PANNEXIN 1 CHANNELS AS A THERAPEUTIC TARGET: STRUCTURE, INHIBITION, AND OUTLOOK

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ABSTRACT: Pannexin 1 (Panx1) channels are transmembrane proteins that release ATP and play an important role in intercellular communication. They are widely expressed in somatic and nervous system tissues, and their activity has been associated with many pathologies such as stroke, epilepsy, inflammation, and chronic pain. While there are a variety of small molecules known to inhibit Panx1, currently little is known about the mechanism of channel inhibition, and there is a dearth of sufficiently potent and selective drugs targeting Panx1. Herein we provide a

review of the current literature on Panx1 structural biology and known pharmacological agents that will help provide a basis for rational development of Panx1 chemical modulators.

INTRODUCTION

Pannexin (Panx) channels are transmembrane proteins that facilitate intercellular communication via the release of ions and small molecules, such as ATP, which diffuse and bind to surface receptors on nearby cells.[1] Panx channels were first identified during a gene database search due to their similarity to invertebrate gap junction proteins, the Innexins (Ix), with which they share 25-33% homology.[2-4] Gap junctions facilitate intercellular communication between adjacent cells when protein 'hemichannels' embedded in the cell membrane dock with those of neighboring cells, forming pores effectively connecting the cytoplasm of the cells.[1, 5] In vertebrates, the Connexin (Cx) protein family are the major gap junction proteins, and these share no sequence homology with Ix and Panx despite having similar functions and protomer structures.[5, 6] Despite their limited homology with Ix, Panx do not form gap junctions under normal levels of expression, and function instead as integral membrane channels which generally localize to non-junctional membrane regions.[6-9]

The Panx protein family consists of three isoforms, Panx1, Panx2, and Panx3, with Panx1 being the most widely expressed and the most thoroughly investigated.[10, 11] The name Pannexin is derived from the Greek *pan* (complete/everywhere) and *nexus* (junction), and these important channel proteins are expressed in most cell and tissue types in the somatic and nervous systems.[3, 5, 10-12] Somatic tissues expressing Panx1 include the heart, skeletal muscle, skin, testes, ovary, placenta, prostrate, thymus, lung, liver, small intestine, pancreas, spleen, colon, blood endothelium and erythrocytes.[3, 5] In the nervous system Panx1 is expressed in the cerebellum, cortex, lens (fiber

cells), retina (retinal ganglion, amacrine and horizontal cells), pyramidal cells, interneurons of the neocortex and hippocampus, amygdala, substantia nigra, olfactory bulb, neurons and glial cells.[3, 11]

Panx1 channels are a principal exporter of ATP, and increased Panx1 expression and stimulation are correlated with an increase in ATP efflux.[13-15] ATP release is involved in a variety of processes including calcium wave propagation, taste sensation, communication between sensory neurons and glia, neutrophil activation and immune defense, and maintenance of vascular tone.[3, 10, 16] Furthermore, the increase in membrane permeability that occurs during apoptosis occurs primarily through Panx1 channels, and the increase of extracellular ATP and UTP recruits phagocytes which clear obsolete cells.[17] Panx1 activity has also been associated with pathological states including pain and inflammation, and diseases such as epilepsy, Crohn's disease, ischemia, and cancer (Figure 1).[13, 18, 19] Viruses such as HIV may be able to exploit Panx pores to gain entry into cells, and it has been hypothesized that some bacterial toxins may cause cell lysis by activating a cascade involving Panx channels.[18] The inhibition of Panx1 has also been shown to alleviate the symptoms of morphine withdrawal[20] and alcohol use disorder[21] in murine models. Conversely, Panx1 expression is reduced in some cancer types and the restoration of Panx1 expression in glioma cells suppressed tumour cell proliferation and motility.[22, 23] Given the range of biological processes that are connected to Panx1 function, selective modulation of Panx channels with small molecules appears to offer a therapeutic target for multiple important human diseases.

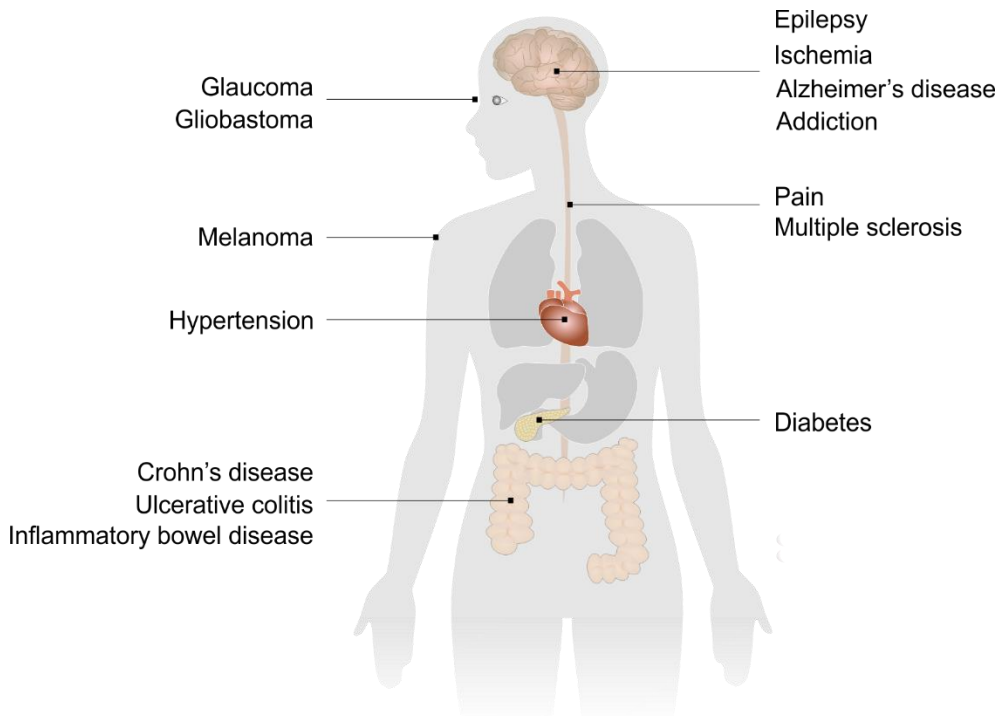


Figure 1. Pathological states associated with Panx1.

PANNEXIN-1 STRUCTURE AND FUNCTION

To inform the development of novel therapeutics, a clear understanding of Panx1 structure, potential binding sites, and chemical interactions between domains is extremely valuable. Each Panx1 protomer possesses four transmembrane domains (TM1-4), two extracellular loops (EL1-2), an intracellular loop (IL1), and cytosolic C- and N-termini.[24-26] The N- and C-terminal regions have not yet been fully resolved, but the four transmembrane regions and both extracellular loops have been recently characterized using cryo-electron microscopic (cryo-EM) imaging.[24-27] EL1 contains a short α-helix (E1H), while EL2 consists of antiparallel β-strands, and these two domains are cross-linked by C66-265 and C84-246 disulfide bridges which are essential for channel function.[24, 26] The C-terminal region contains four α-helices and two loops of unknown topology; this region exhibits low density and intrinsic flexibility which supports the proposed role of this

region as a channel gate.[24] This C-terminal region is thought to inhibit Panx1 activity through amino acid residues 379-391 via a ball and chain mechanism.[12, 28] In this model the C-terminus of the protein acts as a pore plug which can swing open or be cleaved to allow channel opening.[18, 28, 29] It has been demonstrated that Panx1 channels are irreversibly activated by caspase cleavage of the C-terminal region, however, single channel electrophysiological recordings revealed that Panx1 channels still exhibit voltage gating following C-terminal removal.[26] Overlaying cryo-EM images of native Panx1 and C-terminal-cleaved Panx1 revealed that the channel conformation was largely unaltered by the cleavage.[26] This is consistent with the observation that Panx1 forms two distinct open conformations with different permeability depending on the mode of activation.[30]

Until recently it was reported that Panx1 oligomerize as hexamers, but new cryo-EM images of human and frog Panx1 have revealed the channels to be heptameric, with identical subunits arranged around a central symmetry axis (Figure 2).[24, 25, 27] The extracellular pore opening is outlined by TM1 and EL1 while the inner pore opening is primarily outlined by the C-terminal region.[4, 18] The inside of the pore is lined by the transmembrane domains, with TM2 contributing most to the luminal surface while TM3 and TM4 form the periphery of the channel.[25] The extracellular vestibule is primarily lined by hydrophobic and basic residues, and the cytoplasmic vestibule has a net positive electrostatic potential, presumably to attract anions or negatively charged ATP.[25, 27] The narrowest region of the pore is formed by a ring of seven tryptophan residues (W74) located at the N-terminal end of the E1H, and the pore radius at this location is less than 5 Å.[24, 26] In *X. tropicalis* Panx1, R75 from the E1H forms a salt bridge with D81 and a cation-π interaction with W74 on an adjacent helix which likely serve to stabilize the restriction ring.[25, 27] A second, hydrophobic constriction site is formed by I58 near the extracellular end of

TM1, and a third constriction site is outlined by the N-terminal region which extends into the cytoplasm.[26, 27]

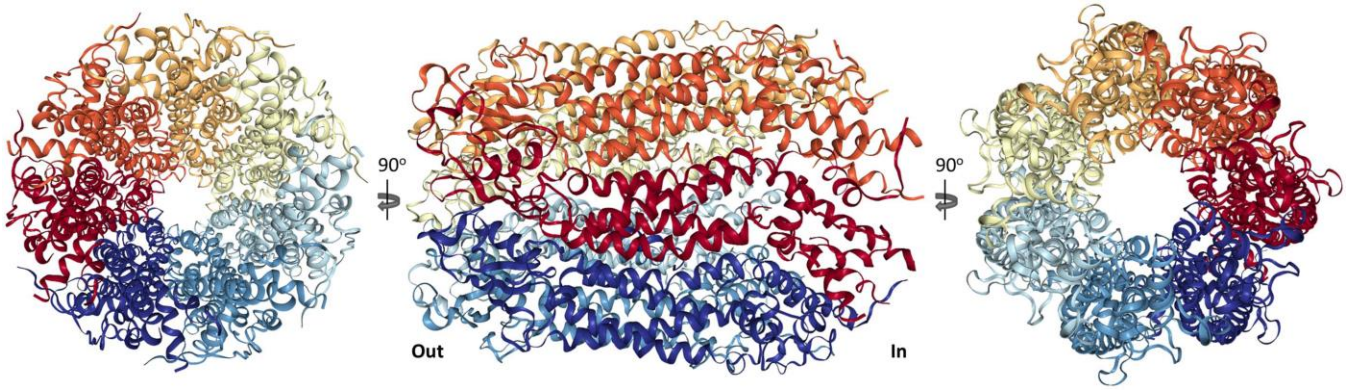


Figure 2. Cryo-EM structure of heptameric human pannexin 1 channel. Each subunit is given a unique color. Left: extracellular view; center: side view; right: intracellular view. (Image from the RCSB PDB (rcsb.org) of PDB ID 6M02 created with NGL).[31]

Panx1 channels generally localize to the cell surface and intracellular membranes, and the trafficking process is dependent on protein glycosylation state and a leucine-rich region within the C-terminal region.[32, 33] Panx1 are N-glycosylated at N254 in EL2, and this is the only identified glycosylation site (Figure 3).[18, 33] Different types of glycosylation are observed in different tissues and subcellular locations, however, most Panx1 localized in the cell membrane exhibit complex glycosylation which may prevent gap junction formation.[18, 34] The prevention of glycosylation was shown to reduce localization to the cell surface without inhibiting protein folding.[33, 35] Notably, deletion of a leucine-rich region within the C-terminal domain decreases glycosylation and cell surface Panx1 expression, but the precise mechanism underlying the observed changes is not yet understood.[32] The C-terminal tail also appears to interact with actin microfilaments which facilitate the trafficking process and stabilize Panx1 at the plasma membrane.[3, 36]

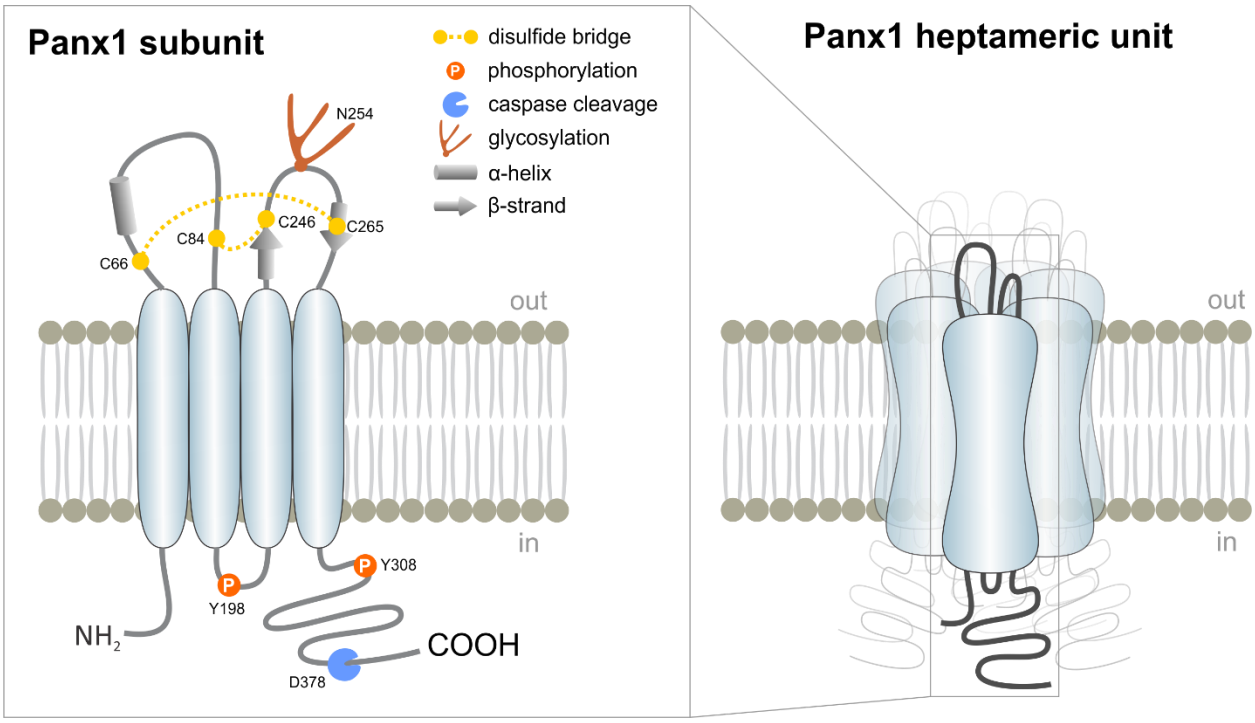


Figure 3. Structure of Panx1 subunit and heptameric channel indicating sites of posttranslational modifications and secondary structures of the extracellular loops.

PHYSIOLOGICAL REGULATION OF PANX1 CHANNELS

Panx1 pores are relatively large and non-selective, allowing transport of both positively and negatively charged compounds and accommodating molecules up to 1.5 kDa in size.[17, 37-39] This makes it critical for Panx1 channel function to be tightly regulated to preserve membrane integrity.[17] However, the positively charged R75 residues at the restriction site appear to impart anion selectivity, and the cation-π interaction with W74 identified through the cryo-EM structure is also involved in this selectivity filter.[27] Panx1 channels are permeable to a wide variety of substrates ranging from small anions (such as fluoride) to medium-sized negatively charged molecules (such as ATP and UTP), as well as cationic and anionic fluorescent dyes.[10, 40] ATP release is correlated with uptake of extracellular dyes to such a great extent that channel function and ATP release are often measured via dye uptake assays.[13] Voltage stimulation via patch-

clamping is another common tool for assessing function, and Panx1 channels exhibit a large single channel conductivity with some investigators reporting conductivities as high as ~500 pS.[9, 14, 40-42] However, relatively high positive voltages are required for this type of stimulation, suggesting that voltage activation is unlikely in vivo.[4, 43] Panx1 channels also exhibit several subconductance states with single-channel unitary conductances below 100 pS.[14, 44] Despite the complexities of interpreting electrophysiological data, patch-clamp recordings provide critical functional data complementary to dye uptake assays, and both are powerful tools for studying Panx1 and evaluating potential therapeutics.

At resting membrane potentials Panx1 channels are closed, but they may become activated in response to stimuli including mechanical stimulation, membrane depolarization, elevated intracellular calcium, elevated extracellular potassium, cleavage of the C-terminal region, activation by various receptor proteins, and Src family kinase (SFK)-mediated phosphorylation.[14, 37, 45] The data on Panx1 stimulation is complex, as different stimuli and combinations thereof cause Panx1 to adopt different conformations with different pore diameters, conductivities, and ion permeabilities.[14, 44, 46] Membrane stretch, due to osmotic pressure or cell distortion, and elevated intracellular calcium levels may trigger calcium waves which propagate to neighboring and distant cells via the diffusion of ATP.[12, 14, 45] Extracellular potassium increases during stroke in response to cerebral artery occlusion as well as during epileptiform (i.e. ictal) activity, and it is reported that potassium binding to the channel may activate Panx1 by altering the conformation of C-terminal moieties leading to an open conformation permeable to ATP.[30, 40] Conversely, voltage stimulation alone induces an open conformation that is highly permeable to chloride but impermeable to cations and ATP.[30, 44, 46, 47] Sequential removal of the C-terminal regions from Panx1 subunits occurs via caspase cleavage during apoptosis, and it is notable that muted Panx1 with truncated C-

termini form channels with similar current-voltage relationships, ion selectivities and inhibitor binding as activated wild-type channels.[17] However, caspase-cleaved Panx1 channels do not exhibit the multiple subconductance states seen in native channels and instead exhibit a maximal unitary conductance of ~95 pS at positive voltages and ~12 pS at negative voltages.[29] It is debated whether the caspase-cleaved channel is permeable to ATP in the absence of other stimuli, but Jin et al. recently used a luciferin/luciferase assay to demonstrate that C-terminal truncated Panx1 are permeable to ATP.[48] Using hexameric Panx1 concatemers, Chiu et al. also showed that sequential C-terminal cleavage events induce stepwise changes in conductivity and channel open probability, presumably corresponding to conformational changes, suggesting that channel activity may be highly tunable in vivo.[12, 17, 29] These intriguing phenomena suggest that Panx1 channels may have multiple, distinct binding sites, and that different inhibitors may cause Panx1 to adopt a fully closed conformation or one of several partially open conformations.

Panx1 activity is also regulated by several families of receptor proteins and disrupting these activation mechanisms may provide an indirect method of inhibiting Panx1. Notably, ATP gated P2X7 receptor activation is a core mechanism for opening Panx1 channels.[16, 38] Prolonged stimulation of these receptors can lead to ATP depletion and cell death.[16] However, millimolar ATP concentrations can also inhibit Panx1 channels, presumably to prevent exhaustion of the cell's ion and energy stores leading to apoptosis.[13, 49] Panx1 are also stimulated by α1-adrenoceptors which respond to phenylephrine and stimulate ATP release in order to regulate arteriolar resistance and blood pressure.[12, 29] The activation of these receptors by phenylephrine occurred in a stepwise, quantized manner in human embryonic kidney cells, similar to activation by caspase cleavage, providing further evidence for highly tunable channel function.[29] Kvβ3, a member of the β-subunit family of voltage-dependent potassium channels, also interacts with

Panx1 at the C-terminal region and rescues channel function following treatment with various inhibitors.[50] Oocytes expressing only Panx1 showed a decrease in current amplitude after application of the reducing agent TCEP, but cells co-expressing Panx1 and Kvβ3 showed an attenuated response.[50] This suggests that Kvβ3 and Panx1 are involved in cell death triggered by hypoxia and ischemia, where intracellular acidification occurs.[50] Activation of *N*-methyl-D-aspartate (NMDA) receptors during stroke also leads to Panx1 opening, often causing neuronal death.[51] Under anoxic conditions Panx1 are also stimulated by SFK-mediated phosphorylation at Y309 (Y308 in mouse).[26, 52] Anoxia and ischemia promote glutamate release from presynaptic terminals and astrocytes, which then stimulates NMDA receptors to activate SFK, which phosphorylate Panx1.[52] Similarly, in vascular smooth muscle cells of resistance arteries Y199 (Y198 in mouse) is phosphorylated to promote Panx1 function.[53]

In addition to the various modes of stimulation in vivo there are several known mechanisms of Panx1 inhibition. Reversible S-nitrosylation at C40 and C346 led to Panx1 inhibition and reduction of ATP release, which is an important negative feedback mechanism in nitric oxide rich tissues in the nervous and vascular systems where blood flow must be matched to tissue oxygen demand.[12, 54] S-nitrosylation was also correlated with increased Panx1 function in hippocampal neurons deprived of glucose and oxygen, but this study relied on a dye leakage assay which could have been confounded by outflow through other Panx isoforms or Cx channels.[12, 54] Intracellular acidification occurs during inflammation of airway epithelial cells, and this also inhibits Panx1 electrical currents in oocytes and inhibits ATP release from airway epithelial cells.[45, 55] Finally, Sandilos et al. showed that the cleaved C-terminal region is capable of inhibiting Panx1 function if it is not able to diffuse from the cell membrane.[28] However, this interaction is nonspecific, and fully scrambled C-termini are still capable of preventing channel conductance.[56]

PHARMACOLOGICAL INHIBITION OF PANX1

A variety of pharmacological agents inhibit Panx1 channels, but due to the complex Panx1 interactome and possible compensatory effects facilitated by Cx, Ix, and other Panx isoforms it is often difficult to isolate effects specific to Panx1.[18, 57] Furthermore, within the Panx family, the N-terminal region is highly conserved while the C-terminal region is variable, and currently little is known about the effects of Panx1 antagonists on Panx2 and Panx3 which share 51% and 62% sequence identity, respectively.[5, 18] Functionally related Cx and Ix proteins are inhibited by many of the same compounds despite their lack of homology, and most Panx inhibitors also bind to unrelated channels, transporters, and non-membrane proteins.[13] Further complicating analyses is the fact that native Panx1 adopts several subconductance states which demonstrate different voltage responses but not ATP release.[13, 14] Therefore, the development of more potent and selective Panx1 inhibitors is an ongoing challenge.

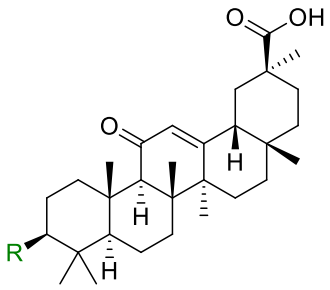
There are currently no known agonists of Panx1, while in total over 30 inhibitors have been identified with IC_{50}'s ranging from 50 nM to 10 mM.[13] These inhibitors consist of gap junction blockers, mimetic peptides, transporter blockers, P2X7 receptor agonists, malaria drugs, and others summarized in existing reviews[13, 58]. Panx1 channels are also indirectly inhibited by P2X7 receptor antagonists, and reversibly inhibited by reducing agents such as TCEP and dithiothreitol, potentially via disruption of the disulfide bridges connecting the extracellular loops.[50] Most inhibitors act reversibly, but Panx1 embedded in the cell membrane is long lived, lasting more than 8 hours following arrest of protein synthesis with cycloheximide, suggesting that Panx inhibition could have longer-lasting effects than Cx which have half-lives of less than 4 hours.[18, 59] In another interesting phenomenon, exposing N2A cells to ATP induced clustering of Panx1 and P2X7R which were then internalized in endosomes for transport to lysosomes or eventual re-expression at

the cell surface.[60] Some of the most well-studied inhibitors are discussed below and their structures are presented in Figure 4 and Figure 5. The modes of inhibition and inhibitor stoichiometry remain to be determined, as well as whether inhibition results from conformational changes induced by ligand binding or by steric blockage of the channel.[49] Furthermore there are few inhibitors with sufficient potency and selectivity to be used as Panx1 targeting drugs and therefore the need for further optimization of a Panx1 specific inhibitor remains.

Some gap junction inhibitors depicted in Figure 4 that also inhibit Panx channels include carbenoxolone (CBX) (**1**), glycyrrhetinic acid (**2**), and flufenamic acid (not shown).[10, 13] Flufenamic acid inhibits both Panx1 and P2X7 receptors, while CBX inhibits Panx1 channels without influencing P2X7 receptors, and exhibits higher selectivity for Panx1 channels than Cx channels.[16] CBX is therefore one of the most commonly used inhibitors.[61] Other inhibitors include the chloride channel blockers DIDS (**3**) and SITS (**4**), which have relatively low IC_{50}'s (see Figure 4) for Panx1, but their inhibition of chloride channels, and the inhibition of P2X7 receptors by DIDS make them less attractive as drug candidates.[62] The food dye known as FD&C Blue No. 1, or Brilliant Blue FCF (BBG FCF) (**5**), is a derivative of the P2X7 receptor antagonist Brilliant Blue G (**7**), but BBG FCF inhibits Panx1 without inhibiting P2X7 receptors.[63] In addition BBG FCF did not inhibit Cx46 or Cx32 hemichannel currents, but was not tested for activity against other Cx.[63] It exhibits weak inhibition of voltage-gated sodium channels at 30 μM, but other off-target effects have not been identified.[64] Fast Green FCF (**6**), which differs from BBG FCF by only one hydroxyl group, had the same IC_{50} and selectivity.[63]

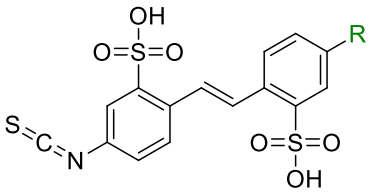
The Panx1 mimetic peptide inhibitor known as ^{10}panx (**8**) is a 10-residue peptide with the sequence WRQAAFVDSY corresponding to residues 74-83 of EC1.[38] Notably, the residues W74 and R75 play a critical role in gating according to cryo-EM images, and both are found in this

sequence.[27] While this peptide is based on a sequence from Panx1, it also inhibits Cx 46 hemichannel currents to a lesser extent.[39] Furthermore, Panx1 channels can be blocked by the Cx 32 mimetic peptide Gap24 (**9**) at higher concentrations.[13] Another commonly used inhibitor is the organic anion transporter blocker probenecid (**10**).[13, 61] Probenecid, historically used as a gout remedy, is now more commonly administered as an adjuvant to antibiotics and chemotherapeutics, exploiting its inhibition of organic anion transporters in the kidney. [61] It has an IC_{50} of ~150 μM for Panx1 channels, and this drug selectively attenuates Panx1 function without affecting Cx function.[61] At concentrations below 1 μM an adaptive phenomenon was observed that was not replicated at higher concentrations, suggesting that there may be multiple binding sites for probenecid.[61] The anti-malarial drug mefloquine (MFQ) (**11**) is one of the most potent Panx1 inhibitors (IC_{50} ~50 nM) in its racemic *erythro* form, but the individual enantiomers have not yet been reported in the literature.[2] There are two other stereoisomers of this compound; (-)-*threo*-MFQ (**12**) has lower inhibitory activity and (+)-*threo*-MFQ is not commercially available and has not been tested.[2] The low IC_{50} lends some selectivity toward Panx over Cx for which MFQ has IC_{50}'s ranging from ~0.3-12 μM.[2] However, MFQ is known to have neurological side effects, thought to be caused by binding of the (-)-e*rythro*-enantiomer to adenosine receptors, but also possibly caused by inhibiting Panx1 given its range of functions in the nervous system.[2] The related anti-malarial drug quinine (**13**) is also capable of inhibiting Panx1, but the higher doses required are sufficient to inhibit Cx channels among other voltage and ligand-gated channels.[65]



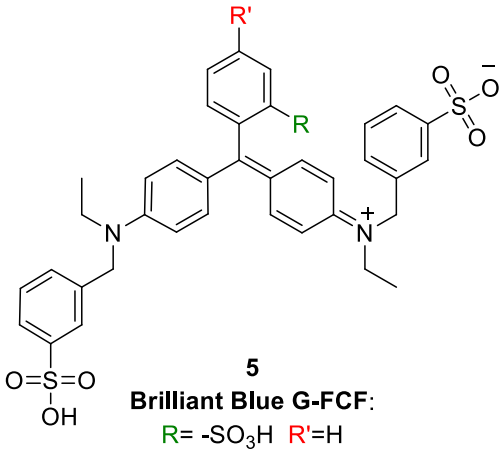
1
Carbenoxolone
R= -OC(O)$CH_2CH_2CO_2H$
5 μM mPanx1

2
Glycyrrhetinic Acid
R= -OH
50 μM (>50% inhib.) xPanx1



3
DIDS
R = -N=C=S
11 μM hPanx1

4
SITS
R = -HNAc
13 μM hPanx1



5
Brilliant Blue G-FCF:
R= -SO_3H R'=H
0.27 μM mPanx1

6
Fast Green FCF:
R= -SO_3H R'=OH
0.27 μM mPanx1

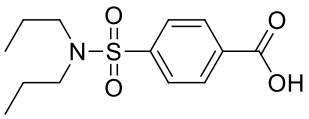
W R Q A A F V D S Y

8
^{10}panx
30-50 μM hPanx1

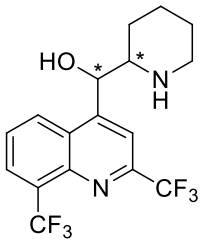
G H G D P L H L E E V K C

9
Gap24
200 μM (>10% inhib.) mPanx1

7
Brilliant Blue G:
R= -H R'=-NH-(*p*)-$OEtC_6H_4$
3 μM mPanx1

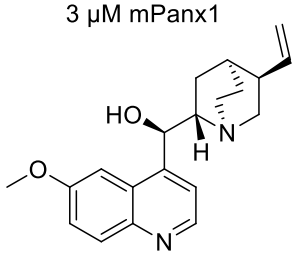


10
Probenecid
150 μM xPanx1



11
(±)-erythro-mefloquine
0.05 μM mPanx1

12
(-)-threo-mefloquine
EC50 = 0.8 μM mPanx1



13
Quinine
150 μM (>20% inhib.) xPanx1

Figure 4. Structures and IC_{50} of some common Pannexin 1 inhibitors. IC_{50} for all compounds indicate inhibition of voltage rectification unless otherwise indicated. (mPanx1: mouse Panx1, xPanx1: *xenopus* Panx1, hPanx1: human Panx1)

Recently, using a high throughput screening method, Ravichandran et al. identified the antibiotic trovafloxacin (**14**) as a potent and selective inhibitor of Panx1 channels with no blockage of Panx2 or Cx43 channels (Figure 5).[18, 66] However, this drug is known to have serious side effects in the central nervous system and kidney through unknown mechanisms.[66] The analogs difloxacin (**15**) and tosufloxacin (**16**) showed lower inhibitory activity, while ciprofloxacin (**17**) and levofloxacin (**18**) did not inhibit Panx1 despite having similar antibacterial activity.[66] The authors suggested that the fluorinated ring at N1 on the quinolone backbone may therefore play an important role in Panx1 binding. Similarly, through a medium-throughput small molecule screen spironolactone (**19**), a common medication for the treatment of resistant hypertension, was shown to directly inhibit Panx1.[67] Spironolactone exerts its anti-hypertensive effects on mineralocorticoid receptor NR3C2 in the kidney and smooth muscle cells, but prolonged use can cause hyperkalemia and gynecomastia as spironolactone is also a partial progesterone receptor agonist.[67] Spironolactone metabolites canrenone (**20**), and 7-α-thiomethylspirolactone (**23**), and the structural analog eplerenone (**24**) showed weak dose-dependent inhibition of Panx1.[67] Conversely, structurally related compounds such as aldosterone (**21**) and progesterone (**22**), and the functionally related drug finerenone (**25**) exhibited no effect on Panx1.[67] Panx1 is also inhibited by the P2X7 receptor agonists ATP (**26**) and benzylbenzoyl-ATP (**27**), and by the P2X7 receptor antagonists suramin, KN-62, and A438079 (not shown).[10, 49]

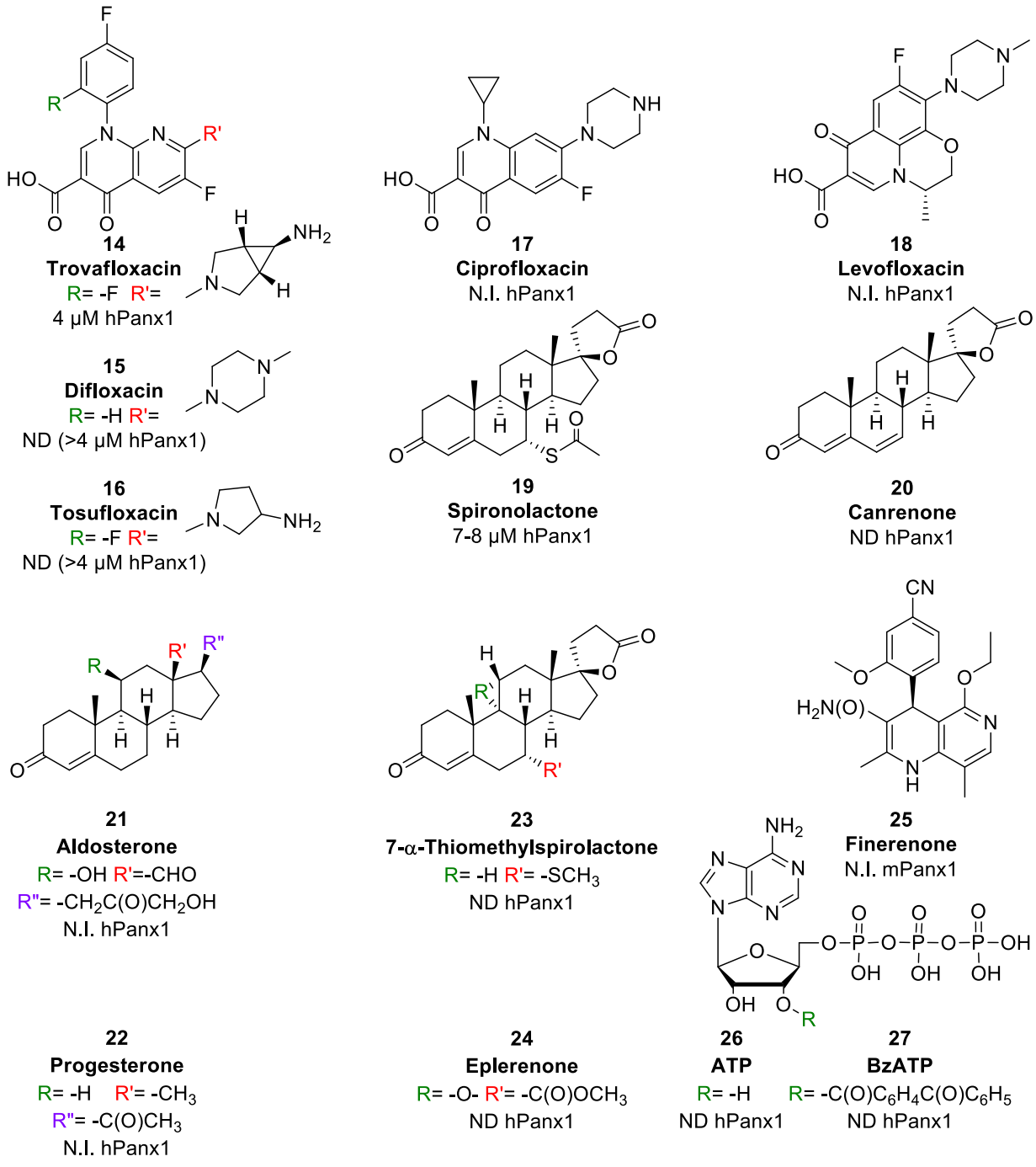


Figure 5. Structures of antibiotics, antihypertensive drugs, P2X7 receptor antagonists, and analogs shown to inhibit Panx1. IC_{50} for inhibition of voltage stimulation are indicated below each structure. (ND: not determined, N.I.: no inhibition, hPanx1: human Panx1, mPanx1: mouse Panx1)

MUTATION STUDIES AND MECHANISMS OF PHARMACOLOGICAL INHIBITION

More information on the mechanisms of Panx1 modulation and inhibition has been inferred through mutation studies. Mouse Panx1 has been used for many of these studies as it exhibits 94% homology with human Panx1 (Figure 6), and can be exogenously expressed in various cell types.[5] Individual cysteine-serine mutations of the four extracellular cysteine residues in mouse Panx1 (C66S, C84S, C245S, and C264S) resulted in nonfunctional channels, indicating that both disulfide bridges connecting the extracellular loops are necessary for normal function.[68] The C66S and C245S mutants also demonstrated impaired membrane localization in N2A cells, as well as a decrease in levels of the fully glycosylated protein which could be the cause of the former observation.[68] Similarly, in mouse Panx1 lysine-alanine substitutions K248A and K265A in EC2 resulted in nonfunctional channels, and these residues are generally conserved between species which implies their potential significance.[49] By using the substituted cysteine accessibility method (SCAM), it was shown in mouse Panx1 that cysteine substitution at seven of the first fifteen residues at the N-terminus also yielded inactive channels suggesting that the N-terminal sequence is critical for normal protein function.[4] Further supporting this assertion, Michalski et al. showed that insertion of W, GS, or AA after the first methionine (M1) in the N-terminal region dramatically increased the voltage sensitivity and open probability (P_o) of both human and mouse Panx1.[43] The cryo-EM structure of *Xenopus* Panx1 shows the N-terminal domains interacting significantly with the IL domains, and together these regions make up an interface between subunits which explains the observation that mutations in these regions dramatically alter protein function.[27]

Figure 6. BLAST sequence alignment of Human and Mouse Panx1. Intracellular residues are red, transmembrane residues are green, and extracellular residues are purple. Non-conserved residues are black.

Several other mutations have been shown to prevent normal Panx1 gating, causing membrane leakage and often cell death. For instance, deletion of residues 21-23 in the N-terminal region of human Panx1 resulted in constitutively open channels without effective gating.[26] In mouse Panx1 a C40S mutation in TM1 had a similar outcome on Panx1 channel opening.[4, 68] K346E and C347S mutations in the C-terminal region also significantly decreased the viability of oocytes expressing mutant mouse Panx1 due to membrane leakage, but these cells could be rescued with CBX treatment.[69] These two residues, as well as the phosphorylation site Y309, are located near the interface of two subunits, therefore disruptions in this region may be capable of inducing conformational changes which cause channel opening or leakage.[26] Mutational analysis has suggested that W74 also plays a critical role in Panx1 gating and ion selectivity, and the cryo-EM structures support this by revealing this residue's position at the restriction ring.[24, 27] A W74A substitution in human Panx1 increased channel activity and ATP efflux, while W74A mutant mouse Panx1 showed up to tenfold greater ATP release following stimulation by potassium gluconate.[24, 70] Furthermore, W74R, W74E, W74A, and W74F mutations in human Panx1

increased sodium permeability relative to the wild-type channel, and decreased anion selectivity.[27] Based on the cryo-EM structure of *Xenopus* Panx1 a cation-π interaction is thought to be possible between W74 and R75 on an adjacent helix, and this feature was also probed through a series of mutations.[27] An R75K mutant showed comparable reversal potentials to wild-type human Panx1, while an R75A mutant showed a loss in chloride selectivity and increase in sodium permeability, and an R75E charge reversal mutant showed an increase in permeability to large anions over chloride.[27]

Mutation studies have also provided insight into possible sites of inhibitor binding. For example, ATP binding to mouse Panx1 appears to involve R75 in EL1 (conserved in human Panx1), as substitution of this residue for negatively charged and neutral amino acids abolished the inhibitory effect of BzATP.[49, 70] Treating *Xenopus* oocytes expressing an R75C/C426S double mutant with the thiol reagent MTSET partially attenuated channel currents, demonstrating that R75 is accessible from the extracellular space and is likely close to the channel pore.[70] This inhibition was assumed to be caused by channel obstruction which indicates that ATP binding might also cause inhibition via steric blockage of the pore.[70] The cryo-EM structures have now confirmed that this residue is close to the extracellular vestibule and that it plays a critical role in determining the channel selectivity.[27] Of the other basic residues in the extracellular region, K248A and K256A substitutions resulted in a total loss of channel activity which prevented determination of their impact on ATP binding.[70] Five other mutations led to >90% attenuation of BzATP's inhibitory effects: W74A in EL1, and S237A, S240A, I247A and L266A in EL2, but whether this effect was mediated by conformational changes to the binding pocket or altered channel gating could not be determined.[70] These same five alanine mutants were tested for inhibition using Brilliant Blue G, and it was found that the inhibitory effect was attenuated for all but the I247A mutant.[63]

Furthermore, the W74A, S237A, S240A, and L266A mutants exhibited five times greater ATP release than oocytes expressing wild-type Panx1.[70]

Other mutation studies have revealed EL1 as a likely target for CBX binding as well, with mutations at residues 67-86 in human Panx1 leading to CBX resistance.[71] It has also been demonstrated that CBX must be applied extracellularly to exhibit its inhibitory effects.[43] Specifically W74 proved to be essential to inhibitor binding as a W74A mutation reversed the effects of conventional inhibitors and induced an increase in voltage-activated channel activity after treatment with CBX, probenecid, and ATP.[71] Even in the absence of voltage stimulation probenecid and CBX had a potentiating effect on the W74A mutant, suggesting that these drugs may activate the mutant Panx1 via EL1 through a mechanism similar to voltage gating.[71] Similarly, individual cysteine substitutions at all residues from 67-86 in EL1 imparted resistance to CBX except for S68C, S71C and A77C.[71] This region was mapped to the cryo-EM structure revealing a groove between EL1 and EL2 that could be a binding pocket for CBX and other inhibitors.[27] To further explore this, Michalski et al. conducted cysteine substitutions at select residues and found that I247, V258 and F262 in EL2 also play a role in Panx1 inhibition by CBX.[27] Together this data supports CBX acting as an allosteric inhibitor of Panx1, potentially binding between EL1 and EL2 in order to inhibit the channel.[27, 71]

OUTLOOK AND DIRECTIONS FOR FUTURE RESEARCH

Due to the modest selectivity of most Panx1 inhibitors, the small window for obtaining selectivity, or the high dosage required utilizing existing drugs, there is an unmet need to develop small molecule inhibitors that are highly potent and selective for Panx1 channels. Further investigation is also needed to elucidate the precise mode of action and the stoichiometry of these inhibitors in order to optimize ligand binding. With cryo-EM structures of human Panx1 now

available, it will be possible to use a rational design approach for optimizing inhibitors to accelerate the development of new small-molecule therapeutics. The effects of Panx1 modulators on other Panx isoforms must also be determined, and the physiological implications of their inhibition must be explored. The possibility of indirectly targeting Panx1 via its complex interactome by disrupting the protein-protein interactions or posttranslational modifications that stimulate Panx1 is also worthy of further study. With new structure and mutation data available, the putative binding site of ATP and other inhibitors may now be confirmed, as well as the composition of the binding site by protomers or by associated subunits. Much remains to be done, but the compilation of structural and pharmacological data will accelerate progress in this area.

CONCLUSION

The selective modulation of Panx channels continues to be a critical area of research as the development of small molecule inhibitors has the potential to provide relief from multiple human conditions. Furthermore, this would relieve many of the difficulties associated with studying channel activity, as it is currently challenging to discriminate effects mediated by Panx1 channels from that of other isoforms and Cx. The data presented herein will help facilitate the process of optimizing Panx1 inhibitors with the potential to be applied as therapies for chronic pain, stroke, epilepsy, addiction, and other areas of unmet clinical need.

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ABBREVIATIONS

Panx1, Pannexin 1; Ix, Innexin; Cx, Connexin; TM1-4, transmembrane regions 1-4; EL1-2, extracellular loops 1-2; IL1, intracellular loop 1; E1H, extracellular loop 1 helix; cryo-EM, cryo-electron microscopy; E1H, α-helix in extracellular loop 1; SFK, Src family kinase; P2X7, P2X purinoceptor 7; TCEP, tris(2-carboxyethyl) phosphine; NMDA, *N*-methyl-D-aspartate; CBX, carbenoxolone; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid ; SITS, 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid; BBG FCF, Brilliant Blue G FCF; MFQ, mefloquine; mPanx1, mouse Panx1; xPanx1, xenopus Panx1; hPanx1, human Panx1;

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