

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech



Anti-prion activities and drug-like potential of functionalized quinacrine analogs with basic phenyl residues at the 9-amino position

Thuy Nguyen^a, Yuji Sakasegawa^b, Katsumi Doh-ura^b, Mei-Lin Go^{a,*}

^a Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore ^b Department of Neurochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan

ARTICLE INFO

Article history: Received 18 January 2011 Received in revised form 2 April 2011 Accepted 4 April 2011 Available online 12 April 2011

Keywords: Cell-based anti-prion activity Quinacrine analogs Drug-like properties Binding to human prion protein fragment PAMPA-BBB permeability Pgp substrate

ABSTRACT

In this paper, we report the synthesis and cell-based anti-prion activity of quinacrine analogs derived by replacing the basic alkyl side chain of quinacrine with 4-(4-methylpiperazin-I-yl)phenyl, (1-benzylpiperidin-4-yl) and their structural variants. Several promising analogs were found that have a more favorable anti-prion profile than quinacrine in terms of potency and activity across different prion-infected murine cell models. They also exhibited greater binding affinities for a human prion protein fragment (hPrP₁₂₁₋₂₃₁) than quinacrine, and had permeabilities on the PAMPA-BBB assay that fall within the range of CNS permeant candidates. When evaluated on bidirectional assays on a Pgp overexpressing cell line, one analog was less susceptible to Pgp efflux activity compared to quinacrine. Taken together, the results point to an important role for the substituted 9-amino side chain attached to the acridine, tetrahydroacridine and quinoline scaffolds. The nature of this side chain influenced cell-based potency, PAMPA permeability and binding affinity to hPrP₁₂₁₋₂₃₁.

© 2011 Elsevier Masson SAS. All rights reserved.

195

MEDI

1. Introduction

Prion diseases (transmissible spongiform encephalopathies) are fatal transmissible neurodegenerative disorders that afflict humans and animals alike. Prion pathology and intervention strategies have been extensively reviewed [1–5]. As yet, there is no effective prophylactic or therapeutic agent for these devastating conditions and the prospect of developing a clinical agent remains daunting, primarily due to the unusual biology of the infectious agent (the prion) and the limited understanding of the structure and mechanistic properties of the putative target. According to the "protein only" or prion hypothesis, infectivity resides in an abnormal isoform of the host-encoded cellular prion protein (PrP^{Sc}) which propagates by imposing its conformation onto the normal, nonpathogenic prion protein (PrP^{C}) expressed in host cells [6].

Numerous compounds have shown anti-prion activity in cell culture models of prion disease. These include sulphonated dyes like Congo red, sulphated glycans (dextran sulphate, pentosan polysulphate), and nitrogen heterocycles (chlorpromazine, quinacrine, quinine) among others [3,4]. However, none have demonstrated sufficient activity to halt disease progression in infected animal models [2–4], and the few that were tested in humans at the post-symptomatic stage of the disease, like flupirtine [7]. quinacrine [8–11], vidarabine [12], amphotericin B [13], amantadine [14], did not significantly alter the clinical course of the disease. In a patient-preference trial that evaluated the safety and efficacy of the 9-aminoacridine quinacrine in human prion disease (PRION-1) [11], quinacrine did not reverse the course of disease, confirming the findings of earlier reports [9,10]. The lack of efficacy was attributed to inadequate accumulation of quinacrine in the brain, due to its removal by P-glycoprotein (Pgp) which is an efflux protein found in the blood-brain barrier [15]. Indeed, when administered orally to prion-infected mice that were deficient in the genes encoding Pgp, brain levels of quinacrine were significantly increased [15,16]. Intriguingly, in one report [16], quinacrine still failed to extend the survival of prion-infected animals which led the authors to speculate that continuous quinacrine treatment promoted the formation of drug resistant prions. This phenomenon could have contributed to the lack of in vivo efficacy of quinacrine and possibly other anti-prion drugs. In spite of its limitations (a Pgp substrate, moderate potency, possible resistance), quinacrine remains a promising lead which on structural modification, has yielded analogs with more potent in vitro anti-prion activity. Notable examples are the bis-acridines [17] and guinacrineimipramine hybrids [18] although the large size (molecular

^{*} Corresponding author. Tel.: +65 6516 2654; fax: +65 6779 1554. *E-mail address:* phagoml@nus.edu.sg (M.-L. Go).

^{0223-5234/\$ –} see front matter \circledcirc 2011 Elsevier Masson SAS. All rights reserved. doi:10.1016/j.ejmech.2011.04.016

weight > 500) and lipophilicity (ClogP > 5) of these potent analogs may hinder their passage across the blood-brain barrier.

In an earlier report, we reported several functionalized monoacridines with anti-prion activities on various prion-infected cell models [19]. Two compounds, an N-(4-methylpiperazinyl)phenylacridine-9-amine (compound 15) and an N-(benzylpiperidinyl) acridine-9-amine (compound 17) (Fig. 1), were particularly outstanding as they reduced or cleared PrP^{Sc} in several cell models at sub-micromolar concentrations that were non-toxic to host cells. Noting that the side chains of these hit compounds have not been associated with anti-prion activity, it is of interest to assess their potential as potent and drug-like fragments with anti-prion activity. To this end, we have prepared analogs of compounds 15 and 17, specifically with modifications at their side chains and evaluated the cell-based anti-prion activities of the resulting compounds. They were also investigated for their binding affinities to a truncated human prion protein (hPrP_{121–123}) to provide some insight into their mode of action. Their in vitro permeabilities determined by the parallel artificial membrane permeation assay (PAMPA), and susceptibility of a representative compound (1) to Pgp efflux were also investigated to gauge the drug-like potential of these target compounds.

2. Chemical considerations

The piperazinylphenyl analog 15 is re-numbered **1** in this report. To investigate the contribution of the 4-methylpiperazine side chain and the substituted acridine ring to anti-prion activity, various analogs were synthesized (Table 1). Modifications to the 4methylpiperazine ring included replacing the methyl group with its ethyl homolog (2), converting the distal basic nitrogen of piperazine to a non-basic amide (3) and inserting a methylene (4) or carbonyl (5) spacer between the piperazine and phenyl rings. The phenyl ring of 1 was replaced by a butynyl-2,3 moiety which is viewed as an aromatic ring equivalent in view of its π -electron density and conformational rigidity [20]. The effect of removing the basic piperazine ring of **1** was investigated in the 9-(N-phenyl) amino analogs 7 and 8.8 was additionally modified so that its 9amino functionality is tertiary. 9 and 10 retained the same 9-amino side chain as 1 but the 2-methoxy-6-chloroacridine ring was replaced by 6-chloro-1,2,3,4-tetrahydroacridine in 9 and 7-chloroquinoline in 10.

As for the benzylpiperidinyl analog 17, it is re-numbered **11** in this report. The side chain was modified by introducing substituents (*p*-CH₃, *p*-Cl, *p*-OCH₃, *p*-CN) with different Hansch σ and Hammett π values to the phenyl ring (**12–15**) and varying the methylene groups (n = 2 or 3) separating the piperidine and phenyl rings (**16–18**) (Table 1). We also rigidified the piperidine of **11** by converting it to a bicyclic ring (**19**) and switched the positions of the piperidine and phenyl rings found in **11** to give **20**. The tetrahydroacridine and quinoline analogs of **11** were also prepared (**21**, **22**).

1 and its analogs (except **6**, **9**) were synthesized by reacting the appropriate amine and 6,9-dichloro-2-methoxyacridine in refluxing

ethanol as solvent [19]. Earlier, we have synthesized **1** by reacting 6,9-dichloro-2-methoxyacridine with 4-(4-methylpiperazin-1-yl) aniline [19]. The aniline was obtained by the Hartwig–Buchwald amination of 1-iodo-4-nitrobenzene with 1-methylpiperazine in the presence of a palladium/BINAP catalyst, followed by catalytic hydrogenation of the nitro group. The same reaction sequence was adopted for the syntheses of 4-(4-ethylpiperazin-1-yl) aniline and 1-[4-(4-aminophenyl)piperazin-1-yl]ethanone which were the reacting amines for **2** and **3** respectively (Scheme 1).

In Scheme 2, commercially available 1-(chloromethyl)-4-nitrobenzene was reacted with 1-methylpiperazine or piperidine in the presence of triethylamine in THF, followed by catalytic reduction of the nitro group with Adam's catalyst. The resulting amines (**25**, **26**) were reacted with 6,9-dichloro-2-methoxyacridine to give **4** and **20** respectively. In a related manner, 4-nitrobenzoyl chloride was reacted with 1-methylpiperazine followed by catalytic reduction to give **27** for the synthesis of **5** (Scheme 3)

2-(4-Chlorobut-2-ynyl)isoindoline-1,3-dione (**28**) was prepared by the Gabriel synthesis of 1,4-dichlorobut-2-yne with potassium phthalimide (Scheme 4) [21]. It was then reacted with 1-methylpiperazine to give **29**, after which the phthalolyl group was removed by hydrazine to give amine **30**. The latter was reacted with 6,9-dichloro-2-methoxyacridine in phenol with heating (120 °C, 4 h) to yield **6**. Aniline and N-methylaniline were reacted with 6,9-dichloro-2-methoxyacridine in refluxing ethanol to give **7** and **8** respectively [19].

3,9-Dichloro-5,6-7,8-tetrahydroacridine (**43**) was synthesized in a one-pot reaction involving 4-chloroanthranilic acid and cyclohexanone [22] (Scheme 5), following which it was reacted with 4-[(4-methylpiperazin-1-yl)methyl]aniline or 1-benzylpiperidin-4-amine in phenol to give **9** and **21** respectively. These amines were also reacted with commercially purchased 4,7-dichloroquinoline to give **10** (refluxing ethanol as solvent) and **22** (phenol as solvent).

11 and its analogs (except **20**) were synthesized by reacting the appropriate amine and 6,9-dichloro-2-methoxyacridine in phenol ($120 \degree C$, 4 h) as solvent [19]. 1-Benzylpiperidin-4-amine (for **11**) was purchased while the amines for **12–18** were synthesized by reacting *t*-butylpiperidin-4-ylcarbamate with a phenalkyl halide in the presence of triethylamine, to give intermediates **31–37**, followed by removal of the protective *t*-Boc moiety by acid hydrolysis to give the desired amine (Scheme 6). The phenalkyl halides were purchased except for 1-chloro-4-(chloromethyl)benzene (**38**) which was obtained by reacting 4-chlorobenzylhydroxy with thionyl chloride in anhydrous dichloromethane.

8-Benzyl-8-aza-bicyclo[3.2.1]oct-3-ylamine (**42**) was synthesized following a reported method [23] with modifications (Scheme 7). Hydrolysis of the ethyl ester of 3-oxo-8-aza-bicyclo [3.2.1]octane-8-carboxylic acid gave the secondary amine (**39**) which was reacted with benzyl chloride to give the ketone **40**. The ketone was derivatized to an oxime **41** and reduced in the presence of Adam's catalyst to give the desired amine **42**. The latter was reacted with 6,9-dichloro-2-methoxyacridine in phenol to give **19**.



Table 1 Structures of **1–22**.



Code	R	Code	R
1	-HN-CH3	11	
2		12	
3		13	
4		14	-HN-CH2-OCH3
5		15	-HN-CH2-CN
6	-HN-CH2-C=C-CH2-N_N-CH3	16	
7	-HN-	17	
8		18	
9		19	
10		20	
		21	
		22	



 $\label{eq:Scheme 1. a) amine, Pd(OAc)_2, BINAP, Cs_2CO_3, anhydrous toluene, 120 ~C; b)~(i)~H_2, Pd/C;~(ii)~6,9-dichloro-2-methoxyacridine, ethanol, reflux, 24 h.$

3. Results

3.1. Anti-prion activity

Anti-prion activity was determined in murine cells that were stably infected with abnormal protease resistant prion protein (PrP^{Sc}) and capable of accumulating PrP^{Sc} in detectable amounts without concurrent cytotoxicity to the host cells. Three prioninfected cell models were investigated, namely ScN2a which comprise mouse neuroblastoma cells (N2a) infected with a mouse adapted scrapie strain (RML), N167 which are N2a cells containing another mouse adapted scrapie strain 22L and F3, a subclone of N2a with higher expression levels of PrP^C and infected with the human prion strain Fukuoka-1. Screening for anti-prion activity involved monitoring PrP^{Sc} by Western blotting after proteinase K digestion of cell lysates. A compound with anti-prion activity will show a concentration-dependent reduction in the signal levels of the immunoblots, from which EC₅₀ (effective concentration required to reduce PrP^{Sc} content to 50% of untreated cells) is then determined. Besides EC₅₀, the full anti-prion activity (FAA) and maximal tolerant concentration (TC) are also obtained. FAA is the estimated lowest concentration required to clear more than 99% of PrP^{Sc} content, while TC is the approximate highest concentration that has no effect on the viability of infected N2a cells. The screening results are presented in Table 2.

The EC₅₀ of the positive control quinacrine on ScN2a was 0.23 μ M, which agreed with reported values on a similar cell model [24,25]. It was also active on N167 (EC₅₀ 0.59 μ M) and F3 (EC₅₀ 1.88 μ M) but failed to clear PrP^{Sc} at concentrations that did not affect the viability of the host cells. Thus, the target end points required of a compound deemed to have a better anti-prion profile than quinacrine are (i) activity across the three cell models, in particular F3 which has been shown to be resistant to drug treatment [19,26]; (ii) ability to clear PrP^{Sc} in all cell models; and (iii) a high tolerant concentration (TC), preferably exceeding that of



Scheme 2. a) (i) amine, TEA, THF, 70 °C, 24 h; (ii) H_2 , PtO₂, 50 psi, overnight; b) 6,9-dichloro-2-methoxyacridine, ethanol, reflux, 24 h.



Scheme 3. a) (i) N-Methylpiperazine, TEA, anhydrous DCM, RT, overnight; (ii) H₂, PtO₂, 50 psi, overnight; b) 6,9-dichloro-2-methoxyacridine, phenol, 120 °C, 24 h.

quinacrine (TC 2.5 μ M). In practical terms, the compound should combine a low EC₅₀ with low FAA across several cell models, while maintaining a high TC on uninfected N2a cells. As seen from Table 2, 1 fulfills most of these criteria except that its tolerant concentration remains comparable to that of quinacrine. As for **11**, it fared less well as it failed to clear PrP^{Sc} in F3 and its potency (EC₅₀) did not exceed that of **1** on the existing cell models.

Among the piperazinylphenyl analogs **1–10**, several members had lower EC_{50} values than **1** on ScN2a but these improved potencies did not extend to N167 or F3. In fact, only 3 analogs (**4**, **5**, **10**) retained activity on all 3 cell models, but with higher EC_{50} values than **1**. Their lower potencies notwithstanding, these compounds have the advantage of being less toxic (TC 4–12 μ M) than **1** and quinacrine.

In spite of the small number of piperazinylphenyl analogs explored here, some broad structure—activity trends may be deduced. First, inserting a spacer moiety (methylene, carbonyl) between the phenyl and piperazine rings retained activity on all 3 cell models. Notably, the analog with the methylene spacer (**4**) exhibited a better anti-prion profile than the carbonyl analog (**5**). Second, homologation of the distal piperazine nitrogen of **1** (4-ethyl in **2** and 4-methylcarbonyl in **3**) adversely affected activity. **2** and **3** are structural isomers of **4** and **5** respectively and their contrasting anti-prion profiles suggest a limited tolerance for even minor modifications to the side chain. Third, the butyn-2,3-yl moiety is a poor substitute for the phenyl ring, as seen from less promising profile of **6**.

We investigated **7** and **8** to determine if a basic substituent on the 9-N-phenyl ring of **1** is essential for activity across the different cell models. The results suggest that the basic substituent contributes to activity. Notably, **7** and **8** had no activity on N167 and F3, but retained potencies on ScN2a (EC₅₀ 0.54, 2.51 μ M).



Scheme 4. a) 1,4-dichlorobut-2-yne, DMF, 100 $^{\circ}$ C, 5 h; b) 1-methylpiperazine, TEA, DCM, rt, overnight; c) NH₂NH₂, EtOH, reflux, 2 h; d) 6,9-dichloro-2-methoxyacridine, phenol, 120 $^{\circ}$ C, 24 h.



Scheme 5. a) cyclohexanone, POCl₃, 0 °C \rightarrow reflux, 2 h; (b) 4-[(4-methylpiperazin-1-yl)methyl]aniline, ethanol, reflux, 24 h; c) 1-benzylpiperidin-4-amine, phenol, 120 °C, 24 h.

Compound **3** (with a basic substituent) showed a similar profile but was many times more potent on ScN2a (EC₅₀ 0.035 μ M) than **7** and **8**. Interestingly, we noted that **8** which is the only compound with a tertiary 9-amino functionality, is also the least active analog in Table 2. Earlier, we found that 9-amino-6-chloro-2-methox-yacridine (primary 9-amino function) was as potent as **1** on ScN2a (EC₅₀ 0.13 μ M) [19] but as seen here, activity declined in **7** (secondary 9-N-phenylamino, EC₅₀ 0.54 μ M) and even more in **8** (tertiary 9-N-methyl-N-phenylamino, 2.51 μ M). Thus, it may be inferred that a tertiary 9-amino function adversely affects antiprion activity. Lastly, replacing the substituted acridine ring of **1** with tetrahydroacridine (**9**) or quinoline (**10**) reduced activity, particularly on F3, although both analogs were noticeably less toxic (TC \geq 10 μ M) on N2a cells compared to **1**.

Unlike the piperazinylphenyl analogs, more members of the Nbenzylpiperidinyl series retained anti-prion activity on all 3 cell models and of these, several are more potent than **11**. The most promising analog is **16** in which the N-benzyl substituent of **11** was replaced by N-phenylethyl. Introducing a *p*-methyl to the N-phenethyl side chain (**17**) or homologation to N-phenpropyl (**18**) are acceptable modifications, but resulted in some loss of potency on F3. Interestingly, a *p*-methyl on the benzyl ring of **11** (**12**) is also permissible, but not substitutents like chloro, methoxy or cyano (**13–15**) which do not have the electron donating and lipophilic character of the methyl group. Rigidifying the piperidine rings (**20**) retained activity. **20** is structurally related to **4** and the acceptable activity profiles of both compounds showed that the dibasic piperazine may be replaced by the monobasic piperidine ring. The



Scheme 6. a) SOCl₂, $0 \circ C \rightarrow RT$, 1 h, anhydrous DCM; b) TEA, THF, reflux, overnight; c) (i) TFA, DCM, rt; (ii) 6,9-dichloro-2-methoxyacridine, phenol, 120 °C, 24 h.



Scheme 7. a) KOH, water, THF, 80 °C, 10 h; b) PhCH₂Cl, TEA, THF, reflux, overnight; c) NH₂OH.HCl, MeOH, Na₂CO₃, rt, 4 h; d)H₂, PtO₂, 50 psi, rt, overnight; e) dichloro-2-methoxyacridine, phenol, 120 °C, 24 h.

tetrahydroacridine (**21**) and quinoline (**22**) analogs were again associated with lower cytotoxicities but unlike the piperazinylphenyl series, both **21** and **22** were active on ScN2a, N167 and F3. They were also equipotent on F3.

3.2. Binding affinity to truncated human prion protein hPrP₁₂₁₋₂₃₁

The binding affinities of the test compounds to a truncated human prion protein comprising the carboxy-terminal polypeptide residues (121-231) was monitored by surface plasmon resonance (SPR). Briefly, the peptide was immobilized on a carboxymethylated dextran sensor chip (CM5) to give a density of approximately 3000 response units (RU). A known concentration of the test compound was then passed over the chip surface and its binding to the immobilized peptide was monitored by a binding response curve (sensorgram) which showed the rates at which the compound associated and dissociated from the peptide. Fig. 2A is a sensorgram of a typical rapidly associating and dissociating ("faston/fast-off") compound (in this case, quinacrine), which is seen from the steep rise and fall of the ascending and descending portions of the plot. The binding capacity of the compound for hPrP₁₂₁₋₂₃₁ is deduced from the maximum response unit (RU_{max}) that is obtained at the end of the association phase and this value is normalized to %RU_{max} by taking into account the molecular weights of the test compound and peptide, the maximum binding response of the peptide (3000 RU units) and assuming a 1:1 stoichiometry in the interaction. Fig. 2B shows the binding response curve of 9 which is a "slow on/slow off" compound. Dissociation is incomplete as seen from the descending portion of the curve which fails to return to the baseline within the monitoring period.

Some test compounds (**6**, **7** and **19**) were not evaluated because of their poor solubilities in phosphate buffer at the test concentration (50 μ M). The remaining compounds interacted with the peptide fragment with a "fast-on/fast-off" profile, except **13** which had a profile that was between that of quinacrine and **9**. The binding parameters of the test compounds are given in Table 3. Also included are the binding affinities of quinacrine and its analogs (**43**–**46**) and 9-amino-6-chloro-2-methoxyacridine (**47**) whose ScN2a EC₅₀ values have been reported earlier [19].

Several investigators have measured the binding affinity of quinacrine to prion protein using SPR [27,28]. In spite of differences in the type of protein (mouse or human prion protein, full length or truncated) and concentration (40 μ M or 100 μ M) used in these assays, the consensus was that quinacrine binds weakly to the prion protein. The classification of compounds as weak or strong binders depends on the experimental conditions employed. Using the carboxy-terminal polypeptide (residues 121–231) of the mouse prion protein immobilized on the CM5 chip, Kawatake et al. [27] classified quinacrine (100 μ M) as a weak binder as its RU was <100. Touil et al. [28] screened structurally diverse compounds (40 μ M) on

Ta	ьı	6	2
Id	DI	e	2

In vitro anti-prion activities of 1–22 on murine neuroblastoma cells infected mouse adapted scrapie strains (ScN2a and N167) and a human prison strain (F3).

Code	ScN2a		N167		F3		ΤC (μM) ^c
	<i>EC</i> ₅₀ (μ <i>M</i>) ^a	<i>FAA (µM)</i> ^b	<i>EC</i> ₅₀ (μ <i>M</i>) ^a	<i>FAA (µM)</i> ^b	<i>EC</i> ₅₀ (μ <i>M</i>) ^a	<i>FAA</i> (µ <i>M</i>) ^b	
1	0.10 (0.08, 0.12)	0.4	0.42 (0.41, 0.43)	1.5	0.68 (0.59, 0.78)	1.5	2.5
2	0.080, (0.063, 0.10)	1	0.96 (0.43, 2.11)	None ^d	None ^d	ND ^e	3
3	0.035 (0.025, 0.049)	0.1	None ^d	ND ^e	None ^d	ND ^e	5
4	0.060 (0.051, 0.069)	0.5	0.35 (0.22, 0.63)	2	0.86 (0.77, 0.96)	3	4
5	1.23 (1.09, 1.39)	5	4.20 (3.81, 4.63)	7	4.10 (3.49, 4.81)	None ^d	12
6	0.027 (0.021, 0.036)	1	0.99 (0.86, 1.14)	3	None ^d	ND ^e	8
7	0.54 (0.36, 0.81)	None ^d	None ^d	ND ^e	None ^d	ND ^e	5
8	2.51 (2.11, 3.00)	5	None ^d	ND ^e	None ^d	ND ^e	8
9	0.082 (0.058, 0.11)	1	None ^d	ND ^e	None ^d	ND ^e	>10
10	0.14 (0.08, 0.25)	1	0.53 (0.41, 0.68)	2	2.04 (1.35, 3.07)	None ^d	10
11	0.42 (0.38, 0.46)	1	0.49 (0.44 0.55)	1.5	0.80 (0.64, 1.00)	None ^d	2
12	0.15 (0.12, 0.19)	3	0.62 (0.49, 0.80)	1	0.63 (0.53, 0.75)	3	5
13	0.28 (0.24, 0.33)	1	0.34 (0.15, 0.78)	3	None ^d	ND ^e	12
14	0.082 (0.063, 0.11)	1.5	0.52 (0.47, 0.58)	2	None ^d	ND ^e	10
15	0.55 (0.49, 0.63)	1	0.14 (0.081, 0.25)	1	None ^d	ND ^e	5
16	0.13 (0.10, 0.16)	0.5	0.23 (0.12, 0.42)	1	0.19 (0.14, 0.26)	2	2
17	0.076 (0.058, 0.10)	1	0.30 (0.14, 0.66)	1	0.69 (0.39, 1.19)	3	3
18	0.093 (0.027, 0.32)	1	0.32 (0.19, 0.54)	1	1.04(0.77, 1.40)	3	4
19	0.054(0.043, 0.067)	2	0.35 (0.21, 0.61)	1	0.54 (0.48, 0.60)	1.5	2
20	0.099 (0.085, 0.11)	0.5	0.51 (0.24, 1.09)	1	0.64 (0.52, 0.79)	3	5
21	0.54 (0.36, 0.80)	1	0.42 (0.32, 0.57)	3	1.19 (0.85, 1.67)	5	5
22	0.15 (0.13, 0.18)	1	0.23 (0.16, 0.33)	2	1.2 (0.88, 1.64)	3	>10
Quinacrine	0.23 (0.22, 0.25)	0.8	0.59 (0.42, 0.82)	None ^d	1.88 (1.64, 2.16)	None ^d	2.5

^a Concentration (μM) required to reduce PrP^{Sc} content to 50% of untreated/control cells from 3 independent determinations. 95% confidence limits are given in italics. ^b Full anti-prion activity (FAA): estimated lowest concentration (μM)to clear more than 99% of PrP^{Sc} content.

^c Tolerant concentration : approximate highest concentration of test compound that had no effect on viability of murine neuroblastoma N2a cells.

^d No effect at non-toxic concentration.

^e Not determined.

human and murine prion proteins immobilized on the same CM5 chip and proposed the following cut-off values, assuming a 1:1 binding mode: multiple site binders >130% RU_{max}, strong binders >50–129% RU_{max} and weak to moderate binders > 5–49% RU_{max}. In that report, % RU_{max} of quinacrine (40 µM) was 36.9% on the truncated human prion protein (residues were not mentioned) and 88.4% on the full length human prion protein. Here, we obtained % RU_{max} of 87.3 for quinacrine (50 μ M) using the truncated human prion protein (residues 121-231) on the CM5 chip. We noted that our experimental conditions were closer to that reported by Touil and coworkers [28]. Moreover, our % RU_{max} for quinacrine (87.3) was approximately twice their reported value (36.9). Hence, we adopted their classification for strong/moderate/weak binders but at twice the % RU_{max} cut-off values. Thus, % RU_{max} for multiple site binders is >260, strong binders 100-259, and weak to moderate binders. <100.

By this criterion, quinacrine is classified as a weak to moderate binder, in line with earlier reports [27,28]. None of the test compounds qualified as multiple site binders. Compounds with the highest binding affinities were the piperazinylphenyl analogs, namely **2** (194), **3** (210), **4** (207) and **5** (232). In comparison, the N-benzylpiperidinyl analogs were generally weaker binders, with a maximum $%RU_{max}$ value of 174 (**16**). The quinacrine analogs (**43**–**46**) which have dialkylaminoalkyl side chains at 9-amino had $%RU_{max}$ values (153–178) which qualify them as strong binders, in marked contrast to quinacrine.

A comparison of % RU_{max} and $EC_{50 SCN2a}$ points to a relationship between binding affinity and potency on ScN2a. For example, the piperazinylphenyl and N-benzylpiperidinyl analogs with the strongest binding affinities were among the more potent anti-prion agents, and interestingly, **8** which had the weakest binding affinity in Table 3, was also the least potent compound on ScN2a. When evaluated by a Spearman bivariate correlation, a significant correlation between the two variables was found, with Spearman rho of -0.470 and p value of 0.018 for the 25 compounds in Table 3. The significant correlation led us to question if there could be a physicochemical basis for the relationship since both affinity and potency measurements were carried out at a pH (7.4) where the compounds were ionized to varying degrees and would have different lipophilicities. Indeed, RU_{max} was significantly correlated to the estimated distribution coefficient of the compounds at pH 7.4 (log $D_{7.4}$), with Spearman rho of -0.501, p = 0.011 for n = 25. The inverse relationship implied that stronger binding affinities were associated with less lipophilic compounds. However, no correlation was observed for EC_{50 SCN2a} and log $D_{7.4}$.

3.3. Evaluation of permeability by PAMPA-BBB

The permeability characteristics of selected compounds were evaluated on the parallel artificial membrane permeation assay (PAMPA) using porcine brain lipids to mimic the lipid barrier of the brain. This assay which is also referred to as PAMPA-BBB, involved measuring the rate at which the test compound passively diffuses across the lipid layer separating the donor compartment (comprising test compound at 30 or 50 µM in phosphate buffer, pH 7.4) from the acceptor compartment filled with the same buffer solution. The concentration of the test compound was then determined in both compartments to give the effective permeability (P_e). Table 3 lists the P_e values of some piperazinylphenyl and N-benzylpiperidinyl analogs and the reference compounds (positive controls) verapamil, quinidine and caffeine. Verapamil and quinidine are representative "high permeability" (or CNS+) compounds while caffeine represents a low permeability (CNS-) compound. The P_e values of caffeine and verapamil compared favorably with those reported by Di et al. [29] using the same method.

Quinacrine was highly permeable across the lipid barrier with a P_e that was comparable to verapamil. The P_e values of the quinacrine analogs **43–46** were also noticeably higher than those of the piperazinylphenyl and N-benzylpiperidinyl analogs. Of the four



Fig. 2. Interactions of compounds with hPrP121–231 (A) Typical sensorgram of quinacrine, an example of a fast on/fast off compound.(B) Typical sensorgram of 9, a slow on/slow off compounds. Spikes seen at the start and end of injections were due to a slight time delay in the reference cell and appeared when reference subtraction was carried out.

piperazinylphenyl analogs evaluated, P_e ranged from 6.13×10^{-6} cm/s to 11.5×10^{-6} cm/s, with a mean of 8.64×10^{-6} cm/s while P_e of the seven N-benzylpiperidinyl analogs in Table 3 varied from 5.28×10^{-6} cm/s to 15.53×10^{-6} cm/s, with a mean of 8.70×10^{-6} cm/s. Thus, permeabilities of piperazinylphenyl and N-benzylpiperidinyl analogs were broadly comparable but lower than **43–46** (mean of 18.17×10^{-6} cm/s). Lower permeabilities notwithstanding, the piperazinylphenyl and N-benzylpiperidinyl analogs are still classified as "CNS+" (high brain penetration) compounds, based on the threshold values proposed by Di and coworkers [29] for "CNS+" ($P_e > 4.0 \times 10^{-6}$ cm/s) and "CNS–" (P_e values < 2.0×10^{-6} cm/s) compounds.

The side chains of the piperazinylphenyl and N-benzylpiperidinyl analogs (except **6**) have in common an aromatic residue, a feature that is absent from the dialkylaminoalkyl side chains of quinacrine and its analogs (**43–46**). The larger size and lipophilicities of side chains with aromatic residues would influence permeability as noted by others [30]. We found P_e to be inversely correlated to logD_{7.4} (Spearman rho –0.674, p 0.003, n = 17) which meant that lipophilic compounds like the piperazinylphenyl and Nbenzylpiperidinyl analogs have lower permeabilities than less lipophilic compounds (**43–46**).

3.4. Compound 1 as a substrate of P-glycoprotein (Pgp)

In spite of its favorable permeability and "CNS+" status, accumulation of quinacrine in the brain is hampered by its status as a substrate of the efflux protein P-glycoprotein (Pgp) [15,16,31]. To determine if the Pgp substrate property is still retained in the present series of compounds, we evaluated a representative compound **1** which has good cell-based anti-prion activity (Table 2) and an acceptable P_e value (8.13 \times 10⁻⁶ cm/s). Briefly, the method involves comparing the bidirectional transport rates of the compound across a layer of Madin–Darby canine kidney (MDCK) cells grown on the insert of a cell culture chamber. The MDCK cells were either parental with normal levels of Pgp (MDCK-WT), or stably transfected with human MDR1 cDNA so that they had higher levels of Pgp (MDCK-MDR1). The rates at which the test compound traverses the cell barrier from the apical (A) to basolateral (B) compartment $(P_{A \rightarrow B})$ and vice-versa $(P_{B \rightarrow A})$ were determined. On normal cells which have constitutional levels of Pgp (MDCK-WT), no significant difference should be detected in the bidirectional transport rates of the test compound even if it is a substrate of Pgp. On the Pgp overexpressing cells (MDCK-MDR1), a Pgp substrate will move across the cells from $A \rightarrow B$ at a slower rate due to efflux by Pgp which is localized at the apical surface of the cells. In the reverse $B \rightarrow A$ direction, the Pgp substrate will be transported more rapidly for the same reason. Thus, the efflux ratio $(P_{B \rightarrow A}/P_{A \rightarrow B})$ of a Pgp substrate is generally >2 [30] while a non-substrate of Pgp will diffuse across the cell layer at nearly comparable rates $(P_{B \rightarrow A})$ $P_{A \rightarrow B} \sim 1$). The bidirectional transport rates for **1** and guinacrine across the monolayer of parental and Pgp overexpressing MDCK cells are presented in Table 4.

The efflux ratio of quinacrine on MDCK-WT cells was 1.2, indicating comparable rates of bidirectional transport across this cell barrier. The ratio increased to 5.1 when measured on MDCK-MDR1 cells, which is in keeping with its status as a Pgp substrate. In the case of **1**, the apparent permeability values were noticeably lower (*ca* 10 fold, on both cell lines) than that of quinacrine, indicating slower diffusion rates for **1**. Nonetheless, its efflux ratio on the parental MDCK-WT cells (1.2) was similar to that of quinacrine. On the Pgp overexpressing cells, permeability in the $A \rightarrow B$ direction was reduced by half (compared to parental cells) but was maintained at similar levels in the $B \rightarrow A$ direction. Hence, efflux ratio of **1** was only 2.4, suggesting that it may have poorer affinity for Pgp than quinacrine.

Mass balance measurements showed that most of quinacrine and **1** (*ca* >50%) could be accounted in the B \rightarrow A direction but to a lesser degree in the reverse (A \rightarrow B) direction. This led us to monitor the degree to which the compounds were retained in the cells and their propensity for adsorption to the experimental set-up. The poor mass balance observed in the A \rightarrow B direction coincided (in 3 out of 4 instances) with higher levels of cell retention of the test compounds (Table 4). Pgp is asymmetrically expressed in cells, with higher levels found on apical surfaces. Both quinacrine and **1** are weak organic bases and may bind to polyanions like RNA, DNA, ATP or accumulate within acidic intracellular compartments [32]. It may be that the higher protein content of the apical surfaces of these cells predisposed these compounds to be retained in greater amounts.

4. Discussion

In an earlier report, we found that replacing the basic alkyl side chain of quinacrine with aromatic residues bearing basic substituents retained and even improved cell-based anti-prion activity [19]. The most promising analogs were **1** and **11** which had sub-micromolar activities on several prion-infected mouse neuroblastoma cell lines, including the more resistant F3 where host cells were

Table 3

Binding responses of test compounds to hPrP121–231 using surface plasmon resonance, permeability characteristics determined by PAMPA-BBB assay and estimated log *D* at pH 7.4

Code	Binding to hPrP121–231		Permeability parameters from PAMPA-BBB assay	$\log D_{7.4}^{c}$	
	Binding response (<i>RU</i> _{max}) ^{a,b} % <i>RU</i> _{max}		Effective permeability $P_e (\times 10^{-6} \text{ cm/s})^{\text{b}}$		
1	130.2 ± 15.1	120.3	8.13 ± 2.75	3.36	
2	184.4 ± 24.1	194.2	6.13 ± 2.03	3.85	
3	224.9 ± 1.8	210.0	8.79 ± 0.37	2.73	
4	215.0 ± 9.7	207.1	ND^d	2.35	
5	248.1 ± 21.7	231.6	ND^d	2.75	
6	ND^d	ND ^c	11.52 ± 1.24	2.35	
7	ND^d	ND ^c	ND^d	3.84	
8	35.0 ± 9.5	43.2	ND^d	5.43	
9	113.8 ± 14.4	127.2	ND^d	4.75	
10	125.7 ± 10.3	165.4	ND^d	3.39	
11	67.5 ± 10.3	65.4	ND^d	3.75	
12	85.2 ± 6.6	69.8	ND^d	4.08	
13	94.5 ± 0.3	98.3	15.53 ± 0.85	4.53	
14	91.8 ± 18.8	159.8	7.54 ± 2.47	3.60	
15	130.0 ± 7.9	109.2	9.15 ± 1.11	3.51	
16	169.5 ± 4.0	173.9	7.68 ± 2.69	3.41	
17	169.6 ± 1.4	147.5	7.39 ± 1.70	4.15	
18	109.6 ± 1.64	114.8	ND^d	4.12	
19	ND ^c	ND ^c	8.31 ± 1.28	2.66	
20	162.7 ± 7.1	144.4	5.28 ± 0.34	3.08	
21	92.1 ± 7.2	138.2	ND^d	5.17	
22	93.9 ± 19.6	113.7	ND^d	3.67	
Quinacrine	101.5 ± 14.3	87.3	19.30 ± 2.52	1.89	
43 ^e	115.5 ± 18.1	153.4	20.98 ± 1.01	1.32	
44 ^e	132.2 ± 3.8	163.6	13.34 ± 2.20	0.96	
45 ^e	140.2 ± 1.7	162.1	21.98 ± 4.56	1.26	
46 ^e	174.2 ± 7.3	177.6	16.37 ± 2.89	1.51	
47 ^e	83.1 ± 17.3	139.0	14.81 ± 4.63	1.97	
Quinidine ^f	ND ^d	ND ^d	11.48 ± 1.19	ND ^d	
Caffeine ^f	ND ^d	ND ^d	2.46 ± 0.33	ND ^d	
Verapamil ^f	ND ^d	ND ^d	18.45 ± 2.26	ND ^d	

^a Binding response was obtained from the maximal response at the end of the association phase from at least 3 experiments on two different chips.

OCH₃

 $^{\text{b}}$ Values are presented as mean \pm standard deviation.

^c Estimated Log *D* at pH 7.4 (ACD/Labs 6.00).

^d Not determined.





 $^{\rm f}$ Reference compounds for PAMPA-BBB assay. Pe values of caffeine and verapamil were reported to be 1.3×10^{-6} cm/s and 16.0×10^{-6} cm/s respectively [29].

stably infected with a human prion strain. In this report, **1** and **11** were structurally modified to give a more coherent lead series with the aim of identifying analogs with greater potency and selectivity, while retaining adequate drug-like profiles that would facilitate their passage across the blood—brain barrier.

Of the two hit compounds, **1** was less tolerant of structural modification, as most of the changes explored did not significantly

improve anti-prion activity. While the modifications investigated here are admittedly limited in scope and diversity, they do serve to underscore the susceptibility of the scaffold to loss of activity arising from minor changes like homologation (2-5) and isosteric replacement (6). In contrast, modifying 11 yielded several analogs with improved anti-prion profiles. The most potent analog identified in this investigation is the phenethyl homolog 16 which,

OCH₃

 NH_2

47

Table 4

Apparent permeability (*P*_{app}), efflux ratio, mass balance, cell retention and % adsorption onto the Transwell apparatus of Quinacrine and **1** across wild type MDCK-WT and Pgp overexpressing MDCK-MDR1 cell monolayers.

Compound	Cell line	$P_{app} (\times 10^{-6} \text{ cm/s})^{a}$		Efflux ratio ^b	Mass balance (%) ^a		Cell retention (%) ^a		Adsorption on Transwell (%) ^a	
		$A \rightarrow B$	$B \rightarrow A$			$B \rightarrow A$	$A \rightarrow B$	$B \rightarrow A$	$A \rightarrow B$	$B \rightarrow A$
Quinacrine	MDCK-WT	$\textbf{2.89} \pm \textbf{0.83}$	$\textbf{3.45} \pm \textbf{1.25}$	1.2	14.6 ± 2.3	51.0 ± 14.4	80.2 ± 9.5	27.7 ± 1.5	5.3 ± 5.0	3.2 ± 2.8
Quinacrine	MDCK-MDR1	1.23 ± 0.05	$\textbf{6.70} \pm \textbf{1.14}$	5.1	$\textbf{34.8} \pm \textbf{10.3}$	$\textbf{75.8} \pm \textbf{17.8}$	$\textbf{35.5} \pm \textbf{6.2}$	24.5 ± 7.3	5.5 ± 3.6	$\textbf{3.9}\pm\textbf{3.1}$
1	MDCK-WT	$\textbf{0.29} \pm \textbf{0.04}$	$\textbf{0.34} \pm \textbf{0.12}$	1.2	11.3 ± 1.5	$\textbf{75.3} \pm \textbf{7.0}$	$\textbf{66.4} \pm \textbf{29.0}$	$\textbf{27.5} \pm \textbf{12.3}$	11.4 ± 8.9	$\textbf{3.5} \pm \textbf{2.5}$
1	MDCK-MDR1	$\textbf{0.14} \pm \textbf{0.02}$	$\textbf{0.34} \pm \textbf{0.10}$	2.4	12.6 ± 10.9	$\textbf{73.0} \pm \textbf{13.9}$	$\textbf{67.0} \pm \textbf{14.4}$	44.2 ± 12.8	$\textbf{6.1} \pm \textbf{3.7}$	3.1 ± 2.5

^a Values were presented as man \pm standard deviation from three independent experiments.

^b Ratio of apparent permeabilities from the basolateral (B) to apical (Å) compartment, to that of apical (Å) to basolateral (B) compartments. Efflux ratio = $P_{app}(B \rightarrow A)/P_{app}(A \rightarrow B)$.

unusual among the other test compounds, was equipotent on both ScN2a and F3 (EC₅₀ 0.13 μ M, 0.19 μ M).

Although the piperazinylphenyl and N-benzylpiperidinyl analogs are distinguished by their N-substituted 9-amino residues, these side chains share common features, namely an aromatic ring and a basic nitrogen-containing heterocycle. The present findings suggest that these features promote, but do not ensure, a broader anti-prion profile. However, in analogs where one but not the other feature is present, the few examples presented here consistently showed no activity on F3 and N167. This was duly observed for **6** and the quinacrine analog **43** [19] (no aromatic ring but with basic moiety) and the piperazinylphenyl analogs **7** and **8** (aromatic ring but without basic moiety).

The acridine ring of **1** and **11** may be replaced by tetrahydroacridine or quinoline. As to whether one scaffold is preferred over the other, the present findings suggest that it is not the scaffold per se, but the side chain attached to it, that influences activity. In this regard, the preferred side chain is the N-(1-benzylpiperidin-4yl) of **11**, as both the tetrahydroacridine (**21**) and quinoline (**22**) analogs had better anti-prion profiles than the corresponding members with the piperazinylphenyl side chain (**9**, **10**). Potency aside, the ring scaffold has a marked effect on cytotoxicity and analogs with the acridine ring were decidedly more cytotoxic (lower TC) than those bearing other heterocyclic rings.

Many investigators have sought to establish a correlation between EC₅₀ derived from cell-based assays and binding affinities to PrP^C as determined from SPR measurements [27,28,33–35]. Compounds that bind to PrP^C are considered more likely to demonstrate an anti-prion effect [28] as the binding step is widely viewed as an important initiating event in prion pathology [1]. Here, we found that $EC_{50 ScN2a}$ of the test compounds (but not EC_{50} F3, possibly due to the smaller number of compounds available for analysis) were significantly correlated (p < 0.05) to the binding parameters %RU_{max} and RU_{max}. In this regard, it was of interest to note that the strong binding affinities of the piperazinylphenyl analogs for the truncated prion protein were linked to their lower lipophilicities (log $D_{7,4}$), or more specifically, to the piperazine ring in the side chain. This is demonstrated in 4 and 20, where replacing the piperazine of 4 with piperidine (20) increased logD_{7.4} from 2.35 to 3.08, and concurrently reduced $\&RU_{max}$ from 207 (4) to 144 (20). As to why a less lipophilic entity is associated with greater binding affinity to PrP^C and by extrapolation, greater anti-prion potency, this question remains to be addressed.

Effective passage across the blood-brain barrier and accumulation in brain tissues are important requirements for anti-prion agents. Quinacrine is known to pass through the blood-brain barrier [36] although accumulation in the brain is curtailed to a significant extent by its efflux by Pgp [15,16,31]. Here we found that the piperazinylphenyl and N-benzylpiperidine analogs had lower PAMPA-BBB permeabilities compared to quinacrine, although their P_e values still qualified them as "high brain penetration" (CNS+) compounds. Like binding affinities, effective permeabilities (P_e) were significantly and inversely correlated to lipophilicities $(\log D_{7.4})$. A closer inspection of the correlation showed that the relationship was driven more by the quinacrine analogs (43-46) rather than the piperazinylphenyl/N-benzylpiperidinyl analogs. The quinacrine analogs had high P_e values (16-22 \times 10⁻⁶ cm/s) and at least 2-fold magnitude lower lipophilicities (log D_{7,4}: 0.96–1.51) than the piperazinylphenyl/N-benzylpiperidinyl analogs. On the other hand, the differences in logD_{7.4} values of the piperazinylphenyl/ N-benzylpiperidinyl analogs are no more than a fold magnitude, and probably too narrow in range to differentiate between their permeabilities.

It is apparent that the 9-(N-substituted) side chain strongly influences the anti-prion and physicochemical profiles of the final compound. The non-aromatic and basic side chains of quinacrine and its analogs (**43–46**) are largely responsible for their lower lipophilicities and in turn, their strong binding affinities for hPrP_{123–231} and good PAMPA permeabilities. Unfortunately, antiprion activities were limited to ScN2a and did not extend to other cell models. The aromatic and basic side chains of the piperazinylphenyl/N-benzylpiperidinyl series resulted in more lipophilic compounds, which are strong hPrP_{123–231} binders and still CNS+ in spite of lower PAMPA permeabilities, but with clearly improved cell-based anti-prion profiles.

Although quinacrine has good permeability across the brain, its accumulation is hampered by Pgp which is expressed at high levels at the blood brain barrier. The bidirectional transport of quinacrine on Pgp overexpressing MDCK-MDR1 cells has an efflux ratio of 5, which is in keeping with its status as a Pgp substrate. Interestingly, 1 traversed the MDCK-MDR1 barrier with an efflux ratio that is half that of quinacrine, indicating that it may be a weaker Pgp substrate.

5. Conclusion

Taken together, the present study shows that the piperazinylphenyl and N-benzylpiperidinyl analogs are promising leads for anti-prion design. The 9-N-substituted side chain of these compounds influence anti-prion activity to a significant degree. It also determines the physicochemical profile, in particular lipophilicity and ionizability as assessed from $\log D_{74}$. Potent and broad ranging anti-prion activities are found in side chains with aromatic residues substituted with basic functionalities. These structural features also diminished effective permeability in PAMPA-BBB by increasing logD_{7.4}, but fortunately still kept the compounds within the permeability range of CNS+ compounds. Their lower permeabilities may be compensated by weaker Pgp substrate properties, as found for 1. If confirmed for other piperazinylphenyl and Nbenzylpiperidinyl analogs, this would enhance their standing as potential drug-like and potent anti-prion templates for further investigation.

6. Experimental

6.1. General experimental methods

¹H NMR and ¹³C NMR spectra were recorded on a Bruker DPX 300 MHz spectrometer and chemical shifts were reported in δ (ppm) relative to the internal standard TMS. Mass spectra (MS, nominal) were collected on an LCQ Finnigan MAT mass spectrometer. Atmospheric pressure ionization (APCI) or electron spray (ionization (ESI)) were used as probes. Reactions were routinely monitored by thin layer chromatography using silica gel 60 F 254 plates from Merck, with UV light as a visualizing agent. Column chromatography was performed using silica gel G (0.04–0.063 mm, Merck). Purity analysis was verified by high-pressure liquid chromatography (HPLC) or by combustion analysis. Combustion analyses (C, H) were determined by Perkin–Elmer PE 2400 CHN/CHNS elemental analyzer by the Department of Chemistry, National University of Singapore. Spectroscopic data, melting points, yields and purities of individual compounds are listed in Appendix 1.

6.2. General procedure for the reaction of 2-methoxy-6,9-

dichloroacridine, and 4,7-dichloroquinoline with amines in ethanol as solvent

A previously described method [19] was followed for the syntheses of **1**–**5**, **7**, **8**, **10** and **20**. Briefly, equimolar quantities of the amine and the acridine/quinoline were dissolved in ethanol, 2 drops of conc. HCl were added and the mixture refluxed for

ca 24 h. On cooling, NaOH (1 M) or ammonia solution (25%) was added, followed by dichloromethane to extract the product. Alternatively, if the product precipitated out of solution on alkalinization, it was removed by filtration and purified by column chromatography.

6.3. General procedure for the reaction of 2-methoxy-6,9-dichloroacridine, 3,9-dichloro-5,6,7,8-tetrahydroacridine and 4,7-dichloroquinoline with amines in phenol as solvent

A previously described method [19] was followed for the syntheses of **6**, **9**, **11**–**19**, **21** and **22**. Briefly, equimolar quantities of the amine and the acridine/quinoline were reacted in phenol at 120 °C for 4 h with stirring. On cooling, dichloromethane was added, followed by a solution of NaOH (1 M). The crude product was extracted with dichloromethane and purified by column chromatography.

6.4. Synthesis of 1-methyl-4-(4-nitrophenyl)piperazine, 1-ethyl-4-(4-nitrophenyl)piperazine (**23**) and 1-[4-(4-nitrophenyl)piperazin-1-yl]ethanone (**24**) by Hartwig-Buchwald amination reaction

The method of Nguyen Thi et al. was followed [19]. Briefly, Cs_2CO_3 , BINAP were added to a flask under argon. A solution of the iodobenzene and amine in dry toluene was added, followed by $Pd(OAc)_2$. The mixture was heated (90 °C) until no trace of the iodobenzene was observed on TLC. On cooling, the reaction mixture was filtered and concentrated to give the crude product which was purified by column chromatography.

6.5. General procedure for catalytic reduction of substituted nitrobenzenes

The substituted nitrobenzenes were reduced to the corresponding anilines by catalytic reduction (50 psi, 10% Pd/C (5% w/w)) on a Parr hydrogenator as previously described [19] The anilines (not characterized) were then condensed with 6,9-dichloro-2-methoxyacridine or 4,7-dichloroquinoline (Section 6.2.) to give **1–3**, **10**.

6.6. Synthesis of 4-[(4-methylpiperazin-1-yl)methyl] benzenamine (**25**) and 4-[(piperidin-1-yl) methyl] benzenamine (**26**)

These amines were required for the syntheses of **4** and **20** respectively. To a solution of 4-nitrobenzylchloride (1 mmol) in anhydrous THF (3 mL) was added 1-methylpiperazine or piperidine (1 mmol) and triethylamine (1.5 mmol, 0.21 mL). The solution was heated at 70 °C overnight. The reaction mixture was then extracted with dichloromethane and water. The organic fractions were combined, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified by column chromatography (hexane/EA 1:4) and characterized by ¹H NMR (Supplementary Information). It was dissolved in 10 mL ethanol, PtO₂ (0.01 g) was added under nitrogen. Hydrogenation was carried out on a Parr hydrogenator at 50 psi for 16 h. The catalyst was then removed by filtration and the filtrate was concentrated in vacuo to give the amine in quantitative yield.

6.7. (4-Aminophenyl) (4-methylpiperazin-1-yl) methanone (27)

This amine was required for the synthesis of **5**. 4-nitrobenzoylchloride (1 mmol, 0.186 g) in anhydrous dichloromethane (5 mL) was reacted with 1-methylpiperazine (1 mmol, 0.11 mL) and triethylamine (1.5 mmol, 0.21 mL) overnight with stirring at room temperature. Workup was carried out as described in Section 6.6 to give 0.249 g of (4-methylpiperazin-1-yl) (4-nitrophenyl) methanone, yellow liquid. ¹H NMR (300 MHz, CDCl₃) δ 2.33 (s, 3H), 2.40 (m, 2H), 2.51 (m, 2H), 3.39 (m, 2H), 3.82 (m, 2H), 7.57 (d, *J* = 8.6, 2H), 8.28 (d, *J* = 8.6, 2H). MS(ESI) *m*/*z* [M + H⁺] 250.1. Reduction to give the title compound was carried out as described in Section 6.6.

6.8. Synthesis of 4-(4-methylpiperazin-1-yl) but-2-yn-1-amine (**30**) [21]

1,4-Dichloro-2-butyne (6 mmol, 0.58 mL) was added to the stirred suspension of potassium phthalimide (3 mmol, 0.556 g) in DMF (5 mL), and heated to 100 °C for 5 h. After cooling, the reaction mixture was extracted with dichloromethane and water. The organic layers were pooled, dried, and concentrated in vacuo. The residue was purified by column chromatography (hexane/ethyl acetate 1:19) to furnish 0.35 g of 2-(4-chlorobut-2-ynyl)isoindoline-1,3-dione (28) (white solid, yield 50%). ¹H NMR (300 MHz, CDCl₃) 4.11 (s, 2H), 4.51 (s, 2H), 7.75 (dd, *J*1 = 3.1, *J*2 = 5.5, 2H), 7.89 (dd, J1 = 3.1, J2 = 5.4, 2H).¹³C NMR (75 MHz, CDCl₃) δ 27.1, 45.7, 46.9, 51.7 (2C), 54.7 (2C), 78.1, 78.4, 123.4 (2C), 131.9 (2C), 134.0 (2C), 166.9 (2C). 28 was reacted with 1-methylpiperazine following the method described in Section 6.6. to give 2-(4-(4-methylpiperazin-1-yl)but-2-ynyl)isoindoline-1,3-dione (29): Yellow solid, 83% yield. ¹H NMR (300 MHz, CDCl₃) δ 2.28 (s, 3H), 2.45 (m, 4H), 2.58 (m, 4H), 3.26 (s, 2H), 4.47 (s, 2H), 7.73 (dd, J1 = 3.1, J2 = 5.4, 2H), 7.88 (dd, $J_{1} = 3.1, J_{2} = 5.4, 2H$). MS(APCI) m/z [M + H⁺] 298.2. A solution of **29** (0.72 mmol, 0.213 g) and hydrazine (0.72 mmol, 0.03 mL) in 1 mL of ethanol was heated at reflux for 2 h. After cooling to 0 °C. phthalhydrazide was removed by filtration. Evaporation of the filtrate gave **30** as a free base. ¹H NMR (300 MHz, CDCl₃) δ 2.30 (s, 3H), 2.49–2.61 (m, 8H), 3.28 (t, J = 1.8, 2H), 3.44 (t, J = 1.8, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 29.7, 31.6, 46.0, 47.2, 52.0 (2C), 53.0, 54.9 (2C). MS(ESI) m/z [M + H⁺] 168.1.

6.9. General method for the synthesis of 1-benzylpiperidine-4amine, 1-phenethylpiperidine-4-amine, 1-phenpropylpiperidine-4amine and their ring substituted analogs

A solution of piperidin-4-yl carbamic acid t-butyl ester (2 mmol), phenylalkyl halide (2 mmol), and triethylamine (6 mmol) in tetrahydrofuran (THF, 5 mL) was refluxed overnight, THF was removed in vacuo, dichloromethane was added and the mixture was extracted with dilute NaHCO₃. The organic fractions were combined, dried over anhydrous Na₂SO₄, concentrated in vacuo, and the residue purified by column chromatography (EA/hexane) to give the carbamic acid t-butyl ester intermediates (31, 33-37) (Supplementary Information). In the case of t-butyl 1-(4-chlorobenzyl)piperidin-4-ylcarbamate (32), 1-chloro-4-(chloromethyl) benzene (38) was synthesized by adding SOCl₂ (15 mmol, 1.1 mL) dropwise at 0 °C to a stirred solution of 4-chlorobenzylhydroxy (5 mmol, 0.713 g) in anhydrous dichloromethane (5 mL). The reaction mixture was allowed to reach room temperature, stirred for 1 h, and then cooled down to 0 °C again. It was then carefully diluted with water, and neutralized with saturated NaHCO₃. The reaction mixture was extracted with dichloromethane and washed with water to afford the product **38** as a yellow oil (0.64 g, 80%) (Supplementary Information).

The carbamic acid tert-butyl ester intermediate was dissolved in dichloromethane (4 mL) and tetrafluoroacetic acid (TFA, 4 mL) was added dropwise. The reaction mixture was neutralized with saturated (5 M) NaOH, extracted with dichloromethane and NaHCO₃ solution to give the desired amine as an oil. The amine was not characterized but reacted immediately with 6,9-dichloro-2-methoxyacridine by the general procedure described in Section 6.3.

6.10. Synthesis of 8-benzyl-8-aza-bicyclo[3.2.1]octan-3-amine (**42**) [23]

A solution of ethyl 3-oxo-8-azabicylo[3.2.1]octane-8-carboxylic acid (2.3 mmol, 0.46 g) in ethanol (1 ml) was mixed with KOH (0.353 g) in water (5 mL) and heated at 100 °C for 3 h. After cooling down to room temperature, the solution was diluted with 20 mL of dichloromethane. The organic laver was dried and concentrated in vacuo to give crude 8-aza-bicyclo[3.2.1]octan-3-one (**39**). ¹H NMR $(300 \text{ MHz}, \text{CDCl}_3) \delta 1.69 \text{ (m, 2H)}, 1.90 \text{ (dd, } 1 = 4.1, 12 = 8.8, 2\text{H}), 2.31$ (m, 1H), 2.36 (m, 2H), 2.56 (m, 2H), 3.87 (m, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 30.0 (2C), 50.8 (2C), 54.9 (2C), 209.7. **39** was reacted with benzyl chloride as in Section 6.6 to give 8-benzyl-8-aza-bicyclo [3.2.1]octan-3-one (**40**). ¹H NMR (300 MHz, CDCl₃) δ 1.63 (m, 2H), 2.16 (m, 4H), 2.72 (m, 2H), 3.50 (m, 2H), 3.76 (m, 2H), 7.34 (m, 5H). $^{13}\text{C}\,\text{NMR}\,(75\,\text{MHz},\text{CDCl}_3)\,\delta\,27.7\,(2\text{C}),\,48.1\,(2\text{C}),\,55.1,\,58.6\,(2\text{C}),\,127.2,$ 128.4 (2C), 128.5 (2C), 138.9, 210.1. MS(APCI) m/z [M⁺] 215.3. To obtain the oxime 41, hydroxylamine hydrochloride (1.7 mmol, 0.117 g) was stirred in methanol (7 mL) at 0 °C. The slurry was treated with Na₂CO₃ (0.09 g) and stirred for 5 min after which 40 (1.36 mmol, 0.29 g) in methanol (1 mL) was added. After stirring for 5 h at room temperature, methanol was removed in vacuo, the residue was treated with dichloromethane and brine, the organic fractions were combined, dried (anhydrous Na₂SO₄), and concentrated to give 0.2 g of the oxime **41** as a white solid (Yield 65%). ¹H NMR (300 MHz, CDCl₃) δ 1.52 (t, J = 9.6, 1H), 1.63 (t, J = 9.6, 1H), 2.04 (m, 2H), 2.14 (d, J = 14.7, 1H), 2.26 (dd, J1 = 2.7, J2 = 12.6), 2.62 (d, J = 14.7, 1H), 2.99 (d, J = 15.6, 1H), 3.36 (b, 2H), 3.66 (s, 2H), 7.25–7.42 (m, 5H). ¹³C NMR (75 MHz, CDCl₃) δ 26.6, 27.5, 31.3, 37.1, 55.5, 57.8, 58.4, 127.0, 128.2 (2C), 128.7 (2C), 138.9, 156.3. MS(ESI) m/ $z [M + H^+]$ 231.1. The oxime **41** was reduced with Adam's catalyst as described in Section 6.6 to give the title compound 42 as a yellow oil (76%) which was not characterized and reacted immediately to give 19 (Section 6.3).

6.11. 3,9-Dichloro-5,6,7,8-tetrahydroacridine (43) [22]

To a mixture of 2-amino-4-chlorobenzoic acid (5 mmol, 0.858 g) and cyclohexanone (5 mmol, 0.52 mL) was carefully added 5 mL of POCl₃ at 0 °C. The mixture was heated under reflux for 2 h, then cooled to room temperature, and concentrated to give a slurry. The residue was diluted with dichloromethane, neutralized with aqueous K₂CO₃, and washed with brine. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo to furnish 1.12 g of **43** as a reddish brown solid (Yield 88%). ¹H NMR (300 MHz, CDCl₃) δ 1.89 (m, 4H), 2.88 (t, *J* = 5.3, 2H), 3.06 (9t, *J* = 5.3, 2H), 7.34 (dd, *JI* = 1.9, *JZ* = 8.9, 1H), 7.89 (m, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 22.1, 22.2, 27.1, 33.4, 123.3, 124.7, 126.8, 127.1, 128.9, 135.1, 141.4, 145.9, 160.5. MS(APCI) *m/z* [M⁺] 252.7.

6.12. Evaluation of cell-based anti-prion activity

The anti-prion activity of test compounds was determined on ScN2a, N167 and F3 cell models as described by Nguyen Thi et al. [19].

6.13. Preparation of human prion protein 121–231 (hPrP_{121–231})

Recombinant PrP (rPrP, human PrP 121-231aa with an Nterminal histidine tag and Factor Xa digestion sequence) was bacterially expressed as inclusion bodies by the *Escherichia coli* protein expression pET system. The inclusion bodies were solubilized in 8 M urea, 25 mM Tris—HCl, 300 mM NaCl, pH 7.5 and then purified by metal chelating chromatography (HiTrap-Ni, GE Health Science). The purified rPrP was refolded by rapid dilution and incubation in 1 M arginine–HCl, 1 mM oxidized glutathione, pH 8.0 at 37 °C overnight. The refolded rPrP was dialyzed against water and concentrated by centrifugal membrane (Amicon Ultra-15, MWCO 3000, Millipore). To remove N-terminal histidine tag, rPrP (0.2 mg/mL) was digested with 0.3 μ g/mL trypsin at 16 °C for 16 h in 25 mM Tris–HCl, pH 7.5. The N-terminal histidine tag-truncated rPrP was purified by anion-exchange chromatography (UNO-Q, 1 mL, Bio-Rad), buffer was changed to 10 mM NaOAc (pH6.0) with Amicon Ultra-15, and stored at -80 °C. Protein concentration was determined by UV measurement and found to be equivalent to 0.79 mg/ml.

6.14. Evaluation of binding affinity by surface plasmon resonance

The assay was performed on a BIAacore 3000 platform (BIAcore, Uppsala, Sweden) following reported methods with modifications [27,28]. The sensor chip CM5 and amine coupling kit were purchased from GE Healthcare Bio-sciences AB (Uppsala, Sweden). The running buffer was prepared by filtering and degassing a phosphate buffer 1×, pH 7.4 containing 2.5% DMSO. Recombinant human prion protein (amino acids 121-231; hPrP121-231, molecular weight 12,544.97 Da) was dissolved in 10 mM sodium acetate buffer, pH 4.5 (10 μ g/ml) and immobilized on the CM5 chip to reach a density of ca 3000 response units (RU) using amine coupling. A reference surface was prepared by treating a blank flow cell in the same way as the flow cell containing the immobilized peptide. Stock solutions of test compounds (2 mM) were prepared in DMSO and diluted to 50 μ M with phosphate buffer 1×, pH 7.4. Each analytical cycle consisted of running buffer for 60 s (stabilization phase), a sample injection of 50 µM in running buffer for 120 s (association phase) and running buffer for 150 s (dissociation phase). Flow rates were kept at 30 µL/min. Surface regeneration of the chip was carried out by injecting 10 mM NaOH (30 s, flow rate of 30 µL/min). After regeneration, the surface was allowed to stabilize for ca 60 s before the next injection. The binding response curve was obtained by subtracting the background signal (from the reference flow cell) from that of the sample flow cell in order to correct for non-specific binding and bulk effects due to the analyte. The binding affinity was taken as the maximum response at the end of the association phase as seen from the binding response curve. Binding was expressed as % RU_{max} which is defined as the % theoretical maximum binding response units (RU) and determined by the following equation assuming a 1:1 stoichiometry:

$$\% RU_{\text{max}} = \left[RU_{\text{compound}} / RU_{\text{immobilized protein}} \right] \\ \times \left[MW_{\text{protein}} / MW_{\text{compound}} \right] \times 100\%$$

6.15. Evaluation of permeability by the PAMPA-BBB assay

The method described by Di et al. [29] was followed with modifications. Porcine polar brain lipid (PBL) (catalog no. 141101) was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Dodecane was obtained from Sigma–Aldrich. The acceptor plate was a 96-well filter plate (MultiscreenTM-IP, catalog no. MAIPN4510, PVDF membrane, pore size is 0.45 μ m) from Millipore (Bredford, USA) and the donor plate was a 96-well plate (catalog no. MATRNPS50, Millipore, Billerica, MA, USA). The 96-well UV plate (catalog no. 3535) was purchased from Corning Inc. (NY, USA). Compounds were dissolved in DMSO at 10 mM, and diluted in phosphate buffer solution 1×, pH 7.4–50 μ M or 30 μ M depending on their aqueous solubilities. An aliquot (300 μ L) of the diluted solution was transferred to the donor well. The filter membrane was coated with 5 μ L of 20 mg/mL PBL in dodecane and the

acceptor well was filled with 150 μ L of the same phosphate buffer solution. The donor plate was carefully aligned to the acceptor plate such that the underside of the membrane remained in contact with buffer solution in the donor wells. The sandwich assemble was incubated in an airtight humid box at room temperature for 10 h. The concentrations of compound in the acceptor, donor and reference wells were determined by UV absorbance at 270 nm using the Tecan Infinite 200 microplate reader. Permeability rate (*Pe*_e) and membrane retention (*R*) were calculated using the following equations:

$$P_{e} = -\frac{2.303V_{A}}{A \cdot t} \left[\frac{1}{1 + r_{v}} \right] \log_{10} \left\{ 1 - \left[\frac{1 + r_{v}^{-1}}{1 - R} \right] \frac{C_{A}(t)}{C_{D}(t = 0)} \right\}$$
$$R = 1 - \frac{V_{A}C_{A}(t) + V_{D}C_{D}(t)}{V_{D}C_{D}(t = 0)}$$

where A is the active surface area of membrane (0.24 cm²), t is the incubation time (s), V_A and V_D are the volumes (cm³) of the acceptor and donor chambers, $r_v = V_D/V_A$, C_A and C_D are drug concentrations in the acceptor and donor chambers.

6.16. Cell-based bidirectional transport assay

Dulbeco's Modified Eagle's medium (DMEM) was purchased from Sigma (MO, USA). Fetal bovine serum was from Hyclone Lab Inc. (Logan, UT, USA). The MDCK-WT (wild type) and MDCK-MDR1 (overexpressing Pgp) were gifts from Dr. Piet Borst (Netherlands Cancer Institute, University of Amsterdam, Netherlands), Western blotting was performed on these two cell lines to confirm the overexpression of Pgp as described [37]. Transwell[®] plates (Cat. no 3401, 12 mm diameter, 0.4 μ m pore size) were obtained from Costar Corp. (Cambridge, MA, USA). The Millicell-ERS system was from Millipore Corp. (Bedford, MA, USA). Transport medium was Hank's balance salt solution (HBSS) from Invitrogen (CA, USA) supplemented with 10 mM HEPES at pH 7.4 (HBSS-HEPES). 10 mM stock solutions of quinacrine and **1** were prepared in DMSO and further diluted to 10 μ M with the transport medium (HBSS-HEPES). The MDCK-MDR1 cell line was grown in DMEM supplemented with 10% fetal bovine serum, 100 mg/L penicillin, and 100 mg/L streptomycin at 37 °C in an atmosphere containing 5% CO₂. For transport assay, cells with passage number 3-8 were seeded at a density of 300,000 cells/well and grown for 4 days in 12-well Transwell® plates. Cell monolayers with transepithelial electrical resistance (TEER) values greater from 120 to 150 Ω cm² were used for the experiment. On the day of assay, fresh medium was added to the cells. Two hours later, they were washed twice and equilibrated with HBSS-HEPES medium for 30 min 500 µL of 10 µM test compounds was added to the apical chamber and 1500 µl of HBSS-HEPES was dispensed to the basolateral chamber for determining apical to basolateral $(A \rightarrow B)$ transport. After 2 h, cells were lysed with 600 µL of lysis solution (acetonitrile:water:formic acid = 70:30:0.1) for 10 min and the cell debris removed by centrifugation (12,000g, 5 min, 4 °C). Both chambers were thoroughly washed with 1 ml of the same lysis solution and this wash solution was tested for presence of compounds adsorbing onto the plastic surface of the Transwell apparatus. Similarly, 500 µL of HBSS-HEPES was introduced to the apical chamber and 1500 µL of the test solution to the basolateral chamber to determine the basolateral to apical $(B \rightarrow A)$ transport. Aliquots (10 µL) were withdrawn from each chamber for quantification by LC/MS/MS (Supplementary Information). The integrity of the monolayer was determined by TEER value and by monitoring the transport of Lucifer yellow, a fluorescent marker for paracellular transport. TEER value was calculated using the following equation:

$$\text{TEER} = \left(R_{\text{cell layer}} - R_{\text{blank}} \right) \times A$$

where $R_{\text{cell layer}} = \text{Resistance}(\Omega)$ of the cell molaye, $R_{\text{blank}} = \text{Resistance}(\Omega)$ of the blank (insert without cells), $A = \text{Effective surface area of the insert (1 cm² for the 12-well insert).$

Cell layers with TEER values in the range of $120-150 \Omega \text{ cm}^2$ were deemed suitable for the bidirectional assay. Apparent permeability (P_{app} , cm/s) was calculated as:

$$P_{app} = J/C_0 = [V_r \cdot C_r]/[A \cdot t \cdot C_0]$$

where *J* is the flux rate, C_0 is the initial concentration in the donor compartment, V_r is the volume of the receiver compartment at the end of the assay, C_r is concentration of compound in the receiver compartment at the end of the assay, *A* is the effective surface area of the insert, *t* is duration of the assay (2 h). Mass balance was determined as

$$MB(\%) = [C_{At} \cdot V_A + C_{Bt} \cdot V_B] / [C_0 V_D] \times 100\%$$

where C_{At} is the concentration of compound in the apical compartment at the end of the assay, V_A is volume of the apical compartment, C_{Bt} is the concentration of compound in the basal compartment at the end of the assay, C_0 is the initial concentration in the donor compartment, V_D is the volume of the donor compartment.

6.17. Statistical analysis

Spearman correlation analysis was carried out using SPSS version 13.0. (SPSS Inc., IL, USA). Log *D* at pH 7.4 was determined with ACD/Labs 6.00 (Advanced Chemistry Development, ON, CN).

Acknowledgments

The authors gratefully acknowledged support by National University of Singapore (NUS) Academic research Fund R148000064112 (to GML) and the Program for the Promotion of Fundamental Studies in Health Science of the NIBIO in Japan, and by the Health and Labor Sciences Research Grant (Research on Measures for Intractable Diseases) from the Ministry of Health, Labor, and Welfare of Japan (to KD and YS). TN was supported by the NUS Ministry of Education Research Scholarship for her graduate studies.

Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.04.016.

References

- A. Aguzzi, A.M. Calella, Prions: protein aggregation and infectious diseases, Physiol. Rev. 89 (2009) 1105–1152.
- [2] C.R. Trevitt, J. Collinge, A systematic review of prion therapeutics in experimental models, Brain 129 (2006) 2241–2265.
- [3] V.L. Sim, B. Caughey, Recent advances in prion chemotherapeutics, Infect. Disord. Drug Targets 9 (2009) 81–91.
- [4] K. Teruya, K. Kawagoe, T. Kimura, C.J. Chen, Y. Sakasegawa, K. Doh-ura, Amyloidophilic compounds for prion diseases, Infect. Disord. Drug Targets 9 (2009) 15–22.
- [5] G. Mallucci, J. Collinge, Rational targeting for prion therapeutics, Nat. Rev. Neurosci. 6 (2005) 23–34.
- [6] S.B. Prusiner, Novel proteinacious infectious particles cause scrapie, Science 216 (1982) 136–144.
- [7] M. Otto, L. Cepek, P. Ratzka, Efficacy of flupirtine on cognitive function in patients with CJD: a double-blind study, Neurology 62 (2004) 714-718.
- [8] S. Haik, J.P. Brandel, D. Salomon, Compassionate use of quinacrine in Creutzfeldt–Jakob disease fails to show significant effects, Neurology 63 (2004) 2413–2415.

- [9] M. Nakajima, T. Yamada, T. Kusuhara, Results of quinacrine administration to patients with Creutzfeldt–Jakob disease, Dement. Geriatr. Cogn. Disord. 17 (2004) 158–163.
- [10] J.F. Martinez-Lage, A. Rabano, J. Bermejo, M.M. Perez, M.C. Guerrero, M.A. Contreras, A. Lunar, Creutzfeldt–Jakob disease acquired via a dural graft: failure of therapy with quinacrine and chlorpromazine, Surg. Neurol. 64 (2005) 542–545.
- [11] J. Collinge, M. Gorham, F. Hudson, A. Kennedy, G. Keogh, S. Pal, M. Rossor, P. Rudge, D. Siddique, M. Spyer, D. Thomas, S. Walker, T. Webb, S. Wroe, J. Darbyshire, A partially randomised patient preference trial to assess the safety and activity of quinacrine in human prion disease: the PRION-1 trial, Lancet Neurol. 8 (2009) 334–344.
- [12] T.W.J. Furlow, R.J. Whitley, F.J. Wilmes, Repeated suppression of Creutz-feldt–Jakob disease with vidarabine, Lancet 2 (1982) 564–565.
- [13] C. Masullo, G. Macchi, Y.G. Xi, M. Pocchiari, Failure to ameliorate Creutzfeldt–Jakob disease with amphotericin B therapy, J. Infect. Dis. 165 (1992) 784–785.
- [14] M.G. Terzano, E. Montanari, S. Calzetti, D. Mancia, A. Lechi, The effect of amantadine on arousal and EEG patterns in Creutzfeldt–Jakob disease, Arch. Neurol. 40 (1983) 555–559.
- [15] Y. Huang, H. Okochi, B.C.H. May, G. Legname, S.B. Prusiner, Quinacrine is mainly metabolized to mono-desethyl quinacrine by CYP3A4/5 and its brain accumulation is limited by P-glycoprotein, Drug Metab. Dispos. 34 (2006) 1136–1144.
- [16] S. Ghaemmaghami, M. Ahn, P. Lessard, K. Giles, G. Legname, S.J. DeArmond, S.B. Prusiner, Continuous quinacrine treatment results in the formation of drug-resistant prions, PLoS Pathog. 5 (2009) e1000673.
- [17] B.C.H. May, A.T. Fafarman, S.B. Hong, M. Rogers, L.W. Deady, S.B. Prusiner, F.E. Cohen, Potent inhibition of scrapie prion replication in cultured cells by bis-acridines, Proc. Natl. Acad. Sci. USA 100 (2003) 3416–3421.
- [18] R. Klingenstein, S. Lober, P. Kujala, S. Godsave, S.R. Leliveld, P. Gmeiner, P.J. Peters, C.J. Korth, Tricyclic antidepressants, quinacrine and a novel, synthetic chimera thereof clear prions by destabilizing detergent-resistant membrane compartments, J. Neurochem. 98 (2006) 748–759.
- [19] H.T. Nguyen Thi, C.Y. Lee, K. Teruya, W.Y. Ong, K. Doh-ura, M.L. Go, Antiprion activity of functionalized 9-aminoacridines related to quinacrine, Bioorg. Med. Chem. 16 (2008) 6737–6746.
- [20] P. Bazzini, C.G. Wermuth, Substituent groups. in: C.G. Wermuth (Ed.), The Practice of Medicinal Chemistry. Academic Press, London, 2008, pp. 445–447.
- [21] H.B. Jeon, Y. Lee, C. Qiao, H. Huang, L.M. Sayre, Inhibition of bovine plasma amine oxidase by 1,4-diamino-2-butenes and -2-butynes, Bioorg. Med. Chem. 11 (2003) 4631–4641.
- [22] M.K. Hu, L.J. Wu, G. Hsiao, M.H. Yen, Homodimeric tacrine congeners as acetylcholinesterase inhibitors, J. Med. Chem. 45 (2002) 2277–2282.
- [23] P. Kutschy, M. Dzurilla, M. Takasugi, M. Torok, I. Achbergerova, R. Homzova, M. Racova, New syntheses of indole phytoalexins and related compounds, Tetrahedron 54 (1998) 3549–3566.

- [24] K. Doh-Ura, T. Iwaki, B. Caughey, Lysosomotropic agents and cysteine protease inhibitors inhibit scrapie-associated prion protein accumulation, J. Virol. 74 (2000) 4894–4897.
- [25] S. Dollinger, S. Lober, R. Klingenstein, C. Korth, P.A. Gmeiner, A chimeric ligand approach leading to potent antiprion active acridine derivatives: design, synthesis, and biological investigations, J. Med. Chem. 49 (2006) 6591–6595.
- [26] A. Kimata, H. Nakagawa, R. Ohyama, T. Fukuuchi, S. Ohta, K. Doh-ura, T. Suzuki, N. Miyata, New series of antiprion compounds: Pyrazolone derivatives have the potent activity of inhibiting protease-resistant prion protein accumulation, J. Med. Chem. 50 (2007) 5053–5056.
- [27] S. Kawatake, Y. Nishimura, S. Sakaguchi, T. Iwaki, K. Doh-ura, Surface plasmon resonance analysis for the screening of anti-prion compounds, Biol. Pharm. Bull. 29 (2006) 927–932.
- [28] F. Touil, S. Pratt, R. Mutter, B. Chen, Screening a library of potential prion therapeutics against cellular prion proteins and insights into their mode of biological activities by surface plasmon resonance, J. Pharm. Biomed. Anal. 40 (2006) 822–832.
- [29] L. Di, E.H. Kerns, K. Fan, O.J. McConnell, G.T. Carter, High throughput artificial membrane permeability assay for blood-brain barrier, Eur. J. Med. Chem. 38 (2003) 223–232.
- [30] E.H. Kerns, L. Di, Blood-brain barrier. in: E.H. Kerns, L. Di (Eds.), Drug-like Properties: Concepts, Structure Design and Methods: From ADME to Toxicity Optimization. Academic Press, Amsterdam, 2008, pp. 122–136.
- [31] S. Dohgu, A. Yamauchi, F. Takata, Y. Sawada, S. Higuchi, Uptake and efflux of quinacrine, a candidate for the treatment of prion diseases, at the blood-brain barrier, Cell Mol. Biol. 24 (2004) 205–217.
- [32] D.S. Miller, A.R. Villalobos, J.B. Pritchard, Organic cation transport in rat choroid plexus cells studied by fluorescence microscopy, Am. J. Phys. 276 (1999) C955–C968.
- [33] J. Hosokawa-Muto, Y.O. Kamatari, H.K. Nakamura, K. Kuwata, Variety of antiprion compounds discovered through an in silico screen based on cellularform prion protein structure: correlation between antiprion activity and binding affinity, Antimicrob. Agents Chemother. 53 (2009) 765–771.
- [34] M.J. Thompson, J.C. Louth, G.K. Greenwood, F.J. Sorrell, S.G. Knight, N.B.P. Adams, B. Chen, Improved 2,4-diarylthiazole-based antiprion agents: switching the sense of the amide group at C5 leads to an increase in potency, ChemMedChem 5 (2010) 1476–1488.
- [35] W. Heal, M.J. Thompson, R. Mutter, H. Cope, J.C. Louth, B. Chen, Library Synthesis and screening: 2,4-diphenylthiazoles and 2,4-diphenyloxazoles as potential novel prion disease therapeutics, J. Med. Chem. 50 (2007) 1347–1353.
- [36] C. Korth, B.C.H. May, F.E. Cohen, S.B. Prusiner, Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease, Proc. Natl. Acad. Sci. USA 98 (2004) 9836–9841.
- [37] H.M. Sim, C.Y. Lee, P.L.R. Ee, M.L. Go, Dimethoxyaurones: potent inhibitors of ABCG2 (breast cancer resistance protein), Eur. J. Pharm. Sci. 35 (2008) 293–306.