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Pharmacology of pannexin channels

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Abstract

Pannexin channels play fundamental roles in regulating inflammation and have been implicated in many diseases including hypertension, stroke and neuropathic pain. Thus, the ability to pharmacologically block these channels is a vital component of several therapeutic approaches. Pharmacologic interrogation of model systems also provides a means to discover new roles for pannexins in cell physiology. Here, we review the state of the art for agents that can be used to block pannexin channels, with a focus on chemical pharmaceuticals and peptide mimetics that act on pannexin 1. Guidance on interpreting results obtained with pannexin pharmacologics in experimental systems is discussed, as well as strengths and caveats of different agents, including specificity and feasibility of clinical application.

Keywords

pannexin; connexin; peptide mimetic; inflammation; hypertension

Introduction

Pannexins (Panx) are proteins that form large-pore, high conductance membrane channels. There are three pannexin isoforms, Panx1, Panx2 and Panx3 [1]. Of these, Panx1 is the most ubiquitously expressed and is the focus of this review. Pannexin channels play central roles in paracrine and autocrine signaling and are thus critical to the control of several physiologic processes, including inflammation [2,3], blood pressure [4,5], pain [6], tumorigenesis [7] and function of the central nervous system [8,9]. Considering the diverse roles for pannexins

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in regulating health and disease, they represent an appealing pharmacologic target. On the other hand, this range of pannexin channel functions also represents one of the challenges in identifying specific pharmacologic strategies to regulate their function.

Pannexin channels are highly permeable and facilitate the diffusion of a broad array of substrates. Classically, Panx1 is most closely associated with ATP secretion and is generally considered anion selective [10], although this is not absolute. For instance, Panx1 channels have been shown to mediate transport of other biologically active molecules and ions, including glutamine, spermidine and possibly calcium, suggesting that they enable general permeation for substrates smaller than1 kDa [11,12]. In this respect, pannexin channels are functionally equivalent to hemichannels formed by connexin family gap junction proteins, however, pannexins and connexins are structurally distinct [1]. Given the functional similarity of pannexin channels and connexin hemichannels, it is important to use agents with the ability to specifically inhibit pannexins or connexins [13].

To date, no intrinsic extracellular ligands have been identified that regulate Panx1 permeability. Instead, Panx1 activity is generally controlled by ionotropic and metabotropic co-receptors that recognize different ligands [14]. For instance, ATP secretion by Panx1 can be stimulated by P2X7 purinergic receptors [15], alpha-adrenoreceptors [16] or TNF-alpha receptors [17,18]. Panx1 has also been associated with N-methyl-d-aspartate (NMDA) receptors in the CNS [19]. Because so many different stimuli can induce Panx1 channel activity, examining the effect of a pharmacologic agent on an output variable in a native system, such as ATP secretion or uptake of fluorescent dyes into cells, does not necessarily distinguish between an interaction with the co-receptor and a direct interaction with the pannexin channel [15]. Given this, heterologous expression systems, such as *Xenopus* oocytes microinjected with mRNA, have been used to measure the effect of agents on pannexin function, using depolarization as a channel opening stimulus [12,20,21].

Chemical agents

There are a number of chemical agents being used to block Panx1 channels (Figure 1; Table 1). A common theme in the initial discovery of pannexin channel inhibitors was to screen previously known ion channel inhibitors, such as chloride channel inhibitors, for the ability to inhibit Panx1 [10,22,23]. While this has proven to identify Panx1 inhibitors, these agents also will have off target effects that need to be considered.

Probenecid

Probenecid is commonly prescribed to prevent gout, promoting uric acid excretion by blocking its reabsorption by transporters present in renal tubules [24]. It was first demonstrated to inhibit Panx1 channels by using electrophysiologic analysis of *Xenopus* oocytes expressing mammalian Panx1 [21]. By contrast, probenecid has little effect on connexin hemichannels, which increases its utility for the study of Panx1 channel function [25–28]. While often used as an agent to inhibit transport activity in Panx1 channels *in vitro*, probenecid is known to broadly inhibit other organic anion transport channels as well [29], which can be a confounding factor in interpreting experiments using probenecid.

Another limitation regarding the use of probenecid is the high dose (in the mM range) required for significant inhibition of Panx1, which undermines its potential for therapeutic use in humans. The exact mechanism of channel inhibition via probenecid has not yet been fully resolved, however biochemical studies have determined the first extracellular loop (ECL1) as a key region required for probenecid to inhibit Panx1 by a gating mechanism [30].

NPPB

5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) is a chloride channel blocker that was also demonstrated to inhibit ATP secretion [31]. Panx1 mRNA microinjected into *Xenopus* oocytes were used to confirm that NPPB directly inhibits Panx1 channels, at a lower concentration than probenecid [21]. The effects of NPPB and probenecid were not additive, suggesting that they may be competing for the same Panx1 binding sites.

Another limitation of NPPB is that it also has the capacity to inhibit connexin hemichannels (e.g. Cx46 and a Cx43/32 chimera), so unlike probenecid, it is not able to be used to distinguish between connexin and pannexin channels [21].

Spironolactone

Spironolactone was discovered using a non-biased, flow cytometry-based screen for Panx1 inhibitors [32,33]. Spironolactone has been used for decades to treat hypertension, and known to act as a mineralocorticoid receptor (MR) antagonist [34]. Since Panx1 has a demonstrated role in regulation of vasoconstriction [16], it seemed likely that Panx1 inhibition could also be part of the mechanism of action for spironolactone in the control of blood pressure. In fact, smooth muscle cell (SMC) specific MR-deficient mice showed decreased blood pressure in response to spironolactone, whereas SMC specific Panx1 deficient mice were unaffected [33]. This was further confirmed in a model of acute hypoxic pulmonary vasoconstriction [35] and spironolactone was also demonstrated to inhibit Panx1 expressed by vascular endothelial cells [18] and in melanoma cells [36]. These studies do not rule out a role for spironolactone in regulating hypertension by attenuating renal MR function [37]. Interestingly, spironolactone metabolites that more specifically target MR are less potent as pannexin channel inhibitors [33], suggesting the potential to produce spironolactone derivatives that more specifically target pannexins.

Carbenoxolone

Carbenoxolone is a derivative of glycyrrhetinic acid, which is known for its anti-inflammatory capacity [38] as well as being a gap junction inhibitor [39]. Bruzonne et al. first showed the micromolar sensitivity of Panx1 and Panx2 to carbenoxolone treatment with mRNA microinjected into *Xenopus* oocytes [20], results that have been confirmed in multiple different experimental systems [26,40–42]. More recently, Michalski et al. identified a putative binding site for carbenoxolone in a region also targeted by probenecid [30]. They then used Cryo-EM to resolve frog Panx1 and determined that the binding site for carbenoxolone is between ECLs 1 and 2, which locks the channel in a closed conformation [43]. The discovery of this binding site suggests that pore blocking is the main mechanism of inhibition of pannexins by carbenoxolone [44]. Although carbenoxolone inhibits both

connexin and pannexin channels, the dose responses for these effects are different [45], which may enable carbenoxolone to be used to distinguish between these two classes of channels [46].

Trovafloxacin

As was the case for spironolactone, trovafloxacin also was discovered using a non-biased screen for pannexin channel inhibitors [32]. Trovafloxacin is specific for Panx1 channels and does not inhibit Panx2 channels or Cx43 gap junctions [32], suggesting utility for this agent as an experimental tool [47]. However, trovafloxacin is known to have serious side effects, including hepatotoxicity [48], which preclude its therapeutic use as a pannexin channel inhibitor.

Mefloquine

Mefloquine was initially discovered through a screening assay of quinine analogs for potential malaria treatments [49]. Its utility as an anti-malarial has diminished due to *Plasmodium* resistance as well as potential neurological complications following mefloquine treatment [50]. Nonetheless, it is still employed in the study of gap junctions and pannexins [51–53]. Nanomolar doses of the racemic erythro form of mefloquine are sufficient for significant inhibition of Panx1 [54], whereas gap junction channels (e.g., Cx36 and Cx50) are inhibited with μM to mM doses [55]. Given this difference in IC_{50}, mefloquine as a high affinity pannexin inhibitor has allowed researchers to distinguish the importance of Panx1 in the context of neurological disorders such as epilepsy and opiate withdrawal [56-58].

Brilliant Blue FCF

Brilliant Blue FCF is a food additive used to color several consumer goods, including processed foods, drinks, and medications. Using the *Xenopus* model system, it has been shown that Brilliant Blue FCF inhibits Panx1 with very high affinity and lacks an effect on connexin hemichannels [59]. Critically, Brilliant Blue FCF does not directly inhibit P2X7 receptors, in contrast to the parent compound Brilliant Blue G which acts on both P2X7 and Panx1 [60]. Although Brilliant Blue FCF has high affinity for the ability to inhibit Panx1, the dark blue color has the potential to interfere with some types of experiments. Also, since Brilliant Blue FCF is a strong pannexin inhibitor, it is not an inert food additive. Given this, efforts are underway to find natural compounds that can be used in consumer products instead of Brilliant Blue FCF [61].

Peptide mimetics

In contrast to chemical agents, peptide mimetics have the potential to specifically target proteins by recognizing specific motifs (e.g., Figure 2; Table 1). This approach has successfully been used to manipulate connexins [13,62]. By analogy, peptides have also been developed that have the capacity to interfere with pannexin function.

^{10}Panx1

The synthetic peptide ^{10}Panx1 was first introduced in 2006 by Pelegrin and Surprenant [63], in which Panx1 was shown to be the functional link between P2X7 receptor large pore

formation and the caspase 1 cascade [63]. The short synthetic ^{10}Panx1 peptide works by blocking amino acid residues 74-83 present in the extracellular loop 1 (ECL1) domain of Panx1. As discussed previously, ECL1, and more specifically the W74 residue in each of the Panx1 subunits, is an essential region for channel function. To date, ^{10}Panx1 has been used in micromolar doses to investigate effects of this ECL1 blockade in a wide variety of cell types including neurons, erythrocytes, T cells, as well as *in vivo* [19,64-66]. Although the peptide sequence is specific to Panx1, current through Cx46 channels was shown to be moderately inhibited by ^{10}Panx1 [67], comparable to the cross reactivity of the connexin mimetic peptides gap^{26} and gap^{27} with the ability to block pannexin channels [67]. The cross-reactivity of these peptides underscores the need to interpret studies using ^{10}Panx1 with caution, and the need to use complementary methods to ensure that pannexin channels are involved.

PxIL2P and Panx$_{308}$

In contrast to peptides made against the Panx1 extracellular loop, both PxIL2P and Panx$_{308}$ were made against regulatory regions of Panx1 on intracellular Panx1 domains. Given this, both PxIL2P and Panx308 require modifications to be cell permeable. This has been accomplished by creating peptide chimeras where the N terminus is conjugated to the cell penetrating TAT (GRKKRRQRRRPQ) sequence. Both PxIL2P and Panx$_{308}$ fused to TAT were shown to enter cells without any other chemical modification or assistance [16,68].

Interestingly, both peptides cover tyrosine residues that were shown to be important in Panx1 channel opening depending on the tissue, PxIL2P targeting the intracellular loop (ICL) domain [16], and Panx$_{308}$ targeting the COOH-tail [68]. PxIL2P covers 10 amino acids starting at K191 and was first described by Billaud et al in a screening for peptides that block alpha-adrenergic constriction of arteries [16]. After multiple peptides were mapped to different intracellular regions of Panx1, PxIL2P was found to provide significant inhibition of vasoconstriction analogous to genetic deletion of Panx1 from smooth muscle cells [16]. The peptide was also found to block Panx1 current and ATP release in a heterologous system, with the Y198 amino acid being of critical importance to the Panx1 channel opening [16]. Further work determined that this effect was due to Src dependent phosphorylation of Y198 [69]. PxIL2P has been used to block Panx1 channel function in multiple cell types [11,33,70].

The Panx$_{308}$ peptide covers 14 amino acids starting at L306 on the COOH tail of the Panx1 monomer [68]. As was the case for Y198, the Y308 residue of Panx1 is also a target for Src phosphorylation, although it is induced in response to neuronal NMDA activation after stroke [68]. Whether Src phosphorylation of Y308 is sufficient to activate Panx1 opening is an open question. Of note, Panx1 is phosphorylated immediately upon NMDA stimulation, however, Panx1 channel activity only occurs after a 10 minute latent period [68]. This suggests a model where Y308 phosphorylation may render Panx1 to be more sensitized to a second stimulus that is required for complete channel opening. Also, the common theme of Src phosphorylation in regulating these two distinct Panx1 domains suggests that both of the PxIL2P and Panx$_{308}$ peptides might act as competitive inhibitors that will prevent phosphorylation of both Y198 and Y308. Whether this is the case has not been directly

tested although it could be determined through the use of phospho-specific anti-Panx1 antibodies.

Conclusion and perspectives

Panx1 has been the focus of this review due to its ubiquitous expression and function. Less is known about the pharmacology of Panx2, which is predominantly expressed in the central nervous system or Panx3 which has been found in bone, cartilage and, potentially, blood vessels [71,72]. To date, there have not been any pharmacologic agents developed that have been shown to specifically block Panx2 or Panx3 and not Panx1. Another caveat related to the pharmacologic manipulation of pannexins is that it has been demonstrated that there are several subconductance states for Panx1 that can be revealed by dose response studies [10,47,73,74] and which are likely to reflect different functional conformations. Panx2 and Panx3 are likely to also have subconductance states and they may differ from those observed for Panx1. Finally, there is a lack of agonists with the ability to stimulate pannexin channel activity. The ability to enhance channel function would certainly have experimental value and could also have therapeutic application.

Thus, considerable caution should be taken when using pharmacologic approaches to assign a functional role for pannexins. Specifically, using a single inhibitor is not sufficient, especially if the experiment does not render definitive inhibition. This problem is compounded in whole animals where Panx1 expression is ubiquitous. For example, Panx1 inhibition in renin secreting cells causes a large release of renin which will increase blood pressure [70], whereas inhibition of Panx1 on smooth muscle cells lowers blood pressure through adrenergic activity [16]. These two processes could offset and give an erroneously negative finding. This also underscores the importance of transgenic mouse models to assist in interpreting pharmacologic studies, especially the use of tissue targeted gene knockouts.

Although the agents described here all have caveats, when their limitations are considered, they can provide insights into roles for pannexins in physiologic processes. We suggest as a best practice to always use multiple complementary pannexin inhibitors (e.g. spironolactone and PxIL2P) to assign a role for pannexin channels in a physiologic process. If the aggregate data (with genetic knockout in cells or animals) still supports an effect of Panx1 channel inhibition, then this provides a strong accumulation of evidence in support of pannexin channels having a functional role.

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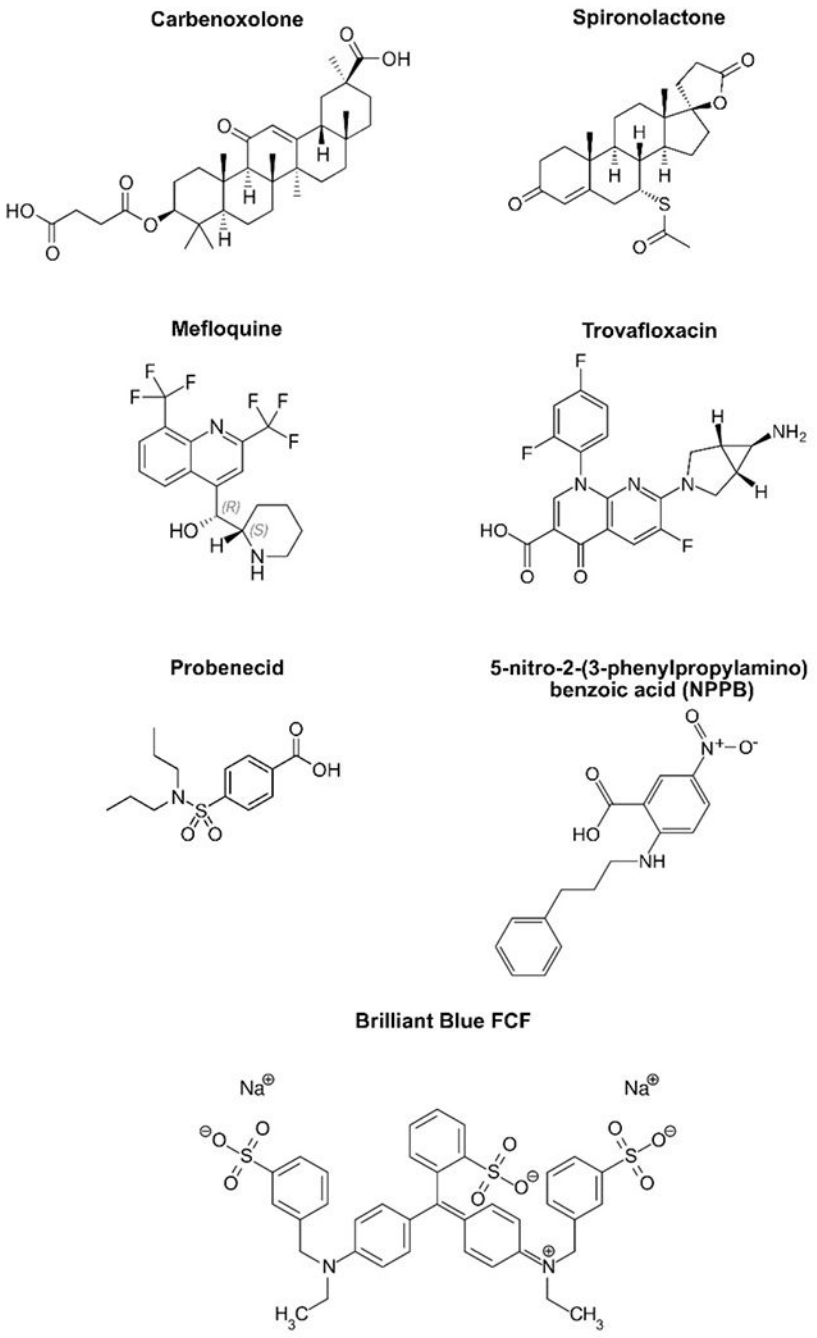

Figure 1. Chemical pannexin inhibitors.

Shown are the structures of representative pannexin channel inhibitors. The structure of trovafloxacin was used under a CC BY-SA 3.0 license (https://commons.wikimedia.org/wiki/File:Trovafloxacin.svg), all other structures were from public domain images.

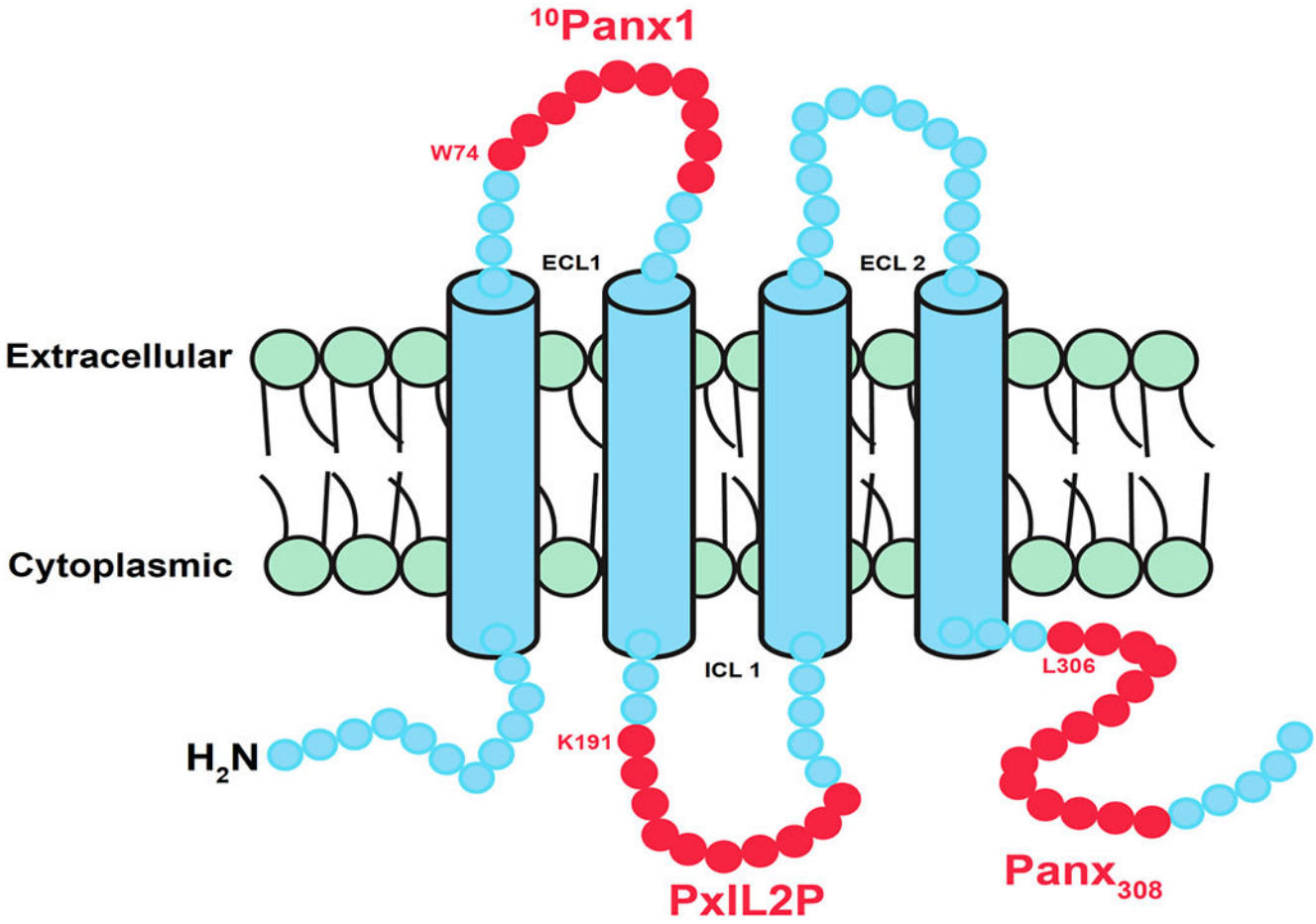

Figure 2. Pannexin domains corresponding to peptide mimetics used to block active Panx1 channels.

The schematic represents one monomer from the heptameric Panx1 channel in blue. Red represents the number of amino acids composing a mimetic peptide made in that region, with the starting amino acid and position noted. ICL is intracellular loop, and ECL is extracellular loop. Note that each circle does not correspond to an individual amino acid.

Table 1.
Current described Pannexin channel inhibitors.

IC_{50} values are from [21,23]

Chemical agents	IC_{50}	First shown to inhibit Pannexin1
Probenecid	150 - 360 μM	Silverman, et al., 2008 [21]
NPPB	21 μM	Silverman, et al., 2008 [21]
Spironolactone	10 μM	Good, et al., 2018 [33]
Carbenoxolone	5 μM	Bruzzone, et al., 2005 [20]
Trovafloxacin	4 μM	Poon, et al., 2014 [32]
Mefloquine	0.05 μM (erythro) 0.8 μM (threo)	Iglesias, et al., 2008 [15]
Brilliant Blue G	3 μM	Qiu and Dahl, 2009 [60]
Brilliant Blue FCF	0.27 μM	Wang, et al., 2013 [59]
Peptide mimetics	**Working concentration**	**First shown to inhibit Pannexin1**
^{10}Panx1 74-WRQAAFVDSY-83	10 μM	Pelegrin and Surprenant, 2006 [63]
PxIL2P 191-KYPIVEQYLK-200	3-20 μM	Billaud, et al., 2015 [16]
$Panx_{308}$ 306-LKVYEILPTFDVLH-319	1-10 μM	Weilinger, et al., 2016 [68].