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Mapping ligand binding pockets in chloride ClC-1 channels through an integrated *in silico* and experimental approach using anthracene-9-carboxylic acid and niflumic acid

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Abstract

Background and Purpose. Although chloride channels are involved in several physiological processes and acquired diseases, the availability of compounds selectively targeting CLC proteins is limited. ClC-1 channels are responsible for sarcolemma repolarization after an action potential in skeletal muscle and have been associated with myotonia congenita and myotonic dystrophy as well as with other muscular physiopathological conditions. To date only a few ClC-1 blockers have been discovered, such as anthracene-9-carboxylic acid (9-AC) and niflumic acid (NFA), whereas no activator exists. The absence of a ClC-1 structure and the limited information regarding the binding pockets in CLC channels hamper the identification of improved modulators.

Experimental Approach. Here we provide an in-depth characterization of drug binding pockets in ClC-1 through an integrated *in silico* and experimental approach. We first searched putative cavities in a homology model of ClC-1 built upon an eukaryotic CLC crystal structure, and then validated *in silico* data by measuring the blocking ability of 9-AC and NFA on mutant ClC-1 channels expressed in HEK 293 cells.

Key Results. We identified four putative binding cavities in ClC-1. 9-AC appears to interact with K231, R421 and F484 within the channel pore. We also identified one preferential binding cavity for NFA and propose R421 and F484 as critical residues.

Conclusion and Implications. This study represents the first effort to delineate the binding sites of ClC-1. This information is fundamental to discover compounds useful in the treatment of ClC-1-associated dysfunctions and might represent a starting point for specifically targeting other CLC proteins.

Keywords: ClC-1 channels, binding sites, docking simulations, patch clamp, skeletal muscle, myotonia congenita

Abbreviations: 9-AC, anthracene-9-carboxylic acid; NFA, niflumic acid; H-bond, hydrogen bond

Introduction

The voltage-dependent ClC-1 chloride channel is principally expressed in skeletal muscle fibres, where it carries the high resting chloride conductance (gCl) responsible for the stabilization of resting membrane potential and proper action potential propagation (Bryant and Morales-Aguilera, 1971; Bryant and Conte Camerino, 1991). Spontaneous loss-of-function mutations in the ClC-1 gene, affecting gating or trafficking, cause dominant and recessive myotonia congenita, the most common ClC-1-associated muscular disease (Lossin and George, 2008; Brugnoni et al., 2013; Desaphy et al., 2013), whereas reduced activity of ClC-1 channels due to post translational modifications and reduced mRNA transcripts can be predictive of conditions of altered skeletal muscle plasticity, such as aging and disuse (Pierno et al., 2013; Pierno et al., 2002; De Luca et al., 1994). ClC-1 loss-of-function and aberrant RNA splicing have also been demonstrated in the skeletal muscles of animal models of myotonic dystrophy (Charlet-B et al., 2002; Mankodi et al., 2002) and Huntington disease (Waters et al., 2013; Miranda et al., 2017).

Despite the robust characterization of ClC-1's physiological role and involvement in pathophysiological conditions, the pharmacology of this and other chloride channels remains poorly defined. As a consequence, no ClC-1 activator exists, and the current management of myotonia congenita relies on unspecific symptomatic therapies often with limited benefit (Cannon, 2015; Imbrici et al., 2016a; Trivedi et al., 2014; Lo Monaco et al., 2014). Among the few ClC-1 inhibitors identified, anthracene-9-carboxylic acid (9-AC) and niflumic acid (NFA) have been widely characterized and used as pharmacological tools to generate animal models of human myotonia or to investigate ClC-1 biophysical properties and modulation (Aromataris et al., 1999; Bryant and Morales-Aguilera, 1971; De Luca et al., 1992; Liantonio et al., 2002; 2003, 2007; Desaphy et al., 2014). Both compounds block heterologously expressed ClC-1 channels from the intracellular side (Liantonio et al., 2007; Steinmeyer et al., 1991; Pusch et al., 2002; Estevez et al., 2003).

Despite the availability of ClC-1 homology models, virtual screening campaigns have never been performed on this protein due to little existing data on ClC-1 binding sites suitable as potential targets for drug-like compounds. In 2003, Estevez and collaborators performed a mutation analysis study based on the structure of the bacterial StClC and on the different sensitivity to 9-AC of ClC-1 and ClC-2 (Dutzler et al., 2002; Estevez et al., 2003). They proposed that 9-AC binds to a partially hydrophobic pocket in ClC-1 close to the chloride binding sites and including a number of residues that possibly influence access and binding to

the pocket. Regarding NFA, a clinically-used non steroidal anti-inflammatory drug belonging to the group of fenamates, the structure-activity studies on this drug and its analogues allowed determining the chemical requisites for channel block (Liantonio et al., 2007), but no information on their binding sites on ClC-1 was inferred.

The aim of this study is therefore to provide an in-depth characterization of the binding pockets in ClC-1, using an integrated computational and experimental approach and exploiting 9-AC and NFA as molecular probes. As a first step, starting from the work by Estevez and co-authors, we identified four putative binding pockets in a homology model of ClC-1 built on the high resolution X-ray structure of the eukaryotic CLC anion-proton exchanger (CmCLC) (Feng et al., 2010; Bennetts and Parker, 2013). Then, 9-AC and NFA were docked on each identified cavity in order to find out, for each ligand, the corresponding binding residues and the mode of binding. The obtained results were validated through functional assays on newly engineered and myotonic ClC-1 mutants (Imbrici et al., 2015b; Portaro et al., 2015). We demonstrated that 9-AC binds to a pore cavity centred on K231 (helix F), F484 (helix N) and R421 (helix L), and that residues located in helices D (G190, L198) and G (G270) may help 9-AC to reach its binding site(s) and block~~ing~~ ClC-1 channels. In addition, we identified for the first time one putative cavity in ClC-1 for NFA binding and pinpointed the critical role of R421 and F484.

Materials and Methods

Mutagenesis and expression of hClC-1 WT and mutant channels

Mutations were introduced into the plasmid pRcCMV-hClC-1 containing the full-length wild-type (WT) hClC-1 cDNA using the QuickChange™ site-directed mutagenesis kit (Stratagene Cloning Systems), as previously described (Desaphy et al., 2013). The complete coding region of the cDNA was sequenced to exclude polymerase errors. The HEK 293 cells (passages 3-10) were transiently transfected with a mixture of the hClC-1 (5 μg) and CD8 reporter plasmids (1 μg) using the calcium–phosphate precipitation method. Cells were examined between 36 and 80 h after transfection. Only cells decorated with anti-CD8 antibody-coated microbeads (Dynabeads M450, Invitrogen) were used for patch-clamp recordings.

Electrophysiology and data analysis

Standard whole-cell patch-clamp recordings were performed at room temperature (~20 °C) using an Axopatch 200B amplifier (Axon Instruments). The composition of the extracellular

solution was (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES, and the pH was adjusted to 7.4 with NaOH. The high-chloride pipette solution contained (in mM): 130 CsCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES, and the pH was adjusted to 7.4 with CsOH. In this condition, the equilibrium potential for chloride ions was about −2.8 mV and cells were clamped at the holding potential of 0 mV. Pipettes were pulled from borosilicate glass and had ~3 MΩ resistance, when filled with the above pipette solutions. Currents were low-pass filtered at 2 kHz and digitized with sampling rates of 50 kHz using the Digidata 1440A AD/DA converter (Axon Instruments). Patches with a series resistance voltage error greater than 5 mV and those with non-negligible leak current were discarded. Chloride currents were recorded ~5 min after achieving the whole cell configuration, to allow the pipette solution to equilibrate with the intracellular solution.

We measured the I-V relationship in high chloride (134 mM) intracellular solutions to enhance current amplitude. To measure the voltage-dependent activity, voltage steps were applied from -150 mV to +150 mV in 10 mV intervals, each followed by a voltage step at -105 mV where tail currents were measured. Voltage steps were applied every 3s to allow complete recovery of current amplitude at the holding potential between two pulses. The steady-state current–voltage relationships were drawn by measuring steady-state current densities (pA/pF) at the end (~390 ms) of each voltage step. To evaluate the on-set and wash-out of drug effects a pulse to -150 and +60 mV was applied every 3s. As mutant channels may affect channel gating, 9-AC and NFA effects were then evaluated in the range of membrane potentials from -150 mV to +150 mV. For the electrophysiological recordings, compounds were daily dissolved in DMSO solutions. DMSO never exceeded 0.2%, a concentration without effect on ClC-1 channels by itself.

Identification of cavities in ClC-1

The FLAPsite algorithm (Henrich et al., 2010; Sirci et al., 2012; Cheng et al., 2007) was used for the identification of cavities within the homology model of ClC-1, recently published by Bennetts and Parker (Bennetts and Parker, 2013) and consisting of the ClC-1 dimer based on the crystallographic coordinates of CmClC (PDB id: 3ORG; Feng et al., 2010). The procedure started by embedding the target protein into a grid with a spatial resolution of 1.0 Å. Two GRID (Goodford et al., 1985) probes were used by the detection routine: the GRID H probe (shape) to identify pocket points and the DRY probe (hydrophobic) for buriedness calculation prioritizing hydrophobic cavities. Once all pockets points were identified by H probe, the next step focused on grid points located within a distance of 4 Å from the closest protein atom,

excluding protein surface points. For the remaining points, a buriedness-index was calculated. The buriedness-index was also weighted by the hydrophobicity computed using the GRID hydrophobic DRY probe. Points with a buriedness-index lower than a pre-determined threshold were discarded. The remaining points were processed by two morphological operations, namely erosion to remove small anomalies (decreasing the size of the cavity) and dilation to fill holes and connect areas (increasing the size of the cavity). By using the GRID hydrophobic probe (DRY), hydrophobic interactions were calculated for each cavity. Cavities showing favourable hydrophobic interactions were prioritized, since they usually bind drug molecules.

Induced fit docking simulations

Flexible receptor docking studies were performed using a multi-stage induced fit docking protocol (IFD) available from the Schrödinger Suite 2016-3 [Schrödinger Release 2016-3, Schrödinger, LLC, New York, NY, 2013]. Both 9-AC and NFA were docked on each identified binding pocket. In particular, a cubic docking grid centered on the centroid of residues belonging on the considered pocket was used. More specifically, 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 protein and ligand were scaled by a factor of 0.5 and ligands were docked using the default Glide SP mode. Then, Prime was used to predict and optimize selected protein side chains. Subsequently, the poses were scored and filtered, and finally ligands were redocked using the Glide SP mode and scored.

To visualize the ClC-1 3D structure and position of mutated residue, the homology model was rendered using Pymol (http://www.pymol.org/).

Data and Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al, 2015). Regarding patch clamp experiments, data were analyzed off-line 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, IC50, 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: $I(c)/I(0) = 1/(1 + c/IC_{50})$, where *c* is the concentration.

Statistical comparisons between the blockade of currents in cells expressing WT ClC-1 and in those expressing mutant ClC-1 were made with Student's unpaired t-tests, with $p < 0.05$

considered to indicate a significant difference. Data subjected to statistical analysis have n of at least five per group. Results are reported as mean ± SEM of the indicated number of cells. Assessment of the effects of 9-AC anor NFA on WT and mutant ClC-1 channels was replicated in two independent cell transfections to ensure the reliability of the measurements. Formal randomization and blinding were not performed because the effect of the test drug was evaluated on the same cell expressing ClC-1 channels in control solution (extracellular solution) and after the application of solutions containing different concentrations of the drug. This might be a limitation of the study.

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., 2015).

Results

Functional effect of 9-AC and NFA on ClC-1 channels expressed in HEK 293 cells

First, we confirmed the direct blocking effect of 9-AC and NFA on ClC-1 channels expressed in HEK 293 cells by recording whole-cell chloride currents before and after the application of the drugs (Figure 1A, B). As shown in Figure 1D, 300 □M 9-AC caused ~ 82% and 79% reduction of steady-state chloride currents at -90 mV and +60 mV, respectively, confirming that ClC-1 inhibition is voltage-dependent (Pusch et al., 2002). This effect was dose dependent and the calculated IC_{50} was ~ 24 ± 5 □M at -90 mV (Figure 1C). Similarly, NFA 300 □M blocked steady-state chloride currents of ClC-1 channels expressed in HEK cells by ~72% and 63% at -90 mV and +60 mV, respectively (Figure 1E); the calculated IC_{50} was ~ 97 ± 18 □M at -90 mV (Figure 1C). Differently from 9-AC (Pusch et al., 2002), NFA block was rapid in onset and completely and quickly reversible upon washout (Figure 1F).

Identification of cavities on ClC-1

Following the protocol described in Methods section, four different cavities were detected in ClC-1:

- P1 including residues M135, D136, Y137, A140, K141, N144, W148, A151 (helix B), N160, F161, W164 (helix C), L223, G224, G226, I227, P228, V229, G230, K231 (helix E), C277, C278, P279, G280, T281, P282 (helix G), A313, F316, R317, L319, A320, N323 (helix I),

A402, T405, F406, P407 (helix K), A415, E417, L418, M419, P420, R421, I424 (helix L), V457, K467 (loop L-M), F484, M485, P486, F488 (helix N), L549 (helix P);

- P2 including residues V187, G188 (loop C-D), M272 (helix G), R377 (helix J), T475, T476, M477 (helix M), I581, V584, K585 (helix R);
- P3 including residues W449, V454, V456, I458, I459, I460 (L-M loop), R496, E500 (helix N);
- P4 including residues R421, I424, S425, F428 (helix L), F488 (helix N), A525, V526, A529 (helix O), C546, L549, T550, G551 (helix P).

The four cavities identified by *FLAPsite* (Henrich et al., 2010; Sirci, et al., 2012; Cheng et al., 2007) are shown in Figure 2. Of note, residues of helix D, F, N and R form the ion transport pathway of ClC-1 channels that occupies especially part of the cavities P1 and P4. Residues R421 (helix L) and F488 (helix N) flap between cavities P1 and P4.

Cavity selection and binding mode prediction

9-AC. A previous mutational study, performed using the structure of bacterial StCLC and mutation analysis, suggested that 9-AC binds to a partially hydrophobic pocket close to chloride conduction pathway in ClC-1. Estevez and collaborators identified S537 between helix O and P as an important residue for 9-AC binding and several additional residues affecting inhibitor sensitivity with high impact, including F484, that falls right within cavity P1 (Estevez et al., 2003). However, the structures of bacterial and the eukaryotic CLC (that we used in the present study) show different conformations in CLC transport regions, which may imply possible differences in drug binding sites (Feng et al., 2010). For this reason, we decided to consider each cavity as a potential 9-AC binding site and performed IFD simulation of 9-AC on P1, P2, P3, and P4 following the protocol described in the section "Material and methods". Interestingly, P1 returned the best docking score (-9.10 kcal/mol). As evident from Figure 3A, the pose with the top-score suggests that 9-AC establishes salt-bridge interactions with K231 (helix F) and R421 (helix L). In addition, a well-oriented hydrogen bond (H-bond) interaction with the backbone of R421 was detected. Note that all these interactions involve the carboxylate moiety of the ligand. Finally, π-π stacking interactions with two residues belonging to helix N, namely F484 and F488, can be also hypothesized (Figure 3A). More specifically, a T-shape configuration (perpendicular aromatic rings) is observed between the aromatic group, of F488 and one aromatic ring of 9-AC. Additionnaly, F484 orients its side chain in a configuration that lets us suggest a sandwich stacking with the central ring of 9-AC (aromatic rings almost parallel). K231, R421 and F484

are all important residues for ClC-1 activity (Imbrici et al., 2015b; Mazon et al., 2012; Fahlke et al., 1997). K231 contributes to the chloride selective pore and K231A shows activation upon hyperpolarization and inverted voltage dependence (Fahlke et al., 1997; 1998). The mutation F484L was found in several Italian families affected by myotonia congenita where it causes a drastic reduction in the open probability of ClC-1 channels (Brugnoni et al., 2013; Imbrici et al., 2015b; Cassone et al., 2016). A molecular dynamics simulation study suggested that mutating F484 affects the stability and frequency of H-bond formation between E232 and Y578 residues (Imbrici et al., 2015b; Bennetts and Parker, 2013), thus impairing the two gating modes of ClC-1 channels, protopore and common gating (Accardi and Pusch, 2000). The mutation R421C in helix L was found to reduce channel expression in a patient affected by myotonia congenita (Mazon et al., 2012; Brugnoni et al., 2013).

NFA. As far as NFA is concerned, no information is available in the literature concerning its putative binding sites in ClC-1. Following the same approach applied to 9-AC, we docked NFA in each of the four detected pockets. In this case, the best docking score (-9.81 kcal/mol) was returned by P4, a small pocket very close to P1 (Figure 2). Interestingly, NFA was predicted to establish interactions with two residues involved in the 9-AC binding and shared by both P1 and P4 cavities, namely R421 (salt-bridge) and F488 (π-π stacking) (Figure 3B).

Identification of 9-AC binding site through mutation analysis

To validate docking results, we made amino-acid substitutions of residues returned from docking simulations and tested 9-AC inhibitory ability on K231A, R421A, F484L and F488A channels using patch clamp. We chose to use 9-AC at the concentration of maximal inhibition for WT channels, that is, 300 μM. As some mutant channels may affect voltage-dependent gating and the inhibition produced by 9-AC is known to be slightly voltage-dependent, we quantified 9-AC sensitivity between _150 and +150 mV. Such a protocol would ensure the assessment of 9-AC block at each channel state including the open one, thus limiting the influence of biophysical changes. Then, for clarity, we chose to report block at two reference potentials -90 and +60 mV (Liantonio et al., 2007; Pusch et al., 2002). Figure 4 shows that K231A, R421A and F484L mutants showed greatly reduced sensitivity to 300 μM 9-AC at the potentials tested compared with WT (Table 1). 9-AC was instead still able to block F488A currents albeit to a minor extent with respect to WT channels (~30 and ~49% reduction of steady-state currents with 300 μM 9-AC at _90 and +60 mV, respectively) (Figure 4, Table 1, Supporting Information Figure S1 and Supporting Information Table S1). For R421A, F484L and to some extent F488A, 9-AC inhibition was stronger at positive voltages.

These experimental findings corroborate the docking studies, providing evidence that 9-AC directly interacts with K231, R421 and F484 within the P1 cavity. To yield additional insights into the 9-AC binding site, we took advantage of three ClC-1 pore mutations that fall in between cavities P1 and P2 and are close to S537 (Estévez et al., 2003). We recently identified these mutations in Italian families affected by myotonia congenita (Portaro et al., 2015; Desaphy et al., 2013; Imbrici et al., 2015). We tested their response to 9-AC 300 μM of G190S, G270V and L198P channels (Figure 5 and Supporting Information Figure S1). G190 falls in the well-conserved motif GSGIPEMK, in helix D, that is important for anion selectivity and single-channel conductance (Dutzler, et al., 2002; Bennetts and Parker, 2013; Skálová et al., 2013). Helix D forms part of the ion conductive pore and is known to interact with helices H and R, and the CBS2 domain, notably being involved in the mechanism of the common gating (Bennetts and Parker, 2013; Skálová et al., 2013). The mutation G190S changes a neutral side chain to a polar one. It induces a dramatic positive shift of open probability resulting in no detectable chloride currents within the physiological range of membrane potentials (Desaphy et al., 2013). 9-AC 300 μM slightly blocked the steady-state currents of the G190S mutant channels (~18 and ~13% at -90 and +60 mV, respectively) (Figure 5, Supporting Information Figure S1 and Table 1). Another mutation located in helix D, near the chloride-binding site, is L198P. This mutation causes a large positive shift in the open probability and in comparison with WT channels reduces the steady-state current densities (Imbrici et al., 2015). L198P mutant channels were blocked by 9-AC, but to a lesser extent than in WT, with steady-state currents reduced by ~26 and ~49% at -90 and +60 mV respectively (Figure 5, Supporting Information Figure S1 and Table 1). G270V occurs in a conserved region of the transmembrane G helix, deep inside the protein and close to the chloride-conducting pathway (Portaro et al., 2015). Consistently, the mutation G270V drastically shifts the voltage-dependence of activation and dramatically reduces chloride current. In these mutant channels, the application of 9-AC 300 μM slowed the kinetics of activation and inhibited steady-state currents approximately twofold less than in WT (~49 and ~45% at -90 and +60 mV, respectively) (Figure 5, Supporting Information Figure S1 and Table 1). Finally, we chose to study a myotonia congenita mutation located in the intracellular CBS1 domain of the protein, thus far from the 9-AC binding site, L628P. This mutant channel shows current amplitude and voltage-dependence very similar to WT proteins (Imbrici et al., 2015). The application of 9-AC 300 μM blocked L628P mutant channels to the same extent as WT channels (Figure 5, Supporting Information Figure S1 and Table 1).

Identification of NFA binding site through mutation analysis

We focused on P4 that returned the highest docking score for NFA binding and included residues R421 and F488. Then, to validate the docking results, using patch-clamp experiments we determined the sensitivity of R421A and F488A channels to 100 and 300 μM NFA. Steady-state R421A currents were less sensitive to 100 and 300 μM NFA (~36 and ~35% inhibition at -90 and +60 mV with 300 μM; Figure 6, Table 1, Supporting Information Figure S2 and Supporting Information Table S1). Conversely, F488A channels responded to NFA to the same extent as WT channels (Figure 6, Table 1, Supporting Information Figure S2 and Supporting Information Table S1). We also tested the ability of NFA to block F484L channels since this residue stands very close to and interacts with F488 (Imbrici et al., 2015). NFA 300 μM inhibited F484L steady-state currents by only ~26 and ~32% (Figure 6, Table 1 and Supporting Information Figure S2). NFA was also able to block K231A mutant channels in pocket P1, thus excluding this lysine from an NFA binding site (Supporting Information Figure S3).

Discussion

To expand the results of previously published studies and provide a solid biochemical basis for ligands binding to ClC-1 channel, we performed cavity analysis and docking simulations on a homology model of ClC-1 and validated the binding pockets identified with electrophysiological experiments on engineered and spontaneous mutant ClC-1 channels, using 9-AC and NFA as probes.

9-AC and NFA block expressed ClC-1 channels

Previous studies indicated that both compounds inhibited ClC-1 channels only when applied from the intracellular side of Xenopus oocytes inside–out patches, suggesting that these drugs interfered with ClC-1 gating and Cl_ permeation by interacting with binding sites facing the cytoplasm (Estévez et al., 2003; Liantonio et al., 2007; Pusch et al., 2002). Here, we applied both 9-AC and NFA from the extracellular side of whole-cell patches from HEK293 cells transfected with ClC-1 and showed that in this experimental condition both compounds were also able to quickly block ClC-1 with comparable IC50 values, suggesting that they can easily cross the more permeable plasma membrane of mammalian cells to reach their binding cavities. The computational and experimental results of the present paper indicate that ClC-1

presents four different binding cavities and that NFA and 9-AC bind preferentially to different, albeit very close, pockets in ClC-1.

9-AC interacts with K231 and R421

Through docking simulations and pharmacological studies, we identified two positively charged residues, K231 and R421, that are predicted to form a salt-bridge and H-bond interaction with the carboxyl group of 9-AC and one aromatic residue, namely, F484, predicted to form π–π stacking interactions with the anthracene moiety of 9-AC in cavity P1. Both K231 and F484 were shown to be important for Cl_ selectivity and belong to helices F and N that point towards the chloride conductive pore (Fahlke et al., 1997; Dutzler et al., 2002; Feng et al., 2010; Bennetts and Parker, 2013; Imbrici et al., 2015). Thus, our results support the hypothesis that 9-AC enters the pore from the intracellular side and blocks the channel through direct interactions with P1 residues, hampering chloride ions from occupying their binding site (pore-blocker mechanism). In agreement with the occurrence of transmembrane binding pockets, L628P in the C-terminal region of ClC-1 is sensitive to 9-AC to a similar extent asWT channels. The F488A mutant still responded to 9-AC block in patch-clamp experiments, despite it being much less sensitive than the WT. It is possible that other residues in the cavity can compensate for the lack of stacking interactions of F488A with the 9-AC anthracene moiety, making this residue less critical. Our experiments also show that a number of pore residues may assist 9-AC binding and block. Indeed, myotonia congenita mutations G190S and L198P (helix D) and G270V (helix G) located in the pore below cavity P1 reduced the sensitivity of ClC-1 to 9-AC. G190S, L198P and G270V shift the voltage-dependence of activation toward positive potentials likely hampering chloride access to its binding site within the pore. In the case of 9-AC, this suggests that conformational changes due to mutated residues might directly interfere with 9-AC approaching its binding cavity or indirectly affect the conformation of the cavity P1.

Several *in silico* functional studies performed using different ClC homology models (from bacterial and eukaryotic transporters EcCLC, CmCLC and bovine ClC-K channel structures) support the idea that channels belonging to the ClC family share similar, albeit not identical, structural features (Feng et al., 2010; Zifarelli et al., 2010; Louet et al., 2017; Park et al., 2017). Moreover, the sequence alignment of ClC proteins (Supporting Information Figure S4) shows that at least four of the putative binding residues identified in this manuscript (G190, K231, F484 and F488) are conserved in other ClC proteins. These observations may help in

the prediction of binding cavities in different channels, although they do not necessarily correlate with sensitivity to the same drug (Feng et al., 2010; Zifarelli et al., 2010; Louet et al., 2017). Similarities exist for instance between ClC-K and ClC-1 binding pockets, thus supporting our conclusions. In particular, the recently published 3D model of the human ClC-Kb channels highlighted the importance of residues D68 (D136 in ClC-1), K165 (K231 in ClC-1), F426 (F484 in ClC-1) and M427 (M485 in ClC-1) for ClC-K function and revealed the existence of an extracellular binding cleft of the protein close to the chloride Sext position similar to pocket P1–P4 in ClC-1 (Louet et al., 2017). Remarkably, residue K165 in ClC-Ks was found to be crucial for the binding of benzofuran derivatives and sartans to these channels (Liantonio et al., 2016; Imbrici et al., 2017) that are, conversely, not sensitive to 9-AC.

With respect to the study of Estévez et al. (2003), our results confirm the role of F484 in helix N in 9-AC binding. Indeed, a similar loss of response to 9-AC had been demonstrated for F484A, F484W and F484C channels (Estévez et al. 2003). Conversely, the S537 residue, which was proposed to bind to 9-AC, was neither present in the P1 binding pocket nor detected by docking simulations among residues that are directly involved in 9-AC sensitivity. We cannot definitely rule out the involvement of S537 in 9-AC inhibition as suggested by chimeric studies performed by Estévez et al. (2003), and it is still possible that S537 mutants may affect 9-AC binding allosterically.

As in similar mutation analysis studies, some ClC-1 mutant channels showed altered biophysical properties (positive shift of the voltage-dependence of the open probability or activation upon hyperpolarization). To limit the possible interference of gating modifications and obtain an adequate measure of drug sensitivity, we quantified drug effect on mutant channels in a wide range of membrane potentials to ensure the assessment of 9-AC and NFA block at each channel state, including the open one. However, we cannot completely exclude the possibility that the blockade produced by 9-AC and NFA may be partially affected by changes in channel gating in addition to changes in binding affinity.

NFA interacts with R421

Combining the same in silico and mutational approach used for 9-AC, we mapped, for the first time, one preferential binding pocket for NFA in ClC-1 (namely P4), which shares only a few amino acids with P1. Docking simulations suggested that the guanidinium moiety of R421 in helix L coordinates the carboxylate group of NFA and that F484 can affect NFA

block These results fit well with earlier ligand-based structure–activity studies aimed at dissecting the structural characteristics of NFA analogues and CPP derivatives required to ensure membrane diffusion and stable block of ClC-1 expressed in X. oocytes. These include a carboxylic function with low pKa and two aromatic rings that form π–π interaction with hydrophobic residues (Liantonio et al., 2003; 2006). The nature of the amino acid residues that we identified in NFA binding pocket P4 is perfectly complementary to the structural requirements of these ligands (Liantonio et al., 2006).

Differently from 9-AC, we did not find any residue that, when mutated, rendered the channel totally insensitive to NFA. NFA is a small molecule and an unspecific ion channel ligand. It binds, as other fenamates, to a wide spectrum of ion channels including Ca2+-activated Cl_ channels, CFTR, ClC-K, TRPA1 and several voltage-gated potassium channels (Liantonio et al., 2006, 2007; Hu et al., 2010; Zifarelli et al., 2010; Klose et al., 2011). For some of these channels, distinct receptor sites have been supposed, activating and inhibitory, the prevalent binding and consequent pharmacological effect depending on NFA concentration, time of application and biological expression system (Liantonio et al., 2006; Imbrici et al., 2014; Garg and Sanguinetti, 2012; Zifarelli et al., 2010). In particular, regarding CLC proteins, amutation analysis study in X. oocytes based on the structure of EcCLC identified extracellular residues in ClC-K that comprise the NFA binding site including L155 (L223 in ClC-1), G345 (G411 in ClC-1) and A349 (A415 in ClC-1) (Zifarelli et al., 2010). Interestingly, the putative binding site of NFA in ClC-K partially overlaps with cavity P1 and is very close to pocket P4 in ClC-1. On the basis of these findings, we cannot exclude the possibility that NFA may fit in other locations, when critical P4 residues are mutated.

Perspectives in pharmacological research

Over the last few years, pharmacological research has turned preferential attention to cation-selective ion channels, for which numerous activators and blockers have been developed. Conversely, chloride-selective channels have been left far behind, in part due to their characteristic double barrelled structure and to screening technical difficulties due to the lack of knowledge of binding sites (Mall and Galietta, 2015). Yet ClC proteins are expressed in most cell types of eukaryotes and many prokaryotes, being involved in acquired and genetic human diseases. Still today, no crystal structure of any ClC channel-inhibitor complexes is available, precluding any attempts to design structure-based drugs and find improved channel modulators. Thus, developing in silico experimental approaches for specifically targeting ClC

proteins with small molecules could have a profound impact in medicine, biotechnology and agriculture. Regarding ClC-1, mapping the putative binding pockets within ClC-1 is, therefore, of utmost importance for the rational design and screening of new and specific inhibitors or activators of this channel type, with therapeutic potential for ClC-1-related disorders. At the same time, given the structural similarities among CLC proteins, the prediction of binding cavities in ClC-1 by computational and experimental techniques for the screening of new ligands can represent a starting point for specifically targeting other CLC proteins and related diseases.

Author contributions.; CA, DS and AF performed the electrophysiological experiments; GFM, ON and FL performed the *in silico* simulations; MRC, DC and J-F D revised the experiments and the paper; PI conceived and wrote the paper. All the authors approved the final version of the manuscript.

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Table 1. Percentage of block of steady-state ClC-1 chloride current at - 90 mV and + 60 mV induced by 9-AC and NFA 300 µM.

	% block, 9-AC 300 µM		**% block, NFA 300 µM**	
	I_{ss} (-90 mV)	**I_{ss} (+60 mV)**	**I_{ss} (-90 mV)**	**I_{ss} (+60 mV)**
WT	81.5 ± 2.9 (5)	79.3 ± 8.8 (5)	72.0 ± 12.4 (5)	62.8 ± 3.9 (5)
K231A	18.8 ± 9.5 (5) *	4.15 ± 7.8 (5) *		
R421A	120.8 ± 9.6 (5) *	24.2 ± 10 (5) *	36.47 ± 10.0 (5) *	34.8 ± 8.7 (5)
F484L	/ (5) *	4.2 ± 2.2 (5) *	26.4 ± 11.5 (5) *	31.7 ± 13.2 (5)
F488A	29.8 ± 4.3 (5) *	48.6 ± 6.5 (5)	55.6 ± 9.0 (5)	63.8 ± 13.8 (5)
G190S	18.5 ± 11.6 (5) *	13.3 ± 11.7 (5) **		
L198P	26.5 ± 11.2 (5) *	49.4 ± 9.7 (5)		
G270V	48.7 ± 8.4 (5)	45.4 ± 4.5 (5)		
L628P	68.3 ± 5.1 (5)	67.6 ± 4.9 (5)		

Data are mean ± SEM of the number of cells indicated in brackets. * $p<0.05$ compared with WT.

Figure legends

Figure 1. Effect of 9-AC and NFA on ClC-1 channels expressed in HEK 293 cells. Representative current traces of ClC-1 channels expressed in HEK 293 cells before and after application of (A) 300 µM 9-AC and (B) 300 µM NFA. Dashed line indicates zero current. The voltage protocol is reported above the current traces. (C) Dose–response relationship of 9-AC and NFA block of ClC-1 steady-state currents measured at _90 mV. Steady-state current–voltage relationship of ClC-1 channels before and after (D) 9-AC and (E) NFA 300 µM. (F) Time course of 300 µM NFA block and washout. Data are mean ± SEM of n = 5 cells. *$P < 0.05$.

Figure 2. Pockets identified in the ClC-1 homology model by the FLAPsite algorithm. The ClC-1 dimer is depicted as a cartoon, with the pockets identified as red (P1), magenta (P2),

green (P3) and yellow (P4) surface representations. The pore cavity identified by CAVER 3.0 is depicted by dots (Chovancova et al., 2012). The C-terminus of both monomers (from residue 598) is not shown for clarity.

Figure 3. Docking simulations of 9-AC and NFA on ClC-1. Top-scored poses of 9-AC (green) and NFA (magenta) in the ClC-1 dimeric structure. (A) Suggested docking site of 9-AC. The zoomed monomer is depicted as a cartoon, while critical amino acid residues (blue) and 9-AC (green) are shown as sticks. Dotted lines depict the salt-bridge interactions between the carboxyl group of 9-AC and amine and guanidinium groups of K231 and R421, respectively, and the H-bond interaction between the backbone of R421 and 9-AC. (B) Suggested docking site of NFA. The monomer is depicted as a cartoon, while critical residues (blue) and NFA (magenta) are shown as sticks. The salt-bridge interaction between the carboxyl group of NFA and the guanidine moiety of R421 is presented as a dotted line.

Figure 4. Effect of 9-AC on steady-state currents of engineered ClC-1 mutants. (A) Representative current traces of ClC-1 mutants expressed in HEK 293 cells before and after the application of 300 μM 9-AC. (B) Steady-state current–voltage relationship of the channels indicated before and after the application of 300 μM 9-AC. Data are mean ± SEM of n = 5 cells. *P < 0.05.

Figure 5. Effect of 9-AC on steady-state currents of myotonia congenita ClC-1 mutants. (A) Representative current traces of the ClC-1 mutants expressed in HEK 293 cells before and after the application of 300 μM 9-AC. (B) Steady-state current–voltage relationship of the channels indicated before and after the application of 300 μM 9-AC. Data are mean ± SEM of n = 5 cells. *P < 0.05.

Figure 6. Effect of NFA on steady state currents of engineered ClC-1 mutants. (A) Representative current traces of the ClC-1 mutants expressed in HEK 293 cells before and after the application of 300 μM NFA. (B) Steady-state current–voltage relationship of the channels indicated before and after the application of 300 μM NFA. Data are mean ± SEM of n = 5 cells. *P < 0.05.