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Original article

Novel inhibitors of the sodium–calcium exchanger: benzene ring analogues of *N*-guanidino substituted amiloride derivatives

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Abstract – A series of *N*-guanidino substituted 2,4-diamino-5-carbonylguanidine molecules related to amiloride were synthesised and evaluated for their ability to inhibit the sodium–calcium exchanger in rat insulinoma cells (RINm5F) and human platelets. Specific chemical pathways were used to prepare the benzene derivatives designed as bioisosteric analogues of the pyrazine derivatives of amiloride. Several so-called 'simplified analogues', where some substituents of amiloride were omitted or replaced, were also prepared and included in the biological evaluation. The inhibitory potency of the sodium–calcium exchanger was screened on both cell types by measuring their effect on $^{45}Ca^{2+}$ uptake. Among the most active compounds, *N*-(2-amino-5-chloro-4-nitrobenzoyl)-*N*′-(1-naphtylmethyl)guanidine ($IC_{50} = 3.4$ μM) was found more active than amiloride ($IC_{50} = 690$ μM) and 3,4-dichlorobenzamil ($IC_{50} = 15.2$ μM), the reference inhibitor. © 2001 Éditions scientifiques et médicales Elsevier SAS

sodium–calcium exchanger / amiloride / RINm5F / human platelets / substituted guanidines

1. Introduction

Calcium plays an important second messenger role in most cell types. This role involves a tight control of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Two processes located at the plasma membrane appear to mediate Ca^{2+} extrusion from cells and take part in the control of $[Ca^{2+}]_i$: the Ca^{2+}-ATPase and the electrogenic sodium–calcium exchanger [1]. Two major types of sodium–calcium exchanger have been described. The first exchanger type (NCX) operates with a stoichiometry of 3 Na^+ for 1 Ca^{2+} [2]. Three genes coding for three different isoforms (NCX1, NCX2, and NCX3) have been identified in mammals [3–5]. NCX1 is by far the most extensively studied and characterised isoform; it is widely distributed [6]. Further variability in this group results from alternative splicing of NCX1, and tissue-specific variants have been identified [7, 8].

The second sodium–calcium exchanger type (RetX or NCKX) was identified and cloned in rod outer segments [9, 10]. It differs from NCX in both functional and molecular terms: for instance, its stoichiometry is different from that of the former because 4 Na^+ are exchanged for 1 Ca^{2+} plus 1 K^+. Two exchanger isoforms NCKX1 and NCKX2 have been identified [11].

Abbreviations: Ac_2O: acetic anhydride; 3,4-DCB: 3,4-dichlorobenzamil; ADP: adenosine 5′-diphosphate; U-46619: 9,11-dideoxy-9α,-11α-methanoepoxy prostaglandin F2α; PMA: phorbol 12-myristate 13-acetate; HEPES: *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; DCC: dicyclohexylcarbodiimide; EGTA: ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris: tris(hydroxymethyl)aminomethane; BSA: bovine serum albumin.

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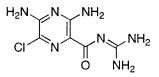
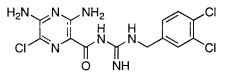
Although the sodium–calcium exchange activity participates in calcium homeostasis in a wide variety of cell types [12], it exerts a prominent role in several cells subject to high Ca^{2+} levels [13]. For instance in the heart [14] and in the pancreatic β-cells [15], sodium–calcium exchange appears to be the main mechanism for Ca^{2+} extrusion. It has also been proposed to play a key role in Ca^{2+} homeostasis of many cell types like neuronal [16], vascular [17] or renal cells [18]. In addition to its major involvement in the Ca^{2+} extrusion mechanism, the sodium–calcium exchange may play a crucial role in cardiac arrhythmogenesis [19–21]. This transport system may be important for the regulation of blood pressure and may thereby play a role in the pathogenesis of salt-dependent essential hypertension [22, 23]. Moreover, the sodium–calcium exchanger may prevent the platelet integrin $\alpha_{IIb}\beta_3$ activation and consequently the platelet aggregation induced by various agonists such as ADP, collagen, thrombin, epinephrine, U46619 and PMA [24, 25]. Increased sodium–calcium exchanger activity has also been reported in brain tissue from Alzheimer's disease subjects [26].

Due to the lack of specific inhibitors of this exchanger, investigation of the sodium–calcium exchanger remains difficult. Among the identified non-specific inhibitors, amiloride and its derivatives constitute a chemically homogeneous series of molecules, with known qualitative structure–activity relationships, as a starting point for further development [27–34] (*figure 1*).

Amiloride, 3,5-diamino-6-chloropyrazinoylguanidine (**1**), was initially demonstrated to inhibit the Na^+ channels present in urinary epithelia and was used as a K^+-sparing diuretic [33]. At higher concentrations, amiloride and its analogues were subsequently shown to inhibit other ion transporters such as the sodium–calcium exchanger [33]. The structure of amiloride has been extensively studied, mainly



amiloride



3,4-dichlorobenzamil

Drug	IC_{50} (μM)		
	Na^+ channel [a]	Na^+/Ca^{2+} exchanger [a]	L-type Ca^{2+} channel [b]
amiloride	0.34	1100	1700
3,4-dichlorobenzamil	0.085	29.7	4.20

[a] Values determined on isolated plasma membrane vesicles prepared from GH_3 rat anterior pituitary cells [33]. [b] Values obtained from $^{45}Ca^{2+}$ influx measurements in GH_3 rat anterior pituitary cells under depolarising conditions [29].

Figure 1. Structure and inhibitory potency of amiloride and 3,4-dichlorobenzamil on ion transporters.

through the pharmacomodulation of the 5-amino moiety, the 6-chloro group or the 2-carbonylguanidine function. At the physiological pH (7.4) the acylguanidine moiety of amiloride **1** is positively charged ($pK_a = 8.7$) [35]. The structural features required for potent inhibition of the sodium–calcium exchanger are the presence of an aryl or an aralkyl on the distal nitrogen of the guanidine function, hydrophobic substituents on the 5-amino moiety, and a chlorine atom on the 6-position of the pyrazine ring. These structural features led to compounds able to inhibit ion transporters like sodium channels and voltage-operated calcium channels (L- and T-type) [28, 29, 35, 37, 38]. For instance, 3,4-dichlorobenzamil (**2**) is the most widely used reference inhibitor of the sodium–calcium exchanger. It was 37 times more potent than amiloride on the sodium–calcium exchanger (*figure 1*).

Despite its lack of specificity, 3,4-dichlorobenzamil (**2**) and its related compounds showed a positive inotropic effect combined with a negative chronotropic activity which was likely the result of the inhibition of sodium–calcium exchange activity [39, 40]. Hence such derivatives might represent a new type of inotropic agent [41, 42].

Searching novel, more potent and more selective inhibitors of the sodium–calcium exchanger, we have explored the bioisosteric replacement of the pyrazine ring of amiloride by a phenyl. The structural requirements described to inhibit the sodium–calcium exchanger were preserved: a carbonylguanidino group with a distal nitrogen substituted by an aralkyl group, two amino moieties and a chloro group in the corresponding positions compared with the structure of amiloride.

The novel and reference compounds were screened (10 μM) for their inhibitory potency on the sodium–calcium exchanger. Their activity was estimated as the inhibition of the intracellular Na^+-dependent $^{45}Ca^{2+}$ uptake by rat insulinoma cells (RINm5F) and human platelets. Dose–response curves were performed for the most active molecules.

2. Chemistry

2.1. Synthesis of amiloride and its N-guanidino substituted pyrazine derivatives

To prepare amiloride **1** and its described pyrazine derivatives **2**–**8** [43], the required *N*-substituted

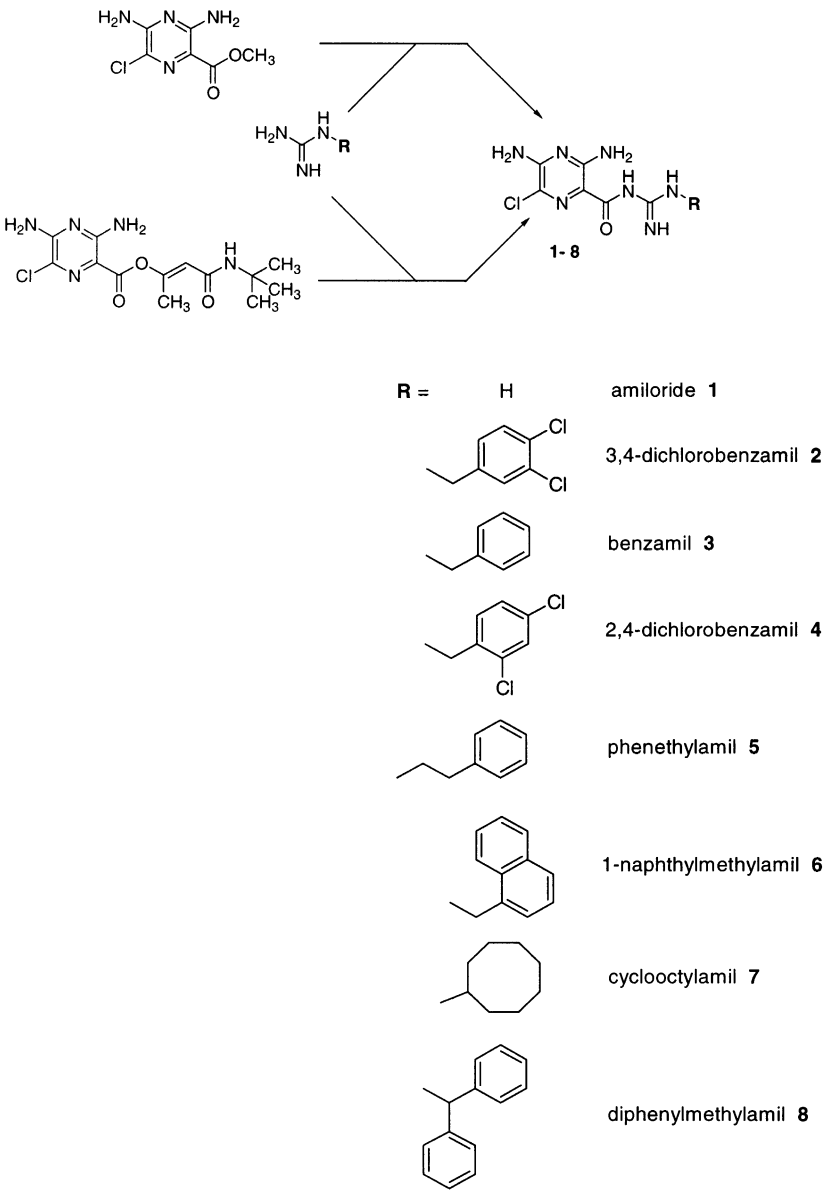


Figure 2. Pyrazine analogues of amiloride.

guanidine reacted with the methyl ester of 3,5-diamino-6-chloropyrazine-2-carboxylic acid [43] or with *N-tert*-butyl-3-[(3,5-diamino-6-chloropyrazine-2-carbonyl)oxy]-crotonamide [44, 45] (*figure 2*). This latter was much easier to handle and gave higher yields. The aralkylguanidines were obtained by using the well-known reaction of the desired amine with a methylthiopseudourea salt or of an aminium salt with cyanamide [46].

2.2. Synthesis of benzene analogues of amiloride and its N-guanidino substituted derivatives

As the methyl ester of 3,5-diamino-6-chloropyrazine-2-carboxylic acid was the key intermediate in the pyrazine series, the preparation of benzene ring analogues of amiloride was planned by a similar reaction between the methyl ester of 2,4-diamino-5-chlorobenzoic acid and

the required guanidine under related conditions. Several variations of the benzoic ester structure had also been foreseen, like amino-protected or nitro compounds, implying additional steps for reduction, protection and deprotection processes. The use of several activated esters was also considered in conjunction with protection/deprotection reactions of the amino substituents. Moreover, many attempts were carried out, often with relatively simple benzoic acid analogues. In particular, the reactions of 2-aminobenzoic acid and 2-amino-5-chlorobenzoic acid were studied and led to molecules which were also included in the pharmacological testing. The most satisfactory chemical pathway involved the synthesis of a benzoxazinone derivative **14** as key intermediate [47] (*figure 3*).

The synthesis of the benzene bioisosteres of amiloride derivatives (**17**–**22**) started from 3-chlorotoluene (**9**) (*figure 3*a). After nitration and reduction of both nitro groups, 2,4-diamino-5-chlorotoluene analogue **11** was acetylated (**12**), and then oxidised by $KMnO_4$ to give

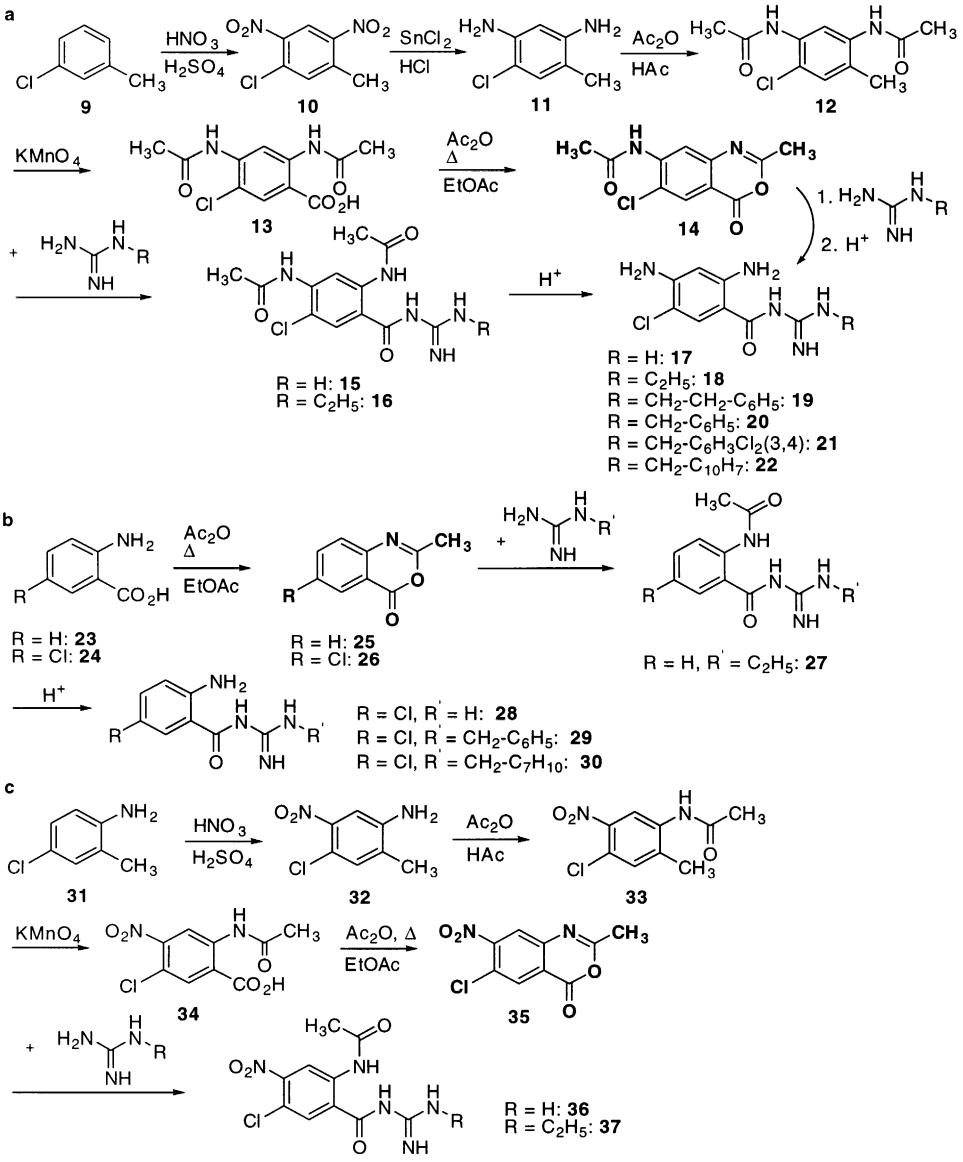


Figure 3. Synthesis of benzene analogues of amiloride **16**–**22**, **27**–**30**, and **36**–**37**.

2,4-diacetylamino-5-chlorobenzoic acid (**13**). By heating this latter in the presence of acetic anhydride, cyclo-dehydration occurred to give *N*-(6-chloro-2-methyl-4-oxo-4*H*-benzo[*d*][1,3]oxazin-7-yl)-acetamide (**14**). This key intermediate reacted with the appropriate guanidine leading to the corresponding acetylated carbonyl-guanidines (**15**, **16**) which underwent acid hydrolysis, generating the benzene amiloride derivatives substituted on the distal nitrogen of the guanidine moiety (**17**–**22**).

2-Aminobenzoic acid **23** and 2-amino-5-chlorobenzoic acid **24** were also employed in a reaction with acetic anhydride in order to produce the corresponding benzoxazinones **25** and **26** which finally reacted with the various guanidines to give molecules **27**–**30** (*figure 3*b).

2-Acetylamino-5-chloro-4-nitrobenzoic acid (**34**) was used in the same sequence of reactions leading to compounds **36** and **37** (*figure 3*c). The conditions of the reaction involving benzoxazinone **35** and the guanidine moiety were very similar. Several alternative synthetic pathways were also investigated to prepare other benzoylguanidines (*figure 4*). They included the reaction between isatoic anhydride **38** and guanidine (*figure 4*a). Generally, isatoic anhydride reacts with nucleophilic reagents but steric hindrance influences the outcome of the reaction [48]. Indeed, the only positive result was obtained with unsubstituted guanidine **39**. The usual preparation of guanidine derivatives from cyanamide and aminium salt was transposed to the acylcyanamide

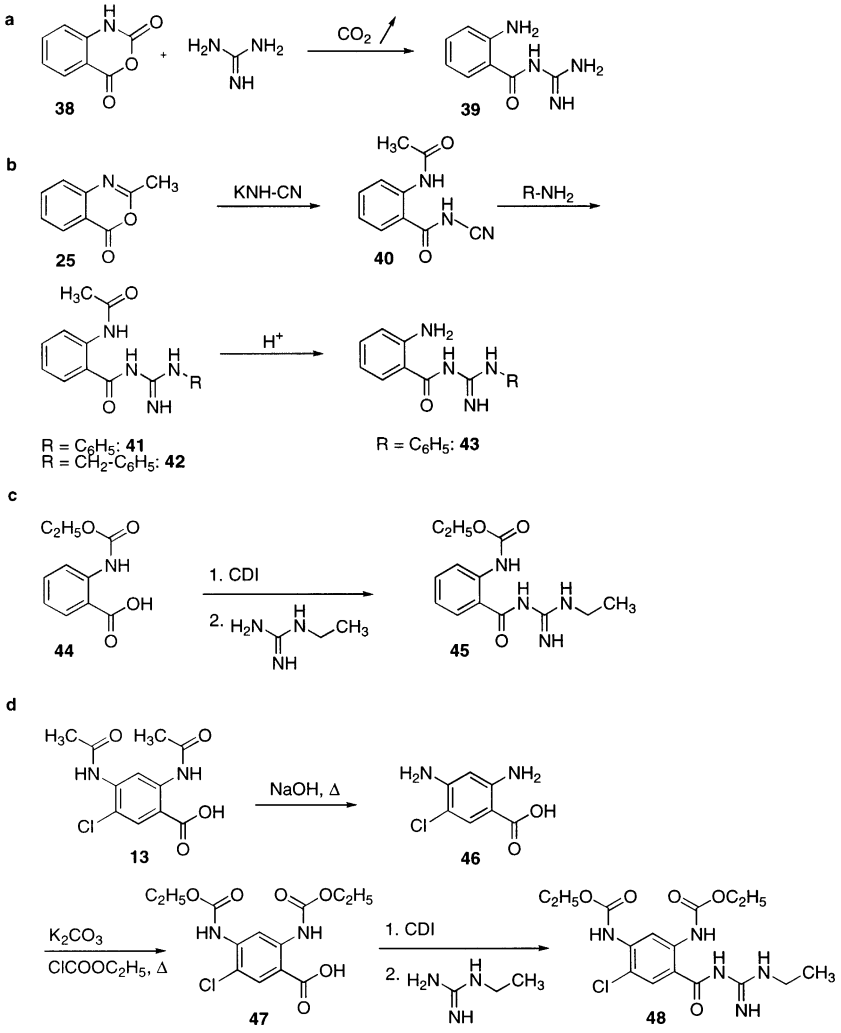


Figure 4. Synthetic pathways of compounds **39**–**54**.

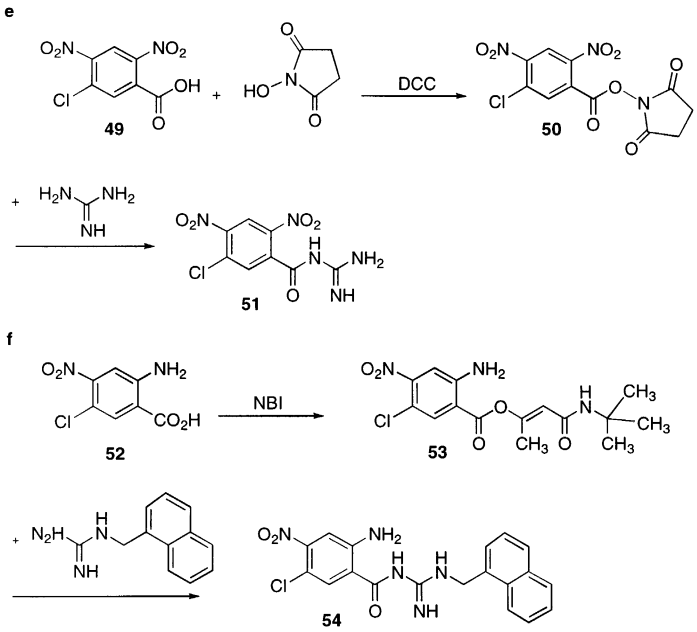


Figure 4. (*Continued*)

40 intermediate [49–52] (*figure 4*b). This approach gave the benzoylguanidines **41**–**43**. *N*,*N*′-carbonyldiimidazole (CDI), a carboxylate activating agent, was used for its high reactivity towards nucleophilic residues [53, 54]. The activated ester reacted with ethylguanidine and led to compound **45** (*figure 4*c). The reaction between 2,4-diethoxycarbonylamino-5-chlorobenzoic acid (**47**) and ethylguanidine gave the benzoylguanidine **48**, but its deacylation was unsuccessful (*figure 4*d). *N*-Hydroxysuccinimide was also used as activating agent of **49** [55], the resulting activated ester reacted with guanidine and gave **51** (*figure 4*e). Finally, the Woodward reagent, *N*-*tert*-butyl-5-methylisoxazolium perchlorate (NBI), employed as activating agent in peptide synthesis [56–58], was successfully used when it reacted with 2-amino-5-chloro-4-nitro-benzoic acid (**52**), but it gave multiple by-products when 2,4-diamino-5-chlorobenzoic acid (**46**) was the starting molecule (*figure 4*f).These alternative methods were generally less convenient than the 'benzoxazinone' pathway (*figure 3*), and could not be applied to a wide variety of *N*-substituted guanidine moieties.

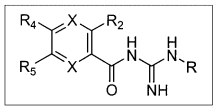
3. Pharmacology

The sodium–calcium exchanger inhibitory potency of the novel compounds and the reference drugs were evaluated on RINm5F cells and on human platelets. The RINm5F cells are rat clonal insulin-producing cells which express two isoforms of the sodium–calcium exchanger type-1, namely NCX1.3 and NCX1.7 [59]. Human platelets exhibit a sodium–calcium exchange activity supported by a K^+-dependent carrier where K^+ is transported with Ca^{2+} [60]. This platelet exchanger is identical to the sodium–calcium retinal exchanger RetX [11]. In both cell types, the sodium–calcium exchange activity was evaluated by measuring the intracellular Na^+-dependent $^{45}Ca^{2+}$ uptake in conditions where the sodium–calcium exchanger worked in reverse mode. The potential inhibitory activity of the tested compounds was assessed at the concentration of 10 μM. For the most active molecules in human platelets, it was determined that their concentration required a decrease of 50% the $^{45}Ca^{2+}$ uptake (IC_{50} values).

4. Results and discussion

In RINm5F cells (*table I*), amiloride (**1**) showed a weak inhibitory potency on $^{45}Ca^{2+}$ uptake. Its pyrazine derivatives bearing a hydrophobic substituent on the guanidine side-chain (**2**–**8**) are more potent. The

Table I. Inhibition of sodium–calcium exchange mediated $^{45}Ca^{2+}$ uptake by RINm5F cells in presence of pyrazine and novel benzene derivatives (10 μM).



Compound	X	R_2	R_4	R_5	R	Inhibition of $^{45}Ca^{2+}$ uptake at 10 μM (%) [a]
1	N	NH_2	NH_2	Cl	H	11.2 ± 4.5
2	N	NH_2	NH_2	Cl	3,4-$ClC_6H_3CH_2$	48.8 ± 4.0
3	N	NH_2	NH_2	Cl	$C_6H_5CH_2$	30.0 ± 2.1
4	N	NH_2	NH_2	Cl	2,4-$ClC_6H_3CH_2$	37.5 ± 1.6
5	N	NH_2	NH_2	Cl	$C_6H_5CH_2CH_2$	25.5 ± 3.4
6	N	NH_2	NH_2	Cl	1-naphtylCH_2	54.8 ± 25.8
7	N	NH_2	NH_2	Cl	cC_8H_{15}	44.1 ± 4.9
8	N	NH_2	NH_2	Cl	$(C_6H_5)_2CH$	14.5 ± 3.8
16	CH	$NHCOCH_3$	$NHCOCH_3$	Cl	C_2H_5	8.8 ± 2.2
17	CH	NH_2	NH_2	Cl	H	NA
18	CH	NH_2	NH_2	Cl	C_2H_5	17.3 ± 4.5
19	CH	NH_2	NH_2	Cl	$C_6H_5CH_2CH_2$	15.3 ± 5.1
20	CH	NH_2	NH_2	Cl	$C_6H_5CH_2$	55.1 ± 14.1
21	CH	NH_2	NH_2	Cl	3,4-$ClC_6H_3CH_2$	68.5 ± 4.8
22	CH	NH_2	NH_2	Cl	1-naphtylCH_2	96.9 ± 2.4
27	CH	$NHCOCH_3$	H	H	C_2H_5	NA
28	CH	NH_2	H	Cl	H	NA
29	CH	NH_2	H	Cl	$C_6H_5CH_2$	17.6 ± 2.5
30	CH	NH_2	H	Cl	1-naphtylCH_2	100.0 ± 4.2
36	CH	$NHCOCH_3$	NO_2	Cl	H	16.6 ± 6.1
37	CH	$NHCOCH_3$	NO_2	Cl	C_2H_5	NA
39	CH	NH_2	H	H	H	NA
41	CH	$NHCOCH_3$	H	H	C_6H_5	37.0 ± 2.0
42	CH	$NHCOCH_3$	H	H	$C_6H_5CH_2$	31.0 ± 4.3
43	CH	NH_2	H	H	C_6H_5	16.4 ± 2.8
45	CH	$NHCOOC_2H_5$	H	H	C_2H_5	NA
48	CH	$NHCOOC_2H_5$	$NHCOOC_2H_5$	Cl	C_2H_5	11.7 ± 5.7
51	CH	NO_2	NO_2	Cl	H	8.9 ± 2.2
54	CH	NH_2	NO_2	Cl	1-naphtylCH_2	100.0 ± 6.3

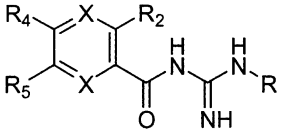
[a] Complete inhibition of Na_0-stimulated $^{45}Ca^{2+}$ uptake is considered as 100%. Mean ± S.E.M.
NA: not active at 10 μM.

highest inhibitory activity was observed when the substituent was a 3,4-dichlorobenzyl (**2**), a 2,4-dichlorobenzyl (**4**), a 1-naphthylmethyl (**6**) or a cyclooctyl (**7**). This is in agreement with published qualitative structure–activity relationships [5]. The absence of chlorine atoms on the benzyl moiety did not result in a total loss of inhibition (**3**). The bioisosteric replacement of the pyrazine by a benzene ring did not disrupt the established structure–activity relationships, and a similar range of activity was observed (*table I*). The benzene counterpart of amiloride **17** was inactive at 10 μM. The benzene derivatives bearing a benzyl (**20**), a 3,4-dichlorobenzyl (**21**) or a 1-naphthylmethyl (**22**) were more potent than their pyrazine counterpart (compare **2**–**21**, **3**–**20** and **6**–**22**). A short alkyl group on the guanidine side-chain (**18**) strongly reduced the biological activity. These results indicated that, to preserve the inhibitory activity, the distal nitrogen of the guanidine had to be substituted with a hydrophobic aromatic or cyclooctyl ring. When using a linker, a methylene was better than an ethylene link which strongly reduced the inhibitory potency whatever the nature of tetrasubstituted ring (compare **3**–**5** and **19**–**20**).

Among the other benzene derivatives (**27**–**54**), the two molecules bearing a 1-naphtylmethyl (**30** and **54**) completely inhibited the $^{45}Ca^{2+}$ uptake. When compared with the potency of **22**, this suggested that the R_4-amino is not required to keep a high inhibitory potency. Indeed, it could be omitted (**30**) or replaced by a nitro (**54**). Here again, the replacement of the R-phenyl (**41**) or the R-benzyl (**42**) by an ethyl (**27**) deleted the biological activity. Other molecules bearing an ethyl- or an unsubstituted-guanidine side-chain appeared as poor inhibitors (**36**, **48**, **51**) or are inactive (**28**, **37**, **39**, **45**).

For human platelets (*table II*), the complete inhibition of the RetX type sodium–calcium exchanger was considered as the inhibition of the $^{45}Ca^{2+}$ uptake in presence of 10 μM 3,4-dichlorobenzamil (**2**), the reference inhibitor. In the pyrazine series, the best results occurred with amiloride derivatives bearing on the guanidine side-chain a 2,4-dichlorobenzyl (**4**), a 1-naphthylmethyl (**6**) or a diphenylmethyl (**8**). Except for **8** and the benzyl derivative (**3**), these results are consistent with those obtained with the RINm5F cells. At 10 μM, amiloride (**1**) was not active on platelet $^{45}Ca^{2+}$ uptake.

Table II. Inhibition of sodium–calcium exchange mediated $^{45}Ca^{2+}$ uptake by human platelets in presence of pyrazine and novel benzene derivatives (10 μM).



Compound	X	R_2	R_4	R_5	R	Inhibition of $^{45}Ca^{2+}$ uptake at 10 μM (%) [a]
1	N	NH_2	NH_2	Cl	H	NA
2	N	NH_2	NH_2	Cl	3,4-$ClC_6H_3CH_2$	100.0 ± 3.4
3	N	NH_2	NH_2	Cl	$C_6H_5CH_2$	NA
4	N	NH_2	NH_2	Cl	2,4-$ClC_6H_3CH_2$	124.1 ± 42.0
5	N	NH_2	NH_2	Cl	$C_6H_5CH_2CH_2$	23.6 ± 3.6
6	N	NH_2	NH_2	Cl	1-naphtylCH_2	91.6 ± 4.6
7	N	NH_2	NH_2	Cl	cC_8H_{15}	21.3 ± 3.0
8	N	NH_2	NH_2	Cl	$(C_6H_5)_2CH$	118.0 ± 3.5
16	CH	$NHCOCH_3$	NH-$COCH_3$	Cl	C_2H_5	NA
17	CH	NH_2	NH_2	Cl	H	13.4 ± 1.8
18	CH	NH_2	NH_2	Cl	C_2H_5	35.5 ± 1.0
19	CH	NH_2	NH_2	Cl	$C_6H_5CH_2CH_2$	23.7 ± 3.0
20	CH	NH_2	NH_2	Cl	$C_6H_5CH_2$	47.6 ± 2.7
21	CH	NH_2	NH_2	Cl	3,4-$ClC_6H_3CH_2$	25.1 ± 3.5
22	CH	NH_2	NH_2	Cl	1-naphtylCH_2	NA
27	CH	$NHCOCH_3$	H	H	C_2H_5	NA
28	CH	NH_2	H	Cl	H	13.4 ± 0.3
29	CH	NH_2	H	Cl	$C_6H_5CH_2$	13.2 ± 3.2
30	CH	NH_2	H	Cl	1-naphtylCH_2	69.4 ± 3.0
36	CH	$NHCOCH_3$	NO_2	Cl	H	NA
37	CH	$NHCOCH_3$	NO_2	Cl	C_2H_5	NA
39	CH	NH_2	H	H	H	NA
41	CH	$NHCOCH_3$	H	H	C_6H_5	9.0 ± 1.3
42	CH	$NHCOCH_3$	H	H	$C_6H_5CH_2$	NA
43	CH	NH_2	H	H	C_6H_5	25.3 ± 0.9
45	CH	$NHCOOC_2H_5$	H	H	C_2H_5	NA
48	CH	$NHCOOC_2H_5$	NH-$COOC_2H_5$	Cl	C_2H_5	NA
51	CH	NO_2	NO_2	Cl	H	NA
54	CH	NH_2	NO_2	Cl	1-naphtylCH_2	110.0 ± 13.6

[a] Inhibition of $^{45}Ca^{2+}$ uptake with 10 μM of 3,4-dichlorobenzamil (**2**) was considered as 100%. Other drugs were used at 10 μM. Mean ± S.E.M. NA = not active at 10 μM.

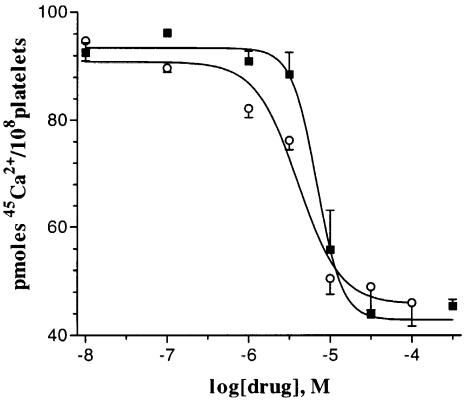


Figure 5. Concentration-response relationships of 3,4-dichlorobenzamil (**2**, ■) and **57** (○).

Table III. Drug concentration reducing of 50% (IC_{50}) the 3,4-dichlorobenzamil-sensitive $^{45}Ca^{2+}$ uptake by human platelets.

Compound	IC_{50} [a] (μmol)
1	690 ± 19
2	15.2 ± 2.6
4	11.2 ± 1.0
6	15.1 ± 2.9
8	25.5 ± 2.2
54	3.4 ± 0.7

[a] Mean ± S.E.M.

The replacement of the pyrazine by a benzene ring resulted in a loss of activity for the 3,4-dichlorobenzyl and for the 1-naphtylmethyl derivatives (compare **2**–**21** and **6**–**22**). Similar sodium–calcium exchanger inhibitory activity of the other benzene derivatives (**16**–**54**) was observed with RINm5F cells and human platelets. While many compounds were equally active on both cellular models, some of them (e.g. **22**) showed a preferential activity for one subtype exchanger NCX or RetX. Such differences between the two cellular models could be attributed to differences in the molecular structures of the two sodium–calcium exchanger type.

The most active compounds (**4**, **6**, **8**, **54**) on human platelets were selected to determine their concentration-response potency and compared to both references, amiloride (**1**) and 3,4-dichlorobenzamil (**2**) (*figure 5*). These curves led to determination of the concentration which decreased by 50% the $^{45}Ca^{2+}$ uptake (IC_{50}) by human platelets in the absence of extracellular Na^+ (*table III*). In the pyrazine series, the 2,4-dichlorobenzyl (**4**, $IC_{50} = 11.2$ μM) and the 1-naphtylmethyl derivatives (**6**, $IC_{50} = 15.1$ μM) were as potent as 3,4-dichorobenzamil (**2**, $IC_{50} = 15.2$ μM). The diphenylmethyl compound (**8**, $IC_{50} = 25.5$ μM) was less active than 3,4-dichlorobenzamil, but 27 times more potent than amiloride ($IC_{50} = 690$ μM). The benzene derivative **54**, bearing a 1-naphtylmethyl on the guanidine side-chain and where the R_4-amino was substituted by a nitro, was the most potent inhibitor ($IC_{50} = 3.4$ μM), four times more active than 3,4-dichlorobenzamil, the reference compound.

5. Conclusion

In conclusion, among the novel derivatives related to the structure of amiloride, several compounds inhibited the sodium–calcium exchange activity. Many structures were found to be active as inhibitors of the sodium–calcium exchanger of both NCX and RetX types. According to the pK_a value of the acylguanidine moiety of amiloride ($pK_a = 8.7$), **3** ($pK_a = 8.1$) and **5** ($pK_a = 8.2$) [36] which are weak inhibitors, the protonated acylguanidine side-chain is probably not the biologically active species at physiological pH (7.4). The results obtained also highlighted that small structural differences in the new compounds allowed modulation of the inhibitory activity of both exchanger types. For instance, the replacement of the NO_2 (**54**) by a NH_2 (**22**) in position 4 did not modify the biological response of the NCX sodium–calcium exchanger (*table I*), while it did suppress the activity on the RetX subtype (*table II*). This substitution reduced the ability of the drug to behave like a H-bond donor for the RetX exchanger, and probably could explain this loss of activity. These novel pharmacological tools will certainly be useful to elucidate the structure–activity relationships and to lead to the discovery of specific inhibitors of the sodium–calcium exchanger.

6. Experimental protocols

6.1. Chemistry

Melting points were determined on a Büchi–Tottoli apparatus and were uncorrected. IR spectra were recorded as KBr pellets on a Perkin–Elmer 1750 FT

spectrophotometer. The ^{1}H-NMR spectra were recorded on a Bruker AW-80 (80 MHz) instrument in $CDCl_3$ or in DMSO-d_6 with HMDS as an internal standard or on a Bruker AW-400 (400 MHz) instrument in DMSO-d_6 with TMS as an internal standard. Chemical shifts are reported in δ values (ppm) relative to internal HMDS or TMS. The abbreviations, e.g. s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, and br. s = broad signal are used throughout. For most amiloride analogues, the high number of protons bound to nitrogen atoms and the hydrate form increase the occurrence of exchange and greatly influence the shift values which depend on the sample concentration and temperature. Elemental analyses (C, H, N) were carried out on 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.

The main pathways leading to amiloride **1** and its pyrazine derivatives (**2**–**8**) have been described [43–45], and the preparation of aralkylguanidine compounds has previously been reported [46].

*6.1.1. 5-Chloro-2,4-dinitrotoluene (**10**)*

The title compound was prepared as previously described [61, 62] (yield: 45%): m.p. 88–89 °C; ^{1}H-NMR (80 MHz, $CDCl_3$) δ 3.70 (s, 3H, C*H*$_3$), 7.60 (s, 1H, 6*H*-phenyl), 8.61 (s, 1H, 3*H*-phenyl); IR (KBr) 3422, 1607, 1583, 1525, 1373, 1343, 834, 740 cm^{-1}. Anal.: $C_7H_5N_2ClO_4$: (C, H, N).

*6.1.2. 2,4-Diamino-5-chlorotoluene (**11**) (adapted from Ref. [61])*

Portionwise 5-chloro-2,4-dinitrotoluene (**10**) (15 g, 0.07 mol) was added to stirred HCl 12N (100 ml). Tin chloride (100 g) was dissolved in HCl 12N (210 ml) and added dropwise to the hydrochloric solution of **10** at 40 °C for about 90 min. After an additional 1 h stirring at 70–75 °C, most of the hydrochloric acid was evaporated under reduced pressure and the residue was diluted with 30% NaOH (50 ml). The alkaline suspension was continuously extracted by $CHCl_3$ overnight. The organic phase was washed with H_2O, dried, filtered and the solvent was evaporated under reduced pressure to yield a brownish residue. Crystallisation from toluene afforded the title compound **11** as yellow needles (5.4 g, 50%): m.p. 119–120 °C; ^{1}H-NMR (80 MHz, $CDCl_3$) δ 1.96 (s, 3H, C*H*$_3$), 3.44 (br. s, 4H, N*H*$_2$), 6.04 (s, 1H, 3*H*-phenyl), 6.80 (s, 1H, 6*H*-phenyl); IR (KBr) 3452, 3361, 1629, 1585, 1433, 1296, 1251 cm^{-1}. Anal.: $C_7H_9ClN_2$: (C, H, N).

*6.1.3. 2,4-Diacetylamino-5-chlorotoluene (**12**)*

The title compound was prepared from 2,4-diamino-5-chlorotoluene (**11**) according to a procedure previously described [61] (yield: 80%): m.p. 260 °C; ^{1}H-NMR (80 MHz, DMSO-d_6): δ 2.04 (s, 3H, C*H*$_3$), 2.09 (s, 3H, C*H*$_3$), 2.15 (s, 3H, C*H*$_3$-phenyl), 7.18 (s, 1H, 6*H*-phenyl), 7.80 (s, 1H, 3*H*-phenyl), 9.01 (s, 1H, N*H*), 9.17 (s, 1H, N*H*); IR (KBr): 3231, 1661, 1526, 1282, 880, 729 cm^{-1}. Anal.: $C_{11}H_{13}N_2ClO_2$: (C, H, N).

*6.1.4. 2,4-Diacetylamino-5-chlorobenzoic acid (**13**)*

In a round-bottom flask, a mixture of $MgSO_4$ (3 g, 0.025 mol) and **12** (10 g, 0.04 mol) in H_2O (200 ml) was stirred and heated at 80 °C. $KMnO_4$ (16 g, 0.1 mol) was added portionwise and the mixture was stirred at 85–90 °C for 1 h. The exceeding $KMnO_4$ was then reduced using $NaHSO_3$; insoluble MnO_2 was removed by filtration of the hot mixture and washed with hot H_2O (2×50 ml). The combined filtrates were acidified with 20% H_2SO_4, and the crude compound **13** precipitated. After standing at 4 °C overnight, it was filtered, washed with H_2O and dried. Crystallisation from ethyl alcohol gave the title compound as white crystals (6.75 g, 60%): m.p. 263–265 °C; ^{1}H-NMR (80 MHz, $CDCl_3$+DMSO-d_6): δ 2.13 (s, 3H, C*H*$_3$), 2.16 (s, 3H, C*H*$_3$), 7.99 (s, 1H, 3*H*-phenyl), 9.23 (s, 2H, 6*H*-phenyl+N*H*), 11.23 (s, 1H, N*H*); IR (KBr): 3416, 3133, 2938, 2686, 1835, 1610, 1583, 1535, 1494, 1401, 877, 823, 793 cm^{-1}. Anal.: $C_{11}H_{11}N_2ClO_4$: (C, H, N).

*6.1.5. N-(6-Chloro-2-methyl-4-oxo-4H-benzo[d][1,3]-oxazin-7-yl)-acetamide (**14**)*

A mixture of ethyl acetate (5 ml), acetic anhydride (10 ml) and **13** (1 g, 0.004 mol) was refluxed for 3.5 h. The mixture was then evaporated under reduced pressure. The white residue formed was crystallised from ethyl acetate to give the title compound **14**, which was filtered, washed with petroleum ether 40–60 °C, and dried (0.65 g, 70%): m.p. 193–195 °C; ^{1}H-NMR (80 MHz, DMSO-d_6) δ 2.18 (s, 3H, C*H*$_3$), 2.30 (s, 3H, C*H*$_3$), 8.14 (s, 1H, 8*H*), 8.25 (s, 1H, 5*H*), 9.69 (s, 1H, N*H* amide); IR (KBr) 3047, 1769, 1646, 1541, 1270, 1212 cm^{-1}. Anal.: $C_{11}H_9N_2ClO_3$: (C, H, N).

*6.1.6. (2,4-Diacetylamino-5-chlorobenzoyl)guanidine (**15**)*

Under nitrogen atmosphere and stirring, NaH (0.8 g of a 60% dispersion, 0.02 mol) was added to a mixture of guanidine hydrochloride (1.91 g, 0.02 mol) and

dimethylformamide (10 ml), and the resulting mixture was heated at 50 °C for 20 min. After cooling to room temperature (r.t.), *N*-(6-chloro-2-methyl-4-oxo-4*H*-benzo[*d*][1,3]oxazin-7-yl)-acetamide (**14**) (2.53 g, 0.01 mol) was added and the mixture stirred for 30 min. Afterwards, it was diluted with H_2O (60 ml) and crude product **15** precipitated. It was collected, washed with H_2O, and dried. It was dissolved in HCl 6N with charcoal. The suspension was filtered and the filtrate diluted with 10% NaOH. After standing at 4 °C for 1 h, the crystalline compound **15** was filtered, washed with H_2O, and dried (1.71 g, 55%): m.p. 263–265 °C (decomposition); ^{1}H-NMR (80 MHz, DMSO-d_6) δ 2.02 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 7.42 (br. s, 4H, *guanidine*), 8.04 (s, 1H, 3*H*-phenyl), 8.84 (s, 1H, 6*H*-phenyl), 9.41 (s, 1H, N*H* amide), 12.79 (s, 1H, N*H* amide); IR (KBr) 3398, 3360, 1656, 1590, 1545, 1339, 1304, 869 cm^{-1}. Anal.: $C_{12}H_{14}N_5ClO_3$: (C, H, N).

6.1.7. *N*-(2,4-Diacetylamino-5-chlorobenzoyl)-*N*′-ethylguanidine (**16**)

The title compound was prepared from **14** and ethylguanidine sulphate as described for **15**. The crude product **16** was dissolved in a mixture of ethyl alcohol–dimethylformamide (3:1), and stirred with charcoal. After filtration, **16** was precipitated by water addition. **16** was filtered, washed with H_2O, and dried (yield 25–35%): m.p. 226–228 °C; ^{1}H-NMR (80 MHz, DMSO-d_6) δ 1.07 (t, 3H, CH$_2$–CH_3), 2.02 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 3.18 (m, 2H, CH_2–CH$_3$), 7.76 (br. s, 4H, *guanidine*), 8.08 (s, 1H, 3*H*-phenyl), 8.84 (s, 1H, 6*H*-phenyl), 9.41 (s, 1H, N*H* amide), 10.54 (s, 1H, N*H* amide); IR (KBr): 3384, 3264, 1677, 1602, 1582, 1502, 1333, 1261 cm^{-1}. Anal.: $C_{14}H_{18}N_5ClO_3$: (C, H, N).

6.1.8. (2,4-Diamino-5-chlorobenzoyl)guanidine dihydrochloride (**17**)

A suspension of **15** (1 g, 0.003 mol) in methanol (25 ml) was bubbled with HCl and stirred for 1 h. After cooling for 2 h, the crystalline compound **17** was collected, washed with diethyl ether, and dried (0.43 g, 45%): m.p. >300 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 6.12 (s, 1H, 3*H*-phenyl), 7.04 (br. s, *guanidine*), 8.00 (s, 1H, 6*H*-phenyl), 8.39 (br. s, 2H, 4-NH_2), 8.62 (br. s, 2H, 2-NH_2); IR (KBr) 3376, 3306, 1676, 1634, 1572, 1538, 1501, 1273, 894, 721 cm^{-1}. Anal.: $C_8H_{10}N_5ClO{\cdot}2HCl$: (C, H, N).

6.1.9. *N*-(2,4-Diamino-5-chlorobenzoyl)-*N*′-ethylguanidine dihydrochloride hydrate (**18**)

A suspension of **16** (1 g, 0.003 mol) in methanol (25 ml) was bubbled with HCl and stirred for 1.5 h. Then, diethyl ether (50 ml) was added and a precipitate was formed. After cooling for 2 h, the crystalline compound **18** was collected, washed with diethyl ether, and dried (0.3 g, 30%): m.p. 192–197 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.18 (t, 3H, CH$_2$–CH_3), 3.34 (m, 2H, CH_2–CH$_3$), 5.90 (br. s, *guanidine*), 6.11 (s, 1H, 3*H*-phenyl), 8.06 (s, 1H, 6*H*-phenyl), 8.72 (br. s, 2H, 4-NH_2), 9.02 (br. s, 2H, 2-NH_2), 9.49 (s, 1H, CON*H*); IR (KBr) 3347, 3292, 3024, 1681, 1631, 1610, 1542, 1475, 1264, 889 cm^{-1}. Anal.: $C_{10}H_{14}N_5ClO{\cdot}2HCl{\cdot}H_2O$: (C, H, N).

6.1.10. *N*-(2,4-Diamino-5-chlorobenzoyl)-*N*′-(2-phenylethyl)guanidine dihydrochloride hydrate (**19**)

Under nitrogen atmosphere and stirring, NaH (0.2 g of a 60% dispersion, 0.005 mol) was added to a mixture of (2-phenylethyl)guanidine hydrochloride (1 g, 0.005 mol) and tetrahydrofuran (30 ml), and the resulting mixture was heated at 50 °C for 20 min. After cooling to r.t., *N*-(6-chloro-2-methyl-4-oxo-4*H*-benzo[*d*][1,3]-oxazin-7-yl)-acetamide (**14**) (1.26 g, 0.005 mol) was added, then the insoluble matter was discarded by filtration and the filtrate was evaporated under reduced pressure. The residue was dissolved in methanol (25 ml) and HCl bubbled. The precipitate formed (**19**) was collected, washed with diethyl ether, and dried (0.31 g, 15%): m.p. 172–177 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.90 (t, 2H, NH–CH$_2$–CH_2-phenyl), 3.56 (m, 2H, NH–CH_2–CH$_2$-phenyl), 5.37 (br. s, *guanidine*), 6.09 (s, 1H, 3*H*-phenyl), 7.22–7.41 (m, 5H, NH–CH$_2$–CH$_2$-*phenyl*), 8.02 (s, 1H, 6*H*-phenyl), 8.82 (br. s, 2H, 4-NH_2), 9.04 (br. s, 2H, 2-NH_2), 9.49 (s, 1H, CON*H*); IR (KBr) 3329, 3289, 3028, 1683, 1629, 1607, 1543, 1520, 1466, 1263, 893, 699 cm^{-1}. Anal.: $C_{16}H_{18}N_5ClO{\cdot}2HCl{\cdot}H_2O$: (C, H, N).

6.1.11. *N*-(2,4-Diamino-5-chlorobenzoyl)-*N*′-benzylguanidine dihydrochloride (**20**)

The title compound was prepared from **14** and benzylguanidine hydrochloride as described for **19** (yield 10–25%): m.p. 300–302 °C (decomposition); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.60 (d, 2H, NH–CH_2-phenyl), 6.10 (s, 1H, 3*H*-phenyl), 6.31 (br. s, *guanidine*), 7.31–7.46 (m, 5H, NH–CH$_2$-*phenyl*), 8.05 (s, 1H, 6*H*-phenyl), 8.93 (br. s, 2H, 4-NH_2), 9.17 (br. s, 2H, 2-NH_2), 9.85

(br. s, 1H, CON*H*); IR (KBr) 3300, 3290, 3023, 1677, 1632, 1609, 1539, 1465, 1262, 880 cm^{-1}. Anal.: $C_{15}H_{16}N_5ClO \cdot 2HCl$: (C, H, N).

*6.1.12. N-(2,4-Diamino-5-chlorobenzoyl)-N′-(3,4-dichlorobenzyl)guanidine dihydrochloride hydrate (**21**)*

The title compound was prepared from **14** and (3,4-dichlorobenzyl)guanidine hydrochloride as described for **19** (yield 15–20%): m.p. 298–300 °C (decomposition); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.62 (d, 2H, NH–CH_2–$C_6H_3Cl_2$(3,4)), 5.80 (br. s, *guanidine*), 6.11 (s, 1H, 3*H*-phenyl), 7.40 (q, 1H, 6′*H*–$C_6H_3Cl_2$(3,4)), 7.67 (d, 1H, 5′*H*–$C_6H_3Cl_2$(3,4)), 7.70 (d, 1H, 2′*H*–$C_6H_3Cl_2$-(3,4)), 8.05 (s, 1H, 6*H*-phenyl), 8.98 (br. s, 2H, 4-NH_2), 9.16 (br. s, 2H, 2-NH_2), 9.94 (s, 1H, CON*H*); IR (KBr) 3341, 3296, 3024, 1693, 1632, 1609, 1544, 1476, 1263, 885, 824, 713 cm^{-1}. Anal.: $C_{15}H_{14}N_5Cl_3O \cdot 2HCl \cdot H_2O$: (C, H, N).

*6.1.13. N-(2,4-Diamino-5-chlorobenzoyl)-N′-(1-naphtylmethyl)guanidine dihydrochloride hydrate (**22**)*

The title compound was prepared from **14** and (1-naphthylmethyl)guanidine hydrochloride as described for **19** (yield 15–25%): m.p. 184–188 °C (decomposition); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.08 (d, 2H, NH–CH_2-naphtyl), 5.66 (br. s, *guanidine*), 6.10 (s, 1H, 3*H*-phenyl), 7.52–8.07 (m, 8H, napthyl+6*H*-phenyl), 9.05 (br. s, 2H, 4-NH_2), 9.26 (br. s, 2H, 2-NH_2), 9.81 (s, 1H, CON*H*); IR (KBr) 3307, 3154, 2926, 1691, 1624, 1540, 1520, 1269, 888, 724 cm^{-1}. Anal.: $C_{19}H_{20}N_5ClO \cdot 2HCl \cdot H_2O$: (C, H, N).

*6.1.14. 2-Methyl-4H-3,1-benzoxazin-4-one (**25**)*

The title compound was prepared from 2-aminobenzoic acid (**23**) according to a procedure previously described [63] (yield 75%): m.p. 72–75 °C; IR (KBr) 2932, 1757, 1694, 1646, 1299, 1266, 1230, 781 cm^{-1}. Anal.: $C_9H_7NO_2$: (C, H, N).

*6.1.15. 6-Chloro-2-methyl-4H-3,1-benzoxazin-4-one (**26**)*

The title compound was prepared from 2-amino-5-chlorobenzoic acid (**24**) according to a procedure previously described [64] (yield 75%): m.p. 119–121 °C; IR (KBr) 3475, 1705, 1657, 1371, 1293, 1230 cm^{-1}. Anal.: $C_9H_6NClO_2$: (C, H, N).

*6.1.16. N-(2-Acetylaminobenzoyl)-N′-ethylguanidine (**27**)*

To a solution of ethylguanidine sulphate (2.72 g, 0.01 mol) in dimethylformamide (10 ml), NaH (0.8 g of a 60% dispersion, 0.02 mol) was added, and the mixture was heated at 50 °C for 20 min. After cooling to r.t., 2-methyl-4*H*-3,1-benzoxazin-4-one (**25**) (1.61 g, 0.01 mol) was added and the mixture stirred for 1 h. Afterwards, it was poured on H_2O (40 ml), the precipitate formed (**27**) was collected, washed with H_2O, and dried. It was purified by dissolution in ethyl alcohol with charcoal. After filtration, **27** precipitated by water addition (1 g, 40%); m.p. 174–178 °C; IR (KBr) 3343, 3184, 1672, 1617, 1593, 1301, 756 cm^{-1}. Anal.: $C_{12}H_{16}N_4O_2$: (C, H, N).

*6.1.17. (2-Amino-5-chlorobenzoyl)guanidine dihydrochloride (**28**)*

Under nitrogen atmosphere and stirring, NaH (0.8 g of a 60% dispersion, 0.02 mol) was added to a mixture of guanidine hydrochloride (1.91 g, 0.02 mol) and dimethylformamide (10 ml), and the resulting mixture was heated at 50 °C for 20 min. After cooling to r.t., 6-chloro-2-methyl-4*H*-3,1-benzoxazin-4-one (**26**) (1.97 g, 0.01 mol) was added and the mixture stirred for 30 min. Afterwards, it was diluted with H_2O (80 ml), the precipitate formed was filtered, washed with H_2O, and dried (1.36 g, 50%). Crude (2-acetylamino-5-chlorobenzoyl)guanidine hydrate was suspended in methanol (15 ml) and HCl was bubbled. After 2 h, the precipitate **28** was collected, washed with diethyl ether, and dried (yield 80–85%): m.p.>300 °C; IR (KBr) 3277, 2807, 1721, 1695, 1623, 1544, 1291, 837 cm^{-1}. Anal.: $C_8H_9N_4ClO \cdot 2HCl$: (C, H, N).

*6.1.18. N-(2-Amino-5-chlorobenzoyl)-N′-benzylguanidine hydrochloride hemihydrate (**29**)*

The title compound was prepared from **26** and benzylguanidine hydrochloride as described for **28** (yield 30–35%). It was crystallised from a mixture of isopropanol–methanol (1:2): m.p. 126–131 °C; IR (KBr) 3438, 3032, 1687, 1619, 1575, 1453, 1243, 837 cm^{-1}. Anal.: $C_{15}H_{15}N_4ClO \cdot HCl \cdot 0.5H_2O$: (C, H, N).

*6.1.19. N-(2-Amino-5-chlorobenzoyl)-N′-(1-naphtylmethyl)guanidine hydrochloride (**30**)*

Under nitrogen atmosphere and stirring, potassium *tert*-butyl oxide (0.62 g, 0.0055 mol) was added to a mixture of (1-naphthylmethyl)guanidine hydrochloride (1.30 g, 0.0055 mol) and dioxane (15 ml), and the resulting mixture was heated at 50 °C for 15 min. After

cooling to r.t., 6-chloro-2-methyl-4*H*-3,1-benzoxazin-4-one (**26**) (0.98 g, 0.005 mol) was added and the mixture stirred for 20 min. Afterwards, the half volume of solvent was evaporated under reduced pressure, the residue diluted with cold H_2O (80 ml), and the precipitate formed was collected, washed with H_2O, and dried. The crude acetylated derivative was suspended in methanol (20 ml) and HCl was bubbled. After 2 h., the precipitate formed was collected, washed with diethyl ether, and dried. Crystallisation from a mixture of isopropanol–methanol (1:1) gave the title compound **30** (yield 20–25%); m.p. 199–201 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.10 (d, 2H, NH-CH_2-naphtyl), 6.87 (d, 1H, 3*H*-phenyl), 7.32 (q, 1H, 4*H*-phenyl), 7.52–8.08 (m, 8H, *napthyl*+6*H*-phenyl), 8.50 (br. s, *guanidine*), 9.22 (br. s, 2H, 2-NH_2), 9.78 (s, 1H, CON*H*); IR (KBr) 3431, 3305, 3094, 1690, 1652, 1620, 1573, 1497, 1454, 1239, 824 cm^{-1}. Anal.: $C_{19}H_{17}N_4ClO{\cdot}HCl$: (C, H, N).

*6.1.20. 4-Chloro-2-methyl-5-nitroaniline (**32**)*

The title compound was prepared from 4-chloro-2-methylaniline (**31**) according to a procedure previously described [65] (yield 45–50%): m.p. 121–123 °C; ^{1}H-NMR (80 MHz, $CDCl_3$) δ 2.16 (s, 3H, CH_3), 3.84 (s, 2H, NH_2), 7.12 (s, 1H, 3*H*-phenyl), 7.17 (s, 1H, 6*H*-phenyl); IR (KBr) 3415, 3336, 1639, 1517, 1356, 1322, 882 cm^{-1}. Anal.: $C_7H_7N_2ClO_2$: (C, H, N).

*6.1.21. 4-Chloro-2-methyl-5-nitroacetanilide (**33**)*

The title compound was prepared from **32** according to a procedure previously described [65, 66] (yield 90–95%): m.p. 133–137 °C; ^{1}H-NMR (80 MHz, $CDCl_3$+DMSO-d_6) δ 2.10 (s, 3H, 2-CH_3), 2.25 (s, 3H, CH_3 amide), 7.25 (s, 1H, 3*H*-phenyl), 8.28 (s, 1H, 6*H*-phenyl), 8.97 (s, 1H, N*H*); IR (KBr) 3256, 1664, 1533, 1347, 1301, 894 cm^{-1}. Anal.: $C_9H_9N_2ClO_3$: (C, H, N).

*6.1.22. 2-Acetylamino-5-chloro-4-nitrobenzoic acid (**34**)*

The title compound was prepared from **33** according to a procedure previously described [66] (yield: 35–40%): m.p. 239–243 °C (decomposition); ^{1}H-NMR (80 MHz, $CDCl_3$+DMSO-d_6) δ 2.09 (s, 3H, CH_3), 8.08 (s, 1H, 6*H*-phenyl), 8.95 (s, 1H, 3*H*-phenyl), 11.01 (s, 1H, N*H*); IR (KBr) 3247, 3187, 3119, 1684, 1648, 1544, 1520, 1364, 710 cm^{-1}. Anal.: $C_9H_7N_2ClO_5$: (C, H, N).

*6.1.23. 6-Chloro-2-methyl-7-nitro-4H-3,1-benzoxazin-4-one (**35**)*

The title compound was prepared from **34** as described for **14** (yield 65–70%): m.p. 149–154 °C; IR (KBr) 3428, 1769, 1646, 1541, 1356, 1310, 1270 cm^{-1}. Anal.: $C_9H_5N_2ClO_4$: (C, H, N).

*6.1.24. (2-Acetylamino-5-chloro-4-nitrobenzoyl)-guanidine hydrochloride (**36**)*

Under nitrogen atmosphere and stirring, potassium *tert*-butyl oxide (1.12 g, 0.01 mol) was added to a mixture of guanidine hydrochloride (0.96 g, 0.01 mol) and dimethylformamide (6 ml), and the resulting mixture was heated at 50 °C for 30 min. After cooling to r.t., 6-chloro-2-methyl-7-nitro-4*H*-3,1-benzoxazin-4-one (**35**) (1.2 g, 0.005 mol) was added and the mixture stirred for 60 min. Afterwards, it was poured on cold H_2O (120 ml), and the yellow precipitate formed was collected, and washed with H_2O. It was purified by dissolution in HCl 6N with charcoal. After filtration, **36** precipitated by 10% NaOH addition. **36** was filtered, washed with H_2O, and dried. Crystallisation from isopropanol gave the title compound (0.25 g, 15%): m.p. 250–255 °C (decomposition); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.21 (s, 3H, CH_3), 7.18 (br. s, *NH*), 8.10 (br. s, N*H*), 8.30 (s, 1H, 6*H*-phenyl), 8.87 (br. s, N*H*), 9.16 (s, 1H, 3*H*-phenyl), 11.01 (s, 1H, CON*H*); IR (KBr) 3434, 3027, 1674, 1628, 1542, 1520, 1343 cm^{-1}. Anal.: $C_{10}H_{10}N_5ClO_4{\cdot}HCl$: (C, H, N).

*6.1.25. N-(2-Acetylamino-5-chloro-4-nitrobenzoyl)-N′-ethylguanidine hydrochloride (**37**)*

Under nitrogen atmosphere and stirring, potassium *tert*-butyl oxide (0.27 g, 0.004 mol) was added to a mixture of ethylguanidine sulphate (0.33 g, 0.002 mol) and dioxane (13 ml), and the resulting mixture was heated at 50 °C for 15 min. After cooling to r.t., 6-chloro-2-methyl-7-nitro-4*H*-3,1-benzoxazin-4-one (**35**) (0.52 g, 0.0022 mol) was added and the mixture refluxed for 2 h. Afterwards, the dioxane was evaporated under reduced pressure, the residue was diluted with cold H_2O (75 ml), and the yellow precipitate formed was collected, washed with H_2O, and dried. It was dissolved in ethyl alcohol, and a HCl saturated diethyl ether solution was added to give the title compound **37**. It was filtered, washed with diethyl ether, and dried (0.24 g, 30%): m.p. 157–158 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.09 (t, 3H, CH_2–CH_3), 2.10 (s, 3H, CH_3), 3.39 (m, 2H, CH_2–CH_3), 8.12 (s, 1H, 6*H*-phenyl), 8.45 (br. s, N*H*), 8.78 (br. s, N*H*), 9.18 (s, 1H, 3*H*-phenyl), 10.81 (s, 1H,

CON*H*); IR (KBr) 3466, 3350, 1679, 1647, 1564, 1533, 1349, 1303, 1226 cm^{-1}. Anal.: $C_{12}H_{14}N_5ClO_4 \cdot HCl$: (C, H).

*6.1.26. (2-Aminobenzoyl)guanidine dihydrochloride (**39**)*

Under nitrogen atmosphere and stirring, NaH (1.2 g of a 60% dispersion, 0.03 mol) was added to a mixture of guanidine hydrochloride (2.88 g, 0.03 mol) and dimethylformamide (10 ml), and the resulting mixture was heated at 50 °C for 20 min. After cooling to r.t., isatoic anhydride (**38**) (1.63 g, 0.01 mol) was added portionwise and the mixture stirred for 3 h. Afterwards, it was diluted with H_2O (50 ml) and extracted three times by a mixture of acetone–$CHCl_3$ (1:1). The organic phases were dried and evaporated under reduced pressure. The residue was dissolved in methanol (10 ml) and HCl was bubbled. After saturation of the solution, it was diluted with diethyl ether (20 ml) and **39** precipitated. It was collected, washed with diethyl ether, and dried (0.38 g, 15%): m.p. 267–268 °C; ^{1}H-NMR (80 MHz, DMSO-d_6) δ 6.05 (br. s, 5H, *guanidine*), 6.52 (m, 1H, 5*H*-phenyl), 6.80 (d, 1H, 3*H*-phenyl), 7.24 (m, 1H, 4*H*-phenyl), 7.94 (d, 1H, 6*H*-phenyl), 8.54 (s, 3H, NH_3^+); IR (KBr) 3377, 3213, 3033, 1711, 1694, 1556, 1494, 1271, 741 cm^{-1}. Anal.: $C_8H_{10}N_4O \cdot 2HCl$: (C, H, N).

*6.1.27. N-(2-Acetylaminobenzoyl)cyanamide (**40**)*

Under nitrogen atmosphere and stirring, cyanamide (1.26 g, 0.03 mol) was added to a solution of potassium *tert*-butyl oxide (3.37 g, 0.03 mol) in dry tetrahydrofuran (30 ml), and the resulting mixture was refluxed for 30 min. After cooling to r.t., **25** (1.61 g, 0.01 mol) was added and the mixture stirred for 30 min. The precipitate formed was collected, washed with tetrahydrofuran and diethyl ether, and dried. It was dissolved in a minimum amount of H_2O, the solution was adjusted to pH 1 with HCl 6N, and the precipitate formed (**40**) was filtered, washed with H_2O, and dried (1.32 g, 65%): m.p. 155–157 °C; ^{1}H-NMR (80 MHz, $CDCl_3$+DMSO-d_6) δ 2.14 (s, 3H, CH_3), 7.06 (m, 1H, 5*H*-phenyl), 7.57 (m, 3H, 3*H*+4*H*-phenyl+N*H*CN), 8.48 (d, 1H, 6*H*-phenyl), 10.63 (s, 1H, N*H*); IR (KBr) 3478, 3154, 2988, 2256, 1666, 1589, 1501, 1431, 1370, 759 cm^{-1}. Anal.: $C_{10}H_9N_3O_2$: (C, H, N).

*6.1.28. N-(2-Acetylaminobenzoyl)-N′-phenylguanidine (**41**)*

Under nitrogen atmosphere and stirring, a solution of aniline (0.23 g, 0.0025 mol) in dry toluene (5 ml) was added to a suspension of **40** (0.51 g, 0.0025 mol) in dry toluene (5 ml), and the resulting mixture was refluxed for 1.5 h. Afterwards, it was cooled to r.t. and allowed to stand at 4 °C for 2 h. The precipitate formed was filtered, washed with petroleum ether 40–60 °C, and dried. Crystallisation from a mixture of ethyl alcohol–H_2O (1:5) afforded the title compound **41** (0.3 g, 40%): m.p. 164–166 °C; ^{1}H-NMR (80 MHz, DMSO-d_6) δ 1.63 (s, 3H, CH_3), 7.10 (m, 3H, 3*H*+5*H*-phenyl+N*H*), 7.31 (s, 5H, *phenyl*), 8.20 (m, 3H, 4*H*+6*H*-phenyl+N*H*), 9.47 (s, 1H, CON*H*), 12.32 (s, 1H, N*H*); IR (KBr) 3364, 3335, 3039, 1661, 1634, 1588, 1552, 1488, 1340, 755, 705 cm^{-1}. Anal.: $C_{16}H_{16}N_4O_2$: (C, H, N).

*6.1.29. N-(2-Acetylaminobenzoyl)-N′-benzylguanidine (**42**)*

The title compound was prepared from benzylamine as described for **41** (yield 20–25%). It was crystallised from a mixture of methanol–H_2O (1:4): m.p. 139–142 °C; IR (KBr) 3429, 3345, 3027, 1672, 1631, 1556, 1495, 1352, 1304, 760, 693 cm^{-1}. Anal.: $C_{17}H_{18}N_4O_2$: (C, H, N).

*6.1.30. N-(2-Aminobenzoyl)-N′-phenylguanidine dihydrochloride (**43**)*

Compound **41** (0.3 g, 0.001 mol) and 70% $HClO_4$ (0.5 ml) were suspended in methanol (10 ml), and the suspension was stirred at r.t. After one hour, it was diluted with H_2O (40 ml) and neutralised with $NaHCO_3$. Afterwards, it was concentrated under reduced pressure and extracted with a mixture of acetone–$CHCl_3$ (1:1) (3×20 ml). The organic phases were dried and evaporated under reduced pressure. The residue was dissolved in methanol (15 ml) and HCl was bubbled until **43** precipitated. It was collected, washed with diethyl ether, and dried (0.23 g, 70%): m.p. 224–226 °C; IR (KBr) 3244, 3099, 2875, 1698, 1641, 1598, 1572, 1496, 1305, 753 cm^{-1}. Anal.: $C_{14}H_{14}N_4O \cdot 2HCl$: (C, H, N, chlorides).

*6.1.31. N-(2-Ethoxycarbonylaminobenzoyl)-N′-ethylguanidine (**45**)*

Under nitrogen atmosphere, *N*-ethoxycarbonylanthranilic acid (**44**) (2.09 g, 0.01 mol) was added to a solution of CDI (1.62 g, 0.01 mol) in dimethylformamide (30 ml), and the mixture was stirred at r.t. for 1 h.

Under nitrogen atmosphere and stirring, NaH (0.8 g of a 60% dispersion, 0.02 mol) was added to a solution of ethylguanidine sulphate (2.72 g, 0.01 mol) in dimethylformamide (15 ml), and the mixture was heated at 50 °C for 20 min. and then allowed to cool to r.t.

Both prepared solutions were mixed and stirred at r.t. for 1 h. Afterwards, the final mixture was poured on H_2O (200 ml), the precipitate formed was collected and washed with H_2O. It was purified by dissolution in HCl 1N at 40 °C with charcoal. After filtration, the filtrate was neutralised with $NaHCO_3$. The precipitate formed (**45**) was filtered, washed with H_2O, and dried (1.11 g, 40%): m.p. 134–136 °C; IR (KBr) 3417, 3354, 3064, 2988, 2901, 1697, 1627, 1602, 1559, 1506, 1458, 1443, 1343, 1249, 762 cm^{-1}. Anal.: $C_{13}H_{18}N_4O_3$: (C, H, N).

*6.1.32. 2,4-Diamino-5-chlorobenzoic acid (**46**)*

A solution of 2,4-diacetylamino-5-chlorobenzoic acid (**13**) (4 g, 0.015 mol) in NaOH 1N (60 ml) was refluxed for 5 h. After cooling to r.t., the medium was adjusted to pH 5 with acetic acid 2N, and a precipitate was formed. After standing at 4 °C for one hour, it was collected, washed with H_2O, and dried. Crystallisation from ethyl acetate gave the title compound **46** (2.52 g, 90%): m.p. 170–172 °C; ^{1}H-NMR (80 MHz, DMSO-d_6) δ 5.62 (br. s, 2H, 4-NH_2), 5.96 (s, 1H, 3*H*-phenyl), 7.42 (s, 1H, 6*H*-phenyl), 8.15 (br. s, 3H, 2-NH_3^+); IR (KBr) 3496, 3382, 3343, 1680, 1626, 1588, 1422, 1290, 1261, 900 cm^{-1}. Anal.: $C_7H_7N_2ClO_2$: (C, H, N).

*6.1.33. 2,4-Diethoxycarbonylamino-5-chlorobenzoic acid (**47**)*

A mixture of 2,4-diamino-5-chlorobenzoic acid (**46**) (1.87 g, 0.02 mol), K_2CO_3 (2.8 g, 0.02 mol), ethyl chloroformate (5.7 ml, 0.06 mol) and dioxane (30 ml) was refluxed for 2 h. Exceeding ethyl chloroformate was evaporated under reduced pressure and insoluble potassium salts were discarded by filtration. The filtrate was diluted with H_2O (90 ml), the precipitate formed was collected, washed with H_2O, and dried. Crystallisation from a mixture of ethyl alcohol–H_2O (5:1) gave the title compound **47** (3.31 g, 50%): m.p. 208–212 °C; ^{1}H-NMR (80 MHz, $CDCl_3$+DMSO-d_6) δ 1.31 (q, 4H, CH_2–CH_3), 4.20 (t, 6H, CH_2–CH_3), 7.84 (s, 1H, 3*H*-phenyl), 7.97 (s, 1H, 6*H*-phenyl), 9.20 (s, 2H, N*H*COOC_2H_5); IR (KBr) 3251, 3107, 1733, 1674, 1526, 1422, 1407, 880 cm^{-1}. Anal.: $C_{13}H_{15}N_2ClO_6$: (C, H, N).

*6.1.34. N-(2,4-Diethoxycarbonylamino-5-chlorobenzoyl)-N′-ethylguanidine (**48**)*

The title compound was prepared from 2,4-diethoxycarbonylamino-5-chlorobenzoic acid (**47**) as described for **45** (yield 20–25%): m.p. 180–184 °C; IR (KBr) 3450, 3410, 3358, 2985, 1727, 1617, 1557, 1486, 1434, 1330, 1248, 1216, 883, 713 cm^{-1}. Anal.: $C_{16}H_{22}N_5ClO_5$: (C, H, N).

*6.1.35. 5-Chloro-2,4-dinitrobenzoic acid (**49**)*

The title compound was prepared from 5-chloro-2,4-dinitrotoluene (**10**) according to a procedure previously described [67] (yield 35–40%): m.p. 180–181 °C; ^{1}H-NMR (80 MHz, $CDCl_3$+DMSO-d_6) δ 8.05 (s, 1H, 6*H*-phenyl), 8.62 (s, 1H, 3*H*-phenyl); IR (KBr) 3436, 2881, 1719, 1542, 1347, 1301, 840 cm^{-1}. Anal.: $C_7H_3N_2ClO_6$: (C, H, N).

*6.1.36. 1-[(5-Chloro-2,4-dinitrobenzoyl)oxy]-pyrrolidine-2,5-dione (**50**)*

5-Chloro-2,4-dinitrobenzoic acid (**49**) (0.74 g, 0.003 mol) was added to a solution of *N*-hydroxysuccinimide (0.38 g, 0.0033 mol) in dry tetrahydrofuran (8 ml), and the mixture was stirred at temperature lowered to −5 to −10 °C. A solution of DCC (0.68 g, 0.0033 mol) in dry tetrahydrofuran (3 ml) was added and the resulting mixture was stirred at r.t. overnight. The suspension was filtered, the insoluble matter discarded, and the filtrate evaporated under reduced pressure. The residue was dissolved in saturated $NaHCO_3$ and extracted with $CHCl_3$ (4×20 ml). The combined organic phases were dried and evaporated under reduced pressure. The residue was crystallised from petroleum ether 100–140 °C to give the title compound **50** (0.46 g, 45%): m.p. 159–160 °C. Anal.: $C_{11}H_6N_3ClO_8$: (C, H, N).

*6.1.37. (5-Chloro-2,4-dinitrobenzoyl)guanidine hydrochloride (**51**)*

Na (0.115 g, 0.005 mol) was added to a suspension of guanidine hydrochloride (0.48 g, 0.005 mol) in a mixture of tetrahydrofuran–ethyl alcohol (1:1) (25 ml), and the resulting mixture was stirred for 20 min at r.t. A solution of 1-[(5-chloro-2,4-dinitrobenzoyl)oxy]pyrrolidine-2,5-dione (**50**) (1.7 g, 0.005 mol) in tetrahydrofuran (15 ml) was added dropwise, and the mixture refluxed for 1.5 h. Afterwards, the solvents were evaporated under reduced pressure, the residue triturated with $CHCl_3$ (30 ml), and the insoluble material discarded by filtration. The filtrate was then extracted with HCl 1N (4×20 ml). The combined acidic phases were evaporated under reduced pressure and the residue was suspended in a mixture of acetone–diethyl ether (3:1). The suspension was filtered, and then the crystalline compound **51** was dried (0.49 g, 30%); m.p. 248–249 °C; ^{1}H-NMR (80 MHz, DMSO-d_6) δ 8.42 (s, 1H, 6*H*-phenyl), 8.84 (br. s,

5H, *guanidine*+3*H*-phenyl), 9.19 (s, 1H, CON*H*); IR (KBr) 3421, 3352, 3080, 1720, 1585, 1532, 1348, 1278, 891, 559 cm^{-1}. Anal.: $C_8H_6N_5ClO_5 \cdot HCl$: (C, H, N).

*6.1.38. 2-Amino-5-chloro-4-nitrobenzoic acid (**52**) (adapted from Ref. [68])*

A mixture of 2-acetylamino-5-chloro-4-nitrobenzoic acid (**33**) (4 g, 0.015 mol), acetic acid (32 ml) and HCl 12N (32 ml) was refluxed for 1 h. After cooling to r.t., it was poured on H_2O (200 ml), and the yellow precipitate formed was filtered, washed with H_2O, and dried. Crystallisation from 10% sodium acetate gave the title compound **52** (2.6 g, 80%); m.p. 268–270 °C; IR (KBr) 3501, 3387, 3069, 1680, 1622, 1583, 1528, 1429, 1363, 1254, 824, 710 cm^{-1}. Anal.: $C_7H_5N_2ClO_4$: (C, H, N).

*6.1.39. N-tert-Butyl-3-[(2-amino-5-chloro-4-nitrobenzenecarbonyl)oxy]crotonamide (**53**)*

NBI (1.2 g, 0.005 mol) was added to a solution of 2-amino-5-chloro-4-nitrobenzoic acid (**52**) (1.08 g, 0.005 mol) and triethylamine (0.5 g, 0.005 mol) in dimethylformamide (5 ml), and the resulting mixture was stirred at r.t. for 2 h. Afterwards, it was poured on iced water (60 ml), and the precipitate formed was collected, washed with H_2O, and dried. Crystallisation from acetonitrile gave the title compound **53** (1.51 g, 85%): m.p. 164–166 °C; IR (KBr) 3506, 3374, 3060, 2967, 2927, 1707, 1682, 1640, 1536, 1437, 1394, 1366, 1343, 1201, 1148, 874, 729 cm^{-1}. Anal.: $C_{15}H_{18}N_3ClO_5$: (C, H, N).

*6.1.40. N-(2-Amino-5-chloro-4-nitrobenzoyl)-N′-(1-naphtylmethyl)guanidine (**54**)*

Under nitrogen atmosphere and stirring, potassium *tert*-butyl oxide (0.31 g, 0.00275 mol) was added to a mixture of (1-naphthylmethyl)guanidine (0.65 g, 0,00275 mol) in dioxane (10 ml) and dimethylformamide (2 ml), and the mixture was heated at 50 °C for 10 min. After cooling to r.t., *N-tert*-butyl-3-[(2-amino-5-chloro-4-nitrobenzenecarbonyl)oxy]crotonamide (**53**) (0.89 g, 0.0025 mol) was added, and the resulting mixture refluxed for 20 min. Afterwards, dioxane was evaporated under reduced pressure and the residue was diluted with H_2O (30 ml). The insoluble material was collected, washed with H_2O, and dried. Crystallisation from ethyl alcohol gave the title compound **54** (0.45 g, 45%): m.p. 230–231 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.93 (br. s, 2H, CH_2-naphtyl), 7.28 (s, 1H, 6*H*-phenyl), 7.51–8.06 (m, 10H, *naphtyl*+3*H*-phenyl+N*H*), 8.67 (s, 2H, 2-NH_2), 9.49 (s, 1H, CON*H*); IR (KBr) 3475, 3416, 1617, 1580, 1556, 1521, 1472, 1360, 1301, 775 cm^{-1}. Anal.: $C_{19}H_{16}N_5ClO_3$: (C, H, N).

6.2. Pharmacology

Ouabain was purchased from Sigma (Brussels, Belgium) and $^{45}CaCl_2$ from NEN Products (Brussels, Belgium).

6.2.1. ^{45}Calcium uptake by RINm5F cells

Cloned insulin-producing RINm5F cells were grown in RPMI medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (from Life Technologies, Merelbeke, Belgium).

The incubation medium was a Krebs–Ringer buffered solution (pH 7.4, 37 °C) bubbled with oxygen (100%), and containing (in mM): NaCl 139, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, Hepes/NaOH 1. If required, NaCl was iso-osmotically replaced by sucrose (241 mM) and HEPES/NaOH by HEPES/KOH, and K^+ concentration was maintained at 5 mM.

Cells (3×10^6) were removed from plates by trypsine and centrifuged (500 *g*, 3 min). The supernatant was removed, and the cells were incubated at 37 °C in the Krebs–Ringer buffer (1 ml) in the presence or absence of drug. After 30 min, the cell suspension was centrifuged (500 *g*, 3 min) and the pellet was incubated at 37 °C for five min in the same medium (1 ml) containing $^{45}CaCl_2$ (200 μCi ml^{-1}). Hence, the effect of these drugs was evaluated after 30 min exposure. At the end of the incubation, the uptake of ^{45}Ca was stopped by addition of 5 ml of the ice-cold Krebs–Ringer solution containing $LaCl_3$ (2 mM) and albumin (20 mg ml^{-1}). The pH was adjusted to pH 7.1. The suspension was centrifuged again 20 min later (500 *g*, 3 min), and the radioactive supernatant was discarded. The cells were suspended in 1 ml of the same ice-cold stop solution. Aliquots (0.1 ml) of this suspension were then placed in polyethylene microcentrifuge tubes and centrifuged (30 s, Beckman Microfuge model B) to deposit the cells in the tip of the tube. After addition of 0.2 ml of a mixture of di-*n*-butylphthalate and di-*n*-nonyl phthalate (10:3), a second centrifugation (30 s) was performed to separate the cells from the medium. The bottom of the tube was cut and transferred to a counting vial containing 5 ml of scintillation fluid (Lumagel, Lumac, The Netherlands). The $^{45}Ca^{2+}$ content was evaluated using a β-counter (Packard Tricarb 460C). The uptake of $^{45}Ca^{2+}$ was expressed as femtomoles of $Ca^{2+}/10^6$ cells with the

specific activity ($^{45}Ca/^{40}Ca$) of the incubation medium. The complete inhibition of Na_0-stimulated $^{45}Ca^{2+}$ uptake was considered as 100%. The results were expressed as means±S.E.M.

6.2.2. ^{45}Calcium uptake by human washed platelets

Platelet-rich plasma (PRP) provided by the Belgian Red Cross was washed three times by centrifugation at 1000 *g* for 10 min in a buffered solution (pH 7.4) containing (in mM): NaCl 140, KCl 5, glucose 10, EGTA 0.2, aspirin 0.1, Hepes 10. To the third washing solution, BSA 0.1% was added, and EGTA was omitted. Finally, washed platelets were suspended in a buffered medium with the following composition (in mM): NaCl 137, KCl 2.7, glucose 5.6, $MgCl_2$ 1, $CaCl_2$ 0.2, NaH_2PO_4 3.3, HEPES 4, Tris ad pH 7.4. Aliquots (150 μl) of washed platelets ($1–2\times10^6$ cells μL^{-1}) were incubated for 30 min at 37 °C with 800 μl of buffered medium containing (in mM): *N*-methyl-D-glucamine 137, KCl 2.7, $MgCl_2$ 1, $CaCl_2$ 0.2, glucose 5.6, NaH_2PO_4 3.3, Hepes 4, HCl ad pH 7.4, ouabain 0.1. When required, 50 μl of this medium were replaced by 50 μl of the tested drug. Afterwards, $^{45}Ca^{2+}$ uptake was initiated by adding 50 μl of $^{45}CaCl_2$ (2–3 μCi ml^{-1}). After 3 h of incubation, the uptake was stopped by diluting the platelet suspension with 5 ml of an ice-cold buffer (in mM: NaCl 137, KCl 2.7, $MgCl_2$ 1, NaH_2PO_4 3.3, EGTA 5, Hepes 4, Tris ad pH 7.4), followed by rapid filtration through a glass-fibre filter (Wathman GF/C). The tube was rinsed twice with the ice-cold buffer. The filters were transferred to a counting vial with 5 ml of scintillation fluid (Lumagel, Lumac, The Netherlands). The $^{45}Ca^{2+}$ platelet content was evaluated using a β-counter (Packard Tricarb 460C). The non-specific radioactivity was considered as the residual radioactivity of a sample immediately filtered and washed after the addition of $^{45}CaCl_2$. It was subtracted from the total radioactivity. 3,4-Dichlorobenzamil was used as a reference inhibitor. The drug potency was calculated according to the following equation: $\%\text{inhibition} = 100-[1-((D-DCB)/(CTRL-DCB))]$, where CTRL and DCB are the intracellular radioactivities measured in the absence and presence of 3,4-dichlorobenzamil (10 μM), respectively. D is the intracellular radioactivity measured in the presence of the drug under study. Each drug was examined in triplicate. Concentration-response relationships were obtained from three independent experiments performed in triplicate. The drug concentration reducing of 50% (IC_{50}) the 3,4-dichlorobenzamil-sensitive $^{45}Ca^{2+}$ uptake was determined by non-linear regression analysis (GraphPad Prism software) and IC_{50} values were expressed as means±S.E.M.

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