOHCH_2$-OH (**32**) ≥ *p*-$O(CH_2)_2OH$ (**30**) > *p*-OH (**16**). We next examined the stereospecificity of **32** and found that the ENaC blocking activity of **33** and **34** were the same as that of racemate **32**. Although this was surprising, it is consistent with the finding that achiral derivatives **30** and **31** were as active as **32** and indicates a lack of stereochemical requirement in this auxiliary binding site.

Discussion

Aerosolized amiloride only transiently and marginally increased MC and pulmonary function in humans.[12,16] These shortcomings as an aerosol therapy indicated that the amiloride lacked sufficient duration and potency to be therapeutically effective. In addition, because the bronchial and renal epithelial sodium channel are structurally and functionally similar,[44] selectivity for this drug class must be achieved by means other than by channel selectivity, or potential hyperkalemia will result from systemic absorption of the more potent ENaC blocker. An effort toward designing more efficacious ENaC blockers

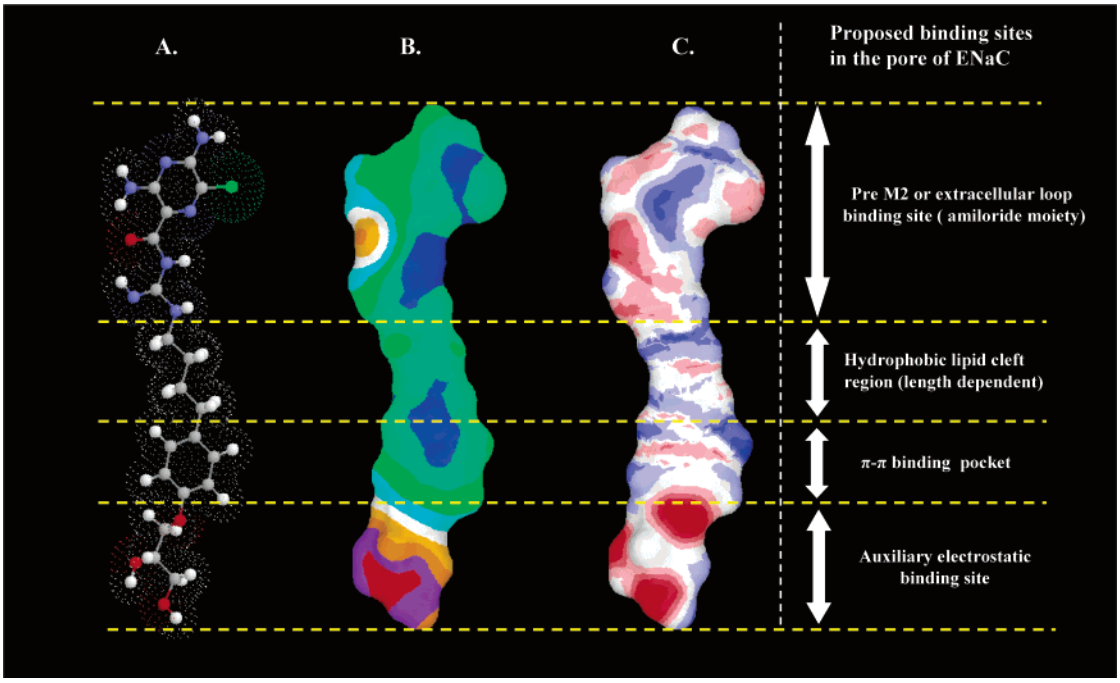


Figure 2. Proposed binding of compound **32** to the ENaC. (A) Compound **32** represented as a ball-and-stick dot model (color scheme: white, H; blue, N; gray, C; green, Cl; and red, O); (B) Lipophilic surface (Gaillard MLP) model (color scheme: red (−0.5 to −0.39); purple (−0.39 to −0.28); orange (−0.28 to −0.17); yellow (−0.17 to −0.06); white (−0.06 to 0.06); cyan (0.06 to 0.17); green (0.17 to 0.28); green blue (0.28 to 0.39); blue (0.39 to 0.50)). (C) Electrophilic surface model; blue is negative, and red is positive. The segments identified by the horizontal dashed yellow lines correspond to the proposed binding regions. The lipophilicity and electrophilicity are normalized.

selective for COPD such as the life-shortening hereditary disease CF was therefore warranted. Conceptually, our approach to this problem included three objectives: first, to increase intrinsic activity (potency) by finding an auxiliary binding site in the channel pocket; second, to increase duration of action by slowing transport across the airway epithelium and/or decreasing channel reversibility; and third, to achieve selectivity by the conversion of the drug to a less active metabolite by systemic or epithelial biotransformation. Alternatively, we could induce a greater fraction of nonrenal elimination of ENaC blockers by making them resemble endogenous metabolites and, thus, reduce renal exposure.

Using a primary bronchial epithelial culture model, we developed an in vitro testing algorithm and defined the structure−activity relationship (SAR) for potency and reversibility (a slower rate of basal current recovery after maximal inhibition) of the new analogues compared to those of amiloride on ENaC expressed in respiratory epithelia. Our approach was to achieve our first two objectives of increasing potency and reducing reversibility. We then sought to identify areas of the molecule that we could manipulate to induce antedrug[43] or soft drug[42] metabolic features that are sensitive to epithelial or systemic biotransformation by producing less potent ENaC blockers and thereby reduce renal side effects. This report describes the design and synthesis of novel ENaC blockers that are the most potent and least reversible reported to date. We found that all of the compounds synthesized in this study were more potent and less reversible than amiloride **1**. The pharmacologic blockade of ENaC increases ASL volume and accelerates MC and, therefore to some extent, limits the efficacy by diluting and physically removing the compound from the targeted protein. One approach to avoid this limitation of efficacy was to reduce reversibility (recovery of activity), which is likely a function of the compound's affinity on ENaC and the on−off rate constants. Therefore, we sought to choose a compound with greatly enhanced potency and better reversibility parameters. A systematic SAR was used to generate a focused 2-substituted acylguanidine analogue library. The results provide new insight on the ENaC blocker SAR and putative auxiliary binding sites for the novel ENaC blockers.

The number of carbon atoms, four being optimum, between one of the terminal nitrogen atoms of the acylguanidinium moiety and the aromatic ring increased intrinsic activity. To extend the invasion hypothesis plug-type model,[25,26] we found that the distance of the acylguanidine moiety and the benzene ring was critical for good activity. This suggests that a length-specific hydrophobic lipid cleft in the ENaC pore in close proximity to the guanidine binding site would support our finding. This notion is further supported by the observation that an oxygen atom in the chain is deleterious to activity, for example, compound **24**. Also, the para-substituted aromatic ring at the end of the straight chain alkyl linker, being essentially planar, could contribute to active site rigidity in the form of a $\pi-\pi$ interaction (auxiliary binding pocket), providing greater potency and less reversibility. Consistent with a $\pi-\pi$ interaction, a regiochemical preference was displayed, where the para position generated the optimal potency for ENaC as the groups increased in size. Finally, the linked alcohols were the most potent ENaC blockers reported to date. The hydroxyl groups in **31**−**34** likely access an additional secondary binding site in an area near the proposed pre-M2 segment or extracellular loop of the α subunit of ENaC. On the basis of these observations, we propose that the following new auxiliary binding regions be added to the invasion hypothesis model: a lipophilic binding region (1) to accommodate the four linear carbon atoms bonded to an aromatic ring (2) anchored by a $\pi-\pi$ interaction followed by a three-four atom spacer (3) into an electrostatic binding site (Figure 2). Each of these regional sites cumulatively contributes about five times in potency relative to that of amiloride, culminating in the highly potent analogues **30**−**34**.

At this point in our study, it was not clear that designing antedrug or soft drugs would lead to systemic selectivity.

However, by designing compounds that resemble endogenous metabolites (**32**−**34**), we provided an alternative means to diminish or eliminate possible hyperkalemia. Preliminary clinical data suggest that **32** systemically becomes less selective for renal elimination (2%) over 24 h[45] than amiloride (87%).[21] This systemic selectivity is a significant feature of the novel ENaC blocker (**32**) and supports the notion of decreasing systemic side effects by including endogenous metabolic structures in the design of compounds.

Conclusions

In summary, we have synthesized a series of more potent, less reversible ENaC analogues than amiloride. To demonstrate that an antedrug or softdrug approach is conceptually possible, we made potential metabolites (**25**−**41**), which are less potent ENaC blockers and, therefore, pose less of a risk in vivo (hyperkalemia) than the parent analogue. We have also provided examples (**32**−**34**) of highly oxidized side chains, which for the most part are not metabolized and are mainly excreted non renally, thus providing an alternate means of achieving selectivity. After a considerable amount of in vitro and in vivo testing, we selected **32** as a clinical candidate. Compound **32** has increased potency by 2 orders of magnitude, reduced reversibility (five times), and decreased selectivity for renal elimination than amiloride. Presently, clinical studies (phase I and II) in CF patients are underway to evaluate **32**, and the results will be published in due course. Our work continues to test our hypothesis and provide additional compounds for COPD and CF as well as for ventilated associated pneumonia (VAP) and provide relief for the dry eyes and dry mouth symptoms associated with the disease known as Sjögren's syndrome.

Experimental Section

General Methods. Proton NMR spectra were recorded on a Bruker WM-360 or 300 UltraShield spectrometer (360 or 300 MHz) using tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in parts per million (ppm) relative to the internal standard TMS. Melting points were measured in capillary tubes on Electrothermal's MEL-TEMP apparatus without correction. Liquid chromatography (LC)/mass spectroscopy (MS) was performed on a Perkin-Elmer Sciex API 100 using one of the following methods. Method A: YMC Pro C8 column, 5 μm, 150 × 4.6 mm; mobile phase A = water + 0.4% acetic acid, B = acetonitrile (MeCN) + 0.4% acetic acid; gradient: 5% B for 1 min, going up to 80% B in 7 min, followed by 100% B for 5 min. Method B: YMC Pro C8 column, 5 μm, 150 × 4.6 mm; mobile phase A = water + 0.4% acetic acid, B = MeCN + 0.4% acetic acid; gradient: 5% B for 1 min, going up to 80% B in 5 min. Method C: Luna C8 (2) column, 5 μm, 150 × 4.6 mm; detector λ = 360 nm; mobile phase A = water + 0.4% acetic acid; B= MeCN + 0.4% acetic acid; gradient: 5% B for 1 min, going up to 80% B in 7 min, followed by washout with 100% B for 5 min.

Analytical HPLC Was Performed by One of the Following Methods. Method A: Shimadzu HPLC 10Avp: Luna C18(2) column, 5 μm, 250 × 4.6 mm; detector λ = 360 nm; gradient: A = water + 0.1% trifluoroacetic acid (TFA); B= MeCN + 0.1% TFA, concentration of MeCN increases from 10 to 60% during a 0−11 min interval and then 60−100% from 11 to 12 min. Method B: Shimadzu HPLC 10Avp: Symmetry C8 column, 150 × 4.6 mm; detector λ = 360 nm; gradient: A = water + 0.1% TFA; B= MeCN + 0.1% TFA, concentration of B increases in the A/B mixture from 10 to 60% during the 0−11 min interval, then B increases to 60−100% from 11 to 12 min. Method C: Gilson HPLC (322 pump, 156 UV−Vis detector), polarity dC18 column, 5 μm, 4.6 × 250 mm; detector λ = 220 nm; 40 °C; gradient: A = water + 0.05% TFA; B = MeCN + 0.05% TFA, 90:10 B/A for 5 min and then to 20:80 B/A over 37 min. Diverse HPLC system, Method D: Atlantis C18 5 μm column, 1.5 mL/min; 95:5 (0.02% TFA: 0.02% TFA MeCN) to 56:44 at 6.5 min to 90% (0.02% TFA MeCN) for 1 min at 40 °C.

Chiral HPLC analysis was performed on a Waters 260 HPLC instrument using the following method. Column: Chiralcel OD (Chiral Technologies), 10 μm, 250 × 4.6 mm; detector: λ = 280 nm; mobile phase = 60% heptane/40% ethyl alcohol containing 1% diethylamine; flow rate: 1 mL/min; run time: 45 min; sample concentration: 1 mg/mL; injection volume: 10.00 μL.

Preparative flash column chromatography was performed on Biotage's Horizon autoseparation equipment using a silica gel cartridge. Preparative HPLC was performed on Gilson CombiChem or on Waters (600 controller, 484 detector) using methods similar to those used in the analytical HPLC. Preparative TLC was performed on Analtech's UNIPLATE prep TLC plates (20 × 20 cm, 1000 μm) and developed by a mixture of dichloromethane, methanol, and ammonia hydroxide (CMA). Combustion analysis was conducted at Quantitative Technologies, Inc. (QTI), Whitehouse, NJ. High-resolution mass spectroscopy analysis was performed at the Center of Functional Genomics, State University of New York at Albany, Albany, NY.

Preparation of Stock Solutions. The concentration of a compound in DMSO was determined using a UV−Vis spectrophotometer (Hitachi U-3010) and the Beer−Lambert Law; the relationship between absorbance and concentration is commonly written as $A = \epsilon * c * l$, where A is the absorbance, ϵ is the millimolar extinction coefficient or molar absorptivity, c is the concentration of the analyte, and l is the length of the absorption path. The novel chemical entities are structurally similar to amiloride, sharing the same core chromophore (pyrazine ring) as that of amiloride; therefore, the extinction coefficient for amiloride was used in calculating the concentration of each compound. When converted to millimolar units, the extinction coefficient for amiloride at a wavelength of 362 nm is 18.6 mM^{-1} cm^{-1} (i.e., a 1 mM solution will generate an absorbance value of 18.6 at 362 nm using a 1 cm cuvette). To determine the concentration of a compound in DMSO, the absorbance of the solution was measured at a defined wavelength of 362 nm using a 1 cm cuvette. Concentration is calculated in millmolar units by substituting the measured absorbance value (A), extinction coefficient (ϵ) for amiloride (18.6 mM^{-1} cm^{-1}), and the path length (1 cm) into the Beer−Lambert Law equation and solving for c (concentration). Because the absorbance values must fall within the linear range of the spectrophotometer, stock solutions were diluted (when appropriate) prior to taking measurements. The measured concentration was then corrected using the appropriate dilution factor to determine the concentration of the compound in the original stock solution. The value obtained for concentration can be converted to mg/mL free base.

General Procedures. Method A: General Procedure for the Preparation of Compounds 2−12. An appropriate alkylamine (1 equiv) was dissolved in a mixture of anhydrous DMF (3 mL) and diisopropylethylamine (1.1 mL). To the solution, powdered 1*H*-pyrazole-1-carboxamidine hydrochloride (2 equiv) was added, and the reaction mixture was stirred at room-temperature overnight followed by the addition of ether (10 mL). The newly formed oil was washed with ether (3 × 10 mL) and dried under vacuum for 36 h. The resulting oil was further dissolved in anhydrous methanol (12 mL). To the solution, sodium methoxide (25 wt % solution in methanol, 1.5 equiv) was added, and the precipitate formed was filtered off. The mother liquor was then concentrated under vacuum (up to 1.5 mL). To the residue, 3,5-diamino-6-chloropyrazine-2-carboxylic acid methyl ester **43a** was added, and the mixture was first stirred at ambient temperature overnight and then heated to reflux for 12 h. The resulting solvent was removed under vacuum, and the residue was treated with water (10 mL). The supernatant was removed, and the resulting oil was washed with water (20 mL) and ether (2 × 10 mL). The thick oil that was obtained was then purified by preparative HPLC. Fractions containing the target compounds were combined and concentrated under reduced pressure. The residue was dissolved in 5 mL of 10% HCl aqueous

solution and evaporated to dryness to give the desired products hydrochloride salt (as yellow powder).

Method B: Coupling of Unprotected Amine with 1-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-2-methylisothiourea Hydriodide (43b). The unprotected amine (1 equiv) was dissolved in anhydrous ethanol (or THF or a mixture of these two solvents; concentration 3 mL/mmol). To the solution, Hunig's base (*i*-Pr_2-EtN, 3 equiv) was added, and the newly formed solution was heated at 65 °C for 15 min. Compound **43b** (1.1 equiv) was then added. The reaction mixture was stirred at 65 °C for an additional 2−3 h, and then cooled to room temperature before being concentrated under vacuum. The resulting residue was chromatographed on silica gel by eluting with CMA. The appropriate fractions were collected and concentrated under vacuum. The desired product (typically a yellow solid) was characterized by spectroscopic methods.

Method C: HCl Salt Preparation of Final Compounds. The purified free base of the final compounds was dissolved in a small amount of methanol (2−3 mL). Concentrated HCl aqueous solution (36.5wt %, 0.5 mL) was added dropwise. The mixture was stirred at room temperature for 30 min, concentrated, and further dried under vacuum. The yellow product was characterized spectroscopically.

Method D: Hydrogenolysis to Cleave the Carbobenzyloxy Protecting Group. The substrate to be reduced was dissolved in methanol or ethanol. The reaction vessel was vacuumed and refilled with argon. The process was repeated three times before the palladium catalyst (10% on charcoal, 50% wet) was added. The reaction was carried out at room temperature under one atmosphere of hydrogen until no further consumption of hydrogen was observed. The reaction vessel was vacuumed and refilled with argon a second time. The catalyst was filtered under vacuum and washed with methanol or ethanol. The filtrate and washings were combined and concentrated under vacuum. The residue was chromatographed, eluting with CMA. The appropriate fractions were collected and concentrated under vacuum. The dry product was then characterized by spectroscopic methods.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-hexylguanidine Hydrochloride (2).** Compound **2** was prepared according to general method A: mp 230−232 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 0.88 (t, J = 8 Hz, 3H, 6-CH_3), 1.26−1.30 (m, 6H, 3-CH_2-4-CH_2-5-CH_2), 1.54 (m, 2H, 2-CH_2), 3.30 (m, 2H, 1-CH_2-N), 7.26−7.60 (br s, 3H, NH_2 + guanidino), 8.78−9.02 (br s, 2H, NH_2), 9.32 (s, 1H, guanidino), 10.60 (s, 1H, guanidino); MS (APCI) *m*/*z* 314 (M + H)$^+$; HRMS (FAB) *m*/*z* 314.1496; calcd, $C_{12}H_{20}ClN_7O$·HCl: 314.1496 (M + H)$^+$. Anal. ($C_{12}H_{20}ClN_7O$·HCl). Calcd, C 41.15, H 6.04 N 27.99; found, C 35.87, H 5.55, N 24.26.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-octylguanidine Hydrochloride (3).** Compound **3** was prepared according to method A: mp 241−244 °C (dec); ^{1}H NMR (360 MHz, DMSO-d_6) δ 0.88 (t, J = 8 Hz, 3H, 8-CH_3), 1.26−1.30 (m, 8H, 4-CH_2-5-CH_2-6-CH_2-7-CH_2-), 1.44 (m, 2H, 3-CH_2), 1.60 (m, 2H, 2-CH_2), 3.20 (m, 2H, 1-CH_2-N), 7.26−7.45 (br s, 2H, NH_2), 7.78 (br s, 1H, guanidino), 8.78−9.02 (br s, 2H, NH_2), 9.32 (s, 1H, guanidino), 10.60 (s, 1H, guanidino); MS (APCI) *m*/*z* 342 (M + H)$^+$. HRMS (FAB) *m*/*z* 342.1805; calcd $C_{14}H_{24}ClN_7O$: 342.1809 (M + H)$^+$. Anal. ($C_{14}H_{24}ClN_7O$·HCl) H. Calcd, C 42.45, N 25.92; found, C 45.73, N 24.06.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-(2-hydroxyethyl)guanidine Hydrochloride (4).** Compound **4** was prepared according to method A: mp 203−205 °C (dec); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.82 (br s, 1H, 2-OH), 3.40 (m, 2H, 1-CH_2-N), 3.62 (m, 2H, 2-O-CH_2), 7.36−7.45 (br s, 3H, guanidino + NH_2), 8.0 (br s, 1H, guanidino), 8.88−9.02 (br s, 2H, NH_2), 9.40 (s, 1H, guanidino), 10.68 (s, 1H, guanidino); MS (APCI) *m*/*z* 274 (M + H)$^+$. HRMS (FAB) *m*/*z* 274.0814; calcd, $C_8H_{12}ClN_7O_2$: 274.0819 (M + H)$^+$. Anal. ($C_8H_{12}ClN_7O_2$·HCl) H. Calcd, C 30.98, N 31.61; found, C 30.50, N 27.58.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-phenethylguanidine Hydrochloride (7).** Compound **7** was prepared according to method A: mp 156−160 °C (dec); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.80 (m, 2H, 2-CH_2-Ar), 3.60 (m, 2H, 1-CH_2-N), 7.20−7.50 (m, 8H, Ar + NH_2 + guanidino), 7.90 (br s, 1H, guanidino), 9.00 (br s, 2H, NH_2), 9.35 (s, 1H, guanidino), 10.60 (s, 1H, guanidino); MS (APCI) *m*/*z* 334 (M + H)$^+$. Anal. ($C_{14}H_{16}ClN_7O$·HCl) H. Calcd, C 45.42, N 26.48; found, C 40.67, N 23.28.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-(3-phenylpropyl)guanidine Hydrochloride (8).** Compound **8** was prepared according to method A: mp 192−196 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.90 (m, 2H, 2-CH_2), 2.68 (t, J = 10 Hz, 2H, 3-CH_2-Ar), 3.35 (m, 2H, 1-CH_2-N), 7.18−7.30 (m, 5H, phenyl), 7.40−7.80 (br s, 3H, NH_2 + guanidino), 8.90−9.06 (br s, 2H, NH_2), 9.48 (s, 1H, guanidino), 10.60 (s, 1H, guanidino); MS (APCI) *m*/*z* 348 (M + H)$^+$. Anal. HRMS (FAB) *m*/*z* 348.1330; calcd, $C_{15}H_{18}ClN_7O$: 348.1339 (M + H)$^+$. Anal. ($C_{15}H_{18}ClN_7O$·HCl). Calcd, C 46.88, H 4.98, N 25.52; found, C 43.43, H 4.43, N 23.29.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-(4-phenylbutyl)guanidine Hydrochloride (9).** Compound **9** was prepared according to method A: mp 160−162 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.50−1.72 (m, 4H, 2-CH_2-3-CH_2), 2.70 (t, J = 7.8 Hz, 2H, 4-CH_2-Ar), 3.35 (m, 2H, 1-CH_2-N), 7.18−7.32 (m, 5H, Ar-), 7.45 (br s, 1H, guanidino), 7.60−7.80 (br s, 2H, NH_2), 9.02−9.18 (br s, 2H, NH_2), 9.42 (s, 1H, guanidino), 10.65 (s, 1H, guanidino); MS (APCI) *m*/*z* 362 (M + H)$^+$. HRMS (FAB) *m*/*z* 362.1504; calcd, $C_{16}H_{20}ClN_7O$: 362.1496 (M + H)$^+$. Anal. ($C_{16}H_{20}ClN_7O$·HCl) H. Calcd, C 48.25, N 24.62; found, C 46.59, N 23.11.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-(5-phenylpentyl)guanidine Hydrochloride (10).** Compound **10** was prepared according to method A: mp 240−243 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.36 (m, 2H, 3-CH_2-), 1.57−1.63 (m, 4H, 2-CH_2- + 4-CH_2-), 2.58 (t, J = 7.8 Hz, 2H, 5-CH_2-Ar), 3.34 (m, 2H, 1-CH_2-N), 7.09−7.30 (m, 5H, Ar-), 7.55(br s, 1H, guanidino), 7.60−7.80 (br s, 2H, NH_2), 8.78−8.92 (br s, 2H, NH_2), 9.26 (s, 1H, guanidino), 10.51 (s, 1H, guanidino); MS (APCI) *m*/*z* 376 (M + H)$^+$. Anal. ($C_{17}H_{22}ClN_7O$·HCl) C, H, N.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-(6-phenylhexyl)guanidine Hydrochloride (11).** Compound **11** was prepared according to method A: mp 126−129 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.38 (m, 4H, 4-CH_2-5-CH_2), 1.72 (m, 4H, 2-CH_2-3-CH_2), 2.62 (t, J = 7.8 Hz, 2H, 6-CH_2-Ar), 3.28 (m, 2H, 1-CH_2-N), 7.18−7.32 (m, 5H, Ar-), 7.45(br s, 1H, guanidino), 7.60−8.00 (br s, 2H, NH_2), 9.02−9.10 (br s, 2H, NH_2), 9.48 (s, 1H, guanidino), 10.68 (s, 1H, guanidino); MS (APCI) *m*/*z* 390 (M + H)$^+$. Anal. HRMS (FAB) *m*/*z* 390.1792; calcd, $C_{18}H_{24}ClN_7O$: 390.1809 (M + H)$^+$. Anal. ($C_{18}H_{24}ClN_7O$·HCl). Calcd, C 50.71, H 5.91, N 23.00; found, C 49.11, H 6.49, N 21.90.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-(8-phenyloctyl)guanidine Hydrochloride (12).** Compound **12** was prepared according to method A: mp 171−173 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.33 (m, 8H, 3-CH_2-4-CH_2-5-CH_2-6-CH_2-); 1.64 (m, 4H, 2-CH_2- and 7-CH_2-); 2.56 (m, 2H, 8-CH_2-Ar); 3.25 (m, 2H, 1-CH_2-N); 7.16−7.27 (m, 5H, Ar-); 7.40 (br s, 2H, NH_2); 8.85 (br s, 1H, guanidino); 8.95 (br s, 2H, NH_2); 9.31 (s, 1H, guanidino); 10.56 (s, 1H, guanidino); MS (APCI) *m*/*z* 418 (M + H)$^+$. HRMS (FAB) *m*/*z* 418.2110; calcd, $C_{20}H_{28}ClN_7O$: 418.2122 (M + H)$^+$. Purity: 99% (method A; t_R = 7.1 min), 97% (method D; t_R = 8.4 min).

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-[2-(4-hydroxyphenyl)ethyl]-guanidine Hydrochloride (13).** Compound **13** was gratefully obtained as a gift from Dr. E. J. Cragoe, Jr.: ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.76 (t, 2H, J = 5.6 Hz, 2-CH_2-Ar), 3.50 (m, 2H, 1-CH_2-N), 6.72 (d, J = 7.6 Hz, 2H, Ar-), 7.10 (d, J = 7.6 Hz, 2H, Ar-), 7.42 (br s, 2H, NH_2), 7.80 (br s, 1H, guanidino), 8.80 (br s, 2H, NH_2), 9.20 (br s, 1H, 4-OH-Ar), 9.30 (s, 1H, guanidino), 10.50 (s, 1H, guanidino); ^{13}C NMR (75 MHz, DMSO-d_6) δ 34.85, 44.48, 111.19, 117.01 (2×C), 122.14, 129.83, 131.25 (2×C), 155.80, 156.65, 157.96, 158.12, 167.47. MS (APCI) *m*/*z* 350 (M + H)$^+$. HRMS (FAB) *m*/*z* 350.1142; calcd, $C_{14}H_{16}ClN_7O_2$: 350.1132 (M + H)$^+$. Anal. ($C_{14}H_{16}ClN_7O_2$·HCl). Calcd, C 43.54, H 4.44, N 25.39; found, C 40.80, H 4.86, N 24.26.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-[3-(4-hydroxyphenyl)propyl]-guanidine Hydrochloride (14).** Pyridine (15 mL) was added dropwise to a cooled (0 °C) solution of 4-(4-

methoxyphenyl)propanol (**44b**) (10.0 g, 0.06 mol), and methanesulfonyl chloride (14.7 g, 0.078 mol) and dry THF (70 mL) was added with adequate stirring. The reaction mixture was stirred at room-temperature overnight. After this time, the solvent was removed under reduced pressure and the residue quenched with 10% HCl (300 mL) and extracted with ethyl acetate. The organic fraction was washed with saturated aqueous $NaHCO_3$ solution and water and dried (anhydrous sodium sulfate). The solvent was removed, and the residual crude ester (8.8 g, 60%, a yellow oil) was used in the following step without purification: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.08 (m, 2H, 2-CH_2-), 2.60 (t, J = 6.9 Hz, 2H, 3-CH_2-Ar), 3.58 (t, J = 6.9 Hz, 2H, 1-CH_2-O), 3.66 (s, 3H, CH_3-SO_3-), 3.98 (s, 3H, 4-MeO-Ar), 6.85 (d, J = 7.8 Hz, 2H, Ar-), 7.03 (d, J = 7.8 Hz, 2H, Ar-). The above oil was dissolved in anhydrous DMF (10 mL). To the solution, sodium azide (3 g, 0.045 mol) was added, stirred at room temperature overnight, and quenched with water (10 mL). The organics were extracted with ethyl ether (3 × 50 mL) and washed with brine, dried (anhydrous sodium sulfate), and concentrated. The residue was chromatographed, eluting with a mixture of ethyl acetate and hexane (20:80, v/v) to afford **45b** in 75% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.90 (m, 2H, 2-CH_2-), 2.65 (t, J = 6.8 Hz, 2H, 3-CH_2-Ar), 3.28 (t, J = 6.8 Hz, 2H, 1-CH_2-N_3), 3.80 (s, 3H, 4-MeO-Ar), 6.85 (d, J = 7.8 Hz, 2H, Ar-), 7.10 (d, J = 7.8 Hz, 2H, Ar-).

The above azide **45b** (5.2 g, 0.027 mol) was dissolved in anhydrous THF (15 mL). To the solution, Ph_3P (11.56 g, 0.0405 mol) and water (0.73 mL) was added, and the reaction mixture was stirred at room temperature overnight and then concentrated under vacuum. The residue was purified by flash chromatography (silica gel, 2:1:0.05 chloroform/ethanol/concentrated ammonium hydroxide) to provide pure amine **46b** (3.2 g, 74%) as a clear oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (m, 2H, 2-CH_2-), 2.50 (m, 4H, 1-CH_2-N + 3-CH_2-Ar), 3.72 (s, 3H, 4-MeO-Ar), 6.85 (d, J = 7.8 Hz, 2H, Ar-), 7.10 (d, J = 7.8 Hz, 2H, Ar-).

The above compound **46b** (2.5 g, 0.015 mol) was dissolved in 48% aqueous HBr solution (5 mL). The solution was heated to reflux for 3 h and then concentrated and further dried under vacuum to afford the desired product **47b** (1.99 g, 75% yield) as a light brown solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.80 (m, 2H, 2-CH_2-), 2.53 (t, J = 6.9 Hz, 2H, 3-CH_2-Ar), 2.78 (m, 2H, 1-CH_2-N), 6.70 (d, J = 7.8 Hz, 2H, Ar-), 7.02 (d, J = 7.8 Hz, 2H, Ar-), 7.80 (br s, 4H, HO-Ar + NH_3^+).

Compound **14** (a yellow solid) was made from **47b** in 31% yield according to the methods B and C: mp 133 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.80 (m, 2H, 2-CH_2), 2.58 (t, J = 6.8 Hz, 2H, 3-CH_2-Ar), 3.35−3.65 (m, 3H, 1-CH_2-N + Ar-OH), 6.70 (d, J = 7.6 Hz, 2H, Ar-), 7.03 (d, J = 7.6 Hz, 2H, Ar-), 7.48 (br s, 2H, NH_2), 8.80 (br s, 1H, guanidino), 8.93 (br s, 1H, guanidino), 9.32 (br s, 2H, NH_2), 10.52 (s, 1H, guanidino); MS (APCI) m/z 364 $(M + H)^+$. HRMS (FAB) m/z 364.1276; calcd, $C_{15}H_{18}ClN_7O_2$: 364.1289 $(M + H)^+$. Anal. ($C_{15}H_{18}ClN_7O_2$·HCl). Calcd, C 45.01, H 4.79, N 24.50; found, C 42.93, H 4.81, N 18.33.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-[3-(4-methoxyphenyl)propyl]-guanidine Hydrochloride (15).** Compound **15** was made from compound **46b** following methods B and C: mp 134 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.85 (m, 2H, 2-CH_2), 2.75 (m, 2H, 3-CH_2-Ar), 3.32 (m, 2H, 1-CH_2-N), 3.78 (s, 3H, 4-MeO-Ar), 6.84 (d, J = 7.6 Hz, 2H, Ar-), 7.20 (d, J = 7.6 Hz, 2H, Ar-), 7.50 (br s, 1H, guanidino), 8.12 (br s, 2H, NH_2), 8.95 (br s, 2H, NH_2), 9.40 (br s, 1H, guanidino), 10.60 (s, 1H, guanidino); MS (APCI) m/z 378 $(M + H)^+$. HRMS (FAB) m/z 378.1431; calcd, $C_{16}H_{20}ClN_7O_2$: 378.1445 $(M + H)^+$. Anal. ($C_{16}H_{20}ClN_7O_2$·HCl). Calcd, C 46.39, H 5.11, N 23.67; found, C 49.81, H 6.14, N 14.42.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-[4-(4-hydroxyphenyl)butyl]-guanidine Hydrochloride (16).** Compound **45c** (a clear oil) was prepared in 66% yield from **44c** using a method similar to that used to obtain compound **45b**: ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.61 (m, 4H, 2-CH_2-3-CH_2-), 2.44 (t, J = 6.8 Hz, 2H, 4-CH_2-Ar), 2.52 (t, J = 7.2 Hz, 2H, 1-CH_2-N_3), 3.78 (s, 3H, 4-MeO-Ar), 6.77 (d, J = 8.1 Hz, 2H, Ar-), 7.05 (d, J = 8.1 Hz, 2H, Ar-).

Following the same procedure used for the preparation of compound **46b**, compound **46c** was prepared in 94% yield from compound **45c**: ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.34 (m, 2H, 3-CH_2-), 1.54 (m, 2H, 2-CH_2-), 2.51 (m, 4H, 1-CH_2-N + 4-CH_2-Ar), 3.70 (s, 3H, 4-MeO-Ar), 6.83 (d, J = 8.1 Hz, 2H, Ar-), 7.08 (d, J = 8.1 Hz, 2H, Ar-).

Following the same procedure used for the preparation of compound **47b**, compound **47c** was prepared from **46c** in 90% yield: MS (APCI) m/z 166 for $C_{10}H_{15}NO$ $(M + H)^+$.

Alternative Synthesis of 47c. 4-(4-Methoxyphenyl)butyric acid (**48**) (450 g, 2.32 mol) was combined with anhydrous THF (4 L) and 4-methylmorpholine (268 mL, 2.43 mol) in a 12 L, three-necked flask, which was equipped with a mechanical stirrer, and placed in an ice−methanol cooling bath and protected under nitrogen atmosphere. Small pieces of dry ice were used to bring the bath temperature below −20 °C. Iso-butylchloroformate was added at a rate so as to not to exceed an internal temperature of −10 °C. After stirring for 30 min at −10 to −20 °C, a 4.7 M solution of ammonia in methanol (990 mL, 4.64 mol) was added. During the addition, the reaction temperature rose to 0 °C. The reaction was allowed to stir for 30 min and stand overnight. The product mixture was transferred to a 22 L separatory funnel with ethyl acetate (6 L) and 10% aqueous sodium chloride solution (1.5 L). The layers were separated and the organic solution washed with the 10% sodium chloride solution (4 × 1 L), followed by brine (3 × 500 mL). The organic layer was dried (anhydrous sodium sulfate), filtered, evaporated, and placed under high vacuum overnight. This afforded 432 g (97%) of pure amide **49** as an off white solid: ^{1}H NMR (300 MHz, CD_3OD) δ 1.81−1.93 (m, 2H, 3-CH_2-), 2.20 (t, J = 7.7 Hz, 2H, 2-CH_2-$CONH_2$), 2.57 (t, J = 7.7 Hz, 2H, 4-CH_2-Ar), 3.74 (s, 3H, 4-MeO-Ar), 6.82 (d, J = 8.7 Hz, 2H, Ar-), 7.09 (d, J = 8.7 Hz, 2H, Ar-); MS (ESI) m/z 194 for $C_{11}H_{15}NO_2$ $(M + H)^+$. 4-(4-Methoxyphenyl)butyramide (**49**) (200 g, 1.0 mol) and THF (300 mL) were combined in a 12 L, three-necked flask, which was equipped with a heating mantle, an internal thermometer, and a reflux condenser. The suspension was slowly mechanically stirred while a 1 M Borane·THF (1 L, 1 mol) solution was dripped in via a pressure equalizing addition funnel over 20 min. Another 2.2 L (2.2 mol) of 1 M Borane·THF complex was dripped in over 20 min. The reaction temperature rose to 45 °C during the addition. The reaction was heated to reflux over 1 h, maintained at reflux for 2 h, and then allowed to cool for 2 h. Methanol (500 mL) was slowly and cautiously dripped into the reaction. Copious H_2 evolution was observed. The reaction was heated at reflux for 2 h and then allowed to cool overnight. The reaction was evaporated and then coevaporated with toluene (500 mL) to afford a thick oil. A 48% aqueous HBr solution (3 L) was slowly and cautiously added to the reaction. Bubbling and foaming were observed during this addition, which was exothermic. After the addition, the reaction was less viscous and was stirred at reflux for 7 h. The reaction was allowed to cool with stirring overnight. The batch was further chilled in an ice bath and then suction filtered to collect an off white solid. The solid was coevaporated with toluene/methanol (1:1, v/v) and then dried under vacuum at 60 °C overnight. This afforded 197 g (77%) of **47c** as an off white crystalline solid: ^{1}H NMR (300 MHz, CD_3OD) δ 1.66 (m, 4H, 2-CH_2-3-CH_2-), 2.57 (t, J = 6.8 Hz, 2H, 4-CH_2-Ar), 2.92 (m, 2H, 1-CH_2-N), 6.70 (d, J = 8.5 Hz, 2H, Ar-), 7.01 (d, J = 8.5, Hz, 2H, Ar-); MS (ESI) m/z 166 for $C_{10}H_{15}NO$ $(M + H)^+$.

Using methods B and C, compound **16** was prepared from compound **47c** in 41% yield as a yellow solid: mp 160 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.56 (m, 4H, 2-CH_2-3-CH_2), 2.48 (m, 2H, 4-CH_2-Ar), 3.35 (m, 2H, 1-CH_2-N), 6.65 (d, J = 7.8 Hz, 2H, Ar-), 6.95 (d, J = 7.8 Hz, 2H, Ar-), 7.50 (br s, 2H, NH_2), 8.75 (br s, 1H, guanidino), 9.05 (br s, 1H, guanidino), 9.33 (br s, 2H, NH_2), 10.55 (s, 1H, guanidino); MS (APCI) m/z 378 $(M + H)^+$. HRMS (FAB) m/z 378.1460; calcd, $C_{16}H_{20}ClN_7O_2$: 378.1445 $(M + H)^+$. Anal. ($C_{16}H_{20}ClN_7O_2$·HCl). Calcd, C 46.39, H 5.11, N 23.67; found, C 45.21, H 5.92, N 18.85.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-[5-(4-hydroxyphenyl)pentyl]-guanidine Hydrochloride (17).** 4-Iodoanisol

50a (10 g, 42 mmol), palladium (II) chloride (0.2 g, 1.1 mmol), and triphenylphosphine (0.6 g, 2.2 mmol) were dissolved in diethylamine (100 mL) then copper (I) iodide (0.5 g, 2.2 mmol), and *N*-Boc-protected 4-pentynyl-1-amine 51 ($n = 3$, 5 mL, 53 mmol) were added. The reaction mixture was stirred at room temperature overnight, and then the solvent was removed under reduced pressure. Ethyl acetate (150 mL) was added to the residue, and the mixture was sequentially washed with 2 N aqueous HCl solution, brine, and water. The organic fraction was isolated and dried (anhydrous sodium sulfate). The solvent was removed under reduced pressure. Product **52a** (7.1 g. 87%) was isolated by flash chromatography (silica gel, 1:2 ethyl acetate/hexanes) as an oily yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H, Boc), 1.88 (m, 2H, 2-CH_2-), 2.53 (t, 2H, $J = 5.7$ Hz, 3-CH_2-alkyne), 3.51 (m, 2H, 1-CH_2-N), 3.72 (s, 3H, 4-MeO-Ar), 6.83 (d, $J = 8.0$ Hz, 2H, Ar-), 7.45 (d, $J = 8.0$ Hz, 2H, Ar-).

A solution of **52a** (7.1 g, 37 mmol) in dry ethanol (150 mL) was placed in a 0.5 L Parr flask. Palladium on carbon (0.92 g, 5% Pd, 50% wet) was added as a suspension in ethanol (25 mL). The reaction mixture was shaken at 50 psi of hydrogen pressure at room temperature for 24 h. After this time, the mixture was filtered through Celite, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (silica gel, 1:3 ethyl acetate/hexanes) to provide **53a** (6.7 g, 92%) as a clear oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (m, 2H, 3-CH_2-), 1.60 (m, 4H, 2-CH_2 + 4-CH_2-), 2.58 (m, 2H, 5-CH_2-Ar), 3.63 (m, 2H, 1-CH_2-N), 3.80 (s, 3H, 4-MeO-Ar), 6.83 (d, $J = 8.0$ Hz, 2H, Ar-), 7.10 (d, $J = 8.0$ Hz, 2H, Ar-).

The HBr salt of **54a** was prepared from **53a** following the same procedure used for the preparation of compound **47a**. The free amine of **54a** (a cloudy oil, 2 g, 80%) was obtained after flash chromatography (silica gel, 6:3:0.1, chloroform/ethanol/concentrated ammonium hydroxide): ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (m, 2H, 3-CH_2-), 1.55 (m, 2H, 4-CH_2-), 1.61 (m, 2H, 2-CH_2-), 2.48 (m, 2H, 1-CH_2-N), 2.58 (m, 2H, 5-CH_2-Ar), 6.68 (d, $J = 8.0$ Hz, 2H, Ar-), 6.98 (d, $J = 8.0$ Hz, 2H, Ar-).

Compound **17** as a yellow solid was made in 39% yield from **54a** following methods B and C: mp 188 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.32 (m, 2H, 3-CH_2), 1.55 (m, 4H, 2-CH_2 and 4-CH_2), 2.45 (m, 2H, 5-CH_2-Ar), 3.29 (m, 2H, 1-CH_2-N), 6.68 (d, $J = 8.0$ Hz, 2H, Ar-), 6.97 (d, $J = 8.0$ Hz, 2H, Ar-), 7.46 (s, 1H, guanidino), 8.00 (br s, 2H, NH_2), 8.97 (br s, 1H, guanidino), 9.46 (br s, 2H, NH_2), 10.55 (s, 1H, guanidino); MS (APCI) *m*/*z* 392 $(M + H)^+$. HRMS (FAB) *m*/*z* 392.1588; calcd. $C_{17}H_{22}ClN_7O_2$: 392.1602 $(M + H)^+$. Anal. ($C_{17}H_{22}ClN_7O_2$·HCl). Calcd, C 47.67, H 5.41, N 22.89; found, C 45.21, H 5.92, N 18.85.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-[4-(5-hydroxyphenyl)butyl]-guanidine Hydrochloride (18).** Compound **52b** was prepared in a manner similar to that used to prepare compound **52a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H, Boc), 2.65 (t, $J = 6.6$ Hz, 2H, 2-CH_2), 3.34 (m, 2H, 1-CH_2-N), 3.72 (s, 3H, 5-MeO-Ar), 4.65 (br s, 1H, NHBoc), 6.83 (d, $J = 7.6$ Hz, 1H, Ar), 6.96 (m, 1H, Ar), 7.03 (d, $J = 7.6$ Hz, 1H, Ar), 7.26 (m, 1H, Ar).

Compound **53b** was prepared from **52b** using a method similar to that used to prepare compound **53a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H, Boc), 1.56−1.78 (m, 4H, 2-CH_2-3-CH_2-), 2.56 (t, $J = 6.4$ Hz, 2H, 4-CH_2-Ar), 3.32 (m, 2H, 1-CH_2-N), 3.72 (s, 3H, 5-MeO-Ar), 4.65 (br s, 1H, NHBoc), 6.76 (m, 3H, Ar-), 7.20 (m, 1H, Ar-).

Compound **54b** was prepared from **53b** following the same procedure used for the preparation of compound **54a**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52 (m, 2H, 3-CH_2-), 1.68 (m, 2H, 2-CH_2-), 2.62 (t, $J = 10.0$ Hz, 2H, 4-CH_2-Ar), 2.72 (m, 2H, 1-CH_2-N), 3.34 (br s, 2H, NH_2), 6.74 (m, 3H, Ar-), 7.20 (m, 1H, Ar-).

Compound **18** was made as a yellow solid from **54b** in 39% yield following methods B and C: mp >215 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52 (m, 4H, 2-CH_2-3-CH_2), 2.51 (m, 2H, 4-CH_2-Ar), 3.30 (m, 2H, 1-CH_2-N), 6.60 (m, 3H, Ar-), 7.10 (m, 1H, Ar-), 7.40 (br s, 2H, NH_2), 7.90 (s, 1H, guanidino), 8.40 (br s, 2H, NH_2), 9.30 (br s, 1H, guanidino), 10.60 (s, 1H, guanidino); MS (APCI) *m*/*z* 378 $(M + H)^+$. HRMS (FAB) *m*/*z* 378.1450; calcd. $C_{16}H_{20}ClN_7O_2$: 378.1445 $(M + H)^+$. Anal. ($C_{16}H_{20}ClN_7O_2$·HCl) H. Calcd, C 46.39, N 23.67; found, C 45.19, N 19.50.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-[4-(2-hydroxyphenyl)butyl]-guanidine Hydrochloride (19).** Compound **52c** was prepared from **50c** in a manner similar to that used to prepare compound **52a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H, Boc), 2.64 (t, $J = 6.3$ Hz, 2H, 2-CH_2), 3.38 (m, 2H, 1-CH_2-N), 3.89 (s, 3H, 2-MeO-Ar), 5.14 (br s, 1H, NHBoc), 6.85 (m, 2H, Ar-), 7.25 (m, 1H, Ar-), 7.37 (d, $J = 7.4$ Hz, 1H, Ar-).

Compound **53c** was prepared from **52c** using a method similar to that used to prepare compound **53a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H, Boc), 1.56−1.62 (m, 4H, 2-CH_2-3-CH_2-), 2.58 (t, $J = 9.4$ Hz, 2H, 4-CH_2-Ar), 3.26 (m, 2H, 1-CH_2-N), 3.83 (s, 3H, 2-MeO-Ar), 4.70 (br s, 1H, NHBoc), 6.87 (m, 2H, Ar-), 7.17 (m, 2H, Ar-).

Compound **54c** was prepared from **53c** following the same procedure used for the preparation of compound **47b** and directly used in the next step without purification.

Compound **19** (a yellow solid) was made in 39% yield from **54c** following general methods B and C: mp 240−242 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.58 (m, 4H, 2-CH_2-3-CH_2-), 2.50 (m, 2H, 4-CH_2-Ar), 3.28 (m, 2H, 1-CH_2-N), 6.70 (m, 2H, Ar-), 7.06 (m, 2H, Ar-), 7.75 (br s, 2H, NH_2), 7.80 (s, 1H, guanidino), 8.70 (br s, 2H, NH_2), 9.10 (br s, 1H, guanidino), 10.47 (s, 1H, guanidino); MS (APCI) *m*/*z* 378 $(M + H)^+$. HRMS (FAB) *m*/*z* 378.1440; calcd. $C_{16}H_{20}ClN_7O_2$: 378.1445 $(M + H)^+$. Anal. ($C_{16}H_{20}ClN_7O_2$·HCl) C. Calcd, H 5.11, N 23.67; found, H 4.42, N 22.36.

***N*-[4-(4-Aminopentyl)butyl]-*N*′-(3,5-diamino-6-chloropyrazine-2-carbonyl)-guanidine Hydrochloride (20).** Compound **52d** was prepared from compound **50d** in a manner similar to that used to prepare compound **52a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H, Boc), 2.66 (t, $J = 6.5$ Hz, 2H, 2-CH_2), 3.40 (m, 2H, 1-CH_2-N), 4.83 (br s, 1H, NHBoc), 7.54 (d, $J = 9.0$ Hz, 2H, Ar-), 8.18 (d, $J = 9.1$ Hz, 2H, Ar-).

Compound **53d** was prepared from **52d** in a method similar to that used to prepare compound **53a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H, Boc), 1.56−1.68 (m, 4H, 2-CH_2-3-CH_2-), 2.53 (t, $J = 7.1$ Hz, 2H, 4-CH_2-Ar), 3.17 (m, 2H, 1-CH_2-N), 3.47 (br s, 2H, 4-NH_2-Ar), 4.46 (br s, 1H, NHBoc), 6.62 (d, $J = 8.3$ Hz, 2H, Ar-), 6.95 (d, $J = 8.3$ Hz, 2H, Ar-).

Compound **54d** was prepared from **53d** following the same procedure used for the preparation of compound **47b** and directly used in the next step without purification.

Compound **20** as a yellow solid was made from **54d** in 39% yield following methods B and C: mp 195−200 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.56−1.64 (m, 4H, 2-CH_2-3-CH_2-), 2.62 (t, $J = 9.6$ Hz, 2H, 4-CH_2-Ar), 3.39 (m, 2H, 1-CH_2-N), 4.99 (br s, 2H, 4-NH_2-Ar), 7.34 (m, 4H, Ar-), 7.66 (br s, 2H, NH_2), 8.06 (s, 1H, guanidino), 8.90 (br s, 2H, NH_2), 9.38 (br s, 1H, guanidino), 10.56 (s, 1H, guanidino); MS (APCI) *m*/*z* 377 $(M + H)^+$. HRMS (FAB) *m*/*z* 377.1607; calcd, $C_{16}H_{21}ClN_8O$: 377.1605 $(M + H)^+$. Anal. ($C_{16}H_{21}ClN_8O$·2HCl). Calcd, C 42.73, H 5.15, N 24.91; found C 38.20, H 5.31, N 21.08.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-[4-(4-methoxyphenyl)butyl]-guanidine Hydrochloride (21).** Compound **21** was prepared as a yellow powder from compound **40c** following methods B and C: mp 108−111 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.56 (m, 4H, 2-CH_2-3-CH_2); 2.56 (m, 2H, 4-CH_2-Ar); 3.32 (m, 2H, 1-CH_2-N); 3.74 (s, 3H, 4-MeO-Ar), 6.85 (d, $J = 7.8$ Hz, 2H, Ar-); 7.10 (d, $J = 7.8$ Hz, 2H, Ar-); 7.46 (br s, 2H, NH_2), 7.85 (br s, 1H, guanidino), 8.98 (br s, 2H, NH_2); 9.46 (s, 1H, guanidino); 10.60 (s, 1H, guanidino); MS (APCI) *m*/*z* 392 $(M + H)^+$. HRMS (FAB) *m*/*z* 392.1604; calcd, $C_{17}H_{22}ClN_7O_2$: 392.1602 $(M + H)^+$. Purity: 99% (method A; $t_R = 6.4$ min), 98% (method D; $t_R = 6.0$ min).

4-{4-[*N*′-(3,5-Diamino-6-chloropyrazine-2-carbonyl)guanidino]-butyl}benzoic Acid Methyl Ester Hydrochloride (22). Compound **45d** was prepared as a clear oil from **44d** in 85% yield following the same method used for the preparation of compound **45a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.68 (m, 4H, 2-CH_2-3-CH_2-), 2.22 (t,

J = 6.4 Hz, 2H, 4-CH_2-Ar), 3.29 (t, J = 6.5 Hz, 2H, 1-CH_2-N_3), 3.92 (s, 3H, 4-MeO_2C-Ar), 7.28 (d, J = 7.8 Hz, 2H, Ar-), 7.98 (d, J = 7.8 Hz, 2H, Ar-).

Compound **46d** was made from **45d** in 53% yield using the same procedure used for the preparation of compound **46b**.

Following methods B and C, compound **22** was made as a yellow solid from **46d** in 25% yield: mp 210 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.59 (m, 4H, 2-CH_2-3-CH_2), 2.71 (m, 2H, 4-CH_2-Ar), 3.23 (m, 2H, 1-CH_2-N), 3.83 (s, 3H, 4-CO_2Me-Ar), 7.40 (d, J = 7.8 Hz, 2H, Ar-), 7.48 (br s, 2H, NH_2), 7.80 (d, J = 7.8 Hz, 2H, Ar-), 8.92 (br s, 2H, NH_2), 9.00 (br s, 1H, guanidino), 9.48 (br s, 1H, guanidino), 10.55 (s, 1H, guanidino); MS (APCI) m/z 420 (M + H)$^+$. HRMS (FAB) m/z 420.1544; calcd, $C_{18}H_{22}ClN_7O_3$: 420.1551 (M + H)$^+$. Anal. ($C_{18}H_{22}ClN_7O_3$·HCl) H. Calcd, C 47.38, N 21.49; found C 46.83, N 18.33.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-[5-(4-methoxyphenyl)pentyl]-guanidine Hydrochloride (23).** Compound **53a** was dissolved in dichloromethane. TFA was added to the solution. The reaction mixture was stirred at room temperature for 1 h, concentrated, and further dried under vacuum. The product was directly used in the next step without purification.

The above crude material was reacted with **43b** using methods B and C to produce desired compound **23** as a yellow solid in 56% yield: mp 132 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.32 (m, 2H, 3-CH_2), 1.55 (m, 4H, 2-CH_2 and 4-CH_2), 2.45 (m, 2H, 5-CH_2-Ar), 3.29 (m, 2H, 1-CH_2-N), 6.68 (d, J = 8.0 Hz, 2H, Ar-), 6.97 (d, J = 8.0 Hz, 2H, Ar-), 7.46 (s, 1H, guanidino), 8.00 (br s, 2H, NH_2), 8.97 (br s, 1H, guanidino), 9.46 (br s, 2H, NH_2), 10.55 (s, 1H, guanidino); MS (APCI) m/z 406 (M + H)$^+$. HRMS (FAB) m/z 406.1742; calcd, $C_{17}H_{22}ClN_7O_2$: 406.1758 (M + H)$^+$. Anal. ($C_{17}H_{22}ClN_7O_2$·HCl) H. Calcd, C 48.84, N 22.17; found, C 44.19, N 19.53.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-[3-(4-methoxyphenoxy)propyl]-guanidine Hydrochloride (24).** 4-Methoxyphenol (**56a**) (10 g, 0.081mol) was dissolved in a mixture of anhydrous THF and DMF (200 mL, 1/1, v/v). To the solution, sodium hydride (60% dispersion in mineral oil, 4.2 g) was added in one portion. The mixture was stirred at room temperature for 1 h before *N*-(3-bromopropyl)phthalimide (**57a**) (23.9 g, 0.089 mol) was added. Stirring was continued overnight. The reaction was quenched with water (50 mL) and partitioned between water and dichloromethane (500 mL, 1/1, v/v). The organic layer was separated, washed with brine, and dried (anhydrous sodium sulfate). After concentration under vacuum, the material was purified by column chromatography (silica gel, 3:1 hexanes/ethyl acetate) to provide **58a** (12 g, 49%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.12 (m, 2H, 2-CH_2-), 3.78 (s, 3H, 4-MeO-Ar), 3.92 (m, 2H, 1-CH_2-N), 3.98 (m, 2H, 3-O-CH_2-), 6.77 (s, 4H, phthalimide), 7.73 (m, 2H, Ar-), 7.85 (m, 2H, Ar-).

Compound **58a** (6 g, 0.019 mol) was dissolved in ethanol (150 mL). Methylamine (2.0 M solution in methanol, 50 mL, 0.1 mol) was added to the solution, and the mixture was stirred at room temperature for 4 h and then concentrated under vacuum. The crude material was purified by column chromatography (silica gel, 3:1: 0.1 chloroform/ethanol/concentrated ammonium hydroxide) to provide **59a** (2.0 g, 58%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.66 (m, 2H, 2-CH_2-), 2.70 (m, 2H, 1-CH_2-N), 3.68 (s, 3H, 4-MeO-Ar), 3.93 (m, 2H, 3-O-CH_2-), 6.85 (s, 4H, Ar-).

Following methods B and C, compound **24** was prepared as a yellow solid from **59a** in 85% yield: mp >98 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.03 (m, 2H, 2-CH_2), 3.32 (m, 2H, 1-CH_2-N), 3.52 (m, 2H, 3-O-CH_2), 3.70 (s, 3H, 4-MeO-Ar), 6.91 (m, 4H, Ar-), 7.46 (br s, 3H, NH_2 + guanidino), 8.93 (br s, 2H, NH_2), 9.48 (s, 1H, guanidino), 10.57 (s, 1H, guanidino); MS (APCI) m/z 395 (M + H)$^+$. Anal. ($C_{16}H_{20}ClN_7O_3$·HCl) H. Calcd, C 44.66, N, 22.79, found, C 41.11, N 18.99.

4-{4-[*N′*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)guanidino]-butyl}benzoic Acid Hydrochloride (25). Compound **25** was made in 65% yield from compound **22** by saponification with lithium hydroxide in THF and purified by chromatography eluting with CMA. The free base was then treated with HCl using method C to make the HCl salt: mp 123−126 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.60 (m, 4H, 2-CH_2-3-CH_2), 2.74 (m, 2H, 4-CH_2-Ar), 3.36 (m, 2H, 1-CH_2-N), 7.30 (d, J = 7.8 Hz, 2H, Ar-), 7.48 (br s, 2H, NH_2), 7.60 (br s, 1H, guanidino), 7.90 (d, J = 7.8 Hz, 2H, Ar-), 8.90 (br s, 2H, NH_2), 9.10 (br s, 1H, guanidino), 9.50 (br s, 1H, guanidino), 12.80 (s, 1H, 4-CO_2H); MS (APCI) m/z 406 (M + H$^+$). HRMS (FAB) m/z 406.1395; calcd, $C_{17}H_{20}ClN_7O_3$: 406.1394 (M + H)$^+$. Anal. ($C_{17}H_{20}ClN_7O_3$·HCl) H. Calcd, C 46.16, N 22.17; found C 41.08, N 19.42.

Sulfuric Acid Mono-(4-{4-[*N*-(3,5-diamino-6-chloropyrazine-2-carbonyl)guanidine]butyl}phenyl)ester (26). To a solution of **16** (0.2 g, 0.5 mmol) dissolved in dry pyridine (5 mL) was added the pyridine sulfur trioxide complex (0.45 g, 2.5 mmol). The reaction mixture was stirred at room-temperature overnight. The precipitate formed was isolated by vacuum filtration and washed with ethyl acetate (2 × 25 mL) to give crude **26** (0.18 g, 39% yield, 87% purity by HPLC). An aliquot of crude **26** (67 mg) was purified by flash chromatography (silica gel, dichloromethane/methanol/concentrated ammonium hydroxide, 6:3:0.1, v/v) to give **26** as a yellow solid (9.3 mg, 4% overall yield): ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.59 (br s, 4H), 2.58 (m, 2H), 3.28 (m, 2H), 7.08 (s, 4H), 7.1−7.9 (m, 6H). MS (ESI) m/z 456 [$C_{16}H_{20}ClN_7O_5S$-H]$^-$. Purity: 95% (method B; t_R = 8.7 min), 88% (method D; t_R = 6.6 min).

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-[4-(4-hydroxymethylphenyl)-butyl]guanidine Hydrochloride (27).** Lithium aluminum hydride (35 mL of a 1.0 M solution in THF, 0.035 mol) was added dropwise to a vigorously stirred solution of **46d** (2.4 g, 0.010 mol) in dry THF (120 mL) cooled at 0 °C. Stirring was continued overnight under a nitrogen atmosphere. To break up the complex, water (6 mL) and 15% NaOH (1.5 mL) were sequentially added dropwise to the cold reaction mixture. The white solid precipitate was filtered off and washed with THF. All organic phases were combined and evaporated. The material was purified by column chromatography (silica gel, 2:1:0.05 chloroform/ethanol/concentrated ammonium hydroxide) to provide **46f** (1.17 g, 64%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (br, 2H, NH_2), 1.54 (m, 2H, 2-CH_2-), 1.70 (m, 2H, 3-CH_2-), 2.60 (m, 4H, 1-CH_2-N + 4-CH_2-Ar), 4.67 (s, 2H, 4-HO-CH_2-Ar), 7.47 (s, 2H, Ar-), 7.60 (s, 2H, Ar-).

Following general methods B and C, compound **27** was prepared as a yellow solid from **46f** in 98% yield: mp >140 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.57 (m, 4H, 2-CH_2-3-CH_2), 2.62 (m, 2H, 4-CH_2-Ar), 3.35 (m, 2H, 1-CH_2-N), 3.73 (br s, 1H, OH−Bn), 4.45 (s, 2H, 4-O-CH_2-Ar), 7.12 (d, J = 7.8 Hz, 2H, Ar-), 7.24 (d, J = 7.8 Hz, 2H, Ar-), 7.67 (br s, 2H, NH_2), 8.85 (br s, 2H, NH_2), 9.98 (br s, 1H, guanidino), 9.32 (br s, 1H, guanidino), 10.55 (s, 1H, guanidino); MS (APCI) m/z 392 (M + H)$^+$. HRMS (FAB) m/z 392.1588; calcd. $C_{17}H_{22}ClN_7O_2$: 392.1602 (M + H)$^+$. Anal. ($C_{17}H_{22}ClN_7O_2$·HCl) H. Calcd, C 47.67, N 22.89; found, C 44.51, N 19.28.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-[3-(4-hydroxyphenoxy)propyl]-guanidine Hydrobromide (28).** The vigorously stirred solution of **24** (80 mg, 0.19 mmol) in 48% HBr (15 mL) was refluxed for 2 h and then cooled to room temperature. The precipitate formed was collected by vacuum filtration, washed with water, and dried under vacuum overnight to provide **28** (52 mg, 52%) as a yellow solid: mp >150 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.99 (m, 2H, 2-CH_2), 3.30 (m, 2H, 1-CH_2-N), 3.96 (t, J = 10.6 Hz, 2H, 3-O-CH_2), 6.77 (d, J = 8.0 Hz, 2H, Ar-), 6.79 (d, J = 8.0 Hz, 2H, Ar-), 7.45 (br s, 2H, NH_2), 8.74 (br s, 1H, guanidino), 8.87 (br s, 2H, NH_2), 9.30 (s, 1H, guanidino), 10.48 (s, 1H, guanidino); MS (APCI) m/z 380 (M + H)$^+$. HRMS (FAB) m/z 380.1239; calcd, $C_{15}H_{18}ClN_7O_3$: 380.1238 (M + H)$^+$. Anal. ($C_{15}H_{18}ClN_7O_3$·HBr). Calcd, C 39.1, H 4.16, N 21.28; found, C 26.65, H 2.92, N 14.11.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N′*-[2-(4-methoxybenzyloxy)ethyl]-guanidine (29).** Compound **58b** was synthesized from **56b** in 72% yield in a manner similar to that used to prepare compound **58a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.32 (m, 2H, 1-CH_2-N), 3.78 (s, 3H, 4-MeO-Ar), 3.92 (m, 2H, 2-CH_2-O),

4.48 (s, 2H, O-CH_2-Ar), 6.77 (d, J = 8.0 Hz, 2H, Ar-), 7.20 (d, J = 8.0 Hz, 2H, Ar-), 7.72 (m, 2H, phthalimide), 7.83 (m, 2H, phthalimide).

Compound **59b** was prepared from **58b** in 56% yield using a method similar to that used to prepare **59a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.78 (m, 2H, NH_2), 2.90 (m, 2H, 1-CH_2-N), 3.60 (m, 2H, 2-CH_2-O), 3.82 (s, 3H, 4-MeO-Ar), 4.48 (s, 2H, O-CH_2-Ar), 6.88 (d, J = 8.0 Hz, 2H, Ar-), 7.27 (d, J = 8.0 Hz, 2H, Ar-).

Using general method B, compound **29** was prepared as a yellow solid in 60% yield from compound **59b**: mp 99−102 °C; ^{1}H NMR (300 MHz, DMSO-d_6 + $CDCl_3$) δ 3.46 (m, 2H, 1-CH_2-N), 3.58 (t, J = 11 Hz, 2H, O-2-CH_2), 3.77 (s, 3H, 4-MeO-Ar), 4.49 (s, 2H, ArCH_2-O), 6.95 (d, J = 7.8 Hz, 2H, Ar-), 7.31 (d, J = 7.8 Hz, 2H, Ar-), 7.18 (s, 1H, guanidino), 8.90 (br s, 2H, NH_2); MS (APCI) m/z 394 (M + H)$^+$. HRMS (FAB) m/z 394.1376; calcd for $C_{16}H_{20}ClN_7O_3$: 394.1394 (M + H)$^+$. Anal. ($C_{16}H_{20}ClN_7O_3$·HCl) H. Calcd, C 44.66, N 22.79; found, C 43.43, N, 20.22.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-{4-[4-(2-hydroxyethoxy)phenyl]-butyl}guanidine Hydrochloride (30).** Compound **52e** was prepared as a brown oil from **50e** in 43% yield in a manner similar to that used to prepare compound **52a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H, Boc), 2.80 (m, 2H, 2-CH_2-), 3.95 (m, 4H, 1-CH_2-N + HO-2′-CH_2-), 4.08 (m, 2H, 1′-CH_2-O-Ar), 6.83 (d, J = 7.8 Hz, 2H, Ar-), 7.35 (d, J = 7.8 Hz, 2H, Ar-).

Compound **53e** was prepared from **52e** as a brown oil using a method similar to that used to prepare compound **53a** and used in the next step without purification.

Compound **55e** was prepared as a clear oil from **53e** in 35% yield using a method similar to that used to prepare compound **55a**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.58 (m, 4H, 2-CH_2-3-CH_2-), 2.55 (m, 2H, 4-CH_2-Ar), 2.73 (m, 2H, 1-CH_2-N), 3.83 (m, 2H, 2′-CH_2-OH), 3.97 (m, 2H, 1′-CH_2-Ar), 6.83 (d, J = 7.8 Hz, 2H, Ar), 7.07 (d, J = 7.8 Hz, 2H, Ar).

Following general methods B and C, compound **30** was prepared as a yellow solid from **55e** in 73% yield: mp 218 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.56 (m, 4H, 2-CH_2-3-CH_2), 2.57 (m, 2H, 4-CH_2-Ar), 3.32 (m, 2H, 1-CH_2-N), 3.70 (t, J = 10.6 Hz, 2H, HO-1′-CH_2-Ar), 3.93 (t, J = 10.8 Hz, 2H, 2′-CH_2-OAr), 4.90 (t, J = 10.8 Hz, 1H, 1′-OH), 6.84 (d, J = 8.0 Hz, 2H, Ar-), 7.12 (d, J = 8.0 Hz, 2H, Ar-), 7.45 (br s, 2H, NH_2), 8.70 (br s, 1H, guanidino), 8.88 (br s, 2H, NH_2), 9.12 (br s, 1H, guanidino) 10.45 (s, 1H, guanidino); MS (APCI) m/z 422 (M + H)$^+$. Anal. ($C_{18}H_{24}ClN_7O_3$·HCl) H. Calcd, C 47.17, N 21.39; found, C 43.53, N 19.90.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-{4-[4-(3-hydroxypropoxy)phenyl]-butyl}guanidine Hydrochloride (31).** 4-(4-Aminobutyl)phenol hydrobromide (**47c**) (197 g, 0.80 mol), water (1 L), 1,4-dioxane (1 L), and sodium bicarbonate (336 g, 4 mol) were combined and stirred while cooled in an ice−methanol cooling bath. Benzyl chloroformate (141 mL, 0.96 mol) was dripped in over 5 min at −2 °C with no appreciable exotherm observed. This was stirred and allowed to warm to room temperature as the cooling bath thawed overnight. An additional quantity of benzyl chloroformate (8 mL, 0.54 mol) was dripped in and allowed to stir for 2 h. The product mixture was then evaporated to approximately 500 mL and transferred to a 2 L separatory funnel with ethyl acetate (decanting the solids away). The aqueous layer was extracted with ethyl acetate (3 × 1 L). The extracts were combined, washed with brine, dried (anhydrous sodium sulfate), filtered, and evaporated to afford 265 g of the crude product. The crude material was crystallized from a mixture of toluene/heptane (1:1, v/v). The crystallized product was suction filtered and washed with toluene/heptane (1:1, v/v). This material was vacuum-dried at 45 °C for 2 h to afford compound **60a** (150 g, 62% yield) as a white crystalline solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43−1.65 (m, 4H, 2-CH_2-3-CH_2-), 2.52 (t, J = 7.4 Hz, 2H, 4-CH_2-Ar), 3.19 (q, J = 6.4 Hz, 2H, 1-CH_2-N), 4.78 (br s, 1H, NHCbz), 5.09 (s, 2H, Cbz), 5.77 (s, 1H, 4-HO-Ar), 6.74 (d, J = 8.5 Hz, 2H, Ar-), 6.98 (d, J = 8.5 Hz, 2H, Ar-), 7.34 (s, 5, Cbz). MS (ESI) m/z 300 (M + H)$^+$.

Compound **60a** (0.3 g, 1.00 mmol) was dissolved in anhydrous DMF (5 mL). To the solution, granular NaOH (48 mg, 1.20 mmol) was added. The mixture was stirred at room temperature for 1.5 h followed by the addition of allyl bromide in one portion. The newly formed mixture was further stirred at room temperature for an additional 4 h and quenched by water (5 mL). The mixture was partitioned between water (50 mL) and dichloromethane (50 mL). The organic layer was separated, washed with brine, dried (anhydrous sodium sulfate), and then concentrated under vacuum. The residue was subject to chromatographic purification, eluting with a mixture of ethyl acetate (0−17%, gradient) and hexanes (100−87%) to afford desired product **61** (0.38 g, 94% yield) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45−1.63 (m, 4H, 2-CH_2-3-CH_3-), 2.56 (t, J = 6.9 Hz, 2H, 4-CH_2-Ar), 3.20 (q, J = 6.9 Hz, 2H, 1-CH_2-N), 4.50 (d, J = 7.3 Hz, 2H, O-CH_2-ally), 4.78 (br s, 1H, NHCbz), 5.09 (s, 2H, Cbz), 5.28 (dd, J = 1.2 Hz, 10.5 Hz, 1H, H_2C═C), 5.43 (dd, J = 1.2 Hz, 10.5 Hz, H_2C═C), 6.08 (m, 1H, C═CH), 6.82 (d, J = 8.5 Hz, 2H, Ar-), 7.08 (d, J = 8.5 Hz, 2H, Ar-), 7.34 (m, 5H, Cbz); MS (ESI) m/z 340 (M + H)$^+$.

Compound **61** (0.38 g, 1.12 mmol) was dissolved in anhydrous THF (5 mL). The solution was cooled to 0 °C in an ice bath, to which the BH_3−THF complex (1.23 mL, 1.0 M solution) was added dropwise over 2 min. The mixture was stirred at 0 °C for 1 h and then at room temperature for 4 h. Water (2 mL) was added to the reaction mixture, followed by aqueous 3 N NaOH solution (2 mL), and finally H_2O_2 (30% aqueous solution, 2 mL). The reaction mixture was further stirred at room temperature overnight and then partitioned between water (20 mL) and dichloromethane (20 mL). The organic layer was separated, washed with brine, dried (anhydrous sodium sulfate), and concentrated under vacuum. The residue was subjected to chromatographic purification, eluting with a mixture of ethyl acetate (25−50%, gradient) and hexanes (75−50%), affording desired product **62** (0.18 g, 45% yield) as an off-white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48−1.70 (m, 4H, 2-CH_2-3-CH_2-), 2.06 (m, 2H, 2′-CH_2-), 2.56 (t, J = 7.4 Hz, 2H, 4-CH_2-Ar), 3.22 (q, J = 6.4 Hz, 2H, 1-CH_2-N), 4.12 (m, 2H, 3′-CH_2-OH), 4.39 (t, J = 5.9 Hz, 2H, Ar-O-1′-CH_2), 4.74 (br s, 1H, NHCbz), 5.11 (s, 2H, Cbz), 6.84 (d, J = 8.5 Hz, 2H, Ar-), 7.06 (d, J = 8.5 Hz, 2H, Ar-), 7.34 (m, 5H, Cbz); MS (ESI) m/z 358 (M + H)$^+$. A 2-hydroxy isomer **63** was also isolated in 14% yield (55 mg, off-white solid): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27 (d, J = 6.4 Hz, 3H, 3′-CH_3), 1.50−1.65 (m, 4H, 2-CH_2-3-CH_2-), 2.38 (d, J = 5.4 Hz, 1H, 2′-HO), 2.52 (t, J = 7.4 Hz, 2H, 4-CH_2-Ar), 3.20 (q, J = 6.4 Hz, 2H, 1-CH_2-N), 3.76 (dd, J = 2.6 Hz, 8.6 Hz, 1H, O-1′-CH_2), 3.94 (dd, J = 2.9 Hz, 9.2 Hz, 1H, 1′-O-CH_2), 4.20 (m, 1H, 2′-CH-O-), 4.72 (br s, 1H, NHCbz), 5.10 (s, 2H, Cbz), 6.84 (d, J = 8.5 Hz, 2H, Ar-), 7.08 (d, J = 8.5 Hz, 2H, Ar-), 7.34 (m, 5H, Cbz); MS (ESI) m/z 358 (M + H)$^+$.

Compound **62** (180 mg, 0.504 mmol) was subject to hydrogenolysis following method D to afford desired product **64** (79 mg, 70% yield) as an off-white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.35 (m, 2H, 2-CH_2), 1.52 (m, 2H, 3-CH_2-), 1.82 (m, 2H, 2′-CH_2-), 2.46−2.56 (m, 4H, 1-CH_2-Ar + 1-CH_2-N), 2.92 (br s, 1H, 3′-OH), 3.56 (m, 2H, 3′-CH_2-OH), 3.98 (t, J = 6.4 Hz, 2H, Ar-O-1′-CH_2-), 4.56 (m, 2H, NH_2), 6.82 (d, J = 8.5 Hz, 2H, Ar-), 7.08 (d, J = 8.5 Hz, 2H, Ar-); MS (ESI) m/z 324 (M + H)$^+$.

Compound **31** was prepared as a yellow solid in 50% yield from **64** using methods B and C: mp 211−213 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.55 (m, 2H, 2-CH_2-3-CH_2-), 1.84 (m, 2H, 2′-CH_2-), 2.52 (m, 2H, 4-CH_2-Ar), 3.30 (m, 2H, 1-CH_2-N), 3.56 (m, 2H, 3′-CH_2-OH), 3.98 (t, J = 6.4 Hz, 2H, Ar-O-1′-CH_2-), 4.58 (t, J = 5.1 Hz, 1H, OH), 6.82 (d, J = 8.5 Hz, 2H, Ar-), 7.12 (d, J = 8.5 Hz, 2H, Ar-), 7.45 (br s, 2H, NH_2), 7.95 (br s, 1H, guanidino), 8.20−8.45 (br s, 2H, NH_2), 9.22 (br, 1H, guanidino), 10.50 (br s, 1H, guanidino); MS (ESI) m/z 436 (M + H)$^+$. HRMS (FAB) m/z 436.1852; calcd, $C_{19}H_{24}ClN_7O_3$: 436.1864 (M + H)$^+$. Anal. ($C_{19}H_{24}ClN_7O_3$·HCl) H. Calcd, C 48.31, N 20.76; found, C 47.41, N 19.89.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbony)-*N*′-{4-[4-(2,3-dihydroxypropoxy)-phenyl]butyl}guanidine Methanesulfonate (32).** A mixture of 4-(4-hydroxyphenyl)butylcarbamic acid benzyl ester (**60**) (30 g, 0.10 mol), glycidol (8.0 mL, 0.12 mol), ethanol (30 mL), and triethylamine (0.7 mL, 0.005 mol) was stirred at reflux for 2 h under the protection of argon. The product mixture was

evaporated, taken up in hot ethyl acetate, and suction filtered through a plug of silica gel, eluting with ethyl acetate. After evaporating to a white solid, the solid was re-crystallized from toluene to afford 21.8 g (58%) of compound **65a**: ^{1}H NMR (300 MHz, CD_3OD) δ 1.42−1.65 (m, 4H, 2-CH_2-3-CH_2-), 2.54 (t, J = 7.5 Hz, 2H, 4-CH_2-Ar), 3.11 (t, J = 6.4 Hz, 2H, 1-CH_2-N), 3.58−3.71 (m, 2H, 3′-CH_2-), 3.88−4.04 (m, 3H, O-1′-CH_2-2′-CH-O-), 5.05 (s, 2H, Cbz), 6.84 (d, J = 8.7 Hz, 2H, Ar-), 7.06 (d, J = 8.5 Hz, 2H, Ar-), 7.32 (s, 5H, Cbz).

Compound **66a** was prepared from **65a** using method D. The crude product was used directly in the following step without purification. A sample of the reaction mixture was dried and characterized by ^{1}H NMR (300 MHz, CD_3OD): δ 1.42−1.55 (m, 2H, 2-CH_2-), 1.55−1.68 (m, 2H, 3-CH_2-), 2.56 (t, J = 7.5 Hz, 2H, 4-CH_2-Ar), 2.65 (t, J = 7.2 Hz, 2H, 1-CH_2-N), 3.58−3.72 (m, 2H, 3′-CH_2-O), 3.89−4.05 (m, 3H, O-1′-CH_2-2′-CH-O), 6.85 (d, J = 8.7 Hz, 2H, Ar-), 7.08 (d, J = 8.7 Hz, 2H, Ar-). Following general method B, the free base of compound **32** was prepared from **66a** in 71% yield.

Preparation of Methanesulfonic Acid Salt. The free base of **32** (10.1 g, 0.022 mol) was suspended in absolute ethanol (200 mL). To the suspension, methanesulfonic acid (1.45 mL, 0.022 mol) was added dropwise to a point where the suspension turned to a practically clear light brown solution. Stirring was continued for an additional 20 min before the undissolved solid was filtered under vacuum. The filter cake was washed with ethanol (2 × 5 mL), and the combined washings and filtrate were slowly added into methyl *tert*-butyl ether (MTBE) (200 mL), which was cooled in a wet ice−methanol bath (at −10 °C). After the addition was complete, the precipitate was stirred for an additional 1 h at the above temperature. The precipitate was filtered under vacuum, washed with MTBE (3 × 50 mL), and dried under vacuum at room temperature overnight to afford desired product **32** (10.3 g, 84% yield) as a yellow solid: mp 92−95 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52−1.64 (m, 4H, 2-CH_2-3-CH_2-), 2.36 (s, 3H, CH_3SO_3H), 2.56 (m, 2H, 4-CH_2-Ar), 3.31 (m, 2H, 1-CH_2-N), 3.42 (m, 2H, 3′-CH_2-O), 3.74−3.82 (m, 2H, O-1′-CH_2-), 3.92−3.98 (m, 1H, 2′-CH_2-O), 4.70 (br s, 1H, OH), 4.92 (br s, 1H, OH), 6.84 (d, J = 8.0 Hz, 2H, Ar-), 7.10 (d, J = 8.0 Hz, 2H, Ar-), 7.45 (br s, 2H, NH_2), 7.90 (br s, 1H, guanidino), 8.88 (br s, 2H, NH_2), 9.16 (br, 1H, guanidino), 10.44 (s, 1H, guanidino); MS (APCI) m/z 452 (M + H)$^+$. Anal. ($C_{19}H_{26}ClN_7O_4 \cdot CH_3SO_3H \cdot H_2O$) C, H, N.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbony)-*N*′-{4-[4-(2(*R*)-2,3-dihydroxy-propoxy)phenyl]butyl}guanidine Methanesulfonate (33).** Compound **65b** was prepared from **60a** in 83% yield as a bright white solid from (*R*)-glycidol in a manner similar to that used to prepare compound **65a**: $[\alpha]_{25}^{D}$: −5.7° (c 1.0, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.40 (m, 2H, 2-CH_2-), 1.54 (m, 2H, 3-CH_2-), 2.52 (m, 2H, 4-CH_2-Ar), 3.00 (m, 2H, 1-CH_2-N), 3.44 (m, 2H, 3′-CH_2-OH), 3.78 (t, J = 6.7 Hz, 2H, 1′-CH_2-O-Ar), 3.94 (m, 1H, 2′-CH-), 4.68 (br s, 2H, 2×OH), 4.93 (s, 1H, NHCbz), 5.00 (s, 2H, CBz), 6.83 (d, J = 7.8 Hz, 2H, Ar-), 7.08 (d, J = 7.8 Hz, 2H, Ar-), 7.35 (m, 5H, Cbz).

Compound **66b** was prepared from compound **65b** in quantitative yield as a white solid in a manner similar to that used to prepare compound **66a**: $[\alpha]_{25}^{D}$: −5.1° (c 0.98, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.32 (m, 2H, 2-CH_2-), 1.54 (m, 2H, 3-CH_2-), 2.55 (m, 2H, 4-CH_2-Ar), 2.87 (m, 2H, 1-CH_2-N), 3.05 (m, 1H, 3′-OH), 3.45 (t, J = 6.8 Hz, 2H, 3′-CH_2-OH), 3.82 (m, 3H, 1′-CH_2-O− + 2′-OH), 3.94 (m, 2H, 1′-CH_2-O-Ar), 6.83 (d, J = 7.8 Hz, 2H, Ar-), 7.08 (d, J = 7.8 Hz, 2H, Ar-).

Following general method B, compound **33** was prepared from **66b** in 58% yield as a yellow solid. Its methansulfonic acid salt was also prepared in 78% yield in a manner similar to that used to prepare compound **32**: mp 169−172 °C (dec); $[\alpha]_{25}^{D}$: −3.73° (c 0.43, MeOH); ee: 100% (chiral HPLC: retention time: 22.21 min); ^{1}H NMR (300 MHz, CD_6OD) δ 1.60−1.76 (m, 4H, 2-CH_2-3-CH_2-), 2.62 (m, 2H, 4-CH_2-Ar), 2.70 (s, 3H, CH_3SO_3H), 3.35 (m, 2H, 1-CH_2-N), 3.66 (m, 2H, 3′-CH_2-OH), 3.92 (m, 2H, 1′-O-CH_2-), 4.03 (m, 1H, 2′-CH-), 6.84 (d, J = 8.8 Hz, 2H, Ar-), 7.10 (d, J = 8.0 Hz, 2H, Ar-); MS (APCI) m/z 452 (M + H)$^+$. Anal. ($C_{19}H_{26}ClN_7O_4 \cdot CH_3SO_3H \bullet H_2O$) C, N. Calcd, H 5.70; found H 5.13.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbony)-*N*′-{4-[4-(2(*S*)-2,3-dihydroxypropoxy)-phenyl]butyl}guanidine Methanesulfonate (34).** Compound **65c** was prepared from (*S*)-glycidol in 55% yield as a bright white solid in a manner similar to that used to prepare compound **65a**: $[\alpha]_{25}^{D}$: +6.4° (c 1.0, MeOH); ^{1}H NMR (300 MHz, CD_3OD) δ 1.44−1.62 (m, 4H, 2-CH_2-3-CH_2-), 2.55 (t, J = 8.9 Hz, 2H, 4-CH_2-Ar), 3.12 (t, J = 8.7 Hz, 2H, 1-CH_2-N), 3.65 (m, 2H, 3′-CH_2-OH), 3.93−4.07 (m, 3H, 2′-CH- + 1′-CH_2-O-Ar), 4.73 (br s, 1H, NHCbz), 5.08 (s, 2H, CBz), 6.83 (d, J = 7.8 Hz, 2H, Ar-), 7.08 (d, J = 7.8 Hz, 2H, Ar-), 7.42 (m, 5H, Cbz).

Compound **66c** was prepared as a white solid from **65c** in quantitative yield in a fashion similar to the synthesis of compound **66a**: $[\alpha]_{25}^{D}$: +12.0° (c 0.26, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.32 (m, 2H, 2-CH_2-), 1.54 (m, 2H, 3-CH_2-), 2.55 (m, 2H, 4-CH_2-Ar), 3.45 (m, 2H, 1-CH_2-N), 3.75 (m, 2H, 3′-CH_2-OH), 3.94 (m, 3H, 1′-O-CH_2- + 2′-CH-), 6.83 (d, J = 8.0 Hz, 2H, Ar-), 7.08 (d, J = 8.0 Hz, 2H, Ar-).

Following general method B, compound **34** was prepared from **66c** in 76% yield as a yellow solid. Its methanesulfonic acid salt was also prepared in 46% yield in the same manner as that used to prepare compound **32**: mp 160−162 °C (dec); $[\alpha]_{25}^{D}$: +12.0° (c 1.0, MeOH); ee: 100% (chiral HPLC: retention time: 26.60 min); ^{1}H NMR (300 MHz, CD_6OD) δ 1.64−1.76 (m, 4H, 2-CH_2-3-CH_2-), 2.62 (m, 2H, 4-CH_2-Ar), 2.70 (s, 3H, CH_3SO_3H), 3.33 (m, 2H, 1-CH_2-N), 3.68 (m, 2H, 3′-CH_2-OH), 3.94 (m, 2H, 1′-O-CH_2-), 4.02 (m, 1H, 2′-CH-), 6.86 (d, J = 8.8 Hz, 2H, Ar-), 7.14 (d, J = 8.0 Hz, 2H, Ar-); MS (APCI) m/z 452 (M + H)$^+$. Anal. ($C_{19}H_{26}ClN_7O_4 \cdot CH_3SO_3H \cdot H_2O$) C, N. Calcd, H 5.7; found H 5.27.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbony)-*N*′-{4-[3-(2,3-dihydroxypropoxy)-phenyl]butyl}guanidine Hydrochloride (35).** Compound **65d** was prepared from **60b** in 58% yield in a manner similar to that used to prepare compound **65a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48−1.70 (m, 4H, 2-CH_2-3-CH_2-), 2.32 (t, J = 3.4 Hz, 1H, 3′-OH), 2.60 (t, J = 7.5 Hz, 2H, 4-CH_2-Ar), 2.86 (d, J = 4.1 Hz, 1H, 2′-OH), 3.20 (t, J = 6.4 Hz, 2H, 1-CH_2-N), 3.70−3.85 (m, 2H, 3′-CH_2-OH), 4.02 (m, 3H, 1′-O-CH_2- + 2′-CH-), 4.30 (br s, 1H, NHCbz), 5.10 (s, 2H, Cbz), 6.76 (m, 3H, Ar-), 7.20 (m, 1H, Ar-), 7.35 (m, 5H, Cbz).

Compound **66d** was prepared from **65d** in a manner similar to that used to prepare compound **66a**. The crude product was used directly in the next step without purification. A sample of the reaction mixture was dried and characterized by ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.38 (m, 2H, 2-CH_2-), 1.58 (m, 2H, 3-CH_2-), 2.60 (t, J = 7.5 Hz, 2H, 4-CH_2-Ar), 2.72 (t, J = 7.2 Hz, 2H, 1-CH_2-N), 3.72−3.88 (m, 2H, 3′-CH_2-OH), 4.08 (m, 3H, 1′-O-CH_2- + 2′-CH-), 6.78 (m, 3H, Ar-), 7.20 (t, J = 2.8 Hz, 1H, Ar-).

Following general methods B and C, compound **35** (HCl salt) was prepared from **66d** in 71% yield as a yellow solid: mp 91−93 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52−1.70 (m, 4H, 2-CH_2-3-CH_2-), 2.60 (t, 2H, J = 6.4 Hz, 4-CH_2-Ar), 3.34 (m, 2H, 1-CH_2-N), 3.42 (m, 2H, 3′-OH + 2′-OH), 3.74−3.86 (m, 3H, 3′-CH_2-2′-CH-), 3.98 (m, 2H, 1′-O-CH_2-), 6.76 (m, 3H, Ar-), 7.18 (t, J = 2.8 Hz, 1H, Ar-), 7.46 (br s, 2H, NH_2), 7.90 (br s, 1H, guanidino), 8.92 (br s, 2H, NH_2), 9.30 (br s, 1H, guanidino), 10.55 (br s, 1H, guanidino); MS (APCI) m/z 452 (M + H)$^+$. Anal. ($C_{19}H_{26}ClN_7O_4 \cdot HCl$) H, N. Calcd. C 45.07; found: C 44.85.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbony)-*N*′-{4-[2-(2,3-dihydroxypropoxy)-phenyl]butyl}guanidine Hydrochloride (36).** Compound **65e** was prepared from **60c** in 84% yield in a manner similar to that used to prepare compound **65a**, except the protecting group in compound **65e** was Boc: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 9H, Boc), 1.49−1.66 (m, 4H, 2-CH_2-3-CH_2-), 2.34 (t, J = 3.4 Hz, 1H, 3′-OH), 2.62 (t, J = 7.5 Hz, 2H, 4-CH_2-Ar), 3.05 (d, J = 4.1 Hz, 1H, 2′-OH), 3.13 (m, 2H, 1-CH_2-N), 3.85 (m, 2H, 3′-CH_2-OH), 4.10 (m, 2H, 1′-O-CH_2-), 4.18 (m, 1H, 2′-CH-), 4.61 (br s, 1H, NHBoc), 6.89 (m, 2H, Ar-), 7.15 (m, 2H, Ar-).

The Boc protecting group in **65e** was cleaved by TFA using the same procedure used for the preparation of compound **55a**. Crude product **66e** was used directly in the next step without purifica-

tion: ^{1}H NMR (300 MHz, CD_3OD) δ 1.67 (m, 4H, 2-CH_2-3-CH_2-), 2.70 (t, J = 7.5 Hz, 2H, 4-CH_2-Ar), 2.90 (t, J = 7.2 Hz, 2H, 1-CH_2-N), 3.78 (m, 2H, 3′-CH_2-OH), 4.08 (m, 3H, 1′-O-CH_2- + 2′-CH-), 6.88 (m, 2H, Ar-), 7.20 (m, 2H, Ar-); MS (APCI) *m*/*z* 240 $(M + H)^+$.

Following general methods B and C, compound **36** was prepared from **66e** in 38% yield as a yellow solid: mp 85−86 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.60 (m, 4H, 2-CH_2-3-CH_2-), 2.59 (m, 2H, 4-CH_2-Ar), 3.34 (m, 2H, 1-CH_2-N), 3.47 (m, 2H, 3′-OH + 2′-OH), 3.86−4.06 (m, 5H, 3′-CH_2-2′-CH- + 1′-O-CH_2-), 6.88 (m, 2H, Ar-), 7.12 (m, 2H, Ar-), 7.46−7.80 (br s, 3H, guanidino + NH_2), 8.80 (br s, 2H, NH_2), 9.25 (br s, 1H, guanidino), 10.51 (br s, 1H, guanidino); MS (APCI) *m*/*z* 452 $(M + H)^+$. HRMS (FAB) *m*/*z* 452.1816; calcd. $C_{19}H_{26}ClN_7O_4$: 452.1813 $(M + H)^+$. Anal. ($C_{19}H_{26}ClN_7O_4$·HCl). Calcd, C 46.73, H 5.57, N 20.08; found, C 45.34, H 6.02, N 19.61.

(4-{4-[*N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)guanidino]-butyl}phenoxy)acetic Acid Hydrochloride (37). Using a method similar to that used for the preparation of compound **70a**, compound **72a** was prepared in 99% yield by the alkylation of **60** (1.00 g, 4.30 mmol) with *tert*-butyl 2-bromoacetate: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H, *t*-Bu), 1.52−1.68 (m, 4H, 2′-CH_2-3′-CH_2), 2.55 (t, J = 6.38 Hz, 2H, 1′-CH_2), 3.20 (m, 2H, 4′-CH_2-N), 4.44 (s, 2H, Ar-O-CH_2-), 4.66 (br s, 1H, NH-CBz), 5.08 (s, 2H, N-CBZ), 6.78 (d, J = 7.80 Hz, 2H, 2-ArH, 6-ArH), 7.04 (d, J = 7.78 Hz, 2H, 3-ArH, 5-ArH).

Compound **73a** was prepared in 93% yield from **72a** using method D: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (s, 9H, *t*-Bu), 1.52−1.66 (m, 4H, 2′-CH_2-3′-CH_2), 2.54 (t, J = 6.38 Hz, 2H, 1′-CH_2), 2.74 (t, J = 6.46 Hz, 2H, 4′-CH_2-N), 4.49 (s, 2H, Ar-O-CH_2), 6.82 (d, J = 7.80 Hz, 2H, 2-ArH, 6-ArH), 7.10 (d, J = 7.78 Hz, 2H, 3-ArH, 5-ArH).

Using method B, compound **74**, a yellow solid, was prepared from compound **73a** in 16% yield: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.42 (s, 9H, *t*-Bu), 1.50−1.66 (m, 4H, 2-CH_2-3-CH_2), 2.56 (t, J = 6.38 Hz, 2H, 4-CH_2), 3.34 (m, 2H, 1-CH_2-N), 4.62 (s, 2H, Ar-O-CH_2), 6.82 (d, J = 7.80 Hz, 2H, 2′-ArH, 6′-ArH), 7.14 (d, J = 7.78 Hz, 2H, 3′-ArH, 5′-ArH). 7.40−7.58 (br s, 2H, NH_2), 7.96 (br s, 1H, guanidino), 8.88−9.06 (br s, 2H, NH_2), 9.36 (s, 1H, guanidino), 10.58 (s, 1H, guanidino); MS (APCI) *m*/*z* 492 $(M + H)^+$.

A mixture of compound **74** (160 mg, 0.30 mmol) in dichloromethane (10 mL) and trifluoroacetic acid (5 mL) was stirred at room temperature for 1 h. The mixture was concentrated under vacuum to almost dryness. The residue was subjected to column chromatography, eluting with a mixture of methanol and dichloromethane (gradient 0−30% methanol, v/v). The product fraction was collected and concentrated under vacuum. The resulting residue was treated with 3% aqueous hydrochloric acid (5 mL) for 15 min with stirring, and concentrated again. This procedure was repeated two more times. The residue was then dried under high vacuum to afford desired product **37** (98 mg, 69%) as a yellow solid: mp 225 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.48−1.66 (m, 4H, 2-CH_2-3-CH_2), 2.55 (t, J = 6.38 Hz, 2H, 4-CH_2), 3.32 (m, 2H, 1-CH_2-N), 4.64 (s, 2H, Ar-O-CH_2), 6.84 (d, J = 7.80 Hz, 2H, 2′-ArH, 6′-ArH), 7.14 (d, J = 7.78 Hz, 2H, 3′-ArH, 5′-ArH). 7.38−7.58 (br s, 2H, NH_2), 7.94 (br s, 1H, guanidino), 8.82−9.04 (br s, 2H, NH_2), 9.30 (s, 1H, guanidino), 10.54 (s, 1H, guanidino); MS (APCI) *m*/*z* 434 $(M + H)^+$. Anal. ($C_{14}H_{22}ClN_7O_4$·HCl) H. Calcd, C 45.77, N 20.76; found, C 41.04, N 18.41.

(4-{4-[*N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)guanidino]-butyl}phenoxy)propionic Acid Hydrochloride (38). Compound **72b** was prepared in 3% yield by the alkylation of **60** (4.17 g, 13.91 mmol) with 3-bromopropionic acid in a method similar to that used to prepare compound **72a**: ^{1}H NMR (300 MHz, CD_3OD) δ 1.47−1.66 (m, 4H, 2′-CH_2-3′-CH_2), 2.56 (t, J = 6.38 Hz, 2H, 1′-CH_2), 2.71 (t, J = 6.21 Hz, 2H, HO_2C-2″-CH-), 3.15 (m, 2H, 4′-CH_2-N), 4.18 (t, J = 6.18 Hz, 2H, Ar-O-1″-CH_2-), 5.05 (s, 2H, N−CBZ), 6.79 (d, J = 7.80 Hz, 2H, 2-ArH, 6-ArH), 7.04 (d, J = 7.78 Hz, 2H, 3-ArH, 5-ArH), 7.34 (m, 5H, Cbz).

Compound **73b** was prepared in 96% yield from **72b** using method D: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.60−1.78 (m, 4H, 2′-CH_2-3′-CH_2), 2.60 (m, 4H, Ar-1′-CH_2, 4′-CH_2-N), 2.88 (t, J = 6.21 Hz, 2H, HO_2C-2″-CH-), 4.20 (t, J = 6.18 Hz, 2H, Ar-O-1″-CH_2-), 6.82 (d, J = 7.80 Hz, 2H, 2-ArH, 6-ArH), 7.10 (d, J = 7.78 Hz, 2H, 3-ArH, 5-ArH).

Using method B, compound **38**, a yellow solid, was prepared from compound **73b** in 52% yield: mp 198−200 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.55−1.72 (m, 4H, 2-CH_2-3-CH_2), 2.52 (m, 2H, Ar-4-CH_2), 2.88 (t, J = 6.21 Hz, 2H, HO_2C-2″-CH-), 3.33 (m, 2H, 1-CH_2-N), 4.18 (t, J = 6.18 Hz, 2H, Ar-O-1″-CH_2-), 6.84 (d, J = 7.80 Hz, 2H, 2′-Ar−H, 6′-ArH), 7.10 (d, J = 7.78 Hz, 2H, 3′-ArH, 5′-ArH), 7.40−7.68 (br s, 3H, CO_2H, NH_2), 7.96 (br s, 1H, guanidino), 8.88−9.06 (br s, 2H, NH_2), 9.36 (s, 1H, guanidino), 10.58 (s, 1H, guanidino); MS (APCI) *m*/*z* 450 $(M + H)^+$. Anal. HRMS (FAB) *m*/*z* 450.1651; calcd, $C_{19}H_{24}ClN_7O_4$·HCl: 450.1656 $(M + H)^+$. Anal. ($C_{19}H_{24}ClN_7O_4$·HCl) N. Calcd, C 46.92, H 5.18; found, C 44.90, H 5.63.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-{4-[3-(2-hydroxyethoxy)phenyl]-butyl}guanidine Hydrochloride (39).** Compound **68a** was prepared via a Sonogashira coupling reaction between compound **67a** and **51**, a reaction similar to that used for the preparation of compound **52e**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.50 (s, 9H, *t*-Bu), 2.58 (t, J = 6.33 Hz, 2H, 3′-CH_2), 3.36 (m, 2H, 4′-CH_2-N), 4.96 (br s, 1H, NHBoc), 6.38 (br s, 1H, 3-OH), 6.82 (dd, J = 8.55 Hz, 3.12 Hz, 1H, 5-ArH), 6.90 (s, 1H, 2-ArH), 6.94 (d, J = 7.56 Hz, 1H, 6-ArH), 7.16 (d, J = 7.8 Hz, 1H, 4-ArH).

The above compound **68a** underwent the hydrogenation reaction in a manner similar to that used for compound **53e** to give compound **69a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (s, 9H, *t*-Bu), 1.50−1.68 (m, 4H, 2′-CH_2-3′-CH_2), 2.56 (t, J = 1.83 Hz, 2H, 1′-CH_2), 3.11 (m, 2H, 4′-CH_2-N), 4.56 (br s, 1H, NHBoc), 6.18 (br s, 1H, 3-OH), 6.66−6.72 (m, 3H, 2-ArH, 4-ArH, 6-ArH), 7.14 (dd, J = 8.33 Hz, 2.82 Hz, 1H, 5-ArH).

A suspension of compound **69a** (3.34 g, 12.60 mmol) and NaH (60% in mineral oil, 0.76 g, 19.00 mmol) in anhydrous THF was cooled by an ice bath and stirred at ~0 °C for 30 min. Tetrabutylammonium iodide (TBAI, 0.46 g, 1.30 mmol) and THP-protected 2-bromoethyl alcohol (3.16 g, 15.00 mmol) were sequentially added to the suspension. The mixture was slowly warmed to room temperature, while stirring continuously overnight and then heated to 50 °C for an additional 3 h. It was then quenched by the slow addition of water (20 mL). The mixture was concentrated under vacuum, and the residue was taken up in dichloromethane (50 mL). The aqueous layer was separated and washed with dichloromethane (3 × 20 mL). All organic layers were combined, dried (anhydrous sodium sulfate), and concentrated under vacuum. The residue was purified by column chromatography, eluting with 10−20% ethyl acetate in hexane to afford compound **70a** (3.83 g, 77%) as a light green solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (s, 9H, *t*-Bu), 1.50−1.70 (m, 10H, 2′-CH_2-3′-CH_2, THP: 2-CH_2-3-CH_2-4-CH_2), 2.60 (t, J = 6.78 Hz, 2H, 1′-CH_2), 3.12 (m, 2H, 4′-CH_2-N), 3.84 (m, 2H, 2″-CH_2-O-THP), 4.18 (m, 4H, 3-Ar-O-1″-CH_2, THP: 5-CH_2), 4.50 (br s, 1H, NHBoc), 4.78 (t, J = 2.65 Hz, 1H, THP: 1-CH), 6.78−6.86 (m, 3H, 2-ArH, 4-ArH, 6-ArH), 7.20 (dd, J = 8.36 Hz, 2.84 Hz, 1H, 5-ArH); MS (ESI) *m*/*z* 394 $(M + H)^+$.

Both Boc and THP protecting groups in **70a** were cleaved by TFA using a method similar to that used for the preparation of compound **55e**. Crude **71a** was used directly without purification. MS (ESI) *m*/*z* 210 $(M + H)^+$.

Compound **39**, a yellow solid, was prepared in 23% yield from compound **71a** using methods B and C: mp 161−163 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.51−1.68 (m, 4H, 2-CH_3-3-CH_2), 2.60 (t, J = 6.33 Hz, 2H, 4-CH_2), 3.36 (m, 2H, 1-CH_2-N), 3.70 (t, J = 5.68 Hz, 2H, 2″-CH_2-OH), 3.96 (t, J = 8.56 Hz, 2H, Ar-O-1″-CH_2), 6.78 (m, 3H, 2′-ArH, 4′-ArH, 6′-ArH), 7. 20 (m, 1H, 5′-ArH), 7.36−7.50 (br s, 2H, NH_2), 8.02 (br s, 1H, guanidino), 8.78−9.02 (br s, 2H, NH_2), 9.32 (s, 1H, guanidino), 10.58 (s, 1H, guanidino); MS (APCI) *m*/*z* 422 $(M + H)^+$. Anal. ($C_{18}H_{24}ClN_7O_3$·HCl) H. Calcd, C 47.17, N 21.39; found, C 46.18, N 20.20.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-{4-[2-(2-hydroxyethoxy)phenyl]-butyl}guanidine Hydrochloride (40).** Compound **40**, a yellow solid, was prepared using the same method as that used for to prepare compound **39**: mp 115 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52−1.71 (m, 4H, 2-CH_2-3-CH_2), 2.58 (t, J = 6.33 Hz, 2H, 4-CH_2), 3.26 (m, 2H, 1-CH_2-N), 3.76 (m, 2H, 2″-CH_2-OH), 3.99 (t, J = 5.04 Hz, 2H, Ar-O-1″-CH_2), 4.81 (t, J = 5.37 Hz, 1H, OH), 6.83 (d, J = 7.29 Hz, 1H, 3′-ArH), 6.94 (d, J = 8.01 Hz, 1H, 4′-ArH), 7.16 (m, 2H, 5′-ArH, 6′-ArH), 7.36−7.50 (br s, 2H, NH_2), 8.02 (br s, 1H, guanidino), 8.78−9.02 (br s, 2H, NH_2), 9.32 (s, 1H, guanidino), 10.58 (s, 1H, guanidino); MS (APCI) m/z 422 $(M + H)^+$. Anal. HRMS (FAB) m/z 422.1719; calcd. $C_{18}H_{24}ClN_7O_3$: 422.1707 $(M + H)^+$. Anal. ($C_{18}H_{24}ClN_7O_3$·HCl) H. Calcd, C 47.17, N 21.39; found, C 45.70, N 20.12.

6-(4-{4-[*N*′-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-guanidino]butyl}phenoxy)-3,4,5-trihydroxytetrahydropyran-2-sodium Carboxylate (41). 2,3,4-Tri-*O*-acetyl-α-D-glucuronic acid methyl ester trichloroimidate (**75**) (1.60 g, 3.3 mmol) was added under an argon atmosphere to **60** (1.50 g, 6.60 mmol) and dissolved in anhydrous dichloromethane (40 mL). The mixture was cooled at −25 °C for 10 min and BF_3·OEt_2 (0.045 mL, 0.33 mmol) was added. The reaction mixture was stirred at −25 °C for 1.5 h and then allowed to warm to −10 °C. The stirring was continued at −10 °C for an additional 1 h before the temperature was raised to 25 °C and continuously stirred for one more hour. The mixture was then quenched with saturated ammonium chloride (25 mL). The product was extracted with dichloromethane (3 × 30 mL). The combined extracts were washed with water (3 × 50 mL) and dried (anhydrous sodium sulfate). The solvent was evaporated and the residue was purified by flash chromatography (silica gel, 1:2 ethyl acetate/hexanes, v/v) to provide **76** (1.50 g, 72%) as a white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41−1.51 (m, 4H, 2′-CH_2-3′-CH_2), 1.99 (s, 9H, 3 x AcO), 2.54 (m, 2H, 1′-CH_2-Ar), 3.10 (m, 2H, 4′-CH_2-N), 3.63 (s, 3H, MeO_2C-), 4.56 (br s, 1H, NHCbz), 4.69 (m, 1H, 6″-CH−CO_2Me), 4.99 (m, 2H, 2″-CH-OAc, 4″-CH-OAc), 5.06 (s, 2H, Cbz), 5.46 (m, 1H, 3″-CH-OAc), 5.60 (m, 1H, 1″-O-CH-O), 6.88 (d, J = 7.82 Hz, 2H, 2-ArH, 4-ArH), 7.12 (d, J = 7.82 Hz, 2H, 3-ArH, 5-ArH), 7.33 (m, 5H, Cbz); MS (APCI) m/z 616 $(M + H)^+$.

Using method D, compound **77** was prepared from **76** in 84% yield as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41−1.51 (m, 4H, 2′-CH_2-3′-CH_2), 2.02 (s, 9H, 3 × AcO), 2.54−2.68 (m, 4H, 1′-CH_2-Ar, 4′-CH_2-N), 3.67 (s, 3H, MeO_2C-), 4.69 (m, 1H, 6″-CH−CO_2Me), 4.99 (m, 2H, 2″-CH-OAc, 4″-CH-OAc), 5.46 (m, 1H, 3″-CH-OAc), 5.60 (m, 1H, 1″-O-CH-O), 6.93 (d, J = 7.82 Hz, 2H, 2-ArH, 6-ArH), 7.18 (d, J = 7.82 Hz, 2H, 3-ArH, 5-ArH).

Following method B, compound **78**, a yellow solid, was prepared in 48% yield from compound **77**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.61 (m, 4H, 2-CH_2-3-CH_2-), 2.05 (s, 9H, 3 × AcO), 2.55 (m, 2H, 4-CH_2-Ar), 3.49 (m, 2H, 1-CH_2-N), 3.71 (s, 3H, MeO_2C-), 4.22 (m, 1H, 6″-CH−CO_2Me), 5.12 (m, 1H, 2″-CH-OAc), 5.34 (m, 3H, 1″-CH-O, 3″-CH-OAc, 5″-CH-OAc), 6.88 (d, J = 7.80 Hz, 2H, 2′-ArH, 4′-ArH), 7.04 (d, J = 7.80 Hz, 2H, 3′-ArH, 5′-ArH); MS (APCI) m/z 694 $(M + H)^+$.

Compound **78** (0.31 g, 0.44 mmol) was added to a mixed solvent of THF and water (1:1, 40 mL). The suspension was cooled to −10 °C. To the suspension was added dropwise an aqueous NaOH solution (1.24 N, 4 mL). The stirring was continued at −10 °C for 1.5 h. After this time, the reaction mixture was allowed to warm to room temperature and THF was removed under reduced pressure. The pH of the remaining solution was adjusted to 6 by 1 N aqueous HCl solution. The precipitate formed upon neutralization was collected by centrifugation and washed with cold water (3 × 20 mL). Compound **41** (0.18 g, 75%) was isolated as a yellow powder after drying under vacuum for 48 h: mp >184 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) 1.56 (m, 4H, 2-CH_2-3-CH_2-), 2.56 (m, 2H, 4-CH_2-Ar), 3.19 (m, 2H, 1-CH_2-N), 3.15−3.40 (br s, 3H, 3 × OH), 3.25 (m, 2H, 6″-CH-CO_2H, 3″-CH-OH), 3.57 (m, 1H, 4″-CH-OH), 4.87 (m, 1H, 5″-CH-OH), 5.10−5.40 (m, 1H, 1″-CH-O), 6.89 (d, J = 7.80 Hz, 2H, 2′-ArH, 6′-ArH), 7.06 (d, 2H, 3′-ArH, 5′-ArH), 7.38−7.58 (br s, 2H, NH_2), 8.82−9.04 (br s, 2H, NH_2) (some peaks for pyrazine and acylguanidine moiety were not shown); MS (APCI) m/z 554 $(M + H)^+$. HRMS (FAB) m/z 554.1757 (Na lost during analysis); calcd. $C_{22}H_{28}ClN_7O_8$: 554.1765 $(M + H - Na)^+$. Anal. ($C_{22}H_{27}ClN_7NaO_8$) H. Calcd, C 45.88, N 17.02; found, C 39.74, N 13.79.

***N*-(3,5-Diamino-6-chloropyrazine-2-carbonyl)-*N*′-[4-(3,4-dihydroxyphenyl)butyl]-guanidine (42).** Using the same method as that used to prepare compound **13**, compound **42** was prepared in 51% yield as a beige solid: mp >154 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52 (m, 4H, 2-CH_2-3-CH_2-), 2.42 (m, 2H, 4-CH_2-Ar), 3.31 (m, 2H, 1-CH_2-N), 6.43 (d, J = 1.86 Hz, 1H, 2′-ArH), 6.61 (m, 2H, 5′-ArH, 6′-ArH), 7.42 (br s, 2H, NH_2), 7.90 (br s, 1H, guanidino), 8.82 (br s, 2H, NH_2), 9.25 (s, 1H, guanidino) 10.52 (s, 1H, guanidino); MS (APCI) m/z 394 $(M + H)^+$; HRMS (FAB) m/z 394.1410; calcd, $C_{16}H_{20}ClN_7O_3$: 394.1394 $(M + H)^+$. Anal. ($C_{16}H_{20}ClN_7O_3$·HCl). Calcd, C 44.66, H 4.92, N 22.79; found, C 43.53, H 5.39, N 18.52.

Materials. Amiloride, benzamil, and phenamil were purchase from Sigma (St. Lois, MO). Compound **13**, was supplied by Dr. E. J. Cragoe, Jr. All other chemicals were of analytical grade, used without purification, and purchased from VWR International or other commercial vendors.

Biological Evaluation and ENaC Assays. The canine bronchial tissue for primary culture was provided by Marshall Bioresources (North Rose, NY) Veterinarian Committee to ensure the humane care and treatment of experimental animals. Briefly, to isolate and culture primary bronchial epithelial cells, canine bronchi were incubated in an MEM medium containing 0.1% protease (Sigma Type XIV) and 50 μg/mL DNase at 4 °C for a minimum of 24 h. Fetal bovine serum (10%) was added to the medium and the epithelial layer scraped and rinsed to improve cell yield. Cells were then centrifuged for 5 min at 500*g*. Re-suspended cells were seeded at a density of 0.4 × 10^6/cm^2 on 0.4 μm porous collagen coated (human placenta type VI Sigma) Snapwell or Transwell (Corning Costar Corp., Cambridge, MA) membranes (1.13 cm^2) and maintained at an air−liquid interface in a hormonally defined medium supplemented with penicillin and streptomyocin.[10] Bronchial epithelial monolayers grown from 6 to 12 days on permeable membrane supports were mounted in modified Ussing chambers (Physiologic Instruments Inc., San Diego, CA). All experiments were performed in Krebs−Ringer bicarbonate solution (KRB) at pH 7.4 containing 140 mM Na^+, 120 mM Cl^-, 5.2 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 2.4 mM HPO_4^{2+}, 0.4 mM $H_2PO_4^-$, 25 mM HCO_3^-, and 5 mM glucose. The epithelium was bathed on both sides with warmed (37 °C) KRB circulated by gas lift with 95% O_2−5% CO_2, maintaining the pH at 7.4. The transepithelial voltage was clamped to 0 mV, except for 0.2-s pulses (+5 mV) every 20 s to calculate R_t. The I_{sc} and R_t values were digitized and recorded on a computer. Data were acquired and analyzed using Acquire and Analysis (V. 1.2) software (Physiological Instruments). The 50% inhibition of I_{sc} concentration (IC_{50}) was calculated from apical drug additions ranging from 10^{-11} to 10^{-4} M (∼ half log increments) and analyzed using nonlinear regression (Prism V.3, Graphpad software Inc). Stocks of ENaC blockers were dissolved in DMSO at a concentration of 10 mM and stored at −20 °C until use.

The percent recovery of I_{sc} from the apical sodium channel blocker exposure was measured 3 min after the third consecutive mucosal bath replacements (KRB), following a full concentration−effect study.

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Supporting Information Available: Analytical results (Table 2), elemental analysis (Table 3), and high-resolution mass spectrometry for compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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