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RESEARCH PAPER

Pharmacovigilance database search discloses ClC-K channels as a novel target of the AT_1 receptor blockers valsartan and olmesartan

Correspondence Dr Paola Imbrici, Department of Pharmacy – Drug Sciences, University of Bari 'Aldo Moro', Via Orabona 4 -70125 Bari, Italy. E-mail: paola.imbrici@uniba.it

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Paola Imbrici, Domenico Tricarico, Giuseppe Felice Mangiatordi, Orazio Nicolotti, Marcello Diego Lograno, Diana Conte* and Antonella Liantonio*

Department of Pharmacy – Drug Sciences, University of Bari 'Aldo Moro', Bari, Italy

*Diana Conte and Antonella Liantonio contributed equally to this work.

BACKGROUND AND PURPOSE

Human ClC-K chloride channels are highly attractive targets for drug discovery as they have a variety of important physiological functions and are associated with genetic disorders. These channels are crucial in the kidney as they control chloride reabsorption and water diuresis. In addition, loss-of-function mutations of *CLCNKB* and *BSND* genes cause Bartter's syndrome (BS), whereas *CLCNKA* and *CLCNKB* gain-of-function polymorphisms predispose to a rare form of salt sensitive hypertension. Both disorders lack a personalized therapy that is in most cases only symptomatic. The aim of this study was to identify novel ClC-K ligands from drugs already on the market, by exploiting the pharmacological side activity of drug molecules available from the FDA Adverse Effects Reporting System database.

EXPERIMENTAL APPROACH

We searched for drugs having a Bartter-like syndrome as a reported side effect, with the assumption that BS could be causatively related to the block of ClC-K channels. The ability of the selected BS-causing drugs to bind and block ClC-K channels was then validated through an integrated experimental and computational approach based on patch clamp electrophysiology in HEK293 cells and molecular docking simulations.

KEY RESULTS

Valsartan and olmesartan were able to block ClC-Ka channels and the molecular requirements for effective inhibition of these channels have been identified.

CONCLUSION AND IMPLICATIONS

These results suggest additional mechanisms of action for these sartans further to their primary AT_1 receptor antagonism and propose these compounds as leads for designing new potent ClC-K ligands.

Abbreviations

AT_1 receptor, angiotensin type 1 receptor; BS, Bartter syndrome

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Introduction

Human **ClC-Ka** (*CLCNKA*) and **ClC-Kb** (*CLCNKB*) channels and their accessory subunit barttin (*BSND*) are expressed in the loop of Henle, distal convoluted tubule and cortical collecting ducts of the kidney, where they contribute to chloride absorption, urine concentration and consequently blood pressure maintenance (Estévez *et al.,* 2001; Krämer *et al.,* 2008; Fahlke and Fischer, 2010). Gain-of-function polymorphisms in *CLCNKA* and *CLCNKB* genes predispose subjects to a form of salt sensitive hypertension, whereas loss-of-function mutations of the *CLCNKB* and *BSND* genes cause type III and type IV Bartter's syndromes (BS), rare conditions characterized by salt and fluid loss, leading to kidney failure and sensorineural deafness in the most severe cases (Simon *et al.,* 1997; Birkenhäger *et al.,* 2001; Jeck *et al.,* 2004; Barlassina *et al.,* 2007; Sile *et al.,* 2009; Sanada *et al.,* 2011). Given the important role of these ClC-K channels in kidney physiology and their consequent association with BS and hypertension, the identification of drugs that target ClC-K channels would be highly desirable. However, investigations of ClC-K channels as a target for drug discovery have been negligible: so far, no drug on the market has proven efficacy towards these channels and patients affected by BS or salt-sensitive hypertension receive symptomatic therapies with limited clinical improvement (Verkman and Galietta, 2009; Loudon and Fry, 2014; Zieg *et al.,* 2016). In the past, by means of ligand- and structure-based approaches, we identified phenyl-benzofuran carboxylic acid derivatives as the most powerful ClC-K channel blockers (Liantonio *et al.,* 2008). Despite still being at a preclinical stage, these compounds revealed diuretic and antihypertensive effects in normotensive and hypertensive rats, suggesting that ClC-K channels may represent key targets in the management of common hypertension (Liantonio *et al.,* 2012; Liantonio *et al.,* 2016). In parallel, we identified niflumic acid, a non-steroid anti-inflammatory drug, as the only known opener of ClC-Ka and ClC-Kb channels expressed in *Xenopus* oocytes but not in mammalian cells (Liantonio *et al.,* 2006; Gradogna *et al.,* 2014; Imbrici *et al.,* 2014).

Current pharmacological research encourages cost-effective approaches favouring the repurposing of drugs already on the market, with known safety and bioavailability in humans, towards novel disease areas (Lavecchia and Cerchia, 2016). The selective optimization of side activities of commercial drugs can also be exploited to re-profile drugs by transforming side activities into novel main effects (Langer and Wermuth, 2012). Indeed, the combination of patch-clamp experiments and pharmacovigilance surveillance has already led us to investigate the atrophic/anti-atrophic effects of openers and blockers of muscle ATP-sensitive K^+ channels in humans and animals (Tricarico *et al.,* 2003a; Tricarico *et al.,* 2010; Mele *et al.,* 2014). On this basis, the aim of this study was to discover novel ClC-K channel ligands among prompt-to-use marketed drugs. Hence, we searched the FDA's pharmacovigilance database, which monitors drug safety, to identify commercial drugs that induce a Bartter-like syndrome as a side effect with the assumption that BS could result from the block of ClC-K channels. Experimental and theoretical studies were carried out to validate and interpret the results. Patch clamp electrophysiology allowed us to demonstrate that the **angiotensin II type 1 (AT_1) receptor** antagonist **valsartan** can effectively block ClC-Ka channels, whereas induced-fit docking simulations (IFD) shed light on the molecular interactions ensuring that binding occurs between ClC-K channels and valsartan. Structure-activity relationship studies allowed us to hypothesize that other AT_1 receptor antagonists having a tetrazole ring and a carboxylic group, such as **olmesartan**, could effectively block ClC-K channels. This information is crucial in the perspective of using the ClC-Ka blockers identified as lead compounds for the development of novel and more potent ClC-K channel ligands, openers and blockers, which could potentially be useful as treatments for ClC-K-associated diseases.

Methods

FDA-AERS database searching

Database searching was performed using the FDA-Adverse Effects Reporting System (FDA-AERS) database (Mele *et al.,* 2014). AERS is a passive surveillance system that accepts spontaneous reports of adverse events following any US licensed drugs from providers, health care workers, and the public. Although AERS cannot usually demonstrate causal associations between drugs and adverse events and does not give the incidence, it can detect signals to be tested with more rigorous methods including an experimental one. Symptoms recorded within AERS report were assigned to one or more coding terms using Coding Symbols for Thesaurus of Adverse Reaction Terms. In this case the specific terms were: BS and pseudo BS (mEDRA database). This process does not employ standardized case definitions. Reports of hospitalization or prolongation of hospitalization, life-threatening illness, persistent or significant disability/incapacity, or certain other medically important conditions are classified as serious. All other reports are coded as non-serious.

Electrophysiology

Human ClC-K channels, rat ClC-K1 and Y98A barttin cDNAs (courtesy of Professor Michael Push and Professor Al George Jr) were subcloned in the pcDNA3. HEK293 cells were transiently transfected with plasmid cDNAs encoding ClC-Ks and accessory subunit in a 1:1 weight ratio using a Ca^{2+} phosphate precipitation method. For the identification of transfected HEK293 cells, a plasmid encoding the CD8 antigen was co-transfected. The transfected cells were identified by microbeads coated with anti-CD8 antibodies (Dynabeads M-450 CD8; Dynal, Great Neck, NY) and were used for electrophysiological recordings. Patch clamp experiments were performed typically 1 day after transient transfection (Imbrici *et al.,* 2014). Whole-cell patch-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Pipettes were pulled from borosilicate glass (Harvard Apparatus, Holliston, MA) and had resistances of 2.2 to 3.2 MΩ. The extracellular solution contained 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$,

1 mM $MgCl_2$, and 5 mM HEPES, whereas the pipette solution contained 120 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES. Both solutions were adjusted to pH 7.4 with NaOH. Patch records for ClC-Ka and ClC-Kb were obtained by stepping the holding potential from 0 mV to various test potentials from −120 to +100 mV for 400 ms. Pulses ended with a tail pulse to −80 mV for 200 ms. For ClC-K1 recording, after a prepulse to 65 mV for 200 ms, channels were stimulated with potentials ranging from −120 to 100 mV with 10 mV increments for 400 ms. Pulses ended with a tail pulse to 65 mV. As a control, we routinely applied a solution containing 100 mM I^- that blocks currents carried by ClC-K channels but not endogenous currents (Imbrici *et al.*, 2014). For the electrophysiological recordings, compounds were daily dissolved in DMSO solutions. DMSO never exceeded 0.2%, a concentration that had no effect on ClC-K channels by itself. Current traces at each potential were filtered at 1 kHz with a 4-pole low-pass Bessel filter and acquired at 5 kHz with pClamp10 program (Axon Instruments, Sunnyvale CA, USA).

Computational details

Valsartan, losartan and olmesartan were subjected to flexible receptor docking studies using a multi stage induced-fit docking (IFD) protocol available from the Schrödinger Suite v2015-3 (Schrödinger Release, 2015). This protocol has been recently proved to be effective at identifying crucial ClC-Ka residues for the binding of benzofuran derivatives (Liantonio *et al.*, 2016). As a first step, all compounds were subjected to LigPrep to properly generate all the tautomers and ionization states at a pH value equal to 7.0 ± 2.0. A cubic docking grid placed on the centroid of residue N68 was used. Notably, an inner box having a side equal to 10 Å and an automatic outerbox were employed. In the first stage, the van der Waals radii of the protein and the ligand were scaled by a factor of 0.5 and ligands were docked using the default Glide Standard Precision (SP) mode (Glide, version 6.8, 2014). Next, Prime was used to predict and optimize the selected protein side chains. The poses were scored and filtered, and redocked using the Glide SP mode. Cartesian coordinates of the protein were extracted from the top-scored poses returned by IFD and used for standard docking studies where the protein held was fixed. More specifically, Glide v.6.5 was used by flagging default settings for extra precision (XP) mode after defining a cubic grid placed on the centroid of residue N68 with the inner box having an edge of 10 Å and an outer box having an edge of 30 Å. All docking simulations were performed using the default Force Field OPLS_2005 (Jorgensen *et al.*, 1996).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). With regard to the FDA-AERS database searching, advanced signal detection and data mining were performed by screening AERS reports among the population. We calculated the ratio of the proportion of a particular term among reports following drug administration to the proportion of the same term among serious reports following all other drugs (proportional reporting ratios, PRR) (Evans *et al.*, 2001). We used the proposed criteria for significant disproportionality (coding terms identified in at least three reports, with a PRR > 2.0 and $\chi^2 > 4.0$) to guide selection of AERS reports for further investigation. No signal is identified, if PRR is =1. The PRR was calculated using the following equation:

$$\mathrm{PRR} = a/(a+c)/b/(b+d)$$

where a is the number of reaction of interest to a given drug, b is the number of reaction of interest for all other drugs reported by the search, c is the number of all other reactions to a given drug, d is the number of all other reactions to all other drugs (Evans *et al.*, 2001). This approach was successfully used to investigate the potential atrophic affects of K_{ATP} channel blockers in humans (Tricarico *et al.*, 2008a; Tricarico *et al.*, 2010; Mele *et al.*, 2014).

For the patch clamp experiments, data were analysed offline by using pClamp 10.3 (Axon Instruments, Sunnyvale CA, USA) and Sigma Plot Software (Systat Software GmbH, Germany). Apparent dissociation constants for drugs showing blocking activity, K_D, were determined by calculating the ratio of the steady-state current in the presence and absence of the drug at different concentrations and fitting the ratios to the equation: $I(c)/I(0) = 1/(1 + c/K_D)$, where c is the concentration.

Statistical analysis was performed using Student's t-test, with $P < 0.05$ considered as significant. Results are reported as mean ± SEM from the indicated number of cells in each experiment. Formal randomization was not employed as in every single experiment the same cell was used to evaluate the effect of the test drug versus the related control. Blinding was not included in the experimental design as the scope of the study was to test whether drugs returned from the FDA-AERS were definitely able to block ClC-K channels *in vitro* and not to compare their potency.

Results

Pharmacovigilance database searching

As a first step, we performed database searching by using the FDA-AERS database. From the analysis of the pharmacovigilance registry, we identified several commercial drugs that induce Bartter-like syndrome as side effect when used to treat patients (Table 1 and Supporting Information Table S1). Up to now, five types of BS have been recognized as due to a mutation: (i) in *SLC12A1* encoding for NKCC2 (type I); (ii) in *KCNJ1* encoding for ROMK (type II); (iii) in

Table 1

Drugs returned from the analysis of the FDA pharmacovigilance database inducing Bartter-like syndrome as a side effect in the indicated number of case reports

BS-inducing drugs	Therapeutic class	Primary pharmacodynamic	Number of BS reports (2011–2012)	PRR	Proposed mechanism of BS syndrome
Furosemide	Loop diuretic	NKCC2 blocker	8 PS	29.90	Inhibits Na/K/2Cl cotransporter mimicking type 1 BS (Reinalter *et al.*, 2004)
Mycophenolate Mofetil	Immuno-suppressant	Inosine Monophosphate-Dehydrogenase inhibitor	7 SS	91.87	Unknown
Prednisone	Anti-inflammatory glucocorticoid	Glucocorticoid receptor agonist	4 PS, 2 SS	11.43	Possibly inhibits Na/K-ATPase similarly to the digitoxigenin derivative rostafuroxin (Lupoli *et al.*, 2015)
Quinapril	Antihypertensive	ACE inhibitor	4 SS	262.04	Unknown
Candesartan Cilexetil	Antihypertensive	Blocks the AT_1 receptor	2 PS, 2 SS	87.85	Unknown
Valsartan	Antihypertensive	Blocks the AT_1 receptor	1 PS, 3 SS	67.85	Unknown
Rituximab	Immuno-suppressant	Monoclonal mAb anti-CD20 B cell	4 SS	35.63	Unknown
Tacrolimus	Immuno-suppressant	Phosphatase calcineurin inhibitor	3 SS, 1 C	35.51	Stimulates the renal Na/Cl cotransporter with hypertension (Lazelle *et al.*, 2016)
Methyl-prednisolone	Antinflammatory, immuno-suppressant	Glucocorticoid receptor agonist	3 SS	26.82	Unknown
Paricalcitol	Synthetic vitamin D analogue	Vitamin D receptor agonist	2 C	1381.68	Increases Ca transport across the epithelial cells and renal phosphate excretion
Calcitriol	Hormone	Vitamin D receptor agonist	2 C	1266.54	Increases Ca transport across the epithelial cells and renal phosphate excretion
Acenocoumarol	Anticoagulant	Vitamin K antagonist	2 C	379.96	Unknown

PS, primary suspected drug; SS, secondary suspected drug; C, concomitant drug; CaSR, calcium sensing receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA

CLCNKB encoding for ClC-Kb (type III); (iv) in *BSND* encoding for barttin (type IV); and (v) in *CASR* encoding for the extracellular calcium sensing receptor (CaS receptor) (type V) (Loudon and Fry, 2014). The identified compounds were further filtered in order to select the best candidates for *in vitro* screening. In this respect, the following criteria were used: (i) high number of reports of Bartter-like adverse reactions; and (ii) physical and/or chemical properties (such as lipophilicity or bulk volume) compatible with *in vitro* screening. To minimize the risk of false positives, all compounds causing BS by a mechanism explicitly unrelated to the block of ClC-K channels were discarded (Reinalter *et al.,* 2004; Chen *et al.,* 2009; Zietse *et al.,* 2009; Lupoli *et al.,* 2015; Lazelle *et al.,* 2016). Examples of these are tobramycin, which belongs to the class of aminoglycosides and causes Bartter-like syndrome by stimulating the CaS receptor (Chen *et al.,* 2009; Zietse *et al.,* 2009), thus mimicking type V BS, and furosemide, a type of loop diuretic that causes Bartter-like syndrome by inhibiting the Na/K/Cl cotransporter, thus mimicking type I BS (Reinalter *et al.,* 2004). Drugs that interact with transporters, enzymes and channels in the kidney and skeletal muscle, including the carbonic anhydrase inhibitors acetazolamide and dichlorphenamide and the of Na/Cl co-transport inhibitor hydrochlorthiazide respectively used in hypokalaemic and hyperkalaemic disorders (Tricarico *et al.,* 2002, 2003b; Dinardo *et al.,* 2012), failed to show the BS signal supporting the specificity of the pharmacodynamic actions of the drugs investigated in determining BS symptoms. Based on these filtering criteria, we finally selected: **mycophenolate mofetil**, **quinapril**, valsartan, **candesartan cilexetil** and **acenocoumarol**.

Evaluation of the blocking efficacy of marketed drugs on ClC-Ka channels

In order to test whether the marketed drugs selected cause Bartter-like symptoms in patients by targeting ClC-K channels, we first expressed ClC-Ka in HEK293 cells and recorded chloride currents through whole-cell patch-clamp before and after the application of 50 μM of each drug (Figure 1A). Previous results showed that the phenyl-benzofuran carboxylic acid derivative SRA-36 is the most powerful ClC-K channel blocker ever known, displaying an

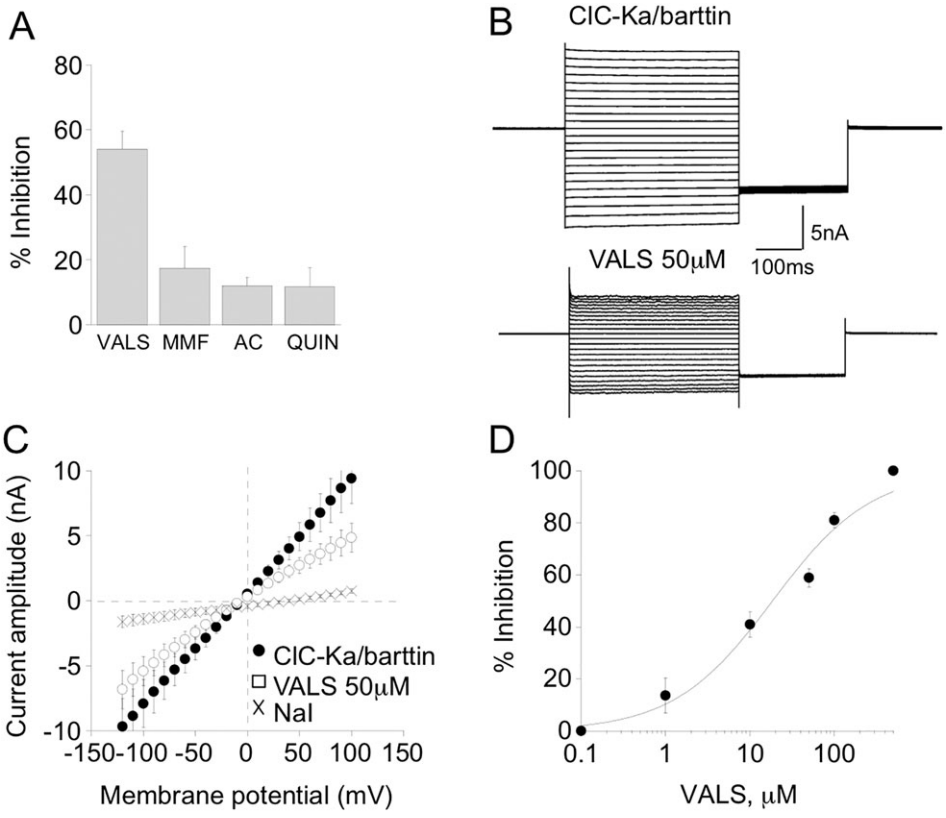


Figure 1

Effect of marketed drugs inducing Bartter-like syndrome on ClC-Ka channels expressed in HEK293 cells. (A) Bar graph showing the % of inhibition of ClC-Ka currents induced by 50 μM of drugs reported to cause BS as a side effect, measured at +60 mV with respect to control solution. Data are mean ± SEM of n = 8 cells. (B) Representative current traces of ClC-Ka/barttin channels before and after the application of valsartan 50 μM in HEK293 cells. (C) I/V plot of steady-state currents of ClC-Ka/barttin channels expressed in HEK293 cells in control condition and after the application of valsartan 50 μM. The residual current is completely blocked by NaI. Data are mean ± SEM of n = 8 cells. At +60 mV chloride currents before and after the superfusion with valsartan 50 μM are significantly different; $P < 0.05$. (D) Concentration-response curve for valsartan. Data are mean ± SEM of n = 8 cells. VALS, valsartan; MMF, micophenolate mophetyl; AC, acenocoumarol; QUIN, quinapril.

IC_{50} = 4 μM (Liantonio *et al.*, 2016). Thus, we investigated the blocking efficacy of the commercial drugs selected at the concentration (50 μM) of SRA-36 that induced a maximal block of ClC-K currents. Micophenolate mophetyl, acenocoumarol and quinapril showed a very low affinity for ClC-Ka channels, inducing less than 20% block at +60 mV (Figure 1). Surprisingly, the same concentration of valsartan caused ~60% reduction of ClC-Ka chloride currents (Figure 1A–C). The inhibition of ClC-Ka by this drug was concentration-dependent with an IC_{50} = 21 μM (Figure 1D). As HEK293 cells lack AT_1 receptors, this effect is probably due to the direct block of the channel.

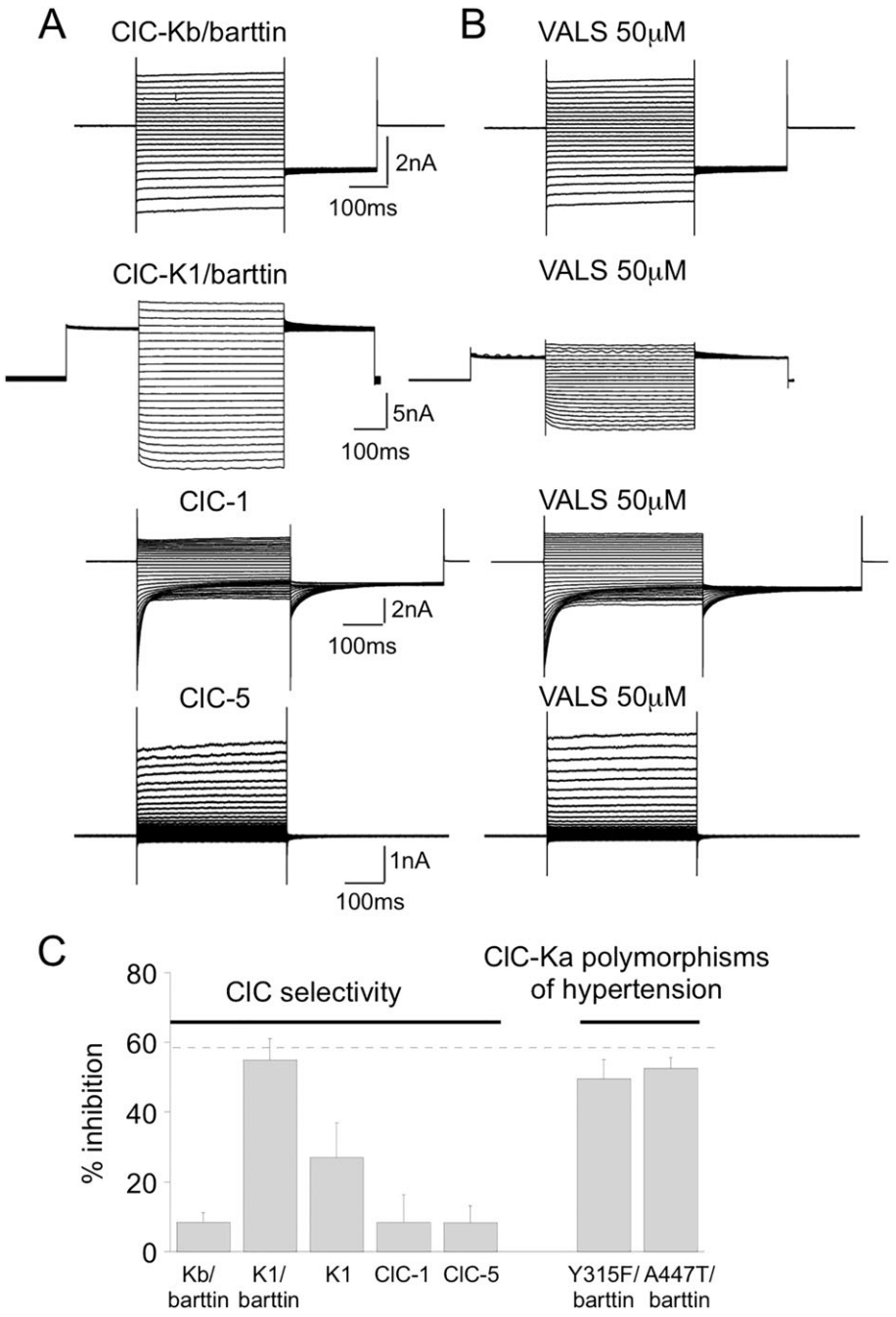


Figure 2

Selectivity of valsartan on ClC-K isoforms and effect on ClC-Ka polymorphisms associated with salt-sensitive hypertension. (A,B) Representative current traces of the indicated ClC channels before (A) and after (B) the application of valsartan 50 μM in HEK293 cells. (C) Bar graph showing the % inhibition of the indicated ClC channels induced by 50 μM valsartan. Dotted line represents effect of valsartan 50 μM on ClC-Ka channels. Data are mean ± SEM of *n* = 8 cells.

Effect of valsartan on other ClC channels and on ClC-Ka polymorphisms associated with salt-sensitive hypertension

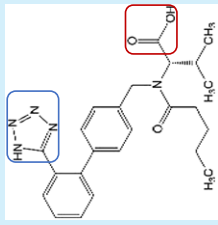
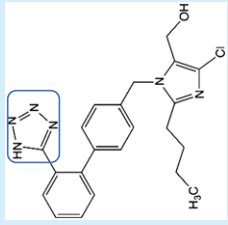
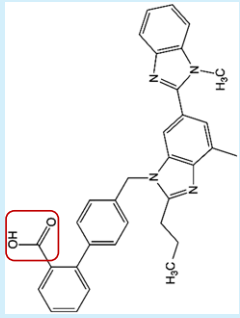
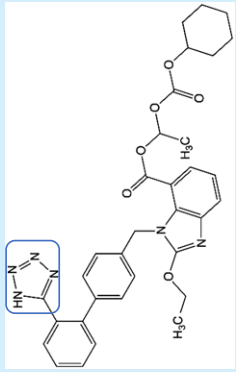
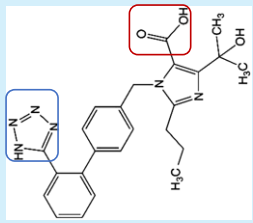
In order to verify whether valsartan was able to block other ClC-K family members, we tested its effect on ClC-Kb/barttin and ClC-K1 channels expressed in HEK293 cells. Of note, to be functional in heterologous expression systems, ClC-Ka and ClC-Kb channels require the accessory subunit barttin that allows their membrane transport and gating (Barrallo-Gimeno *et al.,* 2015). Conversely, the rat ClC-K1 isoform is able to conduct chloride currents even in the absence of barttin, and thus, it represents a useful tool to investigate the contribution of this accessory subunit to the mechanisms of channel pharmacological modulation (Barrallo-Gimeno *et al.,* 2015). As shown in Figure 2, 50 μM valsartan blocked ClC-K1/barttin channels by ~55%, similarly to ClC-Ka. Interestingly, valsartan caused a minor block of ClC-K1 currents in the absence of barttin, suggesting that this accessory subunit might be involved in the blocking effect, at least in our experimental conditions (Figure 2A–C). In contrast, ClC-Kb/barttin channels were much less sensitive to valsartan block, being inhibited by ~40% only at a concentration of 100 μM (Figure 2A–C), whereas the skeletal muscle chloride channel **ClC-1** and the kidney chloride/proton antiporter **ClC-5** were almost insensitive to valsartan block (Figure 2A-C).

Four gain-of-function polymorphisms in the ClC-Ka gene have been associated with salt retention and hypertension (Jeck *et al.,* 2004; Barlassina *et al.,* 2007; Sanada *et al.,* 2011). In order to test whether channels carrying ClC-Ka polymorphisms could be sensitive to valsartan activity, we evaluated the effect of this drug on A447T and Y315F channels. The application of 50 μM valsartan caused a ~50% block of CLC-Ka A447T and Y315F currents, similar to that observed for ClC-Ka WT channels (Figure 2C).

Structure–activity relationship of sartans

Valsartan belongs to a class of antihypertensive drugs acting as AT_1 receptor antagonists. To detect the molecular determinants of valsartan responsible for chloride current inhibition, we then tested other molecules belonging to the same pharmacological class, namely **losartan**, **telmisartan**, candesartan cilexetil and olmesartan to verify their ability to block ClC-Ka channels and measure their efficacy compared to valsartan. As shown in Table 2, these sartans share a common chemical scaffold made by a biphenyl bearing a tetrazole ring at the ortho position. The only exception is telmisartan, where the tetrazole ring is replaced by a carboxylic group, a typical bioisoster showing similar pK_a and logP values (Table 2). We observed that losartan, candesartan cilexetil and telmisartan at 50 μM were very weak blockers of ClC-Ka/barttin channels reducing chloride currents by ~10% to ~25%. Unlike them, valsartan and olmesartan, which contain both the tetrazole ring and the carboxylic group, are by far the stronger blockers of ClC-Ka/barttin channels (Table 2 and Figure 3). It is clear that the efficacious *in vitro* block of ClC-Ka channels can be related to the molecular role played by the carboxylic group and the tetrazole ring of valsartan and olmesartan.

Table 2
Structure–activity studies of sartans

Valsartan (58.9 ± 5.5)	Losartan (12.5 ± 3.9)	Telmisartan (24.5 ± 4.0)	Candesartan cilexetil (11.0 ± 5.9)	Olmesartan (50.5 ± 3.2)
				

Chemical structures of the indicated sartans and % of inhibition of ClC-Ka/barttin currents induced by 50 μM of the indicated sartan. Blue and red squares indicate the tetrazole ring and the carboxylic group respectively. Data are mean ± SEM of $n = 8$ cells.

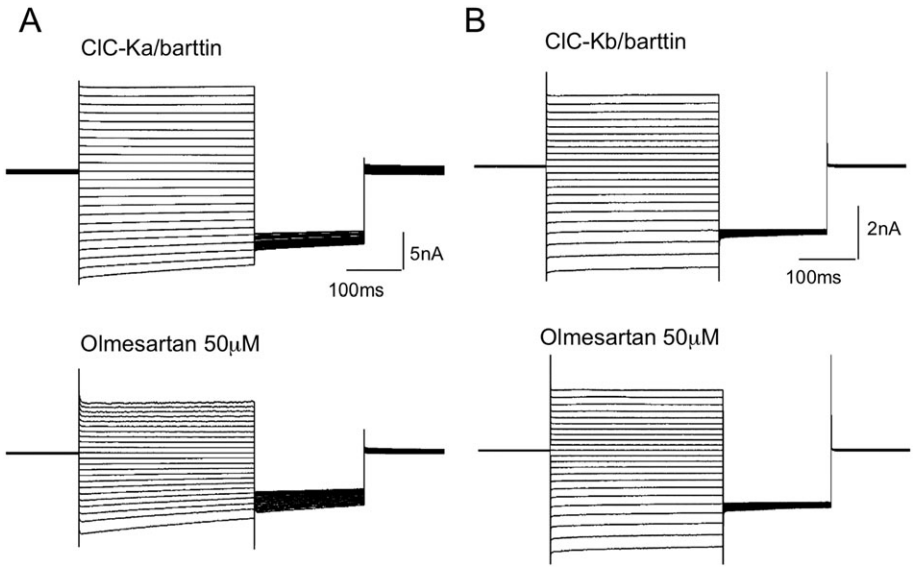


Figure 3

Effect of olmesartan on ClC-Ka and ClC-Kb channels expressed in HEK293 cells. Representative current traces of ClC-Ka/barttin (A) and ClC-Kb/barttin (B) channels before and after the application of olmesartan 50 μM in HEK293 cells.

Molecular docking simulations

By applying structure- and ligand-based approaches, we have recently identified the putative blocking binding site of carboxylic acid benzofuran derivatives on ClC-K channels. In particular, the carboxylic unit of these compounds is necessary to interact with three residues, N68, K165 and H346 located at the extracellular side of the ClC-Ka protein. The strongest ligand-protein interaction is established with K165 (Liantonio *et al.,* 2008; Gradogna and Pusch, 2013; Imbrici *et al.,* 2014; Liantonio *et al.,* 2016). Building on these findings, we performed docking simulations of valsartan, losartan and olmesartan using a homology model of ClC-Ka built on the 3D structure of ClC-ec (Dutzler *et al.,* 2002; Gradogna *et al.,* 2014), in order to hypothesize a reasonable binding mode that complemented the electrophysiological findings. Figure 4A–C show the top-scored poses of all the considered compounds returned by the application of a combined IFD/standard docking protocol (see computational details). As far as valsartan and olmesartan are concerned, the sole predicted ionization state at physiological pH is characterized by two negative charges, resulting from the deprotonation of both the tetrazole ring and the carboxylic acid group. As depicted in Figure 4A and C, these two negatively ionized moieties are able to establish salt bridge interactions with K165, the same positively charged residue crucial for the binding of benzofuran derivatives (Liantonio *et al.,* 2016). In addition, a well-orientated H-bond interaction is established between the carboxylic group of the ligand and N68, while π-π stacking interactions are observed between the proximal aromatic ring of the biphenyl fragment and F347. Finally, in the case of valsartan, hydrophobic contacts are established between the alkyl chain of the butanoic moiety and L253. Taken together, these interactions explain the significant docking score values obtained for both valsartan and olmesartan (−10.77 and −9.05 kcal·mol^{-1} respectively) and indicate the selective and high affinity binding of these sartans towards the ClC-Ka channel, in agreement with the experimental data. Interestingly, a very similar posing was observed in the top-scored docking solution of losartan (Figure 4B). In particular, both the previously described π-π stacking interactions with F347 and hydrophobic contacts with L253 are maintained. Nevertheless, only one ionic interaction occurs, which involves the tetrazole ring and K165, due to the absence of the carboxylic acid group. Thus, the docking studies suggest that the lack of interactions established with N68, which were instead experienced by valsartan and olmesartan, was probably responsible for the reduction in the docking score (−7.18 kcal·mol^{-1}) and for the lower inhibitory capacity towards ClC-Ka, consistent with the experimental data. As shown in Figure S1, the posing predicted by the molecular docking of olmesartan in ClC-Ka is very similar to the X-ray solved conformation observed for the same compound within the AT_1 receptor, available from PDB (entry 4ZUD). Interestingly, this observation confirms the hypothesized binding mode predicted by docking valsartan and olmesartan on ClC-Ka.

Discussion

In this work, we demonstrated that the antihypertensive AT_1 receptor antagonists valsartan and olmesartan are able to block ClC-Ka channels.

Integrated structure–function studies: Implications for the design of high affinity ClC-K ligands

Once it had been assessed that ClC-K channels represent a target for valsartan and olmesartan, we mainly focused on

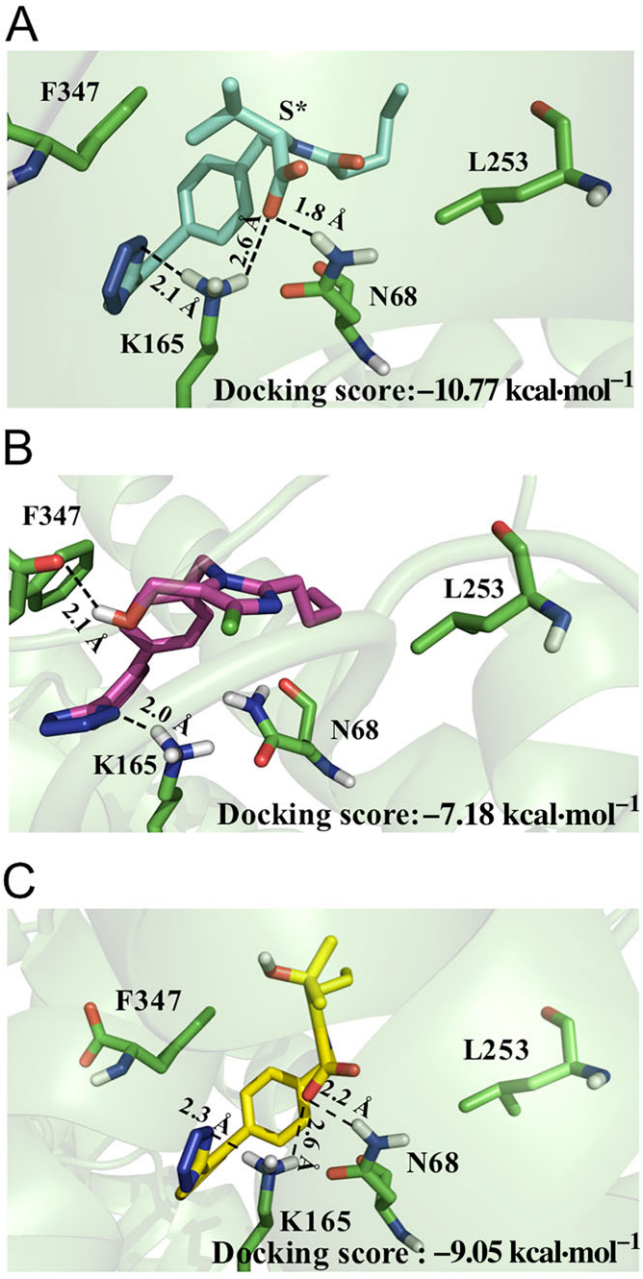


Figure 4

Docking studies of sartans in a homology model of ClC-Ka. Top-scored docking poses of valsartan (A), losartan (B) and olmesartan (C) in ClC-Ka. Important residues and docked ligands are rendered as sticks while the protein is shown as a surface. The H-bond interactions are depicted by a dotted line.

the structural determinants required for the observed block. The aim was twofold: (i) to gain insights into the putative binding site of ClC-K channels; and (ii) obtain valuable information for the future design of new drugs targeting ClC-Ks. As a first step, we carried out docking simulations of valsartan and olmesartan on ClC-Ka channels, assuming the same binding site recently defined for the phenyl-benzofuran carboxylic acid derivatives (Liantonio *et al.*, 2016). Here, we not only confirmed, based on IFD simulations, the hypothesis that valsartan and olmesartan interact with the same residues N68, K165 and H346 of the phenyl-benzofuran carboxylic acid derivatives but, together with the structure–function evaluations *in vitro*, we also provided a wealth of pharmacologically relevant information. Firstly, unlike valsartan and olmesartan, the other sartans tested did not show any appreciable block of ClC-Ka channels. These experiments convincingly showed that both the tetrazole and carboxylic acidic moieties of valsartan and olmesartan are required for an efficacious block of the ClC-Ka current. In turn, as shown in Figure 4B, due to the lack of a carboxylic acidic group, losartan does not interact with N68, hence explaining its weaker capacity to block ClC-Ka channels. In candesartan cilexetil the carboxylic group is masked by the cilexetil ester: this probably explains its reduced block of ClC-Ka *in vitro*, albeit its association with BS symptoms *in vivo*. Thus, differences in the chemical structures of sartans accounted for their different affinities towards the ClC-Ka channel. Secondly, our results also revealed that the sensitivity of ClC-K channels to valsartan depends on the primary sequence of the blocking binding site. Indeed, ClC-Kb channels, characterized by D68 in place of N68, were markedly less sensitive to valsartan and olmesartan, being blocked by 50% only at higher concentrations. Consistently, ClC-1 and ClC-5 channels, presenting the aspartate residue in the corresponding positions, were almost insensitive to valsartan block. This finding can be easily explained by the top-scored poses returned by IFD simulations: differently from N68, D68 that is negatively charged at physiological pH, is repulsed by the carboxylic moiety of valsartan. Coherently, ClC-K1 channels, having both N68 and K165 residues in the binding site, retained the same sensitivity as ClC-Ka channels towards valsartan. Taken together, these results confirmed that both K165 and N68 play a pivotal role in the binding of valsartan and the following block of ClC-Ka channels. The similarity between ClC-Ka and AT_1 receptor binding pockets again supported our *in vitro* and *in silico* observations (Supporting Information Figure S1). Finally, our experiments suggested the possible involvement of barttin in valsartan's blocking effect, as ClC-K1 channels expressed alone were less sensitive to the drug compared to ClC-K1/barttin channels.

Unveiling new activities associated with sartans: Pharmacological and toxicological implications

ClC-K genes have been designated as being among candidate genes predisposing to hypertension (Barlassina *et al.*, 2007; Denton *et al.*, 2013; McCallum *et al.*, 2015) and ClC-K blockers could offer new opportunities to treat this disorder, with a beneficial increase in water diuresis and few systemic effects (Liantonio *et al.*, 2012, 2016). Thus, the discovery of ClC-K ligands could be pivotal for a proper treatment of both rare dysfunctions of ClC-K channels, which still lack specific therapies, and more common diseases, such as hypertension. In terms of medical advice, our results suggest that the herein first observed block of ClC-Ka channels by valsartan and olmesartan may occur as an ancillary pharmacological mechanism of these drugs, possibly concurring to lowering blood pressure, together with the antagonism towards AT_1

receptors. Valsartan is currently used as first line therapy in combination with amlodipine or hydrochlorothiazide to manage hypertension, heart failure and myocardial infarction (Feldman *et al.,* 2015; Stephan *et al.,* 2015). The observation that valsartan is also effective against ClC-Ka and its polymorphisms at least *in vitro*, might open new therapeutic avenues for this drug. Moreover, the finding that other drugs belonging to the same pharmacological class showed very poor inhibitory capacity towards ClC-K channels may give insights into the toxicological profile of sartans, providing helpful suggestions for the clinical management of BS. BS-affected patients are currently treated with non-steroidal anti-inflammatory drugs, potassium supplements, potassium-sparing diuretics and angiotensin inhibitors. While potassium supplements and potassium-sparing diuretics are also used in human and animals affected by hypokalaemia and paralysis (Tricarico *et al.,* 2008b; Cetrone *et al.,* 2014; Imbrici *et al.,* 2016), the angiotensin inhibitors are recommended in BS to reduce high angiotensin II levels (Zieg *et al.,* 2016). Our results suggest the use of losartan or telmisartan in the therapeutic scheme of these patients. In contrast, valsartan could instead exacerbate the clinical phenotype. The lack of any signs of BS in the FDA-AERS database with olmesartan can be explained by the fact that this drug is one of the most recent sartans and has a more limited use, showing indication of use in hypertensive patients and precaution of use in kidney failure (Brousil and Burke, 2003; Omboni *et al.,* 2014).

In conclusion, our multidisciplinary approach combining the exploration of side effects with electrophysiology and docking experiments was successful for the reprofiling of valsartan and olmesartan as new ClC-K ligands. In addition to being of specific interest for discovering new drugs for ClC-K channels, this study represents a successful example of how the time- and cost-effective approach used here can be used as a paradigm to address the rational discovery of ligands targeting ion channels, with enhanced druggability.

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Author contributions

P.I. performed the electrophysiological experiments and wrote the paper; D.T. performed the pharmacovigilance database search; G.F.M. and O.N. performed the molecular docking simulations; M.D.L. revised the paper; D.C. and A.L. conceived and coordinated the work. All the authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Alexander SPH, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015a). The concise guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. Br J Pharmacol 172: 5744–5869.

Alexander SPH, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E *et al.* (2015b). The concise guide to PHARMACOLOGY 2015/16: Other ion channels. Br J Pharmacol 172: 5942–5955.

Barlassina C, Dal Fiume C, Lanzani C, Manunta P, Guffanti G, Ruello A *et al.* (2007). Common genetic variants and haplotypes in renal CLCNKA gene are associated to salt-sensitive hypertension. Hum Mol Genet 16: 1630–1638.

Barrallo-Gimeno A, Gradogna A, Zanardi I, Pusch M, Estévez R (2015). Regulatory/auxiliary subunits of CLC chloride channel/transport proteins. J Physiol 593: 4111–4127.

Birkenhäger R, Otto E, Schürmann MJ, Vollmer M, Ruf EM, Maier-Lutz I *et al.* (2001). Mutation of BSND causes Bartter syndrome with sensorineural deafness and kidney failure. Nat Genet 29: 310–314.

Brousil JA, Burke JM (2003). Olmesartan medoxomil: an angiotensin II-receptor blocker. Clin Ther 25: 1041–1055.

Cetrone M, Mele A, Tricarico D (2014). Effects of the antidiabetic drugs on the age-related atrophy and sarcopenia associated with diabetes type II. Curr Diabetes Rev 10: 231–237.

Chen YS, Fang HC, Chou KJ, Lee PT, Hsu CY, Huang WC *et al.* (2009). Gentamicin-induced Bartter-like syndrome. Am J Kidney Dis 5: 1158–1161.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461–3471.

Denton JS, Pao AC, Maduke M (2013). Novel diuretic targets. Am J Physiol Renal Physiol 305: F931–F942.

Dinardo MM, Camerino G, Mele A, Latorre R, Conte Camerino D, Tricarico D (2012). Splicing of the rSlo gene affects the molecular composition and drug response of Ca2+−activated K+ channels in skeletal muscle. PLoS One 7: e40235. https://doi.org/10.1371/journal.pone.0040235.

Dutzler R, Campbell EB, Cadene M, Chait BT, MacKinnon R (2002). X-ray structure of a ClC chloride channel at 3.0 A reveals the molecular basis of anion selectivity. Nature 415: 287–294.

Estévez R, Boettger T, Stein V, Birkenhäger R, Otto E, Hildebrandt F *et al.* (2001). Barttin is a Cl- channel beta subunit crucial for renal Cl-reabsorption and inner ear K+ secretion. Nature 414: 558–561.

Evans SJ, Aller PC, Davis S (2001). Use of proportional reporting ratios (PRRs) for signal generation from spontaneous adverse drug reaction reports. Pharmacoepidemiol Drug Saf 10: 483–486.

Fahlke C, Fischer M (2010). Physiology and pathophysiology of ClC-K/barttin channels. Front Physiol 1: 155.

Feldman RD, Hussain Y, Kuyper LM, McAlister FA, Padwal RS, Tobe SW (2015). Intraclass differences among antihypertensive drugs. Annu Rev Pharmacol Toxicol 55: 333–352.

Gradogna A, Imbrici P, Zifarelli G, Liantonio A, Camerino DC, Pusch M (2014). I-J loop involvement in the pharmacological profile of CLC-K channels expressed in Xenopus oocytes. Biochim Biophys Acta 1838: 2745–2756.

Gradogna A, Pusch M (2013). Alkaline pH block of CLC-K kidney chloride channels mediated by a pore lysine residue. Biophys J 105: 80–90.

Imbrici P, Liantonio A, Gradogna A, Pusch M, Camerino DC (2014). Targeting kidney CLC-K channels: pharmacological profile in a human cell line versus Xenopus oocytes. Biochim Biophys Acta 1838: 2484–2491.

Imbrici P, Liantonio A, Camerino GM, De Bellis M, Camerino C, Mele A *et al.* (2016). Therapeutic approaches to genetic ion channelopathies and perspectives in drug discovery. Front Pharmacol 7: 121. https://doi.org/10.3389/fphar. 2016.00121.

Jeck N, Waldegger S, Lampert A, Boehmer C, Waldegger P, Lang PA *et al.* (2004). Activating mutation of the renal epithelial chloride channel ClC-Kb predisposing to hypertension. Hypertension 43: 1175–1181.

Jorgensen WL, Maxwell DS, Tirado-Rives J (1996). Development and testing of the OPLS all-atom force field on conformational energetics and properties of organic liquids. J Am Chem Soc 118: 11225–11236.

Krämer BK, Bergler T, Stoelcker B, Waldegger S (2008). Mechanisms of disease: the kidney-specific chloride channels ClCKA and ClCKB, the Barttin subunit, and their clinical relevance. Nat Clin Pract Nephrol 4: 38–46.

Langer T, Wermuth C-G (2012). Selective Optimization of Side Activities (SOSA): a promising way for drug discovery. In: Peters J-U (ed). Polypharmacology in Drug Discovery, First edn. John Wiley & Sons, Inc.: Hoboken, NJ, USA.

Lavecchia A, Cerchia C (2016). In silico methods to address polypharmacology: current status, applications and future perspectives. Drug Discov Today 21: 288–298.

Lazelle RA, McCully BH, Terker AS, Himmerkus N, Blankenstein KI, Mutig K *et al.* (2016). Renal deletion of 12 kDa FK506-binding protein attenuates tacrolimus-induced hypertension. J Am Soc Nephrol 27: 1456–1464.

Liantonio A, Gramegna G, Camerino GM, Dinardo MM, Scaramuzzi A, Potenza MA *et al.* (2012). In-vivo administration of CLC-K kidney chloride channels inhibitors increases water diuresis in rats: a new drug target for hypertension? J Hypertens 30: 153–167.

Liantonio A, Imbrici P, Camerino GM, Fracchiolla G, Carbonara G, Giannico D *et al.* (2016). Kidney CLC-K chloride channels inhibitors: structure-based studies and efficacy in hypertension and associated CLC-K polymorphisms. J Hypertens 34: 981–992.

Liantonio A, Picollo A, Babini E, Carbonara G, Fracchiolla G, Loiodice F *et al.* (2006). Activation and inhibition of kidney CLC-K chloride channels by fenamates. Mol Pharmacol 69: 165–173.

Liantonio A, Picollo A, Carbonara G, Fracchiolla G, Tortorella P, Loiodice F *et al.* (2008). Molecular switch for CLC-K Cl^- channel block/activation: optimal pharmacophoric requirements towards high affinity ligands. Proc Natl Acad Sci U S A 105: 1369–1373.

Loudon KW, Fry AC (2014). The renal channelopathies. Ann Clin Biochem 51: 441–458.

Lupoli S, Salvi E, Barcella M, Barlassina C (2015). Pharmacogenomics considerations in the control of hypertension. Pharmacogenomics 16: 1951–1964.

McCallum L, Lip S, Padmanabhan S (2015). The hidden hand of chloride in hypertension. Pflugers Arch 467: 595–603.

Mele A, Calzolaro S, Cannone G, Cetrone M, Conte D, Tricarico D (2014). Database search of spontaneous reports and pharmacological investigations on the sulfonylureas and glinides-induced atrophy in skeletal muscle. Pharmacol Res Perspect 2: e00028.

Omboni S, Malacco E, Mallion JM, Fabrizzi P, Volpe M (2014). Olmesartan vs. ramipril in elderly hypertensive patients: review of data from two published randomized, double-blind studies. High Blood Press Cardiovasc Prev 21: 1–19.

Reinalter SC, Jeck N, Peters M, Seyberth HW (2004). Pharmacotyping of hypokalaemic salt-losing tubular disorders. Acta Physiol Scand 181: 513–521.

Sanada H, Jones JE, Jose PA (2011). Genetics of salt-sensitive hypertension. Curr Hypertens Rep 13: 55–66.

Schrödinger Release 2015-3: Glide, Schrödinger, LLC, New York, NY, 2015.

Schrödinger Release 2015-3: Schrödinger Suite 2015-3 Induced Fit Docking protocol; Glide, Schrödinger, LLC, New York, NY, 2015; Prime, Schrödinger, LLC, New York, NY, 2015.

Sile S, Velez DR, Gillani NB, Narsia T, Moore JH, George AL Jr *et al.* (2009). CLCNKB-T481S and essential hypertension in a Ghanaian population. J Hypertens 27: 298–304.

Simon DB, Bindra RS, Mansfield TA, Nelson-Williams C, Mendonca E, Stone R *et al.* (1997). Mutations in the chloride channel gene, CLCNKB, cause Bartter's syndrome type III. Nat Genet 17: 171–178.

Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SP *et al.* (2016). The IUPHAR/BPS guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. Nucleic Acids Res 44 (D1): D1054–D1068.

Stephan D, Gaertner S, Cordeanu EM (2015). A critical appraisal of the guidelines from France, the UK, Europe and the USA for the management of hypertension in adults. Arch Cardiovasc Dis 108: 453–459.

Tricarico D, Barbieri M, Laghezza A, Tortorella P, Loiodice F, Conte Camerino D (2003a). Dualistic actions of cromakalim and new potent 2*H*-1,4-benzoxazine derivatives on the native skeletal muscle K_{ATP} channel. Br J Pharmacol 139: 255–262. https://doi.org/10.1038/sj.bjp.0705233.

Tricarico D, Lovaglio S, Mele A, Rotondo G, Mancinelli E, Meola G *et al.* (2008a). Acetazolamide prevents vacuolar myopathy in skeletal muscle of K(+) -depleted rats. Br J Pharmacol 154: 183–190. https://doi.org/10.1038/bjp.2008.42.

Tricarico D, Mele A, Camerino GM, Bottinelli R, Brocca L, Frigeri A *et al.* (2010). The KATP channel is a molecular sensor of atrophy in skeletal muscle. J Physiol 588 (Pt 5): 773–784. https://doi.org/10.1113/jphysiol.2009.185835.

Tricarico D, Mele A, Liss B, Ashcroft FM, Lundquist AL, Desai RR *et al.* (2008b). Reduced expression of Kir6.2/SUR2A subunits explains KATP deficiency in K+−depleted rats. Neuromuscul Disord 18: 74–80.

Tricarico D, Montanari L, Conte Camerino D (2003b). Involvement of 3Na+/2K+ ATP-ase and Pi-3 kinase in the response of skeletal muscle ATP-sensitive K+ channels to insulin. Neuromuscul Disord 13: 712–719.

Tricarico D, Capriulo R, Camerino DC (2002). Involvement of K(Ca2+) channels in the local abnormalities and hyperkalemia following the ischemia-reperfusion injury of rat skeletal muscle. Neuromuscul Disord 12: 258–265.

Verkman AS, Galietta LJ (2009). Chloride channels as drug targets. Nat Rev Drug Discov 8: 153–171.

Zieg J, Gonsorcikova L, Landau D (2016). Current views on the diagnosis and management of hypokalaemia in children. Acta Paediatr 105: 762–772.

Zietse R, Zoutendijk R, Hoorn EJ (2009). Fluid, electrolyte and acid-base disorders associated with antibiotic therapy. Nat Rev Nephrol 5: 193–202.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Table S1 Additional drugs returned from the analysis of FDA pharmacovigilance database inducing Bartter-like syndrome as side effect in the indicated number of case reports.

Figure S1 X-ray solved conformation observed for Olmesartan within AT1 receptor (PDB code: 4ZUD).