Supplemental Digital Content

METHODS

Chemical Synthesis. All tested compounds were synthesized in our laboratory. Column chromatography was performed on ICN Silica Gel 60 Å (63–200 μm) as a stationary phase. Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus and are uncorrected. Mass spectra were recorded on an HP GC/MS 6890-5973 MSD spectrometer, electron impact 70 eV, equipped with HP chemstation or an Agilent LC/MS 1100 Series LC/MSD Trap System VL spectrometer, electrospray ionization (ESI). ^{1}H NMR spectra were recorded in $CDCl_3$ (the use of other solvents is specified) on a Varian-Mercury 300 (300 MHz) spectrometer at room temperature. Chemical shifts are expressed as parts per million (δ). The purity of all tested compounds was > 95%, as confirmed by combustion analysis carried out with an Eurovector Euro EA 3000 model analyzer. Chemicals were purchased from Aldrich (Milan, Italy), AlfaAesar (Karlsruhe, Germany) or Acros (Milan, Italy) and were used without any further purification.

The preparation of tested compounds followed a pathway reported in literature (Fracchiolla et al 2007; Piemontese et al., 2010). The opportune commercially available 5-halogen-2-hydroxybenzophenone was condensed with diethylbromomalonate in the presence of K_2CO_3 in boiling acetone to afford the corresponding ethyl ester intermediate in 65–89% yields. In particular, for this first one-pot cyclization step we used 5-bromo-2-hydroxybenzophenone for entries **3–9**, **11**, **12** and **14**, 5-bromo-2'-chloro-2-hydroxybenzophenone for entries **1**, **10** and **13** or 5,2'-dichloro-2-hydroxybenzophenone for entry **2**, respectively.

For entries **1** and **3–14** the corresponding ethyl 5-bromo-3-aryl-benzofuran-2-carboxylate intermediates were treated with the suitable aryl or alkyl boronic acids or corresponding pinacolate esters in the presence of $Pd(PPh_3)_4$ and K_2CO_3 or Cs_2CO_3 under Suzuki cross-coupling conditions as reported in our previous work. This step was carried out using a microwave reactor which allow to obtain the desired esters intermediates in 10 to 30 minutes and in high yields (Fracchiolla et al., 2008).

All the ethyl esters derivatives were hydrolyzed under alkaline conditions affording the final acids in quantitative yields.

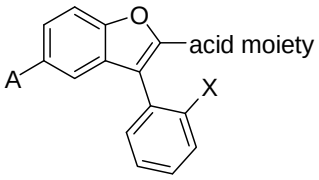
The synthesis of entry **15** was carried out starting from the condensation of 2-bromoacetophenone with the 4-benzylphenol to give the corresponding ketone, whose cyclization in the presence of BCl_3 in anhydrous CH_2Cl_2 afforded the 5-benzylbenzofuran intermediate (Nichols et al., 2010)

This intermediate was reacted with H_2SO_4 in the presence of $(CH_3CO)_2O$, CH_3COOK in ethylacetate and 96% ethanol affording the desired compound as potassium salt in 40% overall yield (Graham et al., 1990).

The entry **16** was obtained in 86% yield by condensation of compound **1** with methanesulfonamide in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC hydrochloride) and 4-(dimethylamino)pyridine (DMAP).

Supplemental Table 1.

Tested Compounds



Entry	Comp.	Acid Moiety	A	X	Formula[a]	recryst. solv.[b]
1	**RT-93**	COOH	Ph	Cl	$C_{21}H_{13}ClO_3$	B
2	**MT-189**	COOH	Cl	Cl	$C_{15}H_8Cl_2O_3$	A
3	**JBL-44**	COOH	Ph	H	$C_{21}H_{13}O_3$	B
4	**SRA-16**	COOH	2-furanyl	H	$C_{19}H_{12}O_4$	A
5	**SRA-31**	COOH	3-furanyl	H	$C_{19}H_{12}O_4$	A
6	**SRA-6**	COOH	4-CH_3O-Ph	H	$C_{22}H_{16}O_4$	A
7	**SRA-29**	COOH	4-Cl-Ph	H	$C_{21}H_{13}ClO_3$	A
8	**SRA-36**	COOH	$PhCH_2$	H	$C_{22}H_{16}O_3$	A
9	**SRA-9**	COOH	$PhCH_2CH_2$	H	$C_{23}H_{18}O_3$	B
10	**SV-12**	COOH	$PhCH_2$	Cl	$C_{22}H_{15}ClO_3$	B
11	**SV-15**	COOH	4-CH_3O-$PhCH_2$	H	$C_{23}H_{18}O_4$	A
12	**SV-21**	COOH	4-CH_3-$PhCH_2$	H	$C_{23}H_{18}O_3$	A
13	**SV-18**	COOH	4-CH_3O-$PhCH_2$	Cl	$C_{23}H_{17}ClO_4$	A
14	**SV-19**	COOH	4-CF_3O-$PhCH_2$	H	$C_{23}H_{15}F_3O_4$	A
15	**OZ-4**	SO_3H[c]	$PhCH_2$	H	$C_{21}H_{15}KO_4S$	B
16	**DNY-15**	$CONHSO_2CH_3$	Ph	Cl	$C_{22}H_{16}ClNO_3S$	A

[a] Elemental analyses for C and H were within ± 0.4% of the theoretical values for the formulas given.

[b] A: hexane/$CHCl_3$; B: hexane. [c] The entry 15 was purified and tested as potassium salt.

pKa determinations. We used the online Advanced Chemistry Development, Inc. software I-Lab 2.0/pKa module to calculate the pKa values for RT-93, SRA-36, SV-12, OZ-4 and DNY-15. The structures were drawn with the ACD/ChemSketch 12 freeware tool and submitted for online calculations to the ACD/I-Lab 2.0/pKa module (v5.0.0.184) at the website ilab.acdlabs.com.

RESULTS

In vitro studies
Structure-activity relationship study

The following chemical modification were performed on the starting compound the 3-phenyl-1-benzofuran-2-carboxylic acid (Figure 2B):

1.Modification of the hydrophobic group at the C-5 position

- *Isosteric ring*

To study the role of the aromaticity requirement at this position, we replaced the C-5 phenyl ring of JBL-44 with an isosteric 2- or 3-furyl moiety, which has an oxygen atom as hydrogen bond acceptor (SRA-16, SRA-31). This maneuver did not increase the inhibition efficacy (Figure 2B-C).

- *Introduction of a p-substituent*

We investigated whether a different electronic cloud density could influence drug activity by introducing in the para position of the phenyl group of JBL-44 a chlorine atom (SRA-29) or a methoxy moiety (SRA-6). Both derivatives showed a 70% block, suggesting that neither an electron withdrawing nor an electron donating group influenced drug potency (Figure 2B-C).

- *Introduction of a spacer alkyl chain*

We separated the aromatic ring at the C-5 position from the benzofuran nucleus with a methylene group (SRA-36, Figure 2B), by introducing a more flexible and bulky substituent (a benzyl group). Importantly, the application of 50 μM SRA-36 significantly blocked CLC-Ka currents by 90% (Figure 3A-B), resulting the K_D of 6.6 ± 1 μM (Figure 2C). The further lengthening of the spacer alkyl chain did not correlate with a further drug potency increase. Indeed, when the methylene group was substituted with an ethylene one (SRA-9, Figure 2B), although drug potency was improved with respect to JBL-44, it was significantly decreased compared to SRA-36 (Figure 2C). These results showed that the drug potency was dependent on the distance between the aromatic ring in C-5 and the benzofuran nucleus, and that the presence of a single methylene group is the optimal condition to confer high blocking activity.

- *Introduction of a p-substituent on the benzyl group*

Having established the critical need for the benzyl group, we next explored the effects of the introduction of a methyl (SV-21) or a methoxy (SV-15) group in the para position of the benzyl moiety of SRA-36 (Figure 2B). Both derivatives were slightly less potent compared to SRA-36, with an inhibition in the 70-85% range (Figure 2C). Since the methyl and methoxy group exert a different effect on the electronic cloud density of the benzyl group, the observed reduced drug potency associated to SV-21 and SV-15 could likely be due to the steric hindrance of the substituent. This hypothesis was further supported by the observation that SV-19, a compound that presented a trifluoromethoxy group in the place of the methoxy group, showed an inhibition below 60 % (Figure 2B-C).

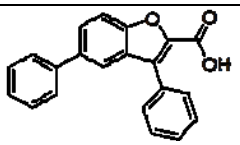
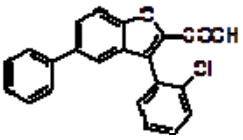
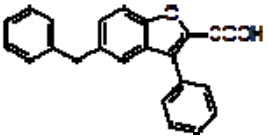
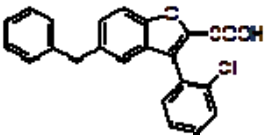
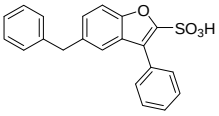
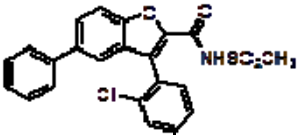
2.Modification of the hydrophobic group at the C-3 position

As previously reported (Imbrici et al., 2014), the presence of an ortho chlorine on the phenyl group at the C-3 position of the furan nucleus (RT-93) increased the blocking potency with respect to JBL-44 (Figure 2C). Since among derivatives with modification of the hydrophobic group at the C-3 position, SRA-36 resulted the most potent, we evaluated the effect of an SRA-36 analogue, in which the chlorine in ortho position of phenyl group at C-3 of benzofuran scaffold was introduced (SV-12, Figure 2B). The combination of the two modifications did not produce any enhancement in the blocking efficacy. Indeed, derivative SV-12 had a similar potency with respect to RT-93 (Figure 2C). The detrimental effect on drug potency of the ortho chlorine substitution is also observed for SV-18 carrying a *p*-substituent on the benzyl group (Figure 2B-C).

3.Modification of the acidic moiety

Since CLC-Ka/K1 is localized both at the apical and basolateral level of the renal epithelium (Uchida and Sasaki, 2005), CLC-K ligands will reach their targets from the luminal fluid, other than from the basolateral fluid. Thus, the acidic group could influence drug activity both by affecting the interaction with the binding site depending on the size of the anionic radius, and by influencing the amount of anionic component that could be present at urinary pH. Furthermore, it has been reported that the block by 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) on CLC-Ka-sustained currents was due to its hydrolysis or polymerisation products, all molecules having sulfonic acid moieties (Matulef et al., 2008). We assess the role of the acidic function by synthetizing the compound with a sulfonic group (OZ-4) or with an N-methylsulfonylcarboxamido group (DNY-15) having respectively a higher and a lower acidity than all the derivatives carrying the carboxylic acid moiety (Supplemental Table 2). Both OZ-4 and DNY-15 derivatives resulted much less potent with respect to SRA-36 and JBL-44 causing an inhibition of only 38 % (Figure 2C), thus indicating that the carboxylic acid moiety plays a pivotal role within benzofuran structures.

Supplemental Table 2
Values of pKa associated to carboxylic, sulfonic and N-methylsulfonylcarboxamido derivatives.

Molecule		pKa
JBL-44		2.5 ± 0.9
RT-93		2 ± 0.9
SRA-36		2.6 ± 0.9
SV-12		2 ± 0.9
OZ-4		1.5 ± 0.4
DNY-15		6.7 ± 0.9

pKa values for each molecule was calculated as above described.

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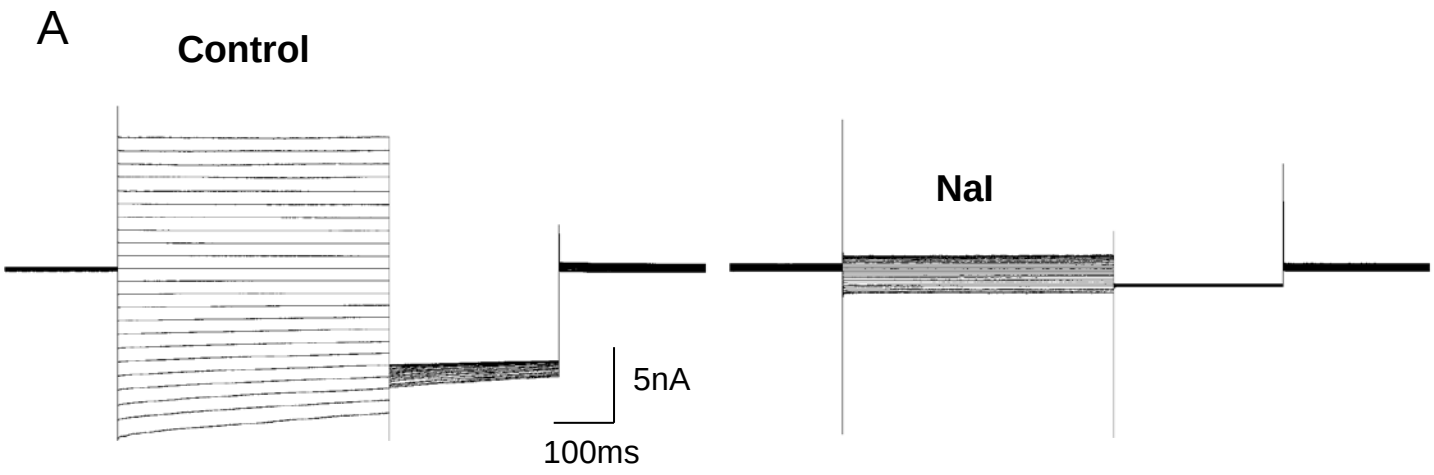
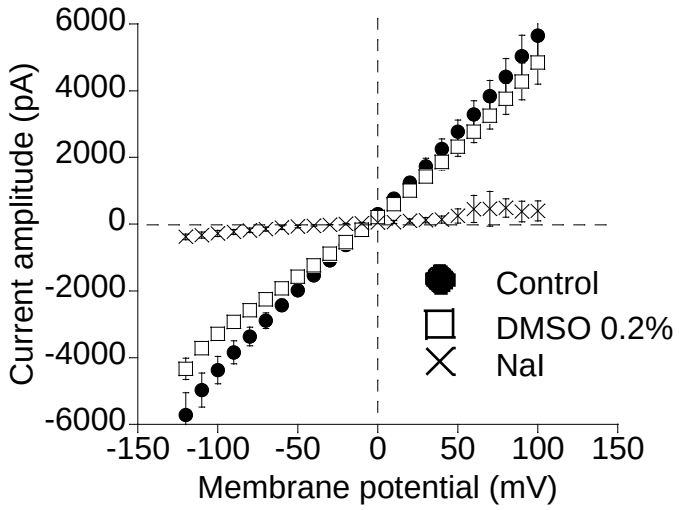
Supplemental Figure 1

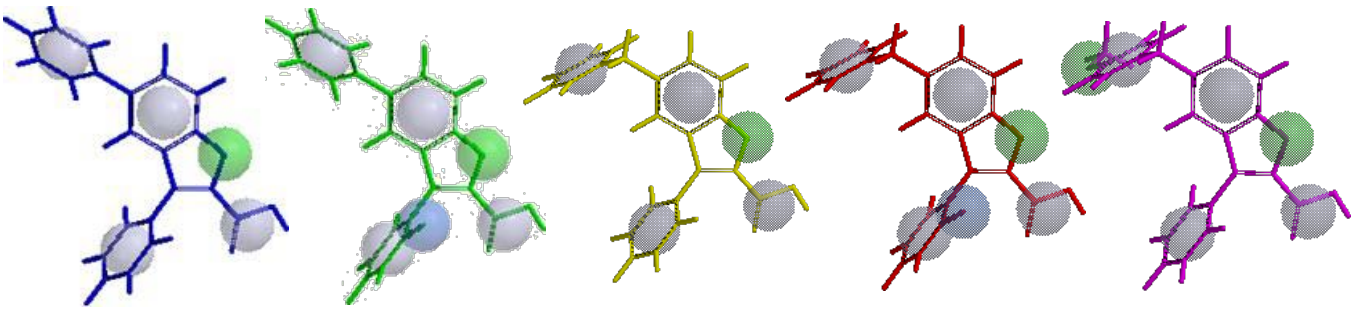




A) Representative CLC-Ka/barttin current traces recorded in the whole cell configuration of patch-clamp from HEK293 transfected cells, before and after the application of 140 mM NaI. Current were evoked from a holding potential of 0 mV to different test potentials ranging from -120 to +100 mV; **B)** Voltage-dependence of mean current amplitude obtained before and after the application of DMSO or 140 mM NaI.

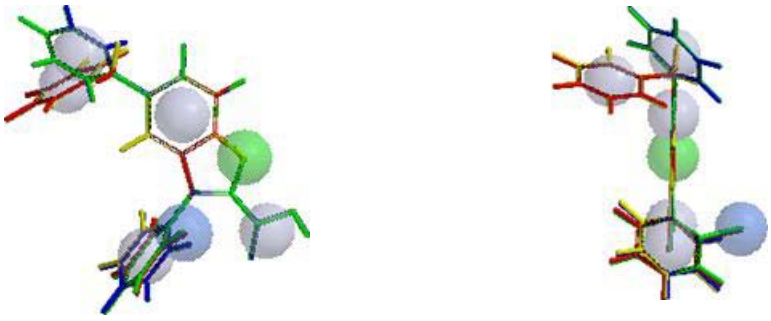
Supplemental Figure 2

A



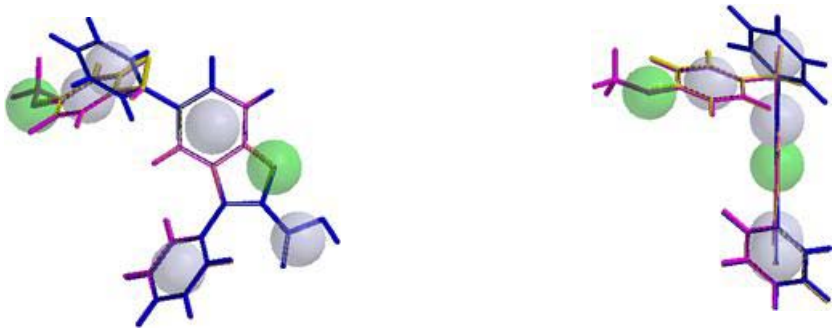
JBL-44 RT-93 SRA-36 SV-12 SV-15

B



Front view 90£ rotated view

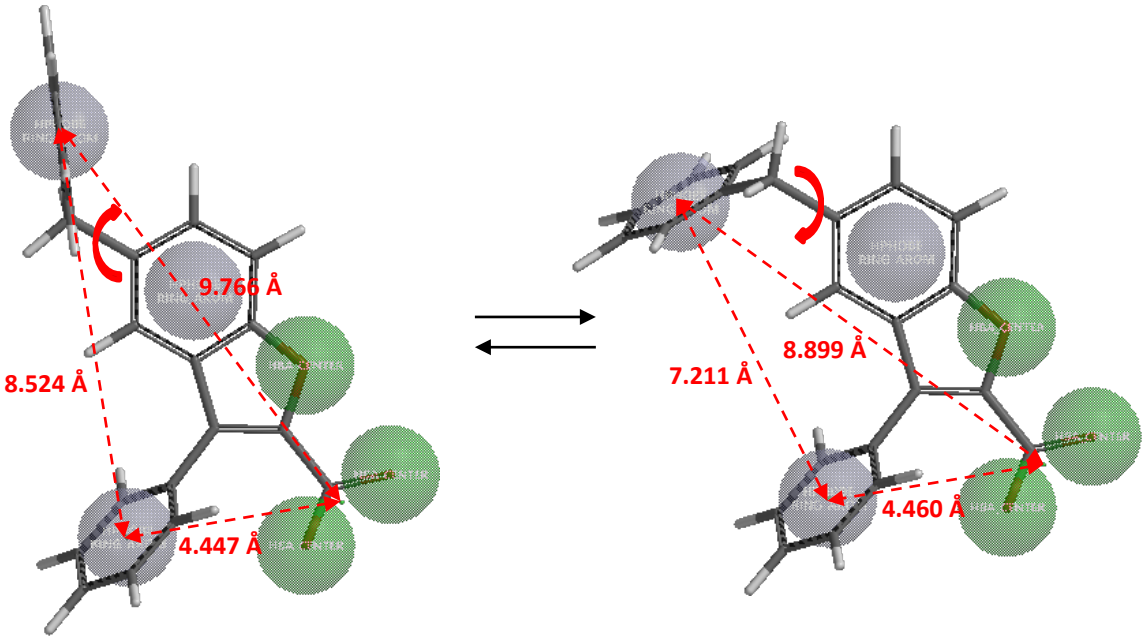
C



Front view 90£ rotated view

Supplemental Figure 2. Modeling study of JBL-44, RT-93, SRA-36, SV-12, SV-15. A) Colored stick models of JBL-44, RT-93, SRA-36, SV-12 and SV-15, with chemical function descriptors CFDs; **B)** overlay of the lowest energy conformations of JBL-44 (blue), RT-93 (green), SRA-36 (yellow) and SV-12 (red); **C)** overlay of the lowest energy conformations of JBL-44 (blue), SRA-36 (yellow) and SV-15 (magenta). The front view and the 90° left rotation view of the molecules overlay are reported.

Supplemental Figure 3



A B

SRA-36 lower energy conformers
(Boltz. distr. A:B = 60:40%)

Supplemental Figure 3. Pharmacophore model. Essential requirements for blocking action as suggested by the preliminary ligand-based investigation. A and B stand for the lower energy conformers obtained for SRA-36 (Boltzmann distribution 60:40%).