

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

Leda Ivanova Bencheva, Marilena De Matteo, Luca Ferrante, Marco Ferrara, Adolfo Prandi, Pietro Randazzo, Silvano Ronzoni, Roberta Sinisi, Pierfausto Seneci, Vincenzo Summa, Mariana Gallo, Maria Veneziano, Antonella Cellucci, Nausicaa Mazzocchi, Andrea Menegon, and Romano Di Fabio

ACS Med. Chem. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acsmchemlett.8b00591 • Publication Date (Web): 07 Feb 2019

Downloaded from <http://pubs.acs.org> on February 8, 2019

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.



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

Leda Ivanova Bencheva,[‡] Marilena De Matteo,[‡] Luca Ferrante,[‡] Marco Ferrara,^{‡,^} Adolfo Prandi,[‡] Pietro Randazzo,[‡] Silvano Ronzoni,[‡] Roberta Sinisi,[‡] Pierfausto Seneci,^{‡,‡} Vincenzo Summa,[‡] Mariana Gallo,[‡] Maria Veneziano,[‡] Antonella Cellucci,[‡] Nausicaa Mazzocchi,[§] Andrea Menegon,^{§,*} Romano Di Fabio^{‡,&,*}

[‡], Promidis, Via Olgettina 60, 20132, Milan, Italy; [§], San Raffaele Scientific Institute, Experimental Imaging Center, ALEMBIC, Advanced Light and Electron Microscopy BioImaging Center, Via Olgettina 60, 20132, Milan, Italy; [&], IRBM Science Park, Via Pontina Km 30.600, 00070 Pomezia (Rome), Italy; [‡], Chemistry Department, Università degli Studi di Milano, Via Golgi 19, I-20133 Milan, Italy. * These two authors share senior authorship.

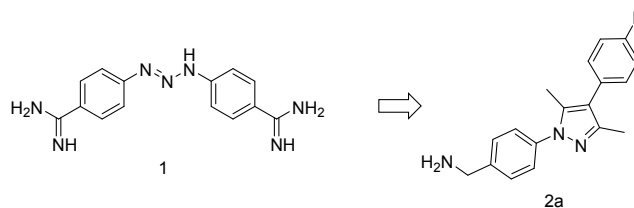
KEYWORDS: ASICs, ion channels, drug discovery, CNS, PNS, cancer.

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 channels⁴ that are highly expressed both in central and peripheral neurons⁵ 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**

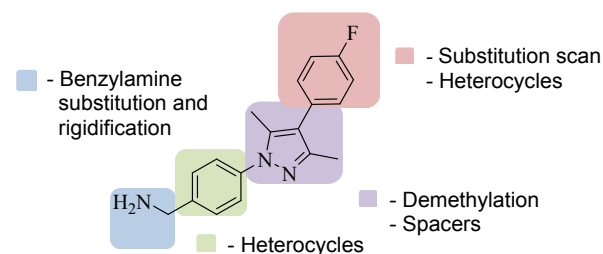


To identify the most appropriate methodology for *in vitro* screening of NCEs, a series of published data^{19,20,21} on both compound **1** and Amiloride, a diuretic drug known to interact with ASICs, specifically drawn our attention and an optical technology based on membrane potential detection by 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

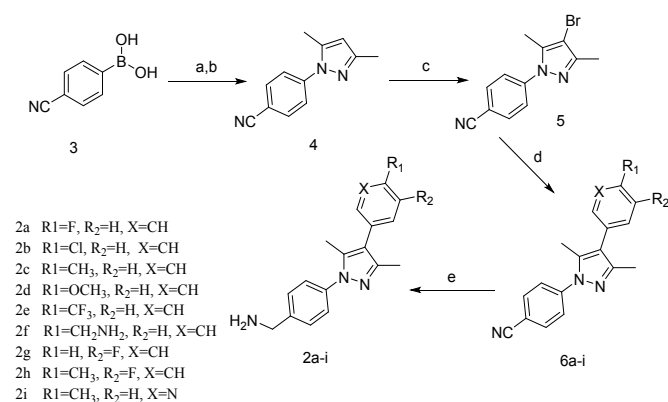
The preliminary structural elaboration of DA, in three sequential steps, enabled the identification, of the 1,4-diaryl, 3,5-dimethylpyrazole derivative **2a** (Chart 1), an early lead compound which was fully characterized in terms of ASICs inhibition. In particular, the linear triazine linker present 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 ($IC_{50} = 18.9 \mu\text{M}$ and $10.9 \mu\text{M}$, 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



^aReagents and conditions: (a) di-*tert*-butyl diazene 1,2-dicarboxylate, Cu(OAc)₂·H₂O, 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(PPh₃)₄, aq. Na₂CO₃, DMF, microwave reactor, 140°C, 15-20 min, 52%-70%; (e) LiAlH₄, 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

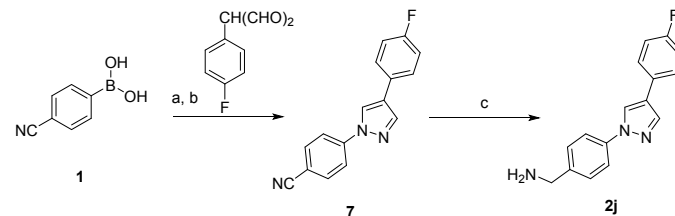
Entry	ASIC1a ^a	ASIC2a ^a	ASIC1a/2a ^a
1 (DA)	56.9 ± 8.9	169.0 ± 18.1	45.2 ± 8.8
2a	>30	18.9 ± 6.9	10.9 ± 1.6
2b	>30	>30	>30
2c	>30	8.8 ± 2.0	>30
2d	>30	16.3 ± 4.6	>30
2e	>30 ^b	>30 ^b	>30
2f	>30	>30	>30
2g	>30	4.3 ± 0.5	>30
2h	>30	>30	>30
2i	>30	>30	>30

^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*-CH₃ or *p*-OCH₃ 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*-CF₃; **2f**, *p*-CH₂NH₂), disubstituted aryls (**2h**, *p*-CH₃, *m*-F) and the presence of a pyridine as phenyl replacement (**2i**, *p*-CH₃, 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



^aReagents and conditions: (a) di-*tert*-butyl diazene-1,2-dicarboxylate, Cu(OAc)₂·H₂O, 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) LiAlH₄, THF, r.t, 1h, 73%.

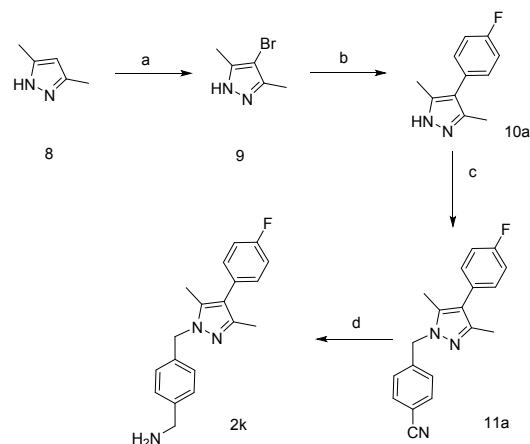
To this aim, *p*-cyanophenyl boronic acid **3** was sequentially treated with di-*tert*-butyl diazene-1,2-dicarboxylate in presence of Cu(OAc)₂·H₂O in MeOH at 65°C for 1h, followed by 2-(4-fluorophenyl)propanedial in 4N HCl in dioxane initially at room temperature for 10 min, then at 80°C for additional 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 ($IC_{50} > 30 \mu\text{M}$ 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 N₁ 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 N₁ 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



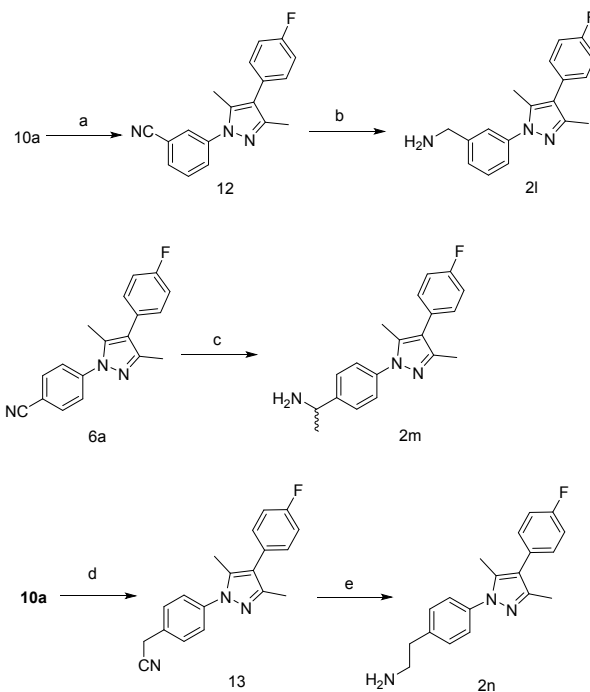
^aReagents and conditions: (a) NBS, EtOAc, sonication, 25–30°C, 15 min, quantitative; (b) 4-(fluorophenyl)boronic acid or 3-(fluorophenyl)boronic acid, Pd(PPh₃)₄, sat. Na₂CO₃, DMF, 140°C, 20 min, 70%; (c) 4-(bromomethyl)benzonitrile, Cs₂CO₃, CH₃CN, 50°C, 10h, quantitative; (d) LiAlH₄, THF, r.t, 1h, 9%.

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, IC₅₀ = 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 protocol²⁶ (microwave reaction, aqueous K₂CO₃ 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

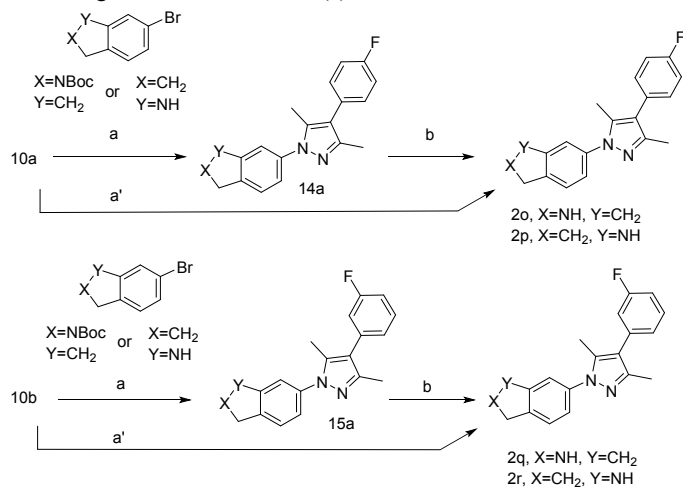


^aReagents and conditions: (a) 3-cyanophenylboronic acid, Cu(OAc)₂·H₂O, pyridine, DMF, 125°C, 3h, 11%; (b) LiAlH₄, THF, r.t, 3h, 31%; (c) MeMgBr, THF, r.t, 5h, then LiAlH₄, THF, 0° to r.t, 13% (two steps); (d) 4-bromobenzyl cyanide, CuI, L-proline, K₂CO₃, DMSO, 140°C, 26h, 33%; (e) NaBH₄, CoCl₂, MeOH, 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^aReagents and conditions: (a) N-Boc-5-bromo isoindoline, CuI,

aqueous K₂CO₃, 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 K₂CO₃, CuI, L-proline, DMSO, microwave, 140°C, 6 h, 44% (**2p**) or 13% (**2r**).

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

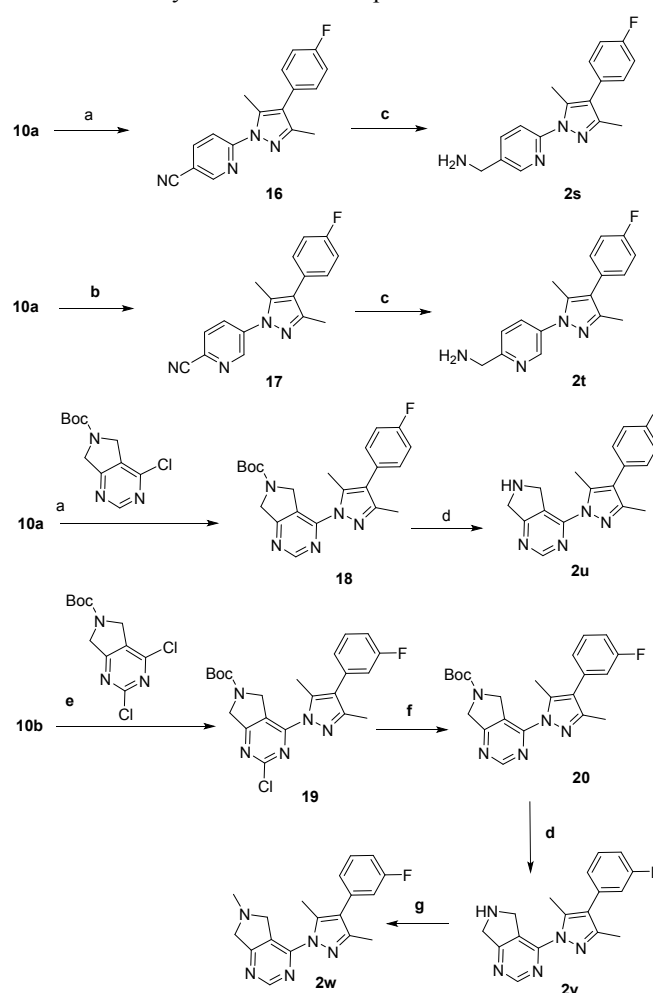
Entry	ASIC1a ^a	ASIC2a ^a	ASIC1a/2a ^a
2a	>30	18.9 ± 6.9	10.9 ± 1.6
2j	>30	>30	>30
2k	>30	16.8 ± 5.4	>30
2l	>30	15.6 ± 2.9	>30
2m	>30	8.7 ± 2.3	>30
2n	>30	11.7 ± 2.7	>30
2o	>30	8.2 ± 1.5	>30
2p	>30	>30	>30
2q	>30	9.9 ± 1.1	11.3 ± 1.0
2r	>30	>30	>30
2s	>30	6.1 ± 1.1	8.5 ± 1.8
2t	>30	>30	>30
2u	>30	17.0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

^a IC₅₀ 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 (IC₅₀ = 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

^aReagents 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, Cs₂CO₃, CuI, 1,2-cyclohexanediamine, microwave, 120°C, 13h, 19%; (c) LiAlH₄, 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°C, 14h, 58%; (f) H₂, TEA, MeOH, 10% Pd/C, 45°C, 1 h; (g) formaldehyde, NaCNBH₃, 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 Cs₂CO₃, 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 ($IC_{50} = 17.0 \mu\text{M}$) 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 Å², 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 $IC_{50} < 10 \mu\text{M}$ 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 30 μM 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**.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Stefano Maiorana (Promidis), Dr. Edith Monteagudo (IRBM Science Park), Dr. Daniel Cicero (IRBM Science Park) and Dr. Annalise Di Marco (IRBM Science Park) for the useful discussion on the synthesis of the described chemical series, the in vitro and in vivo ADME studies, the serum albumin NMR-based analysis and the CYP450 inhibition studies, respectively. In addition, thanks are due to Dr. Maria Rosaria Battista (IRBM Science Park) for the in vitro CYP450 inhibition studies.

AUTHOR INFORMATION

Corresponding Author

* Phone: +390249491701. E-mail: r.difabio@irbm.it

Present Addresses

^ Marco Ferrara: Flamma Innovation Srl, Via Cascina Secchi 217, 24040 Isso (BG), Italy.

Author Contributions

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

Funding Sources

The project was in part funded by the COLLEZIONE DEI COMPOSTI CHIMICI E CENTRO DI SCREENING-CNCCS scarl

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.

REFERENCES

- Chesler, M. Regulation and modulation of pH in the brain. *Physiol. Rev.* **2003**, 83, 1183-1221.
- Wemmie, J. A.; Taugher, R. J.; Kreple, C. J. Acid-sensing ion channels in pain and disease. *Nat. Rev. Neurosci.* **2013**, 14, 461-471.
- Qadri, Y. J.; Rooj, A. K.; Fuller, C. M. ENaCs and ASICs as therapeutic targets. *Am. J. Physiol. Cell. Physiol.* **2012**, 302, C943-965.
- Kellenberger, S.; Schild, L. International Union of Basic and Clinical Pharmacology. XCI. structure, function, and pharmacology of acid-sensing ion channels and the epithelial Na⁺ channel. *Pharmacol. Rev.* **2015**, 67, 1-35.
- Boscardin, E.; Alijevic, O.; Hummler, E.; Frateschi, S.; Kellenberger, S. The function and regulation of acid-sensing ion channels (ASICs) and the epithelial Na⁺ channel (ENaC): IUPHAR Review 19. *Br. J. Pharmacol.* **2016**, 173, 2671-2701.
- Liu, C.; Zhu, L. L.; Xu, S. G.; Ji, H. L.; Li, X. M. ENaC/DEG in Tumor Development and Progression. *J. Cancer* **2016**, 7, 1888-1891.
- Wemmie, J.E. Taugher, R.J.; Creple, C.J. Acid-sensitive ion channels in pain and disease. *Nat. Rev. Neurosci.* **2013**, 14, 461-471.
- 8) Kweon, H.-J.; Su, B.-C. Acid-sensing ion channels (ASICs): therapeutic targets for neurological diseases and their regulation. *BMB Rep.* **2013**, 46, 295-304.
- Li, W. G.; Xu, T. L. ASIC3 channels in multimodal sensory perception. *ACS Chem. Neurosci.* **2011**, 2, 26-37.
- Zhou, Z. H.; Song, J. W.; Li, W.; Liu, X.; Cao, L.; Wan, L. M.; Tan, Y. X.; Ji, S. P.; Liang, Y. M.; Gong, F. The acid-sensing ion channel, ASIC2, promotes invasion and metastasis of colorectal cancer under acidosis by activating the calcineurin/NFAT1 axis. *J. Exp. Clin. Cancer Res.* **2017**, 36, 130.
- Vila-Carriles, W. H.; Kovacs, G. G.; Jovov, B.; Zhou, Z. H.; Pahwa, A. K.; Colby, G.; Esimai, O.; Gillespie, G. Y.; Mapstone, T. B.; Markert, J. M.; Fuller, C. M.; Bubien, J. K.; Benos, D. J. Surface expression of ASIC2 inhibits the amiloride-sensitive current and migration of glioma cells. *J. Biol. Chem.* **2006**, 281, 19220-19232.
- Wu, J.; Xu, Y.; Jiang Y. Q.; Xu, J.; Hu, Y.; Zha, X. M. ASIC subunit ratio and differential surface trafficking in the brain. *Mol. Brain.* **2016**, 9, 4.
- Baron, A.; Lingueglia, E. Pharmacology of acid-sensing ion channels - Physiological and therapeutic perspectives. *Neuropharmacology.* **2015**, 94, 19-35.
- Chen, X.; Qiu, L.; Li, M.; Dürrnagel, S.; Orser, B. A.; Xiong, Z.-G.; MacDonald J. F. Diarylamidines: high potency inhibitors of acid-sensing ion channels. *Neuropharmacology.* **2010b**, 58, 1045-53.
- Lingueglia, E.; Lazdunski, M. Pharmacology of ASIC channels. *Wiley Interdiscip. Rev. Membr. Transp. Signal.* **2013**, 2, 155-171.
- Chen, X.; Orser, B. A.; MacDonald J. F. Design and screening of ASIC inhibitors based on aromatic diamidines for combating neurological disorders. *Eur. J. Pharmacol.* **2010a**, 648, 15-23.
- Zhou, J.; Le, V.; Kalia, D.; Nakayama, S.; Mikek, C.; Lewis, E. A.; Sintim, H. O. Diminazene or berenil, a classic duplex minor groove binder, binds to G-quadruplexes with low nanomolar dissociation constants and the amidine groups are also critical for G-quadruplex binding. *Mol Biosyst.* **2014**, 10, 2724-2734.
- Liu, Y.; Hagan, R.; Schoellerman, J. Dual action of Psalmotoxin at ASIC1a and ASIC2a heteromeric channels (ASIC1a/2a). *Scientific Reports* **2018**, 7179, 1-11.
- Dorofeeva, N. A.; Barygin, O. I.; Staruschenko, A.; Bolshakov, K. V.; Magazanik, L. G. Mechanisms of non-steroid anti-inflammatory drugs action on ASICs expressed in hippocampal interneurons. *J. Neurochem.* **2008**, 106, 429-441.
- Schmidt, A.; Rossetti, G.; Joussen, S.; Gründer, S. Diminazene Is a Slow Pore Blocker of Acid-Sensing Ion Channel 1a (ASIC1a). *Mol. Pharmacol.* **2017**, 92, 665-675.
- Krauson, A. J.; Rooney, J.G.; Carattino, M. D. Molecular basis of inhibition of acid sensing ion channel 1A by diminazene. *PLoS One.* **2018**, 13(5):e0196894.
- Huang, C.-J.; Harootunian, A.; Maher, M. P.; Quan, C.; Raj, C. D.; McCormack, K.; Numann, R.; Negulescu, P. A.; González, J. E. Characterization of voltage-gated sodium-channel blockers by electrical stimulation and fluorescence detection of membrane potential. *Nat. Biotechnol.* **2006**, 24, 439-446.
- Mazzocchi, N.; De Ceglia, R.; Mazza, D.; Forti, L.; Muzio, L.; Menegon A. Fluorescence-Based Automated Screening Assay for the Study of the pH-Sensitive Channel ASIC1a. *J. Biomol. Screen.* **2016**, 21, 372-380.
- Menegon, A.; Pitassi, S.; Mazzocchi, N.; Redaelli, L.; Rizzetto, R.; Rolland, J. F.; Poli, C.; Imberti, M.; Lanati, A.; Grohovaz, F. A new electro-optical approach for conductance measurement: an assay for the study of drugs acting on ligand-gated ion channels. *Sci. Rep.* **2017**, 7, 44843.
- For further details see method for optical measuring variations of cell membrane conductance in EP2457088, WO 2011009825, US 13/386225.
- Heravi, M.M.; Kheilkordi, Z.; Zadsirjan, K.; Heydari, M.; Malmir, M. Buchwald-Hartwig reaction: an overview. *J. Organomet. Chem.* **2018**, 861, 17-104.

