Point of VIEW

ACS chemical biology

New Light on the "Old" Chloride Channel Blocker DIDS

Heike Wulff*

Department of Pharmacology, University of California, Davis, 451 Health Sciences Drive, Davis, California 95616

ABSTRACT 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) has been used as an inhibitor of anion transporters and channels since the early 1970s. A study in this issue shows that DIDS hydrolyzes in aqueous solution and then multimerizes to di-, tri-, tetra-, and pentameric polythioureas, which inhibit both the bacterial ClC-ec1 Cl^-/H^+ exchanger and the mammalian ClC-Ka chloride channel 3−200 times more potently than DIDS itself. The DIDS tetra- and pentamer could potentially act as tethered blockers that simultaneously obstruct both chloride pathways in the dimeric CLC proteins.

CLC proteins are ubiquitously expressed in both prokaryotes and eukaryotes and play crucial roles in chloride transport, volume regulation, organelle acidification, and membrane excitability (*1*). The family contains both *bona fide* chloride channels as well as Cl^-/H^+ antiporters (exchangers) and thus straddles the line between channels and transporters, which was traditionally viewed by biophysicists as an absolute black-and-white divide (*2*). CLC proteins have therefore recently caused a great stir in the biophysical community: it was a surprise that membrane proteins built along similar structural lines can support two ion-transport mechanisms as fundamentally different in terms of their thermodynamics as "downhill" chloride flux following its concentration gradient and Cl^-/H^+ exchange, which moves chloride "uphill" against a concentration gradient (*3, 4*). In contrast to the much better studied potassium channels, where the ion-conducting pathway lies at the interface of the four subunits of the tetrameric channels and is orientated perpendicular to the membrane (*5–7*), CLC proteins are dimers that contain two separate pores that "snake" their way through each subunit (*8, 9*). However, the exact mechanism of chloride flux or transport through these "pores" or permeation pathways is currently not clear and is the focus of intense research.

The human genome contains nine CLC proteins, which serve various physiological functions and potentially constitute novel exciting drug targets for the treatment of hypertension, osteoporosis, and gastrointestinal and renal disorders (*1*). ClC-1 channels are essential for controlling skeletal muscle excitability, and mutations in the ClC-1 gene cause congenital myotonia (muscle stiffness) in humans, mice, dogs, and goats. ClC-2 channels are widely expressed throughout the body and play important roles in volume regulation and transepithelial fluid secretion. The ClC-2 activator lubiprostone (*10*), a bicyclic fatty acid metabolite of prostaglandin E1, is the first clinically used chloride channel modulator that was approved by the U.S. Food and Drug Administration in 2006 for the treatment of chronic constipation. In the kidney and the ear, transepithelial chloride transport is mediated by ClC-Ka and ClC-Kb, two closely related channels, which require barttin as a β-subunit to form functional channels. Mutations in either ClC-Kb or barttin result in Bartter's syndrome III and IV, a defect in urinary concentration ability resembling diabetes insipidus. The remaining five human ClC proteins (ClC-3 to ClC-7) mainly reside in membranes of the endosomal/lysosomal system and in synaptic vesicles and seem to function as Cl^-/H^+ exchanges (*3*) and not as channels, similar to the bacterial ClC-ec1 Cl^-/H^+ exchanger (*4*). The vesicular CLCs are believed to facilitate endocytosis and vesicle acidification, and mutations in ClC-5 and ClC-7 have been shown to underlie Dent's disease (recurrent kidney stones) and osteopetrosis (excessive bone thickening) in both mice and humans.

***Corresponding author, hwulff@ucdavis.edu.**

Published online July 18, 2008

10.1021/cb800140m CCC: $40.75

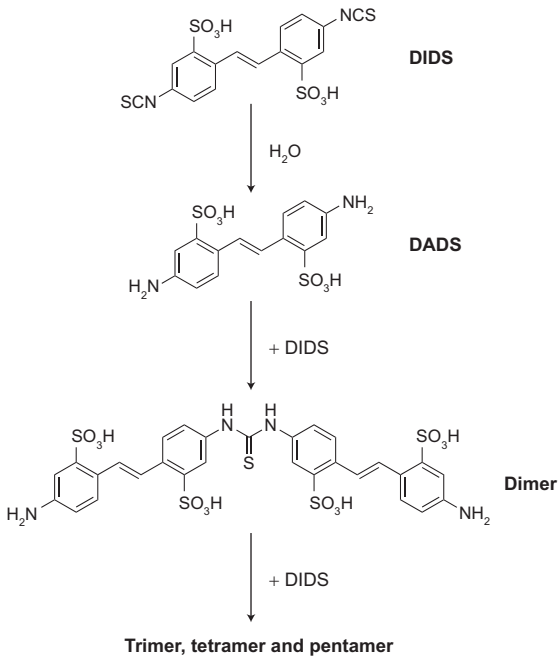

Figure 1. Hydrolysis and subsequent oligomerization of the chloride channel blocker DIDS to polythioureas in aqueous solution.

With the exception of the above-mentioned ClC-2 activator lubiprostone (*10*), CLC chloride channels and exchangers have a poorly developed pharmacology. The "classical" chloride channel blockers, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), niflumic acid, 9-anthracene-carboxylic acid, and *p*-chlorophenoxy-propionic acid, are all of low potency and selectivity, and there is currently an urgent need in the field for potent and selective CLC modulators. For example, the most commonly used tool compound, DIDS, inhibits the ClC-Ka chloride channel with an IC_{50} of 100 μM and the bacterial ClC-ec1 Cl^-/H^+ exchanger with an IC_{50} of ~300 μM and was long known to be unstable in aqueous solution. On page 419 of this issue, Matulef *et al.* (*11*) describe that DIDS forms thiourea oligomers in aqueous solution that inhibit CLC proteins significantly more potently than DIDS itself.

The authors originally were trying to crystallize ClC-ec1 with DIDS bound but soon realized that they first needed to examine the stability of DIDS before attempting to soak DIDS into the crystals or to co-crystallize. While a freshly prepared solution of DIDS in aqueous phosphate buffer rendered a single peak when analyzed by HPLC, solutions that had been left at 50 °C for 48 h no longer contained any detectable DIDS but rather five new, previously undetectable peaks. To their surprise, the authors found that the hydrolyzed DIDS mixture inhibited ClC-ec1 much better than DIDS itself (IC_{50} ~5 μM instead of 300 μM).

Matulef *et al.* therefore isolated each of the five hydrolysis products by semipreparative HPLC and determined their structure by mass spectrometry and ^1H NMR and through independent chemical synthesis. The five fractions corresponded to 4,4′-diaminostilbene-2,2′-disulfonic acid (DADS), a compound in which both isothiocyanate groups of DIDS had been hydrolyzed to primary amines, and four polythioureas containing two, three, four, or five DIDS units. The multimers are very likely the result of a reaction of the amino groups of DADS with the isothiocyanate groups of DIDS to the dimer and subsequently to the higher-molecular-weight trimer, tetramer, and pentamer (Figure 1). Although the amine DADS had no effect on ClC proteins, the oligomers were found to be increasingly potent ClC inhibitors. The pentamer reduced ClC-ec1 activity in flux assays with an IC_{50} of 1.5 μM and blocked ClC-Ka in electrophysiological experiments with an IC_{50} of 500 nM, making it the most potent ClC blocker reported so far. Matulef *et al.* further show that both the tetramer and the pentamer directly bind to ClC-Ka by demonstrating that a single amino acid mutation of a residue on the extracellular side, which had been previously identified to decrease the affinity of DIDS (*12*), completely eliminated inhibition.

Although the tetrameric and pentameric DIDS derivatives identified by Matulef *et al.* are unsuitable as potential drugs because of their high molecular weight and their charge, they constitute extremely valuable new tool compounds with which to study ClC protein function and structure. Interestingly, the fully extended pentamer would be long enough to simultaneously occlude both chloride permeation pathways in the crystal structure of the ClC-ec1 protein (*8, 9*). Matulef *et al.* therefore speculate that the pentamer acts as a tethered blocker and suggest that the 200-fold increase in potency in comparison to DIDS is due to the compound binding to both subunits of the dimeric protein (Figure 2). In this respect, it would be interesting to test in the future whether the hexamer or the heptamer might be even more potent CLC blockers or whether the pentamer already has the optimal length for a tethered CLC blocker. For structural studies, the pentamer will have the great advantage that it is stable and will allow investigators to attempt cocrystallization of ClC-ec1 or other CLCs with the pentamer. On the basis of these structures, it should then be possible to perform structure-guided drug design and eventually obtain more potent and selective CLC modulators. In addition, there is the exciting possibility that a blocker like the pentamer might help stabilize ClC-ec1 in a conformation different from that of the previously crystallized one and might help us to obtain a different "snapshot" of the transport cycle.

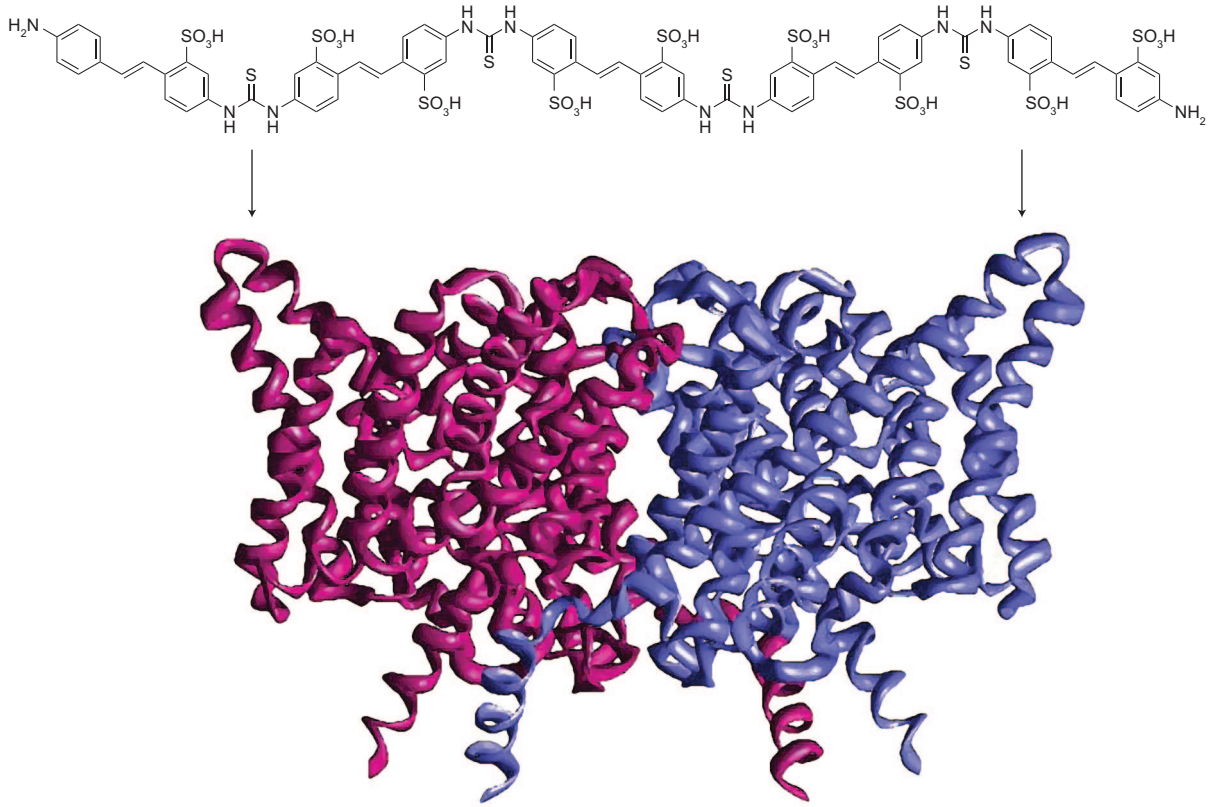

Figure 2. The pentameric DIDS derivative could potentially block both chloride permeation pathways of the dimeric CLC proteins simultaneously (ribbon rendering of the crystal structure of ClC-ec1 (Protein Data Bank 1KpK)).

REFERENCES

1. Jentsch, T. J. (2008) CLC chloride channels and transporters: from genes to protein structure, pathology and physiology, *Crit. Rev. Biochem. Mol. Biol. 43*, 3–36.
2. Miller, C. (2006) ClC chloride channels viewed through a transporter lens, *Nature 440*, 484–489.
3. Scheel, O., Zdebik, A. A., Lourdel, S., and Jentsch, T. J. (2005) Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins, *Nature 436*, 424–427.
4. Accardi, A., and Miller, C. (2004) Secondary active transport mediated by a prokaryotic homologue of ClC Cl^- channels, *Nature 427*, 803–807.
5. Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K+ conduction and selectivity, *Science 280*, 69–77.
6. Long, S. B., Campbell, E. B., and Mackinnon, R. (2005) Crystal structure of a mammalian voltage-dependent Shaker family K+ channel, *Science 309*, 897–903.
7. Long, S. B., Tao, X., Campbell, E. B., and MacKinnon, R. (2007) Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment, *Nature 450*, 376–382.
8. Mindell, J. A., Maduke, M., Miller, C., and Grigorieff, N. (2001) Projection structure of a ClC-type chloride channel at 6.5 Å resolution, *Nature 409*, 219–223.
9. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity, *Nature 415*, 287–294.
10. Lacy, B. E., and Levy, L. C. (2007) Lubiprostone: a chloride channel activator, *J. Clin. Gastroenterol. 41*, 345–351.
11. Matulef, K., Howery, A. E., Tan, L., Kobertz, W. R., Du Bois, J., and Maduke, M. (2008) Discovery of potent CLC chloride channel inhibitors, *ACS Chem. Biol. 3*, 419–428.
12. Picollo, A., Liantonio, A., Didonna, M. P., Elia, L., Camerino, D. C., and Pusch, M. (2004) Molecular determinants of differential pore blocking of kidney CLC-K chloride channels, *EMBO Rep. 5*, 584–589.