Pannexin-1 In Silico Modeling Towards Physiological and Pathological Functioning

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ABSTRACT

The transmembrane pore protein Pannexin-1 (Panx1) forms channels allowing the release of purine nucleotides and participates in processes related to purinergic signaling, including blood pressure regulation, apoptotic cell clearance, and neuropathic pain. Panx1 has also been implicated in several pathological mechanisms, including facilitating HIV entry. While several naturally occurring inhibitors of Panx1 activity have been discovered, elucidating structure-function relationships within the Panx1 oligomer and the ability to design molecules to regulate Panx1 activities has been limited due to the absence of a Panx1 crystal structure. To address this limitation, an *in silico* model of Panx1 was constructed, based on homologous protein channel structures, established physicochemical characteristics and binding properties of the Panx1 channel.

Homologous template protein searches, transmembrane topologies and sequence alignments were subsequently used by MODELLER software to generate Panx1 A-Chain monomer subunits via a multi-template stitching approach. These A-chain subunit models and a GalaxyWeb Panx1 sequence generated A-chain subunit were subsequently used to construct four model homo-oligomers (pentamers, hexamers, heptamers, and octamers) using GalaxyWEB, all oligomeric structures were compared to established biophysical parameters of Panx1 channels. Docking experiments using Medusa Guide were performed using four small molecules and the ^{10}Panx1 mimetic peptide known to block the Pannexin-1 channel. Docking simulation free energy results were used to assess the regional validity of the designed Panx1 model.

The optimum Pannexin-1 model was a hexameric structure exhibiting compact helices and dynamic loops and tails which promoted both pore and oligomer diameters and an overall structure matching Pannexin-1 experimental data. Docking experiments overall exhibited moderate affinity free energy values for Panx1 inhibitors inferring an overall fair modeling accuracy. A high affinity free energy value of -46.38 kcal mol^{-1} was discovered for the mimetic ^{10}Panx1 which partially steric blocks the Pannexin-1 pore region.[2, 8, 66] EL-1/2 docking results demonstrated high (< -30 to -50 kcal mol^{-1}) to poor (> -20 kcal mol^{-1}) affinities. The model suggested these small molecules bound to the EL1/2 regions primarily through steric/hydrophobic and polar interactions. Overall, this model fits with established experimental results and is a reasonable tool for the initial design of compounds which modulate Pannexin-1 gating.   viii.

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Chapter 1

Introduction

1.1 Background of the Problem

Ubiquitously expressed channel transmembrane protein Pannexin-1 controls a multitude of connected homeostatic functions that are interlaced and carried over via a conduit of purinergic receptors ultimately catapulting physiological responses. A deep understanding of these mechanisms is obscure and the Pannexin-1 has not yet been crystallized, moreover current Pannexin-1 pore experimental gating is limited via undiscovered promoters and discovered inhibitors only inferring pore closure via steric block or conformational closure via an elusive Pannexin-1 regional-blocker electro-steric interaction.

1.2 Statement of the Problem (Purpose of the Study)

Therapeutically controlling Pannexin-1 gating will ameliorate a number of health issues, this gating control requires a characterization of pivotal Pannexin-1 uncrystallized regions. The purpose of this study is to complete an in silico reasonable beginning design of these Pannexin-1 pivotal domains that promote channel closure.

1.3 Goals, Objectives and Research Questions of the Study

The overall goal of this research project is to accurately design in silico the uncrystallized Pannexin-1. The objective of this study is to prove this model's accuracy via designing a Pannexin-1 transmembrane protein exhibiting an overall structure matching experimental data and objectified regional accuracy via Dynamic Docking Simulations of known pore blocking partners that bind particular Panx1 electro-steric areas of interest with minimum affinities.

Future goals will include further elucidation of the pivotal residues within Pannexin-1 binding area(s) of interest and development of a preliminary conjugated smart nanoparticle that allows preferential accumulation and conjugation to Pannexin-1 channels (targeted delivery). This smart nanoparticle will promote an enhanced blocking and opening therapeutic efficacy while maintaining a reduced cytotoxicity.[98]

Proposed Future Study Objectives:

1.) Identify conformational changes and host cell membrane depolarization in time during Pannexin-1 protein/channel opening and closing via Molecular Dynamic Simulations.

2.) Generate by computer learning a program predicting binding and functioning of the transmembrane channel as a measure of therapeutic efficiency.

3.) Further characterize the extracellular, intracellular and membrane associated areas of uncrystallized Pannexin-1 and discover the pivotal residues essential for channel opening/closing and trigger mechanisms.

4.) The nano-engineering design of a stealth nanoparticle exhibiting promoter and inhibitor conjugates promoting Pannexin-1 channel opening and closing respectively.

5.) In vitro and in vivo experiments to confirm Molecular Dynamic Simulation in silico results of a designed AuNP and its tuned AuNP-tag allowing the activation of the Pannexin-1 Channel into a closed/open position, (nanoparticle subsequent fragmentation will be accommodated).[99]

Pivotal questions toward this study:

1.) How compact is the modeled pore region and how similar is the overall structural design compared to experimental data i.e. pore and oligomer diameters/oligomerization?

2.) How regionally precise and characterized was the uncrystallized Pannexin-1 transmembrane protein?

3.) What are the inhibitor chemical/protein binding affinities?

Pivotal questions toward future study:

1.) What is the potency and efficacy of the nanoparticle in silico, in vitro and in vivo?

2.) Which residues within the key-domains are responsible for Panx1 closure and opening?

3.) What are the AuNP conjugate Panx1 binding affinities and specificities in silico, in vitro and in vivo?

4.) What will be the nanoparticles, bioavailability, stability, solubility, circulation time/clearance and non-specific toxicity in vivo?[98]

5.) What homeostatic mechanisms may adversely be effected via a blocked or opened Panx1 Channel i.e. immunoregulation etc.?

1.4 Significance of the Study (Need i.e. Theoretical and Practical)

The in silico methodology towards this Pannexin-1 modeling and its energy dockings will contribute theoretical knowledge towards parallel works. Moreover this preliminary design will be a staple to subsequent Molecular Dynamic Simulations towards Pannexin-1 regional refinement and therapeutic Pannexin-1 gating.

The design of an uncrystallized Pannexin-1 to treat disease will practically contribute to Biomedical Informatics Databases characterizing new Pannexin-1 treatment regimens and Pannexin-1 structure and function. For example, Biomedical Informatics databases ViPR (Virus Pathogen Resource), Viral Zone and PDB will fruitfully add Pannexin-1 resource facts.

Table 1.) Pannexin-1 as a new target protein towards HIV prevention will be added to ViPR Biomedical Informatics Database's Antiviral Drug Search.[102]

Table 2.) Pannexin-1 Channel will be added as a HIV Resource Fact demonstrating it as an inter-cascading pivotal event to viral entry.[103]

Table 3.) The Biomedical Informatics Protein Database (PDB) will add Panx1 ATP release sub-structural and functional data.[104]

1.5 Hypothesis

The main hypothesis of this proposal is to demonstrate the accurate in silico design of the uncrystallized Pannexin-1 transmembrane protein via the exhibition of a software visualized best overall structure matching experimental published structural and functional data. Moreover docking simulation free energy results will assess the regional validity of the designed Panx1 model that controls homeostatic gating. This design will be a beginning towards Pannexin-1 therapy via a controlled gating mechanism.

Chapter 2

Literature Review

2.1 Pannexin-1 Structure and Function

Pannexins are a highly conserved transmembrane oligomer protein family encoded via three genes in humans (Panx1, Panx2, Panx3).[2, 8, 21] Pannexin-1 is expressed in at least five inter-species and is ubiquitously plasma membrane expressed (brain, spinal cord, heart, skeletal muscle, skin, testis, ovary, placenta, thymus, prostate, lung, liver, small intestine, pancreas, spleen, colon, blood endothelium, erythrocytes) being highly expressed in the Nervous System and Human Primary Macrophages and CD4$^+$ T-Lymphocytes controlling a multitude of bodily functions.[21, 170] Pannexin-1 may moreover be intracellularly expressed contributing to Ca^{2+} leak signaling mechanisms.[177]

One subunit protein Pannexin-1 gene product has been experimentally inferred to exhibit four ordered compact conserved helical transmembrane domains, three disordered dynamic loops (two extracellular (ELs) and one intracellular (IL)) and two disordered dynamic coils (C-N terminals).[2]
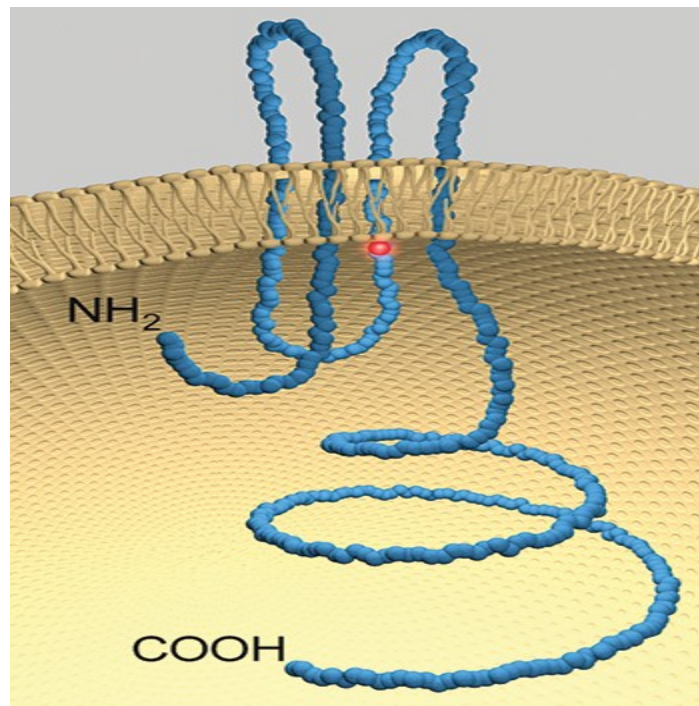
Figure 1.) Experimentally inferred Pannexin 1 with indicated red loop tucked homozygous mutant histidine replacing arginine blocking ATP passage.[6] Ubiquitous Pannexin-1 controls a multitude of bodily functions accordingly blocked ATP-passage may affect a multitude of bodily functions.[5]

Pannexin-1 uHC has been experimentally inferred to be an un-apposed hemichannel homo-oligomer composed of six-glycosylated homologous Pannexin-1 subunit proteins (A-F) forming a hexamer arranged into a large pore exhibiting oligomer and pore cytoplasmic diameters of ~14 nm and ~1.9 nm respectively.[2, 8, 14, 166] Pannexin-1 hemichannels are plasma membrane located and can be opened at the un-apposed cell surface forming non-discriminatory but highly regulated aqueous conduits permeable to ions and small molecules <1 kDa (i.e. ATP, UTP, glutamate, NAD^+, PGE_2). The large size of the Pannexin-1 channel coupled to its lack of selectivity should allow an unhindered efflux of ATP down its concentration and voltage gradients.[8, 173, 188] This flexibility allows the diffusional exchange between the intra- and extracellular compartments constituting a route towards an autocrine/paracrine cellular communication, i.e. Pannexin-1 ATP release and its subsequent hydrolysis triggers an adjacent Purinergic/Adenosine Receptor autocrine conduit towards homeostasis/physiology.[8, 173]
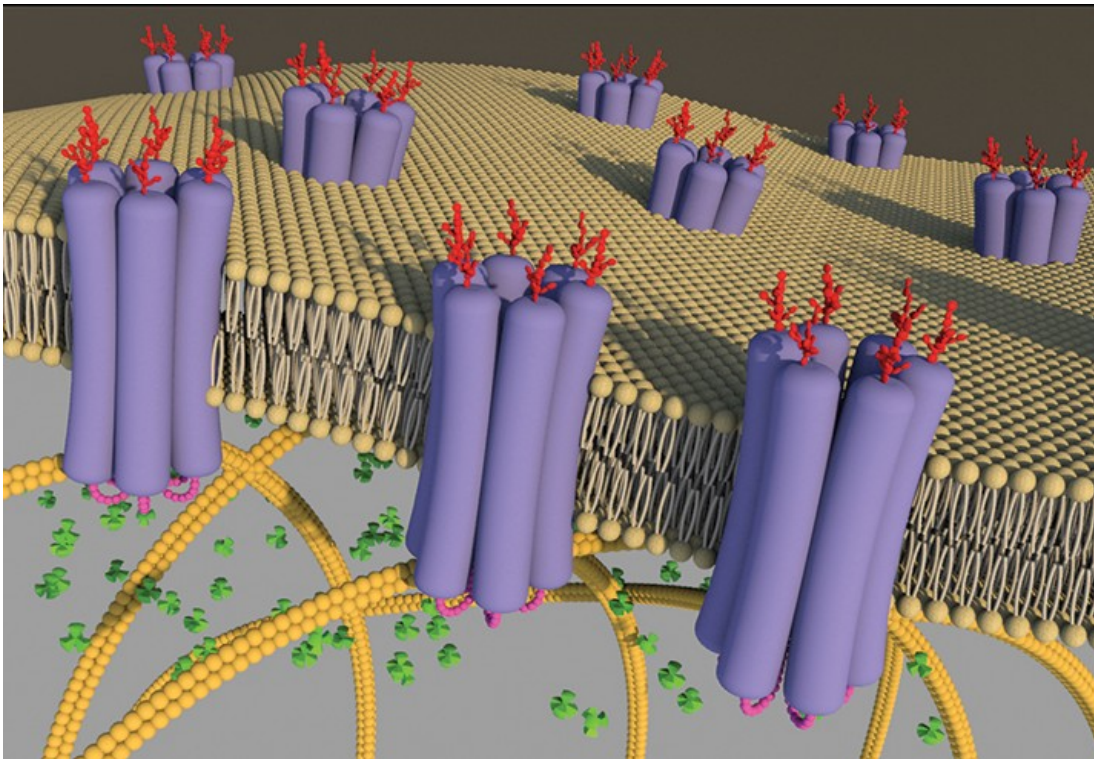
Figure 2.) Experimentally deduced oligomer illustration of six-glycosylated Pannexin-1/homologous subunit proteins arrange into large pore-channels ~1.9 nm in diameter (unopposed hemichannels (uHC)) suitable for 2nd messenger ATP (green-clovers) or other ions and intracellular messengers up to ~1 kDa in molecular mass.[7]

Various pathological and physiological stimuli have been experimentally inferred to open and activate Pannexin-1 channels; i.e. mechanical stimulation, ATP membrane depolarization, activation via different membrane receptors (i.e. HIV gp120/40-CD4 receptor-chemoreceptor complex), hypoxemia, caspase activation C-terminal cleavage, a high concentration of extracellular potassium or intracellular Ca^{2+}, voltage stimulation and pathological membrane to host membrane (i.e. HIV-Host cell membrane) induced stress.[2, 10, 15, 90, 101] Carbon dioxide-mediated acidification, negative ATP feedback and channel blockers probenecid, carbenoxolone (CBX), food dyes Brilliant Blue FCF [BB FCF] and FD&C Green No. 3 (Fast Green FCF) and flufenamic acid close Pannexin-1 channels.[101] How Pannexin-1 senses and integrates such diverse stimuli to form an open and closed channel is yet elusive.

2.1.1 Pannexin-1 Structural and Functional Experimental Evidence

Protein Structure	Panx1 Deduced Structure	Laboratory Evidence
Channel	Mobile Large-Pore Single-Membrane Hemmi-Channels In Vivo, not Double Membrane Gap Intercellular Channels (possible gap junction formation in vitro).	Synthetic rat Panx1 RNA injection results in voltage-activated currents in a non-junctional plasma membrane.[107] Panx1 open gating macroscopic currents via extracellular ligand activator ATP coincide with single plasma membrane channel currents. EM does not exhibit canonical gap junction views in Panx1 cell appositional regions; Panx1 intercellular spacings are 20-50 nm, Cx43 gap junction spacings are 2-5 nm.[108, 172] Immunocytochemistry, detection via light and EM exhibit rodent Panx1 localization within hippocampal and cortical principal neurons to a single plasma membrane not opposing membranes. Residing/operating single blood and airway epithelial cells exhibit Panx1 intracellular and plasma membrane localizations. Whole-cell patch-clamp intercellular conductance recordings upon Panx1 exhibit no significance towards electrical coupling.[109]

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		Fluorescence recovery subsequent to photobleaching exhibits a highly mobile Panx1 at the cell surface in contrast to significantly less mobile gap junction channels.[165]
		Panx1 exhibited surface glycoproteins repel adjacent plasma membranes via steric hindrance precluding Panx1 dockings and gap junction formation.[108]
		Immuno-labeling via specific primary antibodies and secondary gold conjugated antibodies exhibit gold labels on just one side of Panx1.[166]
		Injected synthetic rat Panx1 RNA precipitates nonselective, voltage-activated currents in a non-junctional plasma membrane.
		Panx1 channels are small molecule permeable and blocked via gap-junction blockers indicating hemichannel formation.[107]
		Chemical cross-linking and sedimentation studies indicate Panx1 to exhibit a single membrane channel.[169]
		Panx1 Polarity reversal experimentation indicates dependent hemichannel activity.[108]
		Panx1 homomeric pannexons form functional hemichannels in tissues and functional gap-junctions in Xenopus oocytes.[166]
Topology Orientation	Tetra-Spanning, 1-Intracellular Loop (IL1), 2-Extracellular Loops (EL1/2), In Terminals	Sequence analysis (eg. positive inside rule) indicates a tetra-spanning Panx1 exhibiting an In Topology with 1IL and 2ELs.[169, 210]
		Directed primary antibody 4512 fluorescent conjugate specific to the peptide sequence in the putative first extracellular loop indicates EL1 topology.
		Directed primary antibody 4515 fluorescent conjugate specific to the peptide sequence in the putative carboxyl-terminal portion indicates a C-tail cytosol topology via sole reactions in cryo-sectioned cells.[167]
		Rabbit polyclonal antibodies similarly characterized a Panx1 second extracellular loop (EL2-247) topology.[168]
		Toppred algorithms predicted a Panx1 with four transmembrane domains.[109]
		Membrane topology studies, hydrophobicity plots and secondary structural predictions portend Panx1 to have four transmembrane domains, In Topology and two extracellular loops containing disulfide bonds.

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		* EM images exhibit Panx1 overall topology to match Connexin26.[166] Site-directed mutagenesis of the sole Panx1 surface N-linked glycosylation Asn254 site indicates an EL2 topology via a defunct cell-surface trafficking.[168, 169] Cryo-electron microscopy studies infer a preserved gap junction family cytoplasmic domain cooperating with N and C-terminals during pore-funnel conformation inferring a conserved Pannexin-1 In Topology.[183]
Oligomeric State	Multimer Homomeric Hexamer	Extrapolated data from hemichannel experiments, i.e. ionic selectivity, permeability, voltage gating behavior, indicate Panx1 to exhibit homomeric channel functioning via a large unitary conductance of ~500 pS and a minimum of four substates (5%, 25%, 30% and 90%).[107] Chemical cross-linking and sedimentation studies indicate Panx1 to exhibit a homomeric hexameric structure.[169] DSP cross-linking experiments and N254Q glycosylation-deficient rPanx1 mutants exhibited monomer, dimer, trimer intermediates and an assembled oligomeric primary channel state hexamer rPanx1 respectively.[172] Via a C-terminal truncation mutant Panx1 is predicted to oligomerize to six subunits.[170] Biochemical, co-immunoprecipitation and electrophysiological evidence indicates Panx1/Panx2 heteromeric channels, these heteromers are unstable over time due to a Panx2 symmetric octomeric mismatch (tissue-heteromeric evidence does not exist).[107,166] Current analysis within *Xenopus* oocyte system using rPanx1, rPanx2 and heteromers 3:1, 1:1, 1:3 indicate significant channel activity only within Panx1.[215]
TM Positions	4-TM Domains, 8-Positions	Tmmpred, TMHMM and DNA Star softwares based upon hydrophobicity predicted the following TM segment boundaries for Panx1: TM1, M37-I60 TM2, F108-W127 TM3, L218-S236 TM4, L275-F296[171]
Channel Diameter	Oligomer and Pore Diameters	EM rPanx1 oligomer and pore diameters of ~140 Å and ~19 Å.[166]
Channel Passage	Chemical Flux/Efflux	Panx1 channel ATP efflux determined via luminometry.[173]

		Negative and positive dye uptake indicates a Panx1 channel permeable to both cations and anions.[188] Over-expression systems infer an intracellular Panx1 ER Ca^{2+} leak and Panx1 intercellular Ca^{2+} diffusion.[169]
Channel States; Open/ Closed	Channel Deactivation/ Activation	Voltage-activated Panx1 channel currents are inhibited via food dyes Brilliant Blue FCF [BB FCF] and FD&C Green No. 3 (Fast Green FCF) and Probenecid. Luciferase assays exhibit Panx1 attenuations of ATP release in K+ induced Oocytes via the addition of these same blockers.[69, 70] ATP application exhibits attenuated Panx1 currents indicating an ATP negative feedback loop. (Panx1 EL1 R75 mutant analysis exhibits attenuated ATP inhibitions of Panx1 currents.)[176] Mutational deletions of the C-terminal region distal to the caspase cleavage site indicate a Panx1 C terminus auto-inhibition via current analysis.[174] Experimentally stimulated Glial-like type II cells expressing functional ketanserin-sensitive $5\text{-}HT_2$ receptors increase cytoplasmic Ca^{2+} activating a ketanserin-sensitive inward current thats blocked via CBX indicating high concentration intracellular Ca^{2+} Panx1 activation and CBX inactivation.[175] Fluorescent tracer molecule uptake in erythrocytes indicates Panx1 activated ATP efflux via shear stress, low oxygen content and a high potassium depolarization. CBX addition blocks dye uptake indicating Panx1 inhibition.[167] Ethidium (Etd) dye-up take and subsequent intracellular fluorescence experiments indicate Panx1 hemichannel openings via a membrane- receptor activation (i.e. HIV gp120/40-CD4 receptor-chemoreceptor complex). siRNA of Panx1 or host cell chemoreceptor blockers preclude dye-intake indicating an HIV Panx1 hemichannel activation conduit.[8] Single channel patch clamp tests indicate Panx1 membrane channels to be mechanosensitive ATP conduits.[173]
Channel Structural Analysis	Outer-Pore TM1/EL1, Inner-Pore C-Tail.	Substituted Cysteine Accessibility Method (SCAM) inferred a TM1 and EL1 Panx1 outer pore channel lining and carboxy-terminal inner pore lining.[171]
Protein Localization	Ubiquitously Expressed, Plasma Membrane and ER	Panx1 is expressed in at least five inter-species. Northern Blot Analysis indicates plasma membrane Panx1 expression in the human brain, heart, skeletal muscle, skin, testis, ovary placenta, thymus, prostate, lung, liver, small intestine, pancreas, spleen, colon, blood endothelium and erythrocytes.[170]

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		Overexpression of Panx1 indicates intracellular ER Panx1 permeable expression contributing to Ca^{2+} leak.[177]

Table 4.) Pannexin-1 Structural/Functional Experimental Evidence

2.1.2 Pannexin-1 Phylogeny

Phylogenic and bioinformatics analyses suggests that Pannexin-1, 2 and 3 are paralogs diverged via gene duplication a priori to vertebrate species differentiation sharing their superfamily with the ortholog invertebrate gap junction innexin found in insects and nematodes (gap junctions are membrane proteins connecting the cytoplasms of cells towards communication via permeable ions and small molecules). Vertebrate connexins are similarly functioning gap junctions exhibiting a same tetra-membrane-spanning structure and hexamer channel, however, are not homologous to innexins nor pannexins. Connexins may have diverged first from pannexins and innexins from a distant protein ancestor.[163, 164]

Pannexin sequence homology is closer amongst interspecies than paralogs, i.e. human to mouse Pannexin-1 exhibits a very high sequence homology of 86/94 identical(%)/conserved(%) whereas the most closely related human Pannexin-1 and Pannnexin-2 family members exhibit just 41/59 sequence homology.[168] Pannexin-1 has evolved ubiquitous expression whereas Pannexin-2 and 3 are more limited to the brain and skin respectively, this variable expression may be mostly contributed to glycosylation and Pannexin-tail diversity. Pannexin-1 exhibits Gly-O, Gly-1 and Gly-2 variability promoting a more diverse tissue trafficking whereas Pannexin-2 only exhibits Gly-O limiting its distribution to mostly the ER.[168, 169] The Pannexin N-tails are highly conserved contrarily their C-tails exhibit a great diversity linked to cell localization, function and interactions, i.e. Pannexin-1 and 3 exhibit a shorter C-tail exhibiting a higher LRR HCS consensus sequence than Pannexin-2 resulting in a greater tissue distribution.[192] Pannexin trafficking is moreover connected to oligomerization and embryonic development; Pannexin-2 exhibits heptameric and or octameric states and Pannexin1 and 2 spatio-temporal neural distribution are inversely regulated during development.[169, 107]

Figure 3.) Paralog Pannexin monomers exhibiting similar tetra-spanning transmembranes with In-Tail topology and cysteine homology. C-tail and glycosylation states effect tissue distribution.[159]
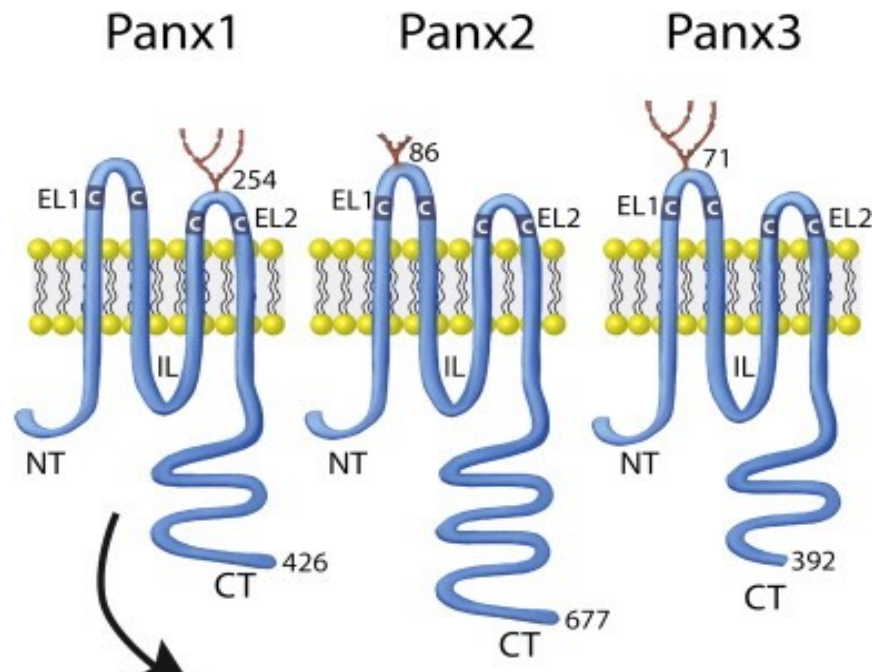
Connexin26 and Innexin6 resemble Pannexin-1 most via topology and sequence respectively.[166, 111] Pannexin-1 and Innexin6 exhibit similar weights and topology diameters.

	Pore Diameter		Oligomer Diameter		Weight
Panx1	1.7-2.1	nm	16.0-12.0nm		48.05 kDa
INX-6	1.9	nm	11	nm	45.13 kDa
Cx26	1.25	nm	8.1	nm	26.22 kDa[179, 166, 207, 208, 209]

Protein	Molecular Weight kDa ~		Single Particle Reconstruction Averages		Diameter Å ~	Pore size Å ~
		Ptcls	Class Avg	Rotational Avg		
Panx1	50	50			120	21
					160	17

Figure 4.) EM images of two grid orientations and two resolutions of clear pore rat Pannexin1 (rPanx1) oligomers, respective diameters exhibited.[16]

2.1.2.1 Pannexin-1 Residue Constraints

Structural and functional constraints restrain residue substitutions that occur during evolution maintaining a properly functioning folded protein (mutations may be compensated via a double-mutational compensation).[135]

Protein Structure	Hemichannel Panx1 Pivotal Residues	Gap Junction Cx26 Pivotal Residues	Gap Junction INX-6 Pivotal Residues	Function
Membrane Juxtaposition	N-linked glycosylated at asparagine 255 in EL2 (mPanx1, site directed mutagenesis, N-glycosidase F treatment)[170]	Non-glycosylated (cryo-electron microscopy)[178]	Non-glycosylated[201]	Glycosylation promotes channel-channel steric hindrance precluding gap junction formation.[170] Glycosylation effects protein structure function and stability.[227]
Extracellular Loop Cysteine-Cysteine Disulfide Bridges	Two-conserved cysteines in each EL; 1 EL1(66, 84), EL2 (246, 265)[187]	Three-conserved cysteines in each EL; EL1(53)-EL2(180), EL1(60)-EL2(174), EL1(64)-EL2(169)[178] (RatCx26 expressed exogenously in *Xenopus laevis*, reduced conductance, site directed mutagenesis)[202]	Two-conserved cysteines in each EL; EL1(58)-EL2(265), EL1 (76)-EL2(248)[179]	Gap junction cysteine-cysteine intramolecular disulfide bonding is a prerequisite towards channel formation.[178, 179] Panx1 cysteine disulfide bonding may modulate channel activity.[187]
Channel Positioning	Mutagenesis substitutions C347S and C40S (*Xenopus laevis* and N2A cells expressing	Mutagenesis G45E Syndromic Deafness, (mutant human Cx26G45E transgenic 14	Point mutagenesis precipitated leakage undiscovered[201]	WT native state closed, Ca^{2+} leak via mutant residue.[182, 184, 187]

	mPanx1 exogenously, increased conductance, dye uptake, site directed mutagenesis)[182, 187]	mouse expression, increased dye uptake)[184]		
N-Terminal Positioning	N-Terminal Insertion M-GS (HEK293 and CHO cells expressing hPanx1 and mPanx1 exogenously, GS insertion, increased conductance)[198]	Mutagenesis M34A Non-Syndromic Deafness and M34Adel2-7(DWGTLQ) (mutant human Cx26M34A exogenously expressed in *xenopus* oocytes, cryo-electron crystallography, reduced dye uptake, zero conductance, X-ray model analysis)[199]	Similar N-terminal blocking channel activity (cryo-EM, modeling via COOT and PHENIX)[179]	Channel Regulation WT Panx1 native state closed via Panx1 N-terminal, N-terminal indirectly pore-blocks via ion selectivity or conductance.[198] WT Cx26 native state open while N-terminus backed into pore, N-terminus mutant channel modulates a pore-closure.[199]
ATP-Channel Flux	EL1 R75 (oocytes expressing mPanx1 exogenously, ATP application, attenuated current and dye uptake, site-directed mutagenesis)[176]	Undiscovered[200]	Undiscovered[201]	Extracellular ATP inhibits an open Panx1 channel via the EL1 R75 residue regulating ATP efflux, a negative feedback loop.[176]
P2XR/NMDA/ Caveolin-Channel Complex-Interaction, Lipid Rafts	P2XR-Panx1 Complex, sites of interaction misunderstood. (Coimmuno-precipitation in endogenous and exogenous expression systems)	Cx26-P2XR/NMDA Complex undiscovered.[200]	Undiscovered[201]	A P2XR/NMDA-channel coupling promotes a sustained channel chemical flux.[189, 190] Lipid Raft Caveolin membrane transports/traffics.[204] Highly concentrated

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	NMDA-Src/ Caevolin Complex-Panx1 unelucidated.[189, 190, 203] Lipid Rafts-Lens Panx1 58/120 kDa Isoforms. (Detergent extraction, methyl-B-cyclodextrin)[217]	Cx26 binds Caveolin-1 Complex, binding sites undiscovered. (293 T cells exogenously expressing Rabbit WT Cx26, immunoprecipit ation, immunofluoresc ence, confocal microscopy)[204]		cholesterol lipid rafts regulate cell signaling.[216]
Caspase Cleavage Site: Motif: DXXD-A/G/S/T/V[194]	376DVVD379 Caspase 3 and 7 at cleavage site D379. Caspase cleavage regulatory region residues K381, T382, A386. (serial deletion, hPanx1 Jurkat cells, caspase dependent current increase) Panx1 mutagenesis TM1 F54C, C-terminal C426S. (hPanx1 exogenously expressed in HEK293T cells, Reversible cysteine cross-linking studies, current increase)[174]	Nonexhibited[200]	Nonexhibited[201]	Caspase cysteine promotes substrate cleavage at ending aspartic acid, integral to apoptosis.[193, 30] Caspase cleavage releases pore-associated C-terminal auto-inhibition, TM1 F54 and C426 binding.[174]
Protein Phosphorylatio nSrc Family Kinase (SFK)	Y10, Y150, Y345 predicted phosphorylation sites, Y199,	Tail phosphorylation undetected.[196]	Seven C-tail putative phosphorylation sites	Post-translational phosphorylation of substrate residues S, T, Y is integral towards a

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	Y309 verified phosphorylation sites. (phosphosite GPS 3.0 predictive software, rPanx1 exogenously expressed in rat N2a cells, site-directed mutagenesis, TAT-Panx$_{308}$ blocker and current reduction)[195, 205]		unverified.[197]	highly regulated/sophisticated gating. SFK modifies residue tyrosine. Panx1 phosphorylation may regulate channel opening directly or indirectly via modulation.[195]
C-Terminus Leucine-Rich repeat (LRR)	Carboxy-Tail (CT) R300-D379 exhibits 5-Highly Conserved Segments (HCS) Solenoidal LRRs. S329-H349 LRR HCS2 (mPanx1 exogenously expressed in HEK293t cells, complementary cell surface oligomerization/ glycosylation state analysis, motif deletion mutants, biotinylation confocal microscopy, EGFP fluorescence, ScanProsite LRR search)[192]	Nonexhibited[200]	Nonexhibited[201]	C-terminus LRR motifs promote cell surface localizations. LRR HCS2 required for cell-surface localization.[192]
Panx1 Blocker: Carbenoxolone (CBX)-Channel	EL1 W74 pivotal residue towards CBX	~~Gap Junction~~ 17	~~Gap Junction~~ 1	CBX modulates WT Panx1 stimulated open state attenuating channel

Interaction	hemichannel block, EL1 Conserved Region residues 67-86 mediate CBX binding. (hPanx1 exogenously expressed in HEK293T cells, mutagenesis CBX current analysis)[15]			activity. W74 acts as a polarity switch.[15] Gap junction cell to cell adhesion precludes extracellular ligand block.
Panx1 Blockers: Probenecid, BB FCF, Fast Green FCF- Channel Interaction	EL1-74, EL2-237, 240, 247, 266 pivotal residues towards chemical hemichannel block. (mPanx1 exogenously expressed in Xenopus oocytes, alanine replacement mutants, mutant current enhancement)[69]	~~Gap-Junction~~ 1	~~Gap-Junction~~	Blockers modulate WT Panx1 stimulated open state attenuating channel activity.[15] Gap junction cell to cell adhesion precludes extracellular ligand block.
Panx1 Blocker: Mimetic [10]Panx1- Channel Interaction	Hexamer pore-region pivotal residues towards mimetic peptide hemichannel block. A possible cryptic binding site(s). (mPanx1 exogenously expressed in Xenopus oocytes, dye and current attenuation)[66]	~~Gap-Junction~~	~~Gap-Junction~~	[10]Panx1 sequence specific block unlikely, WT Panx1 stimulated open state steric block attenuating channel activity. A possible cryptic mimetic peptide Panx1 binding site may exist inducing conformational pore closure.[66] Gap junction cell to cell adhesion precludes extracellular ligand block.

Table 5.) Potential functional and structural residue constraints via experimental evidence.

Non-experimental Pannexin-1 comparative protein sequence analysis amongst uncrystallized homologs further exhibit residue structural and functional constraints.

Table 6.) National Center for Biotechnology Information (NCBI) BLAST Non-Redundant Protein Sequence (nr) Database search exhibiting uncrystallized Pannexin-1 query homologs with comparatively high sequence identities (> ~80%) exhibiting micro-evolutionary constraints, i.e. non-red highlighted residues exhibiting Pannexin-1 functional constraints of the caspase cleavage site DVVD and its downstream associated regulatory regional residues highlighted in light blue.[133]

Table 7.) Phyre[2]'s sequence profiling HHblits' View PSI-Blast Multiple Sequence Alignment tool gathering mostly non-crystallized Panx1 query homologs exhibiting a low (<20%) mutual sequence identity, i.e. yellow highlighted C-residues residues trace macro-evolutionary homologous EL channel structural constraints.[136, 132]

2.2 Purinergic Receptor Structure and Function

Ubiquitously expressed Purinergic (P2) Receptors have affinities for Pannnexin-1 effluxed ATP and its hydrolyzed ADP. P2 is subdivided into Ionotropic P2X and Metabotropic P2Y Purinergic Receptors. P2X exhibits a trimeric structure using seven individual P2X subunits (P2X1-P2X7) and is a ligand gated cation channel/pore promoting Ca^+ influx and K^+ efflux when ATP bound. P2Y exhibits eight P2Y subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) and is coupled to intracellular signaling pathways through heterotrimeric G-proteins.[2, 10] Purinergic signaling is fundamental to many cellular processes (i.e. exocrine/endocrine secretion, neuromodulation) including inflammation and alternatively immunosuppresion eg. Chlamydia infected macrophages are protected from apoptosis via a pathogenic hijacking of extracellular ATP purinergic receptors (HIV similarly promotes itself via purinergic modulation).[1, 2, 8, 10, 14]

Purinergic receptor P2X is activated via extracellular ATP resulting in an increase in intracellular calcium.[9] Mammalian ATP-gated cation channel (P2XR) subunits each contain a large ectodomain, two transmembrane domains and an intracellular N and C termini. Functioning P2XRs are homomeric or heteromeric trimers and contain binding sites involved in activation (orthosteric) and regulation (allosteric). Ectodomains contain three ATP orthosteric agonist binding sites promoting the conformational changes allowing gating, they are located between neighboring subunits and are composed of highly conserved residues. ATP agonist potency is determined via positively charged amino acids lysines coordinating binding of the negatively charged phosphate tail of ATP and aromatic phenylalanine residues coordinating the binding of the ATP adenine ring. Transmembrane domains account for the channel pore and the allosteric regulatory region. The N- and C- domain structures determine the kinetics of receptor desensitization and or pore dilation, critical towards the receptor regulation of ATP (N and C termini may also modulate allosterically).[91]

Purinergic receptor P2Y is activated via extracellular ATP byproducts ADP and UTP.[9] P2Y receptors include an extracellular N-terminus containing several potential N-linked glycosylation

sites, seven transmembrane spanning domains assisting in forming the ligand binding pocket, three extracellular loops, three intracellular loops that participate in G protein coupling and an intracellular C-terminus containing several consensus binding phosphorylation sites for protein kinases.[92] P2Y receptors are G protein-coupled receptors (GPCRs) coupling to heterotrimeric G-Proteins consisting of a Gα subunit that is tightly associated with G$\beta\gamma$ subunits. P2Ys act as guanine nucleotide exchange factors (GEFs) for heterotrimeric G proteins causing dissociation of the G protein from the activated receptor and from its other $\beta\gamma$ subunits.[92] Once released the separated G protein subunits may interact with a variety of effector proteins leading to activation or deactivation of the effector protein ultimately effecting homeostatic/physiological processes.[3, 9, 10, 12, 92]

2.3 AR Receptor Structure and Function

AR Receptors have affinities for Pannnexin-1 effluxed ATP hydrolyzed adenosine. A1, A2A, A2B and A3 are the heterotrimeric guanine nucleotide-binding protein (G protein) coupled metabotropic receptors, each subtype has a unique binding profile, activation profile, subcellular localization and G protein binding preference. The primary sequence variation within GPCR family A is large and the A2A receptor characteristically displays seven-transmembrane α-helices (7TM, helices 1-7), one short membrane associated helix (helix 8), an extracellular amino-terminus (N-terminus), a cytosolic carboxy terminus (C-terminus), three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3).

All subtypes have N-linked glycosylation sites and A1, A2B and A3 subtypes have potential palmitoylation sites at the end of helix-8, the former does not alter ligand binding properties but may target receptors to the plasma membrane, the later does not effect G-protein binding nor receptor down-regulation but does effect receptor degradation subsequent to synthesis. (The depalmitoylation of adenosine A3 receptor increases phosphorylation of the receptor causing a rapid desensitization of the receptor.)

The extramembrane domains display significant structural diversity between subtypes. The extracellular domain of A2A is a funnel-like ligand binding cavity displaying a negatively charged randomly coiled binding cleft primarily formed from ECL2 (extracellular loop 2). ECL2 contains a very short alpha helical segment forming a critical aromatic pie-stacking

interaction between phe168 and bound ligand and is held together via four disulfide bridges. Subtype receptors display affinities towards different agonists and antagonists due to very small sequence variations in the residues of their binding cavities. Only a few residues in the transmembrane domains are highly conserved indicating relevance and importance in conformational changes during activation. As well conserved towards the activation process are hydrogen bonds to internal water molecules connecting the extracellular cavity to the intracellular space, these waters are pivotal towards receptor activation via ligand.
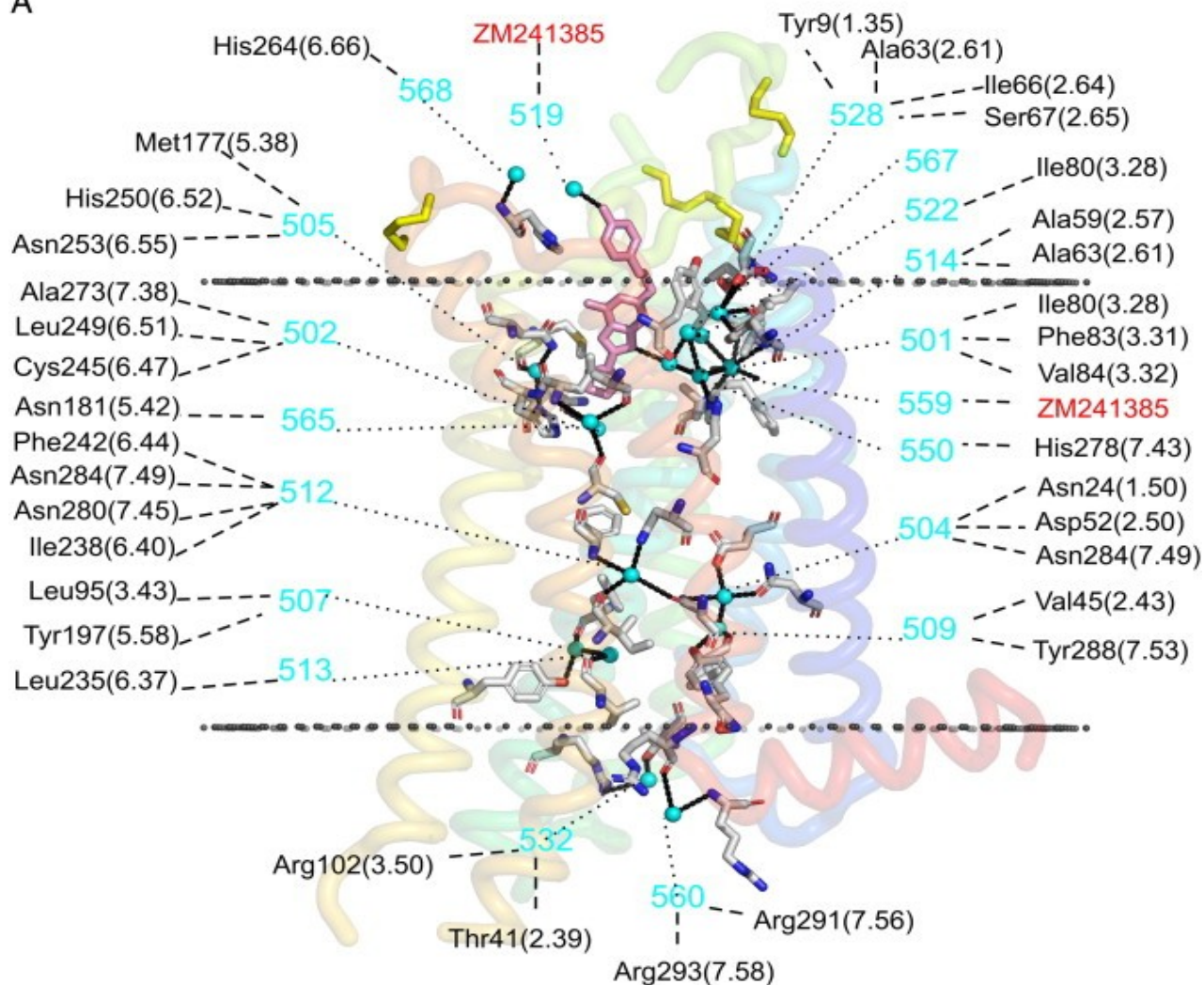
Figure 5.) Three-Dimensional image of human adenosine A2A receptor:antagonist ZM241385. Waters are blue-dots, interacting side-chains are white sticks and bound ZM241385 is a pink stick model.[95]

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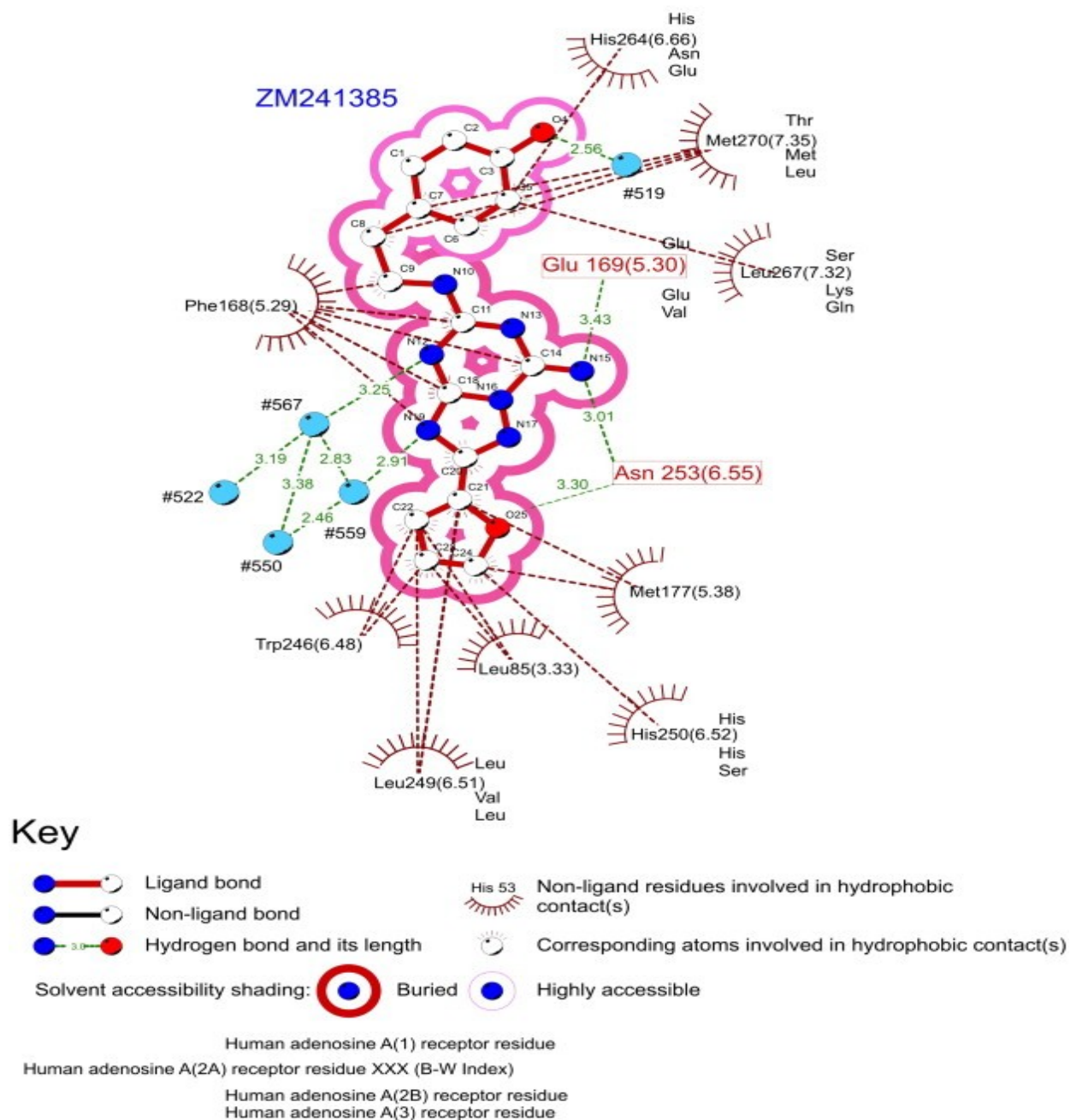
Figure 6.) Human adenosine A2A receptor bound to antagonist ZM241385 in pink. Water molecules #519, #559 and #567 directly interact with antagonist ZM241385. Image displays ECL2's critically flexible non-covalent aromatic pie-stacking interaction between phe168 and bound ligand.[96]

The intracellular domain and carboxy terminus in A2A mediate G-protein binding and signaling. There are several predicted phosphorylation sites within the cytoplasmic and carboxy terminal domains of all four adenosine receptor subtypes.[94] Activation via extracellular adenosine results in an increase in intracellular calcium effecting homeostatic/physiological processes.

Future studies may aim to block the Pannexin-1 channel via a similar antagonistic mechanism exploiting flexible non-covalent bonding connected to a specific water molecule arrangement ultimately reducing negative AR signaling towards homeostatic/physiological processes.[2, 93, 94]

2.4 Pannexin-1 Inhibitors

Pannexin-1 blockers are being used to therapeutically treat disease (eg. CBX is prescribed towards digestive ulcer and inflammation) and study the physiology of Pannexin-1 (eg. Food dyes Brilliant Blue FCF [BB FCF] and FD&C Green No. 3).[69, 20] Although elusive, blocker experimental evidence has pointed to Pannexin-1 pivotal regional areas connected to pore gating, residues EL1 67-86 (FSPSSFS*WRQAAFVDSYCWA) and EL2 (237 (L), 240 (L), 247 (S), 266 (K)).[15. 69, 70] The following four small chemicals and one small mimetic peptide block open Pannexin-1 channels.

2.4.1 CBX

Chimeric Panx1/3 has proven the Pannexin-1 first extracellular loop (EL1) to be of pivotal importance towards chemical Carbenoxolone (CBX)-mediated inhibition. Moreover within EL1, mutant analysis has uncovered variable single aromatic residue position W74 to act as a polarity switch, i.e. CBX enhances currents in a Panx1 W74A point mutant proving CBX to function as a gating modulator of Pannexin-1. Additional cysteine mutagenesis experiments has proven residues 67-86 in Pannexin-1 EL1 to similarly compromise or reverse CBX effects, many of these proven conserved residues are hydrophobic and may therefore mediate a CBX hydrophobic to hydrophobic binding supporting its function.[15]

2.4.2 Probenecid

Chemical Probenecid, used to treat gouty arthritis, is specific to Pannexin-1 channels, however, its inhibitory effects are obscure.[2] Probenecid is hydrophobic and satisfactorily water-soluble and may therefore affect the Pannexin-1 transmembrane regions in addition to the EL1/2 Regions.[70]

2.4.3 Food Dyes

Food dyes Brilliant Blue FCF [BB FCF] and FD&C Green No. 3 exhibit dose selective Pannexin-1 channel inhibition. Alanine mutant studies confirm Pannexin-1 EL1/2 areas to be food dye binding and or gating structures.[69]

2.4.4 [10]Panx1

Pannexin Mimetic Peptide [10]Panx1, a Peptide-Based Inhibitor exhibiting Pannexin-1 EL1 10-residue regional sequence WRQAAFVDSY, most likely blocks Pannexin-1 partially (30% inhibition) via steric channel block not sequence specificity; high inhibiting peptide concentrations, small ion flux and Connexin-mimetic peptide block prove this.[66] Although elusive, [10]Panx-1 may activate a docking gate via a cryptic binding site keeping the hemichannel closed, this has been demonstrated successfully in HIV CD4^{+} T lymphocytes and may therefore function towards the treatment of HIV.[2, 8, 66]

2.5 Pannexin-1 Towards Physiology and Pathology

Pannexin-1 transmembrane protein, ATP and its byproducts and its conduit purinergic receptors effect an array of physiological functions towards homeostasis and pathology eg. blood pressure regulation, infectious disease. Accordingly a comprehension of an array of these conduits towards physiology/pathology will assist in determining nanoparticle gating viability and effects in vivo. For example blocking Pannexin-1 to treat HIV or Neuropathic Pain may deleteriously effect Blood Pressure, apoptosis and inflammatory homeostatic regulation.[2, 35]

2.5.1 Ischemic Stroke

Ischemic Stroke is a dire disease effecting 15 million people worldwide annually resulting in devastating ~33% mortality and disability rates. Neural cell functioning requires an abundance of glucose and oxygen supplied via apropos blood flow. Thrombosis or cerebral artery obstruction precludes the former resulting in severe brain tissue damage via excitotoxicity, peri-infarct depolarizations, inflammation and apoptosis. Pannexin-1 and its conduit Purinergic Receptors are connected to these cell-damaging biochemical cascades.[2]

Anoxia over-activates the NMDA receptor which, via a Src kinase intermediate, opens the Pannexin-1 Channel releasing ATP which activates purinergic receptor opening and irregular intracellular Ca^{2+} influx.[2, 34] This dis-regulated cationic pool subsequently mis-regulates enzymes

which damage neural cell membranes, DNA and structural cytoskeleton.[34, 33] Ischemic Peri-infarct depolarizations and inflammation are furthermore connected to Pannexin-1 channel openings via the NMDA pathway.[33, 32]

2.5.2 Apoptosis

Apoptosis is a natural programmed cell death integral to development and homeostasis, accordingly its mis-regulation can be deleterious. Apoptotic cells releasing ATP attract monocytes towards apoptosis, Pannexin-1 hemichannel is directly responsible for this release via Pannexin-1 intracellular carboxy terminal region caspase cleavage (the Pannexin-1 C-terminus is a dissociable channel blocker).[2, 31, 30]
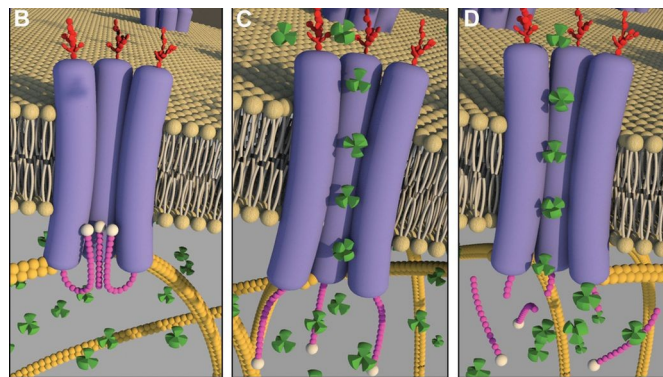
Figure 7.) Pannexin-1 channel opening towards immune cell apoptosis via caspase cleavage.[7]

Pannexin-1 hemichannel Ca^{2+} release located within the ER membranes further control apoptosis via an upgraded Ca^{2+} release out of the ER lumen triggering a Mitochondrial apoptotic conduit.[2, 31]

2.5.3 Atherosclerosis

Chronic Inflammatory Disease Advanced Atherosclerosis develops via a cascade of events involving arterial plaque formation, fatty streaks, arterial wall thickness, oxygen supply reduction; together these may cause vascular disease, ischemic stroke and myocardial infarction. During the progression of Atherosclerosis there is a monocyte recruitment to the arterial inflamed region, Pannexin-1, its ATP byproduct and purinergic receptor conduit are intimately linked to this inflammatory migration event. HIF-1α (Hypoxia-Inducible Factor-1) and its expressed VEGF (Vascular Endothelial Growth Factor) are linked to atherogenesis when exposed to atherogenic conditions, adenosine and its receptors precipitate each of these factors. Accordingly

a Pannexin-1 hemichannel opening and ATP extracellular release and subsequent hydrolysis to adenosine may play an existing role in this adenosine-mediated atherosclerosis mechanism. Contrastingly and importantly if considering Pannexin-1 blockage towards Artherosclerosis or other Pannexin-1 related illnesses, functioning adenosine and its receptors are integral to anti-atherosclerosis mechanisms in the liver.

Atherosclerotic plaques are composed of dysregulated SMC (Smooth Muscle Cell) expressions. ATP purinergic receptor binding stimulates these SMC irregularities, however as in the liver, adenosine byproducts act as endogenous modulators integral to anti-atherosclerosis. Accordingly Pannexin-1 closure to prevent Atherosclerosis may contrastingly aid in the development of atherosclerosis.

ATP purinergic receptor activation yields fibroblast proliferation integral to vascular adventitia and therefore possibly a conduit to atherosclerotic lesion and rupture causing ischemic stroke and myocardial infarction. Accordingly Pannexin-1 may as well be intimately involved in plaque destabilization and fatality.[2]

2.5.4 Blood Pressure Regulation
Blood pressure is highly regulated via an interwoven neural, vascular and renal mechanic network evolved to maintain a proper activity/time dependent blood pressure within physiological limits.[29] Pannexin-1 is an integral vascular source of ATP release within this BP regulating cellular response network heavily relying on purinergic autocrine/paracrine signaling, i.e. vasoconstriction regulation via the Pannexin-1 intracellular loop activated via an $\alpha1$ adrenoreceptor ($\alpha1AR$) signal.[29, 28] Accordingly, similar to apoptosis and inflammation homeostatic regulation, functioning Pannexin-1 is nontrivial.

2.5.5 Neuropathic Pain
Physicochemical neural insult triggers inflammation and sensor-neural remodeling precipitating neuropathic pain. This neuropathic pain conduit may be ameliorated via Pannexin-1 inhibition. Neuropathic pain exhibits homeostatic inflammation and apoptosis amongst neural and immune cells via a Pannexin-1 produced ATP intermediate. Accordingly, blocking Pannexin-1 signaling can be a viable therapeutic treatment towards neuropathic pain prevention.[27]

2.5.6 Synaptic Plasticity Towards Learning

Synaptic plasticity is based on the brain's ability to develop memories and learn via micro-scale changes amongst neural dendrites formed via regulated electrical synaptic activity.[26] Open Pannexin-1 ATP channels maintain an extracellular pool of hydrolyzed byproduct adenosine, a critical nucleotide towards precluding glutamate and methyl-D-aspartate receptor (NMDAR) over-activation and persistent Long-term Potentiation (LTP). Hyper/persistent synaptic activity results in anxiety, sensor-motor gating disabilities and impaired learning and memory. Accordingly Pannexin-1 and its byproduct adenosine are needed to stabilize synaptic plasticity towards memory and learning and may ameliorate CNS Disorders.[25]

2.5.7 Immune-Regulation

Inflammation is a self-defense mechanism to repair, i.e. cytoskeleton rearrangements towards repair. Pannexin-1s' form an ATP, small RNA, SL1, NAD^+ and Ca^{2+} release channel integral towards immune-regulation, eg. Pannexin-1 intracellular ATP efflux promotes a 'Find Me Signal' towards macrophage apoptotic clearance and immune activation and self-deactivation (ATP closing Pannexin-1).[2] Pannexin-1 and its connected Purinergic/AR Receptors are intimately connected to this immune-regulation as well as its deregulation via a pathogenic hijacking, HIV host cell entry and immunosupression is an example of the later.

2.5.7.1 HIV and Pannexin-1 Modulation and Immunosupression

HIV has an innate ability to modulate the host-cell receptor/co-receptor-Pannexin-1-purinergic pathway promoting host-cell entry and later stages of the HIV life-cycle. HIV mediates adjacent Pannexin-1 hemichannel opening in CD4+ T cells and macrophages via the bound complex HIV surface glycoprotein gp120-CD4 receptor and HIV transmembrane glycoprotein gp41-CD4 chemoreceptor.[8] Research cell lines indicate that gp120 signals through chemoreceptor CCR5 activating/opening Pannexin-1 channels resulting in intra to extracellular ATP passage (the molecular model of how HIV opens this channel is elusive and may involve membrane stress). Subsequent extracellular ATP and its hydrolyses promote autocrine activation of adjacent purinergic/AR receptors ultimately promoting HIV entry and its replication via a bi-phasic cascade of intra/extracellular events.[2, 8, 9, 10] Purinergic P2X1 and P2Y2 receptors cause host-cell membrane depolarization and HIV-host-cell membrane fusion and are of pivotal importance towards the early entry stage of HIV infection in immune cells. P2X7 and P2Y1 receptors similarly create a membrane depolarization and participate in late stages of the HIV-life cycle

(P2Y1 may also participate in entry).[2, 9] Ca^{2+} signaling is pivotal to both early and late stage HIV infection, the exact mechanism of the former remains to be elucidated, the later involves a diversity of functions i.e. the promotion of HIV-1 Gag trafficking and viral particle release via Ca^{2+}-Gag-PI(4,5)P2 plasma membrane stabilization. [17, 19]

Purinergic Receptors are HIV indirectly modulated via an open Pannexin-1 ATP efflux. Extracellular ATP released through open Pannexin-1 hemichannels binds to and initially activates P2X1 receptors causing calcium influx (P2X1 is highly sensitive to ATP being activated by low nano-molar concentrations, intracellular calcium pools precipitate via openings in nonselective cation channels, Ca^{2+}-activated K^+ channels, as well) facilitating HIV entry.[10] ATP antagonizes P2X7 poorly and therefore requires an accumulation, accordingly P2X7 is ATP activated subsequent to ATP-sensitive P2X1 activation. P2X7 then subsequently is ATP-activated causing further calcium influx downstream signaling facilitating later stages of the HIV life-cycle.[3, 9, 10] ATP does not antagonize P2Y1 and must be converted to P2Y1's antagonist ADP via extracellular ecto-nucleoside triphosphate diphosphydrolase (E-NTDPase). Once activated via ADP, P2Y1's G-protein conduits a secondary IP3 signal promoting intracellular calcium storage release facilitating HIV later-replication stages (P2Y1 may also promote HIV entry).[3, 8, 9, 10]
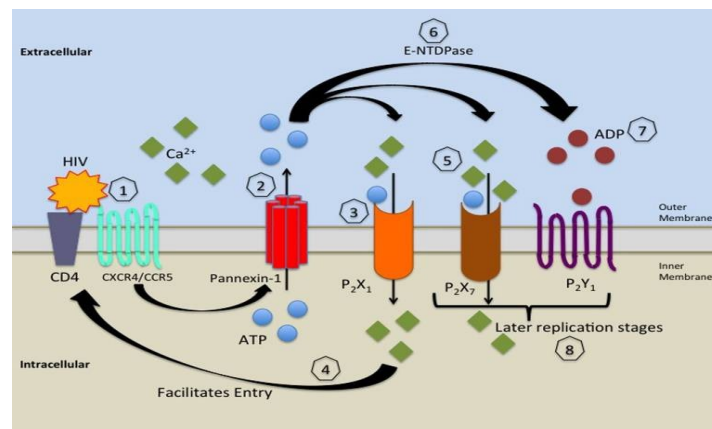
Figure 8.) Proposed model for the role of Pannexin-1 hemichannel and purinergic receptors during HIV infection; open Pannexin-1 channels precipitate HIV infection.[3]

In the case of P2Y2, effector protein proline/tyrosine kinase Pyk2 is activated/phosphorylated (Pyk2Y402*) (P2Y2's Src homology domain 3 (SH3) binding (PxxP) site controls the phosphorylation of tyrosine 402 of Pyk2 (pyk2 may autophosphorylate)) via P2Y2's G-protein

activating mechanism ultimately promoting plasma membrane depolarization, hemifusion and fusion, required for early HIV-1 infection. Pyk2 is a critical effector of HIV-1 infection operating downstream of P2Y2.[10]

Figure 9.) Involvement of Pannexin-1, ATP release, P2Y2 activation, and Pyk2Y402* phosphorylation in early stages of HIV-1 infection (the pathological HIV life cycle via a tyrosine kinase in the hypothetical cascade (pannexin-1—>ATP—> P2Y2—> Pyk2)). Binding of HIV-1 envelope to cellular receptors leads to rapid ATP release from host cells through Pannexin-1 channels. ATP then activates P2Y2 receptors, leading to activation (phosphorylation) of Pyk2 (Pyk2Y402*). P2Y2 and Pyk2 control plasma membrane depolarization, hemifusion, and fusion, which are required for HIV-1 entry-infection.[12]
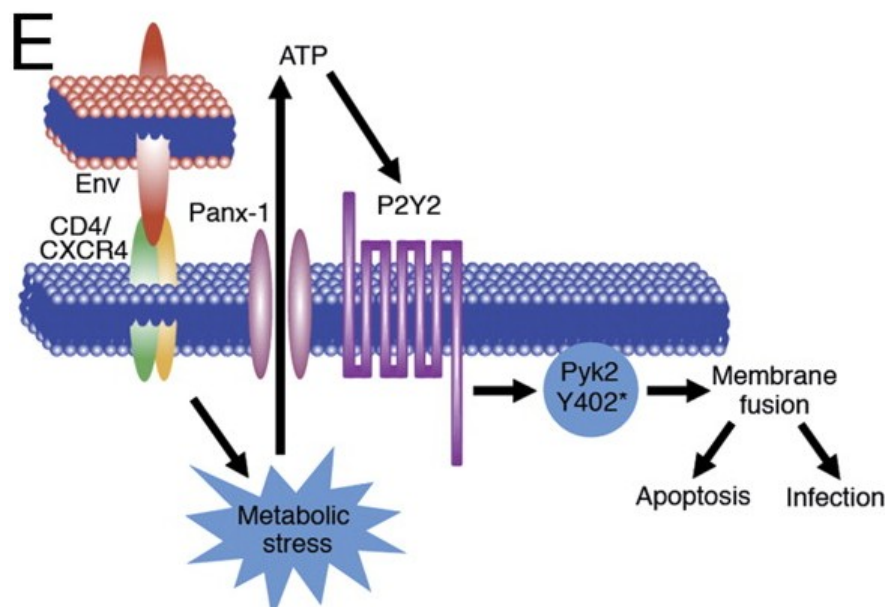
(P2Y2 activation is known to modulate Ca^{2+} influx after nucleotide binding and this may regulate Pyk2 activities and or promote its recruitment into a poly-protein complex that also contains the phosphorylating tyrosine kinase Src. Pyk2 regulates multiple signaling events that may modulate the formation of a signaling complex that contains Src family kinases and actin interacting proteins that reorganize the actin cytoskeleton controlling HIV-1 Env–mediated fusion (P2Y2 itself once activated directly interacts with filamin A, a modulator of the actin cytoskeleton). Although P2Y2 activation induces Pyk2Y402*, the precise mechanisms linking P2Y2 activation and polarization/phosphorylation of Pyk2 remains elusive.[9, 10])

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AR is a G protein coupled receptor that is activated via extracellular adenosine causing an increase in intracellular calcium and activity towards possibly both stages of the HIV life cycle.[2, 9, 10]

Figure 10.)   Ecto-nucleoside triphosphate diphosphydrolase (E-NTDPase) including ecto-ATPase and ATP-diphospho-hydrolase promotes the hydrolysis of ATP to ADP or ADP to AMP (2). Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) promotes the hydrolysis of ATP to AMP (1). AMP is further hydrolyzed by Ecto-5'-nucleotidase/ CD73 (3) to adenosine which activates adenosine receptors (AR).[4]
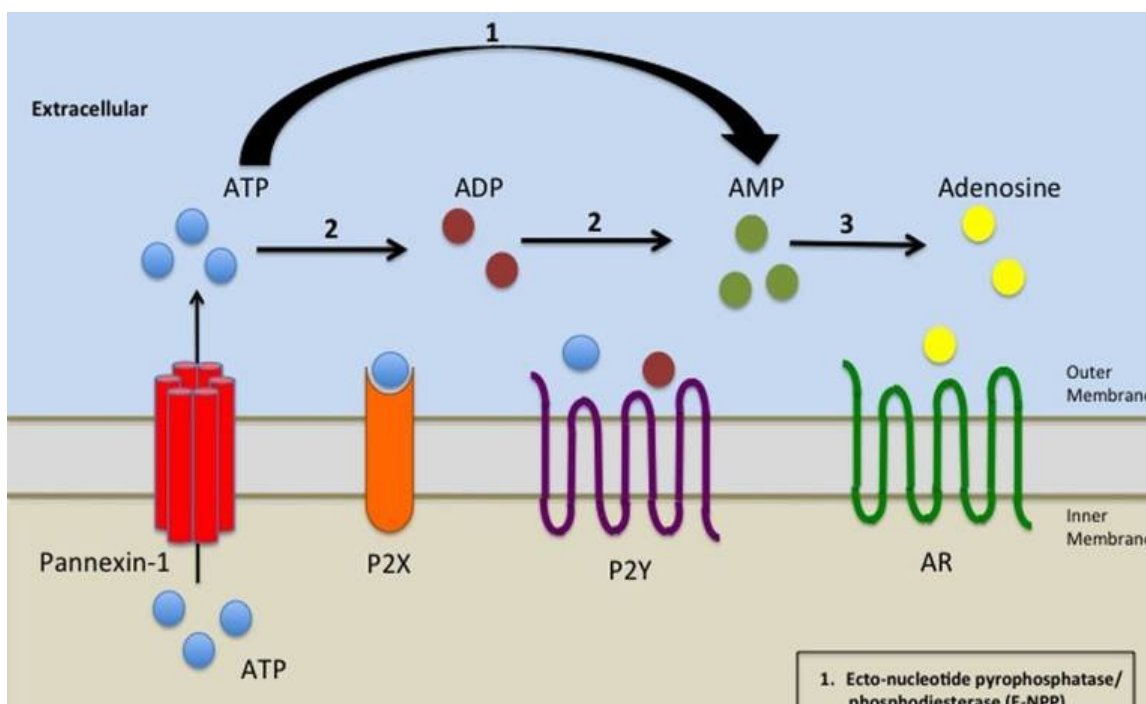
Accordingly elucidating the mechanisms of the uncrystallized Pannexin-1 hemichannel will lead to a therapeutic development towards a highly site-specific inhibitor against Pannexin-1 ultimately combatting HIV infection and replication in immune cells via modal entry attack preventing the spread of HIV.

2.6 Protein Modeling, an Overview

Many proteins' 3-D crystallographic structures are yet to be determined, their elucidation is pivotal towards protein-medicine interactions. In silico molecular modeling may elucidate pivotal misunderstood areas within a protein's 3-D atomic structure, these then determined

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elusive regions may be experimented upon in silico and subsequently in vitro and in vivo leading to prescriptional medicines and cures.

In silico algorithmic methodology based upon prediction methods, use libraries of already crystallized functional, structural and evolutionarily conserved regional protein sequences and secondary structures to discover best/accurate protein models most resembling there 3-D crystallographic structures. In silico modeling methodologies vary from Homology Modeling, when a query protein exhibits a close evolutionary relationship to one selected library template-protein (~>30%) (single target-template alignment approach), to a multiple-template approach when more distant relationships are exhibited and more than one template protein is selected towards modeling. Both methods align the target sequence(s) to the template(s) sequence(s) and use the template(s) 3-D coordinate files towards final protein model production via information from the template structure(s).[85]

2.6.1 Membrane Protein Modeling

Membrane proteins (MP) control a multitude of biological functions ((MP) Pannexin-1 channel opening being a conduit towards fighting infection as well as precipitating HIV infection) and many drugs specifically target these highly functioning membrane proteins controlling their many biological functions.[121] Pannexin-1 channels open and close via diverse stimuli, how it senses and integrates this gating mechanism is illusive and can not be determined solely by its sequence and secondary structure.[15] A whole atomic-level model of Pannexin-1 is necessary exhibiting its precise topological orientation and regional positioning of transmembrane domains (i.e. orientation/positioning and function are connected eg. topology determined Cytoplasmic G-Protein-Coupled-Receptors, intra-membraneous conserved hydrophobic proline pore-gating regulation respectively).[121, 156, 157]
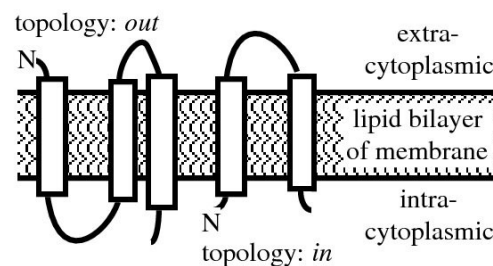
Figure 11.) Transmembrane Topology quasi-rigid cylindrical helices exhibiting 'Out and In Orientations' via N-terminal extra-cytoplasmic or intra-cytoplasmic distribution respectively.[152]
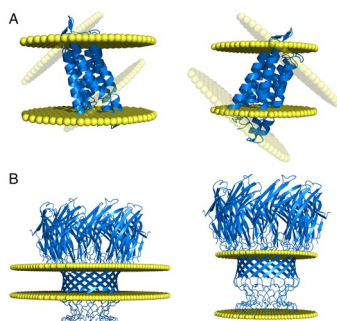
Figure 12.) Residue positioning within membranes is pivotal but difficult to model because of membrane compressibility and protein inclination limited data via experimental determination challenges, crystallized transmembrane proteins lack residue positioning.[153]

Membrane protein determination identifies membrane spanning regions, hydrophobicity predicts this i.e. TM alpha helix stretches with high hydrophobicity have been predicted to be in the membrane sitting in a hydrophobic environment disfavoring any unsatisfied polar atom therefore adopting helical energetically favored compact conformations, this process balances entropy and is overall ordered.[121, 61, 62, 59] Particular amino acids within transmembrane regions are highly conserved and pivotal towards proper biological functioning. Contrarily disordered proteins are highly flexible lacking a single well-defined structure via the containment of mostly electrostatic polar and charged amino acids promoting amino acid repulsion.[54, 55] Disordered proteins play pivotal roles in cell signaling and regulation and are often extra-membranous dynamic/difficult to align/model hydrophilic coils and loops; in the case of transmembrane proteins, these are found in the cytosol and extracellular space as C/N-termini and loop domains. Moreover positive residues are thermodynamically favored to reside in the cytoplasm, a determinant towards topology.[56, 210] Lipid-water interface regions exhibit positively charged residues and the aromatic residues tyrosine and tryptophan. Highly conserved specific-functioning energetically favorable polar residues reside within alpha-helical inner-membrane cores, further determinants towards membrane residue positioning.[156, 100, 211]

Algorithms based upon hydrophobicity, disorder and existing crystallized transmembrane proteins may be used towards identifying query-template transmembrane and topological domain positioning and orientation, pivotal towards a most accurate transmembrane protein design via distinguishing membrane-bound functioning residues from solvent-bound and cytoplasmic from extracellular loops and coils respectively. Membrane protein channel templates are limited,

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however, remotely related proteins may share tertiary features via shared critically conserved residues within membranes assisting positioning and orientational analysis.[221] Via additionally incorporating phylogeny, evolutionary analysis and experimentally determined evidence a best membrane protein query-template topological alignment may be determined.

2.6.1.1 Orientation/Positioning Prediction

Several algorithmic softwares may be cross referenced towards a best predicted orientation and re-entrant helix positioning via topology alignment consensus exhibiting agreement on the orientation of secondary structures and regional positioning of transmembrane domains.

TMPred is a computational algorithm using several weight-matrices towards scoring and is based on the statistical analysis of a database of naturally occurring transmembrane proteins predicting membrane-spanning regions (usually ~21-residues) and their orientation.[60]

TMHMM is a protein transmembrane helices prediction method via a Hidden Markov Model (HMM) predicting unknowns via inference from knowns.[58, 220]

SOSUI predicts transmembrane regions via a Hydrophobicity Analysis for Topology and Probe Helix Method for Tertial Structure; an index of amino acids and amphiphilicity plots of known 3-D structures enable the finding of end-regions of transmembrane helices.[57, 58]

Phyre[2]'s transmembrane helix and topology prediction use a powerful Support Vector Machine (SVM) called memsat-svm predicting TM protein topology via integrating both signal peptide and re-entrant helix prediction, these are combined with Phyre[2]'s integral ability to discriminate between TM and disordered globular proteins.[52]

Solvent exposure algorithms may predict a best re-entrant helix prediction elucidation via loop/coil solvent exposure predictions, i.e. RaptorX's algorithm predicts the disorder of proteins via the machine learning model DeepCNF (Deep Convolutional Neural Fields), CNF captures the interdependency of order/disorder amongst adjacent residues and exploits disordered coils and loops existence in solvent.[53]

2.6.2 Template Selection

BLAST (Basic Local Alignment Search Tool) and HHBlits are algorithmic tools used to selectively discover best template proteins via the comparison of regional similarities amongst already crystalized protein sequences within a sequence database. HHBlits discovers best fit homologs via mutational positional propensies amongst a query and homologs. NCBI BLAST hosts a family of algorithms; protein-protein BLAST (blastp) returning most similar crystallized proteins and Position-Specific Iterated BLAST (PSI-BLAST) is an expanded search of more distant crystallized relatives via a position-specific scoring matrix iterative approach involving multiple alignments, profile construction and arbitrary iteration or convergence.[161, 185] Via calculating statistical significances BLAST and HHBlits output resulting crystallized template proteins, a ~>25% similarity is ideal.[111]

A best NCBI BLAST template is best selected via analyzing resulting BLAST scores, Gaps, Positives, E-Values, Identities and Query-Coverage areas.

Score(S): the score of the alignment (S) is inversely proportional to the amount of substitutions (mismatched nonidentical amino acid template to query substitutes) and gaps (a space introduced into an alignment) within target-template alignments, accordingly a higher score exhibits a greater alignment integrity (scores are BLOSUM generated).[41]
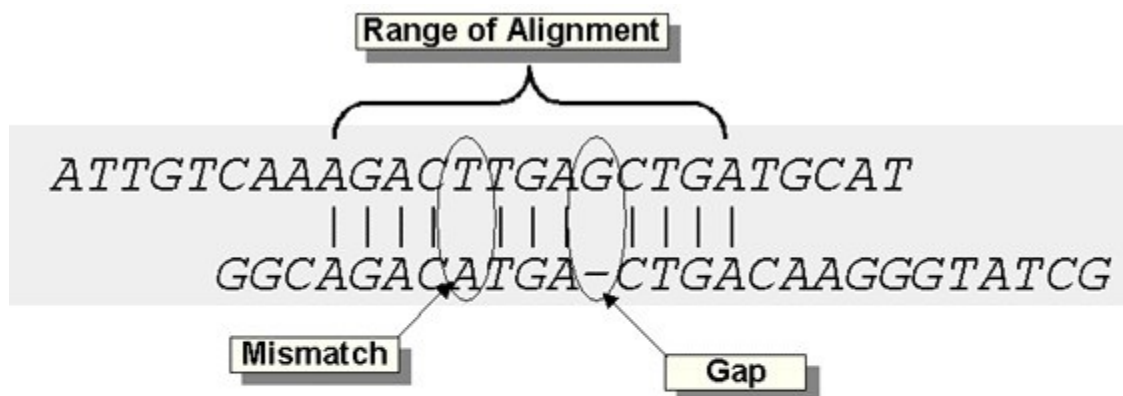
$$S = \sum (\text{identities, mismatches}) - \sum (\text{gap penalties})$$

$$\text{Score} = \text{Max}(S)$$

Figure 13.) BLAST library search example exhibiting a template-target algorithmic alignment, mismatched residues and gaps dilute alignment integrity.[40, 41]

Gap Score: represents the proportionality (gap/query residue number) and accordingly is inversely proportional to alignment integrity.[40]

Positives: exhibit the number of residues that are identical or have similar chemical properties and represent the proportionality (positives/query residue number), accordingly these scores are proportional to alignment integrity.[39]

E-Value: exhibits search chance template hits via size, accordingly E-Value is inversely proportional to match significance, best E-Values are $1e^{-4}$ or below.[40, 39, 155]

Identity Value: exhibits what percent identical the query and template residues are at the same position and represent the proportionality (identical/query residue number), accordingly these scores are proportional to alignment integrity (identities are relative towards query covered i.e. at very low coverage areas an inverse proportionality may be exhibited).[40, 39]

Query Coverage: exhibits the percent the query is aligned, low coverage areas may be significant towards multiple template alignments.[40]

Confidence: query-template homology probability (Phyre[2] HMM-HMM matching).[220, 230]

2.6.3 Target-Template Alignment Selection
Target proteins and more so transmembrane proteins (because of their partially hydrophobic surfaces, flexibility and lack of stability) lack solved protein structures within the Protein Data Bank (PDB), accordingly a single template alignment may be just a beginning approach towards 3D protein model building. Multiple templates threaded on a query sequence increases alignment coverage and better targets domain similarities circumnavigating the dearth of the crystallized protein library. Moreover a multi-template approach allows a better alignment of conserved ordered domains when homology is less than 25% and increases pivotal functional and structural residue alignments.[24, 160] Multiple template approaches may be best aligned via slight manual adjustments to avoid gaps in ordered conserved transmembrane regions and pivotal residues.   36

```
                                   *    *    *        *   *         *
PROTEIN_A|SEQUENCE          ----------------W A V Q Q K N S K A M I A E--------------
TARGET_PROTEIN|SEQUENCE    F R I Y I L L W A V L Q S K N K A A Q Y E R T K N S S
PROTEIN_B|SEQUENCE         F R LNI K N-------------------------------------I T M S N N
                           *   *    *                                           *
```

Figure 14.) Fictitious multiple sequence alignment conserving the target transmembrane region (yellow) and pivotal-residues (lime).[151]

Piecewise pairwise sequence alignment tools are best when query-template sequences exhibit high homology ∼>30%.[123] Multi-Sequence Alignment tool Basic Clustal algorithm aligns via identifying template regions exhibiting functional, structural and evolutionary relationships matching the query.[23] Clustal may similarly be used when more distant homologies <30% are present via exploiting the limited number of protein folds (∼5000) amongst several million existing proteins (∼1-10 billion). Multiple templates, in this case, from various species exhibiting high sequence similarities to query sequence targets may be used regardless of e-values above the threshold.[137]

```
   pannexin-1                    LLYLPPLFWRFAAAPHICSDLKFIME-----------E-------------------LDK
   2ZW3:A|PDBID|CHAIN|SEQUENCE   LVVAAKEVWGDEQADFVCNTLQPGCKNVCYDHYFPISHIRLWALQLIFVSTPALLVAMHV
                                 *:     .*    * .:*. *:   :             .                  :.
```

Figure 15.) Panx1-2ZW3 respective query-template residue pairwise alignment via software Clustal exhibiting pairwise alignment amongst residues, * represents conserved residues, : represents residues with strongly similar chemical properties and . represents residues with weakly similar chemical properties.[120]

Alignment methods perform best alignments via the introduction of gaps i.e. template insertions and query deletions. When aligning target-template(s), template insertions insert amino acid mutations into the molecular model while query deletions delete amino acids from the molecular model (final modeling patches up these defects via PDB library structural local sequence/geometry matching and tweaking).[122] Regardless of these final modeling repairs, insertions and deletions throughout a protein may dilute the integrity of the model with a greater malignancy within ordered/conserved membraneous regions and pivotal residues. Incorporating more rigorous secondary structural algorithms slightly improves gap results and overall alignment in comparison to basic piecewise pairwise sequence alignment tools.

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(Query)	M A I A	Q L A T	E Y V F	S D—L
(Template)	M A I A	Q L L T	E Y—F	S D F L
	MATCH	MISMATCH	INSERTION	DELETION

Figure 16.) Insertions in the template insert mutations into the model whereas deletions in the query delete amino acids from the model.

When remote query-template relationships are exhibited secondary/tertiary structural prediction methods should be used towards alignments. Structural threading exploits evolutionarily conserved secondary structures, these folds of proteins (overall structure) tend to be conserved during evolution (sequences change more quickly than structure) even for low sequence identities of 15%.[122, 121]

Promals3D new and improved alignment uses data from sequence database searches, secondary structural prediction, available homologs with 3D structures and user-defined constraints ultimately predicting more reliable alignments for more distantly related templates.[117]

```
Conservation:                          9    9                                  9         9 9
2ZW3:A_PDBID_CHAIN_SEQUEN   48  ADFVCNTLQPGCKNVCYD----------------------------HYFPISHIRL----WALQLIFV   83
pannexin-1                  59  S--------IGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGNLPLWLHKFFPYILLLFAILL  120
Consensus_aa:                   t.........Ghp.sC@s................................hPl.hh+h....hhLbhIhl
Consensus_ss:                   hh            eeee    hhhhhhhhhhhhhhhhh              hhhhhhhhhhhhhhhhhhh
Conservation:                    9 9                     9 9 9 9
```

Figure 17.) Panx1-2ZW3 respective query-template secondary structural alignment via software Promals3D, Consensus_ss Conserved Helix h Prediction exhibited.[115]

Amongst the better automated BLAST tools and structural prediction alignment servers, Phyre[2] secondary structure (alpha helix, beta sheet, TM helix) prediction predicts protein segments via an HMM neural network trained on known aa sequences and secondary structural folding predictions (there are only 1,000-10,000 naturally occurring protein folds, a relatively low number).[14] Phyre[2] templates are Phyre[2] selected not input.

CNFpred is another powerful protein threading structural prediction program specializing in template discovery (via CNFpred) and template-query alignment with sparse sequence profile, ideal for remote template discovery. Similar to Phyre[2], this alignment algorithm discovers multiple templates to a single target.[65]

A best final alignment may incorporate experimental evidence and constraints, hand-pairwise sequence alignment via multiple templates and topology/structural threading consensus amongst different algorithms.

2.6.4 Protein Molecular Modeling

Highly symmetric homo-oligomers are ubiquitous specifying distinct biochemical and biophysical properties via pivotally reactive sites within oligomeric interfaces, experimental evidence indicates Pannexin-1 to be a symmetric homo-hexamer transmembrane selective gating protein channel highly regulating multiple homeostatic mechanisms i.e. immune reactivity.[130] An accurately modeled Pannexin-1 protein structure is needed towards the optimization of a best *in silico* nanoparticle drug-design whose contents bind to the precisely designed functional alterable Pannexin-1 interface, ultimately altering opening or closure.[122]

Final full 3-D best-molecular protein models may be completed via servers Pymol, MODELLER and GalaxyWeb. The former Pymol is an excellent visualization software tool enabling orientational manipulation, pore and oligomer diameter evaluation and optimization assessment. MODELLER software may generate a new model's 3-D atomic-coordinates when a query-template expresses high or remote homology via a coordinate randomization and repeated minimum score search outputting best molecular 3-D protein models.[85] The later software GalaxyWeb server is an excellent homo-oligomer protein prediction and refinement server specializing in polymer multimerization and orientation via an input monomer. GalaxyHomomer models sequences as well as structural files via homo-oligomer templates from the Protein Database and or the ab initio docking of an experimentally determined or computationally predicted monomer.

Coils and loops lack secondary structure and reside in solvent exposed areas precipitating great conformational flexibility challenging molecular modeling, the later two methods exhibit a final structural refinement towards this highly dynamic nature via ab initio re-modeling and energy minimization.[130] Ab Initio modeling may be physics and or knowledge based using minimization and protein library angle restraint data.[162] Ab Initio modeling exhibits best results in unaligned areas containing less than seven-residues since the geometrical possible number of defined conformations within the known protein structure database past seven-residues becomes inconceivably.[143, 162]

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2.6.4.1 MODELLER Molecular Modeling

MODELLER in silico models a query uncrystallized protein via a comparative modeling technique ultimately predicting a target's 3-D structure via a selected best most similar crystallized template protein(s)'s distance-constraint information and input target-template alignment(s), predicted model errors evaluate the final model.[154] (3D model constructive methodology aside from modeling via a satisfaction of spatial restraints, may model via a 'rigid body modeling' of known atomic subunits or via a 'segment modeling' of conserved atoms within segments, accuracies amongst these three techniques are ultimately similar.)[160]

Constraints and restraints are homology derived and precipitated via a library of proteins induced upon the target within the target-template alignment, additional stereochemical restraints via CHARMM22 force field promote an apropos stereochemistry (examples of restraints are C^α-C^α distances, main-chain and side-chain dihedral angles and hydrogen bonds). Within MODELLER, probability density functions expressing the spatial restraints are smoothly optimized towards a final energy minimization in Cartesian space deriving the final model.

MODELLER models unpredictable loops via multiple methods including an initial two-stem regional library search, molecular mechanics force fields, distance spatial and dihedral angle restraints via library proteins, ab initio modeling and energy minimization.[160. 161] Side-chains are modeled similarly i.e via similar structures and steric and energetic modeling.[161]

Errors towards modeling increase as template-homology decreases; errors may be linked to pivotal side-chain packing, distortions and shifts within correctly aligned regions via structural evolution, regions with no template i.e. insertions, errors due to misalignments, and incorrect templates effecting pivotal residues. Errors may be tolerated if exhibited outside of model pivotal residue docking domains.

Model evaluation follows model building, the model-single.log output file assists in determining which model is best selected via its minimal DOPE potential energy score used to inspect the folds of the model and its runtime errors and restraint violations. MODELLER exhibits different techniques that may be combined to design a most accurate protein model.[160]

2.6.4.1.1 Homology Modeling (Single-Template Approach)

Homology Modeling uses a single-selected template exhibiting homology >30% and is best aligned to the target and input together with the templates known 3-D atomic coordinate file.

```
from modeller import *
from modeller.automodel import *
#from modeller import soap_protein_od

env = environ()
a = automodel(env, alnfile='TvLDH-1bdmA.ali',
              knowns='1bdmA', sequence='TvLDH',
              assess_methods=(assess.DOPE,
                              #soap_protein_od.Scorer(),
                              assess.GA341))
a.starting_model = 1
a.ending_model = 5
a.make()
```

Table 8.) MODELLER software single template input demonstrated via automodel class, automodel object 'a' and set parameters to model-build. Sequence alignment input file 'TvLDH-1bdmA.ali' is the target-template alignment and known '1bdmA' is the template structural file. The above script file, model-single.py, will generate five-models, 'make' method calculates models.[148]

Via a MODELLER input single-alignment and template structural file, similar best molecular 3-D protein models may be calculated and output via a coordinate randomization and repeated minimum score search, one best-fit model may be selected via energy profiling.[85]

```
>> Summary of successfully produced models:
Filename                         molpdf      DOPE score     GA341 score
----------------------------------------------------------------------
Pannexin-1.B99990001.pdb      22649.97656  -241146.95312       0.15862
Pannexin-1.B99990002.pdb      22239.50000  -240925.09375       0.06260
Pannexin-1.B99990003.pdb      22451.53125  -243152.73438       0.16993
Pannexin-1.B99990004.pdb      22131.63477  -240422.79688       0.07378
Pannexin-1.B99990005.pdb      21885.76562  -243731.93750       0.04391
```

Table 9.) MODELLER's 'model-single.log' output file exhibits warnings, errors, input restraints used towards modeling that remain violated in the final model, the last few lines display a summary of five similar models built, here 'Pannexin-1.B99990005.pdb' exhibits a best selection via its best/lowest energy conformational DOPE and molpdf scores.[139, 142]

i.) Filename: contains the coordinates of the model in the PDB format.[139]

ii.) molpdf: objective function score containing the sum of all restraints, lowest value selects the best model. Moldpdf scores are secondary to DOPE scores and are sole scores when MODELLER stitched.[139, 140]   41

iii.) DOPE score: assesses the quality of the selected atoms in the model via DOPE (Discrete Optimized Protein Energy) method, a statistical potential optimized towards model assessment. DOPE scores are based upon energy functions only (not interactions, restraints etc.), selection of the lowest DOPE score selects the best model structure.[138]

iv.) GA341 score: assesses the quality of the model via the GA341 method, a method using a percentage sequence identity between the template and the model as a parameter.[141] Scores range from 0.0 (worst) to 1.0 (native-like) and are secondary to DOPE or SOAP scores in distinguishing 'good' models from 'bad' models.[139]

2.6.4.1.2 Multiple-Template Modeling

When a more remote homology is exhibited a multiple-template approach works best via input multiple atomic coordinate files serving as templates towards a user input query-template multiple-sequence alignment. A multiple template alignment may align a single template to a query target or may align multiple templates to a query target (Clustal). A Multiple Template Method heightens model quality via simply comparing greater amounts of data, this multi-template approach may subtract data from single-template highly exhibited inserted areas ultimately promoting a stitching together of multiple-templates/models.

```
from modeller import *
from modeller.automodel import *

env = environ()
a = automodel(env, alnfile='TvLDH-mult.ali',
              knowns=('1bdmA','2mdhA','1b8pA'), sequence='TvLDH')
a.starting_model = 1
a.ending_model = 5
a.make()
```

Table 10.) MODELLER software multiple-template input demonstrated via automodel class, in this case the alignment input single file 'TvLDH-mult.ali' is the multiple template-query sequence alignment, the knowns input the three template PDB coordinate structural files ('1bdmA', '2mdhA', '1b8pA') towards modeling. Sequence remains the query 'TvLDH', 5-models to be generated.[151]

2.6.4.1.2.1 Multiple-Template Modeling, Single Template per Target

In this multiple template approach via MODELLER a single template is aligned per target resulting in multiple 3-D coordinate parts being stitched together to generate one final complete

protein model, i.e. C_Tail + TM + N_Tail → C_Tail-TM-N_Tail.

a = automodel(env,

               alnfile   = 'align.ali', # alignment filename

               knowns   = ('1', '2', '3'), # codes of the templates

               sequence = 'C_TM_N')   # code of the target

Table 11.) Via automodel class, input alnfile residue sequence corresponds to input known 3-D coordinate files (knowns), sequence remains the target.

The extracted alignment file (align.ali) format for the above stitched modeling example exhibits three-lines per model.

\>P1;1

structureX:1:   1 :10 :::::

CTVMKDVALE--*

(this first line exhibits the protein code after >P1;**1**),

(the second line exhibits 'fields' separated by colons '**:**'),

(the third line exhibits the residue sequence to be modeled **CTVMKDVALE**)[146]

\>P1:2

structureX:2:   1 :15 :::::

--------------------WFLPPLYLLIAFLLL-----------------------------------*

(Field-1 exhibits the specification, (**structureX**: (X-ray))[146]

\>P1;3

structureX:3: 1 :20 :::::

--GQGSSKINELVKLCKYSINE*

(Field-2 exhibits the code to get the structural data, (**:3:**) atomic coordinates to be extracted),

(Fields 3-6 indicate starting (fields 3-4) and ending residues (fields 5-6) of the sequence,

(:   **1 :20 :::::**),

Field 7 is an optional protein name, (eg. :ferredoxin:),

Field 8 is an optional source of protein name, (eg. :Azotobacter vinelandii:),

Field 9 is an optional crystallographic resolution value, (eg. :1.90:),

Field 10 is an optional R-factor value, (eg. :0.19)[146]

>P1;C_TM_N
sequence:C_TM_N:1::45::::0.00: 0.00
CTVMKDVALEWFLPPLYLLIAFLLLGQGSSKINELVKLCKYSINE*
(Field-1 exhibits the specification, (**sequence**: (sequence))[146]

Table 12.) Alnfile exhibiting a stitched technique; C-Tail residue 10 is stitched to TM residue 1 structure 2, TM residue 15 structure 2 is stitched to N-Tail residue 1 structure 3 resulting in a complete C_TM_N 45-residue model.

2.6.4.1.2.2 Multiple-Template Modeling, Multiple Templates per Target
A MODELLER multi-template approach may similarly stitch model via an alnfile exhibiting multiple-coordinates per target residue resulting in model building based on different types of restraints amongst different residues. A Clustal multiple template alignment input promotes this multiple coordinate modeling per target residue.

Table 13.) Clustal Omega multiple template alignment result exhibiting a multiple residue alignment towards Pannexin-3 target residue L162.[83]

2.6.4.2 GalaxyWeb Molecular Modeling

GalaxyWeb's Galaxyhomomer oligomer protein prediction and refinement server specializes in polymer multimerization and orientation via an input monomer or protein sequence (monomers may be designed or PDB crystallized proteins). Both input methods may predict oligomers via detected templates or dockings, both are similarly model refined. Oligomeric states are input or via default server predicted.

Both sequence and monomer input methods firstly detect their oligomeric state via HHsearch within the protein structure database 'pdb70'. Final S-scores are then generated via both HHsearch sequence and secondary structure scores. Five final model oligomeric states and 100 oligomer templates each are generated via this search per method.

Subsequent to monomer or sequence state qualification, five total final oligomer models are built via a sequence and or structure-based similarity approach and or ab initio docking. During a sequence approach a target residue sequence is input and five best S score sequence and structural templates are detected and the sequence is template aligned. Subsequently GalaxyCassiopeia builds oligomer models via the alignment and template structure using Variable Target Function Method (VTFM) optimization (used in MODELLER) combined with FACTS solvation free energy, knowledge-based hydrogen bond energy, dipolar-DFIRE, molecular mechanics bonded and non-bonded energy terms. When a monomer is input the five best S score structure-based detected templates are selected towards oligomer modeling. A structure based method final oligomer model is completed via a superimposition of the input monomer onto each of the detected oligomer templates. Oligomeric templates generate best models when both sequence and monomer input methods lack a combined total of five oligomer templates, the remaining oligomer models are generated via the C_n-symmetry M-ZDOCK ab initio docking of a multiple-template-based GalaxyTBM predicted monomer structure or monomer input respectively. A final ab inito structural refinement re-modeling is promoted via GalaxyLoop and the overall structures are relaxed via GalaxyRefineComplex, three loop/termini refinement regions may be monomer input selected.[130]

Template-based Oligomer Modeling Results (Sequence-based)

Model No	Oligomer template	Number of subunits	Interface area	Sequence identity	Loops/termini that may be refined	Re-submit for refinement
1	3brc	2-mer	2122.0	44.6	15-21	Submit

Template-based Oligomer Modeling Results (Structure-based)

Model No	Oligomer template	Number of subunits	Interface area	Sequence identity	Structure similarity (TM-score)	Loops/termini that may be refined	Re-submit for refinement
2	1zwy	2-mer	798.0	16.6	0.5682	59-74	Submit
3	2car	2-mer	209.9	10.2	0.5230	59-74	Submit
4	3tqu	2-mer	2193.4	9.6	0.5123	59-74	Submit

Ab initio Docking Results

Model No	Number of subunits	Interface area	Docking score	Loops/termini that may be refined	Re-submit for refinement
5	2-mer	628.9	625.491	59-74	Submit

Table 14.) GalaxyHomomer output page via a sequence input method using sequence and structure based template detection. Ab Initio Docking results displaying one of five-model Interface Area and Docking Scores, highest scores are selected. A highest Interface Area Score between adjacent monomer helices exhibits greatest helical energetically favored compact conformations influencing an thermodynamically favorable compact transmembrane pore size. Highest Docking Scores exhibit best cyclical symmetric (C_n) multi-mer structural prediction via M-ZDOCK algorithmic complementarity, electrostatics and desolvation between an input monomer (receptor) and created ligand (receptor rotated).[130, 131]

2.7 Ligand-Target Docking Dynamic Simulation

Molecular Docking Assays are excellent ligand-receptor interaction/complex tools evaluating binding affinities towards drug discovery and protein modeling.[13] Current small molecule docking algorithm MedusaDock endures simultaneous receptor ligand flexibility and adds water molecules explicitly and energy appropriately flexibly bridging the ligand to the protein via a specific best energy ligand (residue conformation) orientation and particular water arrangement promoting a most native ligand-receptor juxtaposition (MedusaScore incorporates a desolvation effect).[47, 11, 147, 220] This MedusaDock local assay restricts itself to the spatial coordinates of the putative binding regions of a < 40-residue target protein and via algorithms generating 5,000-15,000 sampled target Binding Modes (BM), MedusaDock's CHARMM energies qualify a best BM (MedusaScore includes an explicit up to 3 Å hydrogen-bonding model).[47, 64] CHARMM identifies target-ligand bonding via free energy minimization, adds disulfide bridges, protonates

residues at physiological pH etc.. Dock time is proportional to ligand rotatable bonds (<10 rotatable ligand-bonds preferred for best predicting results via lowering BMs) and ligand-target fit (shape complementarity). A software docking's interface client and server set the target and ligand and submit docking at physiological temperature. A greater fraction of the ligand being buried in the target results in a greater docking binding pocket identification and subsequent dock.[64]

In silico docking free energy binding affinities evaluate ligand-target induced fit and molecular modeling regional potentials. Lower in silico free energy minimization values are directly proportional to greater ligand affinities and ligand-receptor hydrophobic interactions are optimized via hydrogen bonding increasing binding affinity and drug efficacy.[220]

Free Energy of Binding is additive:
Formula 1.) $\Delta G_{bind} = \Delta G_{solvent} + \Delta G_{conf} + \Delta G_{int} + \Delta G_{motion}$
where,
$\Delta G_{solvent}$ contribution via solvent, i.e. hydration free energy ΔG_{hyd}.
ΔG_{conf} contribution via conformational changes, i.e. receptor and ligand fee energy changes respectively $\Delta G^r + \Delta G^l$.
ΔG_{int} contribution via proximity, i.e. electrostatic and van der Waals interactions ΔG^{r-l} amongst receptor and ligand.
ΔG_{motion} is the contribution via the motion of receptor and ligand once proximal,
i.e. $\Delta G_{rot} + \Delta G_{t/r} + \Delta G_{vib}$ freezing and internal rotational, translational/rotational and vibrational free energy changes respectively.[46]

Total Energy (REMARK E_total) and Energy subtracting VDWR (REMARK E_without_VDWR) are considered via MedusaDocking Chemical PDB Files when analyzing ligand-receptor binding affinities. (E_without VDWR ameliorates imperfections, i.e. clashing molecular complexes, resulting in a more robust score more closely matching experimentally determined data.)[47] Weak affinities are < -20 kcal mol^{-1} (kcal per mole), < -30 to -50 kcal mol^{-1} exhibit high affinities and > -20 kcal mol^{-1} exhibit poor affinities.[46, 45]

Conclusion

Uncrystallized Pannexin-1 is a highly sophisticated ubiquitously expressed oligomeric transmembrane protein channel regulating homeostasis and physiological diseased states via open or closed gating. Therapeutically controlling Pannexin-1 channel gating may cure dire diseases such as AIDS, be a viable neuropathic pain reliever and is integral to other diseased states, accordingly a Pannexin-1 atomic structural model is needed together with a deep understanding of the interwoven array of Pannexin-1 physiological responses for best Pannexin-1 medicinal purposes.

In silico softwares are today an integral part of medicinal design via the molecular design of proteins coupled to pharmaceutical protein receptor dockings. Various in silico algorithmic protein databases may be combined towards discovering a beginning Pannexin-1 oligomer protein model exhibiting a satisfactory overall structure with reasonably designed medicinal-interacting pivotal domains. This beginning model and its binding partners may be further ameliorated via in silico molecular dynamic simulations, optimal results will control Pannexin-1 gating regulating homeostasis and physiology towards disease prevention.

Chapter 3

Research Method And Design

3.1 Overview and Objectives

3.1.1 Research Overview

A best molecular model of the uncrystallized transmembrane protein Pannexin-1 exhibiting a best overall structure and compact pore region matching experimental data was designed via computer software. In vitro discovered Pannexin-1 channel inhibitor binding events were computationally simulated via a dynamic docking software, these binding/molecular recognition events exhibited overall reasonable energy affinities proving fair pivotal regional accuracy of the Pannexin-1 protein model.

3.1.2 Research Objective

To in silico model a best uncrystallized Pannexin-1 protein exhibiting a fair model accuracy within pivotal domains responsible for its channel closure.

3.1.3 Future Research Objective

Future research will include subsequent refinement of the best host-partner(s), further elucidation of the Pannexin-1 region(s) of interest, key residues, water lattice and the validation of a stealth nano-system that controls Pannexin-1 pore gating. An exhaustive Molecular Dynamic Docking Simulation Assay will exhibit a Pannexin-1 channel pore closure via a host-partner Pannexin-1 pivotal regional binding event. In vitro studies will confirm the former validating a AuNP-conjugate as an effective delivery system and final in vivo studies will confirm Pannexin-1 closure via a stealth delivery nano-system exhibiting final clearance without toxicity.

3.2 Pannexin-1 Modeling, an Overview

Human Pannexin-1's 3-D crystallographic structure is yet to be determined, accordingly building a best Pannexin-1 via in silico 3-D modeling is a best beginning approach. Query sequence Pannexin-1's crystallographic template proteins were discovered via template discovery/alignment algorithms. Template topology consensus and slight hand pairwise matching optimally assisted a best selected multi-template threading alignment approach via

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precisely aligning conserved transmembrane regions and pivotal residues. Template alignments and matching existing crystallographic 3D-structures were subsequently used to MODELLER model an A-chain of several Pannexin-1 alignments. One best Pannexin-1 A-chain subunit's 3-D coordinates were then used to build a best complete symmetric homo-oligomer Pannexin-1 via GalaxyWEB's oligomer prediction server. GalaxyWEB was further used to build a best symmetric similar homo-oligomer of Pannexin-1 via a sequence input. Each complete Pannexin-1 3-D model's pore and overall structure were compared and contrasted towards a best model selection.[85]

3.2.1 Pannexin-1 Template Selection

The template discovery algorithms blastp, PSI-BLAST and HHblits were used to discover the best Pannexin-1 crystallized template proteins, the entire FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN) was input towards this discovery.

MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLISLAFAQEISIGT
QISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGNLPLWLHKFFPYILLLFAILLYLP
PLFWRFAAAPHICSDLKFIMEELDKVYNRAIKAAKSARDLDMRDGACSVPGVTENLGQS
LWEVSESHFKYPIVEQYLKTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFV
CSIKSGILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFRQKTDVL
KVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKVLENIKSSGQGIDPMLLL
TNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQGMNIDSETKANNGEKNARQRLLDS
SC[38]

This template discovery methodology query-ranged the entire Pannexin-1 isoform (1) 1-426 residue sequence to discover best domain templates.

3.2.1.1 NCBI Assay

NCBI blastp Parameters used:

Choose Search Set: Protein Data Bank proteins(pdb) selected towards finding already crystallized protein sets.

Algorithmic Parameters

General Parameters:

Max target sequences: 100

Expect threshold: 10 (chance matches)

Word size: 6 (max)

Scoring Parameters:

Matrix: BLOSUM62

Gap Costs: Existence: 11 Extension: 1

Computational adjustments: Conditional compositional score matrix adjustment

Program: BLASTP 2.9.0

NCBI PSI-BLAST Parameters:

Same as blastp save:

Word size: PSI 3 (max)

PSI/PHI/DELTA BLAST:

PSI-BLAST Threshold: 0.005[111]

Sequence Input:

Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

3.2.1.2 Phyre² HHBlits Assay

Phyre² Parameters Used:

Template Detection: HHpred 1.5.1

Secondary Structure Prediction: Psi-pred 2.5

PSI-Blast 3 Iterations

Disorder Prediction: Disopred 2.4

Transmembrane Prediction: Memsat SVM

Multi-Template Modeling and ab initio: Poing 1.0

Tool: Protein Homology/analogY Recognition Engine V 2.0[134]

Sequence Input:

Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

3.2.2 Pannexin-1 Target-Single Template Alignment Selection

Multiple Biomedical Informatics single template alignment methods (Clustal, Promals3D, Phyre2 and RaptorX/CNFpred) were cross-referenced, these alignments contributed to a final multiple template alignment.

3.2.2.1 Clustal

The basic Clustal pairwise sequence alignment Biomedical Informatics algorithm was firstly run to confirm the best-selected Innexin6 template's remote homology/low identity to Pannexin-1 via a protein library comparison target-template alignment.

Tool Clustal 0(1.2.4)

Clustal Parameter:
CLUSTALW

Sequences Input:

Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Sequence:
>5H1Q:A|PDBID|CHAIN|SEQUENCE
MASQVGAINSVNALISRVFVQPKGDLADRLNSRVTVVILAVSSALLLSSHFIGDPITCWTP
AQFNAQWVNFVNQYCFVHGTYFVPLDQQLAFEEEERTKVSIQYYQWVPYVFALQAFLF
YIPRFIWKAMIAYSGYDLAAAVKYVDRFWSENRDKDDKFKTRLAAFEGRPSVYIWDGIR
LARKKRSRNMALFYTLSTVWQAVNAWIQFYILTQLLDSSIYTLWGPSILGDLLQGNDWQ
TTGHFPRIVHCDFNRRRPASVQLDTVLCVLTLNIYYEKLFIFLWFWLVFVAVVSTVNCFK
WIYYLCNKTKAQKTIKNYLSTAPIKSTISDDQFFSALGEDGLFIMDQMALNLGDIPASYLT
ISMRNICQDFIESEDYIDEERTPFVKSIKHT[44]

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3.2.2.2 Promals3D

Promals3D, a secondary structure predictor towards remote homology, was run via input FASTA Pannexin-1 and Innexin6 sequences, this query-template alignment will exhibit secondary structural alignment features.

Promals3D Parameters used:

identity threshold: 0.6

secondary structure weight: 0.2

amino acid weight: 0.8[117]

Sequences Input:

Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Sequence:

>5H1Q:A|PDBID|CHAIN|SEQUENCE[44]

3.2.2.3 Phyre²

Phyre² Secondary Structural Threading was run to align its best selected templates.

Phyre² Parameters:

(see section: **3.2.1.2 Phyre² HHBlits Assay**)

3.2.2.4 RaptorX/CNFpred

RaptorX Secondary Structural Threading was run to find and align its best selected templates.

RaptorX Parameters Used:

Sequence Input:

Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

3.2.2.5 Consensus Pannexin-1 Structural Threading Alignment

A Methodology Consensus was manually formulated to move towards a best manual multiple

target-template alignment via secondary structural positioning for a target-template expressing a very distant homology i.e. Phyre[2], RaptorX and Promals3D.[105]

3.2.3 Pannexin-1 Orientation and Positioning Selection

A methodology of cross-referencing algorithm prediction servers based upon hydrophobicity, disorder, solvent exposure and existing transmembrane proteins were run and compared to UnitProtKB's Pannexin-1 and Innexin6 algorithmic topological analysis and UnitProtKB's Connexin26 experimental topological analysis. Each topology algorithm ascertains Connexin26/ Innexin6-Pannexin-1 topological similarity amongst themselves and ultimately amongst each-other assisting towards a precise alignments of the four transmembrane regions and topological domains via algorithmic corroboration and consensus. This topological alignment is imperative towards protein transmembrane orientation and positioning modeling via a precisely designed query-multiple template alignment.

3.2.3.1 Modeling Topology

Disordered dynamic hydrophilic coil and loop orientations and ordered membraneous residue positioning in Pannexin-1 and topologically related Connexin26 and best sequence template Innexin6 were methodologically cross-referenced. Connexin26 topology prediction will assist Pannexin-1-Innexin6 alignment via re-entrant residue discovery.

3.2.3.1.1 TMPred

TMPred Topology Prediction Algorithm input Pannexin-1 and Connexin26 topological regional data to be compared against UniProtKB's Pannexin-1 and Connexin26 topological data.

TMPred Parameters:
Output Format: html
Minimum: 14
Maximum: 41
Input Sequence Format: Plain Text[60]

Sequences Input:
Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Connexin26:
>2ZW3:A|PDBID|CHAIN|SEQUENCE[145]

UniProt Pannexin-1:
Topology[110]

UniProt CNX:
Topology[129]

3.2.3.1.2 TMHMM

TMHMM Topology Prediction Algorithm input Pannexin-1 and Connnexin26 topological regional data to be compared against UniProtKB's Pannexin-1 and Connexin26 topological data.

TMHMM Parameters:
Server 2.0
Output Format: Extensive with Graphics[50]

Sequences Input:
Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Connexin26:
>2ZW3:A|PDBID|CHAIN|SEQUENCE[145]

3.2.3.1.3 SOUSI

SOUSI Topology Prediction Algorithm input Pannexin-1 and Connexin26 topological regional data to be compared against UniProtKB's Pannexin-1 and Connexin26 topological data.

SOSUI System: SOSUI engine ver. 1.11[75]

SOUSI Parameters:
Sequences Input:

Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Connexin26:
>2ZW3:A|PDBID|CHAIN|SEQUENCE[145]

3.2.3.1.4 RaptorX

RaptorX Solvent Exposure Prediction Algorithm input Pannexin-1 and Connexin26 topological regional data to be compared against UniProtKB's Pannexin-1 and Connexin26 topological data.

RaptorX System: v1.02[23]

RaptorX Parameters:
Sequences Input:
Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Connexin26:
>2ZW3:A|PDBID|CHAIN|SEQUENCE[145]

3.2.3.1.5 Phyre[2]

Phyre[2] Re-Entrant Helices Prediction Algorithm input Pannexin-1 and Connexin26 topological regional data to be compared against UniProtKB's Pannexin-1 and Connexin26 topological data.

Phyre[2] Parameters Used:
Secondary Structure Prediction: Psi-pred 2.5
Disorder Prediction: Disopred 2.4
Transmembrane Prediction: Memsat SVM
Multi-Template Modeling and Ab Initio: Poing 1.0
Tool: Protein Homology/analogY Recognition Engine V 2.0[134]

Phyre[2]:

Sequences Input:

Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Connexin26:
>2ZW3:A|PDBID|CHAIN|SEQUENCE[145]

3.2.3.1.6 Consensus Topology Determination

Via combining the above topology prediction methods a Pannexin-1 and Connexin26 consensus topology determination methodology was manually created exhibiting Pannexin-1 transmembrane re-entrant helices positions and orientation.

3.2.3.1.7 Structural Threading and Topology Consensus

Via incorporating the former secondary structural alignment consensus method with UniProt Connexin26 topology a Pannexin-1 alignment exhibiting transmembrane helices positioning was predicted, these Pannexin-1 positions will be used towards re-entrant helices alignment.

3.2.3.1.8 Innexin6 Topology

The Innexin6 topology was determined via UniProt sequence algorithmic data and Phyre[2] analysis, this membrane positioning will be used towards a Pannexin-1-Innexin6 transmembrane final multiple template alignment.

UniProt Innexin-6:
Topology[116]

Phyre[2]:
Sequences Input:
Innexin-6 FASTA 389 amino acid sequence provided via UniProtKB-Q9U3N4 (INX6_CAEEL).[82]

57

3.2.4 Pannexin-1 Target-Multiple Template Alignment Selection

The remote Pannexin-1 sequence homology warranted a multiple template approach. Two multiple template modeling approaches were performed and compared for best results, a Clustal multiple template/target approach and a single template/target approach using an Innexin6 structurally threaded top-template.

3.2.4.1 Multiple Template/Target Approach

Pannexin-1 and its six best sequence related templates discovered via the NCBI PSI-BLAST results were FASTA input into CLUSTAL Omega for a multiple template alignment approach.

Tool CLUSTAL 0(1.2.4) Multiple Sequence Alignment[23]

CLUSTAL Parameter:

CLUSTALW

Sequences Input:

Query Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Template Innexin-6, 5H1Q, PDB,

>5H1Q:A|PDBID|CHAIN|SEQUENCE[44]

Template Chain A, 3d Polymerase, 4Y3C_A, PDB,

>pdb|4Y3C|A Chain A, 3d Polymerase

GALERLPDGPRIHVPRKTALRPTVARQVFQPAFAPAVLSKFDPRTDADVDEVAFSKHTSN
QETLPPVFRMVAREYANRVFALLGRDNGRLSVKQALDGLEGMDPMDKNTSPGLPYTTL
GMRRTDVVDWETATLIPFAAERLEKMNNKDFSDIVYQTFLKDELRPIEKVQAAKTRIVD
VPPFEHCILGRQLLGKFASKFQTQPGLELGSAIGCDPDVHWTAFGVAMQGFERVYDVDY
SNFDSTHSVAMFRLLAEEFFSEENGFDPLVKDYLESLAISKHAYEEKRYLITGGLPSGCAA
TSMLNTVMNNIIIRAGLYLTYKNFEFDDVKVLSYGDDLLVATNYQLNFDRVRTSLAKTG
YKITPANKTSTFPLESTLEDVVFLKRKFKKEGPLYRPVMNREALEAMLSYYRPGTLSEKL
TSITMLAVHSGKQEYDRLFAPFREVGVIVPTFESVEYRWRSLFW[128]

Template Chain A, Large Tail Fiber Protein P34, 4UXF_A, PDB,

>pdb|4UXF|A Chain A, Large Tail Fiber Protein P34

MGSSHHHHHHSQDPSQFIRRDIAQTVNGSLTLTQQTNLSAPLVSSSTGEFGGSLAANRTF
TIRNTGAPTSIVFEKGPASGANPAQSMSIRVWGNQFGGGSDTTRSTVFEVGDDTSHHFYS
QRNKDGNIAFNINGTVMPININASGLMNVNGTATFGRSVTANGEFISKSANAFRAINGDY
GFFIRNDASNTYFLLTAAGDQTGGFNGLRPLLINNQSGQITIGEGLIIAKGVTINSGGLTVN
SRIRSQGTKTSDLYTRAPTSDTVGFWSIDINDSATYNQFPGYFKMVEKTNEVTGLPYLER
GEEVKSPGTLTQFGNTLDSLYQDWITYPTTPEARTTRWTRTWQKTKNSWSSFVQVFDGG
NPPQPSDIGALPSDNATMGNLTIRDFLRIGNVRIVPDPVNKTVKFEWVE[127]

Template Chain A, Long-tail Fiber Proximal Subunit, 5NXH_A, PDB,

>pdb|5NXH|A Chain A, Long-tail Fiber Proximal Subunit

SGLVESGTLWDHYTLNILEANETQRGTLRVATQVEAAAGTLDNVLITPKKLLGTKSTEAQ
EGVIKVATQSETVTGTSANTAVSPKNLKWIAQSEPTWAATTAIRGFVKTSSGSITFVGNDT
VGSTQDLELYEKNSYAVSPYELNRVLANYLPLKAKAADTNLLDGLDSSQFIRRDIAQTVN
GSLTLTQQTNLSAPLVSSSTGEFGGSLAANRTFTIRNTGAPTSIVFEKGPASGANPAQSMSI
RVWGNQFGGGSDTTRSTVFEVGDDTSHHFYSQRNKDGNIAFNINGTVMPININASGLMN
VNGTATFGRSVTANGEFISKSANAFRAINGDYGFFIRNDASNTYFLLTAAGDQTGGFNGL
RPLLINNQSGQITIGEGLIIAKGVTINSGGLTVNSRIRSQGTKTSDLYTRAPTSDTVGFWSID
INDSATYNQFPGYFKMVEKTNEVTGLPYLERGEEVKSPGTLTQFGNTLDSLYQDWITYPT
TPEARTTRWTRTWQKTKNSWSSFVQVFDGGNPPQPSDIGALPSDNATMGNLTIRDFLRIG
NVRIVPDPVNKTVKFEWVE[119]

Template Chain A, DNA polymerase lambda, 1NZP_A, PDB,

>pdb|1NZP|A Chain A, DNA polymerase lambda

MAQPSSQKATNHNLHITEKLEVLAKAYSVQGDKWRALGYAKAINALKSFHKPVTSYQE
ACSIPGIGKRMAEKIIEILESGHLRKLDH[114]

Template Chain A, Predicted amidohydrolase, dihydroorotase family, 3CJP_A, PDB,

>pdb|3CJP|A Chain A, Predicted amidohydrolase, dihydroorotase family

MSLIIDGHTHVILPVEKHIKIMDEAGVDKTILFSTSIHPETAVNLRDVKKEMKKLNDVVN
GKTNSMIDVRRNSIKELTNVIQAYPSRYVGFGNVPVGLSENDTNSYIEENIVNNKLVGIGE

59

LTPASGQIKSLKPIFKYSMDSGSLPIWIHAFNPLVLQDIKEIAELCKAFPKVPVILGHMGGN
WMTAVELAKEIQNLYLDTSAYFSTFVLKIVINELPLKCIFGTDMPFGDLQLSIEAIKKMSN
DSYVANAVLGDNISRLLNIEGHHHHHH[113]

3.2.4.2 Single Template/Target Approach

Several slightly adjusted multiple template/target alignments were modeled via the best selected templates and corresponding alignments discovered via Phyre[2] and NCBI PSI-BLAST. The best of these manual input single alnfile alignments used the two Phyre[2] discovered templates (5H1Q, 5H5P) and three of the NCBI PSI-BLAST discovered templates (1NZP, 4UXF, 4Y2C). Conserved transmembrane regions, re-entrant membrane positions and pivotal residue constraints were alignment preserved, consensus contributed to alignments. The Phyre[2] Pannexin-1-Innexin6 threaded alignment (Table 27) remained the top-template and manual adjustments were slight towards maintaining alignment integrity.

MA gapped not maintaining WT N-terminal pore-closure.

Y10 predicted phosphorylation-site S residue neutrally aligned.

C40 WT channel closure aligned moderately via a C-R hydrophobic/polar-charged residue alignment preventing channel leak.

Inner membrane S polar residue constraint maintained, hydrophobic core and ss constraint maintained in TM.

F54 TM1 binding residue of C426 towards C-terminal auto-inhibiton aligned via a F-L conservative hydrophobic-hydrophobic alignment.

Leu (L) more exhibited than Val and Ile, expected in alpha helices (L=25% vs. V and I total contribution=25%).[210]

Positive-inside rule towards alpha helices TM regions and tail topology, N-tail exhibits 12% + charged R and K residues determining an 'In Topology'.[210]

```
                [N-Terminal-Tail-IV=~8%----------------][TM1----------IV=~14%]
PANNEXIN 1(1)MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGIPLLLISLAFAQEISIG (61)
INNEXIN-6 (7)-----AINSVNALISRVFVQPK-------GDLADRLNSRVTVVI-LAVSSALILSSHF-- (53)
                *.::*..:....::..       ..::*::.:::.*:: *:::*:::::::::
                [N-Terminal-Tail-----------------------][TM1--------------
                #
```

#(Micro-Constraints, 100% homology via BLAST_Non-Redundant Protein Sequences (nr) homology)
@(Macro-Constraints, 20% homology via Phyre2_PSI-Blast Pseudo-Multiple Sequence Alignment)

The Pannexin-1 aligned N-Tail coil exhibited expected polar residue end-terminals while TM1 exhibited conserved hydrophobic entrant residues distinguishing N-Tail-TM1 residue boundary positioning.

Polar/Charged Residues (RKDEQNHSTYC)
Hydrophobic Residues (AILMFVPG)
Hydrophobic Residues Center Peaks (AIVL)
Amphipathic Residues Interface Residues (WYM)

(N-Tail)...AVDKMVTC-IAVGLPLL...(TM1)[158]

TM1 ~81% hydrophobic residues (IAVGLPLLLISLAFAQEISIG) with central membrane peaks of (AIVL) ~80%, zero Panx1 TM gaps, and an energetically favorable polar S-residue residing within alpha-helical inner-membrane cores.[100]

TM1-EL1 Boundary exhibits proper hydrophobic-hydrophilic boundary IG-TQ.

Residue constraints near pivotal functional residues aligned, functional residue constraints towards channel gating (W-A, R-Q and 67-86) aligned, structural residue constraints C-C aligned preserving disulfide bridges towards possible channel activity modulation.

Amphipathic Tryptophan W74 acting as a Polarity Switch towards open channel WT Panx1 CBX blocking within conserved region 67-86 is aligned satisfactorily via hydrophobic-hydrophobic A-W alignment.

EL1 R75 binds extracellular ATP inhibiting WT open Panx1, R is polar and aligned to a charged residue Q satisfactorily.

Conserved region towards gating 67-86 aligned well, Identity Value 30% with a high ratio of residue with similar chemical properties and zero gaps.

```
              [EL1---------------------------------IV=21%----------]
PANNEXIN-1(62)TQISCFSPSSFSWRQAAFVDSYCWAA----VQQKNSLQSES---GNLPLWLH(106)
INNEXIN-6 (54)DPITCWTPAQFNAQWVNFVNQYCFVHGTYFVPLDQQLAFEEEERTKVSIQYY(105)
              ::*:*::*.:*::.::.**::**:.    *..:::*..*:   .:.:.::.
              --][EL1------------------------------------------
              #                       ####   #      ##
                  @                    @                   @@
```

Positively charged R and K charged residues and Trp (W) and Tyr (Y) exhibited at boundaries of TMs.[100, 211]

The Pannexin-1 aligned First Extracellular Loop moderately but satisfactorily (considering the loop nature of glycine and proline and amphipathic nature of tryptophan) exhibited expected % charged/polar and Trp (W) interface residues importantly distinguishing proper TM1-EL1-TM2 residue boundary positioning.

Hydrophobic Residues Center Peaks (AIVL)
Amphipathic Residues Interface Residues (WYM)

EL1 ~56% (TQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGNLPLWLH)[158]

Structural ss residues constraints in TM maintained, functional predicted
phosphorylation site Y150 residue constraint maintained along with surrounding
residue constraints.


```
              [TM2—IV=38%----------][IL1------------------
PANNEXIN1(107)KFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY(150)
INNEXIN-6(106)QWVPYVFALQAFLFYIPRFIWKAMIAYS-GYDLAAAVKYVDRF (148)
              :::**:::*.*:*:*:*:*.::*::::*.: .:**.:::::*::
              ----][TM2----------------][IL1------------
              #    #############
              @@     @         @     @
                                                   *
 1NZP_A(279)                                      [Y(279)
```

TM2 exhibited zero gaps with a high IV of 38%.
TM2 highly exhibited expected hydrophobic residues ~84% (KFFPYILLLFAILLYLPPLFW)
with central membrane peaks of (AIVL) ~89% with zero polar central residues and an
expected Trp (W) interface residue.

Y199 verified phosphorylation site aligned via R polar residue alignment,
surrounding residue constraints aligned.


```
              ----------------------------------------------------
PANNEXIN1(151)NRAIKAAKS-ARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYLKTKKNS(206)
INNEXIN6 (169)                              PSVYIWDGIRLARKKRS(185)
                                            -----------------
              ##   #   # # ##  #   ###
                                                   *       *  ** *
               ** * **      *** **        *   **
 1NZP_A(280)  AKAINALKSFHKPVTSYQEACSIPGIGKRMAEKIIEILES(319)
              ------------IV=34%--------------------]
```

Pivotal residue constraint towards blocking the WT open Panx1 via Probenecid L237
is conserved via alignment L-S (this hydrophobic-polar alignment functions since a
serine (or T) R-group exhibits a hydroxyl group contributing H-bonds to the
carbonyl oxygen of the preceding turn of the helix allowing polar S within a
hydrophobic milieu).[212] Functional surrounding residue constraints aligned.

ss conserved TM structural residue constraints aligned, central polar residue
constraints C and Y and surrounding residue constraints aligned.

The Pannexin-1 aligned TM2-IL1-TM3 nicely exhibited re-entrant residue positioning
via the two interface residues W and Y and transmembrane-IL1 boundary hydrophobic
to hydrophilic residue positioning respectively

(TM2)...LYLPPLFW-RFAAAPHI...(IL1)...IIKYISCF-LLTLIIIL...(TM3)[158]

```
                ----IV=13%][---TM3-IV=~10%------]
PANNEXIN1(207)NNLIIKYISCRLLTLIIILLACIYLGYYFSLS(238)
INNEXIN-6(186)RNMALFYTLSTVWQAVNAWIQFYILTQLLDSS(217)
              :*:::.*...::::::::.:::..::*.:::::*
                ---][---TM3-------------][-EL2--
                             #
```

TM3 exhibited zero alignment gaps and highly exhibited an expected hydrophobic residue % and central inner-membraneous conserved polar residues importantly distinguishing proper TM residue positioning.

TM3 ~73% (LLTLIIILLACIYLGYYFSLS) with central membrane peaks of (AIVL) ~71% with one central polar C residue.

EL2 N255S aligned via chemical similarity to preserve asparagine glycosylation site i.e. polar residue alignment.

EL2-L237, L240, S247, K266 are pivotal residues towards a Probenecid and Food Dye blocking of a WT Panx1 open state, well aligned via similar residues.

Conserved Cysteine C246 and C265 in EL2 aligned to preserve disulfide bridges towards possible channel activity modulation.

```
            [----------------------------EL2------IV=10%---------]
PANNEXIN1(239)SL------------------SDEF----VCSIKSGILRNDSTVP-DQFQC--K(266)
INNEXIN6(218) IYTLWGPSILGDLLQGNDWQTTGHFPRIVHCDFNRRRPAS---VQLDTVLCVLT(268)
              .:                  ::.*   .*::::.:  *. *::.*  :
            ----------------------------EL2---------------------
                                              @        @
```

The Pannexin-1 aligned Second Extracellular Loop moderately but satisfactorily (considering the loop nature of glycine and proline) exhibited expected charged and polar residues distinguishing proper TM3-EL2-TM4 residue boundary positioning.

EL2 ~64% (SLSDEFVCSIKSGILRNDSTVPDQFQCK)[158]

```
            [---TM4---IV=26%------]
PANNEXIN1(267)L-I-AVGIFQLLSVINLVVYVLL(287)
INNEXIN6(269) LNIYYEKLFIFLWFWIVFVAVVS(291)
              * * :..:*.:*:::.::*:*:.
              --EL2-][--TM4-Phyre2---
                           @
```

Zero TM4 alignment gaps were exhibited within conserved pivotal transmembrane regions, ss TM4 residue constraints maintained, polar S, N inner membrane residue constraint maintained.

Aligned Pannexin-1 conserved TM4 region ratio highly exhibited expected hydrophobic
residues and central inner-membraneous conserved polar residues importantly
distinguishing proper EL2-TM4-C-Tail residue positioning.

TM4 ~81% (LIAVGIFQLLSVINLVVYVLL) with central membrane peaks of (AIVL) ~71% with
two polar central residues S and N.


```
                          [-------------------------C-Tail-------------------------
                             [R300-D379-----5_HIGHLY_CONSERVED_SEGMENTS(HCS)
                                             [S329-(LRR_HCS2)---
PANNEXIN1(288)APVVVYTLFVPFRQKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLF-----LEENISE(341)
INNEXIN-6(292)TVNCFKWIYYLCN(304)
              ::.::::::::::
              ----][C-Tail-
                   #                                            #
             @
                      *.*:..::*:.:**.**..*:::..:**:..**.*     :.*::.*
4UXF_A (1127)         QGTKTSDLYTRAPTSDTVGFWSIDINDSATYNQFPGYFKMVEKTNE(1172)
                      [-------------NCBIPSI_BLAST---IV=33%---------
```

Y309 verified phosphorylation site aligned via Y-Y alignment, surrounding residue
constraints maintained.

```
                   -------------------EXHIBITING_LRRs-----D379]aligned

                   ---K349]aligned

              --------------------------------------------------
PANNEXIN1(342)VKSYKCLKVLENIKSSGQGIDPM-------LLLTNLGMIKMDVVDGKT (382)
                                                          #    #
              *::.::*::.:*::**.*
4UXF_A(1173)  VTGLPYLERGEEVKSPG(1189)
              ----------------]

4Y2C    (100)              EGMDPMDKNTSPGLPYTTLGMRRTDVVDWET (130)
              :*:***         *::*:***.::****::*
              [---------------------------
```

Y345 predicted phosphorylation-Site Y-L residue aligned.

C347WT_Channel_Closed_aligned conservatively C-Y via a hydrophobic-hydrophobic
residue alignment to prevent channel leak.

376DVVD379 Caspase Cleavage Site for Caspase 3 or 6 at cleavage site D379
aligned.

```
Caspase cleavage regulatory region residues K381, T382, A386 aligned.

Surrounding residue constraints aligned.

                -----C-Tail---IV=5%--------------------------------]
PANNEXIN1(383)----PMSAEMREEQGNQTAELQGMNIDSETKANNGEKNAR----QRLLDSSC(426) 213
4Y2C     (131)ATLIPFAAERLEKMNNK                        (147)
                --IV=40%--------]
                    *::**..*:.:*:
                            .:..:.::..*
4UXF_A(1245)                [PSDIGALPSD](1254)

5H5P    (482)                         SETTANNMRKKGKPNPDQR------(500)
                                      [-----IV=47%-------------]
                                      ***:*** :*:::     **
            #### # ###     #  ######  ####             ###


Surrounding residue constraints maintained.

Cysteine unmatched:

C426 binds TM1 F54 towards C-auto-inhibition, caspase cleavage releases this
binding.
```

Table 15.) A multiple template alignment via two Phyre^2 and three NCBI PSI-BLAST single template/target alignments.[229, 231, 84]

# indicates constraints ascertained via interspecies Pannexin-1 homologs exhibiting high 99% homologies.[222]

@ indicates constraints ascertained via distant homologs exhibiting <20% homologies.[223]

```
⬇ Download ˅    GenPept  Graphics                                       ▼ Next ▲ Previous ◄Descriptions

Chain A, DNA polymerase lambda [Homo sapiens]
Sequence ID: 1NZP_A  Length: 87  Number of Matches: 1

Range 1: 39 to 79 GenPept  Graphics            ▼ Next Match ▲ Previous Match    Related Information
                                                                                Structure - 3D structure displays
Score         Expect  Method                   Identities   Positives   Gaps
28.9 bits(63) 3.6     Composition-based stats. 14/41(34%)   24/41(58%)  1/41(2%)

Query  150  YNRAIKAAKS-ARDLDMRDGACSVPGVTENLGQSLWEVSES  189
            Y +AI A KS  + +    ACS+PG+ + + + + E+ ES
Sbjct   39  YAKAINALKSFHKPVTSYQEACSIPGIGKRMAEKIIEILES  79
```

Table 16.) NCBI PSI-BLAST 1NZP_A selected template-target alignment.[84]

Chain A, Large Tail Fiber Protein P34 [Escherichia virus T4]
Sequence ID: 4UXF_A   Length: 410   Number of Matches: 1
See 14 more title(s) ⌄

Range 1: 248 to 310 GenPept   Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
32.0 bits(71)	1.7	Compositional matrix adjust.	21/63(33%)	28/63(44%)	5/63(7%)

```
Query  301  QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLF-----LEENISEVKSYKCLKVLENIK  355
            Q T   +Y   PT D + F S   ND + YN F     + E  +EV    L+  E +K
Sbjct  248  QGTKTSDLYTRAPTSDTVGFWSIDINDSATYNQFPGYFKMVEKTNEVTGLPYLERGEEVK  307

Query  356  SSG  358
            S G
Sbjct  308  SPG  310
```

Related Information
Structure - 3D structure displays
Identical Proteins - Identical proteins to 4UXF_A

Table 17.) NCBI PSI-BLAST 4UXF_A selected template-target alignment.[84]

Chain A, Genome Polyprotein [Mengo virus]
Sequence ID: 4Y2C_A   Length: 460   Number of Matches: 1

Range 1: 94 to 147 GenPept   Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
32.0 bits(71)	1.5	Compositional matrix adjust.	19/54(35%)	27/54(50%)	4/54(7%)

```
Query  346  KCLKVLENIKSSGQGIDPMLLLTNLGMIKMDVVDGKT----PMSAEMREEQGNQ  395
            + L  LE +    +   P L  T LGM + DVVD +T    P +AE  E+  N+
Sbjct   94  QALDGLEGMDPMDKNTSPGLPYTTLGMRRTDVVDWETATLIPFAAERLEKMNNK  147
```

Related Information
Structure - 3D structure displays

Table 18.) NCBI PSI-BLAST 4Y2C_A selected template-target alignment.[84]

Table 19.) Phyre[2] c5h5pA selected template-target alignment.[86]

3.2.5 Pannexin-1 Molecular Modeling Methodology

MODELLER A-chain subunits were generated via the two former multiple template alignments with various A-chains generated via slight adjustments to the former Table 15 alignment. Single template alignments using Connexin26 and Innexin6 and a multiple template alignment using Connexin26-Innexin6 were similarly A-chain generated. All Coordinate Files were abstracted from the PDB (Connexin26 crystallized state seems to be in an open conformation, Innexin6 crystallized state open or closed state coordinates undetermined).[179,218] GalaxyWeb subsequently oligomerized various pentamers, hexamers, heptamers and octamers of the best generated MODELLER A-chain monomer. These same oligomerizations were similarly GalaxyWeb generated via a Pannexin-1 FASTA sequence input methodology.

3.2.5.1 Multiple Template/Target MODELLER A-Chain

MODELLER modeling of an A-chain Pannexin-1 via a Clustal multiple 6-template alignment and respective PDB-3-D coordinate files.

Tool MODELLER Specific Version Number: 9v15 Installed

Graphic User Interface-None

Database Used-None

Default Parameters all used except number of models generated were 10 instead of one.

```python
# Step 4: model building
#
# This script should produce two models, 1fdx_my.B99990001.pdb and
# 1fdx_my.B99990002.pdb.

from modeller import *
from modeller.automodel import *     # Load the automodel class

log.verbose()
env = environ()  # To get different models from another script

# directories for input atom files
env.io.atom_files_directory = ['./']

a = automodel(env,
              alnfile='align3.ali',       # alignment filename
              knowns=('5h1q', '1nzp', '4uxf', '5nxh', '3cjp', '4y3c'),   # codes
              sequence='Panx1')           # code of the target
a.starting_model= 1                     # index of the first model
a.ending_model  = 10                     # index of the last model
                                        # (determines how many models to calculate)

a.make()                                # do homology modelling
```

Table 20.) MODELLER script used to generate 10 Pannexin-1 models via the align3.ali file noted in results section 4.4.1 Clustal Multiple Template/Target Alignment File and the six-input known 3-D coordinate files (5h1q, 1nzp, 4uxf, 5nxh, 3cjp, 4y3c), Panx1 remains the sequence.[149]

3.2.5.2 Single Template/Target MODELLER A-Chain

MODELLER modeling of an A-chain Pannexin-1 via a single template/target multiple 5-template alignment and respective PDB-3-D coordinate files.

```
# Step 4: model building
#
# This script should produce two models, 1fdx_my.B99990001.pdb and
# 1fdx_my.B99990002.pdb.

from modeller import *
from modeller.automodel import *    # Load the automodel class

log.verbose()
env = environ()  # To get different models from another script

# directories for input atom files
env.io.atom_files_directory = ['./']

a = automodel(env,
              alnfile='align2.ali',        # alignment filename
              knowns=('5H1Q', '1NZP', '4UXF', '4Y2C', '5H5P'),    # codes of the templates
              sequence='Pannexin-1')           # code of the target
a.starting_model= 1                   # index of the first model
a.ending_model  = 10                  # index of the last model
                                      # (determines how many models to calculate)

a.make()                              # do homology modelling
```

Table 21.) MODELLER script used to generate 10 Pannexin-1 models via the align2.ali file noted in results section 4.4.2 Single Template/Target Alignment File and the five-input known 3-D coordinate files (5h1q, 1nzp, 4uxf, 4y2c, 5h5p), Panx1 remains the sequence.[149] (Several other A-chain models were generated via a slightly adjusted align2.ali file.)

3.2.5.3 GalaxyWeb Monomer Input

The multiple template align2.ali file MODELLER generated Panx1 subunit A-chain was subsequently multi-mer modeled via GalaxyWEB's GalaxyHomomer protein homo-oligomer software via five-default setting, 5, 6, 7, and 8 oligomeric state inputs. A default setting and multiple per-mutated ab initio loop/termini refinements were selected and input for each oligomeric state.

Loops or Termini to be Refined (5aa<=len<=20aa, Optional)
can be used only when monomer structure (PDB file) is provided.

Region 1	Start 1	, End 19
Region 2	Start 67	, End 85
Region 3	Start 407	, End 426

Table 22.) Tails (1-19, 407-426) and gating regulating EL1 (67-85) refinement remodeling selected and input.[78]

Python program

MySQL database

GalaxyHomomer Parameters (Coordinate-File Approach):

Inputs:

A-Chain Coordinate Files:

Pannexin-1.B99990001.pdb

Default Setting, 5, 6, 7, 8-Oligomeric State[78]

3.2.5.4 GalaxyWeb Sequence Input

Five-default setting, 5, 6, 7 and 8-oligomeric states and a Panx1 FASTA amino-acid sequence were input into GalaxyWEB's GalaxyHomomer protein homo-oligomer tool.

GalaxyHomomer Parameters (FASTA Approach):

Inputs:

Pannexin-1 FASTA 426 amino acid sequence provided via UniProtKB-Q96RD7-1 (PANX1_HUMAN).[38]

Default Setting, 5, 6, 7, 8-Oligomeric State[78]

3.3 Ligand-Target Docking Dynamic Simulation Method

The discovered four inhibitor chemicals and one mimetic peptide were Medusa Guide-Docked into their in vitro experimental resulting determined Pannexin-1 EL-1, EL-2 and hexamer pore regions, affinities were subsequently determined. This MedusaDock local assay was restricted to the spatial coordinates of the putative binding regions of the best selected hexamer sequence model, all dockings were shape complementary and restricted to <10 rotatable ligand-bonds for best prediction results.[64]

3.3.1 [10]Panx1-Pore Docking Method

The Mimetic [10]Panx1 was Guide-Docked to the Pannexin-1 hexamer pore region.

Tool MedusaDock v2.0

MedusaDock Parameters:

Input:

1.) Select receptor (Pannexin-1 hexamer sequence model uploaded)

2.) Select ligand ([10]Panx1 uploaded)

3.) Binding site set to Pannexin-1 pore-residues S93, Q95, S96 and E97

4.) Constraints set-energy guide docked (no restraints selected); used all default parameters.

5.) 1-10 Rounds Submitted[186]

3.3.2 Target-EL1 Docking Method

All four Pannexin-1 EL-1 binding chemicals (CBX, Probenecid, Brilliant Blue FCF [BB FCF] and FD&C Green No. 3) were guide-docked to the Pannexin-1 EL1 67-86 region.

MedusaDock Parameters:

Input:

1.) Select receptor (Pannexin-1 hexamer sequence model uploaded)

2.) Select ligand (CBX, Probenecid, Brilliant Blue FCF [BB FCF] and FD&C Green No. 3 uploaded)

3.) Binding site set to Pannexin-1 EL1 67-86 region

4.) Constraints set-energy guide docked (no restraints selected); used all default parameters.

5.) 1-10 Rounds Submitted[186]

3.3.3 Target-EL2 Docking Method

Discovered Pannexin-1 EL-2 binding chemicals (Probenecid, Brilliant Blue FCF [BB FCF] and FD&C Green No. 3) were guide-docked to the Pannexin-1 EL2 (237 (L), 240 (L), 247 (S), 266 (K)) targeted regions.

MedusaDock Parameters:

Input:

1.) Select receptor (Pannexin-1 hexamer sequence model uploaded)

2.) Select ligand (Probenecid, Brilliant Blue FCF [BB FCF] and FD&C Green No. 3 uploaded)

3.) Binding site set to Pannexin-1 EL2 (237 (L), 240 (L), 247 (S), 266 (K)) regions

4.) Constraints set-energy guide docked (no restraints selected); used all default parameters.

5.) 1-10 Rounds Submitted[186]

Chapter 4

Results

4.1 Pannexin-1 Templates Selected

NCBI blastp results indicated homolog 5H1Q and 3CJP to be best selected templates. Innexin6 exhibits a best query coverage area with a strong E-value, however, its percent identity value is below 30% inferring a multiple template alignment approach. Although 3CJP exhibits an unfavorable E-value its query-coverage is useful towards a multiple template alignment.

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Chain A, Innexin-6 [Caenorhabditis elegans]	42.4	42.4	26%	9e-04	26.45%	5H1Q_A
☑	Chain A, Predicted amidohydrolase, dihydroorotase family [Clostridium acetobutylicum ATCC 824]	29.3	29.3	3%	9.4	52.94%	3CJP_A

Table 23.) NCBI blastp results.[125]

PSI-BLAST results exhibited a run-iteration of 1 with a max 500 (one iteration was selected via default, a second iteration generated an alignment limited PSSM error i.e. not enough alignments left towards a subsequent iteration).

	Description	Max score	Total score	Query cover	E value	Per. Ident	Accession	Select for PSI blast	Used to build PSSM	Newly added
☑	Chain A, Genome Polyprotein [Mengo virus]	32.0	32.0	11%	1.5	35.19%	4Y2C_A	☐		
☑	Chain A, Genome Polyprotein [Mengo virus]	32.0	32.0	11%	1.5	35.19%	4NYZ_A	☐		
☑	Chain A, 3d Polymerase [Mengo virus]	32.0	32.0	11%	1.5	35.19%	4Y3C_A	☐		
☑	Chain A, Genome Polyprotein [Mengo virus]	32.0	32.0	11%	1.6	35.19%	4NZ0_A	☐		
☑	Chain A, Large Tail Fiber Protein P34 [Escherichia virus T4]	32.0	32.0	13%	1.7	33.33%	4UXF_A	☐		
☑	Chain A, Long-tail Fiber Proximal Subunit [Escherichia virus T4]	31.6	31.6	13%	2.4	33.33%	5NXH_A	☐		
☑	Chain A, DNA polymerase lambda [Homo sapiens]	28.9	28.9	9%	3.6	34.15%	1NZP_A	☐		
☑	Chain A, Large Tail Fiber Protein P34 [Escherichia virus T4]	30.8	30.8	13%	3.9	33.33%	4UXE_A	☐		
☑	Chain A, Long-tail Fiber Proximal Subunit [Escherichia virus T4]	30.4	30.4	13%	4.7	33.33%	5NXF_A	☐		
☑	Chain A, Predicted amidohydrolase, dihydroorotase family [Clostridium acetobutylicum ATCC 824]	29.3	29.3	3%	9.4	52.94%	3CJP_A	☐		

Table 24.) Above PSI-BLAST Pannexin-1 discovered templates exhibiting high E-values albeit apropos towards a Pannexin-1 multiple template alignment approach.[144]

Phyre[2] similarly returned a best Innexin6 template with a high Confidence of 99.9 and satisfactory % i.d. of 21, moreover seventy-one less confident templates were discovered via Phyre[2] including the low confidence scored 5.4 albeit high 60% i.d. c5h5pA to be used towards a multiple template alignment.
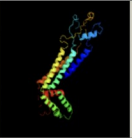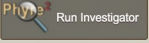
#ⓘ	Template	Alignment Coverage	3D Model	Confidence ⓘ	% i.d.	Template Information
1	c5h1qC	Alignment		99.9	21	**PDB header:**transport protein **Chain:** C: **PDB Molecule:**innexin-6; **PDBTitle:** c. elegans inx-6 gap junction hemichannel Phyre² Run Investigator
66	c5h5pA	Alignment	not modelled	5.4	60	**PDB header:**transcription **Chain:** A: **PDB Molecule:**myelin regulatory factor; **PDBTitle:** crystal structure of myelin-gene regulatory factor dna binding domain

Table 25.) Phyre[2] HH results exhibiting highly confident c5h1qc Innexin6 and c5h5pA to be used towards a Pannexin-1 top template and C-tail multiple template alignment respectively.[97]

4.2 Pannexin-1 Single Target-Template Alignments

Multiple Biomedical Informatics produced the following alignment results to be used towards a multiple template alignment approach.

4.2.1 Clustal

Clustal Innexin6-Pannexin-1 alignment exhibited a BLAST suspected low identity of ~17% (sequence similarities should exhibit >25% identity ratios to avoid substantial alignment errors and lack of template hits[148]).

Table 26.) Clustal Innexin6-Pannexin-1 alignment results (* = Identity Match).[120] Substantial gaps were exhibited within conserved 1st, 3rd and 4th conserved transmembrane regions (yellow-highlights) and pivotal residue Y309 and D379 verified phosphorylation and caspase cleavage sites (orange highlights) respectively.

```
CLUSTAL O(1.2.4) multiple sequence alignment


sp|Q96RD7|PANX1_HUMAN          MA--IAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLISLAFAQEI    58
5H1Q:A|PDBID|CHAIN|SEQUENCE    MASQVGAINSVNALISRVFVQPKGDLAD----RLNSRVTVVILAVS------SALLLSSH    50
                               **  :. : :  .: . :: :*.     .    .*    *. :**.       *  : ..

sp|Q96RD7|PANX1_HUMAN          SIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNS-------LQSESGNLPLWLHKFFPY   111
5H1Q:A|PDBID|CHAIN|SEQUENCE    FIGDPITCWTPAQFNAQWVNFVNQYCFVHGTYFVPLDQQLAFEEEERTKVSIQYYQWVPY   110
                                **   *:*::*:.*. :  . **:.**:.              :.*   :: :  :::.**

sp|Q96RD7|PANX1_HUMAN          ILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVYNRAIKAAKSARDLDMRDGACS   171
5H1Q:A|PDBID|CHAIN|SEQUENCE    VFALQAFLFYIPRFIWKAMIAYSG-YDLAAAVKYVDRFWSENRD-----KDDKFKTRLAA   164
                               :: * *:*:*:* ::*:   *      **   :: :*:.:..   .    :* .::   .:

sp|Q96RD7|PANX1_HUMAN          VPGVTENLGQSLWEVSESHFKYPIVEQYLKTKKNSNNLIIKYISCRLLTLIIILLACIYL   231
5H1Q:A|PDBID|CHAIN|SEQUENCE    FEG---RPSVYIWDGI-----------RLARKKRSRNMALFYTLSTVWQAV----NAWI   205
                               . *   .. . :*:            * **.*.*: : *  . :   :      ::

sp|Q96RD7|PANX1_HUMAN          GYYFSLSSLSDE-----FVCSIKSGIL-----RNDSTVPDQFQCKLIAV-----GIFQLL   276
5H1Q:A|PDBID|CHAIN|SEQUENCE    QFYIL-TQLLDSSIYTLWGPSILGDLLQGNDWQTTGHFPRIVHCDFNRRRPASVQLDTVL   264
                               :*:  :.* *.    :  ** ..:*    :. . .*  .:*.:        :  :*

sp|Q96RD7|PANX1_HUMAN          SVINLVVYVLLAP--VVVYTLFVPFRQKTDVLKVEILPTFDVLHFKSEGYNDLSLYNLF   334
5H1Q:A|PDBID|CHAIN|SEQUENCE    CVLTLNIYYEKLFIFLWFWLVFVAVVSTVNC---------FKWIYYLCNKT---------   306
                               .*:.* :*       : .: :** . ...:        *. ::: .:

sp|Q96RD7|PANX1_HUMAN          LEENISEVKSYKCLKVLEN-----IKSSGQGIDPMLLLTN----LGMI------------   373
5H1Q:A|PDBID|CHAIN|SEQUENCE    --KAQKTIKNYLSTAPIKSTISDDQFFSALGEDGLFIMDQMALNLGDIPASYLTISMRNI   364
                               :  . :*.* .   ::.       *. * * :::: :    ** *

sp|Q96RD7|PANX1_HUMAN          KMDVV-------DGKTPMSAEMREEQGNQTAELQGMNIDSETKA------NNGEKNARQR   420
5H1Q:A|PDBID|CHAIN|SEQUENCE    CQDFIESEDYIDEERTPFVKSIKHTHQA-PDBIDCHAINSEQUENCEMASQVGAINSVNA   423
                               *.:         : :**: .::. :      ::   *:**       : *  *: :

sp|Q96RD7|PANX1_HUMAN          LLDSS-------------------------------------C---------------   426
5H1Q:A|PDBID|CHAIN|SEQUENCE    LISRVFVQPKGDLADRLNSRVTVVILAVSSALLLSSHFIGDPITCWTPAQFNAQWVNFVN   483
                               *:.                                       *

sp|Q96RD7|PANX1_HUMAN          ------------------------------------------------------------   426
5H1Q:A|PDBID|CHAIN|SEQUENCE    QYCFVHGTYFVPLDQQLAFEEEERTKVSIQYYQWVPYVFALQAFLFYIPRFIWKAMIAYS   543


sp|Q96RD7|PANX1_HUMAN          ------------------------------------------------------------   426
5H1Q:A|PDBID|CHAIN|SEQUENCE    GYDLAAAVKYVDRFWSENRDKDDKFKTRLAAFEGRPSVYIWDGIRLARKKRSRNMALFYT   603


sp|Q96RD7|PANX1_HUMAN          ------------------------------------------------------------   426
5H1Q:A|PDBID|CHAIN|SEQUENCE    LSTVWQAVNAWIQFYILTQLLDSSIYTLWGPSILGDLLQGNDWQTTGHFPRIVHCDFNRR   663


sp|Q96RD7|PANX1_HUMAN          ------------------------------------------------------------   426
5H1Q:A|PDBID|CHAIN|SEQUENCE    RPASVQLDTVLCVLTLNIYYEKLFIFLWFWLVFVAVVSTVNCFKWIYYLCNKTKAQKTIK   723


sp|Q96RD7|PANX1_HUMAN          ------------------------------------------------------------   426
5H1Q:A|PDBID|CHAIN|SEQUENCE    NYLSTAPIKSTISDDQFFSALGEDGLFIMDQMALNLGDIPASYLTISMRNICQDFIESED   783


sp|Q96RD7|PANX1_HUMAN          ----------------        426
5H1Q:A|PDBID|CHAIN|SEQUENCE    YIDEERTPFVKSIKHT        799
```

Accordingly although the pairwise Innexin6 alignment exhibits a low identity conventionally unsuitable towards single template homology modeling and moreover exhibits extensive gaps within pivotally conserved residues and membranous regions, this alignment was used towards a top template alignment adjustment within a fold-recognition multiple template alignment threading approach.

4.2.2 Promals3D

Promals3D Innexin6-Pannexin-1 fold alignment results similarly expressed a low identity of just ~14% and gaps within pivotal residues and transmembranes one and four. Accordingly, although Promals3D identity and gap results are unsatisfactory towards a single template alignment modeling methodology, this ss alignment was used towards a threading consensus.[115]

4.2.3 Phyre²

From the previous Phyre² multiple template predictor output, Phyre² aligned via structural threading Innexin6. In this prediction the alignment integrity has been heightened to 21% via Phyre²'s fold recognition algorithm.
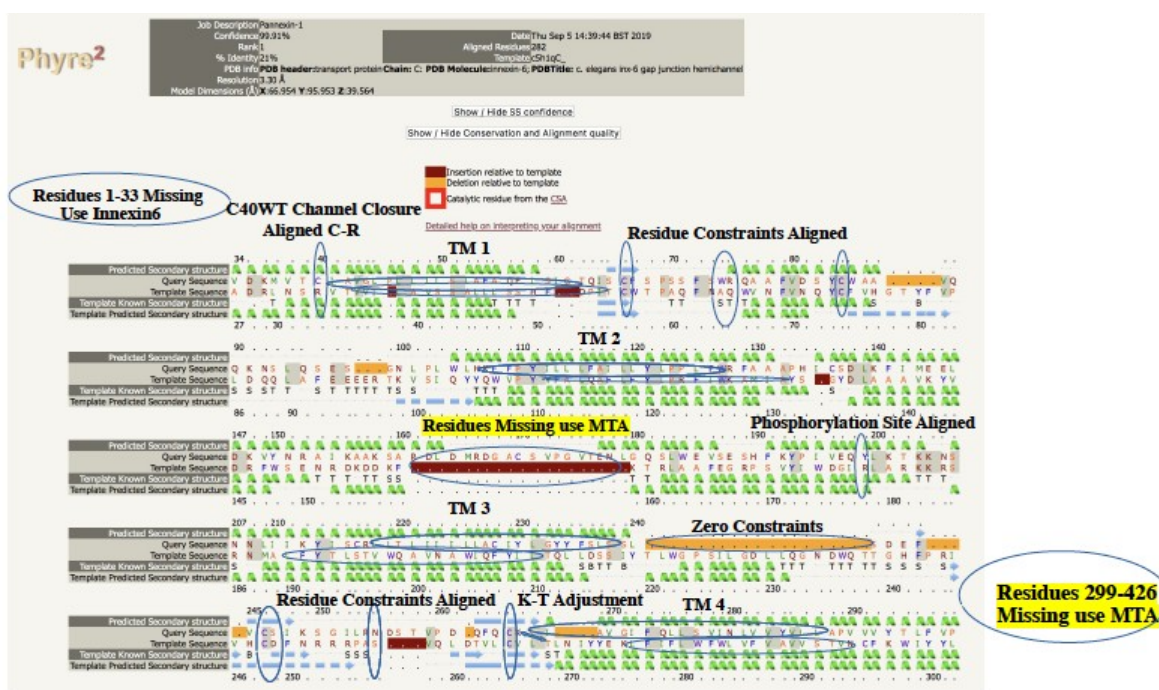
Table 27.) Phyre² Pannexin-1-Innexin6 template alignment secondary structural prediction exhibiting four conserved aligned transmembrane (TM) helices and pivotal residues throughout, bottom K-T to be manually adjusted to preserve residue integrity .[111]

Phyre²'s Pannexin-1-Innexin6 alignment exhibits the best overall coverage area and alignment integrity, however, the Pannexin-1 front and back tail domains were absent. Accordingly this alignment was elected as a top template towards a multiple template alignment approach.

4.2.4 CNFpred

Similar to the previous template discovery methods CNFpred selected Innexin-6 as a best top template. Being a secondary structural predictor, the CNFpred alignment results were similar to Phyre²'s i.e. conserved alignments towards selected constraints. Although this algorithm's alignment results exhibited a low 14% sequence identity it was used towards a consensus structural threading alignment.[63]

4.2.5 Consensus Target-Template Structural Threading

The above single template alignment methods were used towards the development of the below Table 28.) threading agreeing consensus multiple alignment illustrating helices positioning for a query Pannexin-1 exhibiting a distant homology.

```
ALPHAHELIX
TRANSMEMBRANEHELIX
BETASTRAND
BEND
TURN
UNCRYSTALLIZED_TEMPLATE

PANNEXIN 1 (1)MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI (50)
PHYRE2        MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI
PROMALS3D     MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI
RAPTORX       MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI

PANNEXIN 1(51)SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN(100)
PHYRE2        SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN
PROMALS3D     SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN
RAPTORX       SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN

PANNEXIN1(101)LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY(150)
PHYRE2        LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY
PROMALS3D     LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY
RAPTORX       LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY
```

```
PANNEXIN1(151)NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL(200)
PHYRE2         NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL
PROMALS3D      NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL
RAPTORX        NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL

PANNEXIN1(201)KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS(250)
PHYRE2         KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS
PROMALS3D      KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS
RAPTORX        KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS

PANNEXIN1(251)GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR(300)
PHYRE2         GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR
PROMALS3D      GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR
RAPTORX        GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR

PANNEXIN1(301)QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV(350)
PHYRE2         QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV
PROMALS3D      QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV
RAPTORX        QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV

PANNEXIN1(351)LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ(400)
PHYRE2         LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ
PROMALS3D      LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ
RAPTORX        LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ

PANNEXIN1(401)GMNIDSETKANNGEKNARQRLLDSSC                        (426)
PHYRE2         GMNIDSETKANNGEKNARQRLLDSSC
PROMALS3D      GMNIDSETKANNGEKNARQRLLDSSC
RAPTORX        GMNIDSETKANNGEKNARQRLLDSSC
```

4.3 Pannexin-1 Topology

4.3.1 TMPred Topology Prediction

4.3.1.1 Pannexin-1

Prediction parameters:

TM-helix length between 14 and 35

TMPRED.19992.5961.seq[37]
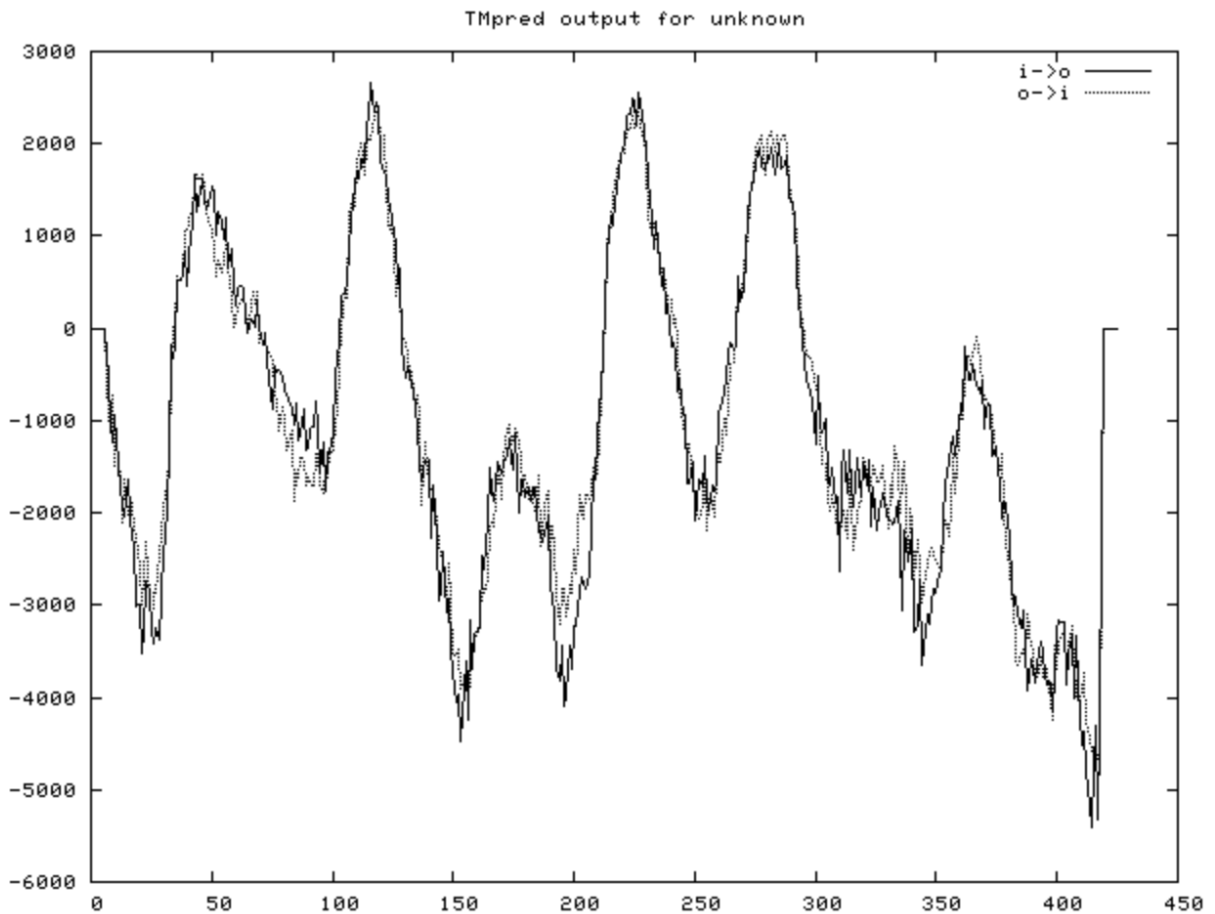
Table 29.) TMPred transmembrane spanning regional inside-outside (i→o), outside-inside (o-→)prediction.[51] In this case my preferred election is towards the former TMPred's alternative model below orienting Pannexin-1's N and C termini in the cytosol scoring 8687 corroborating with UniProtKB's Pannexin-1 topological sequence analysis.

```
-----> STRONGLY prefered model: N-terminus outside
 4 strong transmembrane helices, total score : 8705
 # from    to length score orientation
 1   37    55 (19)     1691 o-i
 2  108   127 (20)     2661 i-o
 3  218   235 (18)     2355 o-i
 4  268   296 (29)     1998 i-o

------> alternative model
 4 strong transmembrane helices, total score : 8687
 # from    to length score orientation
 1   37    54 (18)     1663 i-o
 2  108   127 (20)     2337 o-i
 3  218   235 (18)     2553 i-o
 4  267   296 (30)     2134 o-i
```

Table 30.) TMPred's 'alternative model' with all satisfactory scores of over 500 each corroborates with below UniProtKB's Pannexin-1 algorithmic topological transmembrane positioning.[51]

Topology

Feature key	Position(s)	Description		Actions	Graphical view	Length
Topological domain[i]	1 – 40	Cytoplasmic	Sequence analysis	Add BLAST		40
Transmembrane[i]	41 – 61	Helical	PROSITE-ProRule annotation	Add BLAST		21
Topological domain[i]	62 – 106	Extracellular	Sequence analysis	Add BLAST		45
Transmembrane[i]	107 – 127	Helical	PROSITE-ProRule annotation	Add BLAST		21
Topological domain[i]	128 – 217	Cytoplasmic	Sequence analysis	Add BLAST		90
Transmembrane[i]	218 – 238	Helical	PROSITE-ProRule annotation	Add BLAST		21
Topological domain[i]	239 – 266	Extracellular	Sequence analysis	Add BLAST		28
Transmembrane[i]	267 – 287	Helical	PROSITE-ProRule annotation	Add BLAST		21
Topological domain[i]	288 – 426	Cytoplasmic	Sequence analysis	Add BLAST		139

Table 31.) UniProtKB's Pannexin-1 sequence analysis topology coincides considerably with TMPred's prediction above i.e. oriented N and C termini inside, transmembrane regions and respective extracellular-cytoplasmic residue domains.[110]

4.3.1.2 Connexin26

Prediction parameters:

TM-helix length between 14 and 35

TMPRED.10830.6871.seq[37]

```
-----> STRONGLY prefered model: N-terminus inside
  4 strong transmembrane helices, total score : 7249
 # from    to length score orientation
 1   23    39 (17)     1877 i-o
 2   76    96 (21)     1897 o-i
 3  130   158 (29)     1218 i-o
 4  190   215 (26)     2257 o-i
```

Table 32.) TMPred's Connexin26 STRONGLY preferred model similarly displays oriented cytosolic N and T termini, respective topological domains and lengths and transmembrane positioning as Pannexin-1.[77]

Topology
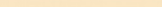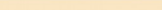
Feature key	Position(s)	Description	Actions	Graphical view	Length
Intramembrane[i]	2 – 13	1 Publication ▾			12
Topological domain[i]	14 – 20	Cytoplasmic 1 Publication ▾			7
Transmembrane[i]	21 – 40	Helical 1 Publication ▾	Add ✎ BLAST		20
Topological domain[i]	41 – 73	Extracellular 1 Publication ▾	Add ✎ BLAST		33
Transmembrane[i]	74 – 94	Helical 1 Publication ▾	Add ✎ BLAST		21
Topological domain[i]	95 – 135	Cytoplasmic 1 Publication ▾	Add ✎ BLAST		41
Transmembrane[i]	136 – 156	Helical 1 Publication ▾	Add ✎ BLAST		21
Topological domain[i]	157 – 189	Extracellular 1 Publication ▾	Add ✎ BLAST		33
Transmembrane[i]	190 – 210	Helical 1 Publication ▾	Add ✎ BLAST		21
Topological domain[i]	211 – 226	Cytoplasmic 1 Publication ▾	Add ✎ BLAST		16

Table 33.) UniProtKB's Connexin26 transmembrane helices via mutational studies, topological lengths and termini cytosolic orientation correlate to TMPred's Connexin26.[129]

4.3.2 TMHMM Topology Prediction

4.3.2.1 Pannexin-1

TMHMM transmembrane prediction results strongly demonstrating positive results of a TM protein via i.), an oriented cytosolic N-terminal via iii.) and topological and transmembrane spanning regions similar to UnitProtKB's Pannexin-1 sequence positioning analysis in Table 31.) above.

i.) line 3.) Exp number of AAs in THMs: 90.50832, any resulting number > 18 very likely predicts a transmembrane protein (or signal peptide).

ii.) line 4.) Exp number, first 60 AAs: 20.66294, is the expected number of amino acids in transmembrane helices in the first 60 amino acids of the complete protein, any number > 3 warns of the N-term being a signal-peptide.

iii.) line 5.) Total prob of N-in: 0.96649, probability that the N-terminal in on the inside of the membrane. In the Pannexin-1 case, this ~97% value agrees with former topological algorithm predictor TMPred's positioning of the N terminal placed in the cytosol.[50]

```
# pannexin-1 Length: 426
# pannexin-1 Number of predicted TMHs:  4
# pannexin-1 Exp number of AAs in TMHs: 90.50832
# pannexin-1 Exp number, first 60 AAs:  20.66294
# pannexin-1 Total prob of N-in:         0.96649
# pannexin-1 POSSIBLE N-term signal sequence
pannexin-1      TMHMM2.0        inside        1     37
pannexin-1      TMHMM2.0        TMhelix      38     60
pannexin-1      TMHMM2.0        outside      61    107
pannexin-1      TMHMM2.0        TMhelix     108    130
pannexin-1      TMHMM2.0        inside      131    212
pannexin-1      TMHMM2.0        TMhelix     213    235
pannexin-1      TMHMM2.0        outside     236    274
pannexin-1      TMHMM2.0        TMhelix     275    297
pannexin-1      TMHMM2.0        inside      298    426
```
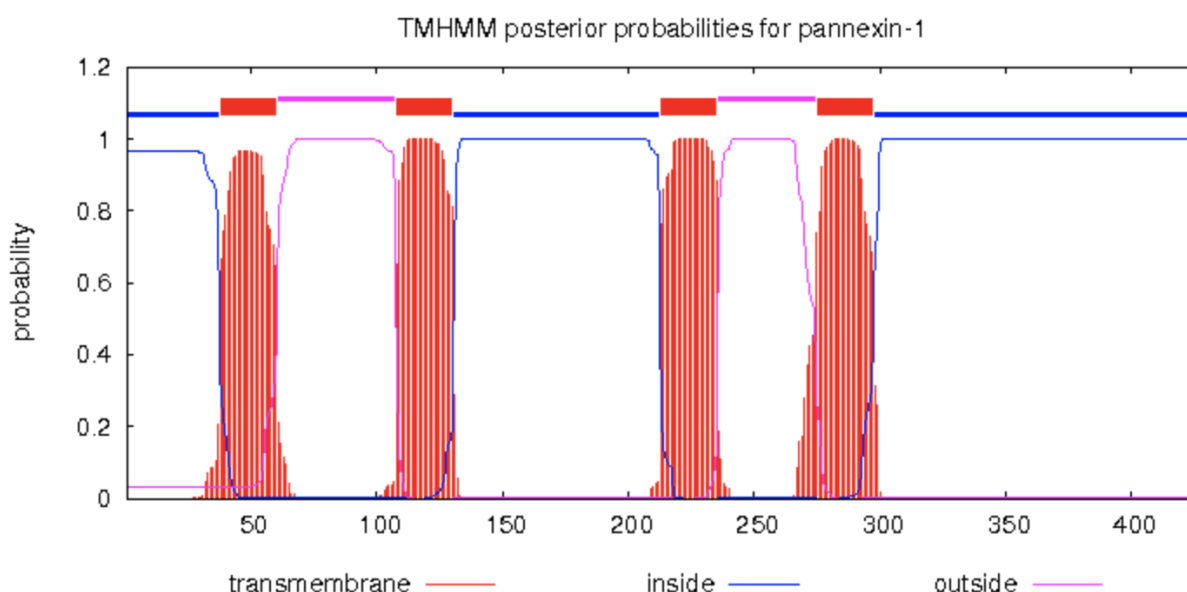

Table 34.) TMHMM topological and transmembrane probabilities similarly corroborating with TMPred's and UnitProtKB's Pannexin-1 sequence analysis positioning.[36]

4.3.2.2 Connexin26

i.) line 3.) Exp number of AAs in THMs: 87.6082, any resulting number > 18 very likely predicts a transmembrane protein (or signal peptide) as Pannexin-1.

iii.) line 5.) Total prob of N-in: 0.99323, probability that the N-term is on the inside of the membrane similar to Pannexin-1.[50]

TMHMM result

[HELP](#) with output formats

```
# 2ZW3:A|PDBID|CHAIN|SEQUENCE Length: 226
# 2ZW3:A|PDBID|CHAIN|SEQUENCE Number of predicted TMHs:  4
# 2ZW3:A|PDBID|CHAIN|SEQUENCE Exp number of AAs in TMHs: 87.6082
# 2ZW3:A|PDBID|CHAIN|SEQUENCE Exp number, first 60 AAs:  20.00686
# 2ZW3:A|PDBID|CHAIN|SEQUENCE Total prob of N-in:      0.99323
# 2ZW3:A|PDBID|CHAIN|SEQUENCE POSSIBLE N-term signal sequence
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        inside      1     20
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        TMhelix    21     40
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        outside    41     75
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        TMhelix    76     98
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        inside     99    131
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        TMhelix   132    154
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        outside   155    192
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        TMhelix   193    215
2ZW3:A|PDBID|CHAIN|SEQUENCE      TMHMM2.0        inside    216    226
```
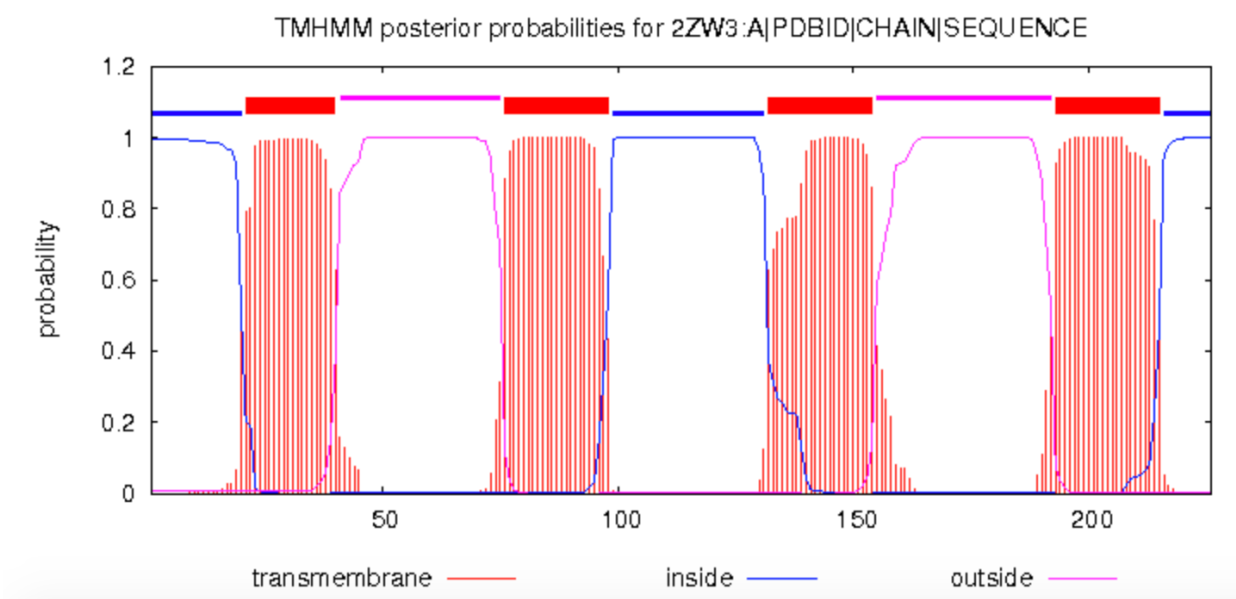

Table 35.) TMHMM's Connexin26 transmembrane and topological domain results are identical to UnitProKB's Connexin26 in Table 33.) and very similar to TMPred's Connexin26.[36]

4.3.3 SOSUI Topology Prediction

4.3.3.1 Pannexin-1

SOSUI Result

Query title : None

Total length : 426 A. A.

Average of hydrophobicity : 0.203521

**This amino acid sequence is of a MEMBRANE PROTEIN
which have 4 transmembrane helices.**

No.	N terminal	transmembrane region	C terminal	type	length
1	38	VTCIAVGLPLLLISLAFAQEISI	60	PRIMARY	23
2	106	HKFFPYILLLFAILLYLPPLFWR	128	PRIMARY	23
3	210	IIKYISCRLLTLIIILLACIYLG	232	PRIMARY	23
4	270	VGIFQLLSVINLVVYVLLAPVVV	292	PRIMARY	23

Table 36.) SOSUI's Pannexin-1 hydrophobicity prediction displays four suggested ordered transmembrane regions corroborating very closely to UnitProtKB's, TMPred's and TMHMM's Pannexin-1 transmembrane sequence positioning.[43]

4.3.3.2 Connexin26

SOSUI Result

Query title : None

Total length : 226 A. A.

Average of hydrophobicity : 0.288053

**This amino acid sequence is of a MEMBRANE PROTEIN
which have 4 transmembrane helices.**

No.	N terminal	transmembrane region	C terminal	type	length
1	20	IGKIWLTVLFIFRIMILVVAAKE	42	PRIMARY	23
2	71	ISHIRLWALQLIFVSTPALLVAM	93	SECONDARY	23
3	139	SIFFRVIFEAAFMYVFYVMYDGF	161	PRIMARY	23
4	193	VFMIAVSGICILLNVTELCYLLI	215	PRIMARY	23

Table 37.) SOSUI's Connexin26 transmembrane positioning results agree with TMHMM's, UnitProtKB's and TMPred's Connexin26 sequence positioning.[88]

4.3.4 RaptorX Topology Prediction

4.3.4.1 Pannexin-1

RaptorX's method results confirmed the existence of coils in Pannexin-1's front and back tails and a loop domain.
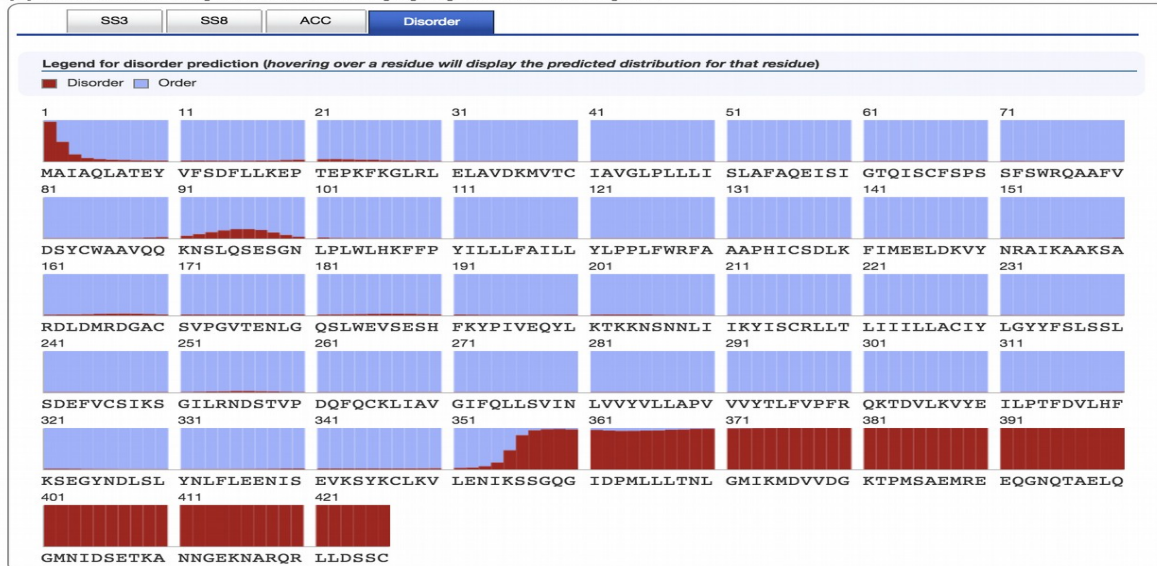
Table 38.) RaptorX prediction suggests disorderly regions in Pannexin-1 front and back tails and topological residues 90-100 inferring coil and loop presence respectively.[42]
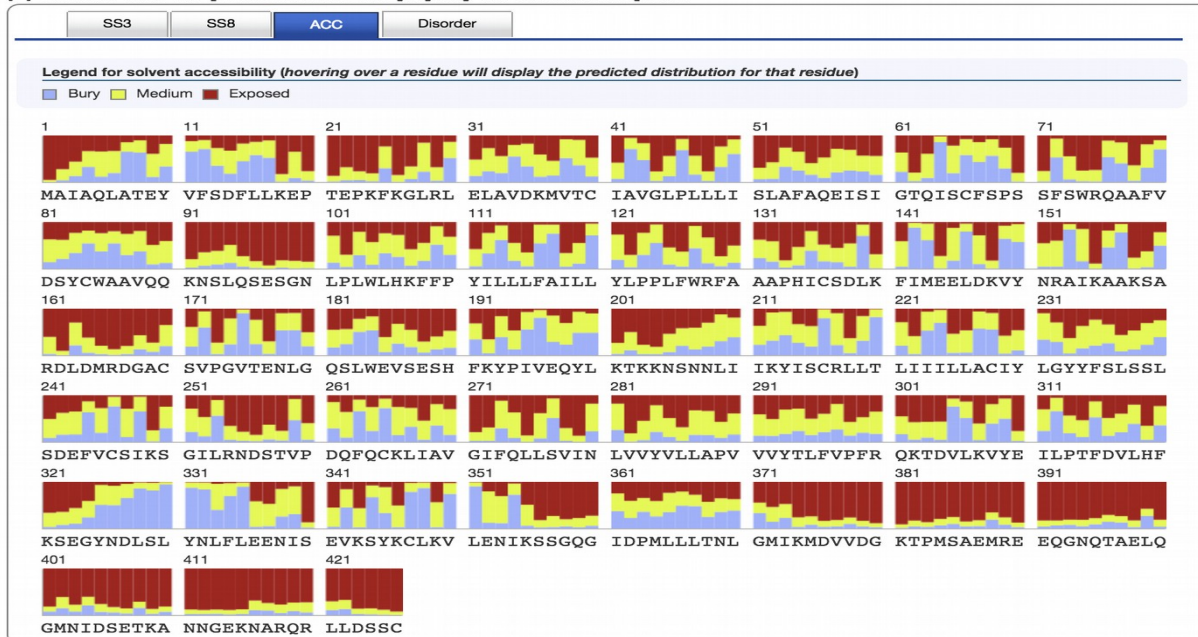
Table 39.) Further RaptorX's strong inference of Pannexin-1 front and back tail coils and a loop region (residues 90-100) respectively via solvent exposure prediction.[42]
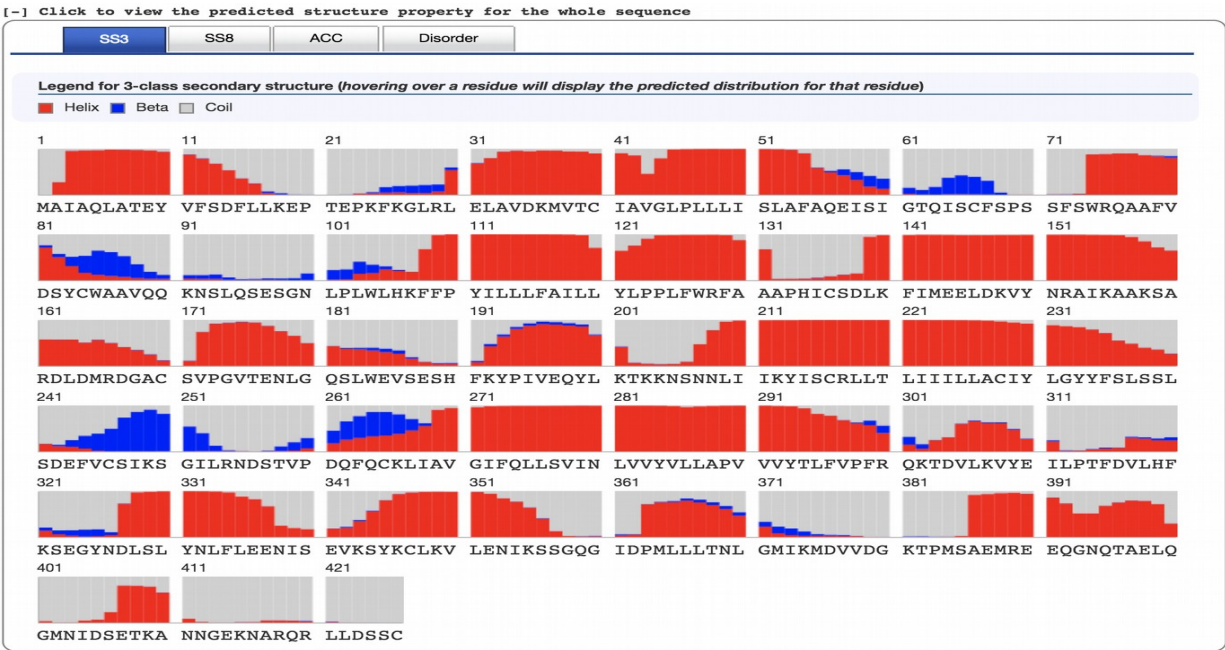
Table 40.) RaptorX 3-class secondary structural output confirming Pannexin-1 disorderly solvent exposed front and back tail coils and topological loop (residues 57-106).[42]

4.3.4.2 Connexin26
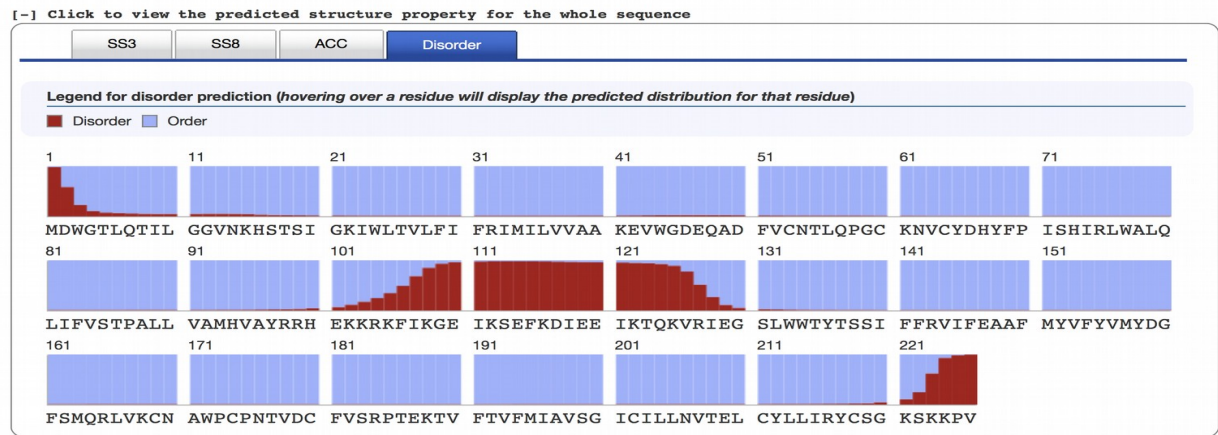
Table 41.) RaptorX prediction suggests disorderly regions in Connexin26 front and back tails (similar to Pannexin-1 coils) and first intracellular loop region (residues ~100-130); confirming TMPred's, UniProtKB's, TMHMM's and SOSUI's Connexin26 topological algorithms and exhibiting symmetry to RaptorX's Pannexin-1 loop presence in its first intracellular domain.[89]

85

Table 42.) RaptorX's strong inference of Connexin26 front and short back tails (similar to Pannexin-1 coils) and first intracellular topological loop region (residues ~100-130) via solvent exposure prediction.[89]

Table 43.) RaptorX 3-class Connexin26 secondary structural output confirming disorderly solvent exposed front and short back tail coils and first intracellular topological loop region (residues ~100-130).[89]

4.3.5 Phyre² Topology Prediction

4.3.5.1 Pannexin-1

Phyre² 's prediction of Pannexin-1 orientation and re-entrant helices agrees considerably with TMPred's, UnitProtKB's, TMHMM's and SOSUI's.
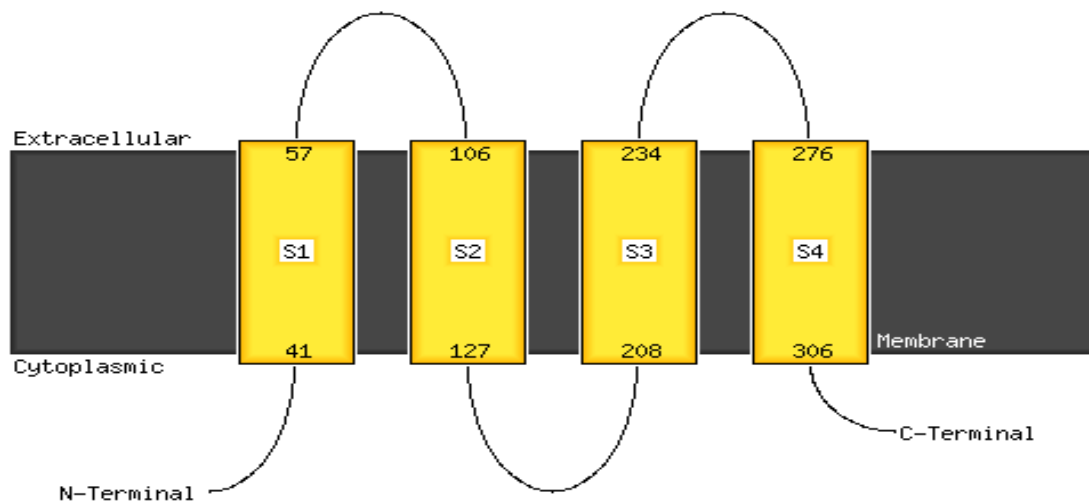
Figure 18.) Phyre² Pannexin-1 transmembrane helices topology prediction.[112]

4.3.5.2 Connexin26

Phyre² prediction exhibiting 4-transmembrane areas, two-cytosolic oriented tails and similar transmembrane residue lengths as Pannexin-1. Phyre² exhibits similar transmembrane residue positions as TMPred's, UnitProtKB's, TMHMM's and SOSUI's Connexin26.
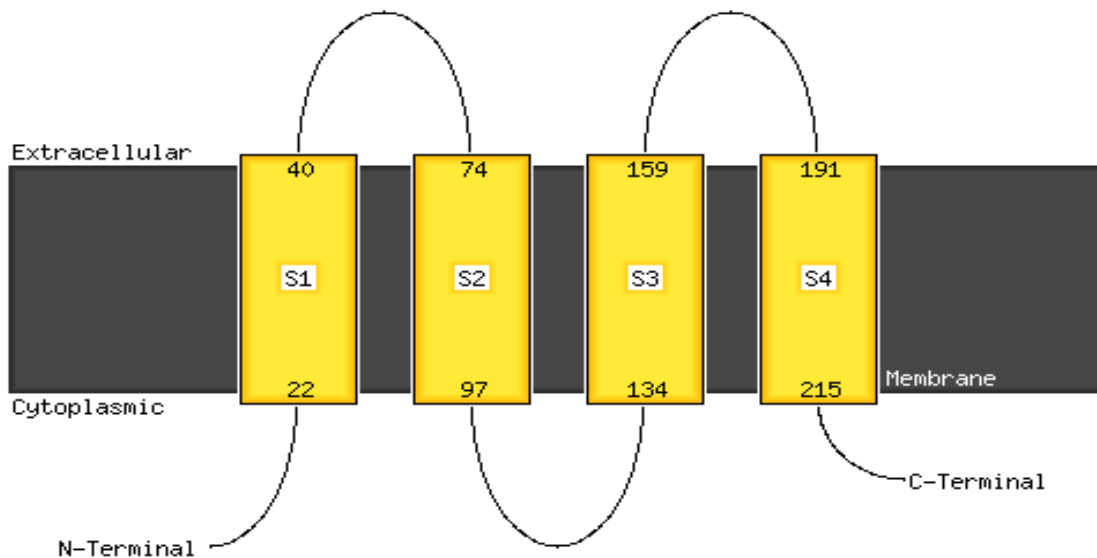
Figure 19.) Phyre²'s Connexin26 transmembrane helices topology prediction.[87]

4.3.6 Topology Consensus

Table 44.) Below is a topological consensus summarizing Pannexin-1 and Connexin26 orientation and positioning agreements amongst themselves and each other via the former topology determining algorithms. (I-O and O-I is inside to outside and outside to inside cell respectively.)

i.) First Cytoplasmic/Topological 'In' N-Terminal Tail Region:

	Algorithm	TM Region	Feature	From	To	Length	Direction	Protein
2	UniProtKB	N-terminal	Cytoplasmic	1	40	40	I	Pannexin-1
3	TMPred	N-terminal	Cytoplasmic	1	36	36	I	Pannexin-1
4	TMHMM	N-terminal	Cytoplasmic	1	37	37	I	Pannexin-1
5	SOSUI	N-terminal	Cytoplasmic	1	37	37	N/A	Pannexin-1
6	Phyre^2	N-terminal	Cytoplasmic	1	40	40	I	Pannexin-1
7	UniProtKB	N-terminal	Cytoplasmic	1	20	20	I	Connexin-26
8	TMPred	N-terminal	Cytoplasmic	1	22	22	I	Connexin-26
9	TMHMM	N-terminal	Cytoplasmic	1	20	20	I	Connexin-26
10	SOSUI	N-terminal	Cytoplasmic	1	19	19	N/A	Connexin-26
11	Phyre^2	N-terminal	Cytoplasmic	1	21	21	I	Connexin-26

ii.) First Helix Transmembrane Region, First Extracellular Second Topological Region and Second Helix Transmembrane Region respectively:

	Algorithm	TM Region	Feature	From	To	Length	Direction	Protein
12								
13	UniProtKB	1	Helical	41	61	21	I-O	Pannexin-1
14	TMPred	1	Helical	37	54	18	I-O	Pannexin-1
15	TMHMM	1	Helical	38	60	23	I-O	Pannexin-1
16	SOSUI	1	Helical	38	60	23	N/A	Pannexin-1
17	Phyre^2	1	Helical	41	57	17	I-O	Pannexin-1
18	UniProtKB	1	Helical	21	40	20	I-O	Connexin-26
19	TMPred	1	Helical	23	39	17	I-O	Connexin-26
20	TMHMM	1	Helical	21	40	20	I-O	Connexin-26
21	SOSUI	1	Helical	20	42	23	N/A	Connexin-26
22	Phyre^2	1	Helical	22	40	19	I-O	Connexin-26
23								
24	UniProtKB	Topological	Extracellular	62	106	45	O	Pannexin-1
25	TMPred	Topological	Extracellular	55	107	53	O	Pannexin-1
26	TMHMM	Topological	Extracellular	61	107	47	O	Pannexin-1
27	SOSUI	Topological	Extracellular	61	105	45	N/A	Pannexin-1
28	Phyre^2	Topological	Extracellular	58	105	48	O	Pannexin-1
29	UniProtKB	Topological	Extracellular	41	75	35	O	Connexin-26
30	TMPred	Topological	Extracellular	40	75	36	O	Connexin-26
31	TMHMM	Topological	Extracellular	41	75	35	O	Connexin-26
32	SOSUI	Topological	Extracellular	43	70	28	N/A	Connexin-26
33	Phyre^2	Topological	Extracellular	41	73	33	O	Connexin-26

34								
35	UniProtKB	2	Helical	107	127	21	O-I	Pannexin-1
36	TMPred	2	Helical	108	127	20	O-I	Pannexin-1
37	TMHMM	2	Helical	108	130	23	O-I	Pannexin-1
38	SOSUI	2	Helical	106	128	23	N/A	Pannexin-1
39	Phyre^2	2	Helical	106	127	22	O-I	Pannexin-1
40	UniProtKB	2	Helical	76	98	23	O-I	Connexin-26
41	TMPred	2	Helical	76	96	21	O-I	Connexin-26
42	TMHMM	2	Helical	76	98	23	O-I	Connexin-26
43	SOSUI	2	Helical	71	93	23	N/A	Connexin-26
44	Phyre^2	2	Helical	74	97	24	O-I	Connexin-26

iii.) Second Cytoplasmic

45								
46	UniProtKB	Topological	Cytoplasmic	128	217	90	I	Pannexin-1
47	TMPred	Topological	Cytoplasmic	128	217	90	I	Pannexin-1
48	TMHMM	Topological	Cytoplasmic	131	212	82	I	Pannexin-1
49	SOSUI	Topological	Cytoplasmic	129	209	81	N/A	Pannexin-1
50	Phyre^2	Topological	Cytoplasmic	128	207	80	I	Pannexin-1
51	UniProtKB	Topological	Cytoplasmic	99	131	33	I	Connexin-26
52	TMPred	Topological	Cytoplasmic	97	129	33	I	Connexin-26
53	TMHMM	Topological	Cytoplasmic	99	131	33	I	Connexin-26
54	SOSUI	Topological	Cytoplasmic	94	138	45	N/A	Connexin-26
55	Phyre^2	Topological	Cytoplasmic	98	133	36	I	Connexin-26

iv.) Third Helix Transmembrane Region, Second Extracellular Fourth Topological Region and Fourth Helix Transmembrane Region respectively:

56								
57	UniProtKB	3	Helical	218	238	21	I-O	Pannexin-1
58	TMPred	3	Helical	218	235	18	I-O	Pannexin-1
59	TMHMM	3	Helical	213	235	23	I-O	Pannexin-1
60	SOSUI	3	Helical	210	232	23	N/A	Pannexin-1
61	Phyre^2	3	Helical	208	234	27	I-O	Pannexin-1
62	UniProtKB	3	Helical	132	154	23	I-O	Connexin-26
63	TMPred	3	Helical	130	158	29	I-O	Connexin-26
64	TMHMM	3	Helical	132	154	23	I-O	Connexin-26
65	SOSUI	3	Helical	139	161	23	N/A	Connexin-26
66	Phyre^2	3	Helical	134	159	26	I-O	Connexin-26
67								
68	UniProtKB	Topological	Extracellular	239	266	28	O	Pannexin-1
69	TMPred	Topological	Extracellular	236	266	31	O	Pannexin-1
70	TMHMM	Topological	Extracellular	236	274	39	O	Pannexin-1
71	SOSUI	Topological	Extracellular	233	269	37	N/A	Pannexin-1
72	Phyre^2	Topological	Extracellular	235	275	41	O	Pannexin-1
73	UniProtKB	Topological	Extracellular	155	192	38	O	Connexin-26
74	TMPred	Topological	Extracellular	159	189	31	O	Connexin-26
75	TMHMM	Topological	Extracellular	155	192	38	O	Connexin-26
76	SOSUI	Topological	Extracellular	162	192	31	N/A	Connexin-26
77	Phyre^2	Topological	Extracellular	160	190	31	O	Connexin-26

89

78								
79	UniProtKB	4	Helical	267	287	21	O-I	Pannexin-1
80	TMPred	4	Helical	267	296	30	O-I	Pannexin-1
81	TMHMM	4	Helical	275	297	23	O-I	Pannexin-1
82	SOSUI	4	Helical	270	292	23	N/A	Pannexin-1
83	Phyre^2	4	Helical	276	306	31	O-I	Pannexin-1
84	UniProtKB	4	Helical	193	215	23	O-I	Connexin-26
85	TMPred	4	Helical	190	215	26	O-I	Connexin-26
86	TMHMM	4	Helical	193	215	23	O-I	Connexin-26
87	SOSUI	4	Helical	193	215	23	N/A	Connexin-26
88	Phyre^2	4	Helical	191	215	25	O-I	Connexin-26

v.) Final Cytoplasmic/Topological C-Terminal Tail Region:

89								
90	UniProtKB	T-Terminal	Cytoplasmic	288	426	139	I	Pannexin-1
91	TMPred	T-Terminal	Cytoplasmic	297	426	130	I	Pannexin-1
92	TMHMM	T-Terminal	Cytoplasmic	298	426	129	I	Pannexin-1
93	SOSUI	T-Terminal	Cytoplasmic	293	426	134	N/A	Pannexin-1
94	Phyre^2	T-Terminal	Cytoplasmic	307	426	120	I	Pannexin-1
95	UniProtKB	T-Terminal	Cytoplasmic	216	226	11	I	Connexin-26
96	TMPred	T-Terminal	Cytoplasmic	216	226	10	I	Connexin-26
97	TMHMM	T-Terminal	Cytoplasmic	216	226	11	I	Connexin-26
98	SOSUI	T-Terminal	Cytoplasmic	216	226	11	N/A	Connexin-26
99	Phyre^2	T-Terminal	Cytoplasmic	216	226	11	I	Connexin-26

4.3.7 Structural Threading Topology Consensus

The former and below structural threading positioning consensus together were used to build a Pannexin-1-Connexin26 alignment exhibiting accurate re-entrant membrane residue positioning to be used towards building a re-entrant residue accurate final multiple template alignment.

```
ALPHAHELIX
TRANSMEMBRANEHELIX
BETASTRAND
BEND
TURN
UNCRYSTALLIZED_TEMPLATE

PANNEXIN 1 (1)MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI (50)
PHYRE2         MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI
PROMALS3D      MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI
RAPTORX        MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLI
UniProt(TM_1)                                    IAVGLPLLLI

CONNEXIN-26    MDWGTLQTILGGVNKHSTSIGKIWLTVLFIFRIMILVVAAKEVWGDEQAD
UniProt(TM_1)                   GKIWLTVLFIFRIMILVVAA

PANNEXIN 1(51)SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN(100)
PHYRE2         SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN
PROMALS3D      SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN
RAPTORX        SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN
UniProt(TM_1) SLAFAQEISIG

CONNEXIN-26    FVCNTLQPGCKNVCYDHYFPISHIRLWALQLIFVSTPALLVAMHVAYRRH
UniProt(TM_2)                       LWALQLIFVSTPALLVAMHVAYR

PANNEXIN1(101)LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY(150)
PHYRE2         LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY
PROMALS3D      LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY
RAPTORX        LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY
UniProt(TM_2)        KFFPYILLLFAILLYLPPLFW

CONNEXIN-26    EKKRKFIKGEIKSEFKDIEEIKTQKVRIEGSLWWTYTSSIFFRVIFEAAF
UniProt(TM_3)                       LWWTYTSSIFFRVIFEAAF
```

90

```
PANNEXIN1(151)NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL(200)
PHYRE2        NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL
PROMALS3D     NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL
RAPTORX       NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEVSESHFKYPIVEQYL


CONNEXIN-26   MYVFYVMYDGFSMQRLVKCNAWPCPNTVDCFVSRPTEKTVFTVFMIAVSG
UniProt(TM_3) MYVF                       UniProt(TM_4)VFMIAVSG


PANNEXIN1(201)KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS(250)
PHYRE2        KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS
PROMALS3D     KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS
RAPTORX       KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS
UniProt(TM_3)             LLTLIIILLACIYLGYYFSLS


CONNEXIN-26   ICILLNVTELCYLLIRYCSGKSKKPV

UniProt(TM_4) ICILLNVTELCYLLI

PANNEXIN1(251)GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR(300)
PHYRE2        GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR
PROMALS3D     GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR
RAPTORX       GILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR
UniProt(TM_4)            LIAVGIFQLLSVINLVVYVLL

PANNEXIN1(301)QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV(350)
PHYRE2        QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV
PROMALS3D     QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV
RAPTORX       QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV

PANNEXIN1(351)LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ(400)
PHYRE2        LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ
PROMALS3D     LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ
RAPTORX       LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ

PANNEXIN1(401)GMNIDSETKANNGEKNARQRLLDSSC                       (426)
PHYRE2        GMNIDSETKANNGEKNARQRLLDSSC
PROMALS3D     GMNIDSETKANNGEKNARQRLLDSSC
RAPTORX       GMNIDSETKANNGEKNARQRLLDSSC
```

Table 45.) Above a Topology Consensus Alignment of Pannexin-1 and Connexin26 exhibiting transmembrane helices positioning via ss methods, CONNEXIN-26 PDB crystallized data and UniProt experimental transmembrane positioning.[110, 105, 106]

Table 46.) A re-entrant helices alignment with slight residue adjustments amongst the Pannexin-1-Connexin26 topological relatives exploiting threading/ss and transmembrane positioning consensus. Note all four transmembrane regions exhibit zero gaps and align via default all intra-extracellular domains. TMs express high hydrophobic residue %s with hydrophobic central peak residues and amphipathic W interface residues, loops and coils exhibit charged and polar residues. Residue constraints are maintained eg. residue K266 pivotal towards chemical blocker interactions properly topologically exhibited in the EL2 domain not TM4 precluding an Innexin6 4th TM residue misalignment. Similarly pivotal polar residues 39T and 40C are properly aligned to a non-membraneous TM1 conserving 40C action as a channel closer.

K266 EL2 Positioning
Polar Residues
Hydrophobic Residues

ALPHAHELIX
TRANSMEMBRANEHELIX
BETASTRAND
BEND
TURN
UNCRYSTALLIZED CONNEXIN-26 PORTION

PANNEXIN 1 (1)MAIAQLATEYVFSDFLLKEPTEPKFKGLRIELAVDKMVTQIAVGLPLLLI (50)
CONNEXIN-26   --------------------MDWGTLQTILGGVNKHSTSIGKIWLTVLF
                                             * *   * *   *   *

PANNEXIN 1 (51)SLAFAQEISIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGN(100)
CONNEXIN-26   IFRIMILVVAAKEVWG-------DEQADFVCNTLQPGCKNVCYDHYFPIS
                                          * **

PANNEXIN1(101)LPLWLHKFFPYILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVY(150)
CONNEXIN-26   -HIRLWALQLIFVSTPALLVMHVAYRRH--------------------
                        *   *   *   *   *

PANNEXIN1(151)NRAIKAAKSARDLDMRDGACSVPGVTENLGQSLWEV**SE**SHFKYP**I**VE**Q**YL(200)
CONNEXIN-26   EKKRKFIKG--------------------------------------
                      *   *   EIK**SE**FKDIEE**I**KTQ

PANNEXIN1(201)KTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKS(250)
CONNEXIN-26   -KVRIEGSLWWTYTSSIFFRVLFEAAFMYVFYVMYDGFSMQRLVKCNAWP
                       *   *   *   *

PANNEXIN1(251)GILRN-----DSTVPDQFQCHLIAVGIFQLLSVINLVVYVLRAPVVVYTLFVPFR(300)
CONNEXIN-26   CPNTVDCFVSRPTEKTVFTVFMIAVSGICILLNVTELCYLLIRY------------
                     *   *   *** *   *   *   CSGKSKKPV

PANNEXIN1(301)QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKV(350)
CONNEXIN-26   --

PANNEXIN1(351)LENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQ(400)
CONNEXIN-26   --

PANNEXIN1(401)GMNIDSETKANNGEKNARQRLLDSSC   (426)
CONNEXIN-26   --------------------------

Transmembrane-4 Panx1 Re-Entrant Residue Prediction

4.3.8 Innexin6 Topology

Innexin6 below exhibiting four spanning transmembranes with respective positions.
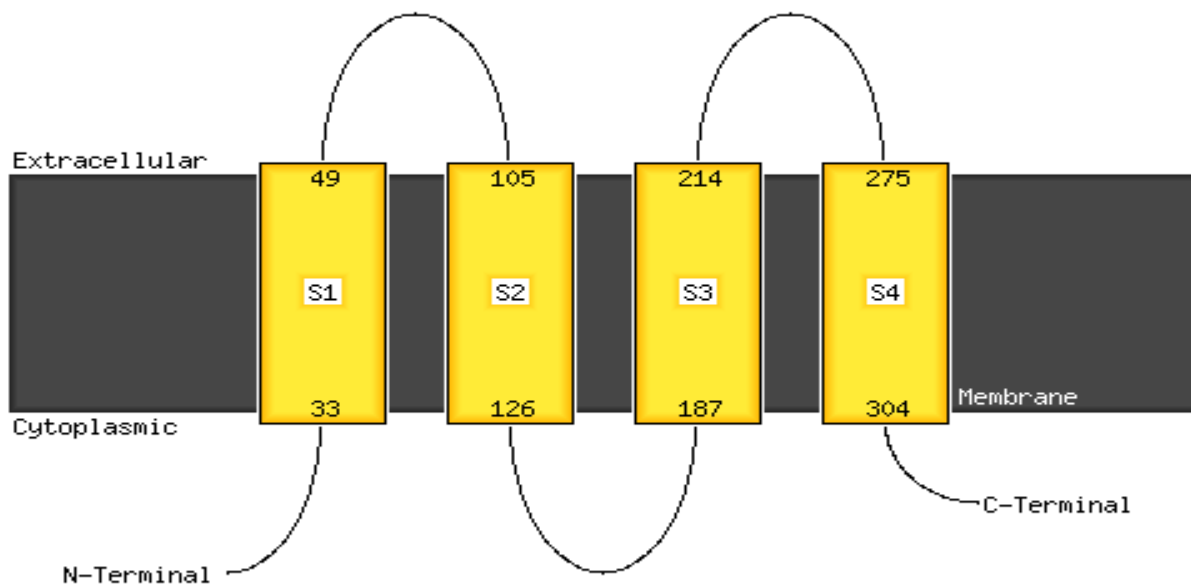
Figure 20.) Phyre2 Innexin6 transmembrane topology helix prediction in accordance with below Innexin6 UnitProtKB sequence analysis.[82]

Topology

Feature key	Position(s)	Description	Actions	Graphical view	Length
Transmembrane[i]	36 – 56	Helical 🗘 PROSITE-ProRule annotation ▾	🏠 Add 🔍 BLAST		21
Transmembrane[i]	111 – 131	Helical 🗘 PROSITE-ProRule annotation ▾	🏠 Add 🔍 BLAST		21
Transmembrane[i]	190 – 210	Helical 🗘 PROSITE-ProRule annotation ▾	🏠 Add 🔍 BLAST		21
Transmembrane[i]	276 – 296	Helical 🗘 PROSITE-ProRule annotation ▾	🏠 Add 🔍 BLAST		21

Table 47.) UniProtKB's Innexin6 positions via algorithmic sequence analysis.[116]

4.4 Pannexin-1 Multiple Template Alignments

Two multiple template alnfiles generated for MODELLER input.

4.4.1 Multiple Template/Target Alignment File

```
>P1;5h1q
structureX:5h1q:    7 :A:369 :A:::-1.00:-1.00
-----------------------AINSVN----------------ALISRVFVQPK
GD--LADRLNSRVTVVILAVSSALLLSSHF--DPITCWTPAQFNAQWVNFVNQYCFVHGT
YFVP---L---DQQLAFEEEE-------RTKVSIQYYQWVPYVFALQAFLFYIPRFIWKA
MIAYSGYDLAAAVKYVDRFWSENRDKD---DKFKTRL---------AAFEGRPSVYIWDG
IRLARKKRSRNMALFYTLSTVWQAVNAWIQFYILTQLLDSSIYTLWGPSILGDLL-----
--------------------------QGNDWQTTGHFPRIV-------------HCDFN
RRRPAS-------------------VQLDTVLCV-------LTLNIYYEKLFIFLWFWLV
FVAVVSTVNCFKWIYY-LCN-KTKAQKTIKNYLSTAPIKSTISDDQF----FSALGEDGL
FIMDQMALNLGDIPAS------------YLTISMRNICQDF-----------------
---------I------------------------------------------------
----------------------------------------------------------
------------------------------------------*


>P1;4uxf
structureX:4uxf:   893 :A:1289:A::::
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
-----------PSQFIRRDIAQTVNGSL-TLTQQTNLSAPLVSSSTGEFGGSLAANRTFT
IRNTGAPTSI----VFEKG-----------PASGANPAQSMSIRVWGNQFGGGSDTTRST
VFEVGDDTSHHFYSQRN--------KDGNIAFNINGTVMPININASGLMNVNG--TATFG
R------SVTANGEFISKSANAFRAINGDYG-----F-FI--RNDA-SNTYFL-------
LTAAGDQTGGFNGLRPLLIN------NQSG---------------------QITIG-EGL
IIAKGVTINSGGLTVNSRIRSQGTKTSDLYTRAPT-SDTVGF-----------WSIDIN-
----------DSATYNQFPGYFKMV-EKTNEVTGLPYLERGEEVKSPGTLTQFGNTLDSLY
QDWITYPTTPEARTTRWTRTWQKTKNSWSSFVQVFDGGNPPQ----------PSDIGALP
SDN---ATMGNLTIRDFLRIGNVRIVPDPVNKTVKFEWVE---*


>P1;5nxh
structureX:5nxh:   744 :A:1289:A::::
--------------------EANETQRGTLRVATQVEAAAG----------T--LDNVLITPK
K---LLGTKSTEAQEGVI--KVATQSETVTGTSANTAVSPKNLKWIAQSEP---TWAATT
AIRGF--VKTSSGSITFVGNDTVGSTQDLELYEKNSYAVSPYELNR-VLANYLPLKAKAA
DTNL--LDGLDSSQFIRRDIAQTVNGSL-TLTQQTNLSAPLVSSSTGEFGGSLAANRTFT
IRNTGAPTSI----VFEKG-----------PASGANPAQSMSIRVWGNQFGGGSDTTRST
VFEVGDDTSHHFYSQRN--------KDGNIAFNINGTVMPININASGLMNVNG--TATFG
R------SVTANGEFISKSANAFRAINGDYG-----F-FI--RNDA-SNTYFL-------
LTAAGDQTGGFNGLRPLLIN------NQSG---------------------QITIG-EGL
IIAKGVTINSGGLTVNSRIRSQGTKTSDLYTRAPT-SDTVGF-----------WSIDIN-
----------DSATYNQFPGYFKMV-EKTNEVTGLPYLERGEEVKSPGTLTQFGNTLDSLY
QDWITYPTTPEARTTRWTRTWQKTKNSWSSFVQVFDGGNPPQ----------PSDIGALP
SDN---ATMGNLTIRDFLRIGNVRIVPDPVNKTVKFEWVE---*


>P1;3cjp
structureX:3cjp:    3 :A:264:A::::
----------------LI-----IDGHTHVILPVEKHIKIMDEAGVDKT-----ILFSTS
I--------HPETAVNLR--D----------------------VKKEM----------
KKLNDVVNGKTNSMIDVRR-NSIKELTNVIQAYPSRYVGFGN----------VPVGLSEN
DTNS--YI------------------------EENIVNNKL-------------------VG
IGELTPASGQ----IKSLK-----------PI-FKYSMDSGSLPIWIHAFNPLVLQ---D
IKEIAEL-CKAFPKVP-----VILGHMGGSNWMTAVEL------AKEIQNLYLD-TSAY-
----------------------FSTFV----------------------LKI-------
VI-NELPLKCIF---GT------------------------DMPFGDL----------
------QLSIEA------IKK-M--SND------------------------------
-------------------SYVANA-VLGDNISR------------------------LL
N---------------------------------------------------------
-------------------I---------------------*
```

93

```
>P1;4y3c
structureX:4y3c:   1 :A:460:A:::::
-GAL-----------ERL-----PDG---------PRIHVPRKTALRPT--VARQVFQPA
FAPAVLSKFDPRTDADVD--EVAF-----SKHTSNQETLPPVFRMVAREY----------
ANRVFALLGRDNGRLSVKQ--ALDGLEGMDPMDKNTSPGLPY-----------TTLGMRRT
DVVD--WETATLIPFAAERLEKMNNKDFSDIVYQTFLKDEL----------------RP
IEKVQAAKTR----IVDVP-----------PF-EHCILGRQLL------------------
----GKF-ASKFQTQPGLELGSAIGCDPDVHWTAFGVA------MQGFERVYDVDYSNFD
STHSVAMFRLLAEEFFS-EENGFDPLVKDYL-----E-SLAISKHAYEEKRYL-------
IT-GGLPSGCAA---TSMLN-----TVMNNIIIRAGLYLTYKNFEFDDVKVLSYG-DDL
LVATNYQLNFDR------VRTSL--AKTGYKITPA-NKTSTFPLESTLEDVVFLKRKFKK
EGPLYRPVMNREALEAMLSYYRPG-TLSEKLTSITMLAVHSGK----------QEYDRLF
A-----------------------------------------------------------
------------------PFREVGVIVPTFESVEYRWRSLFW*


>P1;1nzp
structureX:1nzp:   242 :A:327:A:::::
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------AQ----------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------------------------PS-SQKATNHNLHITEKLEVLAKAYSV
QGDKWRALGYAKAINALKSFHKPV-TSYQEACSIPGIGKR--------------MAEKII
E-----------------------------------------------------------
--------------------ILESGHLRK-LDH----------*


>P1;Panx1
sequence:Panx1:1    : :426    ::::::
---MAIAQLATEYVFSDFL----------LKEPTEP-----KFKGLRLELAVDKMVTCIA
V----------GLPLLLI--SLAFAQEIS-IGTQISCFSPSSFSWRQAAFVDSYCWAAVQ
QKNS---LQSESGNL--------------PLWLHK---FFPYILLLFAILLYLPPLFWRF
AAA---PHICSDLKFIMEELDKVYNRAIKAAKSARDL-----------------------
------------------D----------MRDGACSVPGV--------------TE-----
------------------------NLGQSLWEVSESHFKYP-------------------
----------IVEQYLKTKKNSNNLII-KYISCRLLTLIIILLACIYLGYYFS-------
LSSLSDEFVCSI--KSGILRNDSTVPDQFQCKLIAVGIFQLLS--------VINLV-VYV
LLAPVVV---YTLFVP--F-RQKTDVLKVYEILPT-FDVLHFKSE---------------
------------GYNDLSLYNLFLEENISEVKSYKCLKVLENIKSSG------QGIDPML
LLT--------------------NLGMIKMDVVDGKTPMSAEMREEQGNQTAELQGMN
IDSETKANNGEKNARQ------RLLDSSC------------*
```

Table 48.) A CLUSTAL 6-template result alignment file designated 'align3.ali'.[213]

4.4.2 Single Template/Target Alignment File

```
>P1;5H1Q
structureX:5H1Q:   7 :A:369 :A:::-1.00:-1.00
------AINSVNALISRVFVQPK-------GDLADRLNSRVTVVI-LAVSSALLLSSHF
--DPITCWTPAQFNAQWVNFVNQYCFVHGTYFVPLDQQLAFEEEERTKVSIQYYQWVPY
VFALQAFLFYIPRFIWKAMIAYS-GYDLAAAVKYVDRF---------------------
--------------------PSVYIWDGIRLARKKRSRNMALFYTLSTVWQAVNAWIQF
YILTQLLDSSIYTLWGPSILGDLLQGNDWQTTGHFPRIVHCDFNRRRPAS---VQLDTV
LCVLTLNIYYEKLFIFLWFWLVFVAVVSTVNCFKWIYYLCN------------------
------------------------------------------------------------
------------------------------------------------------------
----------*


>P1;1NZP
structureX:1NZP:   279 :A:319:A:::::
------------------------------------------------------------
------------------------------------------------------------
---------------------------------YAKAINALKSFHKPVTSYQEA
CSIPGIGKRMAEKIIEILES----------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
----------*
```

94

```
>P1;4UXF
structureX:4UXF:   1127 :A:1254:A:: ::
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
-------------------------------------------QGTKTSDLYTRAPTSDTV
GFWSIDINDSATYNQFPGYFKMVEKTNEVTGLPYLERGEEVKSPG--------------
--------------------------------PSDIGALPSD---------------
----------*

>P1;4Y2C
structureX:4Y2C:    100 :A:147:A::::
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
---------------------------------------------EGMDPMDKNTSPGL
PYTTLGMRRTDVVDWETATLIPFAAERLEKMNNK----------------------
----------*

>P1;5H5P
structureX:5H5P:    482 :A:500:A::::
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------------------
--------------------------------------------SETTANNMRKKGKPN
PDQR------*

>P1;Pannexin-1
sequence:Pannexin-1:      : :      : ::: 0.00: 0.00
MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLISLAFAQEIS
IGTQISCFSPSSFSWRQAAFVDSYCWAA----VQQKNSLQSES---GNLPLWLHKFFPY
ILLLFAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVYNRAIKAAKS-ARDLDMRDGA
CSVPGVTENLGQSLWEVSESHFKYPIVEQYLKTKKNSNNLIIKYISCRLLTLIIILLAC
IYLGYYFSLSSL-------------------SDEF----VCSIKSGILRNDSTVP-DQF
QC--KL-I-AVGIFQLLSVINLVVYVLLAPVVVYTLFVPFRQKTDVLKVYEILPTFDVL
HFKSEGYNDLSLYNLF-----LEENISEVKSYKCLKVLENIKSSGQGIDPM-------L
LLTNLGMIKMDVVDGKT----PMSAEMREEQGNQTAELQGMNIDSETKANNGEKNAR--
--QRLLDSSC*
```

Table 49.) A 5-template result single alignment file designated 'align2.ali' (Methods Table 15.), query remains the sequence.[150]

4.5 MODELLER A-Chain Models

MODELLER calculated ten similar 3-D models for each of the multiple template alignments, one best-fit model of each was selected via energy profiling. The A-chain monomer based on the former align2.ali single template/target alignment took precedence towards oligomerization.

4.5.1 Multiple Template/Target Monomer

Because Clustal is a pairwise alignment algorithm not aligning via secondary structural folds, imperative towards transmembrane protein Pannexin-1 alignment, its generated A-chain monomer exhibited a topology not matching template Connexin26 and Innexin6 EM ribbon generated topology models.

4.5.2 Single Template/Target Monomer

The align2.ali file target template alignment based on secondary structural folds took precedence towards generating a best A-chain monomer exhibiting an expected tetra-transmembrane configuration with respective coiled tails and three-loops, all other slightly adjusted single template/target alignments, similar to Clustal, generated skewed topologies.

>> Summary of successfully produced models:

Filename   molpdf

Pannexin-1.B99990001.pdb   3512.81323
Pannexin-1.B99990002.pdb   3717.61914
Pannexin-1.B99990003.pdb   4068.33667
Pannexin-1.B99990004.pdb   3745.99121
Pannexin-1.B99990005.pdb   3679.26147
Pannexin-1.B99990006.pdb   3874.66064
Pannexin-1.B99990007.pdb   3978.54883
Pannexin-1.B99990008.pdb   3668.39819
Pannexin-1.B99990009.pdb   3823.51099
Pannexin-1.B99990010.pdb   4078.27271

Table 50.) Of the 10 Pannexin-1 A-chain monomers generated via MODELLER using the align2.ali file, structural file Pannexin-1.B99990001.pdb was elected as the best model via its lowest moldpdf score of 3512.81323.[159]
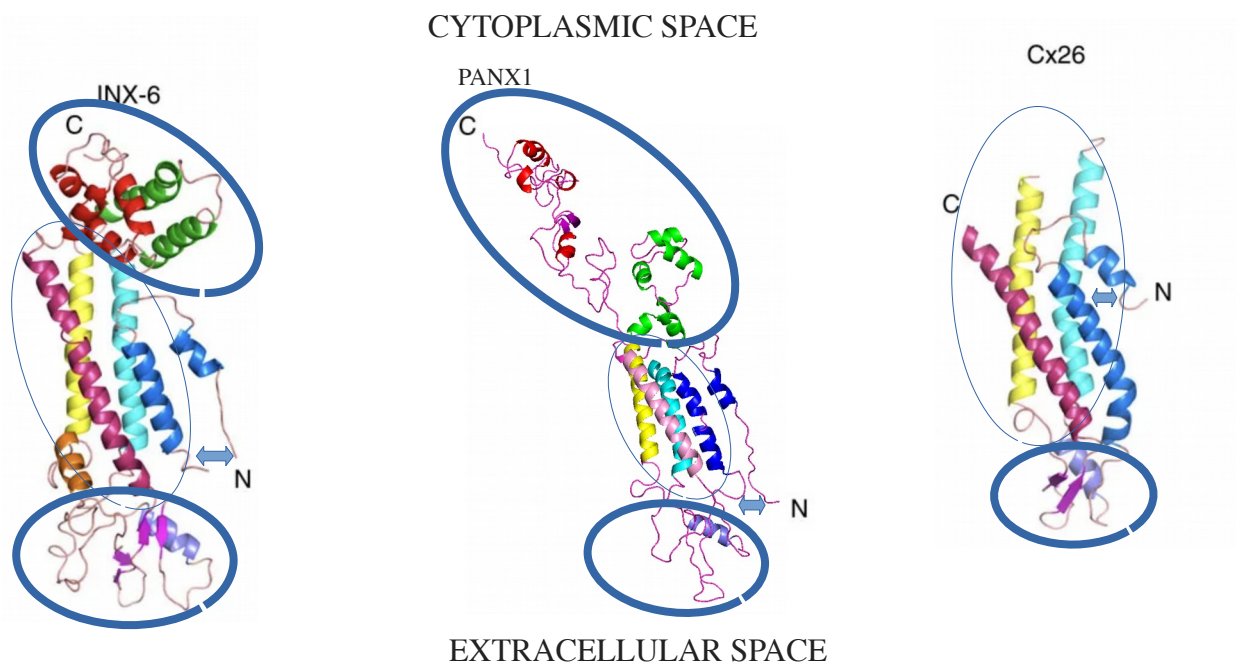
CYTOPLASMIC SPACE

EXTRACELLULAR SPACE

Figure 21.) Pymol visual of best elected Pannexin-1 A-chain based on the align2.ali file alignment exhibiting 4-symmetrically compact transmembranes (thin blue ellipses) and loops and tails (thick blue ellipses) (TM1-N-tail helix blue, TM2 cyan, TM3 yellow, TM4 pink, EL1 helix purple, IL1 helices green, c-tail helices red, beta sheets magneta, primary structure light magenta) comparable to respective sequence and topology templates Innexin6 and Connexin26 (Innexin6 and Connexin26 illustrated via EM ribbon models.) Note the N-tail tucked within the membraneous region (blue double-arrow) as experimentally determined, residue positions match experimental data.[63, 22, 171, 198]

4.5.3 Single/Multiple Template-Target Monomers
Single template aligned Innexin6 and Connexin26 and multiple template aligned Innexin6-Connnexin26 generated unsuccessful skewed topological A-chains.

4.6 Galaxy Oligomer Models
Input A-chain subunit Pannexin-1.B99990001.pdb and refinement settings Tails (1-19, 407-426), gating regulating EL1 (67-85) (Table 22.) Galaxyhomomer generated a best elected hexamer amongst all oligomers and refinements generated.[78] Although this hexamer exhibited symmetry,

a compact outer-pore region and satisfactory diameters, its inflated lower pore-structure and loops and coils exhibiting steric clashes precluded this model from subsequent docking selection. Galaxy FASTA sequence inputs similarly generated an overall best elected hexamer oligomer and overall best Pannexin-1 model exhibiting consistent satisfactory diameters and an overall structure matching experimental data, this model was subsequently selected towards docking simulations. (MolProbity model validation software exhibited a 78th percentile score out of a best 100% towards clashes, rotamer and Ramachandran evaluations for the sequence input generated Panx1 hexamer, the monomer input generated hexamer's MolProbity score was substantially lower.)[219]

Adding to the bulk of the literature reports supporting a hexamer structure, both the sequence and monomer input generated oligomer modeled pentamers, heptamers and octamers (via oligomeric state selection and default setting) exhibited structural problems at the subunit interfaces and steric clashes within the coils and tails resulting in pore and oligomer diameters not fitting reported sizes, moreover generated oligomers outside of the hexamers exhibited inner pore lining TM1 and EL1 domains not matching SCAM experimental data.

4.6.1 GalaxyHomomer Hexamers

Both monomer and sequence inputs resulted in an Ab Initio Docking methodology via default due to zero oligomer templates discovered within the structural database.

4.6.1.1 Galaxy Scores

Ab initio Docking Results

Model No	Number of subunits	Interface area	Docking score	Loops/termini that may be refined	Re-submit for refinement
1	6-mer	11958.7	1126.893	N/A	Submit
2	6-mer	14887.0	1062.338	N/A	Submit
3	6-mer	9655.0	947.809	N/A	Submit
4	6-mer	8308.4	924.505	N/A	Submit
5	6-mer	9890.0	890.033	N/A	Submit

Table 51.) Ab initio docking results displaying interface area and docking scores towards a best selected Pannexin-1 hexamer generated via an input monomer, best score 2 was selected.[78]

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Ab initio Docking Results

Model No	Number of subunits	Interface area	Docking score	Loops/termini that may be refined	Re-submit for refinement
1	6-mer	16432.5	1330.357	251-257	Submit
2	6-mer	13422.6	1274.161	251-257	Submit
3	6-mer	13675.6	1041.152	251-257	Submit
4	6-mer	6187.8	884.699	251-257	Submit
5	6-mer	7913.5	801.861	251-257	Submit

Table 52.) Ab initio docking results displaying interface area and docking scores generated via a FASTA input, highest score 1 was selected.[76]

4.6.1.2 Monomer vs. FASTA Hexamers

The FASTA input GalaxyHomomer more closely matched Pannexin-1 experimental data.

|-----------------------14 nm----------------------| |-----------------------16 nm----------------------|
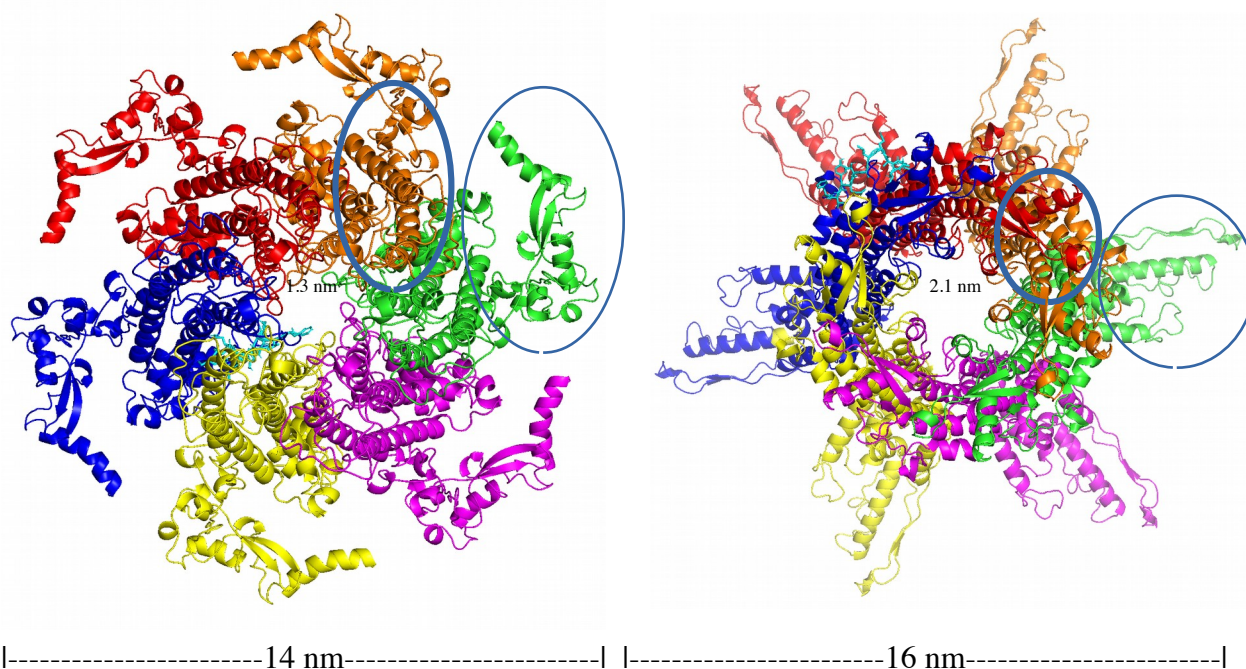
Figure 22.) Pymol visual of monomer and FASTA Galaxy generated hexamers respectively viewed from the extracellular space. Both exhibit fair diameters, monomer membraneous tucked N-terminal (monomer residues 1-12 cyan stick model) exhibited, the FASTA model's lower helices exhibit a much greater thermodynamically favorable compact conformation (thick blue ellipses) with substantially more optimized tails and loops (thin blue ellipses).[73]

99
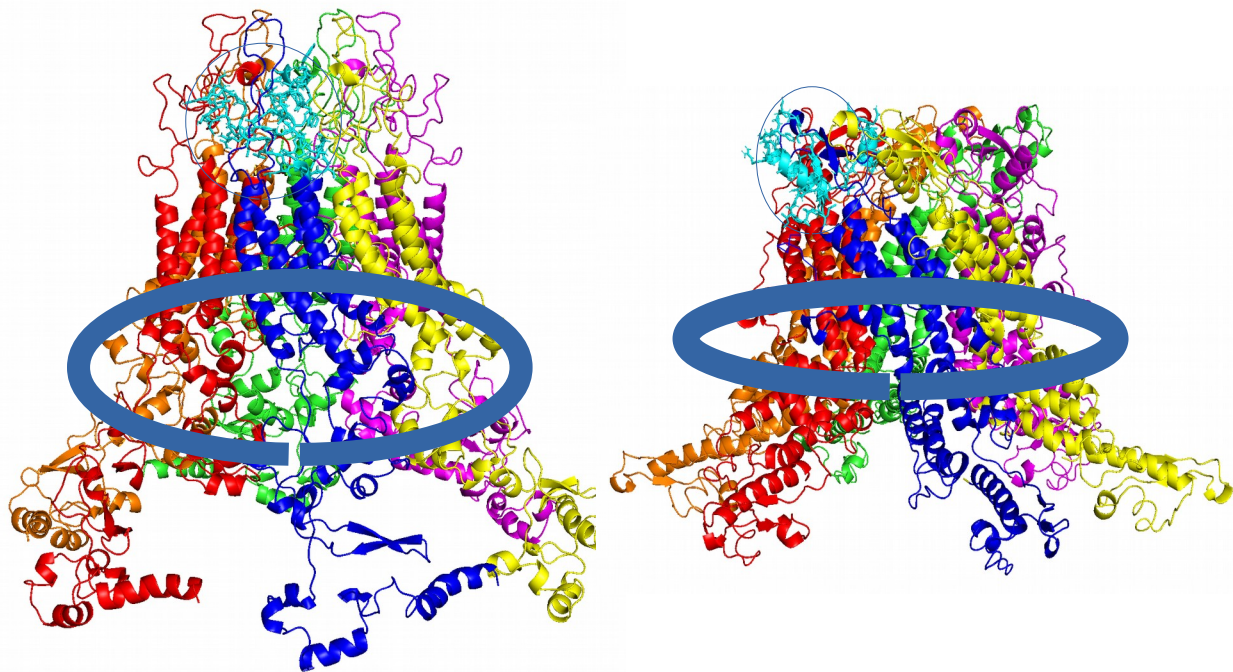
Figure 23.) Respective monomer and FASTA Galaxy generated Pannexin-1 models side-view via Pymol exhibiting residues 57-107 EL1 in stick cyan in apropos extracellular space. FASTA input exhibits a greater lower compact helices conformation precipitating a more compact transmembrane region throughout (thick blue-ellipses). EL1 regions via the FASTA version exhibit a greater optimized conformation via charged residue repulsions within the loops (thin blue-circles).[79]
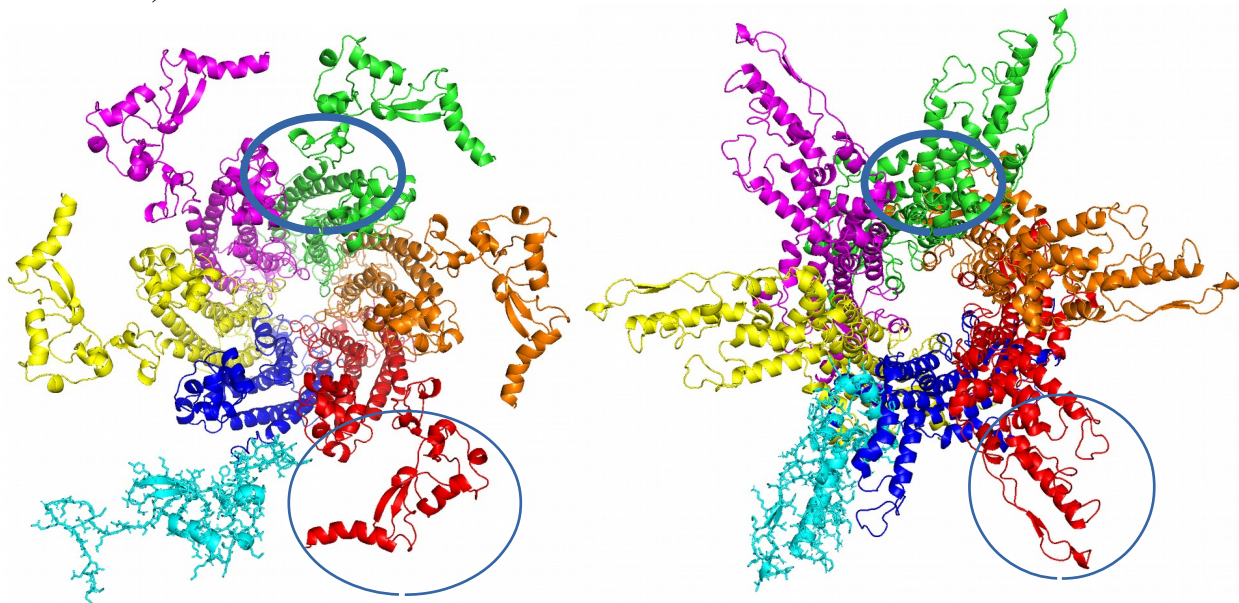
Figure 24.) Cytoplasmic view, respective monomer and FASTA generated hexamers with FASTA model generation exhibiting substantially greater lower helices compaction (thick blue-circles) and greater coil/tail electrostatic repulsion (thin blue-circles) promoting consistent pore and oligomer sizes respectively and a better overall thermodynamic structure (residues 306-426 C-tail as cyan stick model.)[80]
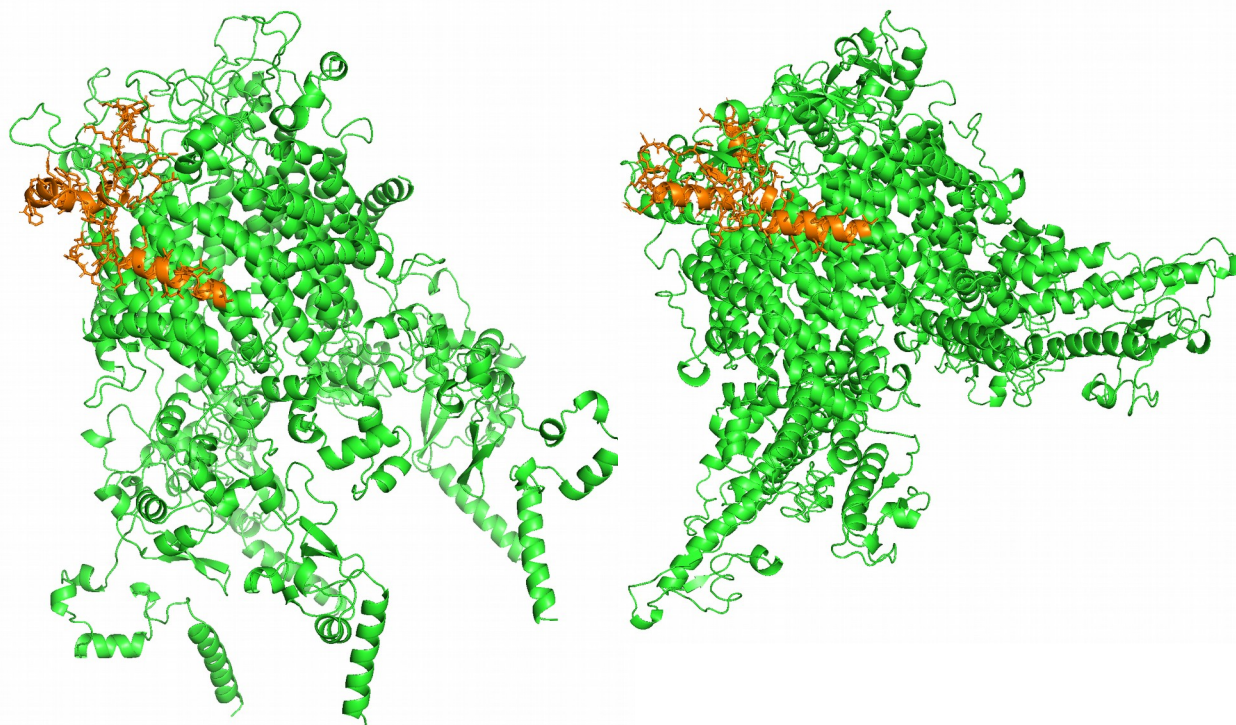
Figure 25.) Monomer and FASTA hexamer elected models respectively exhibiting a TM1 and EL1 outer pore lining (orange stick model) matching experimental SCAM data.[81]
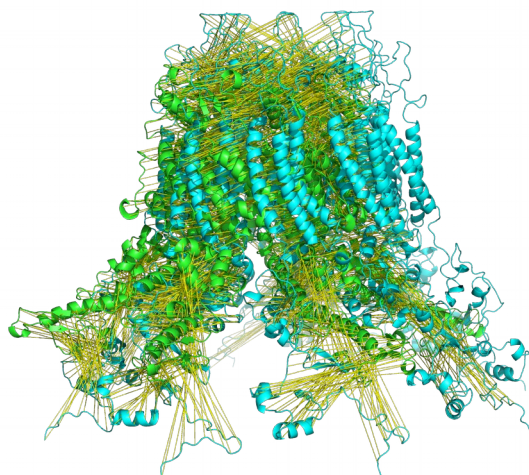
Figure 26.) Pymol monomer and sequence based hexamers superimposed exhibiting residue-residue correspondence (yellow-sticks). Green sequence based hexamer exhibiting a consistently compact pore region and optimized loops and tails, cyan monomer based hexamer exhibits steric clashes amongst its loops and coils and inflated lower transmembrane helices.
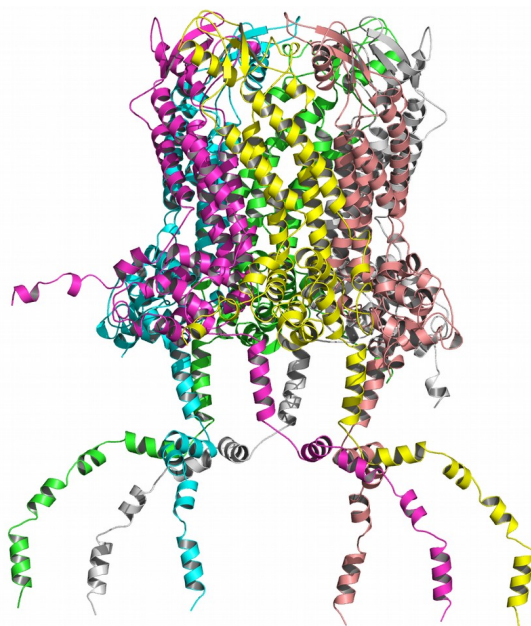
Figure 27.) GalaxyHomomer Pannexin-1 FASTA input method predicted symmetrical Pannexin-1 hexamer homo-oligomer model exhibiting consistently compact intra-membraneous helices with dynamic disordered optimized tails and loops, side-view.[74]
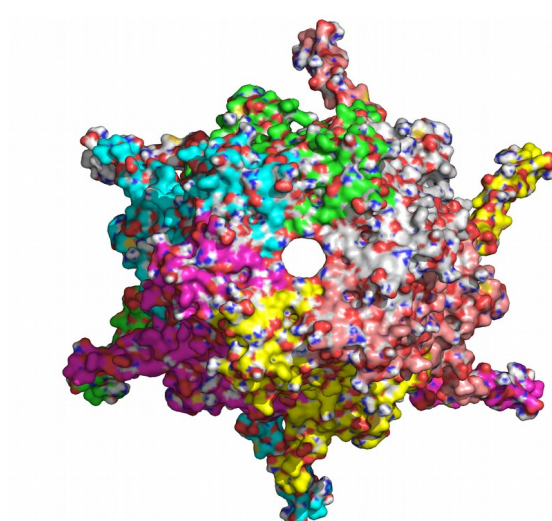
Figure 28.) GalaxyHomomer FASTA input method predicted symmetrical Pannexin-1 hexamer model viewed from the extracellular space exhibiting an apropos compact pore size via energetically favorable compact helical conformations.[72]
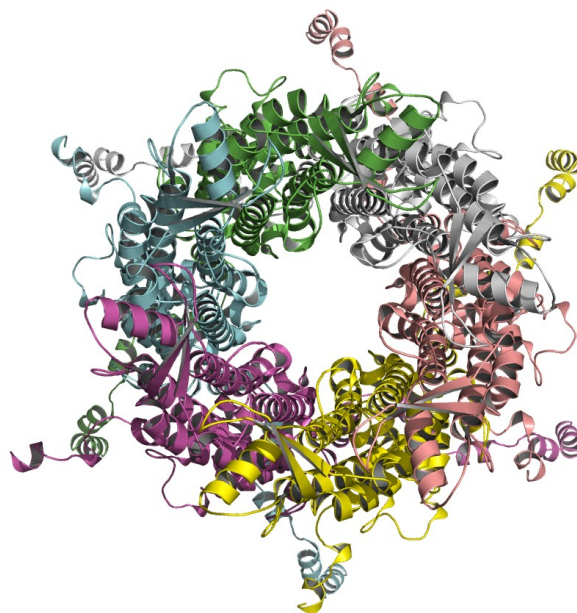
Figure 29.) GalaxyHomomer FASTA input method predicted symmetrical Pannexin-1 hexameric model viewed from the extracellular space exhibiting dynamic disordered optimized tails and loops and highly compact helices resulting in oligomer and consistently compact pore sizes matching Pannexin-1 experimental data.[71]

4.7 Ligand-Target Experimental and Docking Dynamic Simulation Results
4.7.1 Experimental Results
Experimental alanine mutant results proved food-dye and probenecid binding sites and or gating structural activity within the Panx1 EL1-74 and EL2-237, 240, 247, 266 residues.[69]

Experimental mutant results proved CBX binding sites and or gating structural activity within the Panx1 EL1-74 and Conserved Region EL1 67-86.[15]
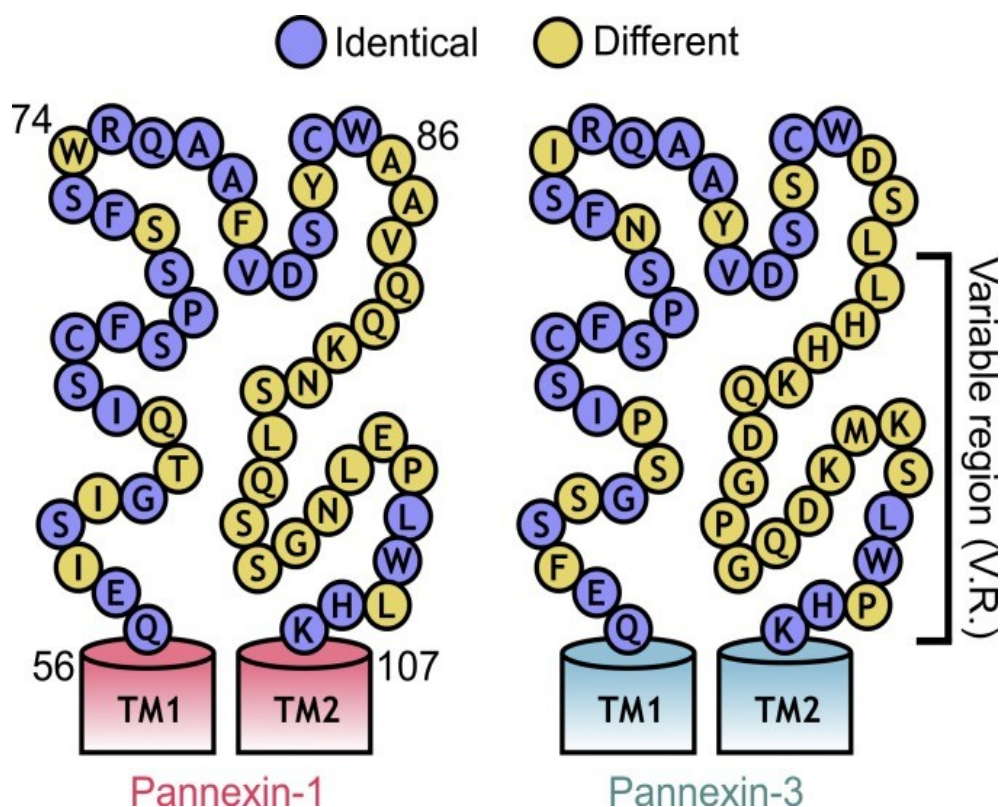
Figure 30.) Pannexin-1 Extracellular Loop Region 1 (EL1) with both conserved and non-conserved residues outlined. CBX has proven that the conserved region promotes Pannexin-1 gating activity.[67]

Experimental dye and channel current results have demonstrated the [10]Panx1 mimetic peptide to most likely steric block the hexamer pore region ([10]Panx1 may activate a docking gate via a cryptic binding needing further elucidation).[2, 8, 66]

4.7.2 In Silico Results

In silico dockings overall exhibited weak to high affinity binding free energy values using the proven partners known to experimentally block the Pannexin-1 channel inferring an overall fair regional modeling accuracy. Similar to Adenosine Receptor activation, conserved internal water molecules pivotally connect the extracellular cavity to the intra-Pannexin-1 space via H-bonding

to ligands and conserved Panx1 residues promoting a most native ligand-receptor apropos juxtaposition (MedusaDock via default discovers and docks to a best/lowest energy ligand binding residue-specific conformation). Ligand-receptor dockings also exhibited electrostatic or polar interactions and steric effective nonbonding and steric match via a steric augmentation of products exhibiting a smallest steric strain.[222, 224, 226]

4.7.2.1 Pore Docking Results

The Mimetic [10]Panx1 docked to the modeled hexamer pore region hydrogen-bonding and steric effectively nonbonding to residues S93, Q95, S96 and E97, four Panx1 subunits exhibited bonds. Mimetic [10]Panx1 exhibited a high binding affinity inferring a high pore modeling accuracy, ligand binding partially steric blocked the hexamer pore-region matching experimental data.
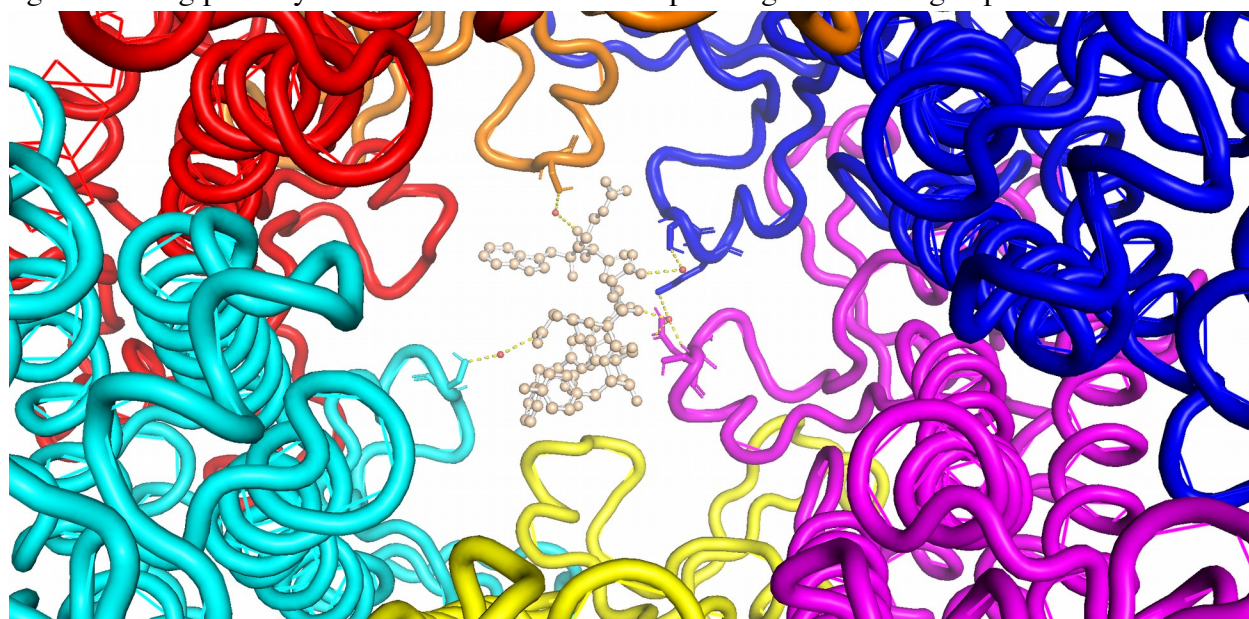
Figure 31.) A cytoplasmic view of Mimetic [10]Panx1 bound to the Panx1 non-conserved EL1 Pore Region via a hydrogen-bonding water arrangement (red-dots are water molecules).[126]

High binding affinity exhibited in the Panx1-EL1 pore region:
PAX_10PANX.pdb
REMARK E_total: -44.4728 kcal mol^{-1}
REMARK E_without_VDWR: -46.38 kcal mol^{-1} [186]

4.7.2.2 EL-1 Docking Results

EL-1 docking results exhibited outer Pannexin-1 polar interactions and nonbonding steric effects amongst all four-chemicals. Binding position indicates a gating allosteric event.
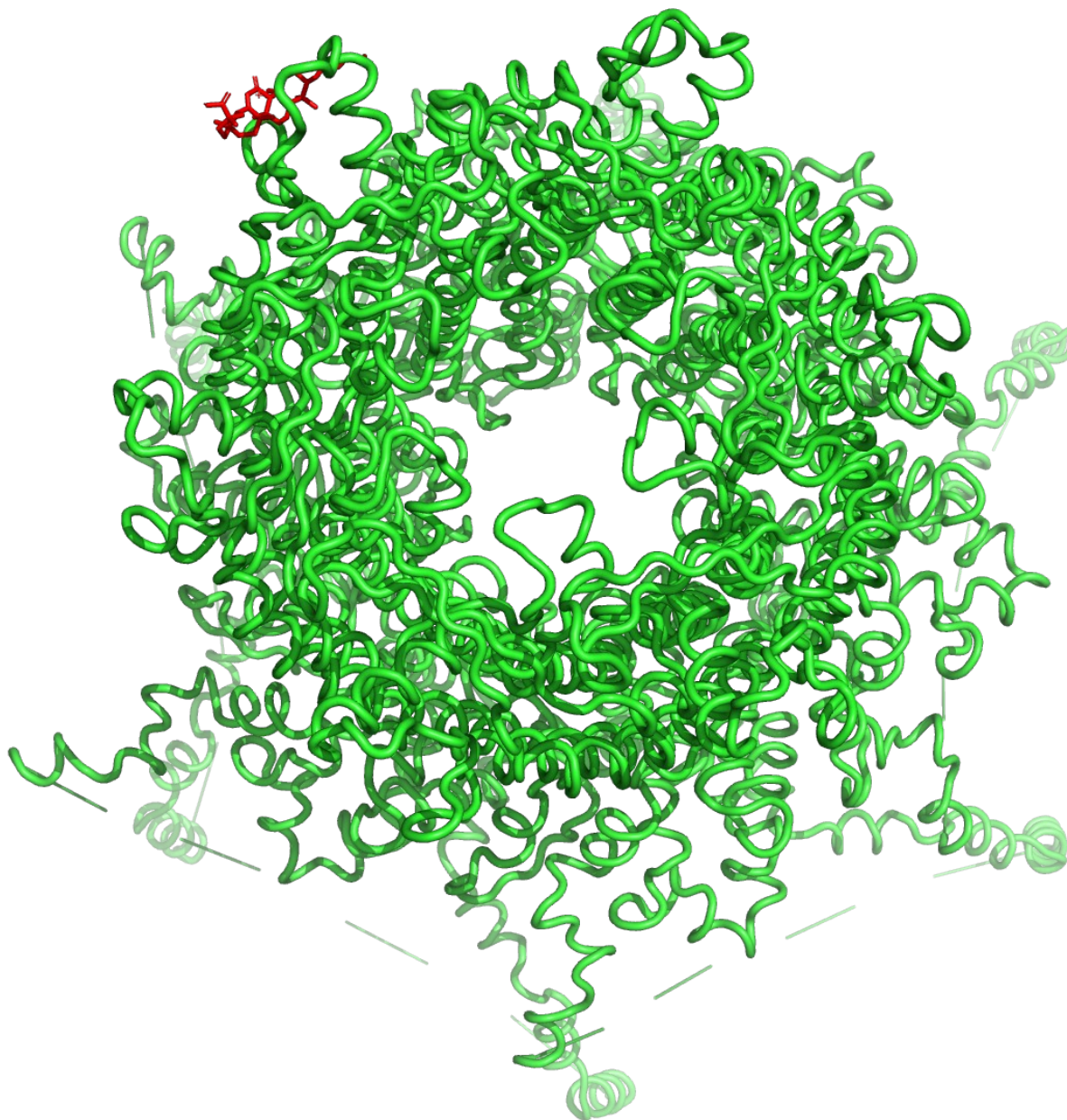
Figure 32.) CBX nonbonding steric effect on the outer Panx1 EL-1 experimentally proven region.[49]
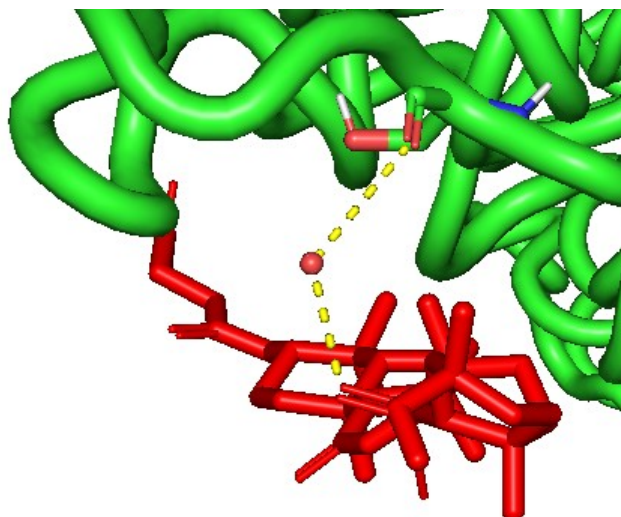
Figure 33.) Above Pannexin-1-CBX interaction inflated exhibiting 3 Å H-bonding red-dot water molecule to CBX, a polar interaction of water to pivotal residue 68 > 3.1 Å in the EL1 and CBX hydrophobic to Panx1 hydrophobic residue steric effective nonbonding.[48]

Poor to high binding affinities exhibited in the Panx1-EL1 region inferred an overall fair EL1 modeling accuracy.

PAX_Carbenoxolone.pdb
REMARK E_total: -23.4778 kcal mol^{-1}
REMARK E_without_VDWR: -25.3025 kcal mol^{-1}

PAX_brillianblue.pdb:
REMARK E_total: -27.5987 kcal mol^{-1}
REMARK E_without_VDWR: -29.5413 kcal mol^{-1}

PAX_Fastgreen.pdb
REMARK E_total: -31.5298 kcal mol^{-1}
REMARK E_without_VDWR: -34.8731 kcal mol^{-1}

PAX_Probenecid.pdb
REMARK E_total: -15.3546 kcal mol^{-1}
REMARK E_without_VDWR: -15.5225 kcal mol^{-1} [186]

4.7.2.3 EL-2 Docking Results

Probenecid and green and blue food dyes docked to the experimentally determined Panx1 EL2-237, 240, 266 region via polar interactions and nonbonding steric effects.

Poor to high binding affinities exhibited in the Panx1-EL2 region inferred an overall fair EL2 modeling accuracy.

PAX_Probenecid.pdb
REMARK E_total: -15.949 kcal mol^{-1}
REMARK E_without_VDWR: -16.0732 kcal mol^{-1}

PAX_Fastgreen.pdb
REMARK E_total: -26.6799 kcal mol^{-1}
REMARK E_without_VDWR: -31.2314 kcal mol^{-1}

PAX_brillianblue.pdb
REMARK E_total: -31.8357 kcal mol^{-1}
REMARK E_without_VDWR: -33.294 kcal mol^{-1} [186]

Chapter 5

Discussion

5.1 Interpretation of Main Findings

Pannexin-1 is a ubiquitous transmembrane protein regulating homeostasis and pathology via autocrine/paracrine channel signaling. The significance of solving this beginning Pannexin-1 structure in silico will contribute to subsequent studies further elucidating the physicochemical characteristics, binding properties and gating mechanisms of the Panx1 channel. Together these contributions along with a deeper understanding of Pannexin-1's homeostatic conduits will lead to a therapeutic gating controlling a multitude of physiological functions.

Experimental evidence indicates the Pannexin-1 to be a symmetrical un-apposed hemichannel homohexamer. An input MODELLER generated Panx1 sub-unit and Panx1 amino acid sequence GalaxyHomomer generated hexamers most closely matching established documented experimental evidence, both exhibited topologies and orientations similarly matching published data. All other oligomeric states generated exhibited structural problems at the subunit interfaces and steric clashes within the coils and tails resulting in pore and oligomer diameters not fitting reported sizes. Between the input sub-unit and sequence based hexamers, the later exhibited a best overall structure via a consistently thermodynamic compact pore region with highly optimized coils and loops.

A GalaxyHomomer sequence input method generates monomers via a multiple-template based GalaxyTBM tool. GalaxyTBM generated a more favorable potential docking sub-unit than MODELLER via its overall approach of least contaminating templates, conservative core modeling and MODELLER CSA heightened optimization. GalaxyTBM specially selected a 2HDI_B template compatible to its selected top template 5h1q (Innexin6). Moreover 2HDI_B was the only other template selected covering residues (353-426), this precluded template contamination via conflicting constraints upon convergence precipitated when greater than three templates are selected (the MODELLER generated A-chain used five-templates). GalaxyTBM models conserved cores first and ab initio models ULR (Unreliable Local Regions) loops and coils subsequently increasing modeling accuracy (MODELLER models all regions

simultaneously). GalaxyTBM updated MODELLER CSA more rigorously optimizes (minimizes forces between atoms) in multiple template cases minimizing conflicting template restraints/contamination during model building resulting in a better converged monomer.[223]

Pannexin-1 pore closure can be precipitated in vitro via a mimetic peptide steric block or via a conformational change within the pore region via Pannexin-1 chemical inhibitors. Overall Medusa flexible guide docking results within this study exhibited moderate ligand-receptor affinities using experimentally determined data inferring a fair regional modeling accuracy. Medusa explicit water addition and each ligand exhibiting <10 rotatable bonds further strengthened results via exhibiting a more closely resembling native ligand-receptor juxtaposition and a lowering of binding modes respectively.

Mimetic [10]Panx1 exhibited a high affinity (-46.38 kcal mol^{-1}) to the Pannexin-1 pore region. Moreover, via Pymol Visualization, this docking event exhibited experimentally inferred steric block. Accordingly, the designed Pannexin-1 model pore region within this study is non-trivial. The precise design of the conserved region of the Panx1 Extracellular Loop Region 1 (EL1) is pivotal, particularly non-conserved residue W74, due to its proven Pannexin-1 pore gating activity via conformational change. Food dyes and CBX together exhibited fair docking affinities in this region pushing the validity of the Panx1 EL1 model, however, Probenecid's affinity of -15.5225 kcal mol^{-1} may warrant a greater EL1 elucidation. CBX is a known and proven regulator of Pannexin-1 closure via EL1 binding sites and gating structural activity. CBX docking is nonbonding steric/hydrophobic and polar, hydrophobic interactions stabilize ligand receptor binding and hydrogen bonding optimizes these hydrophobic effects.[220] Together these two weak intermolecular interactions increased ligand receptor binding affinity enough to infer a fair EL1 regional modeling. Similarly exhibiting chemical inhibitor binding sites and gating structural activity, the Panx1 EL2 region was overall docked reasonably well via chemical inhibitors inferring a fair modeling of the EL2 region. Probenecid's poor affinity value of -16.0732 kcal mol^{-1} similarly may warrant EL2 model refinement or may alternatively strengthen EL1 and EL2 model results via Probenecid docking affinity value corroboration i.e. in this case maybe a finely tuned steric nonbonding conformation may be required to successfully trigger a finer gating mechanism.

Chapter 6

Conclusion

6.1 Final Statement

The main hypothesis of this study is predicated upon the modeling of a yet uncrystallized transmembrane protein called Pannexin-1 that controls a multitude of functions towards physiology and homeostasis. Pannexin-1 was designing in silico exhibiting a thermodynamically compact pore region, expected overall hexameric structure and validated regional accuracy. The pivotal regional domains responsible for gating were validated via overall moderate in silico docking affinities exhibited by various chemicals and one mimetic peptide known to experimental block the Pannexin-1 channel. This proven protein design may be used in future studies to control Pannexin-1 gating to combat disease.

6.2 Limitations

Pannexin-1 experimental blocking action is yet elusive and may be only gating regulated, the modeled Panx-1 EL1/2 regions are located distal to the pore. The largest inhibitor, Mimetic [10]Panx1 exhibiting a high affinity and pore steric block in silico, may instead bind a cryptic site regulating pore-gating. Moreover [10]Panx1 being a larger ligand may have generated false positives via force field scoring inaccuracies being proportional to ligand-receptor contact area.[47] Inaccurately placed ligand-receptor interface waters may additionally have generate false positive affinity values.[225]

This preliminary data is yet limited and expresses a beginning Pannexin-1 model. The Pannexin-1 model is not co-crystallized and therefore does not include any potentially pivotal water molecule arrangements within the Panx1 model structure which would transfer its 3-D space adding to its 3-D accuracy. Pannexin-1 pore conformational closure via the former inhibitors has not yet been exhibited via Molecular Dynamic Simulations, accordingly this designed Pannexin-1 model requires further validation exhibiting structural changes towards pore closure.

6.3 Future Research

This investigated software designed Pannexin-1 hexamer model is a start towards future Pannexin-1 regional-inhibitor/promoter modifications. A lengthy and exhaustive subsequent study using Molecular Dynamic Simulations will discover the pivotal residue inhibitor/promoter/ and arranged water electro-steric interactions involved in Pannexin-1 gating via exhibiting structural changes within the channel. Subsequently a nanoparticle may be engineered exhibiting a tag that triggers a Panx1 conformational closure, this development will lead to in vitro and in vivo testings.

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