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Prions and Transmissible Spongiform Encephalopathy (TSE) Chemotherapeutics: A Common Mechanism for Anti-TSE Compounds?

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ABSTRACT

No validated treatments exist for transmissible spongiform encephalopathies (TSEs or prion diseases) in humans or livestock. The search for TSE therapeutics is complicated by persistent uncertainties about the nature of mammalian prions and their pathogenic mechanisms. In pursuit of anti-TSE drugs, we and others have focused primarily on blocking conversion of normal prion protein, PrP^{C}, to the TSE-associated isoform, PrP^{Sc}. Recently developed high-throughput screens have hastened the identification of new inhibitors with strong in vivo anti-TSE activities such as porphyrins, phthalocyanines, and phosphorthioated oligonucleotides. New routes of administration have enhanced beneficial effects against established brain infections. Several different classes of TSE inhibitors share structural similarities, compete for the same site(s) on PrP^{C}, and induce the clustering and internalization of PrP^{C} from the cell surface. These activities may represent a common mechanism of action for these anti-TSE compounds.

Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are infectious neurodegenerative syndromes of mammals that include bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) of deer and elk, scrapie in sheep, and Creutzfeld−Jakob disease (CJD) in humans. TSEs have incubation periods of months to years but after the appearance of clinical signs are rapidly progressive, untreatable, and invariably fatal. Attempts to develop therapeutic strategies for these diseases are hobbled by gaping holes in the understanding of the transmissible agent (or prion) and the pathologic consequences of its propagation in the host. Nonetheless, recent studies have placed tighter limits on the nature of TSE infectivity, suggested salient features of TSE neurotoxicity, and revealed new anti-TSE compounds and treatment regimens that prolong the lives of infected individuals.

The Nature of TSE Infectivity: Protein-Only Prions?

The full molecular nature of TSE infectivity and its propagation mechanism remain unclear. One critical component appears to be an abnormal form of prion protein called PrP^{Sc}. PrP^{Sc} is defined loosely by its apparent association with TSE infectivity but, otherwise, has variable properties and is poorly understood structurally.[1] Usually, if not always, PrP^{Sc} is multimeric and has greater β sheet secondary structure and protease resistance than normal PrP (PrP^{C}). Relative protease resistance is often used practically to discriminate PrP^{Sc} from PrP^{C} and gives rise to the operationally defined alternative term, PrP-res. PrP^{Sc} is made post-translationally from the normal protease-sensitive prion protein. The mechanism of this conversion is not well understood but involves the ability of multimeric PrP^{Sc} to bind PrP^{C} and induce a conformational change as PrP^{C} is recruited into the growing PrP^{Sc} multimer.

The prion hypothesis posits that PrP^{Sc} is the only necessary component of TSE infectivity.[2] Efforts to test this hypothesis have led to recent reports of the in vitro generation of TSE prions.[3,4] Synthetic truncated prion protein (PrP) fibril preparations were shown to accelerate disease when inoculated into transgenic mice that vastly overexpress the same truncated PrP construct.[4] However, these fibrils were not infectious for normal mice and thus were $\geq 10^{8}$-fold less infectious than bona fide PrP^{Sc}. Although it was concluded that prions had been synthesized from recombinant PrP^{C} alone, the lack of controls leaves open the possibility that the recipient transgenic mice were spontaneously making prions.

In contrast, others have shown compelling evidence for continuous serial amplification of robust TSE infectivity in cell-free reactions containing crude brain homogenate.[3] This landmark result virtually eliminates the possibility that replication of an agent-specific nucleic acid genome is required. However, these studies also do not prove the "prion protein-only" model for TSE infectivity because many other host-encoded molecules besides PrP were present in the reaction.

The Most Infectious Prion Protein Particles

A fundamental question with many neurodegenerative protein misfolding diseases is whether large fibrillar

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deposits or smaller subfibrillar oligomers are the prime causes of disease.[1] To address this question with respect to TSE diseases and characterize the basic infectious unit of TSE infectivity, we have fractionated infectious PrP-containing aggregates by flow field-flow fractionation and compared their infectivity per unit protein (i.e., specific infectivity).[5] Nonfibrillar particles between about 300−600 kDa (mass equivalent to ~14−28 PrP molecules) had much higher specific infectivity than larger fibrils or smaller oligomers (≤5-mers) of PrP. These most infectious particles were ~25 nm in diameter, consistent with particles detected previously in filtration[6] and field flow fractionation[7] experiments. In our analyses, the infectivity levels were nearly proportional to the concentration of *particles* rather than protein, suggesting that as long as PrP^{Sc} oligomers are above a minimal size, they are similarly infectious in vivo.[5] Accordingly, per unit mass, smaller particles are more infectious than larger ones. Although the predominant protein constituent of the "most infectious" particles was PrP, it remains possible that other molecular constituents are important.

Thus, our results also fall short of providing firm support for a protein-only nature of mammalian prions. On the contrary, it seems just as plausible to argue that host-derived molecules besides PrP might be required for robust TSE infectivity. For example, there is growing evidence that sulfated glycosaminoglycans (GAGs),[8−10] nucleic acids, or both could be essential, at least as cofactors in pathological PrP conversion.[11−13] Indeed, as discussed below, compounds such as these, or analogues thereof, can interact with PrP, alter its conformation, and have potent anti-TSE activities. Nonetheless, these findings support the emerging view that with many protein aggregation diseases, smaller nonfibrillar oligomers are more pathological than large fibrils or clusters of fibrils (plaques).

Neuropathologic Mechanisms

Although the enigmatic PrP^{Sc} multimer seems almost certain to be a major component of the transmissible agent, it is not necessarily the main neurotoxin of TSE diseases. Alternative forms of PrP have also been observed that may play primary roles in neuropathogenesis (reviewed in ref 1). Furthermore, there is evidence that the neuropathology of TSE infections is greatly enhanced by the presence of PrP^{C} [14,15] and, more specifically, PrP^{C} that is anchored to cellular membranes by its glycophosphatidylinositol (GPI) anchor.[16] In scrapie-infected transgenic mice expressing only anchorless PrP^{C}, PrP^{Sc} (PrP-res) and TSE infectivity are propagated, but the resulting neuropathological and clinical effects are dramatically reduced.[16] Thus, it is likely that in addition to being the substrate for PrP^{Sc} formation, GPI-anchored PrP^{C} somehow transduces or potentiates the neurotoxicity of TSE infections.

Prophylactic and Therapeutic Strategies

Despite fundamental uncertainties regarding the infectious agent, its replication mechanism, and neuropathological manifestations, a number of anti-TSE interventions have been pursued. An important but elusive goal is to be able to treat the disease after the appearance of clinical signs. This will most likely involve some combination of inhibiting PrP^{Sc} formation, destabilizing existing PrP^{Sc}, blocking neurotoxic effects of the infection, and promoting the recovery of lost functions in the central nervous system (CNS). Another worthwhile goal is to reduce the risk of infection in the first place by neutralizing sources of infection, blocking infections via the most common peripheral routes, or blocking neuroinvasion from the periphery. Although immunotherapies are being pursued with some tantalizing results,[17,18] we have focused primarily on chemotherapeutic approaches. Although no clinically proven anti-TSE drug has been developed, significant progress has been made, especially in identifying compounds with prophylactic activity.

In Vitro Screens for Anti-PrP^{Sc} Compounds

Most TSE drug discovery efforts to date have attacked PrP^{Sc} accumulation.[17] Our usual approach has been first to screen for inhibitors using TSE-infected cell cultures and then to test the most promising inhibitors against scrapie infections in rodents. Higher throughput screens have enabled the testing of thousands of compounds against multiple strains of murine and sheep scrapie in cell cultures.[19,20] Recent development of the first deer cell line chronically infected with CWD has enabled us to begin screening compounds for activity against this cervid TSE disease as well.[21] Unfortunately, no cell lines are available that are infected with BSE or human CJD, despite the great significance of these TSEs to public health and agriculture. The importance of testing compounds against multiple TSEs in multiple cell types is indicated by the striking species and strain specificities of PrP^{Sc} inhibitors that have been observed already.[19,20]

Testing in Animals

A much slower process in TSE drug development is the testing of compounds against infections in animals. Despite possible problems with strain and species dependence of anti-TSE compounds, most in vivo testing has been done in rodents, which allow for much faster and less expensive screening than is possible in the natural, large-animal host species. Drug treatments initiated after high-dose intracerebral inoculations test for potential therapeutic activities in hosts with established CNS infections, the most difficult challenge in TSE therapeutics. Often it is also of interest to test for prophylactic protection against lower dose inoculations by peripheral routes (e.g., intraperitoneal).

Anti-TSE Compounds

A growing list of compounds has been reported to have anti-TSE activity in vitro and in vivo (Table 1). Of those that are known to inhibit PrP^{Sc} accumulation in TSE-infected cell cultures, many, but not all, also have pro-

Table 1. Compounds with in Vivo Anti-TSE Activity

class or compound	examples	inhibit PrP^{Sc} in infected cell culture	activity prior to or soon after ip TSE inoculation	activity post-ic TSE inoculation or clinically	refs
sulfonated dyes	Congo red, suramin	+	+	+	40,55,56
sulfated glycans	pentosan polysulfate, dextran sulfate	+	+	+	52,57,58,59
polyoxometalates	HPA23	+	+	-	59,60
cyclic tetrapyrroles	porphyrins, phthalocyanines	+	+	+	23,24,25,34
polyene antibiotics	amphotericin B, MS8209	+	+	+	28,29,61
quinolines	mefloquine, quinine, quinidine	+	-	±	31,33,62
metal chelators	penicillamine	+	+	?	63
DMSO		+	±	±	24,64
flupirtine		+	?	+	65
tetracyclines	doxycycline	-	±	-	66,67
peanut oil		?	+	?	68
prednisone		?	+	?	69
phosphorothioate oligonucleotide		+	+	?	26,27

phylactic anti-scrapie activity against peripheral (e.g., intraperitoneal) infections in vivo. The most effective examples, such as, pentosan polysulfate,[22] certain cyclic tetrapyrroles (cTPs),[23−25] and phosphorothioated oligonucleotides (PS-ONs)[26,27] can more than triple survival times of rodents inoculated intraperitoneally with high scrapie titers (e.g., 10^3-10^4 lethal doses) and completely protect animals receiving lower titers. In contrast, few compounds are known to have any beneficial effects if treatment is initiated after infection of the CNS. Many of the test compounds that are effective prophylactically have problems with blood−brain barrier penetration due to high molecular weight, charge, or both. Exceptions include the polyene antibiotics,[28,29] which have significant toxicity problems. Much attention has been given to the anti-malarial drug quinacrine, which has anti-scrapie activity in cell culture,[30] crosses the blood−brain barrier, and is being administered to numerous CJD patients. However, there is no clear evidence that quinacrine is effective in vivo. We have found that the same is true of mefloquine (another anti-malarial drug),[31] curcumin (unpublished results), and a number of other CNS-permeable compounds that potently inhibit PrP^{Sc} formation in cell culture.[32] In the absence of evidence of anti-TSE efficacy in vivo, it is hard to understand the rationale for continued clinical trials of quinacrine against CJD.

Delivery of Anti-TSE Compounds into the Brain

To bypass the blood−brain barrier, Doh-Ura and colleagues have used osmotic pumps to deliver PrP^{Sc} inhibitors such as pentosan polysulfate directly to the brains of rodents via intraventricular cannulas.[33] As a result, significant extensions of scrapie incubation period were observed even with treatments directed against established CNS infections. Based on those results, similar intraventricular administrations of pentosan polysulfate have been initiated in human CJD patients, but the effects of such treatments are not clear.

cTPs, that is, porphyrins and phthalocyanines (Figure 1), are among the most promising of the anti-TSE compounds. Compounds of this class are PrP-res inhibitors in cultured cells infected with sheep scrapie, mouse scrapie, and mule deer chronic wasting disease.[20,21,23] As noted above, cTPs can have strong prophylactic anti-scrapie activity rivaling that of pentosan polysulfate.[24,25] Although some porphyrins are thought to cross the blood−brain barrier to some extent, this may not be true of our cTPs that are the most effective when used prophylactically or in cell cultures.

To test the efficacy of these compounds against CNS infections, we have directly injected cTPs into the brain as a crude substitute for Doh-Ura's sophisticated intraventricular osmotic pumping technique.[34] When weekly injections of the anionic Fe(III)*meso*-tetra(4-sulfonatophenyl)porphine (Fe-TSP) were initiated 2 weeks after a high dose (10^6 lethal doses) intracerebral scrapie inoculation, the survival times increased by an average of 51%. Interestingly, indium- and zinc-bound TSP and various metal complexes of a cationic porphyrin *meso*-tetra(4-*N*,*N*,*N*-trimethylanilinium)porphine (TMP) had no statistically significant effects in the same experiment. In another experiment, porphyrins were mixed directly with the scrapie brain inoculum just prior to intracerebral injection to test for an ability to mask or decontaminate infectivity. Interestingly, Fe-TSP was less active in this protocol than Fe-TMP, which increased survival times as if the inoculum were diluted by 10^3-10^4.

Structure−Activity Relationships of cTPs

Compounds from each class of cTP in Figure 1 have shown anti-TSE activity in cell-free PrP conversion reactions, cell cultures, and animals.[20,21,23−25,34] Many different types of structures were active, whereas others with seemingly similar structures were much less active. The results obtained thus far suggest that for anti-TSE activity, numerous permutations of cTP structure can often be

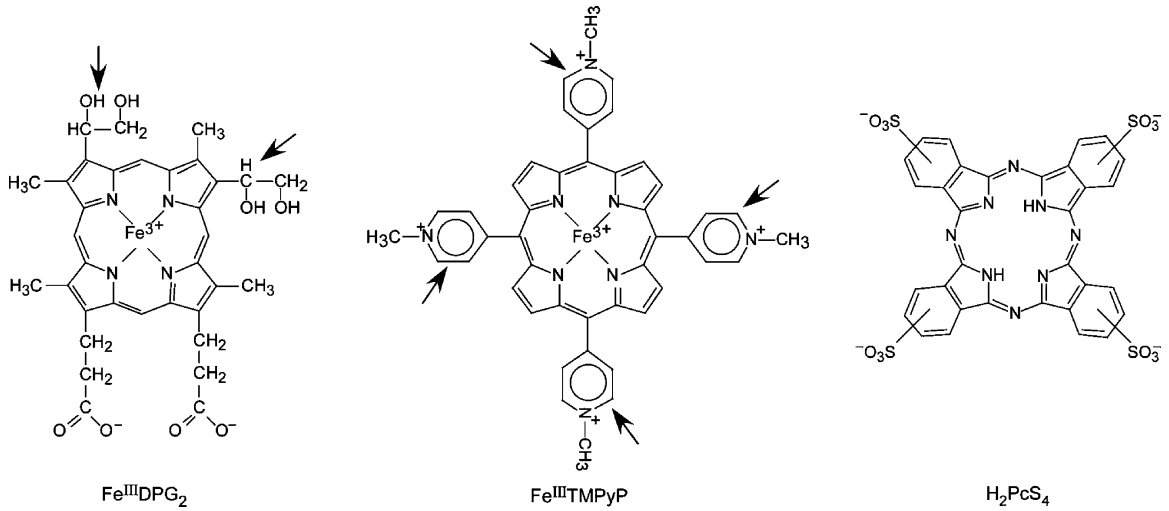

FIGURE 1. Representative cyclic tetrapyrrole (cTP) structures with anti-TSE activity. The cTPs most extensively studied have structures related to these. On the left, iron(III) deuteroporphyrin IX 2,4-bis(ethylene glycol), designated Fe^{III}DPG$_2$, represents one of many deuteroporphyrin IX derivatives with different substituents at the 2 and 4 ring positions (indicated by arrows). In the center, iron(III) *meso*-tetra(4-*N*-methylpyridyl)-porphine (Fe^{III}TMPyP) represents synthetic porphyrins that possess aryl substituents (denoted by arrows) on the linking meso carbons but no peripheral ring substituents on pyrrole moieties. Aryl substituent variations include cationic 4-*N*,*N*,*N*-trimethylanilinium and anionic phenyl-4-sulfonates (not shown). On the right, phthalocyanines with one to four sulfonic acid peripheral substituents are represented by phthalocyanine tetrasulfonate (H$_2$PcS$_4$). The structure shown does not designate specific binding sites for each sulfonate group in that the preparations we have used were mixtures of isomers.

tolerated, but their influence can depend on other structural elements and the type of anti-TSE assay employed. Such differences include peripheral ring substituents and centrally bound metals.

One property that appears to correlate with anti-TSE activity is the ability to assemble into supramolecular aggregates. Aggregation of many phthalocyanines and porphyrins to dimers, trimers, and higher-order oligomers in aqueous media is well-known. The extent of such self-aggregation is influenced by cTP structure and concentration, as well as the solution conditions.[35,36] Certain cTPs can also occupy sites on proteins, nucleic acids, and other polymers as both monomers and π-stacked aggregates.[35,37] In solution, aggregate formation could affect cTP tissue bioavailability, whereas assembly on the surface of a biopolymer such as PrP^C or PrP^{Sc} could block PrP conversion, propagation of infectivity, or both.

Comparison of anti-TSE activity with self-aggregation propensity for various metal PcS$_4$'s (Figure 1) supports a relationship between the two properties. Specifically, the Al^{III} derivatives exhibited much lower anti-TSE activities in vitro than did metal-free PcS$_4$ or several other metal PcS$_4$'s.[23] At the same time, the Al^{III} derivative has a lower tendency to aggregate in aqueous media than the others.[36] Further studies are needed to test the role of supramolecular assembly in cTP anti-TSE activities. Fortunately, a variety of techniques can be used to monitor the nature of cTP interactions with themselves and with proteins.[35,36] Furthermore, the use of cTPs in several other medical areas has provided useful information on the biodistribution, toxicity, retention, and methods of administration of cTPs. Particularly notable are the frequently low toxicities of cTPs.[37−39]

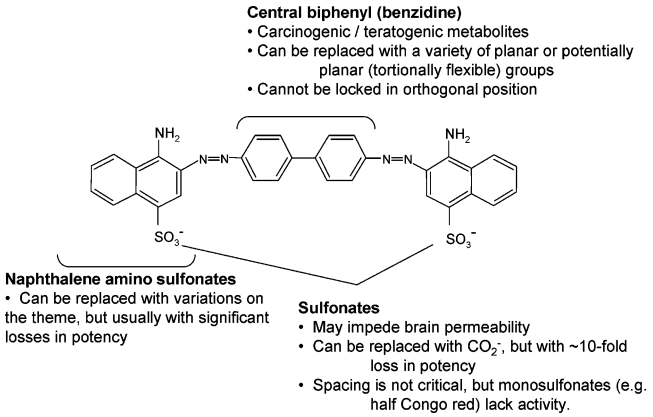

FIGURE 2. Structure−activity relationships of Congo red and analogues.

Structure−Activity Relationships with Other Anti-TSE Compounds

Like the cTPs, several other types of inhibitors of PrP^{Sc} accumulation that we have identified are planar, highly conjugated, multi-ringed molecules that are likely to have the ability to form π-stacked aggregates or similar interactions with planar nonionic surfaces on PrP molecules. Those with the best activity in vivo also tend to have one or more charged or polar moieties on the edges of the planar ring system. For example, the prototypic PrP^{Sc} inhibitor Congo red[40−42] is a sulfonated dye (Figure 2) that is thought to form stacked aggregates within proteins such as RNA polymerase[43] and immunoglobulins[44] (Figure 3C).

Also notable are the observations that oligonucleotides, which contain polyanionic backbones and π-stacked bases, bind to PrP^C and induce conformational changes.[11,12,45] More to the point are observations of PrP^C binding, PrP^{Sc} inhibition, and anti-TSE activity by phos-

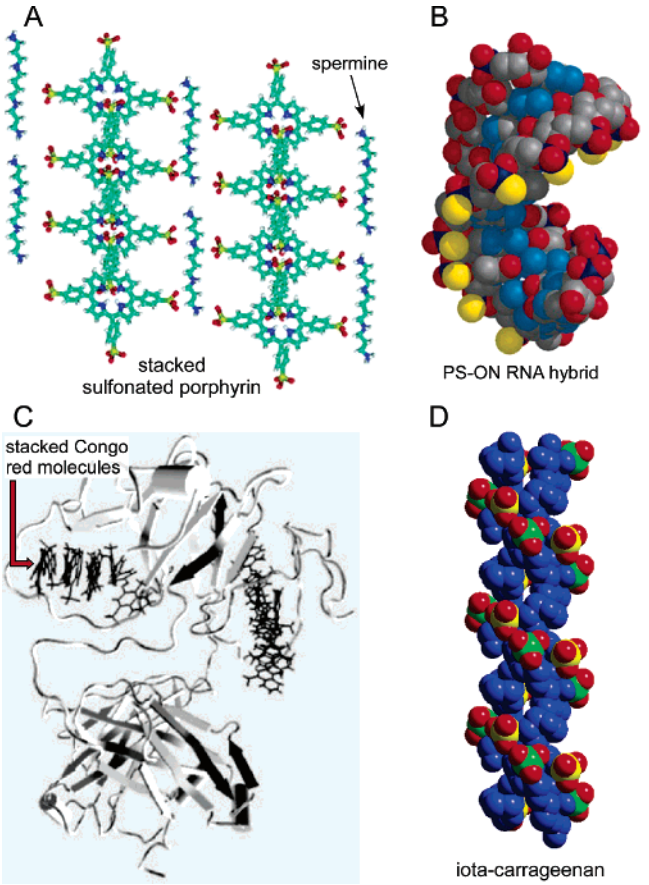

FIGURE 3. Structural similarities among different classes of anti-TSE compounds. Like the phosphorothioated oligonucleotides (PS-ONs) and sulfated glycans, planar π-stacked supramolecular aggregates of sulfonated cTPs and dyes can be extended structures with periodic negative charges and hydrophobic surfaces. Panel A shows a molecular model of tetrakis(4-sulfonatophenyl)porphyrin molecules stacked in the "J" grouping in association with the polyamine, spermine. Reproduced with permission from ref 70. Copyright 2005 Royal Society of Chemistry. Panel B shows molecular graphics of a 10-base phosphorothioate oligonucleotide hybridized with a complementary 10-base RNA. Reproduced with permission from ref 71. Copyright 2003 Biophysical Society. Panel C shows a molecular dynamics simulation of four Congo red molecules stacked in a pocket of immunoglobulin L chain λ. Reproduced with permission from ref 44. Copyright 2005 Wiley Interscience. Panel D shows an X-ray diffraction-based double-helical structure of iota-carrageenan[46] (courtesy of S. Janaswamy & R. Chandrasekaran, Purdue University).

phorothioated oligonucleotides (PS-ONs).[26,27] The importance of the extended oligomeric character of PS-ONs was indicated by the strong dependence of activity on polymer length.[27] PS-ON inhibition was also dependent upon the phosphorothioate modification of the oligonucleotide backbone, which adds hydrophobicity to the polymer, but was mostly independent of base composition. Even sulfated glycan inhibitors such as pentosan polysulfate, a polysaccharide containing ~12−18 pentose disulfate sulfate units, and iota-carrageenan, a double helical sulfated glycosaminoglycan,[46] have structural analogies to both PS-ONs and stacked oligomers of sulfonated dyes and anionic cTPs, namely, repeated negative charges and hydrophobic domains (Figure 3).

A Common Inhibitor Binding Site on PrP

These analogies raise the possibility that the anionic cTPs, sulfonated dyes, PS-ONs, and sulfated glycans exert their inhibition by binding to PrP molecules at the same or overlapping sites. Indeed, competitive binding studies have shown that sulfated glycans compete with Congo red[47] and PS-ONs[27] for binding to PrP^C. It is tempting to speculate that the dimensions of this common inhibitor binding site on PrP^C corresponds approximately to a PS-ON 25-mer because inhibitory activity is reduced substantially with shorter PS-ON polymers.[27] In that case, multiple cTPs, sulfonated dyes, and other planar aromatic molecules might stack together to mimick polymeric PS-ONs or sulfated glycans (Figure 4). The display of multiple alternating anionic and nonpolar surfaces by such oligomeric inhibitors suggests that the binding site on PrP^C should include repeated cognate cationic and nonpolar surfaces. Such surfaces might be provided by the five octapeptide repeats and additional pseudorepeats in the flexible amino-terminal domain. Each repeat contains a cationic histidine residue and an aromatic tryptophan (or tyrosine) residue. The histidines might pair with anionic substituents on the edges of the inhibitors, while the tryptophan side chains could interact with nonpolar surfaces and even intercalate between planar aromatic regions of inhibitor molecules (Figure 4). Analyses of the sulfated glycan binding site on PrP^C by several groups have produced evidence for the involvement of residues in three different segments of the amino acid sequence: the highly cationic amino-terminal residues, the octapeptide repeats, and a more carboxy-terminal site containing residues 110−128, with differing views as to which residues are most important.[48−50] We expect that the residues involved in binding different classes of anionic PrP^{Sc} inhibitors might vary somewhat, depending on the size and specific nature of the particular inhibitor. For instance, long sulfated glycans or PS-ONs might be able to bind to residues in all three segments of PrP^C, while the smaller planar aromatic inhibitors might have a preference for interacting with the tryptophan side chains of octapeptide repeats. In addition, planar aromatic inhibitors with anionic substituents might also be able to π-stack against themselves while forming ion pairs with adjacent PrP^C molecules as depicted in the figure at the amino-termini of the PrP^C molecules.

Whatever the precise PrP binding mechanism(s), one net effect of these inhibitors in several cases is the aggregation of PrP^C in cells. For instance, it is known that pentosan polysulfate,[49] sulfonated dyes,[51] and the PS-ONs[27] cause PrP^C to cluster on the surface of cells and then become internalized. Furthermore, we have found that Congo red and cTPs (R. Kodali and B. Caughey, unpublished data) can cause aggregation of recombinant PrP^C. Hence, in the model depicted in Figure 4, we show PrP^C molecules being pulled together by the inhibitors. In each case, it seems plausible for these inhibitors to serve as a bridge between PrP^C molecules. With this in mind, it is noteworthy that activity is eliminated by cutting

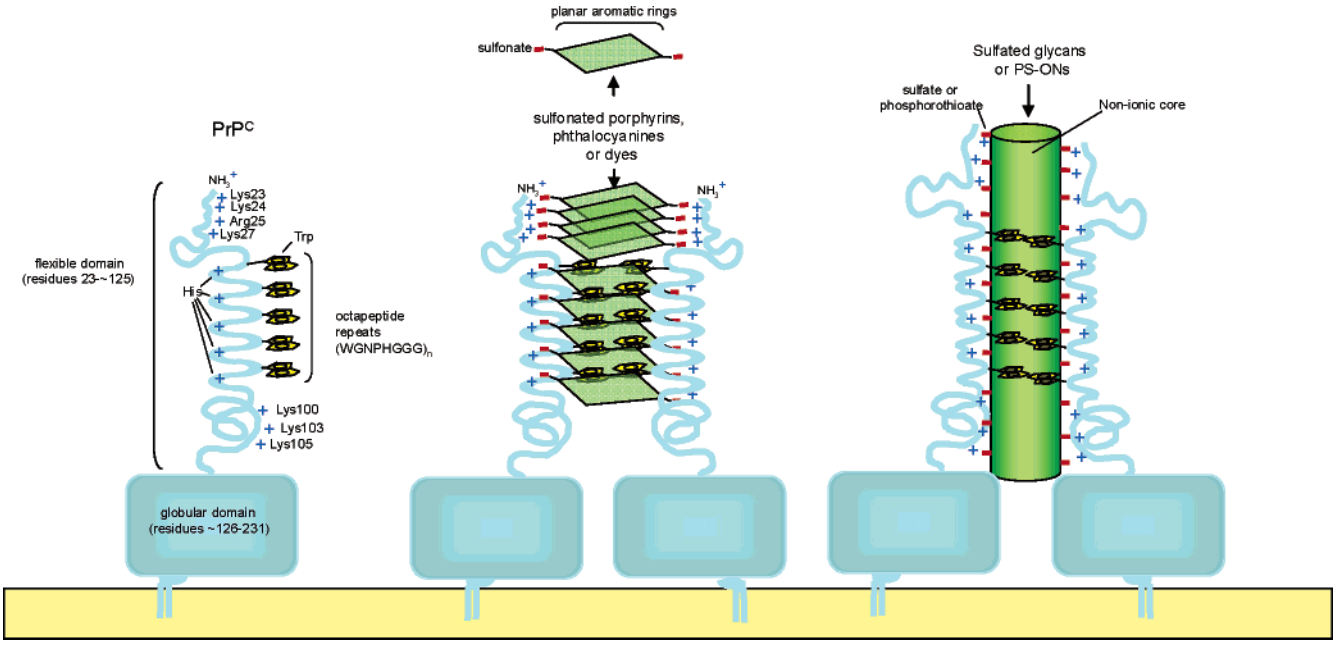

FIGURE 4. Model of possible interactions between PrP^C and various PrP^{Sc} inhibitors that cause PrP^C aggregation. The left panel shows diagrammatic PrP^C structure emphasizing the planar aromatic tryptophan side chains in the octapeptide repeats and cationic residues in regions that have been implicated in sulfated glycan binding as described in the main text. In the middle panel, planar aromatic sulfonated inhibitors such as the sulfonated porphyrins, phthalocyanines, and azo dye molecules (e.g. Congo red) are shown to be stacked directly against one another while ion-pairing with cationic residues at the amino-terminus, and co-stacked with tryptophan (Trp) side chains in the octapeptide region while ion-pairing to histidine (His) residues. In the right panel, extended polyanionic inhibitors such as sulfated glycans and phosphorothioated oligonucleotides are also shown to bind via similar ion pairs and hydrophobic interactions with aromatic side chains in the octapeptide repeats. These interactions could result in dimerization (as shown) or higher order clustering of PrP^C molecules as has been observed on the cell surface with several of these types of inhibitors.

Congo red in half[41] (see Figure 2) or removing a third ring system in some planar aromatic polyphenols.[19] Such molecules may lack sufficient planar aromatic area to be able to bind two PrP^C molecules at once. Although for simplicity we show the dimerization of PrP^C, the formation of higher order PrP^C aggregates might well be induced in a similar fashion by the inhibitor molecules or their supramolecular aggregates. Alternatively, it remains possible that aggregation of PrP^C is not mediated directly by the inhibitor molecules as depicted in the model but by induction of aggregation-prone conformations in PrP^C. At the cellular level, the PrP^C aggregation caused by these classes of inhibitors may lead to sequestration of PrP^C in a state or subcellular location that is incompatible with conversion to PrP^{Sc}.

Implications for Physiological Mechanisms of PrP Function and Conversion

The fact that several different structural classes of PrP^{Sc} inhibitors share certain properties, PrP binding sites, and abilities to cause PrP aggregation and internalization begs the question of how these phenomena might relate to the normal function of PrP^C and the mechanism of conversion to PrP^{Sc}. More specifically, it seems likely that these inhibitors bind to a site normally reserved for physiological ligands that are important in the conversion to PrP^{Sc}. Prime candidates for such ligands are sulfated glycosaminoglycans such as heparan sulfate, which bind to PrP^C,[47,52] associate with PrP^{Sc} deposits in vivo,[53] and support PrP conversion.[8,9] Consistent with this view is the observation that many of the PrP^{Sc} inhibitors discussed above can be viewed as glycosaminoglycan analogues or mimics. If PrP molecules interact with polyanions, then it is also reasonable to expect that the polycationic inhibitors (e.g., branched polyamines[54] and cationic cTPs[23,34]) could mask cellular polyanionic molecules such as GAGs that must bind to induce and stabilize the conversion of PrP^C. Polycations might also interact directly with PrP, possibly via bridging cations. In addition, crucial interactions with other cellular ligands and surfaces might be directly or indirectly affected by inhibitor binding. While such effects may block PrP^{Sc} formation, they might also have negative consequences relating to functions of PrP^C. Hopefully, further studies of the normal and disease-associated interactions and functions of PrP isoforms will suggest new and improved therapeutic strategies for the TSE diseases.

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