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Commentary

Pharmacological approaches to prion research

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Abstract

The ''protein-only'' mechanism by which infectious agents of prion diseases such as Creutzfeldt–Jakob disease and bovine spongiform encephalopathy replicate remains undetermined. The identification of several distinct classes of prion inhibitors has created an opportunity to investigate the mechanism of prion formation using pharmacological tools. These new inhibitors include substituted tricyclic derivatives, tetrapyrrole compounds, cysteine protease inhibitors, branched polyamines, and specific antibodies. Each inhibitor class contains at least one active compound that inhibits prion propagation in cell culture at sub-micromolar concentrations and several structurally related, inactive compounds. Work with branched polyamines and specific antibodies has already provided insight into the kinetics and cell biology of endogenous prion clearance mechanisms. Other anti-prion compounds do not appear to bind directly to the prion protein. Detailed investigation of the mechanism of drug action of these compounds may lead to the identification of novel prion propagation factors.

Keywords: Prion; Pharmacology; Dendrimers; Antibodies; Tetrapyrrole; Tricyclic

1. Introduction

Prions are the infectious agents of fatal neurological diseases such as Creutzfeldt–Jakob disease, bovine spongiform encephalopathy, and scrapie [1]. A wealth of evidence supports the hypothesis that, unlike conventional infectious agents, prions lack informational nucleic acids and are instead composed primarily of an abnormally folded protein designated PrP^{Sc} [2]. The existence of an infectious agent that lacks nucleic acid defies the central dogma of molecular biology, and the mechanism of prion propagation remains unknown. During infection, new PrP^{Sc} molecules are formed by an induced conformational change of a normal host protein designated PrP^{C}. PrP^{C} is a host-encoded 33- to 35-kDa GPI-anchored glycoprotein expressed predominantly in neurons [3,4]. Whereas PrP^{C} is composed of 42% α-helix and 3% β-sheet, PrP^{Sc} is composed of 30% α-helix and 43% β-sheet [5,6]. The secondary structure of PrP^{Sc} causes it to be relatively protease-resistant and insoluble in buffer conditions that preserve prion infectivity [7]. The insolubility of PrP^{Sc} molecules and their tendency to aggregate into amyloid fibrils have hindered attempts to study the process of prion replication directly using biochemical techniques. A method to amplify PrP^{Sc} molecules *in vitro* using repeated cycles of sonication and incubation has been reported recently, but it remains unknown whether the protease-resistant PrP molecules generated by this technique are infectious [8]. It is likely that non-biochemical approaches will be needed to investigate the mechanism of prion replication.

An opportunity to use pharmacological techniques to study the mechanism of prion formation now exists because several novel anti-prion compounds have been identified recently. In the past, the mechanisms of many other biochemical and cell biological processes have been investigated successfully using selective inhibitors. For instance, the potent opioid antagonist naloxone was used to identify the opiate receptor [9]. Studies of the drug action of penicillin revealed the mechanism of bacterial cell wall synthesis [10]. We propose that the recently identified inhibitors of prion propagation could be used in a similar fashion as tools to characterize the molecular pathways required for prion propagation.

The newly discovered anti-prion compounds can be grouped into distinct classes, each with their own pharma-

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Abbreviations: PrP, prion protein; PrP^{C}, cellular prion protein; PrP^{Sc}, scrapie prion protein; GPI, glycophosphatidylinositol; PPI, polypropyleneimine; PAMAM, polyamidoamine; ScN2a, scrapie-infected neuroblastoma; DOPSA, 2,3-dioleyloxy-*N*-[2(sperminecarboxyamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; mAb, monoclonal antibody; PcTS, phthalocyanine tetrasulfonate(s); T(*N*-Me-4-Py)P, *N*-methyl pyridine; T(4-Py)P, tetra-pyridyl porphine(s).

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cology: branched polyamines, specific blocking antibodies, substituted tricyclic derivatives, cysteine protease inhibitors, and tetrapyrrole compounds. In this commentary, we briefly review the pharmacology of each inhibitor class and discuss how these compounds could potentially be exploited as tools to elucidate the cell biology and molecular mechanism of prion formation. We confine our discussion to the experimental pharmacology of novel anti-prion compounds because ongoing efforts to develop these compounds into therapeutic agents have been reviewed elsewhere [11].

2. Branched polyamines

Branched polyamines such as PPI and PAMAM dendrimers cure ScN2a cells at non-cytotoxic concentrations [12,13]. These compounds potently promote the clearance of PrP^{Sc} from the endosomes and lysosomes of ScN2a cells within 3 hr of administration [12]. A mixture of purified prion rods with PPI generation 4.0 at pH < 4.5 renders PrP^{Sc} molecules protease-resistant and dissolves the rod architecture, showing that branched polyamines act by binding directly to PrP^{Sc} [12]. A unique feature of branched polyamine compounds is that they promote the clearance of pre-existing PrP^{Sc}. The rate of PrP^{Sc} clearance mediated by branched polyamines provides a high baseline value against which the kinetics of endogenous clearance mechanisms can be compared, e.g. the polyamine-mediated PrP^{Sc} clearance rate $T_{1/2} = 3$ hr in ScN2a cells, whereas blocking antibodies and acridine derivatives require ~2 days to lower endogenous PrP^{Sc} levels. These observations provide a more refined estimate of the endogenous PrP^{Sc} clearance rate in ScN2a cells, previously measured by pulse-chase labeling as >1 day [14].

The potency of branched polyamines for denaturing PrP^{Sc} depends on several molecular features. Structure–activity experiments revealed that both branching architecture and high surface density of terminal primary amines were required for dendrimers to denature PrP^{Sc} *in vitro* and also to cure ScN2a cells in culture [13]. For instance, PAMAM generation 4.0 and PPI generation 4.0 clear PrP^{Sc} from ScN2a cells with an IC_{50} of 9 nM, whereas PAMAM-OH generation 4.0 fails to alter PrP^{Sc} levels even at a 1 μM concentration (Table 1) [13]. Liposomes containing 20 nM DOPSA also induce degradation of PrP^{Sc} in ScN2a cells [15]. Like dendrimers, DOPSA liposomes possess a high surface density of branching adducts capped with primary amines that are essential for PrP^{Sc} clearing activity.

3. Blocking antibodies

Several specific anti-PrP recombinant Fab molecules and the anti-PrP mAb 6H4 inhibit PrP^{Sc} formation, but not PrP^{C} expression, in ScN2a cells [16,17]. Transgenic expression of 6H4 also blocks prion neuroinvasion in mice [18]. The most potent blocking Fab, D18, inhibits PrP^{Sc} formation in ScN2a cells at a concentration of 6 nM, whereas R72, a different high-affinity anti-PrP Fab, fails to alter PrP^{Sc} levels at a >400 nM concentration (Table 1) [16]. Peptide mapping studies indicate that D18 specifically recognizes PrP residues 132–156, R72 recognizes residues 152–163, and mAb 6H4 recognizes residues 144–152 [19,20]. These maps suggest that antibody binding to residues 144–152 inhibits PrP^{Sc} formation. This region coincides with helix A of recombinant hamster PrP 90–231 produced in *Escherichia coli*, which was identified as residues 144–155 by NMR spectroscopy [21]. Peretz *et al*. [16] propose that helix A participates in the binding between PrP^{C} and PrP^{Sc}. Consistent with this hypothesis, amino acid substitutions in helix A contribute to interspecies prion transmission barriers [22–25].

4. Substituted tricyclic derivatives

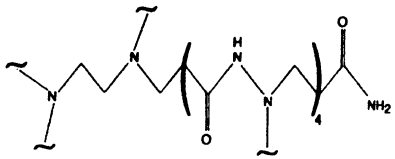
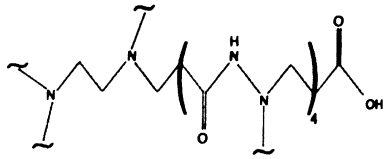
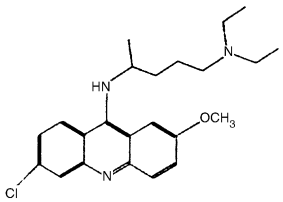
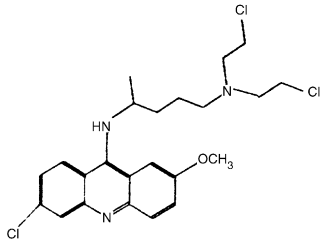
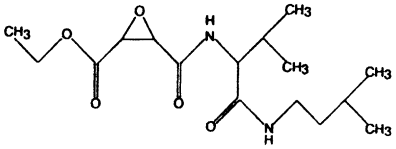
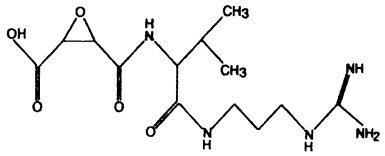
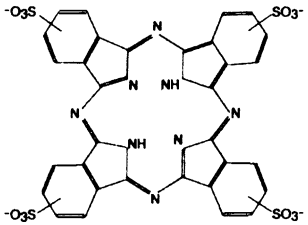
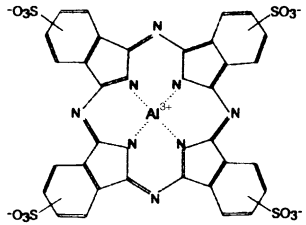
Quinacrine, an antimalarial acridine derivative, has been shown to inhibit PrP^{Sc} formation in ScN2a cells with an IC_{50} of 0.3–0.4 μM [26,27]. A comparative analysis using 24 structurally related acridine and phenothiazine derivatives revealed that the nitrogen atom at the 9 position of the tricyclic scaffold and the length and composition of the aliphatic side chain all contributed to inhibitory potency [27]. For example, quinacrine is 10 times more potent at inhibiting PrP^{Sc} formation than chlorpromazine, which has a sulfur atom at the 9 position of the tricyclic scaffold. Modification of the aliphatic side chain to generate quinacrine mustard completely abolishes anti-prion activity (Table 1) [27].

Quinacrine has been used clinically to treat malaria, giardiasis, and trypanosomiasis. Its anti-trypanosomal effect is mediated by inhibition of trypanothione reductase, but no biochemical target for quinacrine has been identified in mammals [28]. Doh-ura *et al*. [26] showed that quinacrine did not alter the formation of protease-resistant PrP in a cell-free conversion assay, and Korth *et al*. [27] claimed that quinacrine does not directly alter the conformation of PrP^{Sc} when mixed with purified prion rods. Both groups showed that quinacrine does not alter the expression of PrP^{C}. Furthermore, inhibition of PrP^{Sc} formation by tricyclic derivatives requires at least 2 days to take effect, similar to the kinetics of inhibition displayed by blocking antibodies. Taken together, these results indicate that quinacrine blocks PrP^{Sc} formation by binding to a novel target that plays an essential role in prion replication.

5. Cysteine protease inhibitors

Doh-ura *et al*. [26] reported that the selective, membrane-permeable cysteine protease inhibitor (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester

Table 1
Pharmacology of newly identified anti-prion compounds

Compound	IC_{50} (μM)	Structure/specificity	Compound	IC_{50} (μM)	Structure/specificity	Reference
PAMAM G4.0	0.009		PAMAM-OH G4.0	>0.7		[13]
Fab D18	0.006	PrP residues 132–156	Fab R72	>0.4	PrP residues 152–163	[16]
Quinacrine	0.3		Quinacrine mustard	>5		[27]
E-64d	0.5		E-64	~4		[26]
PcTS-metal free	0.5		PcTS-Al^{3+}	>10		[29]

(E-64d) inhibited PrP^{Sc} accumulation in ScN2a cells with an IC_{50} of 0.5 μM (Table 1). The membrane-impermeant compound L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine (E-64) was 8-fold less potent and leupeptin was 50-fold less potent than E-64d in inhibiting PrP^{Sc} accumulation. Fifteen micromolar E-64d did not change the apparent molecular size of either PrP^{C} or PrP^{Sc}, suggesting that its mechanism of action does not involve inhibition of a PrP protease. E-64d also did not alter the expression level of PrP^{C}, showing that its inhibitory effect is post-translational [26]. Finally, E-64d did not inhibit the formation of protease-resistant PrP in a cell-free conversion assay, indicating that its target is a molecule distinct from PrP^{C} [26].

6. Tetrapyrrole compounds

Several porphyrin and phthalocyanine compounds block PrP^{Sc} formation in ScN2a cells [29]. These structurally related compounds each contain four pyrrole rings arranged in a 2-fold symmetrical pattern. Effective inhibitors of PrP^{Sc} formation include various phthalocyanine tetrasulfonates (PcTS); sulfonate- and glycol-substituted deuteroporphyrins (DP); and meso-tetrasubstituted porphines (TSP) [29]. The most potent phthalocyanine inhibitors identified were metal-free PcTS and PcTS-Fe^{3+}. Metal-free PcTS blocked PrP^{Sc} formation in ScN2a cells with an IC_{50} of 0.5 μM, whereas PcTs-Al^{3+} was ineffective at a 10 μM concentration (Table 1) [29]. The most potent deuteroporphyrin identified was DP(glycol)$_2$-Fe^{3+}, which inhibited PrP^{Sc} formation with an IC_{50} of 1 μM. Metal-free DP(glycol)$_2$ was less effective than the iron-complexed compound. The most potent TSP was the *N*-methyl pyridine compound T(*N*-Me-4-Py)P-Fe^{3+}, which inhibited PrP^{Sc} formation with an IC_{50} of 0.5 μM. Metal-free T(*N*-Me-4-Py)P was less effective than T(*N*-Me-4-Py)P-Fe^{3+}. Interestingly, metal-free tetra-pyridyl porphine T(4-Py)P was a better inhibitor than T(*N*-Me-4-Py)P. By extrapolation, it is possible that iron-complexed T(4-Py)P-Fe^{3+} would be a more potent inhibitor than T(*N*-Me-4-Py)-P-Fe^{3+}, but the anti-prion potency of metal-complexed T(4-Py)P has not been reported.

Tetrapyrrole compounds do not affect the biosynthesis of PrP^{C}, nor do they inhibit the cell-free formation of protease-resistant PrP-res *in vitro* [29]. These observations suggest that specific tetrapyrroles block PrP^{Sc} formation by binding to a factor other than PrP.

7. Structure-based inhibitors

Using rational structure-based drug design, Perrier *et al.* [30] identified two inhibitors of PrP^{Sc} accumulation in ScN2a cells. Their strategy was based on the availability of an NMR structure of recombinant PrP and the discovery that PrP residues 168, 172, 215, and 219 form a binding site that mediates inhibition of prion formation by dominant-negative PrP alleles. A computational search in the available chemicals database (ACD) of 210,000 compounds for those that might mimic the dominant-negative binding site yielded 63 potential inhibitors. These compounds were screened for their ability to block PrP^{Sc} formation in ScN2a cells, resulting in the identification of the inhibitor 2-amino-6-[(2-aminophenyl)thio]-4-(2-furyl)pyridine-3,5-dicarbonitrile (Cp-60), with an IC_{50} of 18 μM [30]. Although Cp-60 is a relatively weak inhibitor of prion formation, chemical modification of the Cp-60 backbone may eventually produce a compound that potently binds to an unidentified cellular factor responsible for mediating dominant-negative inhibition of prion formation by specific polymorphic PrP alleles.

8. Inhibitors as new reagents for prion research

The availability of several classes of prion inhibitors effective at sub-micromolar concentrations creates a new set of tools to investigate the mechanism of prion formation. Within each class, there are specific chemical requirements for inhibitory activity, and some compounds are more potent than others (Table 1). Non-inhibitory and weakly inhibitory compounds within each chemical class provide useful pharmacological controls for all the methods employing potent inhibitors discussed below.

The mechanism of inhibition has been established for two classes of inhibitors, blocking antibodies and branched polyamines. Blocking antibodies bind to PrP^{C} and prevent PrP^{Sc} formation, whereas branched polyamines bind to and denature PrP^{Sc} in acidic compartments. Studies of these two inhibitor classes have already provided some insight into the kinetics, structural biology, and cell biology of prion formation.

The mechanism of indirect inhibitors of PrP^{Sc} formation has not been investigated thoroughly. These inhibitors, such as acridine derivatives and tetrapyrrole compounds, could be used to identify factors other than PrP required for prion formation. Since these compounds have been shown not to interact directly with PrP and yet can inhibit PrP^{Sc} formation completely, they must act on at least one other cellular factor required for prion formation. The simplest hypothesis is that these inhibitors bind directly to a factor or factors that promote prion propagation. Identification of such factors would represent a major advance towards understanding the mechanism of prion formation.

Radioligand binding is a simple technique that could be used to characterize and facilitate the isolation of receptors that specifically bind compounds such as quinacrine or PcTS. Many other receptors and enzymes have previously been characterized and isolated using radioligand binding techniques. To demonstrate specificity, radioactive binding

to specific sites in crude extracts should not be displaceable by compounds such as quinacrine mustard or PcTS-Al^{3+}, in accordance with the inability of these compounds to inhibit PrP^{Sc} formation in ScN2a cells. A useful property of quinacrine as a ligand is its intrinsic fluorescence. Fluorescence could potentially be used instead of radioactivity to track quinacrine binding, obviating the need for radioactive synthesis. The tetrapyrroles PcTS, T(*N*-Me-4-Py)P, and T(4-Py)P could also be traced without radioactive organic synthesis. Since iron-bound tetrapyrrole compounds are at least as potent as metal-free tetrapyrroles in inhibiting PrP^{Sc} formation, each metal-free compound could simply be coordinated with isotopic iron to generate radioactive ligands.

Photoaffinity labeling is a variation of the radioactive/fluorescent binding method that creates covalent label attachment, permitting subsequent purification of the bound receptor under denaturing conditions. Chemical addition of photoreactive groups to radioactive acridine or tetrapyrrole derivatives could generate photoaffinity ligands for their corresponding receptors. It is noteworthy that the inhibitor E-64d is an epoxy-based suicide substrate that acts by covalently attaching to the active site cysteine of specific proteases. Thus, E-64d may be a useful irreversible affinity label that does not require chemical addition of a photoreactive group.

Another strategy to purify novel prion propagation factors using indirect anti-prion inhibitors is affinity chromatography. Compounds such as quinacrine or tetrapyrroles could be covalently coupled to resins and used to isolate specific receptors from crude extracts. After washing such columns with inactive compounds such as quinacrine mustard, bound receptors could be specifically eluted with the active inhibitor.

The availability of structure–potency profiles also creates opportunities to test specific hypotheses about inhibitory mechanisms for each class of compounds. For instance, one could measure the effect of various acridine derivatives on endosomal function in ScN2a cells. A correlation between the potency of each compound at inhibiting endosomal function and its potency at blocking PrP^{Sc} formation would support a role for endosomal function in PrP^{Sc} formation.

The success of each of the techniques described here depends on selective binding. In particular, it would be wise to use the highest affinity ligand from each class for binding and chromatography experiments. The potencies of indirect inhibitors of PrP^{Sc} formation are all within the sub-micromolar range, and compounds with affinities $\sim$300 nM can be used successfully for all the techniques described above. Furthermore, measured inhibitory potencies represent minimum affinities of chemical compounds for their receptors. If either delivery or inhibitory activity of anti-prion compounds in ScN2a cells is inefficient, the true binding constant K_d could be significantly lower than the IC_{50} value. In either case, the creation of even more potent inhibitors of PrP^{Sc} formation through chemical modification would be desirable.

9. Summary

The pharmacology of prion disease is emerging. The study of branched polyamines and blocking antibodies has already provided insights into the kinetics and cell biology of PrP^{Sc} clearance. Several other classes of prion inhibitors have been identified recently, and their mechanisms of action await elucidation. Such studies may lead to the identification of factors and/or pathways required for prion replication. Using this knowledge, additional chemical modification and compound screening may yield novel anti-prion compounds with even higher affinity for their drug targets.

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