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## *Review:*

# In silico strategies on prion pathogenic conversion and inhibition from $PrP^{C}$ –$PrP^{Sc}$

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**Article highlights**

- No suitable anti-prion drug has been identified so far.
- Efficiency of anti-prion compounds was based on multifactorial nature of the disease.

- Pocket-D is the most important binding pocket for prion inhibition and conversion from $PrP^{C}$-$PrP^{Sc}$.
- The salt bridges between $Arg^{156}$-$Glu^{196}$ and $Arg^{156}$- $His^{187}$ play an important role in prion folding.
- Presence of oxymethyl groups and electro-negative nitrogen enhance anti-prion activity.
- Pharmacophore analysis gives us more knowledge of drug binding to $PrP^{C}$ hotspots.
- Conformations of amyloid fibrils and protein oligomers are very important for future anti-prion drug discovery.

***Introduction***: To date, various therapeutic strategies identified numerous anti-prion compounds and antibodies that stabilize $PrP^{C}$, block the conversion of $PrP^{C}$-$PrP^{Sc}$ and increased effect on $PrP^{Sc}$ clearance. However, no suitable drug has been identified clinically so far due to the poor oral absorption, low blood–brain-barrier [BBB] penetration, and high toxicity. Although some of the drugs were proven to be effective in prion-infected cell culture and whole animal models, none of them increased the rate of survival compared to placebo.

***Areas Covered:*** In this review, the authors highlight the importance of *in silico* approaches like molecular docking, virtual screening, pharmacophore analysis, molecular dynamics, QSAR, CoMFA and CoMSIA applied to detect molecular mechanisms of prion inhibition and conversion from $PrP^{C}$-$PrP^{Sc}$.

***Expert opinion***: Several *in silico* approaches combined with experimental studies have provided many structural and functional clues on the stability and physiological activity of prion mutants. Further, various studies of *in silico* and *in vivo* approaches were also shown to identify several new small organic anti-scrapie compounds to decrease the accumulation of $PrP^{res}$ in cell culture, inhibit the aggregation of a $PrP^{C}$ peptide, and possess pharmacokinetic characteristics that confirm the drug-likeness of these compounds.

**Key words**: Prion, Docking, Molecular Dynamics (MD), QSAR (Quantitative Structure Activity Relationship), CoMFA (Comparative Molecular Field Analysis), CoMSIA (Comparative Molecular Similarity Indices).

## 1. Introduction

Prion disease is characterized to be lethal for both humans and animals. They occur by the deposition of an abnormal proteinase K-resistant isoform $PrP^{Sc}$ or $PrP^{res}$ in the brain [1] [2]. Studies have shown that prion disease arises when the normal cellular protease sensitive form of prion protein, $PrP^{C}$ [$PrP^{sen}$], which is rich in α-helix, is converted into an abnormally folded,

disease-related isoform $PrP^{Sc}$, which is beta rich [3]. Studies have shown that this processes of conversion from $PrP^{C}$-$PrP^{Sc}$ takes place through an intermediate form of $PrP^{C}$ represented as PrP* with the help of another protein named as protein X [4] [5] [6]. Once the conversion starts, the deposition of $PrP^{sc}$ will increase enormously causing the disease invariably fatal [4]. Currently, no effective therapy or vaccine exists due to long incubation periods ranging from months to decades without showing any signs of the disease. Consequently, numerous studies have been directed towards the development of therapeutics for preventing the conversion of $PrP^{c}$ to $PrP^{sc}$ involved in neurodegeneration despite, the lack of a detailed understanding of the cellular mechanism of prion propagation. To date, various compounds like quinacrine and its structurally related tricyclic anti-depressants [7-9], statins [10], pyrazolones [11], indole-3-glyoxylamides [12, 13], and pyridyl hydrazones [14] including 'Compound B', have been shown to reduce $PrP^{sc}$ accumulation in a cell culture model of prion diseases. Later, pyrazolone compound has been shown to be up to 130 fold more effective compared to quinacrine in inhibiting the accumulation of $PrP^{sc}$ [15]**.** In addition, larger polyanionic or polycationic molecules [e.g., dendritic polyamines of PAMAM] were reported to exhibit anti-prion activity in cells [16] [17]. Except for PAMAM, none of the approved drugs or experimental compounds were reported to lower levels of $PrP^{Sc}$ in stationary-phase cells [18]. Once the therapeutic activity of Congo red was discovered, more amyloid dye derivatives and glucoseaminoglycan mimetics have been used as possible candidates for treating prion diseases [19] [20]. Studies also shown that a new class of amyloidophilic chemicals, styrylbenzoazole derivatives was shown as effective as anti-prion compounds with a more discrete labeling of amyloid deposition in brain tissues affected by prion diseases, which have better penetration through the blood-brain barrier [21] [22]. The compound "GN8" could interact with N-terminal domain of $PrP^{C}$. However, the studies of the chemical shift changes caused by "GN8" binding show that the major binding region is located at C-terminal domain [23]. The compounds, 2-aminothiazoles that represent a promising new class of drug leads for prion diseases were also discovered that improve metabolic stability and permeability in mice. Some of these inhibitors show stronger inhibitory activities toward SHaPrP [24]. In contrast, a variety of compounds with a large structural diversity was identified as high potent inhibitors and accelerators of $PrP^{C}$ [25]. Although the two compounds, tacrolimus and aztemizole were already marketed as anti-prion drugs, they were withdrawn from the US market because of possible neurotoxicity and rare cardiac arrhythmias when used at elevated levels. Micromolar treatment of furamidine derivative DB772 on sheep microglial using sheep derived prion strains showed the minimal effect on cell viability and near-maximal anti-prion activity [26]. Initial medicinal chemistry efforts have also identified four aryl amides differing in their N-linked aryl groups doubled the survival of prion-infected mice. However, none of these compounds has shown efficacy against CJD (Creutzfeldt–Jakob disease) prions [27]. Recently, drug-like, brain-penetrant iron tetrapyrrole derivative showed inhibition of prion replication and $PrP^{C}$ mediated toxicity. Nevertheless, these studies are still under investigation [28]. Thus, the current challenge of developing the most efficient compounds was based on multifactorial nature of the disease which is difficult to understand experimentally. This

review will provide the necessary information for future therapeutic research, both in laboratory models and in clinical trials.

## 2. In silico studies of anti-prion compounds

The molecular docking strategy is a standard high-throughput screening method of choice to filter anti-prion compounds *in silico*. Using rational structure-based drug design, two inhibitors of $PrP^{Sc}$ accumulation in ScN2a [scrapie-infected mouse neuroblastoma] cells were identified that specifically bind to $PrP^{C}$ residues: $Gln^{168}$, $Gln^{172}$, $Thr^{215}$, and $Gln^{219}$. Moreover, *in silico* screening of 210,000 compounds for their ability to block $PrP^{Sc}$ formation in ScN2a cells yielded 63 potential inhibitors, resulting in the identification of the inhibitor with an $IC_{50}$ of 18 μM [29] **[29]**. However, none of the compounds identified in the ScN2a cell culture system were proven effective in prion-infected mouse models. Out of 1050 pyridine dicarbonitriles screened, 45 compounds were selected for synthesis. Finally, in vitro screening using surface plasmon resonance has selected a total of 19 compounds bound to different conformers of prion protein [30]. The most effective compound 'GN8' fits into the pocket-C between the α1-β2 loop and α2 to α2-α3 loop created by distant residues $Asn^{159}$ and $Glu^{196}$ and inhibits the formation of $PrP^{Sc}$ [23] **[Fig.1]**. Fragment molecular orbital calculations also proved that four amino acids $Asn^{159}$, $Gln^{160}$, $Lys^{194}$, and $Glu^{196}$ are important for the bridging conformation of the GN8-$PrP^{C}$ complex [31]**.** By using these studies several binding poses were predicted, in agreement with NMR studies using docking and all-atom MD refinements. The calculated dissociation of free energy [7.8 ± 0.9 kcal/mol] agrees with experimental dissociation constant [Kd] of 3.9 μM, corresponding to $\Delta G^{0}$ = -7.5 kcal/mol [32]. Based on their binding-free energies, a set of anti-prion compounds were classified into five categories as: [I] binders and effective, [II] low binders and effective, [III] binders and not effective, [IV] low binders and not effective, and [v] accelerators [25]. Screening a library of 149 water soluble metabolites identified thiamine as a prion ligand with a binding constant of ~60 μM using a combination of 1D NMR, fluorescence quenching and surface plasmon resonance. Pharmacophore analysis using computer-aided docking and molecular dynamics, revealed the common features of interaction with other thiamine binding proteins [33]. Docking studies also revealed that thiamine binding to pocket-B between α1 and L1 is similar to other thiamine binding proteins [34] **[Fig.1]**. Further studies on 2-aminothiazoles have shown that the compounds with quinoline bind with higher affinity to pocket-D between α1 and α2 and α3 loop than isoquinoline and naphthalene groups [35] **[Fig.1]**. Previous studies also showed that tetracycline strongly binds to solvent exposed functional sidechains of threonine's 190-193 on α2 [36]. Recently, Kamatari and co-workers classified anti-prion compounds based on four potential molecular mechanisms of action: [I] specific conformational stabilization of $PrP^{C}$; [II] nonspecific stabilization; [III] promotion of $PrP^{C}$ aggregation and precipitation [IV] interactions with $PrP^{Sc}$ or membrane proteins [37]**.** The methoxychalcones and oxadiazoles that were active in reducing $PrP^{res}$ levels by more than 50%

at a 1μM concentration in cell culture was shown to interact directly with $PrP^C$**.** Anti-prion compounds against murine $PrP^C$ revealed that most prevalent binding modes occurred between α2 and the antiparallel β-sheet [38]. Virtual screening followed by cluster analysis identified two compounds BMD42-29 and BMD42-35 with strong interactions in the "GN8" binding site [39]. Some of these ligand protein complexes were further studies using molecular dynamics and montecarlo simulation studies to see the effect of ligand on prion protein stability.

## 3. Molecular Dynamics (MD) on prion pathogenic conversion

MD simulations of human $PrP^C$ revealed that both wild type and mutant Glu200Asp maintained the native protein structure, whereas Glu200Lys partially unfolds [40]. Under the strongly acidic condition, tertiary structure becomes more compact after 10-ns simulations stabilized by parallel secondary structures and a large number of new, non-native contacts between the side chains. Protonation of $Asp^{202}$ and $Glu^{196}$ disturbs the stability of the native fold by eliminating a single negative charge at one of the key sites. Such changes in the tertiary structure were not observed in the simulations with higher temperature. According to these studies, the most fluctuations of the human prion protein occur in the mutant model [PDB: 2K1D] at "GN8" binding pocket with residues ranging from $Thr^{190}$ to $Lys^{194.}$ Homology modelling and structural dynamics of the buffalo $PrP^C$ mutant [$BufPrP^C$] at residue 143 have shown five hydrogen bonds and a strong salt bridge between $Asp^{178}$–$Arg^{164}$ [O–N] keeping the β2–α2 loop intact. Mixed Monte Carlo and MD simulations of the human prion protein mutant Asp178Asn could cross a free-energy barrier that resulted in the unfolding of α1 due to the loss of a specific hydrogen bond between α1 and α3, involving residues $Tyr^{149}$ and $Asp^{202}$ [43]. Non-Markovian *metadynamics* method showed that antiparallel β-sheet in the pathogenic Asp178Asn mutant is significantly weaker than in the wild-type mouse $PrP^C$ [44]. Furthermore, the structural instability was shown larger with higher RMSD (Root Mean Square Deviation) in Asp178Asn mutant compared with wild type with a stable Cation–π interaction [45]. When $His^{187}$ is mutated to Arginine, the hydrophobic core of $PrP^C$ is exposed due to a breakdown of the salt bridge between $His^{187}$–$Arg^{156}$ [N–O] linking α-helices α2 and α1. The protonation of $His^{187}$ leads to loss of interaction between two PrP subdomains. Parallel simulations at pH 2 showed an intermediate stable β-rich structure in the formation of $PrP^{Sc}$, indicating that misfolding may precede dimerization [46]. In the presence of Trimethylamine N-Oxide, simulations at lower pH also showed lower helical content and higher β-sheet yielding a $PrP^{Sc}$-like state [47]. Mutant structural studies of Ala117Val globular domains [109-228 and 90-228] finally showed an increase in the β-sheet compared with wild type. Essential collective dynamics revealed that the β-strand β1, and the loop β1-α1, exhibit relatively high levels of variability, dynamical disorder and local flexibility**.** When applied to ovine $PrP^C$, the α2α3 dimer interface shows strong intra-molecular and inter-molecular correlations relative to the β-sheet dimer interface [48]. By combining mutagenesis and molecular dynamics on OvPrP, the conformationally stable β-sheet was observed as the possible nucleus of oligomerization, which is in good correlation with deploymerization kinetics of purified α2α3 oligomers [49]. Recent MD simulations on

monomeric soluble state of mouse $PrP^C$ suggest that $Tyr^{169}$ stabilizes the $3_{10}$-helical conformation of the β2-α2 loop more than the single-point mutants Tyr169Gly, Tyr169Ala, Tyr169Phe, Arg164Ala, Phe175Ala, and Glu178Ala [51]. Binding of "GN8" to flexible spots on α2 near $Glu^{196}$ prevents urea-induced denaturation of $PrP^C$ [41]**.** Further studies using MD simulations showed that NPR-053 and -056 bind to same "GN8" binding site of $PrP^C$ around the residues $N^{159}$, $Q^{160}$, $K^{194}$ and $E^{196}$ [42]. The energy calculations based on MM-GBSA [Molecular mechanics with generalized Born and surface area solvation] estimated the primary binding mode of Congo red and GNNQQNY (Pocket A in Fig.1) protofibril to be more stable than the secondary binding mode by -5.7 kcal/Mol. Solid-state nuclear magnetic resonance analyses followed by MD simulations of luminescent conjugated polythiophenes revealed that anionic side chains interacted with regularly spaced cationic residues of amyloid fibrils**.** Interestingly, the most favorable binding energy obtained was shown to be highly effective therapeutically [50]. Overall, these studies predict the importance of salt bridges between $Arg^{156}$-$Glu^{196}$ and $Arg^{164}$-$Asp^{178}$, and $Arg^{156}$- $His^{187}$ in stabilizing $PrP^C$

## 4. QSAR, CoMFA and CoMSIA studies of anti-prion compounds

QSAR studies of 2-aminothiazoles indicated that asymmetric molecules having high nitrogen content and low propensity to form hydrogen bonds are highly potent anti-prion compounds. In addition, 3D-QSAR of tetracycline derivatives revealed the presence of hydroxyl groups, electron donors, alkylamine substitution and NMe2 group in a non-epi configuration are predicted to possess anti-fibrillogenic activity [52]. Further, studies using CoMFA and CoMSIA maps reveal that the compounds with oxymethyl groups and electro-negative nitrogen are highly favorable to enhance anti-prion activity [35]. Recently, it was concluded that anti-prion activities of small molecules are greatly influenced based on shape of the molecular surface area, distribution of charge, ability to form contacts, and the presence of nitrogen atoms [53]. These results predict that electronegative nitrogen plays an important role in anti-prion activity of small molecules computationally.

## 5. Conclusion

Although extensive research has been done on prion disease, a suitable method of diagnosing the prion disease is yet to be discovered. The promising therapeutic that was identified for preventing prion disease was proved to be disappointing when subsequently tested *in vivo* for increasing the rate of survival. To compensate experimental studies, *in silico* strategies were used to identify several characteristics of folding pathway and protein aggregation on a molecular level. These studies could provide useful information for *in silico* drug discovery against prion disease targeting $PrP^C$. Undoubtedly, the pharmacophore analysis of $PrP^C$-ligand complex obtained using molecular docking gives us a more accurate understanding of drug binding to hot spots of $PrP^C$. Further advanced studies should be developed in future to evaluate these effects in different experimental models of disease using NMR of the compounds–$PrP^C$ complexes.

## 6. Expert Opinion

Despite the multipronged approach to tackle the conversion of $PrP^{C}$ to $PrP^{Sc}$, there is no effective medication for the transmitted prion disease due to longer incubation periods without showing any signs of the disease. Only few methods exist to detect $PrP^{Sc}$ in the brain of CWD (Chronic Wasting Disease) in animals besides using neuropathological and immune-histochemical methods after death. Peripheral administration of many compounds in prion infected model of vCJD (Variant Creutzfeldt–Jakob disease) in humans was also not shown to be effective. Due to the difference in mammalian and yeast $PrP^{C}$ sequences, a yeast-based screen was not proven useful even though the compounds diminish the propagation of yeast prion proteins [PSI+] & [URE3] [54] [55] [56] [57]. Compounds that were identified in cell-free conversion assays and neuroblastoma-derived N2a cell line are of potential interest, but they are not qualified as drugs due to the lack of efficiency in crossing the BBB. Intra-ventricular infusion of pentosan polysulfate showed adverse effects such as hematoma formation at higher levels. Even though congo red was shown anti-prion activity in an *in vivo* model, the benzidine structure makes it unsuitable for animal or human use because of its carcinogenic and toxic properties [58]. Later, Congo red analogs showed much effective in tissue culture with limited effect *in vivo* [59] [60]. Furthermore, PrP amyloid imaging ligands not only showed anti-prion clearance in cell culture but also showed some effectiveness in Tg20 PrP over-expressing transgenic mice *in vivo* [21] [14]. However, the incubation period was not extended significantly in Tg7 mice and wild-type hamsters infected with 263K $PrP^{Sc}$. Additionally, anti-prion compounds identified in ELISA-based assay utilizing ScN2a cells do not show direct interaction with recombinant PrP [61]. Recent studies on conjugated polythiophenes in prion-infected mice increased the survival rate by only 8%. Detecting the underlying mechanism of these identified anti-prion compounds will be one of the key steps to be further optimize them as molecular chaperones in treating amyloid related diseases. To achieve this goal, several diagnostic methods, namely, protein misfolding cyclic amplication, conformation-dependent immunoassay, dissociation-enhanced lanthanide fluorescent immune assay, capillary gel electrophoresis, fluorescence correlation spectroscopy, flow microbed immono assay, optical Fiber Immunoassay [SOFIA] and real-time quaking-induced conversion [RT-QuIC] etc. were developed precisely to detect $PrP^{Sc}$ sensitivity [62] [63] [64] [65]. However, these assays are selective for compounds that inhibit $PrP^{res}$ formation. Simultaneously, synthetic peptides that were used to inhibit the conversion [$PrP^{sen}$-$PrP^{res}$] have shown the same biochemical properties like non-inhibitory peptides with β-sheets and sedimental $PrP^{sen}$ aggregates [66]. Antibody-mediated therapy using Fab fragments appeared to be promising in animal models but the delivery across the blood-brain barrier became a major challenge due to its shorter half-life [67]. Moreover, vaccine treatment for prion disease is not a good strategy as they need to be given before an infection starts. Although, RNAi approach delayed the onset of disease, all the animals used throughout the study died eventually. Expressing siRNA in mouse embryonic stem cells and neural precursors can be of use in differentiating to specific neuronal type on the site of brain damage, these therapies are still in the experimental phase of development. Due to these failures of time consuming experiments, computational strategies were applied to study the prion aggregation at atomic resolution. These studies indicated that formation of a α-sheet as a common structural transition [68] [69] [70] [71] [72]. Since all atom simulations are computationally expensive, multi-scale modelling is used for

easy comparison of the experimental data by taking the information from coarse-grained models for all atoms as constraints [73] [74] [75]. To avoid the problem of missing important information about critical nuclei, a discontinuous algorithm was utilized for doing MD simulations containing ~100 peptides. The calculated inter-molecular interactions between $PrP^{C}$ and its peptides will show the way to further development of new anti-prion and amyloid fibril inhibitors. Since the potential binding sites of $PrP^{C}$ are broadly distributed, wide range of anti-prion compounds can be detected using virtual screening irrespective of binding affinities [76]. Moreover, ex vivo screening resulted in a novel anti-prion compound, termed "GN8" that works as a chemical chaperone. In contrast, a variety of compounds that was screened computationally with a large structural diversity have therapeutic efficacy against $PrP^{Sc}$ at a rate of 2%. Some of these compounds stabilize $PrP^{C}$ conformation and act as possible candidates for the chemical chaperones [25]. The compound designed using a 3D pharmacophore model of $PrP^{C}$-GN8 complex inhibits $PrP^{Sc}$ with a stronger binding affinity in a high-throughput misfolded protein detection assay than other compounds reported to date [39]. Using both CoMFA and CoMSIA in combination with fluorescence quenching studies, we showed that the compound [N-[4-[3, 4-dimethoxyphenyl]-1, 3-thiazol-2-yl] quinolin-2-amine] binds to pocket-D similar to "GN8" binding site with a $K_d$ value of 46.4 μM. In the same study, we also showed that 1-Substituted bicyclic compounds are more potent than 2-substituted naphthalene [35]. The pymol plugin "NAGARA" that was recently developed, identified several novel anti-prion compounds, including tegobuvir which was approved clinically for HCV infection [77]. Based on these available data, it was expected that *in silico* drug design against the binding pocket of $PrP^{C}$ would be a valuable tool for initial screening of potential anti-prion drugs from huge compound libraries. This provides clues about the small molecules interfering in the regulation of pathogenic conversion in prion infected cell cultures. Although these compounds were more helpful for drug design, these drugs have to be additionally validated using *in vitro* and *in vivo* assays with prion-infected animals. Due to the costs and time consuming, compounds that bind to specific pocket of $PrP^{C}$ will only be synthesized for further evaluation [23] [38]. Still there is a possibility of missing some effective compounds that do not bind to the C-terminal domain of $PrP^{C}$ or that have other molecular targets besides $PrP^{C}$ [61]. If an alternative target for such compounds is $PrP^{Sc}$, the three-dimensional structures of $PrP^{Sc}$ aggregates in the form of dimers, trimers and oligomers should be determined in urgency with the help of supercomputers [78] [79]. This is not an easy task, and deserves attention from the scientific community, especially on the part of biophysicists and computational biologists. Although the selected drugs against $PrP^{C}$ and $PrP^{Sc}$ were effective in infected cell lines of different prion strains, they did not increase the survival time of prion-infected mice [42]. This result reinforces the need for a thorough pharmacokinetic assessment of the most promising molecules. These findings based on both experimental and computational research indicate that prion propagation may be strongly inhibited by targeting auxiliary proteins like plasminogen along with $PrP^{C}$ in future drug discovery. However, the biggest challenge is still underway to discover 1. How the conversion of $PrP^{C}$-$PrP^{Sc}$ cause's prion disease. 2. To diagnose the disease before significant brain damage occurs. 3. The ability of the treatment to distinguish between self and non-self and access to CNS via the blood-brain barrier. 4. How the auxiliary proteins involved in prion protein conversion from $PrP^{C}$-$PrP^{Sc}$. Future studies in the upcoming years may clarify issues about the biological pathways that are dominant or decisive for the process and how it is triggered. In addition, earlier

detection methods of the prion disease may be developed in the future for effective immunotherapy. At present, we are particularly interested to see the pathogenic conformations of amyloid fibrils and protein oligomers in neurodegenerative diseases. My personal opinion is that the molecules designed *in silico* should be tested in distinctive biologic assays at the same time with the normal and scrapie form of prion protein to see the effect of each molecule in different environmental conditions. The molecule which possessed more or less similar biological effect with different prion strains should be taken as lead compound for further optimization to become a clinical candidate. In this process, we strongly believe that various *in silico* approaches will address some of the fundamental unanswered questions in prion biology, especially in the area of protein oligomerization for developing better prion disease models, and suggest some possible therapeutic targets and pharmacological agents respectively.

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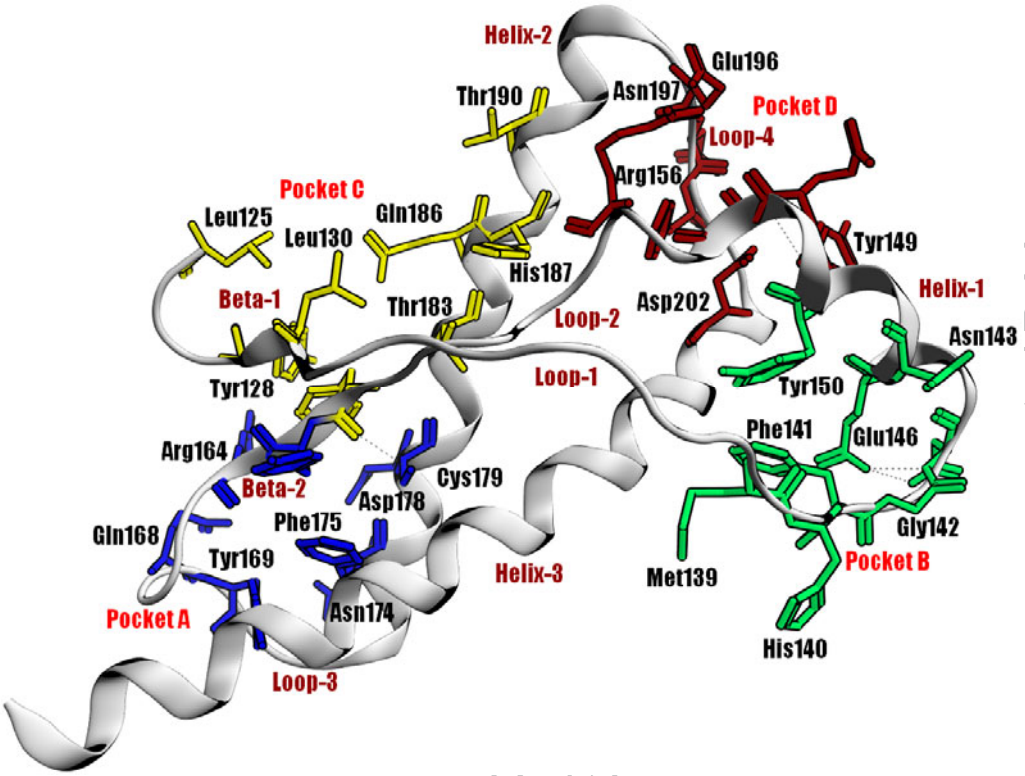


**Fig.1**. Three-dimensional structure of cellular prion protein SHaPrP [PDB: 1B10] predicted using MOE software (Chemical Computing Group Inc, Canada). Alpha helices and beta sheets were shown in white color. Loops are represented as L1, L2, L3 and L4. Residues in the binding pockets were represented in stick mode. Binding pockets [A-D] are represented as Pocket A (Blue), Pocket B (Green), Pocket C (Yellow) and Pocket D (Maroon). Residues in pocket A, B, C and D are represented in blue, green, yellow and maroon colors.

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