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Antiprion activity of functionalized 9-aminoacridines related to quinacrine

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ABSTRACT

A library of functionalized 6-chloro-2-methoxy-(N^9-substituted)acridin-9-amines structurally related to quinacrine were synthesized and evaluated for antiprion activity on four different cell models persistently infected with scrapie prion strains (ScN2a, N167, Ch2) or a human disease prion strain (F3). Most of the compounds were distinguished by the side chain attached to 9-amino of the acridine ring. These were dialkylaminoalkyl and phenyl with basic groups on the phenyl ring. The most promising compound was 6-chloro-2-methoxy-*N*-(4-(4-methylpiperazin-1-yl)phenyl)acridin-9-amine (**15**) which had submicromolar EC_{50} values (0.1–0.7 μM) on all cell models, was able to clear PrP^{Sc} at non-toxic concentrations of 1.2–2.5 μM, and was more active than quinacrine in terms of EC_{50} values. Other promising compounds were **14** (a regioisomer of **15**) and **17** which had a 1-benzylpiperidin-4-yl substituent attached to the 9-amino function. Activity was strongly dependent on the presence of a substituted acridine ring, which in this library comprised 6-chloro-2-methoxy substituents on the acridine ring. The side chains of **14**, **15**, and **17** have not been previously associated with antiprion activity and are interesting leads for further optimization of antiprion activity.

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1. Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of infectious and fatal neurodegenerative diseases that include Creutzfeldt–Jakob disease (CJD) in humans, and scrapie, bovine spongiform encephalopathy, cervid chronic wasting disease among others, in animals.[1–4] These diseases may arise spontaneously, have a genetic origin or are acquired through infection. TSEs are characterized by the accumulation of protease-resistant aggregates of an altered isoform of the cellular prion protein (PrP^C) called scrapie prion protein (PrP^{Sc}) in the central nervous system and the lymphoreticular system. Unlike PrP^C, PrP^{Sc} has a multimeric structure with a high β sheet content. It can assemble into fibrils and plaques, a process that bears some similarity to the abnormal protein aggregation encountered in protein misfolding diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, except that PrP^{Sc} is also infectious. There is presently no clinically proven anti-TSE drug although significant progress has been made in identifying compounds with prophylactic activity.[5–7] Besides the general requirements of potency coupled with low toxicity, a successful antiprion drug must have good blood–brain permeability and the ability to halt or substantially modify pathogenesis late in the course of the disease.

Quinacrine, a 9-aminoacridine derivative, has been used for many years as an antimalarial agent.[8] It was found to inhibit PrP^{Sc} formation in a cell-based model of prion infection at submicromolar EC_{50} values,[9,10] but failed to demonstrate activity in scrapie-infected mice.[7,11,12] Nonetheless, it was used in a few patients with CJD on compassionate grounds, but was found to be only transiently beneficial in these cases.[13,14] In spite of these shortcomings, there is sustained interest in the antiprion activity of substituted 9-aminoacridines that are structurally related to quinacrine. May and co-workers[15] found bis-acridines like compound A (Fig. 1) to have more potent in vitro antiprion activity than quinacrine. Klingenstein and co-workers[16] observed the synergistic antiprion effects of quinacrine and iminodibenzyl-derived antidepressants, and this led to the synthesis of hybrid molecules like quinpramine and compound B (Fig. 1) which had 5- to 15-fold improved antiprion potencies over quinacrine in cell-based assays.[17] Investigations into the structure–activity relationships of quinacrine showed that antiprion activity was greatly influenced by several structural features, namely, the length of the alkyl linker attached to the 9-amino functionality, the substituents on the distal tertiary amino group of the alkyl side chain, and the substitution pattern on the acridine ring.[10,18]

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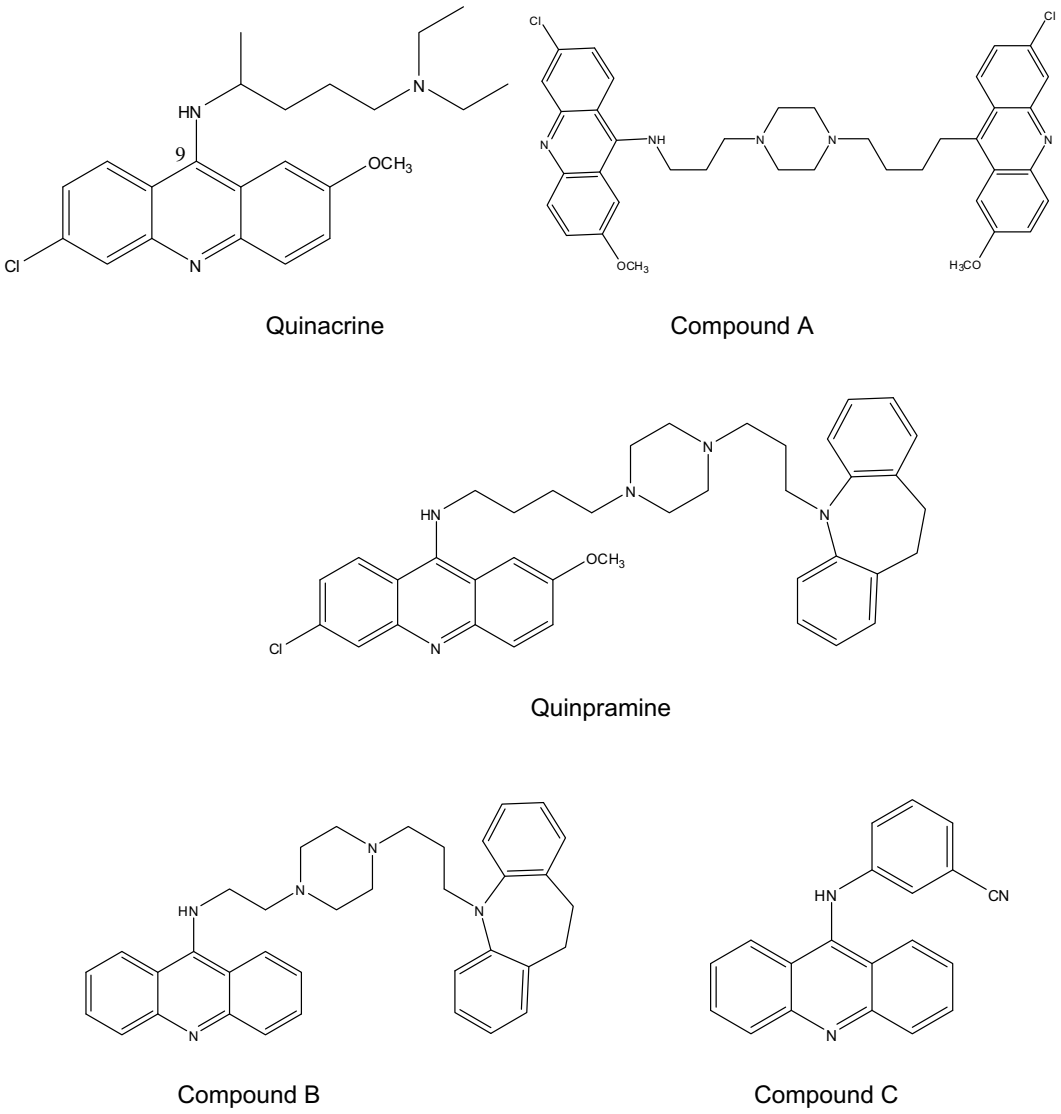


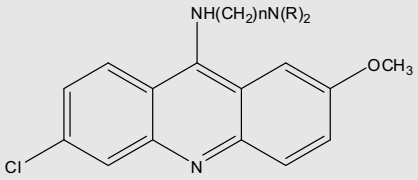
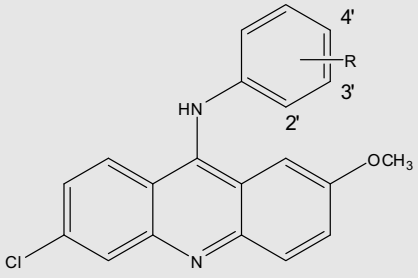
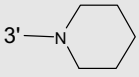
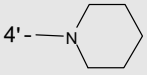
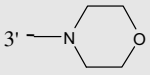
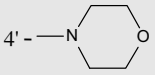
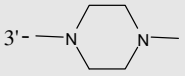
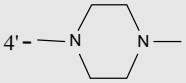
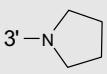
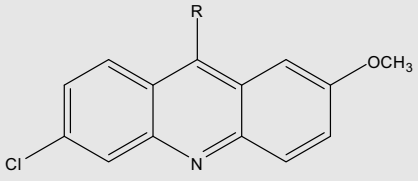
Figure 1. Structures of quinacrine and some antiprion acridines.

The latter feature was also identified as an important determinant of cellular cytotoxicity, with substituents like 3-fluoro-6-methoxy-4-methyl associated with greater cytotoxicity as compared to the 2-methoxy-6-chloro groups found on the acridine ring of quinacrine.[18] Cope and co-workers synthesized several substituted *N*-phenylacridin-9-amines and found electron withdrawing groups on the *N*-phenyl ring to be particularly favorable for activity. The most promising compound in their series (compound C, Fig. 1) had an EC_{50} of 1.0–2.5 μM on the scrapie mouse brain (SMB) cell model.[19] Taken together, these reports underscore the antiprion potential of the 9-aminoacridine template. For this reason, we have focused our medicinal chemistry efforts on 9-aminoacridines in an attempt to identify compounds with greater antiprion potencies and lower cytotoxicities than quinacrine. Here, we report the synthesis of a library of 6-chloro-2-methoxy-(N^9-substituted)acridine-9-amines and the evaluation of their antiprion activities on four different prion-infected cell models.

2. Chemistry

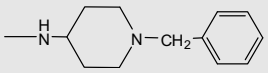
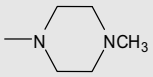
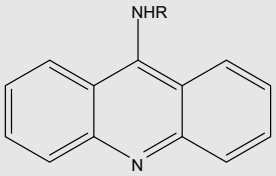
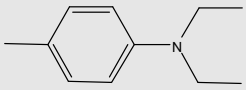
The synthesized compounds were broadly classified into four groups as shown in Table 1. The first group of compounds (**1–4**) were structurally related to quinacrine but with modifications on the side chain attached to the amino group at C^9 of the acridine ring, namely, the alkyl chain length between the nitrogen atoms of the side chain and the alkyl groups on the terminal nitrogen. Group 2 compounds (**5–16**) were 6-chloro-2-methoxy-*N*-phenyl acridin-9-amines, differentiated by the type of basic groups attached to the phenyl ring. These were *N*′,*N*′-dimethylamino, *N*′,*N*′-diethylamino, 1-piperidinyl, 1-pyrrolidinyl, 4-morpholinyl, and 4-methylpiperazin-1-yl, attached to 2′, 3′, or 4′ positions on the phenyl ring. Compounds with this motif have been associated with antimalarial, antitrypanosomal and cytotoxic activities,[20–22] but have not been widely investigated for antiprion activity. Group 3 consisted of miscellaneous compounds, namely, **17** which had a 1-benzylpiperidin-4-yl ring attached to the 9-amino functionality, **18** where the 9-amino functionality was part of the 4-methylpiperazin-1-yl ring, and **19** where a simple 9-amino group was present. Group 4 comprised only two compounds (**20, 21**) with unsubstituted acridine rings, in contrast to Groups 1–3 compounds which had 6-chloro-2-methoxy substituents. Compounds **20** and **21** are the unsubstituted equivalents of compounds **9** and **19**, respectively, and a comparison of their activities would provide some useful information on the role of ring substitution for activity.

Table 1
Antiprion activity of quinacrine and library compounds on ScN2a cells

Compound	Substituent	EC_{50} (μM)[a]	FAA (μM)[b]	TC (μM)[c]	SI[d]
	 Group1				
1	$n = 2$, R = C_2H_5	0.021 (0.019–0.023)	0.1	4	190
2	$n = 3$, R = C_2H_5	0.14 (0.11–0.17)	0.3	1	7
3	$n = 4$, R = C_2H_5	0.15 (0.12–0.19)	0.4	1	7
4	$n = 3$, R = CH_3	0.11 (0.09–0.13)	0.3	1	9
	Quinacrine	0.23 (0.22–0.25)	0.8	2.5	11
	 Group 2				
5	2′-$N(CH_3)_2$	0.25 (0.22–0.28)	0.8	5	20
6	3′-$N(CH_3)_2$	0.32 (0.26–0.39)	None[e]	2	6
7	4′-$N(CH_3)_2$	0.51 (0.34–0.77)	None[e]	2	4
8	3′-$N(C_2H_5)_2$	1.01 (0.85–1.21)	2.5	3	3
9	4′-$N(C_2H_5)_2$	0.48 (0.24–0.93)	1.5	2	4
10		0.18 (0.15–0.22)	0.5	4	22
11		4.24 (3.67–4.90)	7	9	2
12		0.9 (0.75–1.08)	None[e]	3	3
13		1.28 (1.16–1.42)	3	4	3
14		0.29 (0.26–0.33)	1	4	14
15		0.1 (0.08–0.12)	0.4	2.5	25
16		1.06 (0.95–1.18)	None[e]	2	2
	 Group 3				

(*continued on next page*)

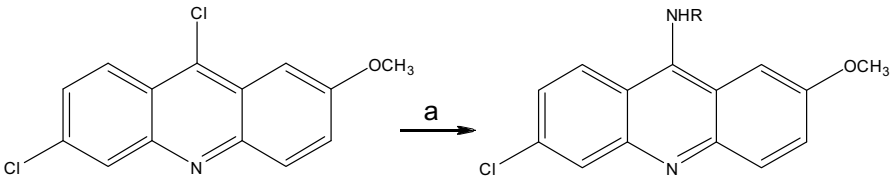
Table 1 (*continued*)

Compound	Substituent	EC_{50} (μM)[a]	FAA (μM)[b]	TC (μM)[c]	SI[d]
17		0.42 (0.38–0.46)	1	2	5
18		0.36 (0.29–0.45)	1.8	4	11
19	$-NH_2$	0.13 (0.12–0.14)	0.4	4	30
	 Group 4				
20		0.24 (0.16–0.36)	None[e]	0.4	2
21[f]	H	None[e]	None[e]	2.5	–

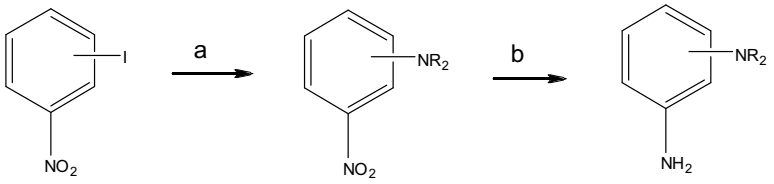
[a] Concentration required to reduce PrP^{Sc} content to 50% of untreated ScN2a cells, average of no less than three independent determinations. 95% Confidence intervals are given in brackets.
[b] Full antiprion activity: estimated lowest concentration required for more than 99% reduction of PrP^{Sc}.
[c] Tolerant concentration: estimated maximal concentration that had no toxic effect on cell growth to confluency.
[d] Selectivity index = TC/EC_{50}.
[e] Could not be determined at non-toxic concentrations of test compound.
[f] Purchased from Sigma–Aldrich Chemical Company.

The target compounds were synthesized by the selective displacement of the chlorine atom at position 9 of 6,9-dichloro-2-methoxyacridine by a nucleophilic primary amine (Scheme 1). The reaction was carried out in ethanol under weakly acidic conditions. The displacement reaction occurred only at C^9 and not at C^6, because of the proximity of C^9 to the electron withdrawing acridinium nitrogen as well as the stability of the resulting intermediate. Phenol has been employed as solvent, in which case a phenoxide intermediate was formed in situ and subsequently displaced by the incoming amine.[23,24] In our hands, both solvents gave comparable yields but workup procedures were generally easier with ethanol. In cases where yields in ethanol were unsatisfactory, a change to phenol provided a favorable solution.

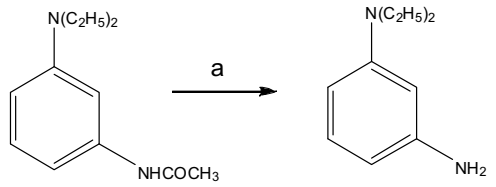
Many of the amines used in the condensation reaction were prepared from iodobenzene in a palladium-coupled Hartwig–Buchwald amination reaction (Scheme 2). 4-(3-Nitrophenyl)morpholine, 1-methyl-4-(3-nitrophenyl)piperazine (and its 4-isomer), 1-(3-nitrophenyl)piperidine, 1-(3-nitrophenyl)pyrrolidine, and *N*′,*N*′-dimethyl-3-nitroaniline were obtained in this way in fairly good yields. The aromatic nitro function of these compounds was subsequently reduced by palladium catalyzed hydrogenation to give the desired aniline which was subsequently reacted with the 4,6-dichloro-2-methoxyacridine as shown in Scheme 1. A different



Scheme 1. Reagents and conditions: (a) RNH_2, ethanol, HCl, reflux, 24 h or phenol, 100 °C (1 h), followed by addition of amine, 4–5 h, 100 °C.



Scheme 2. Reagents and conditions: (a) Amine (pyrrolidine, piperidine, 1-methylpiperazine, morpholine, or *N*,*N*-dimethylamine), $Pd(OAc)_2$, BINAP, Cs_2CO_3, anhydrous toluene, 120 °C; (b) H_2, 10% Pd/C (5% w/w), 50 psi.



Scheme 3. Reagent and conditions: (a) Concd HCl, reflux, 2 h.

route was adopted for N^1,N^1-diethylbenzene-1,3-diamine, which was prepared from the acid hydrolysis of the commercially available *N*-(3-(diethylamino)phenyl)acetamide (Scheme 3). N^1,N^1-Dimethylbenzene-1,2-diamine was prepared by reacting 1-chloro-2-nitrobenzene with hexamethylphosphoramide (HMPA), followed by catalytic reduction of the aromatic nitro group (Scheme 4).

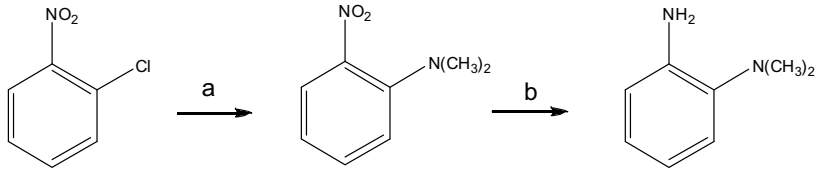
3. Results

3.1. Antiprion activity against ScN2a cells

ScN2a cells are murine neuroblastoma cells (N2a) that have been persistently infected with the scrapie prion strain RML. This cell model is widely used for the screening of antiprion candidates, resulting in the identification of several promising target compounds like acridines and phenothiazines,[10] chrysoidine,[25] and quinoline derivatives.[26,27] The method involved incubating the test compound with the ScN2a cells until confluency was attained (3 days), after which the cells were lysed to give lysates that were treated with proteinase K and analyzed by immunoblot for PrP immunoreactivity. PrP^{Sc} is resistant to proteinase K digestion and shows characteristic bands in the immunoblot. The signal levels of these bands are reduced in the presence of a compound with antiprion activity. Figure 2 shows the results of representative immunoblots obtained at different concentrations (0.1–2.0 μM) of compound **15**.

EC_{50} which is the effective concentration of test compound required to reduce PrP^{Sc} content to 50% of untreated ScN2a cells was determined by measuring the intensities of the immunoreactive signals at different concentrations of test compound. The results were plotted to give a sigmoidal curve from which EC_{50} was obtained. A representative plot is given in Figure 3.

Table 1 lists the antiprion effects of the test compounds expressed in terms of their (i) EC_{50} values, (ii) full antiprion activity (FAA) which is the approximate concentration required to clear more than 99% of PrP^{Sc} content, and (iii) maximal tolerant concentration (TC) which is the approximate highest concentration to have no toxic effect on uninfected N2a cells. The ratio of the



Scheme 4. Reagents and conditions: (a) Hexamethylphosphoramide, 150 °C, 24 h; (b) H_2, 10% Pd/C (5% w/w), 50 psi.

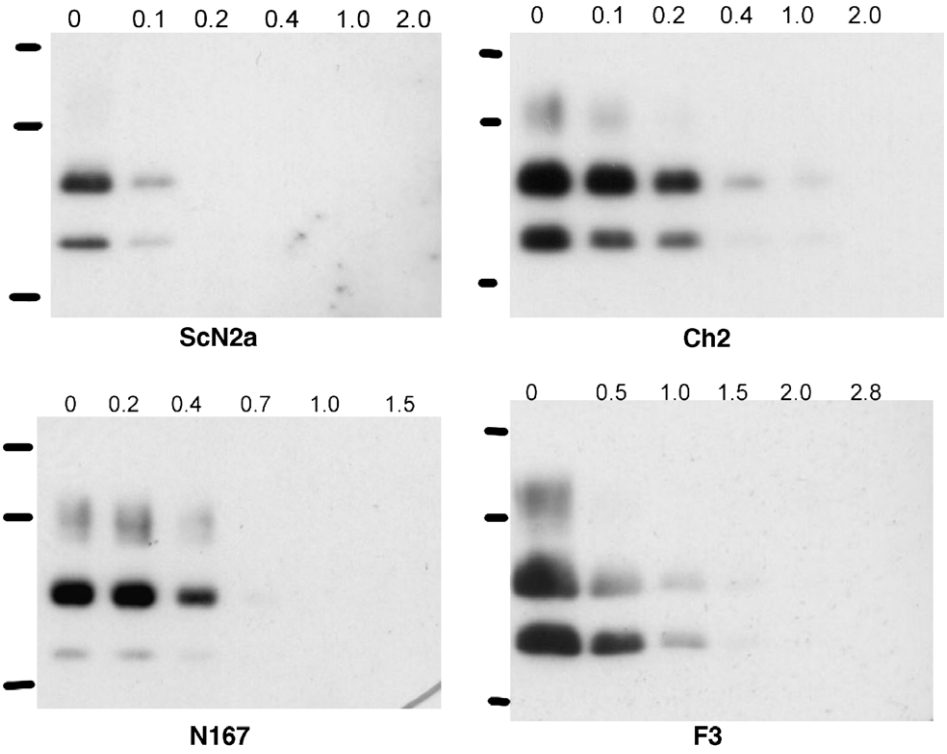


Figure 2. Immunoblots of PrP^{Sc} formation in the presence of compound **15** in ScN2a, Ch2, N167 and F3 prion-infected cell models. Apparent molecular masses based on migration of protein standards are 45.7, 32.5 and 18.4 kDa (from top to bottom). The top most line in each panel gives the concentration (μM) of **15**. The first column on the right ('0') represents cells that were not treated with **15**.

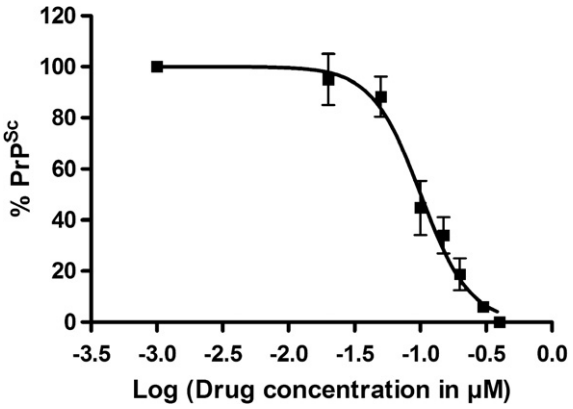


Figure 3. Plot of % PrP^{Sc} formation (equivalent to intensities of immunoreactive signals relative to untreated cells) in ScN2a cells treated with different concentrations of compound **15**. EC_{50} was obtained from the descending portion of the sigmoidal curve using a commercial software program GraphPad Prism 4.03.

tolerant concentration (TC) to EC_{50} gives the selectivity index, which is a useful measure of the selective activity of the compound.

Quinacrine was used as the reference compound in these experiments. Its EC_{50} was found to be 0.23 μM. This value compares favorably with those reported by others like Doh-ura et al.[9] (EC_{50} 0.4 μM under similar experimental conditions) and Dollinger et al.[17] (EC_{50} 0.3 μM, incubation period of 7 days).

As seen from Table 1, many compounds had submicromolar EC_{50} values that were comparable to quinacrine. Compound **1** was particularly interesting because of its potency (EC_{50} 0.021 μM, almost 10 times lower than quinacrine) and exceptionally high selectivity index (SI = 190). The activity of **1** was found to be sensitive to the length of the side chain separating the two amino functions in the side chain. In **1**, the amino functions are separated by two carbon atoms ($n = 2$). As more carbon atoms were introduced, activity and selectivity declined, as seen from its homologues, compounds **2** ($n = 3$) and **3** ($n = 4$). On the other hand, activity was less affected by the type of substituents on the distal amino function. Thus for the same alkyl chain length ($n = 3$), no difference in activity was noted for compounds **2** (*N*′,*N*′-diethylamino) and **3** (*N*′,*N*′-dimethylamino).

The Group 2 compounds were *N*-phenylacridin-9-amines with different basic substituents attached to the *N*-phenyl ring. A cursory examination of antiprion activity in this Group (Table 1) suggests that among the basic heterocycles attached to the *N*-phenyl ring, the 1-methylpiperazine ring present in compounds **14** and **15** was preferred to other basic rings like piperidine (**10, 11**), pyrrolidine (**16**), or morpholine (**12, 13**). Mention should also be made of compounds **5, 10**, and **15** which had significantly good selectivities (SI ⩾ 20) compared to quinacrine (SI = 11) but EC_{50} values (0.1–0.25 μM) that were just comparable to quinacrine (0.23 μM).

The antiprion activities of the Group 3 compounds **17** and **18** were broadly similar to most members in Group 2. Thus we inferred that the substitution of the 9-amino functionality with phenyl (Group 2) or a non-aromatic heterocyclic ring (Group 3, namely **17, 18**) did not markedly affect antiprion activity. The activity of compound **19** which has an unsubstituted 9-amino functionality was unexpectedly good (EC_{50} 0.13 μM, SI = 30), in sharp contrast to its ring-unsubstituted analogue **21** (9-aminoacridine) which was the only compound in the entire library with no detectable activity on ScN2a cells. Another ring-unsubstituted compound **20** was also found to have a poor antiprion profile, providing additional support to the view that an unsubstituted acridine ring was detrimental to activity.

3.2. Antiprion activity against other prion-infected cell models

Besides ScN2a, the compounds were also evaluated on other prion-infected cell models as described in previous studies.[28,29] These were N167 which were mouse neuroblastoma cells (N2a) infected with the scrapie prion strain 22L, and Ch2 which were mouse neuroblastoma cells (N2a#58) that overexpressed PrP^{C} (3–5 times more than N2a cells) and infected with the scrapie prion strain RML. The third cell model F3 was N2a#58 cells infected with the mouse-adapted human prion strain Fukuoka-1. Only some compounds were screened against these additional cell models and they were chosen based on their selectivity indices (SI) compared to quinacrine in ScN2a cells: **1, 15**, and **19** had better selectivities (SI > 20), **14** and **18** were comparable to quinacrine, while **11** and **17** had poorer selectivities (SI < 10). The antiprion activities of these compounds are given in Table 2.

Table 2 clearly shows that antiprion activity varied according to the cell model employed for testing. On the N167 cells, compounds **1** and **19** had no measurable EC_{50} values, in contrast to their strong antiprion activities against ScN2a cells. The most promising compounds on the N167 cell model (**15** and **17**) were also not particularly outstanding against ScN2a cells. On the N167 model, **15** and **17** had EC_{50} values (0.42 and 0.49 μM) that were comparable to quinacrine (0.59 μM) but they were able to clear PrP^{Sc} at 1.5 μM, a feature that was not observed for quinacrine.

A comparison of EC_{50} values against Ch2 and ScN2a showed that most compounds were less potent against the PrP^{C} overexpressing Ch2 cell model. A notable exception was **17** which retained the same level of activity (EC_{50} 0.4 μM) on both Ch2 and ScN2a. Compounds **14** and **15** were also interesting in that they were

Table 2
Antiprion activities of quinacrine and selected compounds against different prion-infected cell lines

Compound	Prion-infected cell lines							
	ScN2a		N167		Ch2		F3	
	EC_{50}[a]	FAA[b]	EC_{50}[a]	FAA[b]	EC_{50}[a]	FAA[b]	EC_{50}[a]	FAA[b]
1	0.021	0.1	None[c]	None[c]	0.7 *(0.41–1.19)*	3	None[c]	
11	4.24	7	4.29 *(3.90–4.73)*	None[c]	None[c]	ND[d]	ND[d]	ND[d]
14	0.20	1	1.19 *(0.92–1.53)*	3	0.38 *(0.31–0.48)*	1.5	1.49 *(1.34–1.65)*	3.5
15	0.10	0.4	0.42 *(0.41–0.43)*	1.5	0.22 *(0.19–0.27)*	1.2	0.68 *(0.59–0.78)*	1.5
17	0.42	1	0.49 *(0.49–0.55)*	1.5	0.41 *(0.32–0.51)*	1	0.80 *(0.64–1.00)*	None[c]
18	0.36	1.8	None[c]	None[c]	>2	ND[d]	None[c]	None[c]
19	0.13	0.4	None[c]	None[c]	None[c]	None[c]	ND[d]	ND[d]
Quinacrine	0.23	0.8	0.59 *(0.42–0.82)*	None[c]	0.46 *(0.40–0.54)*	2	1.88 *(1.64–1.00)*	None[c]

[a] Concentration (μM) required to reduce PrP^{Sc} content to 50% of untreated cells from three independent determinations. 95% confidence limits are given in italics.
[b] Full antiprion activity: estimated lowest concentration (μM) required for more than 99% reduction of PrP^{Sc}.
[c] No data were obtained at non-toxic concentrations.
[d] Not determined.

more active (lower EC_{50} and FAA values) than quinacrine on the Ch2 cell model.

The compounds were also tested on the F3 cells which are PrP^C over-producing mouse neuroblastoma cells (N2a#58) infected with a mouse-adapted human prion strain. Here, only quinacrine **14**, **15**, and **17** retained antiprion activity, but with EC_{50} values that were 2- to 5-fold higher than those determined against Ch2 cells. Potency measured in terms of EC_{50} was of the order **15** (0.68 μM) > **17** (0.80 μM) > **14** (1.49 μM) > quinacrine (1.88 μM). Only **14** and **15** were able to clear the cells of PrP^{Sc} formation.

4. Discussion

Based on the screening results given in Tables 1 and 2, compounds **14, 15**, and **17** were found to be the most promising candidates. These compounds had measurable EC_{50} values in the low to submicromolar range on four different prion cell models that were persistently infected with scrapie prion strains (ScN2a, N167, Ch2) or a human disease prion strain (F3). In addition, they were able to clear PrP^{Sc} formation at low micromolar concentrations, except for **17** against F3 cells. These compounds were either comparable or more active than quinacrine on most of these cell models.

The antiprion profile of compound **15** was of particular interest because its activity was restricted to a narrow EC_{50} range (0.1–0.7 μM). It had a good selective index of 25 and was able to clear PrP^{Sc} formation at FAA values of about 1.5 μM. In addition, its antiprion activity appears to be better than that of quinacrine on all four cell models. Structurally, **15** is *N*-phenylacridin-9-amine with a (4-methylpiperazin-1-yl) ring attached to the 4′ position of the phenyl ring. Some structure–activity trends may be deduced for **15**. First, relocating 4-methylpiperazin-1-yl from 4′ to 3′ gave **14**, another promising compound which retained activity on all prion-infected cell models but at higher EC_{50} and FAA values than **15**. Second, replacing 4-methylpiperazin-1-yl in **15** with piperidin-1-yl gave **11**, which had sharply reduced activity on ScN2a and N167 cells. Another observation was that bypassing the phenyl ring and directly linking the 4-methylpiperazine ring to acridine, as in **18**, adversely affected activity. Lastly, the results from ScN2a cells indicated that 4-methylpiperazin-1-yl was the preferred substituent, compared to other basic moieties like piperidin-1-yl, morpholin-1-yl and pyrrolidin-1-yl. Taken together, these observations underscore the antiprion potential of appropriately substituted *N*-phenylacridine-9-amines, as exemplified by **14** and **15**. Compound **15** in particular has a promising antiprion profile which warrants further investigation.

The antiprion activities of other *N*-phenylacridin-9-amines have been reported earlier by Cope and co-workers,[19] but they were tested on a different cell model (SMB) which makes comparison with the present results difficult. Nonetheless, it was noted that one compound in their study (**1o**, N^1-(acridin-9-yl) benzene-1,3-diamine) which had a primary amino function on the phenyl ring did not fare as well as other compounds in their library. If we extrapolate from our present SAR, activity of **1o** may be improved by introducing substituents on the acridine ring or by having a bulkier basic group like tertiary amine or a basic heterocycle on the phenyl ring. As we have shown, compounds **20** and 21 with no substituents on the acridine ring did not fare as well as their ring substituted analogues (**9** and **19**), and the most promising compounds in our library (**14, 15, 17**) have tertiary substituted amino groups.

Mention should also be made of compound **17** which was not particularly active against ScN2a (EC_{50} 0.42 μM) and had a low selectivity index (5). In spite of these limitations, **17** retained the same level of activity against N167 and Ch2 cells (EC_{50} 0.41–0.49 μM) which was in contrast to quinacrine, **14** and **15**, which had lower activities against N167 and Ch2 cells. Compound **17** was also active against the mouse-adapted human prion strain Fukuoka-1 (EC_{50} 0.8 μM) but failed to clear PrP^{Sc} formation in this cell model. Structurally, **17** has a 1-benzylpiperidin-4-yl side chain attached to the 9-amino of acridine and this entity has not been previously associated with antiprion activity. It would be of interest to see if structural modifications of this side chain could overcome some of the limitations associated with **17**.

5. Conclusions

We have shown that structural modification of the 9-substituted amino side chain of quinacrine resulted in several promising compounds (**14, 15**, and **17**) with good antiprion potencies and selectivities on four prion-infected murine cell models, including a mouse-adapted human prion strain (F3). Compounds **14** and **15** are 6-chloro-2-methoxy-(*N*-substituted)phenylacridin-9-amines with 4-methyl piperazin-1-yl side chains attached to different positions on the phenyl ring. In compound **17**, the 9-amino functionality of 6-chloro-2-methoxy-9-aminoacridine is substituted with 1-benzylpiperidin-4-yl. Both basic side chains have not been previously associated with antiprion activity and are interesting leads for further modification. Further investigations would involve evaluating their activities in vivo, and to assess their permeability across the blood–brain barrier.

6. Experimental

6.1. Chemistry

Melting points were determined in open glass capillary tubes on a Gallenkamp melting point apparatus and were not corrected. ^1H NMR and ^{13}C 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 were collected on LCQ Finnigan MAT mass spectrometer with chemical ionization (APCI) or electron spray ionization (ESI) 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) from Merck. The purity of final compounds was verified by high pressure liquid chromatography (HPLC) (Supplementary information) 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.

All chemicals were purchased from Sigma–Aldrich Chemical Company (MO, USA), except piperidine and *N*,*N*-dimethyl-3-nitroaniline, which were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Alfa Aesar (MA, USA), respectively.

6.1.1. General procedure for Hartwig–Buchwald amination

1-Iodo-3 (or 4)-nitrobenzene (2.49 g, 10 mmol) and the amine (1-methylpiperazine, pyrrolidine, piperidine, morpholine) (25 mmol) were dissolved in dry toluene (30–50 ml) in the presence of argon in a round-bottomed flask. The solution was transferred via a canula under positive pressure to another flask which contained cesium carbonate (8.15 g, 25 mmol) and *rac*-BINAP (rac-2,2′-bis(diphenylphosphino)-1,1′-binaphthyl) (0.50 g, 0.8 mmol), which were earlier weighed into the flask in the presence of argon. $Pd(OAc)_2$ (palladium (II) acetate) (0.013 g, 0.06 mmol) was quickly added to the flask and the reaction mixture was heated to 90 °C in an oil bath, with stirring. The reaction was stopped when traces of the iodonitrobenzene were not

observed on TLC. After cooling to room temperature, the mixture was filtered and the organic phase concentrated under reduced pressure. The residue was purified by column chromatography using as mobile phase ethylacetate/hexane (1:1) which was increased stepwise to ethyl acetate/methanol/ammonia (9:1:0.1).

6.1.1.1. 4-(3-Nitrophenyl)morpholine. Orange solid. Yield 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.25 (t, 4H, J = 5.3) 3.89 (t, 4H, J = 5.3), 7.18 (dd, $J1$ = 1.9, $J2$ = 8.4, 1H) 7.40 (t, J = 8.1, 1H) 7.71 (m, 2H).

6.1.1.2. 1-Methyl-4-(3-nitrophenyl)piperazine. Red solid. Yield 92%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.30 (s, 3H) 2.58 (t, J = 5.3, 4H) 3.30 (t, J = 5.3, 4H) 7.37 (m, 2H) 7.66 (m, 2H).

6.1.1.3. 1-Methyl-4-(4-nitrophenyl)piperazine. Red solid. Yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.36 (s, 3H) 2.56 (t, J = 4.3, 4H) 3.44 (t, J = 4.3, 4H) 6.83 (d, J = 9.4, 2H) 8.13 (d, J = 9.4, 2H).

6.1.1.4. 1-(3-Nitrophenyl)pyrrolidine. Orange solid. Yield 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 2.06 (t, J = 6.6, 4H) 3.34 (t, J = 6.6, 4H) 6.80 (dd, $J1$ = 2.1, $J2$ = 8.0, 1H) 7.32 (m, 2H) 7.46 (dd, $J1$ = 1.7, $J2$ = 8.0, 1H). MS (ESI, MeOH) m/z $[M+1]^+$ 193.4.

6.1.1.5. 1-(3-Nitrophenyl)piperidine. Orange liquid. Yield 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.64 (m, 2H) 1.72 (m, 4H) 3.27 (t, J = 5.3, 4H) 7.18 (dd, $J1$ = 2.3, $J2$ = 8.3, 1H) 7.34 (t, $J1$ = 2.3, $J2$ = 8.3, 1H) 7.60 (dd, $J1$ = 1.6, $J2$ = 8.0, 1H) 7.71 (t, J = 2.1, 1H). MS (ESI, MeOH) m/z $[M+1]^+$ 207.3.

6.1.2. N^1,N^1-Dimethyl-2-nitrobenzene

Hexamethylphosphoramide (2.69 g, 15 mmol, 2.5 ml) was added to 1-chloro-2-nitrobenzene (0.4 g, 2.5 mmol) in a flask under argon and heated at 150 °C for 24 h. The reaction mixture was diluted with water (≈20 ml), extracted with ether, after which the ether fraction was extracted with 4 M HCl (4× 10 ml). The acidic fraction was made alkaline with 4 M NaOH, extracted with ether (4× 20 ml), and dried over anhydrous Na_2SO_4. After removal of ether under reduced pressure, the residue was recrystallised in ethanol to give yellow crystals. Yield 40%. ^{1}H NMR ($CDCl_3$) δ 3.09 (s, 6H) 7.10 (d, J = 1.5, 1H) 7.57 (d, J = 1.5, 1H) 7.88 (s, 1H).

6.1.3. General procedure for catalytic reduction of the aromatic nitro functionality

The nitrobenzene was dissolved in ethanol and the catalyst (10% palladium on carbon) (5% w/w) was added under nitrogen. Hydrogenation was carried out on a Parr hydrogenator at 50 psi for 12–18 h. At the end of the process, the catalyst was removed by filtration and the filtrate is concentrated in vacuo. The product was obtained quantitatively and used immediately for the next step of reaction.

6.1.4. General procedure for the reaction of 6,9-dichloro-2-methoxyacridine with amines in phenol

This method was used for the syntheses of **1–5, 8, 9, 17, and 18**. 9-Dichloro-2-methoxyacridine (0.28 g, 1 mmol) was added to melted phenol (0.94 g, 10 mmol) and stirred at 100 °C for 1 h before addition of the amine (1.1 mmol). Stirring was continued for 3–4 h at 120 °C under nitrogen. On cooling to room temperature, diethylether was added to the reaction mixture. The resulting precipitate was either removed by vacuum filtration or extracted with dichloromethane. It was dissolved in an appropriate solvent and purified by column chromatography. The final compound was recrystallized from methanol or converted to a HCl salt with ethereal HCl.

6.1.5. N^1-(6-Chloro-2-methoxyacridin-9-yl)-N^2,N^2-diethylethane-1,2-diamine dihydrochloride (1)

6,9-Dichloro-2-ethoxyacridine and N^1,N^1-diethylethane-1,2-diamine were reacted in phenol to give **1** as a yellow solid in 74% yield. Mp 252–254 °C (lit. 257–259 °C). ^{1}H NMR (CD_3OD) δ 1.41 (t, J = 7.2, 6H) 3.39 (q, J = 7.2, 4H) 3.80 (t, J = 6.4, 2H) 4.06 (s, 3H) 4.63 (t, J = 6.4, 2H) 7.55 (dd, $J1$ = 2.7, $J2$ = 9.3, 1H) 7.67 (t, J = 8.3, 1H) 7.80 (dd, $J1$ = 4.2, $J2$ = 9.2, 1H) 7.86 (d, J = 2.7, 1H) 8.01 (d, J = 3.0, 1H) 8.49 (dd, $J1$ = 1.5, $J2$ = 9.4, 1H). MS (ESI, MeOH) m/z $[M^+]$ 358.1. Anal. ($C_{20}H_{24}N_3OCl.2HCl.H_2O$) C calcd 53.51, found 53.33; H calcd 6.24, found 6.03.

6.1.6. N^1-(6-Chloro-2-methoxyacridin-9-yl)-N^3,N^3-diethylbenzene-1,3-diamine (8)

3-(*N*,*N*-Diethylamino)acetanilide (1.2 g, 5.8 mmol) and 2.5 ml concentrated HCl were heated to 100 °C for 2 h. On cooling, the pH of the solution was adjusted to 4 with 2 M NaOH. The product, N^1,N^1-diethylbenzene-1,3-diamine, was not isolated or purified but directly reacted with 6,9-dichloro-2-methoxyacridine (1.61 g, 5.8 mmol) in phenol as described under the general procedure. The product was obtained as an orange solid in 41% yield and was converted to the HCl salt with ethereal HCl. Mp 274–276 °C. ^{1}H NMR (CD_3OD) δ 1.10 (t, J = 7.0, 6H) 3.38 (q, J = 7.0, 4H) 3.67 (s, 3H) 6.63 (d, J = 7.7, 1H) 6.69 (t, J = 2.0, 1H) 6.79 (dd, $J1$ = 2.3, $J2$ = 8.5, 1H) 7.33 (t, J = 8.1, 1H) 7.41 (dd, $J1$ = 2.0, $J2$ = 9.4, 1H) 7.56 (d, J = 2.5, 1H) 7.64 (dd, $J1$ = 2.6, $J2$ = 9.3, 1H) 7.82 (d, J = 9.3, 1H) 7.88 (d, J = 1.9, 1H) 8.25 (d, J = 9.4, 1H). MS (ESI, MeOH) m/z $[M^+]$ 406.3. Anal. ($C_{24}H_{24}N_3OCl{\cdot}HCl{\cdot}1/4H_2O$) C calcd 64.50, found 64.56; H calcd 5.64, found 5.79.

6.1.7. N^9-(1-Benzylpiperidin-4-yl)-6-chloro-2-methoxyacridin-9-amine (17)

Compound **17** was obtained as a yellow solid in 48% yield. Mp 136–138 °C ^{1}H NMR ($CDCl_3$) δ 1.74 (d, J = 9.4, 2H), 2.05 (t, J = 9.4, 4H), 2.90 (d, J = 11.7, 2H), 3.51 (s, 2H), 3.70 (b, 1H), 3.96 (s, 3H), 4.34 (b, 1H), 7.19 (d, J = 2.5, 1H), 7.30 (m, 4H), 7.34 (d, J = 2.1, 1H) 7.37 (d, J = 2.0, 1H), 7.44 (dd, $J1$ = 2.6, $J2$ = 9.4, 1H), 8.01 (t, J = 9.5, 2H), 8.10 (d, J = 1.8, 1H). MS (ESI, MeOH) m/z $[M^+]$ 432.4.

6.1.8. General procedure for the reaction of 6,9-dichloro-2-methoxyacridine with amines in ethanol

This method was used for the preparation of compounds **6, 7, 10–16, 19, and 20**. 6,9-Dichloro-2-methoxyacridine (2 mmol) and the reacting amine (0.56 g, 2 mmol) were dissolved in ethanol (30–50 ml) and 2 drops of concentrated HCl were added. The mixture was refluxed and the course of reaction followed by TLC until little or no starting material was detected (around 24 h). On cooling to room temperature, the reaction mixture was diluted with cold water and neutralized with 28% v/v ammonia solution. The precipitate was collected by vacuum filtration, washed with distilled water, and purified by column chromatography (gradient elution with ethyl acetate/hexane 1:1 to ethyl acetate/methanol/ammonia 9:1:0.1). If neutralization (with ammonia or 1 M NaOH) did not result in precipitation of the desired product, the alkaline solution was extracted with dichloromethane. The organic layer was washed with brine, concentrated in vacuo, and purified by column chromatography.

6.1.9. 6-Chloro-2-methoxy-*N*-(3-(4-methylpiperazin-1-yl)phenyl)acridin-9-amine (14)

6,9-Dichloro-2-methoxyacridine and 3-(4-methylpiperazin-1-yl)aniline (obtained from reduction of 1-methyl-4-(3-nitrophenyl)piperazine) were reacted in ethanol to give **14** as a red solid in 72% yield. Decomposed at 114 °C. ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H), 2.50 (t, J = 4.4, 4H), 3.11 (t, J = 4.5, 4H), 3.78 (s, 3H), 6.30 (d, J = 7.6, 1H), 6.40 (s, 1H), 6.43 (s, 1H), 6.55 (d, J = 8.3, 1H), 7.13 (d, J = 5.8,

2H), 7.44 (d, J = 10.3, 1H), 7.98 (d, J = 8.9, 1H), 8.07 (d, J = 9.0, 1H), 8.17 (s, 1H). MS (ESI, MeOH) m/z [M^+] 433.2.

6.1.10. 6-Chloro-2-methoxy-*N*-(4-(4-methylpiperazin-1-yl)phenyl)acridin-9-amine (15)

6,9-Dichloro-2-methoxyacridine and 4-(4-methylpiperazin-1-yl)aniline (obtained from reduction of 1-methyl-4-(4-nitrophenyl)piperazine) were reacted in ethanol to give **15** as a yellow solid in 65% yield. Mp 190.9–192.2 °C. ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H), 2.56 (t, J = 4.8, 4H), 3.12 (t, J = 4.6, 4H), 3.66 (s, 3H), 6.82 (b, 4H), 7.08 (s, 1H), 7.15 (d, J = 9.1, 1H), 7.32 (d, J = 9.4, 1H), 7.85 (d, J = 9.2, 1H), 7.94 (d, J = 9.0, 1H), 8.02 (s, 1H). MS (ESI, MeOH) m/z [M^+] 431.9.

6.1.11. 6-Chloro-2-methoxyacridin-9-amine dihydrochloride (19)

6,9-Dichloro-2-methoxyacridine (0.028 g, 0.1 mol) was dissolved in 200 ml of methanol and refluxed for 2 h with a solution of sodium methoxide, prepared by dissolving sodium (0.003 g, 0.12 mol) in 50 ml of methanol. The precipitated sodium chloride was removed by filtration and the filtrate treated with water to precipitate the desired product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.01 (s, 3H) 4.21 (s, 3H) 7.38 (d, J = 2.7, 1H) 7.47 (m, 2H) 8.09 (d, J = 9.5, 1H) 8.19 (m, 2H). The product, 6-chloro-2,9-dimethoxyacridine, was dissolved in 20 ml of alcohol and treated with ammonium chloride (0.64 g, 0.12 mol) dissolved in 2 ml of water. The mixture was maintained at 60–70 °C for 2 h. Compound **19** was obtained as a yellow solid by filtration. (85% yield). Mp 341 °C. ^{1}H NMR (300 MHz, CD_3OH) δ 8.71–7.20 (m, 6H), 3.96 (s, 3H). MS (ESI, MeOH) m/z [M]$^+$ 258.1.

6.1.12. N^1-(Acridin-9-yl)-N^4,N^4-diethylbenzene-1,4-diamine (20)

6,9-Dichloro-2-methoxyacridine and N^1,N^1-diethylbenzene-1,4-diamine were reacted in ethanol as described in the general procedure. Product was obtained as a brownish red solid in 88% yield. Mp 227–228 °C. ^{1}H NMR ($CDCl_3$) δ 1.09 (t, J = 6 Hz, 6H) 3.25 (q, J = 6 Hz, 4H) 6.56 (d, J = 9 Hz, 2H) 6.84 (d, J = 9 Hz, 2H) 7.21–7.16 (m, 2H) 7.57 (t, J = 9 Hz, 2H) 7.94 (t, J = 9 Hz, 4H). MS (ESI, MeOH) m/z [M^+] 341.2.

6.2. Antiprion activity assay

The assay was performed as described previously.[26,28,29] Briefly, the ScN2a, N167, Ch2, and F3 cells were grown in minimal essential medium (Opti-MEM, Invitrogen) supplemented with 10% fetal calf serum. Approximately 2×10^6 cells were seeded into each well of a six-well plate. Stock solutions of the test compounds were prepared in DMSO and were added to the wells when cells were seeded. The final concentration of DMSO in each well was kept at <0.5% v/v. When the cells reached confluency after 3 days, they were examined microscopically (10× magnification) for signs of abnormal appearances. The medium was then removed from each well by aspiration, the cells were rinsed with cold PBS (2 ml) and treated with lysis buffer (500 μl) which consisted of 0.5% Nonidet P-40 and 0.5% sodium deoxycholate in PBS. The solution was transferred to safe-lock tubes for centrifugation at 6000g, 5 min, 4 °C. The supernatant was treated with 5 μl of proteinase K (1 μg/μl, Merck) for 30 min (37 °C), cooled on ice (2–3 min), followed by addition of phenylmethanesulfonyl fluoride (PMSF, 5 μl of 0.1 M solution) to stop the reaction. Then, 20 μl of glass fog solution 1% (Qbiogene Inc.) was added. The mixture was rolled over for 5 min and then centrifuged at 13,000g for 2 min. After removal of supernatant, the protein precipitate was dispersed in 20 μl of sample loading buffer and denatured for 5 min at 95 °C. On cooling to room temperature, the protein samples were loaded on to polyacrylamide SDS–PAGE gel for electrophoresis. Detection of PrP was done as described previously[29,30] using SAF83 (1:5000; SPI-Bio, France), a primary antibody against a human PrP fragment (amino acids 142–160), followed by an alkaline phosphatase-conjugated secondary antibody (anti-mouse IgG H&L, 1:20,000; Promega). Immunoreactive signals were visualized with CDP-Star detection reagent (Amersham) and were analyzed densitometrically with the ImageJ program (National Institute of Health, Bethesda, USA). Three independent assays were performed for each concentration of test compound.

6.3. Statistical analysis

EC_{50} and its 95% confidence intervals were obtained by nonlinear regression using the sigmoidal dose–response equation from GraphPad Prism 4.03.

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Supplementary data

Experimental procedures and analytical data for compounds **2**–**5**, **9**, **18**, **6**, **7**, **10**–**13**, **16**. Determination of purity of final compounds **5**–**8**, **10**–**20** by HPLC. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.05.060.

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