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Synthesis and Biological Evaluation of Aroylguanidines Related to Amiloride as Inhibitors of the Human Platelet Na^+/H^+ Exchanger

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Abstract—Pyridine and benzene bioisosteres of amiloride were synthesized and evaluated for their inhibitory potency against the sodium–hydrogen exchanger (NHE) involved in intracellular pH regulation. The inhibition of NHE was determined by using the platelet swelling assay (PSA) in which the swelling of human platelets was induced by their incubation in an acid buffer (pH 6.7). Additionally, the inhibitory potency of the most active compounds was assessed by measuring the inhibition of the EIPA-sensitive $^{22}Na^+$ uptake (UIA) by human platelets after intracellular acidosis. The results indicated that several benzene derivatives and compounds bearing an carbonylguanidine moiety in the *meta* position of the pyridine nitrogen were much more potent than amiloride (PSA:$IC_{50}=43.5$ μM; UIA:$IC_{50}=100.1$ μM), but less than EIPA, a pyrazine NHE inhibitor (PSA:$IC_{50}=0.08$ μM; UIA:$IC_{50}=0.5$ μM). In both biological assays (2-amino-5-bromo-pyridine-3-carbonyl)guanidine (**32**) was the most active molecule (PSA: $IC_{50}=0.8$ μM, UIA : $IC_{50}=0.8$ μM). Our investigations demonstrated that the replacement of the pyrazine ring of amiloride by a pyridine or a phenyl ring improved the NHE inhibitory potency (phenyl > pyridine > pyrazine).

Introduction

In many cell types, including platelets, several mechanisms take part in the maintenance of intracellular acid-base homeostasis.[1,2] One of the predominant mechanism by which cells adapt to intracellular acidosis is through the Na^+/H^+ antiport system (NHE). To date, six mammalian isoforms of the Na^+/H^+ exchanger have been identified and characterized (NHE-1–NHE-6).[3–6] The most widely distributed subtype is the amiloride-sensitive Na^+/H^+ antiporter (NHE-1) which is involved in intracellular pH (pH_i) homeostasis, cell volume control, and in cell signaling.[7] Isoform NHE-1, the main Na^+/H^+ exchanger in platelets, mediates the electroneutral exchange of intracellular H^+ for extracellular Na^+ with a 1:1 stoichiometric relationship using the transmembrane Na^+ gradient established by Na^+/K^+ATPase.[8] The structure and the functional implications of NHE-1 and other isoforms have been recently reviewed.[9,10] The activity of the Na^+/H^+ antiporter, especially NHE-1, can be modulated by a number of stimuli such as growth factors, hormones and neurotransmitters, as well as by changes in cell volume and mechanical stimuli.[11] Although the activation of NHE is essential for the restoration of physiological pH, an excessive stimulation of NHE results in an increase of intracellular Na^+ concentration and a subsequent activation of Na^+/K^+ATPase, with a consecutive increase of energy consumption. Since cellular Na^+ and Ca^{2+} transport is coupled via the Na^+/Ca^{2+} exchanger, which depends on the Na^+ gradient, the high intracellular Na^+ levels lead to raised intracellular Ca^{2+}.[8,12,13] This cellular Ca^{2+} overload is assumed to be involved in ischemic and reperfusion injuries, like arrhythmias, myocardial contracture, stunning and tissue necrosis.[14,15] In addition, the abnomalities in the

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activity of the NHE are implicated in the pathogenesis and/or pathophysiological processes such as essential hypertension,[16–18] diabetes mellitus,[19,20] postischemic dysfunction, and cellular death. Moreover, raised NHE activity in kidney and vascular smooth muscle could play a critical role in the etiology of hypertension,[20–22] vascular smooth muscle cell proliferation[23] and renal disorders.[24] Recently, the inhibition of the NHE has been considered as an useful approach to limit Ca^{2+} influx and avoid its deleterious consequences during cardiac ischemia and reperfusion.[25]

Consequently, to attenuate the harmful consequences of an excessive NHE activation, several inhibitors were developed (Fig. 1). Beside its inhibitory potency against the Na^+/Ca^{2+} exchanger and Na^+ channels, amiloride is a NHE inhibitor. Pharmacomodulation of amiloride generated more potent and more specific NHE inhibitors: the 5-*N*-substituted pyrazine derivatives closely related to the structure of amiloride (Fig. 1). Later, benzoylguanidines were developed (e.g., cariporide, HOE 694, EMD-96785 and FR-183998). These drugs are highly potent and markedly selective inhibitors of NHE-1 without acting on other ion carrier or pH regulatory systems.[26,27]

Pharmacological development of NHE inhibitors for cardiac therapy focused on selective NHE-1 inhibitors. These agents, as well the earlier nonspecific amiloride derivatives, have shown excellent cardioprotective properties. Thus, the NHE inhibitors were expected to be efficient tools for preventing high intracellular Na^+ levels and concomitant Ca^{2+} overload.[28–30]

The success of NHE inhibitors in experimental studies has led to clinical trials in high risk patients with coronary artery disease as well in patients with acute myocardial infarction. These NHE inhibitors attenuated both acute and chronic responses to myocardial post-infarction resulting in the evolution of heart failure.[31–34] These data suggest a potentially effective new therapeutic perspective for the treatment of heart failure with NHE-1 inhibitors.

Since the nature of groups R and R′ (Fig. 1) has been extensively studied in the amiloride family, we have examined the influence of bioisosteric replacement of the pyrazine ring by a pyridine or a phenyl, and the effect of other substitutions on the aromatic ring (Fig. 2).

Chemistry

In a previous work,[35] we reported the preparation of derivatives of amiloride where their pyrazine ring was substituted by a phenyl. Here, pyridine compounds (**12**, **14**, **28, 30**–**33**) structurally related to amiloride were prepared (Table 1). The strictly pyridine bioisosteres of amiloride (**12**, **28**) differ by the position of the pyridinic nitrogen. The preparation of **12** started from 2-amino-6-methylpyridine (Scheme 1). The nitration of this product led to 6-methyl-2-nitraminopyridine **1**.[36,37] The one-pot nitration of **1** with an excess of nitric acid (3 equivalents) gave 2-amino-6-methyl-3,5-dinitropyridine **2**.[38] The synthesis of 2-hydroxy-6-methyl-3,5-dinitropyridine **3** by diazotation of **2** was carried out under the conditions used by Berrie et al.[39] This step was required by the presence of a high chloride concentration which could not directly generate 2-chloro-6-methyl-3,5-dinitropyridine **4**.[40] This latter was obtained by chlorination of **3** with a mixture of $PCl_5/OPCl_3$ according to Czuba method.[41] After reduction of both nitro groups,[42] the 3,5-diamino-2-chloro-6-methylpyridine **5** was acetylated (**6**), and then oxidized by $KMnO_4$ to 3,5-diacetylamino-6-chloro-pyridine-2-carboxylic acid **7**.

In a first procedure, this intermediate gave the methyl ester **8** or **9**, which could not lead to the desired compound **12**. In a second approach, **7** was hydrolyzed in alkaline conditions, providing the 3,5-diamino-6-chloropyridine-2-carboxylic acid **10** with high yields (70–75%). The carboxylic derivative **10** was then esterified by means of *N*-*tert*-butyl-5-methylisoxazolium perchlorate (NBI)[43] to give the crotonamide intermediate **11** which finally reacted with guanidine to give **12**. A third

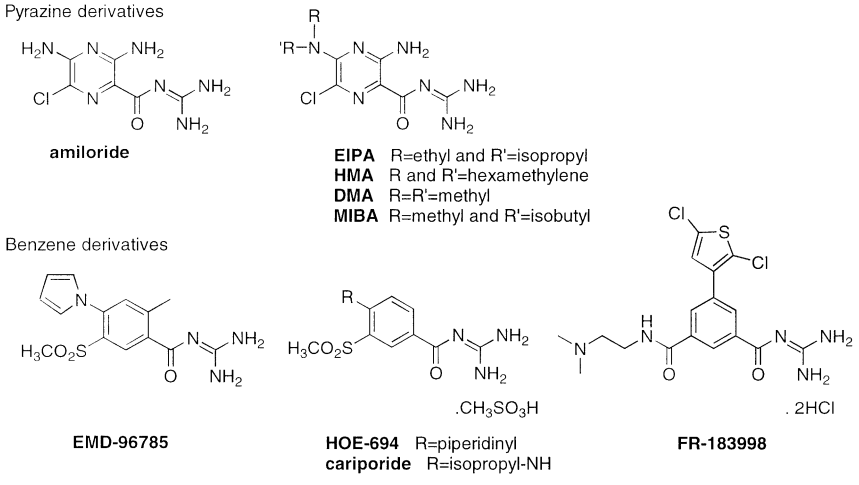


Figure 1. Inhibitors of the Na^+/H^+ exchanger.

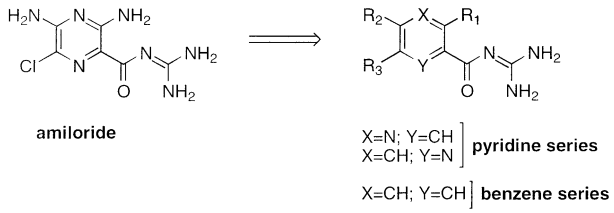
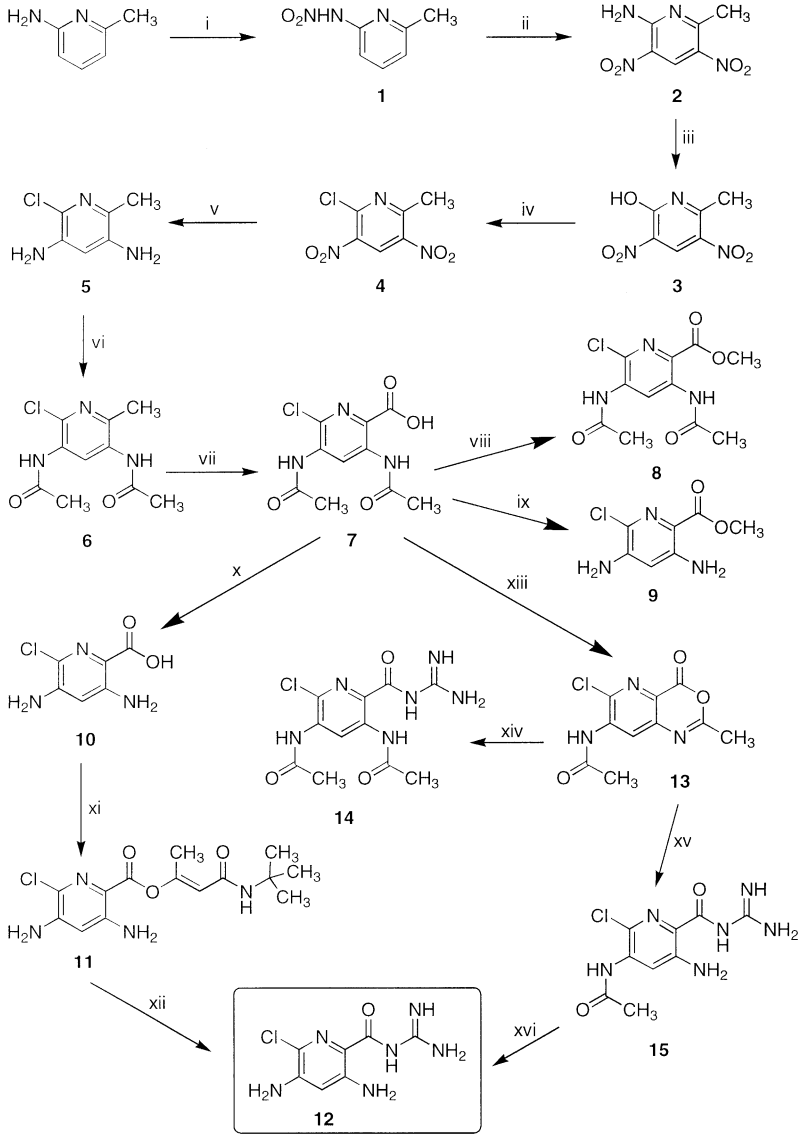


Figure 2. Pyridine and benzene derivatives of amiloride.

pathway involved the cyclization of **7** into *N*-(6-chloro-2-methyl-4-oxo-4H-pyrido[3,2-d]-1,3-oxazin-7-yl)-acetamide **13**, which reacted with guanidine to generate the diacetylated and acetylated carbonylguanidine compounds **14** and **15**, respectively. This latter underwent acidic hydrolysis to give the pyridine bioisostere **12**. However, this hydrolysis occurred with poor yields (25–30%). Alternative pathways[44] such as the synthesis of *N*-hydroxysuccinimide esters or trifluoroacetic protection of amino groups have also been investigated but did not succeed to reach final compound **12**.

The synthesis of the other pyridine bioisostere **28** started from **16** obtained from 2-methylglutaronitrile following Fritz procedure (Scheme 2).[45]

Although, the preparation of intermediates **17** and **18** was previously described,[46] a mixture of both molecules was always generated, and required fractional crystallization. The 2,5,6-trichloropyridine-3-carboxylic acid **21** was produced by three different ways: the oxidation of **17** by $KMnO_4$, the catalytic oxidation of a mixture of bromide derivatives (**19** and **20**), and the oxydative process of **23** which was obtained from 2-methyleneglutaronitrile according to Mutterer procedure.[47] In the last two cases, the yields were of 50 and 80%, respectively. Indeed, the conversion of a polyhalogenated pyridine to its carboxylic acid derivative by $KMnO_4$ is



Scheme 1. Reagents: (i) H_2SO_4/HNO_3, −20 °C; (ii) H_2SO_4; (iii) $NaNO_2/H_2SO_4$; (iv) $PCl_5/OPCl_3$; (v) $SnCl_2/HCl$; (vi) Ac_2O; (vii) $KMnO_4$; (viii) CH_2N_2; (ix) CH_3OH/H_2SO_4; (x) NaOH; (xi) Et_3N/DMF/NBI; (xii) guanidine.HCl/*tert*-ButOK; (xiii) Ac_2O/EtOAc; (xiv) guanidine:HCl/Na; (xv) guanidine.HCl/Na/HCl; (xvi) HCl/H_2O/EtOH.

known to give low yields.[48,49] As observed,[47] an attempt to prepare the acid **27** by reaction of **21** with NH_4OH led to decarboxylation (**22**). The carboxamide **25** was generated from the acyl chloride **24**, and the substitution of both chlorine atoms gave the 2,6-diamino-5-chloro-pyridine-3-carboxamide **26** with satisfactory yields (65%). Alkaline hydrolysis led to the key intermediate **27**. This latter was then converted by reaction with *N*,*N'*-carbonyldiimidazole (CDI) into its corresponding ester, which reacted with guanidine to provide the pyridine bioisostere **28**.

Some compounds, related to **28**, where the amino groups are substituted by chlorine were synthesized (Scheme 3).

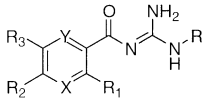
Hence, the 2,6-dichloropyridine-3-carboxylic acid **29** was prepared by *ortho*-lithiation according to the procedure reported by Radinov.[50] The carboxylic acids **21** and **29** were transformed by condensation with CDI into their corresponding esters, which then reacted with guanidine to provide the desired molecules **31** and **30**, respectively. An attempt to obtain **31** via the methyl ester was unsuccessful. Compound **32** was obtained by bromination of (2-amino-pyridine-3-carbonyl)guanidine as previously described by Cragoe.[51]

Finally, some 'simplified' pyridine (**33**) and benzene (**40**, **42**) derivatives were prepared according to known procedures.[52,53]

Results and Discussion

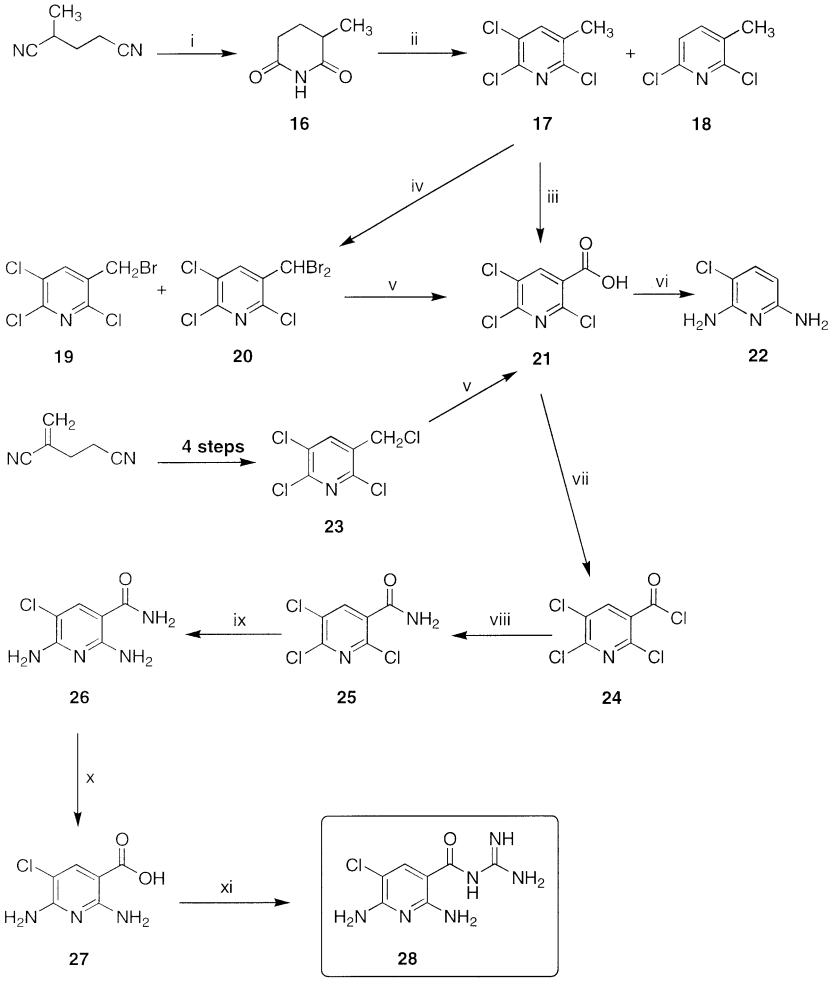
To measure the effect of the synthesized compounds on the NHE activity, the human platelet swelling assay (PSA) was used.[54,55] Addition of platelet-rich plasma (PRP) to an acid propionate buffer led to the activation of the NHE which uptakes extracellular Na^+ and extrudes cytoplasmic H^+. To restore *iso*-osmotic conditions, the cytosolic accumulation of Na^+ is accompanied by a cell swelling due to the influx of water. This platelet swelling induced a decrease of the optical density (OD) of the platelet suspension.[16,18] This OD decrease is related to the value of extracellular pH. Indeed, at pH 6.7, EIPA (10 μM), a specific NHE inhibitor, completely abolished the decrease of OD and prevented the platelet swelling (Fig. 3). Other molecules acting on Na^+ carriers such as ouabain (inhibitor of Na^+/K^+ATPase pump) or furosemide (inhibitor of the symport $Na^+K^+2Cl^-$) were investigated, and failed to modify the OD decrease, and thus the platelet swelling.

Table 1. Chemical structures and inhibitory potency of the platelet Na^+/H^+ exchanger



Compound	X	Y	R_1	R_2	R_3	R	IC_{50} (μM) ± SEM	
							PSA[a]	$^{22}Na^+$ uptake[b]
12	CH	N	NH_2	NH_2	Cl	H	24.0 ± 2.0	
14	CH	N	$NHCOCH_3$	$NHCOCH_3$	Cl	H	9.9 ± 0.9	
28	N	CH	NH_2	NH_2	Cl	H	1.2 ± 0.5	1.3 ± 0.4
32	N	CH	NH_2	H	Br	H	0.85 ± 0.05	0.8 ± 0.1
31	N	CH	Cl	Cl	Cl	H	2.8 ± 0.4	5.3 ± 0.6
30	N	CH	Cl	Cl	H	H	NA	
33	N	CH	H	H	H	H	NA	
34[d]	CH	CH	NH_2	NH_2	Cl	H	0.8 ± 0.08	16.0 ± 3.8
35[d]	CH	CH	NH_2	NH_2	Cl	C_2H_5	NA	
36[d]	CH	CH	NH_2	H	Cl	H	0.5 ± 0.04	1.8 ± 0.4
37[d]	CH	CH	NH_2	H	H	H	NA	
38[d]	CH	CH	NO_2	NO_2	Cl	H	1.1 ± 0.3	10.6 ± 0.1
39[d]	CH	CH	Cl	H	NO_2	H	0.7 ± 0.4	5.6 ± 2.1
40	CH	CH	H	Cl	Cl	H	1.3 ± 0.5	2.7 ± 0.5
41	CH	CH	Cl	H	NH_2	H	NA	
42	CH	CH	H	H	H	H	NA	
43	CH	CH	$NHCOCH_3$	NO_2	Cl	H	0.11 ± 0.1	3.6 ± 2.2
44	CH	CH	$NHCOCH_3$	NO_2	Cl	C_2H_5	24.4 ± 0.2	
45	CH	CH	$NHCOCH_3$	H	H	H	66.1 ± 4.0	
Amiloride (1)	N	N	NH_2	NH_2	Cl	H	43.5 ± 4.5	100.1 ± 32
EIPA	N	N	NH_2	$(C_2H_5)NCH(CH_3)_2$	Cl	H	0.08 ± 0.03	0.5 ± 0.1
DMA	N	N	NH_2	$N(CH_3)_2$	Cl	H	0.8 ± 0.04	
HMA	N	N	NH_2	$N(CH_2)_6$	Cl	H	0.2 ± 0.01	3.3 ± 0.4
Cariporide	CH	CH	H	$(CH_3)_2CH$	SO_2CH_3	H	0.12[c]	
EMD-96785	CH	CH	CH_3	pyrrole	SO_2CH_3	H	0.03[c]	

[a]Drug concentration to achieve half-maximal inhibition of acid-induced swelling of human platelets.
[b]Drug concentration to achieve half-maximal inhibition of the EIPA-sensitive $^{22}Na^+$ uptake by human platelets. NA = no activity at 10 μM.
[c]Data from ref. 56.
[d]Synthesis of compounds **34**–**39**, **41**, **43**–**45** previously described in ref 35.

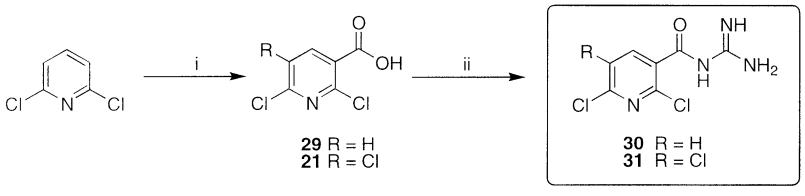


Scheme 2. Reagents: (i) H_2SO_4/CH_3COOH; (ii) PCl_5; (iii) $KMnO_4$; (iv) $NBS/CCl_4/UV$; (v) $H_2SO_4/HNO_3/HgNO_3/CuSO_4.5H_2O$; (vi) NH_4OH; (vii) $SOCl_2/DMF$; (viii) NH_4OH; (ix) $NH_4OH/NH_3(g)$; (x) NaOH; (xi) guanidine.HCl/*tert*-ButOK/CDI.

The inhibitory potency of the novel compounds was evaluated in the PSA and compared to amiloride and to other potent and specific NHE inhibitors such as EIPA, HMA and DMA (Fig. 1). Concentration–response curves (Fig. 4) were drawn to determine the concentration required to prevent of 50% the platelet swelling (IC_{50}) induced by a propionate buffer (pH 6.7) (Table 1).

The first pyridine bioisostere of amiloride bearing the carbonylguanidine side chain in *ortho* of the pyridine nitrogen (**12**, $IC_{50} = 24.0$ μM) and its *N*-diacetyl derivative (**14**, $IC_{50} = 9.9$ μM) were more active than amiloride which was confirmed to be a poor inhibitor of NHE (**1**, $IC_{50} = 43.5$ μM). The second pyridine bioisostere bearing the carbonylguanidine moeity in the *meta* position (**28**) was much more efficient ($IC_{50} = 1.2$ μM). The deletion of the R2 amino group (**32**) or the replacement of both amino moeities by a chlorine atom (**31**) preserved the activity if a halide was present in R3 (compare **30** and **31**). The unsubstituted (pyridyl-3-carbonyl)guanidine **33** was inactive.

The benzene counterpart of amiloride (**34**) was 54 times more potent ($IC_{50} = 0.8$ μM) than its parent. It was as active as the pyridine bioisostere **28** and DMA ($IC_{50} = 0.8$ μM), a pyrazine NHE inhibitor. Compared to **34**, the compound with an ethyl group on the distal nitrogen of the guanidine (**35**) showed a complete loss of inhibitory activity. In the benzene series (**34**–**45**), the removal of the R2 amino group (**36**) also maintained the activity ($IC_{50} = 0.5$ μM) if a chlorine atom was present in R3 (e.g., **36** and **37**). The deletion of both amino moieties and their replacement by two nitro (**38**) or one chlorine (**39**, **40**) preserved the potency ($0.7 < IC_{50} < 1.3$



Scheme 3. Reagents: (i) *n*-BuLi/diisopropylamine/CO_2; (ii) guanidine.HCl/*tert*-butOK/CDI.

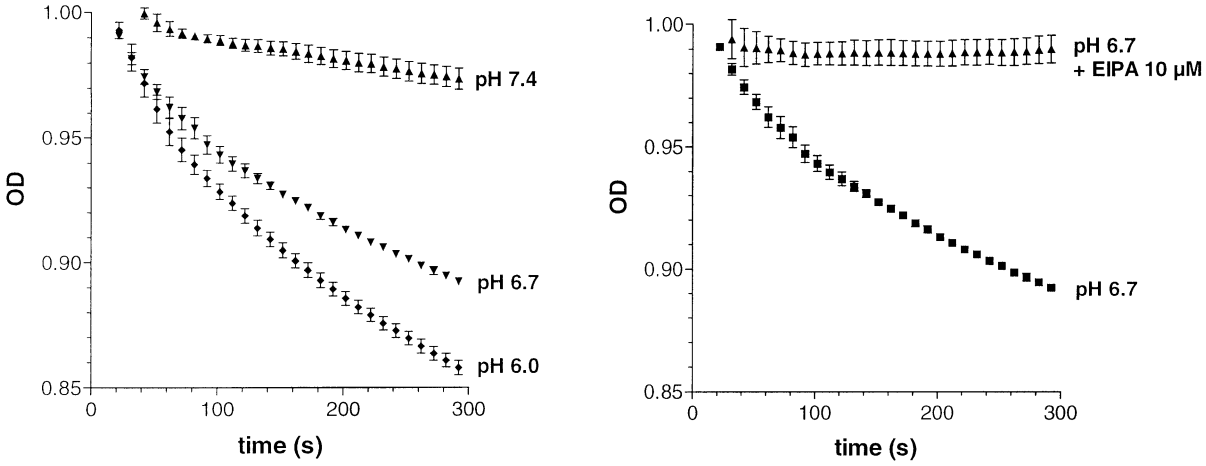


Figure 3. Effect of extracellular pH (left panel) and EIPA (right panel) on the platelet swelling expressed as the decrease of the relative optical density (OD at 680 nm) of a platelet suspension.

µM). Once more, the replacement of the nitro group (**39**) or of the chlorine (**40**) in R3 by an amino (**41**) led to a marked loss of activity. The unsubstituted benzoylguanidine (**42**) was inactive.

With respect to these considerations, and as observed for the pyridine derivatives, a chloro or a nitro group was required in R_3 to inhibit the NHE (c.f., **36**, **37** and **41**). As shown in the pyridine series, an acetylamino in R1 (**43**–**45**) enhanced the potency (c.f., **14** and **43**). The substitution on the distal nitrogen of the guanidine with an ethyl strongly reduced the biological response (c.f., **43** and **44**). These results indicated that the nature of the aromatic ring and the position of the heterocyclic nitrogen strongly influenced the NHE inhibitory potency following the sequence phenyl (**34**) > pyridine (**28** > **12**) > pyrazine (amiloride).

For the reference drugs, and for the most active compounds (IC_{50} < 3 µM), the inhibitory potency of NHE inhibitors was also evaluated by their ability to reduce the EIPA-sensitive $^{22}Na^+$ uptake by human platelets suspended at pH 6.7. After 5 and 10 min of incubation with EIPA (10 µM), the platelet $^{22}Na^+$ uptake was reduced by 31 and 33%, respectively. The EIPA concentration–response curve was drawn and led to calculate the concentration required to reduce of 50% (IC_{50}) the so-called EIPA-sensitive $^{22}Na^+$ uptake (Fig. 5).

The *meta*-carbonylguanidine pyridine derivatives (**28**, **31**, **32**) were much more potent ($0.8 < IC_{50} < 5.3$ µM) than amiloride ($IC_{50} = 100.1$ µM). As observed in the platelet swelling assay, **32** remained the most active molecule in this series. The benzene derivatives (**34**, **36**, **38**, **39**, **40**, **43**), active in the swelling assay, reduced dose-dependently the EIPA-sensitive $^{22}Na^+$ uptake. As observed for EIPA and HMA, their IC_{50} value was 2–30 times higher than in the PSA. In contrast to the results obtained in the PSA, the benzene bioisostere of amiloride (**34**) was less active than the pyridine bioisostere **28**.

Conclusion

In conclusion, this study demonstrated that the replacement of the pyrazine ring of amiloride by a pyridine or a phenyl ring improved the NHE inhibitory potency.

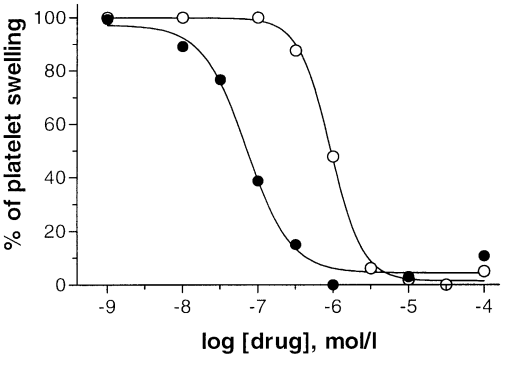


Figure 4. EIPA (●) or **34** (○) reduced the NHE activity expressed as the human platelet swelling induced by an acid buffer (pH 6.7).

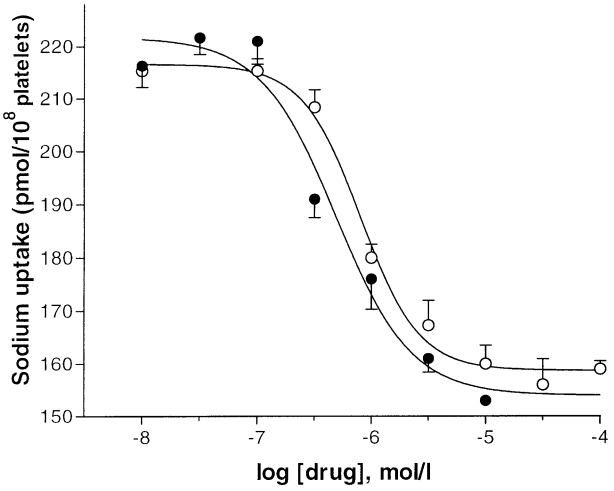


Figure 5. Human platelet sodium uptake inhibited by EIPA (●) or 32 (○). Each point is the mean and SEM of the response in at least three different experiments.

To preserve the biological activity, a nitro or a halide group was required in the R3 position. For the pyridine derivatives, the nitrogen of the heterocycle should be in the *meta* position of the carbonylguanidine side–chain. Taking into account these results, and as compared to the structure of EIPA and other 5-*N*-substituted amiloride derivatives, the synthesis of pyrid-3-yl carbonylguanidine compounds bearing a *N*-substituted nitrogen in R2 should improve the inhibitory potency of NHE. Finally, further functional experiments are warranted to confirm their therapeutic interest.

Experimental

Drugs and reagents

Stock solutions (0.5 mM) of DMA, EIPA, HMA (all from Sigma, Belgium) or amiloride derivatives were prepared in DMSO. The final concentration of DMSO was minimized (maximum:2%) so that it did not affect the measurements. ^{22}NaCl (37 Mbq) was purchased from NEN (Brussels, Belgium) and ouabain from Sigma (Brussels, Belgium). Furosemide was a generous gift from Pharmachim (Jemeppe, Belgium).

Chemistry

Melting points were determined on a Büchi-Tottoli apparatus in open capillary tubes and are uncorrected. IR spectra were recorded as KBr pellets on a Perkin–Elmer 1750 FT spectrophotometer. The ^{1}H NMR spectra were taken on either a Bruker AW-80 (80 MHz) in $CDCl_3$ or in DMSO-d_6 with HMDS as the internal standard and a Bruker AW-400 (400.13 MHz) instrument in DMSO-d_6 with TMS as the internal standard, at 25 °C; chemical shifts are reported in δ values (ppm) relative to internal HMDS or TMS. The abbreviation s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, and br. s = broad singulet are used throughout. For most amiloride analogues, the high number of protons bound to nitrogen atoms increases the occurence of exchange and greatly influence the shift values. The off-decoupling ^{13}C NMR spectra were measured with a Bruker DRX 400 Avance spectrometer (100.62 MHz) in DMSO-d_6, at 25 °C. Chemical shifts are reported in δ values (ppm) with TMS as the internal standard. Elemental analyses (C, H, N, S in%) were performed with a Carlo-Erba EA 1108-elemental analyser and were within ± 0.4% of the theoretical values. All reactions were routinely checked by TLC on silica gel Merck 60F 254 plates.

3,5-Diamino-2-chloro-6-methylpyridine (5). Portionwise 2-chloro-6-methyl-3,5-dinitropyridine **4** (7.5 g, 35 mmol) was added to a suspension of tin chloride (42 g, 186 mmol) in HCl 12 N (110 mL). After stirring for 90 min at room temperature, the mixture was heated at 100 °C for 45 min. After cooling, it was concentrated under reduced pressure, the residue brought to pH 12 with NaOH 5N, and continously extracted with $CHCl_3$ overnight. The organic layer was washed with water, dried and evaporated under reduced pressure to afford a solid brown residue. The crude product **5** was crystallized from toluene. The precipitate was filtered, washed with petroleum ether 40–60 °C, and dried (3.3 g, 60%): mp 170–172 °C; IR 3460, 3375, 3301, 1618, 1477, 1449, 1427, 1245, 1212, 871, 741 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$ + DMSO-d_6, HMDS), δ 2.26 (s, 3H, C*H*$_3$), 3.49 (s, 2H, 3-N*H*$_2$), 3.74 (s, 2H, 5-N*H*$_2$), 6.36 (s, 1H, 4*H*-pyridine). Anal. calcd for $C_6H_8N_3Cl$: C 45.73, H 5.12, N 26.66; found: C 46.05, H 5.23, N 26.57.

3,5-Diacetylamino-2-chloro-6-methylpyridine (6). A solution of 3,5-diamino-2-chloro-6-methylpyridine **5** (7.5 g, 48 mmol) in acetic anhydride (100 mL, 1.06 mol) was stirred for 30 min at room temperature until formation of a white precipitate. The medium was then poured on ice (100 g) and stirred for 30 min. Afterwards, it was neutralized with 30% NaOH to pH 5, and then to pH 7 with saturated $NaHCO_3$. After cooling overnight, the crude compound **6** was filtered, washed with water, and dried. Crystallization from toluene gave the title compound (10.3 g, 90%): mp 238–240 °C; IR 3306, 3270, 3016, 1667, 1536, 1499, 1432, 1373, 1241, 681 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$ + DMSO-d_6, HMDS), δ 2.07 (s, 6H, NH–CO–C*H*$_3$), 2.33 (s, 3H, C*H*$_3$), 8.26 (s, 1H, 4*H*-pyridine), 9.48 (s, 2H, N*H*–CO–CH$_3$). Anal. calcd for $C_{10}H_{12}N_3O_2Cl$: C 49.70, H 5.00, N 17.39; found: C 49.45, H 5.26, N 17.66.

3,5-Diacetylamino-6-chloro-pyridine-2-carboxylic acid (7). A mixture of 3,5-diacetylamino-2-chloro-6-methylpyridine **6** (10 g, 41 mmol) and $MgSO_4$ (3 g, 25 mmol) in water (200 mL) was stirred and heated at 80 °C. $KMnO_4$ (16 g, 101 mmol) was added portionwise, and the resulting mixture was stirred for 1 h. Afterwards, the excess of $KMnO_4$ was reduced by $NaHSO_3$, the manganese dioxide (MnO_2) filtered, and washed with hot water (2 × 50 mL). The combined filtrates were adjusted to pH 1 with 20% H_2SO_4. Left overnight at 4 °C, the formed precipitate was collected, washed with water, and dried. Crystallization from ethanol gave the title compound **7** (6.75 g, 60%): mp 212–215 °C; IR 3533, 3346, 3245, 1712, 1690, 1584, 1537, 1475, 1371, 1247, 1228, 1194, 723, 667 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$ + DMSO-d_6, HMDS), δ 2.19 (s, 3H, 5-NH–CO–C*H*$_3$), 2.33 (s, 3H, 3-NH–CO–C*H*$_3$), 5.36 (s, COO*H* + H_2O), 9.35 (s, 1H, 5-N*H*–CO–CH$_3$), 9.68 (s, 1H, 4*H*-pyridine), 11.07 (s, 1H, 3-N*H*–CO–CH$_3$). Anal. calcd for $C_{10}H_{10}N_3O_4Cl$: C 44.21, H 3.71, N 15.47; found: C 43.96, H 3.98, N 15.76.

Methyl 3,5-diacetylamino-6-chloro-pyridine-2-carboxylic ester (8). The decomposition of methylnitrosourea (4.1 g, 40 mmol) into NaOH 5 N gave diazomethane, which was extracted with diethyl ether (10 mL). The diazomethane solution was then added dropwise to a solution of 3,5-diacetylamino-6-chloro-pyridine-2-carboxylic acid **7** (2.7 g, 9.9 mmol) in a mixture ethanol–dimethylformamide (1:1, 100 mL), and the temperature was kept under 10 °C. After warming to room temperature, the medium was concentrated under reduced pressure, and diluted with water (100 mL). The pure compound **8** was filtered, washed with water, and dried (1.85 g, 65%): mp 197–199 °C; IR 3321, 3277, 1710, 1688, 1586, 1523,

1476, 1229, 1190, 1117, 741 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$ + DMSO-d_6, HMDS), δ 2.03 (s, 3H, 5-NH–CO–C*H*$_3$), 2.10 (s, 3H, 3-NH–CO–C*H*$_3$), 3.74 (s, 3H, C*H*$_3$ ester), 9.25 (s, 1H, 4*H*-pyridine), 9.51 (s, 1H, 5-N*H*–CO–CH_3), 10.41 (s, 1H, 3-N*H*–CO–CH_3). Anal. calcd for $C_{11}H_{12}N_3O_4Cl$: C 46.25, H 4.23, N 14.71; found: C 46.03, H 4.32, N 15.02.

Methyl 3,5-diamino-6-chloro-pyridine-2-carboxylic ester (9). Under nitrogen atmosphere, H_2SO_4 (d = 1.84; 1.8 mL, 34 mmol) was added dropwise to a suspension of 3,5-diacetylamino-6-chloro-pyridine-2-carboxylic acid **7** (1 g, 3.7 mmol) in methanol (20 mL).The reaction mixture was refluxed for 3 h. After cooling, methanol was evaporated under reduced pressure and crushed ice (30 g) was added. The medium was then neutralized to pH 7 with saturated $NaHCO_3$. After standing at 4 °C for 2 h, the precipitate formed was filtered, washed with water, and dried. Crystallization from toluene gave the title compound **9** (0.19 g, 25%): mp 211–213 °C; IR 3475, 3436, 3367, 3337, 3040, 1676, 1619, 1434, 1273, 1122, 1066, 696 cm^{-1}; ^1H NMR (80 MHz, DMSO-d_6, HMDS), δ 3.65 (s, 3H, C*H*$_3$ ester), 5.96 (s, 2H, 5-N*H*$_2$), 6.31 (s, 1H, 4*H*–pyridine), 6.50 (s, 2H, 3-N*H*$_2$). Anal. calcd for $C_7H_8N_3O_2Cl$: C 41.70, H 4.00, N 20.84; found: C 41.58, H 4.26, N 21.12.

3,5-Diamino-6-chloro-pyridine-2-carboxylic acid (10). A solution of 3,5-diacetylamino-6-chloro-pyridine-2-carboxylic acid **7** (2 g, 7.4 mmol) in NaOH 1 N (60 mL) was refluxed for 3 h. After cooling to room temperature, pH was adjusted to 5 with acetic acid 2 N. The precipitate formed **10** was collected, washed with water, and dried (1.0 g, 75%): mp 192–194 °C; IR 3481, 3442, 3350, 2925, 1688, 1614, 1430, 1255, 690 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6 + CF_3COOD, TMS), δ 6.37 (s, 1H, 4*H*–pyridine); ^{13}C NMR (400 MHz, DMSO-d_6, TMS), δ 105.82 (C_4), 116.04 (C_6), 124.18 (C_2), 145.84 (C_5), 149.84 (C_3), 168.45 (C=O). Anal. calcd for $C_6H_6N_3O_2Cl$: C 38.42, H 3.22, N 22.40; found: C 38.76, H 3.49, N 22.11.

***N-tert*-Butyl-3-[(3,5-diamino-6-chloro-pyridine-2-carbonyl)-oxy]crotonamide (11).** NBI (1.2 g, 5 mmol) was added to a mixture of 3,5-diamino-6-chloro-pyridine-2-carboxylic acid **10** (0.94 g, 5 mmol) and triethylamine (0.5 g, 5 mmol) in dimethylformamide (5 mL), and the resulting mixture was stirred for 2 h at room temperature. Afterwards, it was poured into ice-water (60 mL), and the precipitate formed was filtered, washed with water, and dried. Crystallization from acetonitrile afforded the title compound **11** (1.14 g, 70%): mp 121–122 °C; IR 3451, 3399, 3344, 3311, 3069, 1708, 1677, 1632, 1614, 1543, 1364, 1275, 1242, 1218, 1154, 1092, 1058, 735, 704 cm^{-1}; ^1H NMR (80 MHz, DMSO-d_6, HMDS), δ 1.09 (s, 9H, *N*-*tert*-butyl), 1.86 (s, 3H, C(C*H*$_3$)=CH), 5.42 (s, 1H, C(CH_3)=C*H*), 6.03 (s, 2H, 5-N*H*$_2$), 6.34 (s, 1H, 4*H*–pyridine), 6.55 (s, 2H, 3-N*H*$_2$), 7.00 (s, 1H, CON*H*). Anal. calcd for $C_{14}H_{19}N_4O_3Cl$: C 51.46, H 5.86, N 17.14; found: C 51.29, H 5.98, N 17.36.

(3,5-Diamino-6-chloro-pyridine-2-carbonyl)guanidine hydrochloride hydrate (12). Method 1. Under nitrogen, a mixture of guanidine hydrochloride (0.48 g, 5 mmol) and potassium *tert*-butyl oxide (0.56 g, 5 mmol) in dioxane (10 mL) was heated at 50 °C for 20 min. After cooling to room temperature, *N*-*tert*-butyl-3-[(3,5-diamino-6-chloro-pyridine-2-carbonyl)-oxy]crotonamide **11** (0.82 g, 2.5 mmol) was added, and the resulting mixture was refluxed for 1 h. After cooling, dioxane was evaporated under reduced pressure and water (30 mL) was added. After stirring for 30 min, the crude product **12** was collected, washed with water, and dried. It was purified by dissolution in methanol or isopropanol with charcoal. The suspension was filtered, and the filtrate diluted with a HCl saturated diethyl ether solution to give the title compound. It was filtered, washed with diethyl ether, and dried (0.21 g, 30%)

Method 2. A mixture of (3-amino-5-acetylamino-6-chloro-pyridine-2-carbonyl)guanidine hydrochloride hydrate **15** (0.94 g, 3 mmol), ethanol (4 mL) and HCl 6 N (6 mL) was refluxed for 2 h. After cooling, ethanol was evaporated under reduced pressure, water (5 mL) added, and the solution heated at 60 °C with charcoal. After filtration, the filtrate was kept at 4 °C for 3 h, and the precipitate formed **12** was filtered, washed with diethyl ether, and dried (0.25 g, 30%): mp 184–185 °C; IR 3390, 3336, 3287, 3187, 1692, 1674, 1628, 1536, 1244, 709 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, TMS), δ 6.45 (s, 1H, 4*H*–pyridine), 6.92 (br. s, *guanidine*), 8.53 (br. s, 4H, N*H*$_2$); ^{13}C NMR (400 MHz, DMSO-d_6, TMS), δ 104.99 (C_4), 114.77 (C_6), 124.73 (C_2), 147.07 (C_5), 150.48 (C_3), 155.54 (C=NH), 166.62 (C=O). Anal. calcd for $C_7H_{12}N_6O_2Cl_2$: C 29.70, H 4.27, N 29.68; found: C 29.39, H 4.58, N 29.92.

***N*-(6-Chloro-2-methyl-4-*oxo*-4H-pyrido[3,2-*d*]-1,3-oxazin-7-yl)-acetamide (13).** A mixture of 3,5-diacetylamino-6-chloro-pyridine-2-carboxylic acid **7** (1.36 g, 5 mmol), acetic anhydride (20 mL, 213 mmol) and ethyl acetate (10 mL) was refluxed for 4 h. After cooling, ethyl acetate was evaporated under reduced pressure, and the solution was kept at −18 °C for 2 h. The precipitate formed was filtered, washed with ethyl acetate, and dried. Crystallization from the mixture ethyl acetate–petroleum ether 40–60 °C (1:3) gave the title compound **13** (0.85 g, 70%): mp 215–218 °C; IR 3454, 3354, 3086, 1764, 1713, 1642, 1555, 1506, 1451, 1383, 1358, 1228, 1202, 1180 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$ + DMSO-d_6, HMDS), δ 2.15 (s, 3H, C*H*$_3$), 2.34 (s, 3H, C*H*$_3$), 8.50 (s, 1H, 8*H*), 9.70 (s, 1H, CON*H*). Anal. calcd for $C_9H_8N_3O_3Cl$: C 44.74, H 3.34, N 17.39; found: C 45.03, H 3.63, N 17.56.

(3,5-Diacetylamino-6-chloro-pyridine-2-carbonyl)guanidine hydrate (14). Guanidine base was prepared by consecutive addition of Na (0.63 g, 27.5 mmol) and guanidine hydrochloride (2.64 g, 27.5 mmol) to dry methanol (50 mL), and the solution was refluxed for 30 min under nitrogen atmosphere. After cooling at room temperature, *N*-(6-chloro-2-methyl-4-*oxo*-4H-pyrido[3,2-d]-1,3-oxazin-7-yl)-acetamide **13** (1.37 g, 55 mmol) was added, and the resulting mixture was stirred for 45 min at room temperature. Afterwards, methanol

was evaporated under reduced pressure and water (100 mL) was added. The precipitate formed was filtered, washed with water, and dried. It was purified by dissolution in methanol with charcoal. The suspension was filtered, and the filtrate diluted with water to give the title compound **14** (2.92 g, 32%): mp 244–247 °C (decomposition); IR 3346, 3245, 1712, 1690, 1584, 1537, 1475, 1371, 1247, 1228, 1194, 723 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, TMS), δ 2.19 (s, 3H, 5-NH–CO–CH_3), 2.33 (s, 3H, 3-NH–CO–CH_3), 6.83 (s, 3H, *amidine*), 9.35 (s, 1H, 5-N*H*–CO–CH_3), 9.68 (s, 1H, 4*H*–pyridine), 10.41 (s, 1H, 3-N*H*–CO–CH_3), 11.07 (s, 1H, CO–N*H*). Anal. calcd for $C_{11}H_{15}N_6O_4Cl$: C 39.95, H 4.57, N 25.41; found: C 40.12, H 4.68, N 25.13.

(3-Amino-5-acetylamino-6-chloro-pyridine-2-carbonyl)-guanidine hydrochloride hydrate (15). Guanidine base was prepared by consecutive addition of Na (0.23 g, 10 mmol) and guanidine hydrochloride (0.96 g, 10 mmol) to dry methanol (20 mL), and the solution was refluxed for 30 min under nitrogen atmosphere. After cooling at room temperature, *N*-(6-chloro-2-methyl-4-*oxo*-4H-pyrido[3,2-*d*]-1,3-oxazin-7-yl)-acetamide **13** (0.50 g, 20 mmol) was added, and the resulting mixture was refluxed for 75 min. Afterwards, methanol was evaporated under reduced pressure, water (40 mL) added, and the mixture triturated for 15 min. The precipitate formed was collected, and washed with water. It was dissolved in HCl 1N (50 mL) at 50 °C with charcoal. The suspension was filtered, and the filtrate kept at 4 °C for 2 h. The precipitate formed **15** was filtered, washed with water, and dried (0.13 g, 40%): mp 255 °C; IR 3355, 3192, 1700, 1651, 1579, 1550, 1495, 1442, 1418, 1376, 1228, 778 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, TMS), δ 2.11 (s, 3H, 5-NH–CO–CH_3), 6.92 (s, 2H, 3-NH_2), 8.22 (s, 1H, 4*H*-pyridine), 8.83 (s, 3H, *amidine*), 10.66 (s, 1H, 5-N*H*–CO–CH_3), 10.97 (s, 1H, CON*H*). Anal. calcd for $C_9H_{14}N_6O_3Cl_2$: C 33.24, H 4.34, N 25.85; found: C 33.13, H 4.16, N 26.05.

3-Methyl-2,6-piperidinedione (16). A mixture of H_2SO_4 (d = 1.84; 160 mL, 3 mol), acetic acid (d = 1.049; 1000 mL, 17.5 mol) and α-methylglutaronitrile (85% purity; 257 g, 2.4 mol) was stirred at room temperature, and a solution of acetic acid (d = 1.049; 200 mL, 3.5 mol) in water (63 mL) was then added dropwise for 1 h. The reaction medium was heated to 130 °C and this temperature was sustained for an additional hour. Afterwards, it was cooled at 4 °C overnight, $(NH_4)_2SO_4$ was filtered off, washed with acetic acid, and the filtrate was concentrated under reduced pressure. The residue formed (470 g) was collected, poured into water (1.5 L), and the solution was adjusted to pH 5 with Na_2CO_3 (120 g). The first crop of crude product **16** was filtered, and dried (96.5 g). The filtrate was extracted with $CHCl_3$ (3×300 mL) and washed with water. The combined organic layers were dried and evaporated under reduced pressure to give a second crop of **16** (269 g). The different crops were crystallized from water or from a mixture of toluene–petroleum ether 40–60 °C (1:5) to give the title compound **16** (yield: 60–65%): mp 91–92 °C; IR 3197, 3094, 2993, 2974, 2960, 2937, 2883, 1713, 1703, 1649, 1639, 1463, 1444, 1364, 1339, 1315, 1249, 1203, 1185 cm^{-1}. Anal. calcd for $C_6H_9NO_2$: C 56.68, H 7.13, N 11.02; found: C 56.72, H 7.08, N 11.07.

2,5,6-Trichloro-3-methylpyridine (17). A well-powdered mixture of 3-methyl-2,6-piperidinedione **16** (23.3 g, 183 mmol) and phosphorus pentachloride (240 g, 1.2 mol) was quickly introduced in a flask fitted with a large reflux condenser and slowly heated to 150 °C for 2 h, until a viscous liquid was formed. The hot mixture was then poured on crushed ice (1 kg), and kept at 4 °C overnight. The precipitate formed **17** was filtered, washed with iced water, and dried (16.2 g, 45%). An analytical sample of **17** was obtained by fractional crystallization from a mixture ethanol–petroleum ether 40–60 °C (1 :8): mp 94–96 °C; IR 3446, 3053, 2968, 2628, 1810, 1631, 1566, 1532, 1442, 1387, 1336, 1235, 1212, 1177, 1102, 1038, 1009, 927, 907, 825, 720, 707, 669, 536, 520 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$, HMDS), δ 2.28 (s, 3H, CH_3), 6.55 (s, 1H, 4*H*–pyridine). Anal. calcd for $C_6H_4NCl_3$: C 36.68, H 2.05, N 7.13; found: C 36.72, H 1.81, N 7.19.

2,5,6-Trichloro-3-bromomethyl-pyridine (19) and 2,5,6-Trichloro-3-dibromomethyl-pyridine (20). A vigorously stirred mixture of 2,5,6-trichloro-3-methylpyridine **17** (20 g, 102 mmol), *N*-bromosuccinimide (50.9 g, 286 mmol), carbon tetrachloride (200 mL) was refluxed and illuminated with a 60-watt photoflood lamp for 4 h. The succinimide formed was then removed by filtration. The filtrate was evaporated under reduced pressure and the yellow oil residue was purified by flash column chromatography on silica gel with *n*-hexane as eluent to give **19** and **20**. **19**: mp 51–52 °C; IR 3468, 3028, 1567, 1532, 1438, 1392, 1350, 1220, 1174, 1129, 1092, 940, 927, 878, 744, 719, 670, 590, 552 cm^{-1}; ^1H NMR (80 MHz, DMSO-d_6, HMDS), δ 3.28 (s, 2H, CH_2Br), 8.38 (s, 1H, 4*H*–pyridine). Anal. calcd for $C_6H_3NCl_3Br$: C 26.17, H 1.10, N 5.09; found: C 26.47, H 1.30, N 5.07. **20**: mp 47–48 °C; IR 3011, 1566, 1525, 1385, 1347, 1215, 1187, 1174, 1150, 1084, 941, 905, 753, 735, 703, 671, 662, 595, 550 cm^{-1}; ^1H NMR (80 MHz, DMSO-d_6, HMDS), δ 7.26 (s, 1H, C*H*Br_2), 8.44 (s, 1H, 4*H*–pyridine). Anal. calcd for $C_6H_2NCl_3Br_2$: C 20.34, H 0.57, N 3.95; found: C 20.64, H 0.79, N 3.99. Generally, the residue (**19** and **20**) was used without any further purification.

2,5,6-Trichloro-pyridine-3-carboxylic acid (21). Method 1. Portionwise $KMnO_4$ (7.6 g, 48 mmol) was added to a suspension of 2,5,6-trichloro-3-methylpyridine **17** (3.9 g, 20 mmol) in water (250 mL), and the resulting mixture was heated at 90 °C for 6.5 h. Afterwards, the manganese dioxide (MnO_2) was filtered off and washed with hot water (2×50 mL). The combined filtrates were concentrated to 20 mL and adjusted to pH 1 with HCl 12 N. After standing at 4 °C overnight, the crude product was collected, washed with a minimum amount of iced water, and dried. Crystallization from water gave the title compound **21** (1.37 g, 30%). The unreacted starting material was recovered from MnO_2 cake by extraction with acetone (150 mL). The yield of derivative **21** was then calculated on the basis of the quantity of reacted starting material

Method 2. A mixture of 2,5,6-trichloro-3-bromomethyl-pyridine **19** and 2,5,6-trichloro-3-dibromomethyl-pyridine **20** (30.9 g), $HgNO_3$ (1.51 g, 5.7 mmol), $CuSO_4.5H_2O$ (0.67 g, 2.7 mmol) in H_2SO_4 (d = 1.84; 73 mL, 1.4 mole) was stirred and heated at 110 °C. Dropwise HNO_3 (d = 1.52; 40 mL, 0.97 mol) was added for 90 min and the temperature was maintained at 130 °C. 15 min later, the medium was poured on crushed ice (±300 g), and the precipitate formed was filtered, and dried. It was dissolved in saturated $NaHCO_3$ (200 mL) with charcoal. The suspension was filtered, and the filtrate acidified with HCl 12 N to give the crude product **21**. It was collected, washed with iced water, and dried. Crystallization from water afforded the title compound **21** (yield 40–50%): mp 160–162 °C; IR 3070, 2631, 1711, 1694, 1566, 1520, 1380, 1329, 1271, 1235, 1176, 1156, 1086, 920, 783, 759, 718, 675, 643, 503 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3 + CF_3COOD$, TMS), δ 8.40 (s, 1H, 4*H*–pyridine); ^{13}C NMR (400 MHz, DMSO-d_6, TMS), δ 128.58 (C_5), 129.39 (C_3), 142.91 (C_4), 145.87 (C_6), 149.06 (C_2), 164.37 (C=O). Anal. calcd for $C_6H_2NO_2Cl_3$: C 31.82, H 0.89, N 6.18; found: C 31.91, H 1.13, N 6.26.

2,5,6-Trichloro-pyridine-3-carboxamide (25). A mixture of 2,5,6-trichloro-pyridine-3-carboxylic acid **21** (8.6 g, 38 mmol), thionyl chloride (4.9 mL, 7.9 g, 67 mmol), benzene (16 mL) and dimethylformamide (0.08 mL) was refluxed for 2.5 h. Afterwards, the reaction medium was evaporated under reduced pressure, the residue dispersed in dry toluene (25 mL) and the solvent eliminated under reduced pressure to give **24** (7.6 g, 81%). This step was repeated twice. A solution of 2,5,6-trichloropyridine-3-carboxylic acid chloride **24** (7.6 g, 31 mmol) in dry dioxane (10 mL) was then added to 10% NH_4OH (500 mL), and the mixture was stirred at 4 °C for 1 h. The precipitate formed **25** was filtered, washed with cold water, and dried (6.3 g, 90%): mp 151–153 °C; IR 3391, 3181, 1662, 1567, 1522, 1423, 1374, 1321, 1172, 1085, 911, 715, 659, 600 cm^{-1}. Anal. calcd for $C_6H_3N_2OCl_3$: C 31.96, H 1.34, N 12.42; found: C 31.99, H 1.05, N 12.40.

2,6-Diamino-5-chloro-pyridine-3-carboxamide (26). A mixture of 2,5,6-trichloro-pyridine-3-carboxamide **25** (2.0 g, 44 mmol) and a 25% NH_4OH solution (60 mL) saturated with NH_3 was placed in a stainless-steel autoclave and heated at 130 °C for 12 h. After cooling at room temperature, the solid residue formed was filtered, washed with water, and dried. Crystallization from acetone gave the title compound **26** (5.35 g, 65%): mp 181–184 °C; IR 3495, 3425, 3346, 3215, 1698, 1646, 1607, 1526, 1482, 1396, 1314, 1265, 1087, 1033, 788, 671, 563 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, TMS), δ 6.41 (br. s, 2H, $CONH_2$), 7.16 (br. s, 4H, NH_2), 7.85 (s, 1H, 4*H*–pyridine). Anal. calcd for $C_6H_7N_4OCl$: C 38.62, H 3.78, N 30.02; found: C 38.61, H 3.89, N 29.78.

2,6-Diamino-5-chloro-pyridine-3-carboxylic acid (27). A suspension of 2,6-diamino-5-chloro-pyridine-3-carboxamide **26** (5.35 g, 29 mmol) in NaOH 4 N (80 mL) was refluxed for 1 h. After cooling at room temperature, the medium was diluted with water (40 mL), adjusted to pH 1 with HCl 6 N and kept at 4 °C overnight. The precipitate formed **27** was collected, washed with cold water, and dried (4.9 g, 90%): mp 159–160 °C; IR 3487, 3470, 3333, 3288, 3147, 2393, 1633, 1603, 1580, 1545, 1428, 1364, 1338, 1300, 1233, 1126, 1062, 1034, 941, 830, 796, 702, 598 cm^{-1}; ^1H NMR (80 MHz, DMSO-d_6, HMDS), δ 6.46 (br. s, 2H, 6-NH_2), 6.90 (br. s, 3H, 2-$NH_2 + HN^+$), 7.65 (s, 1H, 4*H*–pyridine). Anal. calcd for $C_6H_6N_3O_2Cl$: C 38.42, H 3.22, N 22.40; found: C 38.57, H 3.37, N 22.30.

(2,6-Diamino-5-chloro-pyridine-3-carbonyl)guanidine (28). Guanidine base was prepared by consecutive addition of potassium *tert*-butyl oxide (0.28 g, 2.5 mmol) and guanidine hydrochloride (0.24 g, 2.5 mmol) to dry mixture of dimethylformamide–dioxane (1:1; 10 mL). Under nitrogen atmosphere, the mixture was heated at 50 °C for 20 min, and then KCl filtered off. 2,6-Diamino-5-chloro-pyridine-3-carboxylic acid **27** (0.47 g, 2.5 mmol) was added to a solution of CDI (0.41 g, 2.5 mmol) in dimethylformamide (10 mL), and the mixture was stirred at room temperature for 1 h. This latter was added to the guanidine filtrate, and the final mixture was stirred at room temperature for 5 h. Afterwards, the solvents were evaporated under reduced pressure, and the residue suspended in cold water (20 mL). The crystalline solid formed was filtered, washed with water, and dried. It was purified by fractional cristallization from methanol to give the title compound **28** (0.13 g, 22%): mp > 300 °C; IR 3466, 3419, 3390, 3350, 3264, 3168, 1644, 1591, 1520, 1395, 1367, 1309, 1232, 1105, 1033, 945, 894, 805, 730, 603, 519, 474 cm^{-1}; ^1H NMR (400 MHz, DMSO-$d_6 + D_2O$, TMS), δ 8.02 (s, 1H, 4*H*–pyridine). Anal. calcd for $C_7H_9N_6OCl$: C 36.77, H 3.97, N 36.76; found: C 36.42, H 3.79, N 36.48.

2,6-Dichloro-3-pyridine-carboxylic acid (29). A solution of *n*-butyllithium (0.25 mol) in hexane (100 mL) was added dropwise to a cold (−80 °C) and freshly prepared solution of diisopropylamine (35 mL, 0.25 mol) in tetrahydrofuran (200 mL), and the medium was stirred for 1 h. A solution of 2,6-dichloropyridine (37 g, 0.25 mole) in tetrahydrofuran (100 mL) was then added by portions, and the resulting mixture was stirred at −80 °C for 30 min. After warming at 0 °C, it was quickly poured on a large excess of carbogene in diethyl ether (1 L), left until CO_2 production ceased, and then water was added (500 mL). The aqueous layer was extracted with diethyl ether (2×250 mL) and mixed with charcoal. The suspension was filtered, and the filtrate adjusted to pH 1 with HCl 12 N. After standing at 4 °C overnight, the precipitate formed was collected and dried. Crystallization from toluene afforded the title compound **29** (28.8 g, 60%): mp 145–147 °C; IR 3427, 3090, 2970, 2652, 2550, 1722, 1578, 1542, 1454, 1438, 1406, 1342, 1283, 1245, 1171, 1144, 1052, 904, 851, 780, 756, 668, 603, 569 cm^{-1}. Anal. calcd for $C_6H_3NO_2Cl_2$: C 37.53, H 1.57, N 7.29; found: C 37.24, H 1.88, N 7.27.

(2,6-Dichloro-pyridine-3-carbonyl)guanidine hydrate (30). The title compound was prepared from 2,6-dichloro-3-pyridine-carboxylic acid **29** (1 g, 5 mmol) as described for **28**. It was crystallized in dioxane (0.3 g, 24%): mp > 300 °C; IR 3430, 3074, 2156, 1670, 1615,

1573, 1529, 1472, 1368, 1340, 1201, 1146, 1074, 906, 846, 815, 791, 678, 577 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, TMS), δ 7.55 (d, 1H, 5*H*–pyridine), 8.03 (d, 1H, 4*H*–pyridine); ^{13}C NMR (400 MHz, DMSO-d_6, TMS), δ 123.84 (C_5), 135.84 (C_3), 141.89 (C_4), 146.48 (C_6), 148.65 (C_2), 163.23 (C=NH), 174.89 (C=O). Anal. calcd for $C_7H_8N_4O_2Cl_2$: C 33.49, H 3.21, N 22.31; found: C 33.76, H 3.25, N 21.95.

(2,5,6-Trichloro-pyridine-3-carbonyl)guanidine (31). The title compound was prepared from 2,5,6-trichloro-3-pyridine-carboxylic acid **21** (0.72 g, 3 mmol) as described for **28**. It was crystallized in dioxane (97 mg, 12%): mp >300 °C; IR 3370, 1668, 1599, 1564, 1521, 1454, 1347, 1317, 1168, 1105, 970, 937, 836, 798, 656, 617 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, TMS), δ 6.95, 8.00 (br. s, 3H, *guanidine*), 8.35 (s, 1H, 4*H*–pyridine); ^{13}C NMR (400 MHz, DMSO-d_6, TMS), δ 128.93 (C_5), 136.52 (C_3), 141.48 (C_4), 144.61 (C_6), 146.31 (C_2), 163.13 (C=NH), 173.12 (C=O). Anal. calcd for $C_7H_5N_4OCl_3$: C 31.43, H 1.88, N 20.94; found: C 31.23, H 2.14, N 20.69.

(2-Amino-5-bromo-pyridine-3-carbonyl)guanidine (32). The title compound was prepared by bromination of (2-amino-pyridine-3-carbonyl)guanidine (1.5 g, 8.4 mmol) as previously described by Cragoe[51] (1.0 g, 45%): mp >300 °C; IR 3481, 3437, 3334, 1638, 1604, 1561, 1505, 1464, 1400, 1358, 1327, 1234, 918, 849, 806, 694 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, TMS), δ 8.06 (d, 1H, 6*H*-pyridine), 8.28 (d, 1H, 4*H*–pyridine); ^{13}C NMR (400 MHz, DMSO-d_6, TMS), δ 104.63 (C_5), 116.52 (C_3), 141.60 (C_4), 151.33 (C_6), 158.54 (C_2), 162.92 (C=NH), 175.01 (C=O). Anal. calcd for $C_7H_8N_5OBr$: C 32.58, H 3.12, N 27.14; found: C 32.75, H 3.29, N 26.96.

General procedure for the preparation of 'simplified' aroylguanidines

Compounds **33**, **40**, **42** were synthesized by condensing the appropriate methyl ester with the guanidine under reflux or by reaction between the desired acid chloride and the guanidine at room temperature.[52,53] Guanidine base was always freshly prepared from the hydrochloride salt using Na, NaH or potassium *tert*-butyl oxide.

(Pyridine-3-carbonyl)guanidine dihydrochloride (33). The title compound was prepared from methyl pyridine-3-carboxylic ester according to the general procedure (yield: 19%): mp 254–256 °C (decomposition). Anal. calcd for $C_7H_{10}N_4OCl_2$: C 35.46, H 4.25, N 23.63; found: C 35.72, H 4.52, N 23.34.

(3,4-Dichlorobenzoyl)guanidine hydrochloride (40). The title compound was prepared from (3,4-dichloro)-benzoyl chloride according to the general procedure (yield: 21%): mp 215–218 °C. Anal. calcd for $C_8H_8N_3OCl_3$: C 35.78, H 3.00, N 15.65; found: C 36.02, H 3.32, N 15.52.

Benzoylguanidine hydrochloride (42). The tiltle compound was prepared from methyl benzoic acid ester according to the general procedure (yield: 18%): mp 211–214 °C. Anal. calcd for $C_8H_{10}N_3OCl$: C 48.13, H 5.05, N 21.05; found: C 48.33, H 5.24, N 21.35.

Pharmacology

Human platelet swelling assay (PSA). The NHE inhibitory potency of the novel drugs was assessed by their ability to reduce the human platelet swelling induced by a propionate buffer (pH 6.7).[16,18,27] A solution of the tested compound (20 μL) was added to 1.73 mL of propionate buffer (in mM: sodium propionate 140, glucose 10, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 20 qs ad pH 6.7) contained in a spectrophotometer cuvette. Then, 0.25 mL of human platelet rich plasma (PRP from the Belgian Red Cross) containing $1–2\times10^8$ cells/mL was added. The suspension was stirred and the change in optical density (OD) was recorded each 10 s for 5 min at 680 nm (Perkin-Elmer lambda 20 double-beam spectrophotometer). During the experiment, the suspension was maintained at 25 °C. The decrease of OD corresponded to a monoexponential curve following the equation $OD_{(t)} = OD_{t=0}\ e^{-kt}$ where t is the time (in s) corresponding to the recorded OD and k is the decrease rate constant.[16] For each drug, the concentrations were plotted against their corresponding k values. The maximum platelet swelling was measured in absence of any drug. The minimum swelling was observed in presence of EIPA (10 μM) and is the result of a completely inhibited NHE. Sigmoidal curves were drawn by non-linear regression analysis (GraphPad Prism software). They led to calculate the drug concentration (IC_{50}) which decrease of 50% the platelet swelling. For each molecule, the measurements were performed in triplicate. The IC_{50} values are expressed as mean ± SEM.

$^{22}Na^+$ uptake inhibition assay (UIA). The $^{22}Na^+$ uptake inhibition assay used human washed platelets.[56] The uptake of $^{22}Na^+$ started when platelet-rich plasma (0.23 mL) was added to 0.75 mL of sodium propionate buffer (pH 6.7) containing 1–2 μCi of $^{22}Na^+$ (specific radioactivity 37 MBq) and to 0.02 mL of buffer containing the drug. The incubation medium contained ouabain (0.1 mM) to prevent sodium ejection through the Na^+/K^+ ATPase. After 5 min. incubation at 25 °C, 4 mL of ice-cold buffer (containing in mM: choline chloride 100, NaCl 15.4, Tris 45, HCl qs ad pH 7.4) was added, the sample rapidly filtered through a glass-fibre filter (Whatman GF/C), and the tube rinsed twice with ice-cold buffer. The filter was placed in plastic scintillation vial containing an emulsion-type scintillation mixture (4 mL) and the radioactivity was measured using a β-counter (Packard Tricarb 460C). Three independant $^{22}Na^+$-influx experiences were performed in quadruplicate for each compound. The residual cell radioactivity measured in presence of EIPA (10 μM) was considered as non relevant of the sodium uptake through the NHE. This EIPA-insensitive sodium uptake was 153 pmol/10^8 platelets. The control was considered as the sodium uptake in absence of drug (216 ± 5 pmol/10^8 platelets). The sodium uptake data were plotted against drug concentrations. Sigmoidal curves were drawn by a non-linear regression analysis (GraphPad

Prism software). They led to determine the drug concentration (IC_{50}) required to decrease of 50% the EIPA-sensitive sodium uptake induced by intracellular acidosis (pH 6.7). The IC_{50} values are expressed as mean ± SEM. (GraphPad Prism software).

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