Challenges of finding novel drugs targeting the K-Cl cotransporter

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Abstract

Human-disease causing mutations and genetically-modified mouse models have established the importance of KCC2 and KCC3 in nervous system physiology. These two proteins mediate the electroneutral cotransport of K⁺ and Cl⁻ ions across the neuronal membrane. Disruption of KCC2 function affects inhibitory synaptic transmission with consequences for epilepsy, pain perception, and potentially some neuropsychiatric disorders; whereas disruption of KCC3 affects both central and peripheral nervous systems resulting in psychosis and peripheral neuropathy. Until recently, the KCC field has suffered from an almost complete lack of pharmacological tools with which to probe cotransporter function. The only available tools being the very poorly potent loop diuretics (e.g. furosemide EC₅₀ = 6×10⁻⁴ M). In order to address this deficiency, efforts that focused on the discovery of KCC modulators have been undertaken. This work has resulted in the discovery of novel inhibitory compounds that are up to 4 orders of magnitude more potent (EC₅₀ = 6×10⁻⁷ M) and with increased specificity. While useful for ex vivo studies, these tools possess poor pharmacokinetic properties, severely limiting their utility in vivo. In addition, only a few agents acting on regulatory molecules have been identified as putative KCC activators. Thus, further research is required to develop tools suitable to advance our understanding of how KCC modulation may be useful for the treatment of disease.

Keywords

K-Cl cotransport; KCC2; KCC3; inhibitory neurotransmission; epilepsy; pain; neuropathy

K-Cl cotransport (or KCC) mediates the Na⁺-independent movement of K⁺ and Cl⁻ ions across cell membranes. Because of the tightly coupled ratio of 1 cation for 1 anion translocated across the membrane per transport cycle, KCC-mediated transport is electrically silent. K-Cl cotransport uses the energy stored in the K⁺ gradient (high K⁺ inside the cell while low K⁺ outside), which is maintained by the Na⁺/K⁺-ATPase pump, to drive Cl⁻ against its electrochemical potential. Thus, by using the large outward gradient of K⁺, K-Cl cotransport functions as a Cl⁻ ‘pump’ and maintain Cl⁻ values lower than electrochemical

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Notes

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potential equilibrium. The resultant Cl\(^-\) gradient is important to neurons, as ligand-gated (GABA/glycine) Cl\(^-\) channels hyperpolarize the membrane, thereby decreasing the propensity for neurons to trigger action potentials. Among the four K-Cl cotransporters, the neuronal-specific KCC2 is by far the best studied. In rodents, expression of KCC2 is low at birth and increases postnatally, concomitantly with the disappearance of giant GABA depolarizing potentials, and the appearance and strengthening of inhibitory GABA responses. Both mutations in human KCC2 and decreased KCC2 expression in genetically-modified mouse models result in brain hyperexcitability and epilepsy (reviewed in \(^1\)). Note that KCC2 is also a potential target for the modulation of pain signals as excitability of spinal cord relay neurons is also affected by inhibitory inputs. KCC3 is likewise expressed in large projection neurons and interneurons in the CNS, and similarly to KCC2, its expression is low at birth and increases significantly during the postnatal period. Absence of KCC3 in hippocampal neurons also leads to a shift in the Cl\(^-\) reversal potential, although the shift is much smaller than the one observed with inactivation of KCC2. This might be due to the fact that KCC3 function, unlike KCC2, is mostly silent under isosmotic conditions. Thus, the role of KCC3 in neurons is still poorly understood. Loss-of-function mutations in KCC3 lead to peripheral nerve disease, mental retardation, schizophrenia-like symptoms, and variable degree of agenesis of corpus callosum\(^2\), thus highlighting the importance of this isoform of the K-Cl cotransporter for CNS and PNS physiology.

Pharmacology of K-Cl cotransporter started concurrently with the identification of the K-Cl cotransport as a distinct functional transport unit. K-Cl cotransport was originally described in red blood cells as a furosemide-sensitive, swelling- and NEM-activated Cl\(^-\) dependent K\(^+\) transport mechanism. In contrast to the Na-K-2Cl cotransporter which is inhibited by very low (0.5 – 5 \(\mu\)M) concentrations of bumetanide and furosemide, KCC is inhibited by 2–3 order of magnitude higher concentrations of these loop diuretics (Figure 1). We now believe that cell swelling and N-ethylmaleimide affect intracellular molecules that modulate the activity of the cotransporter and not the transport protein directly; this will be briefly discussed later. Bumetanide and furosemide inhibition of KCC occurs across many species, which is not the case for KCC inhibition by stilbene derivatives. Indeed, sheep red blood cell KCC, in contrast to human RBC K-Cl cotransport, is sensitive to DIDS, H2DIDS, and SITS.\(^3\) Like for furosemide, the authors found that inhibition of KCC by DIDS and H2DIDS was significantly enhanced by external K\(^+\). Interestingly, they also found that the cotransporter was mostly insensitive to 1 mM SITS in absence of external Rb\(^+\)/K\(^+\), but completely inhibited by this stilbene derivative in the presence of 10 mM external Rb\(^+\)/K\(^+\). Whether this K\(^+\)-dependency indicates that binding of the stilbenes occurs at a site close to the site of loop diuretic binding still needs to be determined. Now that sequence information exists for all K-Cl isoforms in multiple species, chimeric and mutagenesis strategies can be devised to identify the stilbene binding site(s). K-Cl cotransport is also inhibited by DIOA, a [(dihydropindenyl)oxy]alkanoic acid, with an IC\(_{50}\) around 10 \(\mu\)M.\(^4\) Until our discovery of the first potent and selective KCC inhibitors, published in 2009, DIOA was widely used in preference to furosemide due to its much higher potency, especially in \textit{in vivo} experiments, with the caveats that the alkanoic acid compound had poor specificity and affected cell viability.
In 2008, we performed a large-scale high-throughput screen designed to identify new direct-acting modulators of KCC2 function. The screen was based on the ability of the transporter to carry the monovalent cation thallium (Tl⁺) via the K⁺ binding site and the availability of cell-permeant indicators to emit enhanced fluorescence upon Tl⁺ binding. The screen was performed using HEK293 cells overexpressing rat KCC2 in the presence of ouabain to prevent Tl⁺ uptake through the Na⁺/K⁺-ATPase pump. Because we incubated the cells with the compounds for a relatively short (8 min) pre-incubation period, the screen was not intended to capture regulatory molecules that affect KCC2 function. However, the screen was designed to allow both increases and decrease in fluorescent signal relative to untreated controls thereby providing the possibility to detect activators and inhibitors, respectively. The primary screen tested 234,560 samples at a nominally 10 μM concentration. While a large number of compounds were identified in this primary screen as stimulators of Tl⁺ uptake, none withstood testing against HEK293 cells not expressing KCC2. Note that in contrast to enzymes, channels, and membrane receptors like G-protein coupled receptors, no positive allosteric modulators have to date been described for solute carriers.

In contrast to the unspecific effect of the compounds that stimulated Tl⁺ uptake in our primary screen, many inhibitory compounds passed the test of specificity. Our lead compound was N-(4-methylthiazol-2-yl)-2-(6-phenylpyridazin-3-ylthio)acetamide or ML77 (Figure 1) with an IC₅₀ that was 3 orders of magnitude more potent than furosemide. A structure activity relationship study revealed that the thiazole moiety was essential for activity and that several alkyl substitutions in the 4 position of the thiazole were tolerated. A follow-up study addressed the drug metabolism and pharmacokinetic profile of ML77. Unfortunately, high plasma protein binding and high clearance following intravenous administration in rats, makes this compound poorly suitable for in vivo studies. Note that specificity of ML077 within the four K-Cl cotransporter isoforms has not yet been addressed, with the exception of KCC3 function, which we showed was equally affected by addition of ML077.

Additional chemical modifications were made to the ML77 chemical structure to improve potency and pharmacokinetic properties. A compound with a 9-times higher potency (IC₅₀ of 61 nM) was identified. As seen in Figure 1, the VU0463271 compound featured an N-cyclopropyl amide group at the N-Me amide position. Unfortunately, VU0463271 was also found to have poor pharmacokinetic properties. A similar effort was done at UCB in Belgium using MDCK cells overexpressing rat KCC2 and ⁸⁶Rb tracer uptake as the functional assay. Benzyl prolinate derivatives were identified as selective and effective inhibitors of the cotransporter in the submicromolar range (Figure 1).

Binding of ML77 to KCC2 in relationship to external K⁺ and Cl⁻ ions was investigated using rapid equilibrium transport kinetics. It was found that the effect of ML77 on KCC2 transport at different external K⁺ concentrations is consistent with a model of competitive inhibition, i.e. binding of the inhibitor and K⁺ were mutually exclusive, likely because they compete for the same site. Thus, binding of ML077 to KCC2 prevented the binding K⁺, thereby abolishing transport. This behavior was quite different from the binding of furosemide to the sheep red blood cell K-Cl cotransporter, which is more consistent with a non-competitive model with respect to K⁺. In the case of furosemide, like in the case of...
DIDS, inhibition was enhanced by the presence of external Rb⁺/K⁺. These data suggests that ML077 likely binds a different binding site, than furosemide and stilbenes derivatives.

Because disruption of KCC2 function is detrimental to neuronal circuitries, inhibitors would definitely not be appropriate therapeutic agents. In contrast, agents that would enhance KCC2 function and facilitate GABAergic inhibition could be beneficial. Unfortunately, as previously mentioned, no allosteric potentiators of solute transporters have been described to date. It may be that the structure/function of KCC preclude direct potentiation of the transporter. Thus, other pathways or mechanisms need to be considered. One such mechanism could be trafficking of the transporter to and from the plasma membrane. Indeed KCC2 transporters have a relatively short lifespan at the cell surface, and thus, any factor that would disrupt protein internalization would result in accumulation of KCC2 in the membrane. Such a compound, CLP257 was identified in a screen of 92,500 small molecules and Cl⁻ efflux measured through a Cl-sensitive green fluorescent protein derivative, Clomeleon in neuron-like NG108 cells. Increased cell surface expression of KCC2 was demonstrated in BDNF-treated (the neurotrophin is known to induce down-regulation of KCC2) rat spinal cord slices exposed to CLP257 and derivatives.⁹ While the mechanism has not yet been fully resolved, one can propose that the compound inhibits internalization of the cotransporter (Figure 1). Specificity of the CLP257 effect on KCC2 trafficking and function is still in need of independent confirmation. An alternative mechanism leading to KCC2 activation would by preventing its phosphorylation by inhibitory protein kinases. KCC2 and KCC3 are inhibited by phosphorylation of two very specific and conserved threonine residues located at the cytosolic carboxyl-terminus: Thr906 in KCC2 and Thr991 in KCC3 (site 1) and Thr1007 in KCC2 and Thr1048 in KCC3 (site 2). While the kinase affecting site one has not yet been identified, the WNK/SPAK kinases are thought to phosphorylate site 2. As indicated in Figure 1, this is likely the mechanism by which cell volume or N-ethylmaleimide enhances cotransporter function via inhibition of these inhibitory kinases. Thus, finding compounds that specifically inhibit the kinases involved in KCC phosphorylation will result in cotransporter activation. Several compounds have been identified as inhibitors of the WNK/SPAK signaling cascade.¹⁰⁻¹¹ Whether these compounds lead to KCC2 activation still needs to be demonstrated.

As KCC2 and KCC3 receive more and more attention due to the discovery of human disease-causing mutations,¹⁻²,¹² increased emphasis is being placed on finding small molecules that modulate cotransporter activity. Discovery of such modulators may pave the way for treatment KCC-related diseases as well as other conditions including epilepsy, anxiety, and others.

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Figure 1. Direct inhibitors and indirect activators of KCC function
K-Cl cotransporter in the plasma membrane exists in two states: phosphorylated or inactive and de-phosphorylated or active. Inhibitors - written in blue - are thought to bind directly to the cotransporter, whereas activators - labeled in green - are believed to inhibit the kinase(s) silencing the cotransporter. CLP257 increases cell surface expression of KCC2. For each factor affecting KCC activity we added a date (in parenthesis) of first publication.