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Cation/proton antiporters: novel structure-driven pharmaceutical opportunities

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Cation/proton antiporters (CPAs) regulate cells' salt concentration and pH. Their malfunction is associated with a range of human pathologies, yet only a handful of CPA-targeting therapeutics are presently in clinical development. Here, we discuss how recently published mammalian protein structures and emerging computational technologies may help to bridge this gap.

CPAs: physiological functions and association with human pathologies

Membrane-residing CPAs exchange protons with cations (mainly sodium and potassium) across the cell membrane, thereby balancing intracellular ion concentration and maintaining pH homeostasis. Thus, CPAs are involved in many cellular- and organism-level biological processes, both directly and indirectly [1,2]. Thirteen CPA paralogs are known to be expressed in humans (Box 1); these antiporters share a common fold, characteristic of most known CPAs [1]. As CPAs modulate both cytoplasm and organelle homeostasis, and can also alter the extracellular environment, their malfunction is associated with numerous human pathologies. For example, overactivation of Na+/H+ exchanger (NHE) 1, the predominant isoform in the heart and skeletal muscles, has been associated with calcium accumulation via the Na^+/Ca^{2+} exchanger and with pH abnormalities in cardiomyocytes, leading to cardiac hypertrophy and heart failure [2,3]. NHE1 may also promote tumor invasiveness in numerous types of cancer, by locally modifying intracellular and extracellular pH [2]. Another isoform, NHE3, expressed in the gastrointestinal tract, kidney, and urinary bladder, is associated with intestinal inflammation [2,3]. Mutations in the NHE6 gene are linked to Christianson syndrome (a disorder affecting the nervous system) and are associated with epilepsy, ataxia, intellectual disabilities, and autistic behavior [2,3].

Challenges in developing CPA-targeting drugs

Though the aforementioned findings suggest that CPA-targeting therapeutics should have substantial pharmaceutical potential, only a few have reached clinical development. To our knowledge, only one CPA-targeting drug is currently FDA approved: Ardelyx's IBSRELA®, used to treat irritable bowel syndrome (IBS) [2][i]. IBSRELA's active ingredient, tenapanor hydrochloride, is an NHE3 inhibitor that reduces sodium absorption from the small intestine and colon. This action triggers intestinal water secretion, alleviating IBS-associated constipation[i]. Pharmaceutical companies have also attempted to develop NHE1 inhibitors to treat cardiovascular pathologies, but despite promising results in animal models, these efforts failed to culminate in pharmaceutical drugs [2]. In particular, cariporide, developed by Sanofi Aventis to alleviate effects of myocardial ischemia, reached Phase 3 clinical trials but did not show significant efficacy over placebo for patients who underwent coronary artery bypass surgery, or for patients at risk of acute coronary syndrome [2]. Merck's eniporide and Pfizer's zoniporide were also discontinued for similar reasons [2]. Rimeporide, originally developed by Merck Serono to treat congestive heart failure, was repurposed by EspeRare for treating cardiorespiratory complications in patients with Duchenne muscular dystrophy [2]. Rimeporide passed Phase 1 safety and pharmacokinetics trials in 2019, but we do not know if it entered Phase 2 clinical trials.

Two main factors are likely to explain the difficulty in developing CPA-targeting drugs: first, like many other solute carriers, CPAs are understudied in comparison with other gene families [4]. Effective targeting of a CPA requires understanding of its physiological functions; its tissue, cell, and sub-cell expression profiles; its regulation mechanisms; and the exact ways in which it is involved in human pathologies. These aspects are still elusive for most human CPA paralogs.

A second major obstacle is the lack of structural data. Until 2020, the only existing experimental structures of CPAs were of bacterial and archaeal origins. These structures are of limited utility in the two main *in silico* approaches for drug discovery, the so-called receptor-based and ligand-based methodologies. The receptor-based approach relies on the three-dimensional structure of the target protein to computationally screen binders that appear geometrically and electrostatically complementary to the binding/active site. Naturally, the applicability of this method is contingent on the availability of high-quality structural data regarding the focal (human) protein. The ligand-based approach, in turn, entails constructing models of candidate drug molecules, called pharmacophore hypotheses, on the basis of features of ligands that are known to bind to the target protein; these models are then used to identify specific drug candidates. This approach is inherently limited in the case of CPAs, as their natural substrates are simple monatomic ions, meaning that the corresponding chemical search space is highly restricted. This basic limitation is further exacerbated by the lack of human-specific structural data that might assist in the discovery of CPA binders.

Box 1. Human CPAs

The 13 human CPA paralogs are classified into three families, based on sequence similarity [2]: SLC9A, which includes Na+/H+ exchangers 1–9 (NHE1–9); SLC9B, consisting of Na+/H+ antiporters 1 and 2 (NHA1-2); and SLC9C, comprising NHE10 and NHE11. All paralogs share a conserved antiporter membrane domain comprising 13 or 14 transmembrane helices that are organized in two functional subdomains: a mobile core subdomain, harboring the cation/proton binding site, and a stationary dimerization subdomain (Figure IB). For the conserved domain, sequence identity between the paralogs in each family ranges between ~30% and ~80% but can be as low as ~11% across families (Figure IA), which nevertheless share the same fold (Figure IB). The highly conserved CPA fold and the availability of experimental structures result in relatively high-quality models produced by AlphaFold (AF) (Figure IB). Extensive sequence and phylogenetic analyses reveal that all CPAs, human included, share a highly conserved sequence motif that facilitates cation/proton transport [1]. Aside from the conserved membrane antiporter domain, human CPAs also feature highly diverse auxiliary domains. For example, different SLC9A members feature intracellular C-terminal domains of different lengths, which are largely unstructured.

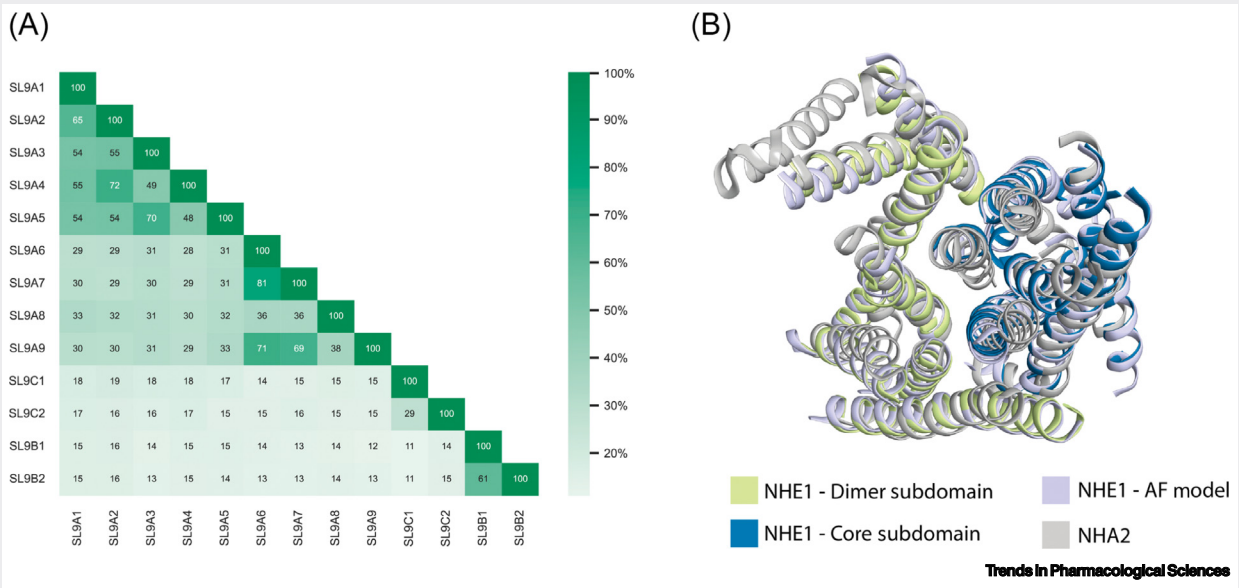

Figure I. Human cation/proton antiporters (CPAs) share a common fold, despite different degrees of sequence identity. (A) A matrix showing the % sequence identity of the membrane domain among human CPAs. (B) Cryo-electron microscopy (cryoEM) structure of human NHE1 [Protein Data Bank (PDB) entry 7DSW] with the dimerization subdomain colored yellow and the core subdomain colored blue. Loops were removed for clarity. NHE1's structure is superimposed onto an NHE1 model produced by AlphaFold (AF) without the use of templates and the cryoEM structure of human NHA2 (PDB entry 7B4L). AF performs well in predicting NHE1 structure, with a root-mean-square deviation of 1.3 Å when superimposing the membrane domains. With 15% sequence identity only, NHE1 and NHA2 share the same fold, nevertheless.

Newly published human CPA structures could advance *in silico* guided drug development

Encouragingly, the capacity to use structure-based *in silico* methods to identify CPA-targeting drugs is likely to improve, as five mammalian CPA structures have recently emerged, including three human structures (Box 2) [5–8]. As anticipated, the mammalian antiporters share the known CPA fold. However, they also feature unique characteristics, such as additional transmembrane helices and auxiliary extramembrane regulatory domains. These additions, which might have evolved to facilitate the more intricate regulatory mechanisms found in higher animals [2], provide opportunities for discovery of selective drugs.

Consider, for example, the human NHE1 structure, determined in complex with the selective inhibitor cariporide and the regulatory protein calcineurin B-homologous protein 1 (CHP1) [8]. NHE1 is the main CPA isoform present in human heart and nerve cells [8]. Cariporide binds NHE1 in an outward-open conformation with its guanidine group interacting with D267, which facilitates proton and ion transport [1,8]. Interacting with D267 and other residues in the core domain, cariporide outcompetes Na^+ binding to NHE1 (see Figure I in Box 2). Interestingly, cariporide also interacts with the dimerization domain and hinders conformational changes associated with the alternate-access transport mechanism. Comparisons of the apo and holo NHE1 structures reveal that the protein undergoes a conformational change and adopts a distinct cariporide-bound conformation. The structure, combined

with available data on mutations affecting cariporide binding and a comparative analysis of other human NHE paralogs and of related compounds, can provide a valuable foundation for the development of selective CPA drugs.

Another drug discovery strategy could be based on targeting the NHE1–CHP1 protein–protein interface. CHP1, an EF-hand calcium-binding protein, is an obligatory binding partner that sustains NHE1's basal activity and regulates its cell-surface expression and degradation [2,8]. Disrupting the NHE1–CHP1 interaction decreases NHE1 activity [2,8]. A similar approach should be applicable to NHE3, another human CPA that binds CHP1, and whose structure, in complex with CHP1, was also recently determined [7]. Pharmaceutically targeting the mostly unstructured and flexible C terminus domain of CPAs presents a challenge and obtaining additional structural data on the interactions between this domain and regulatory proteins can greatly aid these efforts.

NHE3's structure presents two other features that offer drug development opportunities. First, a C-terminal helix-loop-helix motif appears to insert into the funnel that leads to the antiporter's binding site in the inward-facing conformation, blocking it and autoinhibiting NHE3 [7] (see Figure I in Box 2). The loop interacts with D192 and other conserved negatively charged residues in the protein's core [1,7]. This loop, of 12 residues, and particularly the 635–638 fragment, can serve as a basis for designing cyclic peptides and peptidomimetics (compounds mimicking natural peptides), to modulate NHE3's activity. Lastly, NHE3's structure also revealed two phosphoinositide-like lipid molecules (PIs) bound to a cytoplasmic peripheral groove (see Figure I in Box 2). PIs enhance NHE3's transport activity [7] and the PI binding site could potentially accommodate small molecules that allosterically modulate NHE3's activity.

These recent developments come at a particularly opportune moment in terms of the state of the field of computational biology, as discussed in what follows.

Exploiting AlphaFold (AF) to model missing human CPA structures

Currently, only three of the 13 human CPAs have been structurally solved and even in these, significant portions of the extra-membrane domains are still missing. However, the release of AF [9], a machine-learning algorithm for predicting highly accurate protein models, can be exploited to model the missing CPA structures. The conservation of the CPA fold across multiple phyla [1], including human paralogs (Box 1), as well as the inclusion of prokaryotic CPA structures in AF's original training set, suggests that AF should be suitable for CPA structure prediction. Reassuringly, AF accurately modeled the membrane domain of the newly published human CPAs, not included in the original training set (structures published until April 30, 2018) [10]. Compared with the respective cryo-electron microscopy structures, AF's predictions for NHE1 [Protein Data Bank (PDB) entry 7DSW] and NHE3 (PDB entry 7X2U), generated without using templates, produced root-mean-square deviations (RMSDs) of 1.3 Å and of 1.2 Å, respectively. Notably, for Na+/H+ antiporter (NHA) 2 (PDB entry 7B4L), AF modeled an opposite conformation, resulting in an RMSD of 3.6 Å.

One should keep in mind, though, that virtual screening and docking are sensitive to side-chain positioning, which is notoriously difficult to model. Fortunately, AF models provide reliable metrics, such as predicted local distance difference test (pLDDT) scores, for assessing confidence levels corresponding to specific regions. For instance, the average pLDDT score for residues within a radius of 10 Å from the aspartate binding site in the NHE1 model is approximately 90, indicating high precision. Post-modeling refinement and sampling techniques, such as molecular dynamics (MD) and induced fit, can further enhance accuracy of protein–ligand models. Furthermore, AF models can be used for computing relative free energy of binding of small drug-like molecules within the free-energy perturbation framework, widely used in computer-aided drug design. A benchmark shows that results using AF models are comparable with those obtained with crystal structures [11].

AF models can also enhance experimental procedures for structure determination, so there is hope for the emergence of more mammalian/human CPA structures soon, preferably in multiple conformations and in complex with effector proteins. Such

Box 2. Newly published mammalian CPA structures

Five mammalian CPA structures have recently emerged: the SLC9A members NHE9 from *Equus caballus* (EcNHE9) [6], human NHE1 (HsNHE1) [8] and NHE3 (HsNHE3) [7], alongside the SLC9B member NHA2 from both bison (BbNHA2) and human (HsNHA2) [5]. The structures feature unique characteristics that distinguish them from prokaryotic homologs. The cryoEM density map of BbNHE9 showed a low-resolution unique ~50-residue extracellular domain connecting the second and third transmembrane helices [6]. The structures of both human NHE1 and NHE3 were determined in complex with the calcineurin B-homologous protein CHP1, which binds to a site on their C-terminal cytosolic domain (Figure IA) [7,8]. Additionally, NHE1's structure was determined in complex with the inhibitor cariporide (Figure IB). The structure of NHE3 displays an autoinhibited conformation in which a helix-loop-helix motif from the protein's C terminus is inserted into the funnel that leads to the binding site (Figure IC). The structure also shows an interaction with two PI molecules (Figure ID). Finally, both the bison and human NHA2 structures revealed an additional 14th N-terminal helix that gives rise to a unique dimerization interface (Figure IE,F).

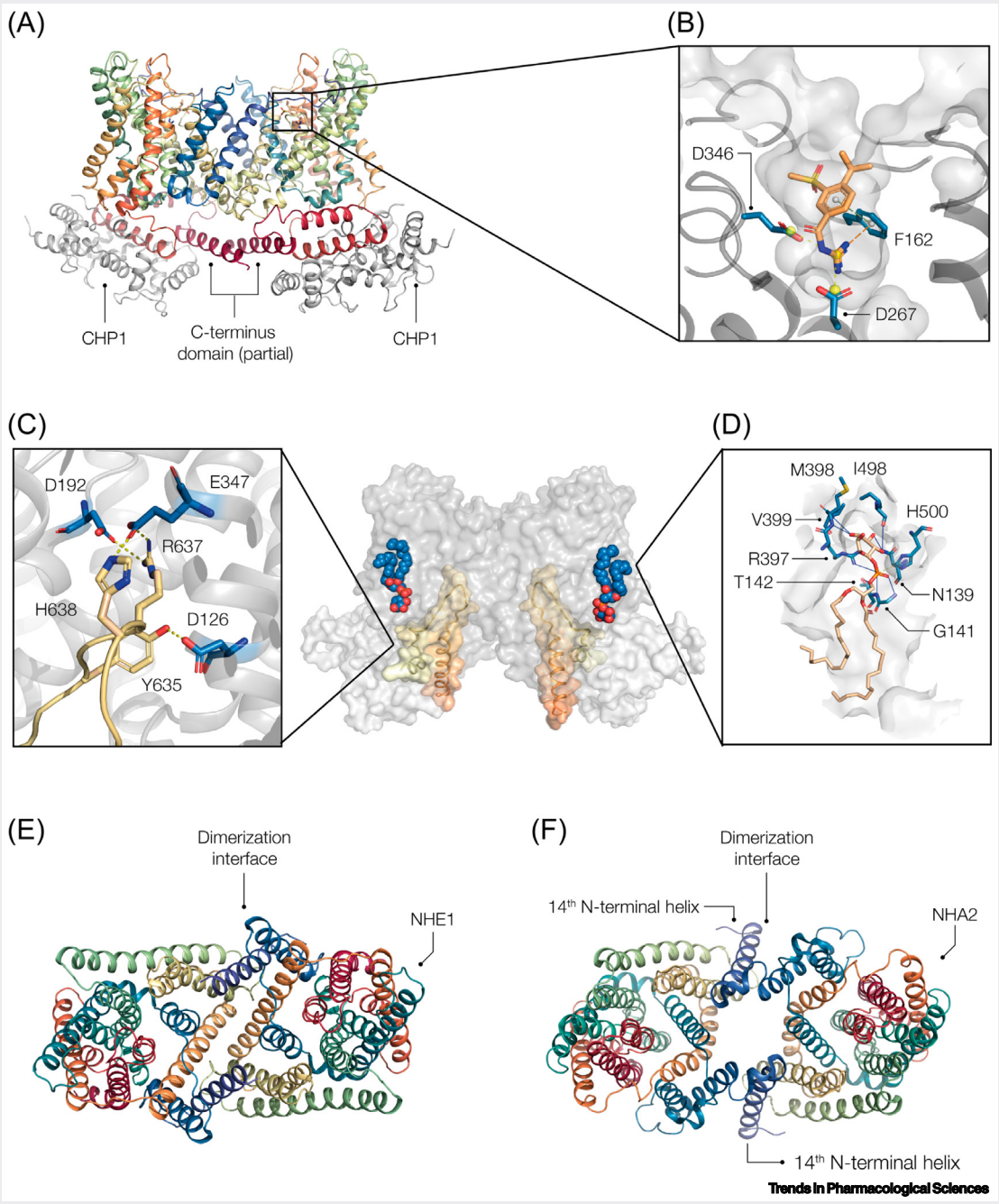

Figure I. Unique characteristics of mammalian cation/proton antiporters (CPAs). (A,B) The cryo-electron microscopy (cryoEM) structure of human NHE1 in complex with CHP1 and cariporide [Protein Data Bank (PDB) entry 7DSX]. (A) NHE1's cytoplasmic C-terminal domain (red) is depicted bound to CHP1 (gray). (B) A detailed view of the interactions between cariporide and NHE1. Salt bridges are indicated with yellow dashed lines, cation–π interactions with orange dashed lines, and pi-stacking with green dashed lines. D267 is the binding site for both protons and cations. (C,D) The cryoEM structure of autoinhibited human NHE3 in complex with CHP1 and PIs (PDB entry 7X2U). (C) A close-up view of the interactions between amino acids on the helix-loop-helix motif and negatively charged residues in the antiporter's core. Salt bridges are denoted as in (B). (D) A close-up view of the polar interactions between a PI and the cytoplasmic peripheral groove. Hydrogen bonds are denoted by blue lines and salt bridges are denoted as in (B). (E,F) Compared with NHE1 and other human CPAs (B), NHA2 has an additional N-terminal 14th transmembrane helix that gives rise to a unique dimerization interface (C). Figures were produced using PyMOL and PLIP (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index).

structures could be critical for designing new drugs, as exemplified by the recently determined human structures, discussed in the previous subsection.

Exploring conformational dynamics using simulations and machine learning may enable allosteric targeting of CPAs

In CPAs, conformational changes facilitate ligand binding and transport and allow regulation and interactions with other proteins. Allosterically targeting CPAs at sites other than the cation/proton binding site can stabilize metastable states, which is advantageous for targeting specific paralogs. Such stabilization could further allow for finer functional modulation of CPAs in addition to reducing off-target binding and side effects.

Many common methods for structure prediction and determination are limited in their capacity to reveal all relevant conformations. However, several computational methods may fill this gap. For example, MD simulations have become a standard tool in studying proteins' structure–function relationships [12]. For CPAs, MD simulations can be used to study conformational dynamics of proteins embedded in their native lipid bilayer environment. Membrane models are becoming more accurate, yielding more realistic insights concerning protein–lipid interactions, dimerization, and dynamics. MD-derived data can then be exploited using molecular docking and other computer-aided drug design methods, such as those discussed earlier. Simulations can reveal cryptic binding sites to be targeted.

Recent developments in machine learning applications further enable rigorous and robust analysis of the enormous outputs of MD simulations. For example, Markov models are employed to identify metastable states in MD trajectories and analyze the kinetics of complex systems [13,14]. Deep learning-based generative models, which learn a given data distribution and can generate new data with some variations, are now utilized to study protein dynamics. For example, application of a Boltzmann generator model to short MD trajectories of a small protein in two distinct states enabled previously unseen intermediary states to be sampled [15,16]. Similarly, variational autoencoders were recently used to exhaustively explore the phase space of a highly complex protein-membrane system by coupling macro- and micro-scale simulation techniques, thus bridging the gap between simulation timescales and biological timescales [17,18].

Concluding remarks

The recently published mammalian CPA structures, combined with advances in computational biology, such as the development of AF and machine learning approaches to biological research, offer exciting opportunities to advance therapeutic targeting of CPAs. But many challenges remain. Primarily, we should invest heavily in linking CPAs to specific pathologies and establishing how abnormalities in CPA function, expression patterns, and regulation promote disease onset and progression. Such efforts will go hand in hand with acquiring better understanding of the unique biological role of each of the 13 known human paralogs.

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Declaration of interests

None declared by the authors.

Resources

[i]https://ir.ardelyx.com/news-releases/news-release-details/ardelyx-receives-fda-approval-ibsrelar-tenapanor-nhe3-sodium

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