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Letter

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Identification of Isoform 2 Acid-Sensing Ion Channel Inhibitors as Tool Compounds for Target Validation Studies in CNS

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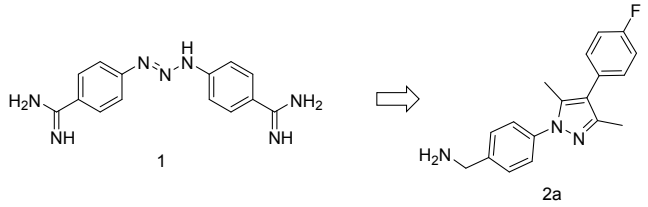
KEYWORDS: ASICs, ion channels, drug discovery, CNS, PNS, cancer.

ABSTRACT: Acid-sensing ion channels (ASICs) are a family of ion channels permeable to cations and largely responsible for the onset of acid-evoked ion currents both in neurons and in different types of cancer cells, thus representing a potential target for drug discovery. Owing to the limited attention ASIC2 has received so far, an exploratory programme was initiated to identify ASIC2 inhibitors using Diminazene, a known *pan*-ASIC inhibitor, as a chemical starting point for structural elaboration. The performed exploration enabled the identification of a novel series of ASIC2 inhibitors. In particular, compound **2u** is a brain penetrant ASIC2 inhibitor endowed with an optimal pharmacokinetic profile. This compound may represent a useful tool to validate in animal models *in vivo* the role of ASIC2 in different neurodegenerative central nervous system pathologies.

The variation of proton concentration in tissues is a tightly controlled process.[1] In particular, a decrease of pH has been observed both in physiological conditions, i.e. control of neuronal functions by proton-mediated signalling, and pathological conditions.[2,3] Notably, acidification occurring in pathological conditions was found to recruit acid-sensing ion channels (ASICs), a family of proton-activated ion channels[4] that are highly expressed both in central and peripheral neurons[5] and in different types of cancer cells,[6] thus representing a potential target for drug discovery.[3,7] ASICs are voltage-insensitive ion channels belonging to the ENaC/DEG channel super-family, which includes epithelial Na^+ channels (ENaC) and degenerins (DEG). Four ASICs genes (*ASIC1-4*) and two specific splice variants for ASIC1 and ASIC2 (a and b) have been described in mammals to date. ASIC1a, ASIC2a, and ASIC2b are primarily expressed in central nervous system (CNS) neurons, while all subunits are expressed in the peripheral nervous system (PNS). ASIC1a, ASIC 2a and ASIC3 subunits assemble to form both homotrimeric and heterotrimeric channels, whereas ASIC2b and ASIC4 only contribute to forming heteromeric channels with other ASIC subunits.[7,8] In terms of electrophysiology, while ASIC1a undergoes rapid inactivation, for ASIC2 and ASIC3 a non-inactivated current was observed, potentially relevant in chronic pathologies. Thus far, both ASIC1 and ASIC3 have been extensively studied,[9] while ASIC2 has received much less attention. Notably, ASIC2 have recently been proposed as relevant target in some forms of cancer[10,11] whereas, in combination with ASIC1 subunits, appear to play a key role in neuronal physiopathology.[12] Several natural peptides and synthetic small molecules, i.e. diminazene **1** (DA, Chart 1), an anti-infective veterinary drug, have been described as ASICs inhibitor.[13,14,15] However, the latter compound shows both poor target and ASIC isoform specificity, along with negligible blood-brain barrier (BBB) penetration, hence limiting its usage as therapeutic agent both for CNS and PNS pathologies.[16,17] Hence, an exploratory projects was initiated, using DA as chemical starting point, to obtain brain penetrant ASIC2 inhibitors as useful tool compounds for target validation studies in CNS. To this aim, being ASIC1a and ASIC2a the most highly expressed ASIC subunits in CNS neurons, the new chemical entities (NCEs) synthesized were specifically tested for their effects on murine homotrimeric ASIC1a and ASIC2a and on the heterotrimeric ASIC1a/2a.[18]

Chart 1. Structure of diminazene (DA) 1 and early lead 2a

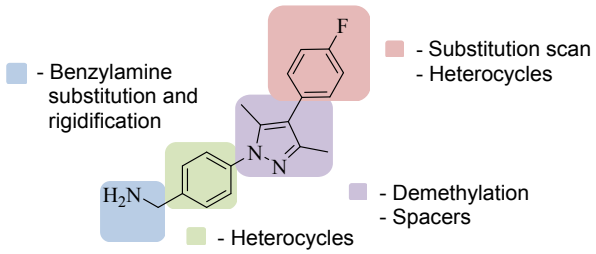


To identify the most appropriate methodology for *in vitro* screening of NCEs, a series of published data[19,20,21] on both compound **1** and Amiloride, a diuretic drug known to interact with ASICs, specifically drawn our attention and an optical technology based on membrane potential detection by voltage-sensitive dyes (VSDs) [22,23,24,25] was proposed. This original assay format showed an adequate throughput performance and ability to efficiently predict the inhibitory effect of ASICs inhibitors. Moreover, additional evidence suggested the use of optic based assays, owing to the membrane potential sensitivity of ASIC1a binding affinity to small molecules.[20]

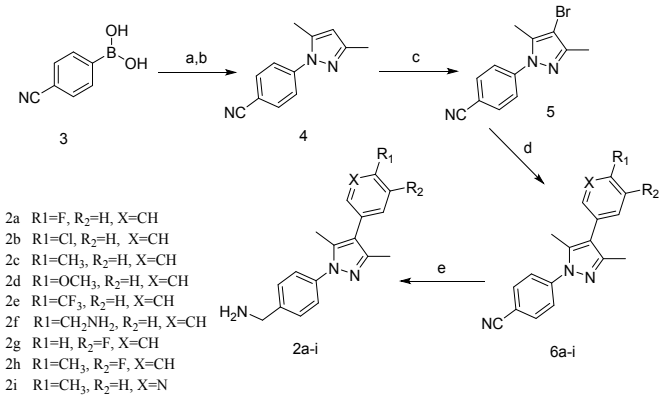
The preliminary structural elaboration of DA, in three sequential steps, enabled the identification, of the 1,4-diaryl, 3-5-dimethylpyrazole derivative **2a** (Chart 1), an early lead compound which was fully characterized in terms of ASICs inhibition. In particular, the linear triazene linker present in DA was initially replaced by a 1,3-disubstituted 5-membered heterocycle, with the aim to rigidify the molecular core. Then, to reduce the basic character of the molecule, with the aim to improve drug-like character and possibly secure BBB permeability, one of the two amidine functions was successfully removed, Finally, an initial exploration was made on the effect of the substitution of the terminal phenyl ring.

As shown in Table 1, compound **2a** exhibited greater *in vitro* activity than compound **1** and comparable activity on ASIC2a and ASIC1a/2a (IC_{50} = 18.9 μM and 10.9 μM, respectively), while being inactive on ASIC1a. Based on these preliminary encouraging results, the rapid "4 points" analoging exploration strategy, as depicted in Chart 2, focused on the sequential elaboration of the two aryl moieties ("pink and green"), the heterocycle core ("fuchsia") and the suitable rigidification/masking of the potentially metabolically labile terminal benzyl function ("cyan"), was envisioned.

Chart 2. Structural optimization of early lead 2a



Scheme 1. Variations on the 4-aryl substituent: compounds 2a-i[a]



[a]Reagents and conditions: (a) di-*tert*-butyl diazene 1,2-dicarboxylate, $Cu(OAc)_2{\cdot}H_2O$, MeOH, 65°C, 1h; (b) pentane-2,4-dione, 4N HCl in dioxane, r.t., 10 min then 80°C, 10 min, 76% (two steps); (c) NBS, EtOAc, sonication, 25-30°C, 15 min, 80%; (d) substituted aryl/pyridinyl boronic acid, $Pd(PPh_3)_4$, aq. Na_2CO_3, DMF, microwave reactor, 140°C, 15-20 min, 52%-70%; (e) $LiAlH_4$, THF, r.t, 0.5-3 h, 18-75%.

At first, following the synthetic strategy shown in Scheme 1 nine "pink" analogues **2a-i** were synthesized**.** In particular, p-cyanophenyl boronic acid **3** was reacted with di-*tert*-butyl diazene 1,2-dicarboxylate and 2,4-pentanedione, to obtain the 3,5-dimethyl pyrazole **4**. The following bromination reaction with NBS led to the 4-bromo analogue **5**, which was coupled with 9 different aryl or heteroaryl substituted boronic acids. The resulting 1-(p-cyanophenyl) pyrazoles **6a-i** were reduced to the corresponding benzylamines **2a-i** with lithium aluminium hydride in moderate to good overall yields.

The inhibition of ASICs constructs by compounds **2a-i** is reported in Table 1.

Table 1. Compounds 2a-i: ASICs inhibition[a]

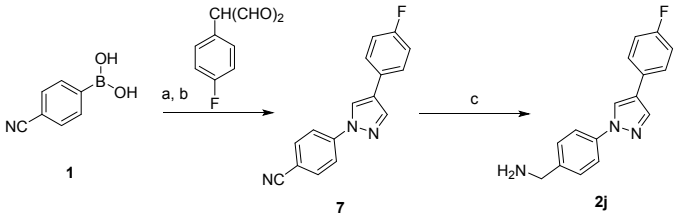
Entry	ASIC1a [a]	ASIC2a [a]	ASIC1a/2a [a]
1 (DA)	56.9 ± 8.9	169.0 ± 18.1	45.2 ± 8.8
2a	>30	18.9 ± 6.9	10.9 ± 1.6
2b	>30	>30	>30
2c	>30	8.8 ± 2.0	>30
2d	>30	16.3 ± 4.6	>30
2e	>30[b]	>30[b]	>30
2f	>30	>30	>30
2g	>30	4.3 ± 0.5	>30
2h	>30	>30	>30
2i	>30	>30	>30

[a] IC_{50} were determined as described in the Supplementary Information; they are expressed in μM and are the average value of at least n=3 independent experiments ± SEM.

As shown in Table 1, monosubstituted compounds **2c** and **2d**, bearing a p-CH_3 or p-OCH_3 respectively, mostly retained the activity of the pyrazole lead compound **2a**, while isoform-selectivity for ASIC2a *vs* ASIC1a/2a was improved. Compound **2g**, the m-F analogue of **2a**, was the most potent and selective compound of this series. Notably, larger and/or charged functions (**2e**, p-CF_3; **2f**, p-CH_2NH_2), disubstituted aryls (**2h**, p-CH_3, m-F) and the presence of a pyridine as phenyl replacement (**2i,** p-CH_3, X=N) led to inactive compounds.

Then, the influence of the 3,5-dimethyl substituents present on the pyrazole core was evaluated by synthesizing the corresponding "fuchsia" des-methyl analogue **2j** (Scheme 2).

Scheme 2. Scaffold hopping: des-methyl compound 2j[a]



[a]Reagents and conditions: (a) di-*tert*-butyl diazene-1,2-dicarboxylate, $Cu(OAc)_2{\cdot}H_2O$, MeOH, 65°C , 1h; (b) 2-(4-fluorophenyl)propanedial, 4N HCl in dioxane, r.t., 10 min then 80°C, 10 min, 56% two steps; (c) $LiAlH_4$, THF, r.t, 1h, 73%.

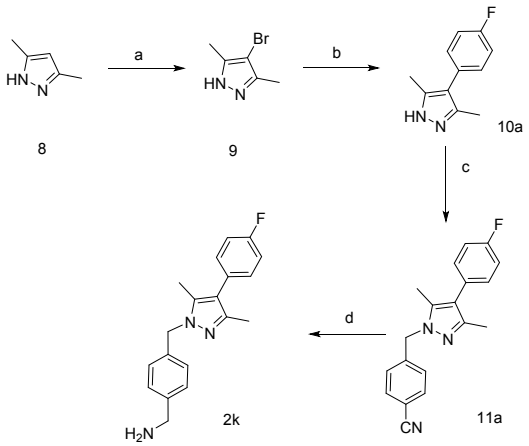
To this aim, p-cyanophenyl boronic acid **3** was sequentially treated with di-*tert*-butyl diazene-1,2-dicarboxylate in presence of $Cu(OAc)_2{\cdot}H_2O$ in MeOH at 65°C for 1h, followed by 2-(4-fluorophenyl)propanedial in 4N HCl in dioxane initially at room temperature for 10 min, then at 80°C for additional 10 min to give intermediate **7** in 56% yield. Then, reduction of the cyano group led to the corresponding benzylamine **2j** with lithium aluminium hydride in 73% yield.

Compound **2j** was found inactive in terms of ASICs inhibition (IC_{50} > 30 μM on all ASICs constructs, Table 2), pointing out the

relevance of both methyl groups for the recognition of the receptor binding site.

To acquire additional SAR information, a methylene spacer was introduced between the N_1 of the pyrazole moiety ("fuchsia" compound **2k**, Scheme 3) by bromination and Suzuki coupling on 3,5-dimethyl pyrazole **8**, followed by alkylation at the N_1 position of 3,5-dimethyl pyrazole derivative **10a** with p-CN benzyl bromide, and by final reduction of the nitrile group with lithium aluminium hydride in poor, unoptimized yields.

Scheme 3. Spacer introduction: compound 2k[a]



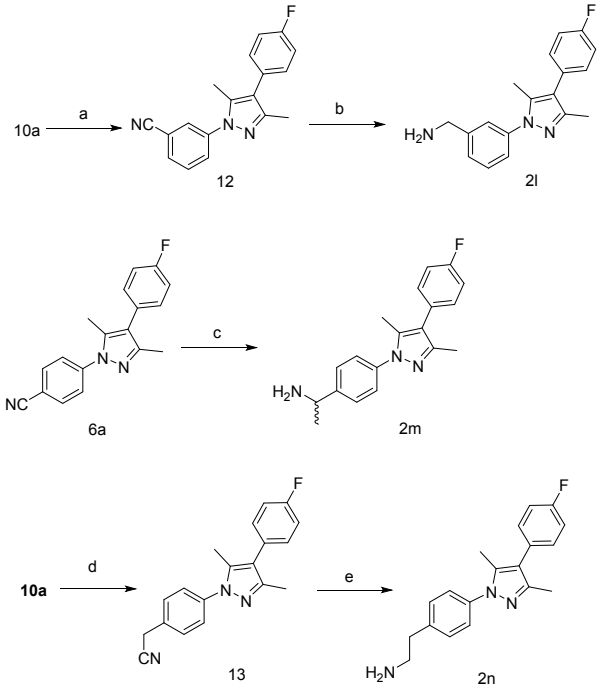
[a]Reagents and conditions: (a) NBS, EtOAc, sonication, 25-30°C, 15 min, quantitative; (b) 4-(fluorophenyl)boronic acid or 3-(fluorophenyl)boronic acid, $Pd(PPh_3)_4$, sat. Na_2CO_3, DMF, 140°C, 20 min, 70%; (c) 4-(bromomethyl)benzonitrile, Cs_2CO_3, CH_3CN, 50°C, 10h, quantitative; (d) $LiAlH_4$, THF, r.t, 1h, 9%.

When compound **2k** was tested for its ability to inhibit ASICs a comparable activity and isoform-selectivity was observed for ASIC2a with respect to compound **2a** (Table 2, IC_{50} = 16.8 μM and 18.9 μM, respectively).

Our attention was then focused on the "blue" exploration by initially moving the p-benzylamine moiety from *para* to *meta* position of the phenyl ring (compound **2l**, Scheme 4), by introducing a methyl group at the benzylic position (compound **2m**, Scheme 4), and by C-1 homologation (compound **2n**, Scheme 4).

In particular, as for the synthesis of **2l**, 4-(p-fluorophenyl)-3,5-dimethyl pyrazole **10a** was N-arylated with m-cyanophenyl boronic acid using copper(II) acetate; the resulting 1-(m-cyanophenyl) pyrazole **12** was reduced to the corresponding benzylamine **2l** with lithium aluminium hydride in unoptimized poor yields. Compound **2m** was synthesized by reacting previously described 1-(p-cyanophenyl) pyrazole **6a** with methylmagnesium bromide in reducing conditions. Finally, compound **2n** was prepared from 4-(p-fluorophenyl)-3,5-dimethyl pyrazole **10a** which was N-arylated with p-cyanomethyl phenyl bromide, according to Buchwald-Hartwig reaction experimental protocol[26] (microwave reaction, aquous K_2CO_3 and DMSO, CuI and L-proline, 140°C, 26 h). The resulting pyrazole intermediate **13** was then reduced with sodium borohydride in the presence of cobalt (II) chloride, to obtain the corresponding target benzylamine **2n**.

Scheme 4. Variations on the benzylamine: compounds 2l-n[a]



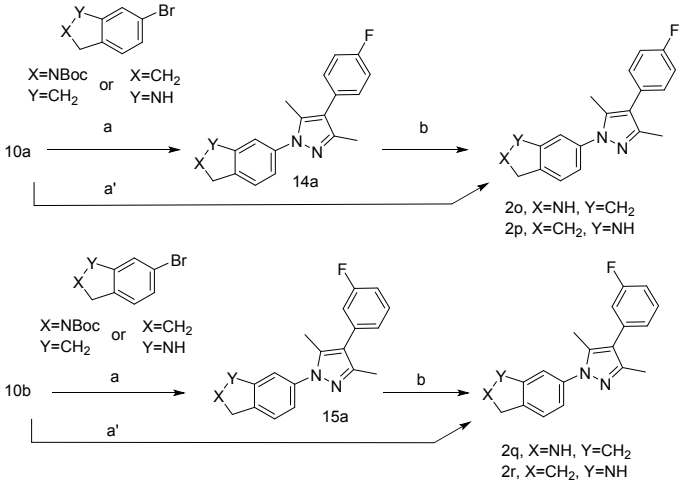
[a]Reagents and conditions: (a) 3-cyanophenylboronic acid, $Cu(OAc)_2{\cdot}H_2O$, pyridine, DMF, 125°C, 3h, 11%; (b) $LiAlH_4$, THF, r.t, 3h, 31%; (c) MeMgBr, THF, r.t, 5h, then $LiAlH_4$, THF, 0° to r.t, 13% (two steps); (d) 4-bromobenzyl cyanide, CuI, L-proline, K_2CO_3, DMSO, 140°C, 26h, 33%; (e) $NaBH_4$, $CoCl_2$, MeOH, r.t, 1h, 25%.

Compounds **2l**, **2m** and **2n** mostly maintained the activity of the early pyrazole lead **2a**, and showed high ASIC2a isoform-selectivity (Table 2), whereas the nitrile intermediates **6a** was inactive, pointing out the relevance of the presence of the primary amine function.

The "blue" exploration was expanded by constraining the amine function within a 5-membered ring. To this aim, compounds **2o-r** bearing a more symmetrical (**2o**, **2q**) or unsymmetrical amine (**2p**, **2r**) and a p-F (**2o**, **2p**) or m-F substituent at the 4-phenyl ring (**2q**, **2r**) were prepared. The synthesis of this sub-series of compounds is reported in Scheme 5. Namely, previously described 4-(p-fluorophenyl)-3,5-dimethyl pyrazole intermediate **10a**, and 4-(m-fluorophenyl)-3,5-dimethyl pyrazole intermediate **10b** (prepared as **10a** in Scheme 3, using m-fluorophenyl boronic acid) were N-arylated with N-Boc-5-bromo isoindoline, using CuI in basic conditions in a microwave reactor.

The resulting N-Boc-protected 4-(p-fluorophenyl) pyrazoles **14a** and **15a** were obtained in 42% and 15% unoptimized yield, respectively. Then, removal of the N-Boc protecting group in acidic conditions afforded the corresponding target compounds **2o** and **2q** in 32% and 63% yields, respectively. Intermediates **10a** and **10b** underwent the same coupling reaction with 6-bromo indoline, obtaining the corresponding target compounds **2p** and **2r** in 44% and 13% yield, respectively (Scheme 5).

Scheme 5. Rigidification of the benzylamine: compounds 2o-r[a]



[a]Reagents and conditions: (a) N-Boc-5-bromo isoindoline, CuI, aqueous K_2CO_3, L-proline, DMSO, microwave, 75°C, 4 h then 140°C, 13 h, 42% (**14a**) or 15% (**15a**); (b) 4N HCl in dioxane, 30min, r.t., 32% (**2o**) or 63% (**2q**); (a') 6-bromo indoline, aqueous K_2CO_3, CuI, L-proline, DMSO, microwave, 140°C, 6 h, 44% (**2p**) or 13% (**2r**).

Table 2. Compounds 2k-w and 6a: ASIC inhibition[a]

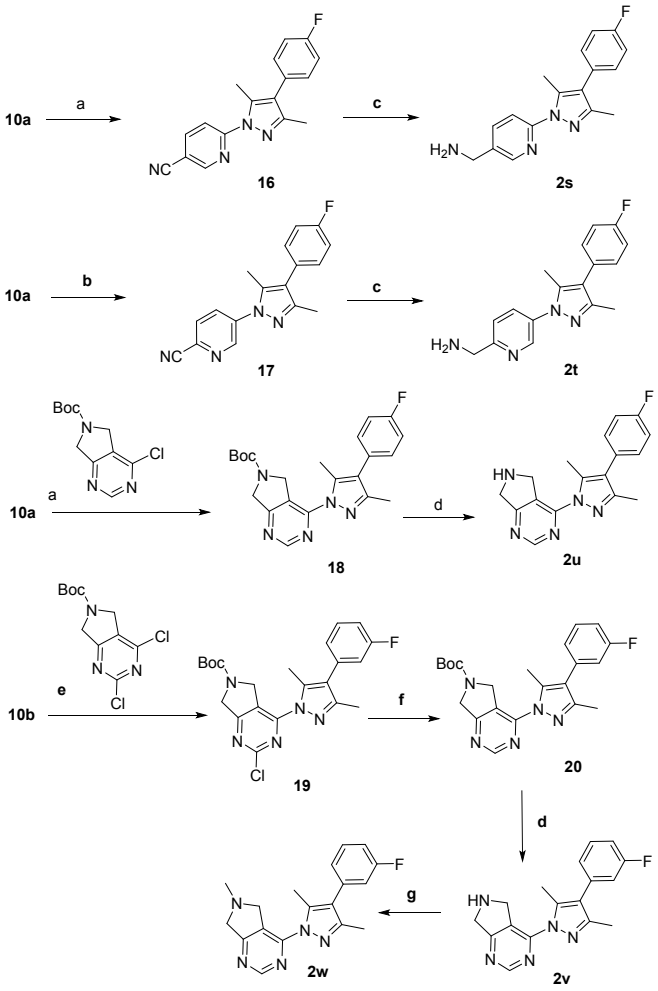
Entry	*ASIC1a*[a]	*ASIC2a*[a]	*ASIC1a/2a*[a]
2a	>30	18.9 ± 6.9	10.9 ± 1.6
2j	>30	>30	>30
2k	>30	16.8 ± 5.4	>30
2l	>30	15.6 ± 2.9	>30
2m	>30	8.7 ± 2.3	>30
2n	>30	11.7 ± 2.7	>30
2o	>30	8.2 ± 1.5	>30
2p	>30	>30	>30
2q	>30	9.9 ± 1.1	11.3 ± 1.0
2r	>30	>30	>30
2s	>30	6.1 ± 1.1	8.5 ± 1.8
2t	>30	>30	>30
2u	>30	17.0 ± 4.7	>30
2v	>30	>30	>30
2w	>30	>30	>30
6a	>30	>30	>30

[a] IC_{50} were determined as described in the Supplementary Information; they are expressed in μM and are the average value of at least n=3 independent experiments ± SEM.

The inhibition of ASICs constructs by "blue" compounds **2o-r** is reported in Table 2. In particular, the isoindole derivative **2o** exhibited good in vitro activity and complete ASIC2a isoform-selectivity. Conversely, compound **2q** inhibited both ASIC2a and ASIC1a/2a isoforms (IC_{50} = 9.9 μM and 11.3 μM, respectively). Notably, the corresponding indoline derivatives **2p** and **2r** were inactive.

Finally, the phenyl ring bearing the benzyl amine function was replaced ("green" exploration") by a 2-pyridine (**2s**) and a 3-pyridine (**2t**) moiety, whereas dihydropyrrolo[3,4-d]pyrimidine homologues **2u** and **2v** and the N-Me derivative of the latter compound **2w** were prepared. Their synthesis is depicted in Scheme 6.

Scheme 6. Phenyl substitution: compounds 2s-w[a]



[a]Reagents and conditions: (a) 60% NaH, DMF, 0°C, 30 min then 6-fluoronicotinonitrile, r.t, 1h (98%, **16**) or tert-butyl 4-chloro-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidine-6-carboxylate, r.t., 1 h (9%, **18**); (b) 5-bromo-2-cyanopyridine, Cs_2CO_3, CuI, 1,2-cyclohexanediamine, microwave, 120°C, 13h, 19%; (c) $LiAlH_4$, THF, r.t, 1h, 4% (**2s**), 16% (**2t**); (d) 4N HCl in dioxane, r.t, 1-3h, 8% (**2u**), 19% (**2v**); (e) 2/1 THF/DMF, DIPEA, microwave, 150°, 14h, 58%; (f) H_2, TEA, MeOH, 10% Pd/C, 45°C, 1 h; (g) formaldehyde, $NaCNBH_3$, MeOH, r.t, 1 h, 57%.

Previously described 4-(p-fluorophenyl)-3,5-dimethyl pyrazole intermediate **10a** was smoothly N-arylated with p-cyano-2-pyridyl fluoride in basic conditions (NaH in DMF); the resulting 1-(p-cyano-2-pyridyl) pyrazole **16** was reduced to the corresponding benzylamine derivative **2s** with lithium aluminium hydride in unoptimized, poor yields. Instead, the corresponding m-F analogue **2t** was synthesized from **10a** and 5-bromo-2-cyanopyridine using Cs_2CO_3, CuI and 1,2-cyclohexanediamine. This coupling reaction was run in a microwave reactor for 13 h at 120°C, and afforded cyano intermediate **17**, which was then reduced to amine **2t** in a poor, unoptimized 16% yield. Alternatively, compound **10a** was N-arylated with *tert*-butyl 4-chloro-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidine-6-carboxylate as seen earlier; the resulting N-Boc-protected pyrazole **18** was deprotected in acid conditions to yield the target compound **2u** in poor, unoptimized yields. Then,

intermediate **10b** was transformed into the target compound **2v** by N-arylation followed by removal of the Cl atom by hydrogenolysis to give intermediate **20**, which was finally deprotected in acid conditions to the corresponding NH-free pyrazole **2v**. Finally, this compound was N-methylated to yield compound **2w** in good yields (Scheme 6).

As shown in Table 2, none of the compounds **2s-w** inhibited ASIC1a. The presence of a 2-pyridyl ring was well tolerated by ASIC2a (**2s**). Conversely, the presence of a 3-pyridyl ring led to complete inactivity (**2t**). The dihydropyrrolo[3,4-d]pyrimidine derivative **2u** was an ASIC2 inhibitor (IC_{50} = 17.0 μM) selective against the ASIC1a/2a heterodimer. Surprisingly, its close congeners **2v** and **2w**, bearing a 4-(m-fluorophenyl) substitution, resulted to be completely inactive.

Compound **2a**, **2o** and **2u** were profiled in terms of early physicochemical and ADME properties. As to in vivo PK in mice priority was given to the characterization of **2u** over **2o** owing to its more drug-like physicochemical features (cLogP = 2.6 and 4.1; TPSA = 56 and 29 $Å^2$, for **2u** and **2o**, respectively). The summary of both the in vitro and in vivo characterization studies performed is reported in Table 3.

Table 3. ADME profiling: compounds 2a, 2o and 2u

Compound/Assay	**2a**	**2u**	**2o**
cLogP [a]	3.9	2.6	4.2
TPSA ($Å^2$) [a]	44	56	30
Solubility (μg/ml)[b]	73	32	56
PPB (%)[c]	93	94	95
h-ERG (IC_{50}, μM)	>30	>30	>30
CYP450 (IC_{50}, μM)[d]	0.4 [1A2]	0.8 [1A2]	0.2 [1A2] 4.8 [2D6]
Cl (ml/min/Kg)[e]	224	34	NT
Vd (l/Kg)	50.9	1.4	NT
C_{max} (μM)	0.09	2.6	NT
F (%)	60	100	NT
B/P ratio [f]	61	1.3	NT

[a] calculated logP and topological polar surface area; [b] kinetic solubility at pH 7.4; [c] % of bound compound to human serum albumin measured by NMR-based analysis; [d] only CYP450 isoforms showing IC_{50} <10 μM are reported; [e] in vivo PK studies were performed at 1 mg/Kg, i.v. and at 3 mg/Kg, p.o.; [f] brain penetration studies were performed at 1 mg/Kg, i.v. and B/P was calculated from 2h to 8h after dosing.

As to physicochemical descriptors, compound **2u** was significantly less lipophilic than **2a** and exhibited a greater TPSA value. Both compounds showed acceptable kinetic solubility and plasma protein binding (PPB). No inhibition of hERG was observed up to 30μM concentration. In terms of inhibition of CYP450 isoforms, compound **2a** was found to inhibit two different isoforms, i.e. 1A2 and 2D6, although at different extents, whereas **2u** inhibited only the 1A2 isoform. Particularly relevant were the differences observed in the in vivo pharmacokinetics in mice. Both compounds were tested at 1 mg/kg, i.v. and at 3 mg/Kg, p.o. Notably, **2a** was highly cleared following i.v. administration (Cl = 224 ml/min/Kg), but widely distributed in tissues (Vd = 50.9 l/Kg), resulting in a low C_{max} after oral administration (C_{max} =0.09 μM). However, being highly brain penetrant (B/P = 61), a relevant total brain concentration was observed after the administration of 1 mg/Kg dose, i.v. (C_{max} =2.46 μM). Conversely, compound **2u** showed a more balanced pharmacokinetic profile, owing to a sizable enhancement of the metabolic stability (Cl = 34 ml/min/Kg) with respect to compound **2a**, an appropriate tissue distribution (Vd=1.4 l/Kg), complete absorption after oral administration (F=100%) along with a significantly higher exposure p.o. with respect to **2a** (C_{max} = 2.6 μM), and good brain penetration (B/P = 1.3). The relevant improvement of pharmacokinetic profile of **2u** *vs* **2a** was most likely due to the lack of the basic, metabolically labile primary benzylamine function present in compound **2a**, which was appropriately masked in **2u** within the constrained dihydropyrrolo[3,4-d]pyrimidine bicyclic moiety.

In conclusion, the described exploratory strategy enabled the identification of novel ASIC2 inhibitors. In particular, the "cyano" optimization approach, focused on the stabilization of the terminal benzylamine function, allowed to obtain compounds 20 and 2u as selective ASIC2a the *in vivo*-compliant lead compound **2u**. This compound, owing to its relevant drug-like character and balanced pharmacokinetic profile (including brain penetration), may represent a valuable tool compound to validate p.o. the role of ASIC2 in vivo in animal models of different type of CNS pathologies. In addition, **2u** can be seen as a foundation molecule for future optimization studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures for the synthesis and the analytical characterization of both key intermediates and final compounds **2a-w**, **6a**, the *in vitro* screening of compounds **2a-w, 6a** and in vivo PK studies of compounds **2a** and **2u**.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest

ABBREVIATIONS

ASICs, acid-sensitive ion channels; DA, diminazene; ENaC, epithelial Na^+ channels; DEG, degenerins; CNS, central nervous system; PNS, peripheral nervous system; NCE, new chemical entity; VSD, voltage-sensitive dyes; BBB, blood-brain barrier; TPSA, topological polar surface area; PK, pharmacokinetic.

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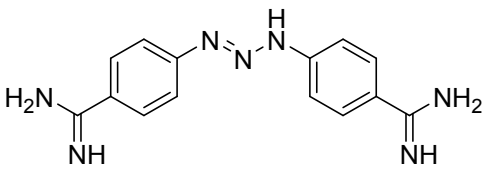
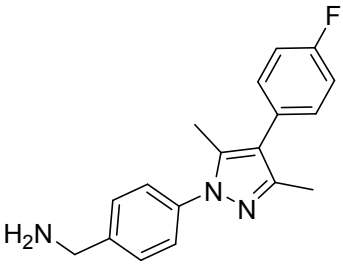
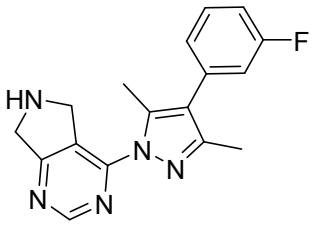


1 (Diminazene)



2a



2u