Investigations of Pharmacologic Properties of the Renal CLC-K1 Chloride Channel Co-expressed with Barttin by the Use of 2-(p-Chlorophenoxy)Propionic Acid Derivatives and Other Structurally Unrelated Chloride Channels Blockers

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Abstract. CLC-K chloride channels are expressed in the kidney, where they play a pivotal role in the mechanisms of urine concentration and Na\(^+\)/\(H^+\) reabsorption. The identification of barttin as an essential \(\beta\)-subunit of CLC-K channels allowed performance of a pharmacologic characterization of wild-type CLC-K1 expressed in \textit{Xenopus} oocytes. To this end, a series of 2-(p-chlorophenoxy)propionic acid (CPP) derivatives were screened using the two-microelectrode voltage-clamp technique. Several chemical modifications regarding the phenoxy group of the side chain (elimination of the oxygen atom or of methylenic groups, substitutions of the chlorine atom) did not alter the drug blocking activity, with five different derivatives showing a similar potency. Among these, a derivative of CPP carrying a benzyl group on the chiral center in the place of the methyl group represented the minimal structure for blocking CLC-K1. It inhibited the channel from the extracellular side with an affinity in the 150 \(\mu\)M range. The blocking potency of this compound is fourfold increased by lowering the extracellular chloride concentration, suggesting that the drug interacts with the channel pore. Concomitantly, the effect of some “classical” Cl\(^-\) channel blockers (9-anthracencarboxylic acid, 2-(phenylamino)benzoic acid, iminodibenzoic acid, niflumic acid, 5-nitro-2-(3-phenylpropylamino)benzoic acid, 4,4‘-diisothiocyanato-2,2‘-stilbenedisulfonic acid disodium salt, and 4-acetamido-4‘-isothiocyanato-2,2‘-stilbenedisulfonic acid disodium salt) was screened. 4,4‘-Diisothiocyanato-2,2‘-stilbenedisulfonic acid disodium salt was the only one capable of blocking CLC-K1 with a potency similar to the CPP derivative, although in an irreversible manner. The newly identified substances provide a useful tool to investigate the biophysical and physiologic role of these renal channels and a starting point for the development of therapeutic drugs with diuretic action.

CLC-K chloride channels are selectively expressed in the kidney and the inner ear, where they are important for transepithelial salt transport. In humans, two highly homologous isoforms are known, indicated as CLC-Ka and CLC-Kb, whereas CLC-K1 and CLC-K2 are the related isoforms in rodents. Within the nephron, CLC-Ka/K1 and CLC-Kb/K2 are localized in the ascending limb and thick limb of Henle’s loop, respectively (1). In addition, the presence of putative CLC-K channels also in the distal convoluted tubule was recently hypothesized (2). The pivotal role of CLC-K channels in renal physiology is evidenced by the observations that mutations of CLC-Kb cause Bartter’s syndrome type III, a kidney disease characterized by severe salt wasting and hypokalemia (3). Although no mutations of CLC-Ka have been found in humans up to now, it is probably important for the mechanisms of urine concentration. This conclusion is based on the observation that CLC-K1 knockout mice showed nephrogenic diabetes insipidus with a massive vasopressin-insensitive water loss likely as a result of a reduced hypertonicity in the kidney medulla (4,5). Other than in kidney, CLC-K channels are expressed in the inner ear, where they are involved in the mechanisms of the endolymph production that is essential for sound signal transduction (6,7).

Recently, a two-transmembrane segment protein, barttin, that seems to be an essential \(\beta\)-subunit of CLC-K channels in native tissues (7) has been identified (8). Barttin strongly
increased the functional plasma membrane expression of rat and human CLC-K channels in a heterologous system (7). Co-immunoprecipitation experiments revealed a physical association between CLC-K channels and barttin (9,10). Corroborating the idea that the presence of barttin is fundamental for the correct physiologic function of CLC-K channels in native tissues, mutations of the gene encoding for the β-subunit lead to Bartter’s syndrome type IV, a disease characterized by renal failure and deafness (8). The identification of barttin as an essential β-subunit now allows exploration of their biophysical and pharmacologic properties.

In a previous study (11), we realized a preliminary pharmacologic characterization of CLC-K channels by screening the effect of a series of derivatives of 2-p-chlorophenoxy propionic acid (CPP), a specific ligand of the muscle CLC-1 channel (12,13). The bis-chlorophenoxy derivatives of CPP resulted in the unique class capable of blocking with micromolar affinity the current sustained by CLC-K chimeras as well as by wild-type CLC-K1 co-expressed with barttin (11).

Starting from one of these structures (derivative N3; see Figure 1) and using the two-microelectrode voltage-clamp technique, here we perform a structure–activity relationship study to define the molecular requisites to block the CLC-K1 channel. The identification of the pharmacophore moieties really will allow the discovery of high-affinity ligands. Thus, we tested the requirements of the two aromatic moieties of the molecule for inhibitory activity and identified derivative N11, an analogue of CPP carrying a benzyl group on the chiral center in the place of the methyl group, as the minimal structure for blocking CLC-K1 currents from the extracellular side. The binding site is located in the pore of the channel. Furthermore, we concomitantly screened the effect of some of the “classical” Cl− channel blockers (9-anthracenecarboxylic acid [9-AC], 2-(phenylamino)benzoic acid [DPC], iminodibenzoic acid, niflumic acid [NFA], 5-nitro-2-(3-phenylpropylamino) benzoic acid [NPPB], 4,4’-disothiocyanato-2,2’-stilbenedisulfonic acid disodium salt [DIDS], and 4-acetamido-4’-isothiocyanato-2,2’-stilbenedisulfonic acid disodium salt [SITS]). DIDS was the only one capable of blocking CLC-K1 with a potency similar to derivative N11, although in an irreversible manner. These newly identified substances provide a useful tool to investigate the biophysical and physiologic role of these renal channels and a starting point for the development of possible therapeutic drugs.

**Materials and Methods**

**Expression of CLC-K1 Channel in Xenopus laevis Oocytes**

Wild-type rat CLC-K1 (14) was co-expressed with human barttin. We used the activating mutant Y98A of barttin enhancing surface channel expression (7). Oocyte expression and electrophysiologic measurements were performed as described previously (13). Briefly, voltage-clamp data were acquired at room temperature (21 to 25°C) using the Pulse program (HEKA, Lambrecht, Germany) and a custom amplifier. Currents were recorded in the standard solution containing (in mM) 90 NaCl, 10 CaCl2, 1 MgCl2, and 10 HEPES at pH 7.3. In the solution with lower [Cl−], NaCl was replaced by 180 mM sucrose.

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<th>Compound</th>
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<td>N2 (CPV)</td>
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**Figure 1.** CLC-K1 inhibition by 2-(p-chlorophenoxy)propionic acid (CPP) derivatives. The columns from left to right are as follows: compound, number used in the text for indicating each derivative; structure, chemical structure of each derivative; inhibition, Kd value because it would require large amounts of drug, and the precise value was not of interest for the aims of this study.

We used sucrose to test the drug effect in low extracellular [Cl−] without evaluating possible interferences with other anion.

The following pulse protocol was used: From a holding potential of −30 mV, after a prepulse to 60 mV for 100 ms, voltage was stepped from −140 to 80 mV in 20-mV increments for 200 ms and followed by a constant pulse to −100 mV. Apparent dissociation constants, Kd, were determined by calculating the ratio of the steady-state current in the presence and in the absence of the drug and fitting the ratios to the equation I(I0)/I(I0) = 1/(1 + c/Kd), where c is the concentration (equation 1). Errors in figures are indicated as SEM. For drugs showing an affinity ≥1 mM, we did not determine the accurate Kd value because it would require large amounts of drug, and the precise value was not of interest for the aims of this study.

**Drugs**

**CPP Derivatives.** All tested compounds were synthesized in our laboratory as racemate mixtures according to procedures previously reported: CPP (N1) (15); CPV (N2) and CPPA (N12) (16); N11 (17); N3, N6, N9, and N10 (18); N4, N5, and N7 (19). Derivative N8 was...
prepared according to the procedure reported for N3 starting from diethyl-2-(4-chlorophenoxy)malonate and 1-methanesulfonyl-3-methoxy propane. Finally, the single enantiomers of derivative N11 were obtained with procedures detailed elsewhere (20).

“Classical” Chloride Channels Blockers. All drugs used were purchased from Sigma-Aldrich. The substances used were 9-AC, DPC, iminodibenzoic acid, NFA, NPPB, SITS, and DIDS. Compounds were prepared daily in aqueous bicarbonate or DMSO stock solutions, and the final concentrations were obtained by appropriate dilution with the solution used for the electrophysiologic recordings.

Results
Effect of CPP Derivatives: Structure–Activity Relationship
In agreement with our previous study (11), the CPP derivative N3, which has two chlorophenoxy groups, blocked CLC-K1 rapidly and in a reversible manner with a K_D of 119 ± 22 μM and 181 ± 40 μM at 60 mV and −140 mV, respectively (see Figure 1). Starting from this compound, we evaluated the effect of a series of analogues modified in different parts of the molecule (Figure 2) to define the structural requisites to block CLC-K1. The potency of the compounds was evaluated at 60 mV and −140 mV to reveal a possible voltage dependence of block.

Substitutions on the Aromatic Ring 1 of Derivative N3. To define the role of the chlorine atom of the aromatic ring 1 of derivative N3, we evaluated the effect of derivative N4 and N5 in which it was eliminated or substituted with a methoxy group, respectively. Figure 3A illustrates the reduction of CLC-K1 currents, at a positive and a negative voltage, observed after application of the compounds at a concentration of 200 μM. Both derivatives (N4 and N5) were less potent compared with N3, with a K_D value at 60 mV in the 350 to 450 μM range (Figure 1).

Substitutions on the Aromatic Ring 2 of Derivative N3. The same chemical modifications on ring 2 did not produce similar detrimental effect. Indeed, only the substitution of the chlorine atom with a methoxy group (N6) reduced the drug-blocking activity (Figure 3B). The substitution with an H-atom (derivative N7) did not change the drug potency (see Figure 1 for the K_D values).

Substitution of the Aromatic Ring 2 of Derivative N3 with a Methyl Group. For eliminating any doubts regarding the pivotal role of the ring 2, this phenyl group was substituted with a methyl group. Application of derivative N8 at a concentration of 200 μM did not produce any reduction of CLC-K1–sustained currents (Figure 3C), and the estimated K_D was larger than 1 mM. Thus, it seems that the aromatic ring 2 represents an important pharmacophore moiety.

Change of the Length of the Side Alkyl Chain of Derivative N3. To investigate the role of the alkyl chain, we evaluated the effect of derivative N9 in which the spacer alkyl chain was shortened to only one methylenic group and of
derivative N10 in which it was lengthened to five methylenic groups with respect to N3. Both derivatives blocked CLC-K1 currents with a similar potency as derivative N3 (Figure 4A). This shows that the capability of inhibiting the CLC-K1 channel is independent on the length of the alkyl chain and that the presence of only one methylenic group is sufficient to confer drug-blocking activity.

Elimination of the Oxygen Atom of the Phenoxy Group 2 of Derivative N3. Because the shortening of the alkyl chain to one methylenic group (N9) as well as the elimination of the substituent on the aromatic ring 2 (N7) did not alter drug activity while the presence of the aromatic ring 2 was fundamental, we evaluated the effect of derivative N11 in which a simple benzyl group was introduced on the chiral center in the place of the second chlorophenoxy function. It is interesting that this compound was able to produce a block of CLC-K1 currents with the same features and even with a slightly increased potency with respect to the more structurally complex molecule N3 (Figure 1). Furthermore, the presence of the methylenic group that distances the aromatic ring from the chiral center is very important: When the benzyl group was substituted with a phenyl one (N12), the drug potency significantly decreased with respect to derivative N11 as well as N3 (Figures 1 and 4B).

Mechanism of Action of Derivative N11

The most active derivatives include N3, N7, N9, N10, and N11, and all of these compounds are almost equipotent with a $K_D$ at 60 mV at approximately 120 μM. Among these, derivative N11 represented the minimal molecular structure capable of inhibiting CLC-K1. Thus, we characterized in more detail the mechanism of block for this compound.

Voltage Dependence of Inhibition by Derivative N11.

At a concentration of 200 μM, derivative N11 markedly decreased CLC-K1 currents both at positive and negative potentials, even if the block was more pronounced for outward currents. Both the onset of the effect and the recovery of the current upon removal of the drug were very rapid (Figure 5A). Voltage dependence of block was assessed by measuring the magnitude of the steady state at voltages between $-140$ and 80 mV. In particular, the mean amplitude of the inward steady-state current at $-140$ was $-6.7 \pm 1.2$ and $-3.4 \pm 0.7$ μA in the absence and presence of 200 μM derivative N11, respectively, whereas there was a greater effect on the outward steady-state current at 60 mV decreasing from $13.2 \pm 3.1$ to $4.7 \pm 1.2$ μA. The concentration dependence of block by the compound N11 could be well fitted by a simple titration curve at $-140$ mV and at 60 mV (Figure 6), suggesting a simple 1:1 binding. The rapid effect and washout in voltage-clamp recordings indicated that derivative N11 does not enter the cells acting from the inside but rather acts from the outside as has been demonstrated for derivative N3 in a previous study (13).

Dependence of Derivative N11 Inhibition on the Extracellular Chloride Concentration.

To test for a possible pore location of the binding site, we investigated the dependence of block on the extracellular $Cl^-$ concentration. As expected, lowering extracellular $Cl^-$ from 100 mM to 22 mM leads to a decrease of CLC-K1 $Cl^-$ currents in control conditions (Figure

Figure 4. Influence of the length of the side alkyl chain and importance of the oxygen atom of the second phenoxy group. (A) The shortening (N9) or the elongation (N10) of the side alkyl chain did not change the potency with respect to derivative N3. Each bar represents the apparent $K_D$ calculated at 60 mV for the indicated derivative. (B) Comparison between the effect produced by derivative N12 and derivative N3. Voltage-clamp traces were elicited using the pulse-protocol described in Materials and Methods. For clarity, only the current traces corresponding to 60 mV and $-140$ mV are shown.

Figure 5. Reversible and irreversible block produced by compound N11 (A) and 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (B), respectively.
More important, the potency of derivative N11 was much larger in low Cl\(^-\) compared with high Cl\(^-\) with apparent \(K_D\) values of 21.4 ± 1.5 \(\mu M\) and 45.3 ± 15 \(\mu M\) at 60 mV and -140 mV, respectively, values that are a factor of approximately 4 smaller than those in high Cl\(^-\) (Figure 7C). These results indicate that extracellular Cl\(^-\) ions antagonize the activity of derivative N11, suggesting that the drug-binding site is probably located within or close to the pore.

**Stereoselectivity.** All CPP derivatives were tested here as racemate mixtures. To determine whether one or both enantiomers were responsible for drug-blocking activity, we solved racemate derivative N11 and evaluated the effect of the single enantiomers. As shown in Figure 8A, at 200 \(\mu M\), each enantiomer was able to produce a decrease of CLC-K1 currents, albeit the S(-)-enantiomer resulted in the more potent one. The features of the mechanism of action for each enantiomer resembled that of the racemate derivative N11, i.e., the block appeared rapidly, was more pronounced at positive potentials (Figure 8A), and was quickly reversible upon washout (data not shown). At both potentials at which the \(K_D\) was calculated, the S(-)-enantiomer was fourfold more potent than the R(+) -enantiomer (Figure 8B).

**Effect of Other “Classical” Chloride Channels Blockers**

To evaluate the sensitivity of the CLC-K1 channel to other chloride channel blockers, we also screened the effect of a series of molecules that are commonly used as chloride channels blockers. NPPB, NFA, and DPC blocked the currents sustained by CLC-K1 with \(K_D\) values in 250 to 400 \(\mu M\) range at 60 mV, where 9-AC, iminodibenzoic acid, and SITS were completely ineffective.

It is interesting that DIDS turned out to be the most potent inhibitor among this class of compounds, capable of blocking in a voltage-dependent manner CLC-K1 chloride currents with a potency comparable to that of derivative N11 (Figure 9). In Figure 5B is shown the effect of the application of DIDS at a concentration of 200 \(\mu M\). In contrast to derivative N11 (Figure 5A), the effect of DIDS was irreversible upon washout.

**Discussion**

Many unresolved questions about the function of the chloride channels belonging to the CLC family are partially caused by the lack of specific pharmacologic agents (1). This is especially true for the renal CLC-K channels for which the unsuccessful attempts to express them in heterologous systems (21) has impeded *in vitro* biophysical and pharmacologic studies. The identification of barttin as a \(\beta\)-subunit of CLC-K channels has recently shed light on some unexplored aspects of

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*Figure 6.* Concentration and voltage dependence of the block of CLC-K1 by compound N11. The dose–response relationship of the block at 60 and -140 mV was determined. The solid lines are fits to Eq. 1 with the \(K_D\) values reported in the text.

*Figure 7.* Effect of extracellular Cl\(^-\) concentration on the block of CLC-K1 mediated by compound N11. Voltage-clamp traces corresponding to 60 mV and -140 mV are shown before (solid line) and after (dashed line) application of 50-\(\mu M\) compound N11 in high (A) and low (B) extracellular Cl\(^-\) concentration. The \(K_D\) values shown in C were obtained from paired experiments from oocytes that were individually subjected to high and low chloride solutions. Thus, the \(K_D\) value in high Cl does not include all of the data used for the value reported in Figure 1, explaining the slight difference between the two numbers.
these channels, particularly regarding the biophysical features and consequently the physiologic role (7,9).

Previously, we demonstrated that bis-chlorophenoxy derivatives of CPP inhibit currents sustained by CLC-K chimeras as well as by CLC-K1 co-expressed with barttin (11). Searching the structural requisites to bind and block CLC-K1, in this study, we initially evaluated the effect of derivatives of N3 with modifications mainly regarding the aromatic moieties of the drug. By this structure-activity study, we concluded that the presence of the chlorine atom on the ring 1 plays an important role probably because, depauperating the electronic cloud of the aromatic ring, it allows a π-π interaction between this portion of the molecule and a specific hydrophobic pocket located on the channel protein CLC-K1. The aromatic ring 2 is an important determinant for the drug-blocking activity, as indicated by the complete inactivity of derivative N8, and its structural requirements are different with respect to those of the aromatic ring 1. Indeed, the steric hindrance and/or the enrichment of the electronic cloud of the aromatic ring due to the methoxy group (N6) could hinder a proper drug interaction with the binding site. Unexpected, the change of the length of the alkyl chain, either the shortening (N9) or the elongation (N10), did not alter the drug activity.

All of these findings prompted us to evaluate the effect of derivative N11 having a simple benzyl function on the chiral center in the place of the more complex chlorophenoxy group. It is interesting that this derivative fully conserved the capability of blocking CLC-K1 showing KD values in 100 to 150 µM range. The further elimination of the methylenic group of the benzyl moiety (N12) caused a marked attenuation in the potency, whereas the replacement of the aromatic ring with an alkyl group, CPP (N1) and CPV (N2), led to a complete lack of drug activity. These data indicate that for drug activity on CLC-K1, the presence of a second aromatic group, spaced from the chiral center by an alkyl chain formed at least by one methylenic group, is indispensable.

Thus, among the tested CPP derivatives, N11 represents the minimal structure capable of inhibiting CLC-K1. The benzyl...
group binds probably within a hydrophobic pocket. Because the structurally more complex derivatives N3, N7, N9, and N10 were equipotent with respect to N11, it is reasonable to hypothesize that the part of the binding site that interact with ring 2 does not have a rigid conformation but that it can also accommodate a more bulky aromatic group.

The rapid onset and the reversibility of the block suggest that N11 and the other derivatives bind to a site that is accessible from the extracellular side. Thus, the sidedness of action of derivative N11 resembles that of N3, for which only extracellular application led to CLC-K1 block in excised patch clamp recordings (11).

The inhibitory activity of derivative N11 was markedly increased when the Cl\(^{-}\) concentration was lowered, indicating that the binding site is strictly related to the permeation pathway and suggesting that a possible competition between drug and Cl\(^{-}\) ions could take place. Furthermore, evaluating the effect of the single enantiomers, we demonstrated that the block mediated by derivative N11 is stereoselective because the S(-)-enantiomer was fourfold more potent with respect to R(+) -enantiomer. In contrast, it is well known that the effect of CPP on CLC-1 is stereospecific as indicated by the observation that only the S(-)-enantiomer is responsible for drug activity (12,13). The stereoselective but not fully stereospecific effect of derivative N11 leads to the speculation that the structural requirements to interact with the relevant binding site of this renal channel are not particularly strict. That a certain number of derivatives (N3, N7, N9, and N10) showed the same potency of derivative N11 further corroborates this hypothesis. Based on the three-dimensional structure of bacterial CLC homologues (22,23), a mapping of the binding site of these derivatives using site-directed mutagenesis will help to understand the mechanism of drug-channel interaction.

Among the classical chloride channel blockers, DIDS was the most effective one in blocking CLC-K1. This is also in line with a previous study in which extracellular DIDS blocked CLC-K1 as well as CLC-K chimeras (21).

Although the sensitivity of CLC-K1 to DIDS is very similar to that observed for derivative N11, the mechanism of action differs significantly from that of derivative N11 and similar derivatives: Block by DIDS is almost completely irreversible upon washout. DIDS inhibits anion channels in an unspecific manner, acting frequently on more than one class of channels such as Ca\(^{2+}\)-activated Cl\(^{-}\) channels, volume-regulated anion channels, or cystic fibrosis transmembrane conductance regulator (CFTR) (1). The irreversibility could arise from a covalent binding to the ε-amino group of lysines by the isothiocyanate moieties (24,25). The lack of effect of the strictly related stilbene derivative SITS would lead to the hypothesis that both isothiocyanate groups of DIDS are necessarily involved in such an interaction with lysine residues located on the extracellular side of the channel protein.

In contrast to 9-AC and iminodibenzoic acid that were completely ineffective on CLC-K1, DPC, NPBB, and NFA showed blocking activity, albeit with a reduced potency with respect to derivative N11. NPBB and particularly DPC seemed to have a high affinity for Cl\(^{-}\) channels of the basolateral membrane of the thick ascending limb (26). In addition, it has been reported that intracellular application of NPPB and DPC blocked Cl\(^{-}\) currents of native mouse distal convoluted tubule, which are presumably sustained by CLC-K channels (2). Thus, the heterogeneous structures of these drugs as well as the different sidedness of drug action found on native tissues suggests that the binding site for these molecules may be somewhat unspecific and different from that of derivative N11.

CLC-K1 is mainly expressed in the thin ascending limb and is thought to be one main determinant of the efficiency of urinary concentration. Thus, CLC-K1 could represent an interesting pharmacologic target to obtain a diuretic effect, and derivative N11 could be a candidate starting compound. In this regard, it is interesting to notice that the activity of derivative N11 is specific for the renal CLC-K1 channel. Indeed, this derivative binds CLC-1 with a much smaller potency with respect to CPP, producing only a slight inhibition of native gCl of rat skeletal muscle fibers as well as of expressed CLC-1 channel (19). Recently, the inhibitor binding site for 9-AC and the more simple clofibrate derivative p-chloro phenoxy-acetic acid (CPA) have been mapped on CLC-1 (27), CPA binds to a hydrophobic pocket, formed by several aromatic residues, in which a serine in position 537 plays a key role in determining drug affinity. This inhibitor binding site is localized close to the chloride binding site and is accessible only from the intracellular side. This CPA binding site is most likely completely distinct from the binding site for derivative N11 on CLC-K1 that is directly accessible from the extracellular side.

Regarding the specificity of drug action, preliminary experiments show that derivative N11 blocked the human isoform CLC-Ka with a potency and a mechanism of action very similar to that observed for CLC-K1. Surprising is that it was completely ineffective on human CLC-Kb (unpublished results). This selective drug activity among the two CLC-K isoforms evidences that derivative N11 could represent on the one hand a useful tool to distinguish these highly homologous chloride channels and on the other hand a therapeutic drug to control diuresis without severe NaCl losing and devoid of side effect on the inner ear.

Loop diuretics, such as furosemide or bumetanide, act from the luminal side in the nephron, like most diuretics. In light of our results identifying the presence of an extracellular binding site and taking into account the apical and the basolateral localization of CLC-K1 channels in the nephron, CLC-K1 channels could represent a useful alternative target to obtain a diuretic effect. CLC-K1 inhibitor may reach the specific target via the luminal fluid as well as via the basolateral fluid. This latter possibility could ensure drug activity also in condition of decreased renal blood flow or renal failure in which instead the therapeutic effectiveness of the furosemide-like diuretics could be seriously compromised.

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