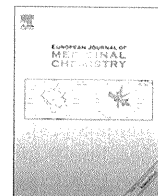


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Original article

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

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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-1-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 (hPrP_{121–231}) 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 hPrP_{121–231}.

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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, non-pathogenic 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 sulphated 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 quinacrine-imipramine hybrids [18] although the large size (molecular

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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 mono-acridines with anti-prion activities on various prion-infected cell models [19]. Two compounds, an N-(4-methylpiperazinyl)phenyl-acridine-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 4-methylpiperazine 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 9-amino 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 phthaloyl 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 °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**.

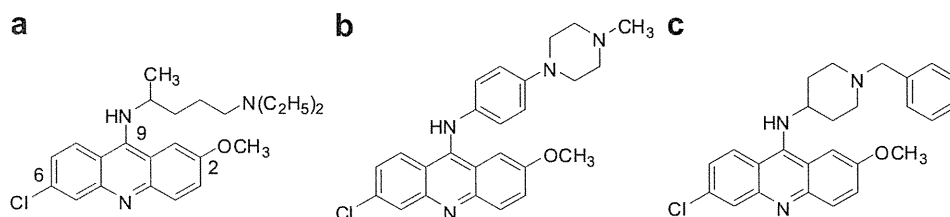
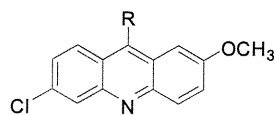
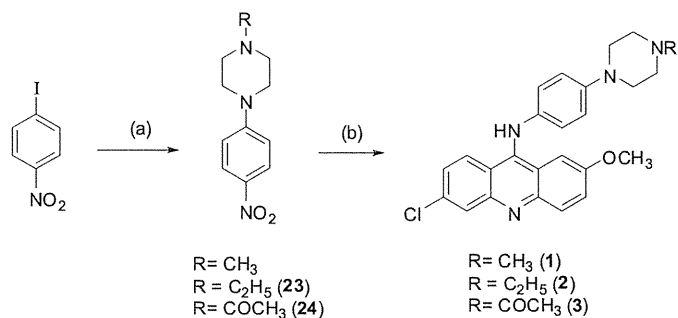


Fig. 1. (a) Quinacrine (b) Compound 15 [19] (c) Compound 17 [19]. In the present report, compounds 15 and 17 are referred to as **1** and **11** respectively.

Table 1
Structures of 1–22.

Code	R	Code	R
1		11	
2		12	
3		13	
4		14	
5		15	
6		16	
7		17	
8		18	
9		19	
10		20	
		21	
		22	



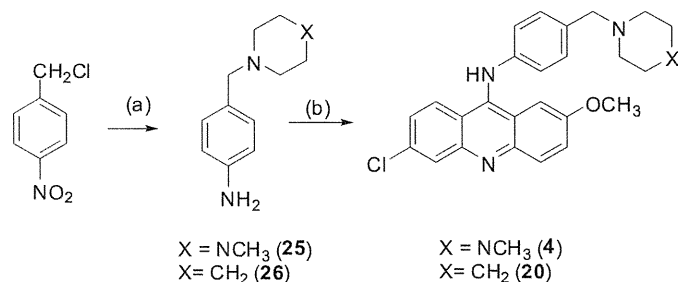
Scheme 1. a) amine, Pd(OAc)₂, BINAP, Cs₂CO₃, anhydrous toluene, 120 °C; b) (i) H₂, Pd/C; (ii) 6,9-dichloro-2-methoxyacridine, ethanol, reflux, 24 h.

3. Results

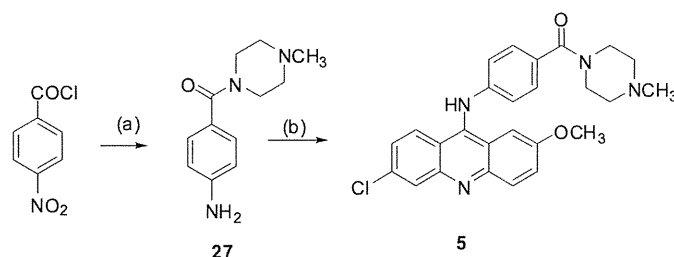
3.1. Anti-prion activity

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 prion-infected 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 μM, which agreed with reported values on a similar cell model [24,25]. It was also active on N167 (EC₅₀ 0.59 μM) and F3 (EC₅₀ 1.88 μM) 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) H₂, PtO₂, 50 psi, overnight; b) 6,9-dichloro-2-methoxyacridine, ethanol, reflux, 24 h.



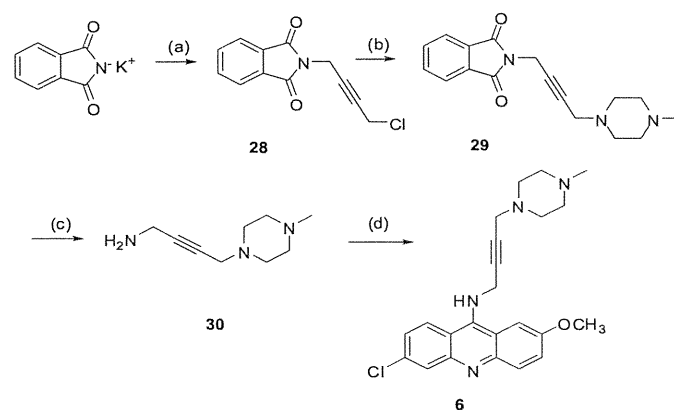
Scheme 3. a) (i) N-Methylpiperazine, TEA, anhydrous DCM, RT, overnight; (ii) H₂, PtO₂, 50 psi, overnight; b) 6,9-dichloro-2-methoxyacridine, phenol, 120 °C, 24 h.

quinacrine (TC 2.5 μM). 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.

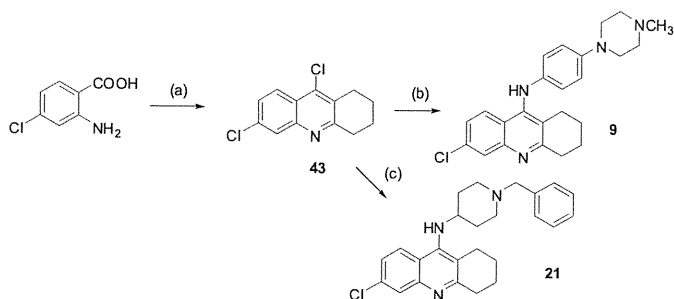
Among the piperazinyphenyl analogs **1–10**, several members had lower EC₅₀ values than **1** on ScN2a but these improved potencies did not extend to N167 or F3. In fact, only 3 analogs (**4**, **5**, **10**) retained activity on all 3 cell models, but with higher EC₅₀ values than **1**. Their lower potencies notwithstanding, these compounds have the advantage of being less toxic (TC 4–12 μM) than **1** and quinacrine.

In spite of the small number of piperazinyphenyl 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 μM).



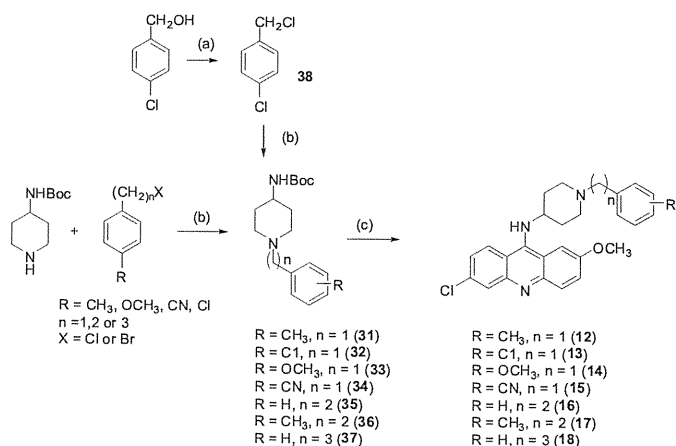
Scheme 4. a) 1,4-dichlorobut-2-yne, DMF, 100 °C, 5 h; b) 1-methylpiperazine, TEA, DCM, rt, overnight; c) NH₂NH₂, EtOH, reflux, 2 h; d) 6,9-dichloro-2-methoxyacridine, phenol, 120 °C, 24 h.



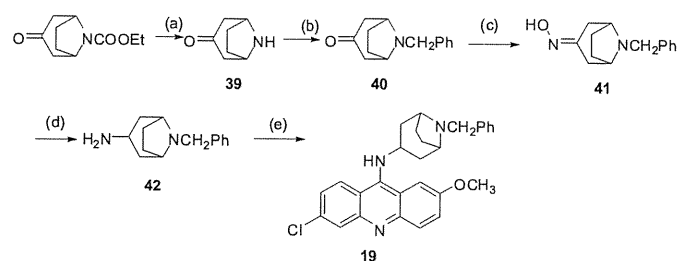
Scheme 5. a) cyclohexanone, POCl_3 , $0^\circ\text{C} \rightarrow$ reflux, 2 h; (b) 4-[(4-methylpiperazin-1-yl)methyl]aniline, ethanol, reflux, 24 h; (c) 1-benzylpiperidin-4-amine, phenol, 120°C , 24 h.

Compound **3** (with a basic substituent) showed a similar profile but was many times more potent on ScN2a (EC_{50} $0.035\ \mu\text{M}$) 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-methoxyacridine (primary 9-amino function) was as potent as **1** on ScN2a (EC_{50} $0.13\ \mu\text{M}$) [19] but as seen here, activity declined in **7** (secondary 9-N-phenylamino, EC_{50} $0.54\ \mu\text{M}$) and even more in **8** (tertiary 9-N-methyl-N-phenylamino, $2.51\ \mu\text{M}$). Thus, it may be inferred that a tertiary 9-amino function adversely affects anti-prion 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 ($\text{TC} \geq 10\ \mu\text{M}$) on N2a cells compared to **1**.

Unlike the piperazinyphenyl analogs, more members of the N-benzylpiperidinyl 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 substituents like chloro, methoxy or cyano (**13–15**) which do not have the electron donating and lipophilic character of the methyl group. Rigidifying the piperidine ring (**19**) and switching the positions of the phenyl and 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) SOCl_2 , $0^\circ\text{C} \rightarrow$ 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) PhCH_2Cl , TEA, THF, reflux, overnight; c) $\text{NH}_2\text{OH}\cdot\text{HCl}$, MeOH, Na_2CO_3 , rt, 4 h; d) H_2 , PtO_2 , 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 piperazinyphenyl 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 hPrP_{121–231}

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 (“fast-on/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 hPrP_{121–231} 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 $\% \text{RU}_{\text{max}}$ 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 ($50\ \mu\text{M}$). 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 EC_{50} 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 ($40\ \mu\text{M}$ or $100\ \mu\text{M}$) 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 ($100\ \mu\text{M}$) as a weak binder as its RU was <100 . Touil et al. [28] screened structurally diverse compounds ($40\ \mu\text{M}$) on

Table 2In vitro anti-prion activities of **1–22** on murine neuroblastoma cells infected mouse adapted scrapie strains (ScN2a and N167) and a human prion strain (F3).

Code	ScN2a		N167		F3		TC (μM) ^c
	EC ₅₀ (μM) ^a	FAA (μM) ^b	EC ₅₀ (μM) ^a	FAA (μM) ^b	EC ₅₀ (μM) ^a	FAA (μM) ^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	None ^d	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)	3	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 μM) 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 piperazinyphenyl 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 EC₅₀ ScN2a points to a relationship between binding affinity and potency on ScN2a. For example, the piperazinyphenyl 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 EC₅₀ 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 piperazinyphenyl 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 piperazinyphenyl and N-benzylpiperidinyl analogs. Of the four

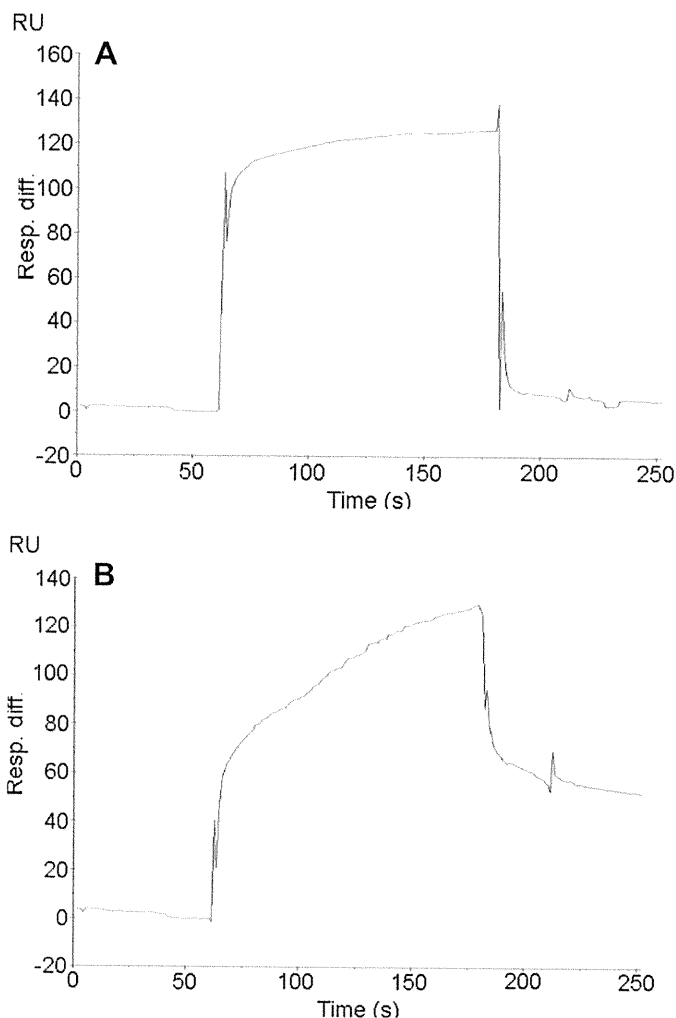


Fig. 2. Interactions of compounds with hPrP121–231 (A) Typical sensorgram of quinacrine, an example of a fast on/fast off compound. (B) Typical sensorgram of **9**, a slow on/slow off compound. Spikes seen at the start and end of injections were due to a slight time delay in the reference cell and appeared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

piperazinyphenyl 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-benzylpiperidiny 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 piperazinyphenyl and N-benzylpiperidiny analogs were broadly comparable but lower than **43–46** (mean of 18.17×10^{-6} cm/s). Lower permeabilities notwithstanding, the piperazinyphenyl and N-benzylpiperidiny 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 $< 2.0 \times 10^{-6}$ cm/s) compounds.

The side chains of the piperazinyphenyl and N-benzylpiperidiny 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 $\log D_{7.4}$ (Spearman rho -0.674 , p 0.003, $n = 17$) which meant that lipophilic compounds like the piperazinyphenyl and N-benzylpiperidiny 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 (8.13×10^{-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 quinacrine 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.

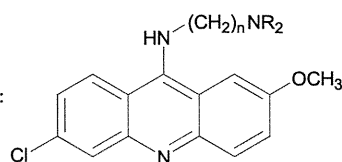
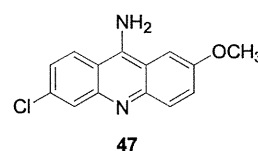
Mass balance measurements showed that most of quinacrine and **1** (ca $>50\%$) could be accounted in the B \rightarrow A direction but to a lesser degree in the reverse (A \rightarrow B) direction. This led us to monitor the degree to which the compounds were retained in the cells and their propensity 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 3Binding 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 <i>D</i> _{7.4} ^c
	Binding response (<i>RU</i> _{max}) ^{a,b}	% <i>RU</i> _{max}	Effective permeability <i>P</i> _e (×10 ⁻⁶ cm/s) ^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.^b Values are presented as mean ± standard deviation.^c Estimated Log *D* at pH 7.4 (ACD/Labs 6.00).^d Not determined.^e Structures of **43**–**47**::**43** n=2, R=C₂H₅**44** : n= 3, R = CH₃**45**: n= 3, R=C₂H₅**46**: n=4, R= C₂H₅**47**^f Reference compounds for PAMPA-BBB assay. *P*_e 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,

Table 4Apparent 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	<i>P</i> _{app} (×10 ⁻⁶ cm/s) ^a		Efflux ratio ^b	Mass balance (%) ^a		Cell retention (%) ^a		Adsorption on Transwell (%) ^a	
		A→B	B→A		B→A	A→B	B→A	A→B	B→A	
Quinacrine	MDCK-WT	2.89 ± 0.83	3.45 ± 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	6.70 ± 1.14	5.1	34.8 ± 10.3	75.8 ± 17.8	35.5 ± 6.2	24.5 ± 7.3	5.5 ± 3.6	3.9 ± 3.1
1	MDCK-WT	0.29 ± 0.04	0.34 ± 0.12	1.2	11.3 ± 1.5	75.3 ± 7.0	66.4 ± 29.0	27.5 ± 12.3	11.4 ± 8.9	3.5 ± 2.5
1	MDCK-MDR1	0.14 ± 0.02	0.34 ± 0.10	2.4	12.6 ± 10.9	73.0 ± 13.9	67.0 ± 14.4	44.2 ± 12.8	6.1 ± 3.7	3.1 ± 2.5

^a Values were presented as mean ± standard deviation from three independent experiments.^b Ratio of apparent permeabilities from the basolateral (B) to apical (A) compartment, to that of apical (A) to basolateral (B) compartments. Efflux ratio = *P*_{app}(B→A)/*P*_{app}(A→B).

unusual among the other test compounds, was equipotent on both ScN2a and F3 (EC_{50} 0.13 μ M, 0.19 μ M).

Although the piperazinyphenyl 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 piperazinyphenyl 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-4-yl) of **11**, as both the tetrahydroacridine (**21**) and quinoline (**22**) analogs had better anti-prion profiles than the corresponding members with the piperazinyphenyl 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 EC_{50} 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 piperazinyphenyl 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 $\log D_{7.4}$ 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 piperazinyphenyl 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 piperazinyphenyl/N-benzylpiperidinyl analogs. The quinacrine analogs had high P_e values ($16\text{--}22 \times 10^{-6}$ cm/s) and at least 2-fold magnitude lower lipophilicities ($\log D_{7.4}$: 0.96–1.51) than the piperazinyphenyl/N-benzylpiperidinyl analogs. On the other hand, the differences in $\log D_{7.4}$ values of the piperazinyphenyl/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, anti-prion activities were limited to ScN2a and did not extend to other cell models. The aromatic and basic side chains of the piperazinyphenyl/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 piperazinyphenyl 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 $\log D_{7.4}$. 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 $\log D_{7.4}$, 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 piperazinyphenyl and N-benzylpiperidinyl 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 alkalization, 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, Cs₂CO₃, 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, J₁ = 3.1, J₂ = 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, J₁ = 3.1, J₂ = 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-4-amine, 1-phenethylpiperidine-4-amine, 1-phenpropylpiperidine-4-amine 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-azabicyclo[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 layer was dried and concentrated in vacuo to give crude 8-aza-bicyclo[3.2.1]octan-3-one (**39**). ¹H NMR (300 MHz, CDCl₃) δ 1.69 (m, 2H), 1.90 (dd, *J*₁ = 4.1, *J*₂ = 8.8, 2H), 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). ¹³C NMR (75 MHz, CDCl₃) δ 27.7 (2C), 48.1 (2C), 55.1, 58.6 (2C), 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, *J*₁ = 2.7, *J*₂ = 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, *J*₁ = 1.9, *J*₂ = 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 N-terminal 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 μg/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 BIAcore 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; hPrP_{121–231}, molecular weight 12,544.97 Da) was dissolved in 10 mM sodium acetate buffer, pH 4.5 (10 μg/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 μM 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:

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

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 μm) 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 μM or 30 μM depending on their aqueous solubilities. An aliquot (300 μL) of the diluted solution was transferred to the donor well. The filter membrane was coated with 5 μL of 20 mg/mL PBL in dodecane and the

acceptor well was filled with 150 μL 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 (P_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 cm^2), t is the incubation time (s), V_A and V_D are the volumes (cm^3) 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

Dulbecco'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 μm 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 μM 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% CO_2 . 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 Ωcm^2 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→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→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:

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

where $R_{\text{cell layer}}$ = Resistance (Ω) of the cell monolayer, R_{blank} = Resistance (Ω) of the blank (insert without cells), A = Effective surface area of the insert (1 cm^2 for the 12-well insert).

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

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

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

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

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).

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Appendix. Supplementary data

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

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Cardiac Positron-Emission Tomography Images With an Amyloid-Specific Tracer in Familial Transthyretin-Related Systemic Amyloidosis

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We report the case of a 32-year-old man who had suffered from orthostatic syncope and body weight loss since he was 27 years old. As years passed by, he also showed muscle weakness and abnormal sensations in both legs, hyporeflexia in 4 limbs, and autonomic failure (impotence, urinary and fecal incontinence, and edema in lower limbs) suggesting the presence of peripheral somatic and autonomic polyneuropathy. His mother, mother's father, and mother's paternal aunt also had similar symptoms. Both the sensory nerve action potential and the sensory nerve conduction velocity of his right sural nerve were low (1.26 μ V and

47.2 m/s, respectively), and the motor nerve conduction velocity of his right tibial nerve was 41.1 m/s (normal >45 m/s). A DNA test on the man disclosed a missense mutation in the transthyretin gene (Ser50Arg), which is relatively rare in familial transthyretin-related systemic amyloidosis.^{1,2} Transthyretin-immunoreactive amyloid deposition was demonstrated in the biopsied gastroduodenal mucosa (Figure 1). Echocardiography showed a markedly thickened ventricular wall (thickness of interventricular septum 22.3 mm [normal <12 mm]) with normal wall motion (fractional shortening 37.6% [normal 28–42%]), indicating that he also had cardiac

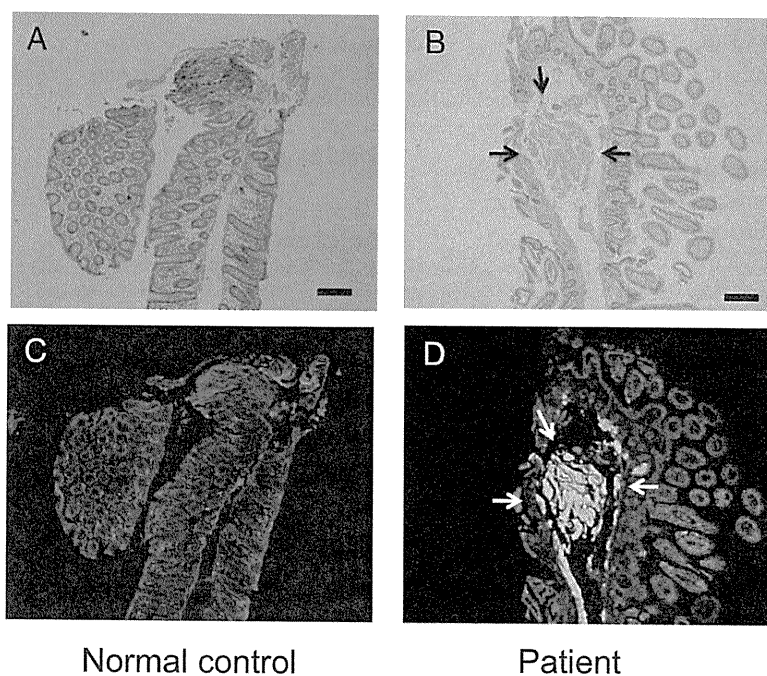


Figure 1. Detection of amyloid deposition in the intestines. Congo red (**A** and **B**) and BF-227 (**C** and **D**) clearly detect transthyretin in the submucosal space of the small intestine of the patient. Scale bars, 100 μ m.

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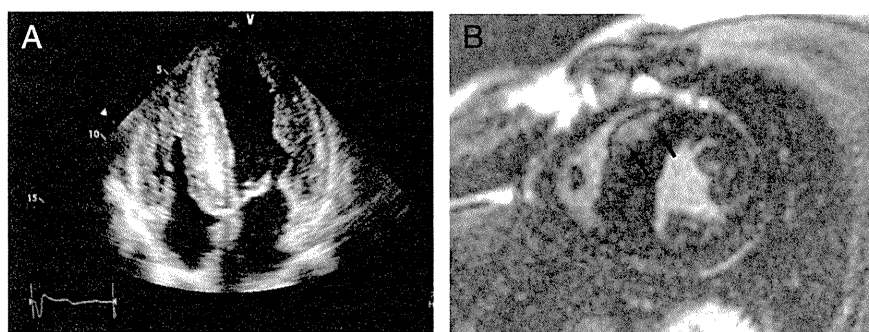


Figure 2. **A**, Echocardiographic finding. Four chamber views show symmetrical thickening of ventricular walls and septum with hyperrefractile myocardial echo (the so-called granular sparkling appearance). **B**, Contrast magnetic resonance imaging with gadolinium. Focal late gadolinium enhancement is visible (arrows).

amyloidosis (Figure 2A). Contrast magnetic resonance imaging³ revealed focal late gadolinium enhancement in the thickened ventricular wall (Figure 2B). The patient had been treated with orthotopic live-donor liver transplantation when he was 31 years old to alleviate and prevent exacerbation of his neuronal and cardiac symptoms. His condition, including the neurological disability, gradually improved, and he started to work again 10 months after liver transplantation.

In order to visualize amyloid deposition in the myocardium, the patient underwent a cardiac positron-emission tomography study with [¹¹C]-BF-227 that sensitively and specifically binds to aggregated amyloid fibrils.⁴ The positron-emission tomography images revealed significantly robust retention of [¹¹C]-BF-227 in the patient's heart compared with that of the normal control (Figure 3). Biopsy specimens from the patient's duodenum also showed higher signals of BF-227 compared with that of the normal control (Figure 1, C and D). The present result provides evidence that our amyloid-specific positron-emission tomography tracer, [¹¹C]-BF-227, can successfully detect amyloid deposition in the heart. Several molecules, such as ^{99m}Tc-aprotinin and ^{99m}Tc-labeled phosphate derivatives, have been investigated to visualize cardiac amyloidosis.² None of the previous tracers, however, could specifically bind to aggregated amyloid, which forms a β -pleated sheet structure. In any of the amyloidogenic disorders, such as transthyretin-related systemic amyloidosis and Alzheimer's disease, it is surmised that the monomer of the amyloid protein itself is not very toxic, whereas misfolded oligomers could cause damage to human organs.¹⁻⁴ It is therefore truly important to detect the accumulation of real amyloid fibrils for the early and accurate diagnosis of amyloidosis. To our knowledge, this is the first report

showing the usefulness of a β -pleated sheet structure-specific positron-emission tomography in investigating visceral organ amyloidosis.

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Disclosures

None.

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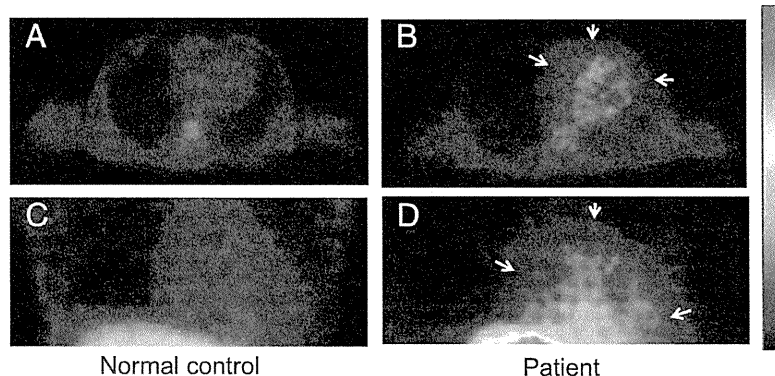


Figure 3. [¹¹C]-BF-227 positron emission tomography succeeds in visualization of amyloid deposition in the heart. Axial and coronal images are **A** and **B** and **C** and **D**, respectively. Arrows indicate high signals of [¹¹C]-BF-227 in the heart of the patient (**B** and **D**).



Synthesis of [^{11}C]interleukin 8 using a cell-free translation system and L-[^{11}C]methionine

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Abstract

Positron emission tomography (PET), which requires a compound labeled with a positron emitter radioisotope as an imaging probe, is one of the most useful and valuable imaging modalities in molecular imaging. It has several advantages over other imaging modalities, particularly in sensitive and quantitative investigations of molecular functions and processes *in vivo*. Recent advances in biopharmaceuticals development have increased interest in practical methods for proteins and peptides labeling with positron emitter radioisotope for PET molecular imaging. Here, we propose a novel approach for preparing positron emitter-labeled proteins and peptides based on biochemical synthesis using a reconstituted cell-free translation system. In this study, [^{11}C]interleukin 8 (IL-8; MW 9.2 kDa) was successfully synthesized by the cell-free system in combination with L-[^{11}C]methionine. The *in vitro* biochemical reaction proceeded smoothly and gave maximum radioactivity of [^{11}C]IL-8 at 20 min with a radiochemical yield of 63%. Purification of [^{11}C]IL-8 was achieved by conventional cation exchange and ultrafiltration methods, resulting in enough amount of radioactivity with excellent radiochemical purity (>95%) for small-animal imaging. This study clearly demonstrates that cell-free protein production system combined with positron emitter-labeled amino acid holds great promise as a novel approach to prepare radiolabeled proteins and peptides for PET imaging.

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Keywords: Interleukin 8; Imaging; Positron; Carbon-11; Cell-free protein synthesis; Unnatural amino acid

1. Introduction

Biological research is moving from the gathering of genomic sequences to protein expression for analysis of sequence function and utility. In accordance with this sequence-to-function paradigm shift, there have been great advances in biotechnology and bioengineering that enable the preparation of large quantities of numerous active proteins using simple yet sophisticated approaches [1,2]. These technologies are rapidly spreading not only in many research fields, such as chemical biology, structural biology and pharmaceutical biology, but also in drug discovery and development. Especially, pharmaceutical peptides and pro-

teins, including biotechnology-derived antibody, are becoming increasingly important as source of major new drugs [3,4].

In addition to these advances in biotechnology and its applications, molecular imaging has come to be regarded as a key method for characterization of biomolecular functions and roles *in vivo*. Basic research and technology on molecular imaging have made great advances in recent years, and their application to drug development is highly expected [5]. One of the most useful and valuable imaging modalities in molecular imaging is positron emission tomography (PET), which uses a compound labeled with a positron emitter radioisotope as imaging probe. Positron emission tomography has several advantages over other imaging modalities, particularly in sensitive and quantitative investigations of molecular functions and processes *in vivo*. In addition, PET has a wide range of imaging objects extending from small animals to humans [6]. The use of appropriate positron emitter-labeled compounds is critical

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for PET molecular imaging because the characteristics of each labeled probe can define specific imaging information. Considering recent advances in biopharmaceuticals, positron emitter labeling of biomolecules for PET imaging is increasingly becoming important.

To date, several methods for labeling peptides and proteins with positron-emitting nuclides have been reported [2,7]. For instance, biomolecules can be labeled by conjugation with a prosthetic group containing a positron emitter, such as *N*-succinimidyl-4- ^{18}F fluorobenzoate through acylation reaction, ^{18}F maleimide through formation of a thioether bond and ^{18}F fluoroalkyne through the click reaction [8]. Conjugation of a metal chelator, such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, with biomolecules followed by chelating of a positron emitter metal, such as ^{64}Cu or ^{68}Ga , also affords radiolabeled products [9,10]. In addition, biomolecules can be directly radiolabeled with iodine-124 in the presence of an oxidizing agent, such as Iodogen or chloramine T [11]. Essentially, all of these conventional labeling methods are used to directly or indirectly attach a radioisotope to peptides or proteins based on a chemical reaction.

In this article, we propose a novel approach for synthesis of positron emitter-labeled proteins and peptides based on a biotechnology method. We focused on a cell-free protein synthesis system for preparation of radiolabeled peptides and proteins. The principle of our approach is to combine a cell-free system with a positron emitter-labeled amino acid. One of the most refined cell-free protein synthesis system is the PURE system, whose components are completely reconstituted purified essential elements from *Escherichia coli* with the composition of each component arbitrarily determined [12]. According to our approach, we chose interleukin 8 (IL-8) as an example to synthesize a radiolabeled chemokine using a commercially available PURESYSYSTEM and ^{11}C MET [13,14]. Interleukin 8, a chemotactic factor that attracts neutrophils, basophils and T-cells, but not monocytes, and is involved in neutrophils activation, is released from several types of cells in response to inflammatory stimulus [15]. Since neutrophils express IL-8 receptors on their surface, positron emitter-labeled IL-8 would have the potential to be a PET probe for inflammation imaging. In fact, $^{99\text{m}}\text{Tc}$ - and ^{131}I -labeled IL-8 is used for detection of infection and inflammation in clinical research [16,17]. In this study, we investigated the possible use of a cell-free protein synthesis system for preparation of ^{11}C IL-8 as a PET probe.

2. Material and methods

2.1. Synthesis of IL-8 using a noncellular in vitro protein synthesis system

We synthesized recombinant human IL-8 protein in a cell-free system as described previously [1]. Plasmid DNA used for the synthesis of IL-8 was kindly gifted by Dr. A. Ametani

(Post Genome Institute Co. Ltd.). The polymerase chain reaction (PCR) product used for the synthesis was prepared in our laboratory by two-step PCR method. In brief, in the first PCR step, we amplified cDNA using IL-8 specific primers with adaptor sequences at the 5' terminus. Regulatory sequences, such as T7 promoter, epsilon sequence and Shine–Dalagarno sequence, were attached in the second PCR step (Fig. 1).

Protein synthesis reaction was performed as described in PURESYSYSTEM (currently available from BioComber Co. Ltd.) manufacturer's instructions. PURESYSYSTEM is a novel reconstituted protein synthesis system kit that consists of about 30 purified enzymes necessary for in vitro transcription, translation and energy recycling. The kit reagents also contains *E. coli* 70S ribosome, amino acids, NTPs, *E. coli* tRNA and energy recycling system, so the target protein can be synthesized just by addition of the template DNA to the reaction with a short reaction time. According to this method, IL-8 was synthesized by addition of plasmid DNA of IL-8 to the kit reagents. To confirm the production of IL-8, the reaction samples were subjected to 15%–25% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a polyvinylidene difluoride membrane. The membrane was then incubated with polyclonal antibody against human IL-8 (MBL) (1:1000). After rinsing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Pierce). The blot was developed using ECL reagents (Millipore) and detected using an image analysis system, LAS-1000 (Fujifilm).

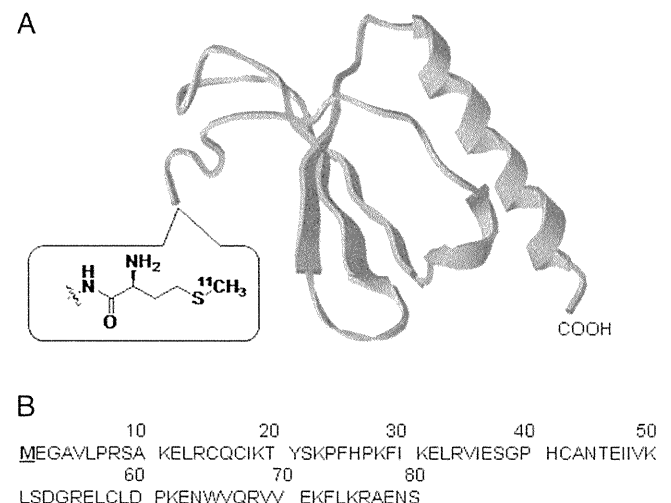


Fig. 1. A schematic illustration and an amino acid sequence of ^{11}C IL-8. (A) Cartoon representation of ^{11}C IL-8 structure estimated by SWISS-MODEL [18–20]. ^{11}C MET was site-specifically incorporated into the N-terminal of IL-8 because methionine was added to the amino acid sequence of IL-8 as a starting amino acid for protein synthesis. (B) An amino acid sequence of IL-8 used for ^{11}C IL-8 synthesis. A methionine residue was added to the N-terminus of IL-8 to initiate translation in a cell-free protein synthesis system. Underlined section represents the additional methionine.

2.2. Radiosynthesis of [^{11}C]IL-8 using a noncellular in vitro protein synthesis system

2.2.1. Radiosynthesis of L-[^{11}C]methionine

L-[^{11}C]Methionine ([^{11}C]MET) was synthesized using two precursors, L-homocysteine thiolactone hydrochloride (Sigma) and L-homocysteine (ABX). The former precursor was used for on-column preparation of [^{11}C]MET [21]. Briefly, L-homocysteine thiolactone hydrochloride (6 mg) dissolved in EtOH-1 M NaOH (1/1) solution (0.4 ml) was loaded onto a Sep-Pak Plus tC18 cartridge (Waters), and [^{11}C]methyl iodide was passed through the cartridge. The radioactive product was then eluted with 0.5% acetic acid (3 ml) and collected in a flask. The eluate was evaporated to dryness, and the residue was dissolved in distilled water (1 ml). The latter precursor, on the other hand, was used to prepare a chloride-free solution of [^{11}C]MET [22]. In brief, salt-free L-homocysteine (1 mg) was dissolved in 0.2 M NaOH (60 μl), and [^{11}C]methyl triflate converted from [^{11}C]methyl iodide was bubbled in the precursor solution. The reaction mixture was then neutralized with 0.2 M Na_2HPO_4 (71 μl). Average specific activity of carbon-11 ligands at the end of the synthesis when the same methylation methods were used at CYRIC. A very small fraction of synthesized [^{11}C]MET was used for further experiments due to the limited reaction volumes of a commercially available protein translation system.

2.2.2. Radiosynthesis and purification of [^{11}C]IL-8

[^{11}C]MET (100 μl , 74–202 MBq) and DNA plasmid (20 μl , 4 μg) were added to a mixture solution containing Sol A (200 μl) and Sol B (80 μl) of the PURESYSYSTEM ADVANCE WITHOUT METHIONINE KIT and then incubated at 37°C for an appropriate period of time. After incubation, the reaction was stopped with RNase (4 μl , 200 μg). [^{11}C]IL-8 was isolated from the crude solution in two steps using a cation spin column and a centrifugal filter device.

Firstly, the crude solution mixed with 0.1 M HEPES buffer (pH 7.6) was loaded onto a strong cation exchange spin column ($-\text{SO}_3^-$; Waters) and centrifuged (1500 $\times g$, 5 min). After washing with 0.1 M NaCl-HEPES (1500 $\times g$, 5 min), [^{11}C]IL-8 was eluted with 0.5 M NaCl-HEPES from the column (1500 $\times g$, 5 min). Secondly, the eluate was placed into a centrifugal filter device (YM-50, Millipore) and centrifuged (10,000 $\times g$, 6–8 min) in order to remove other constituents from in vitro translation system. These purification processes required approximately 25 min.

2.2.3. Autoradiography of SDS-PAGE

After SDS-PAGE of radiolabeled samples from each process, the gel was exposed to a BAS-MS2025 imaging plate (Fujifilm) for 30 min. Autoradiographic images were obtained using a BAS-5000 phosphor imaging instrument (Fujifilm). Radiochemical yields and purities of [^{11}C]IL-8 were determined by analyzing the gel autoradiograms. After decay of the radioactivity, the gels were stained by

SYPRO Red (Invitrogen), and fluorescence images were obtained by FLA-2000 (Fujifilm).

2.3. Whole-body imaging of [^{11}C]IL-8 in mice

All animal experimental protocols described in this report were approved by the Laboratory Animal Care Committee of Tohoku University. Whole-body distribution of [^{11}C]IL-8 in mice was evaluated with a planar positron imaging system (PPIS) (Hamamatsu Photonics K.K.) [23]. Focal plane images were constructed from coincidence data collected by opposing planar detectors, achieving data acquisition with higher sensitivity and finer time resolution than conventional animal PET such as microPET. Mice anesthetized with isoflurane were positioned prone on an acrylic plate and placed on the midplane between the two opposing detectors arranged in a horizontal mode. Synthesized [^{11}C]IL-8 at a dose of about 0.7 MBq was injected intravenously into the mouse via the tail vein. Data were acquired with a 1-min time frame interval for 60 min after injection with six summation images created every 10 min.

3. Results and discussion

Nonradiolabeled IL-8 was successfully synthesized using a cell-free translation system (PURESYSYSTEM), which is a unique reconstituted system consisting of a set of independently prepared enzymes, translation factors, ribosome, ATP, tRNA and amino acids necessary for protein synthesis [12]. As such, the composition of each component of this system can therefore be arbitrarily determined. This characteristic has a great advantage because the components of amino acids necessary for protein synthesis can easily be modified. Expression of IL-8 using this system was examined by SDS-PAGE and Western blotting (Fig. 2A) and showed a band at about 10 kDa. These findings confirm

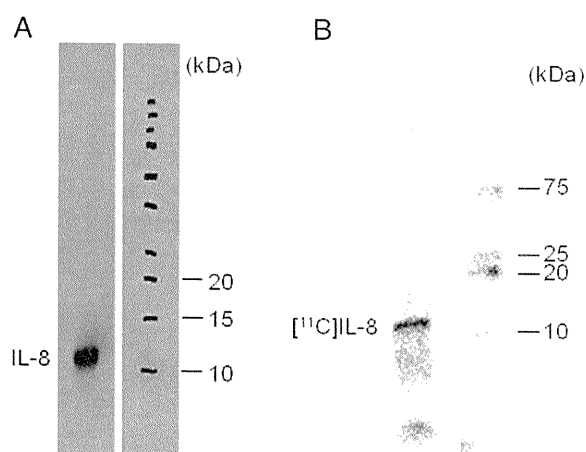


Fig. 2. Synthesis of IL-8 using the PURE system. Confirmation of production of nonlabeled and [^{11}C]IL-8 by SDS-PAGE with Western blotting (A) and ARG (B).

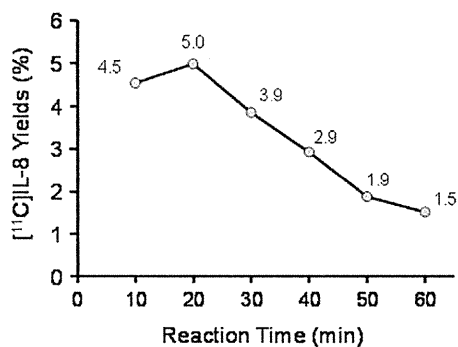


Fig. 3. Optimization of reaction time for [¹¹C]IL-8 synthesis. Non-decay-corrected yields of [¹¹C]IL-8 at the end of synthesis at the different reaction times (min) of in vitro translation system with 25-min purification processes are shown in the graph.

our successful preparation of IL-8 using PURESYSYSTEM and are in accordance with previous results [1].

Next, we specially prepared a set of natural amino acids lacking only methionine of PURESYSYSTEM to efficiently incorporate [¹¹C]MET into the protein synthesis. Production of [¹¹C]IL-8 was examined by SDS-PAGE and autoradiography (ARG). As shown in Fig. 2B, a radioactive band at about 10 kDa corresponding to IL-8 was clearly identified, which is consistent with Western blotting data (Fig. 2A). These results strongly suggest that [¹¹C]IL-8 was successfully synthesized by this cell-free protein synthesis system.

In view of practical use of this labeling method, we examined the reaction time to obtain maximum radioactivity of [¹¹C]IL-8 at the end of synthesis (EOS) including the 25 min of purification step. As shown in Fig. 3, the non-decay-corrected yield of [¹¹C]IL-8 from [¹¹C]MET reached a maximum of 5.0% after 20-min reaction time, and then the yield decreased with time based on carbonyl-11 half-life ($T_{1/2}$: 20.4 min). The decay-corrected radiochemical yield of [¹¹C]IL-8 after 20-min reaction time was determined to be 63% on average ($n=4$) by SDS-PAGE ARG. Given 45 min of total synthesis time, [¹¹C]IL-8 could be obtained in a non-decay-corrected yield of 13% at the EOS. The difference between the experimental yield (5.0%) and the calculated yield (13%) could be caused by the loss of [¹¹C]IL-8 during the purification.

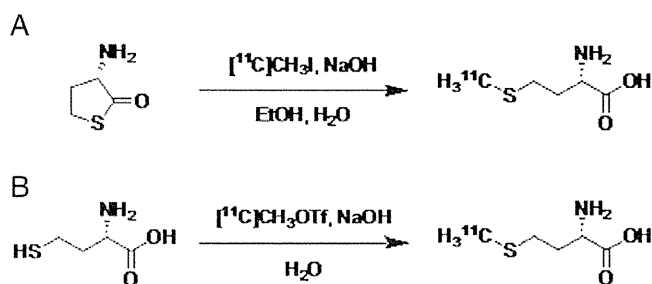


Fig. 4. Synthesis of [¹¹C]MET from different precursors. (A) Radiosynthesis scheme of [¹¹C]MET from L-homocysteine thiolactone and [¹¹C]methyl iodide. (B) Radiosynthesis scheme of [¹¹C]MET from L-homocysteine and [¹¹C]methyl triflate.

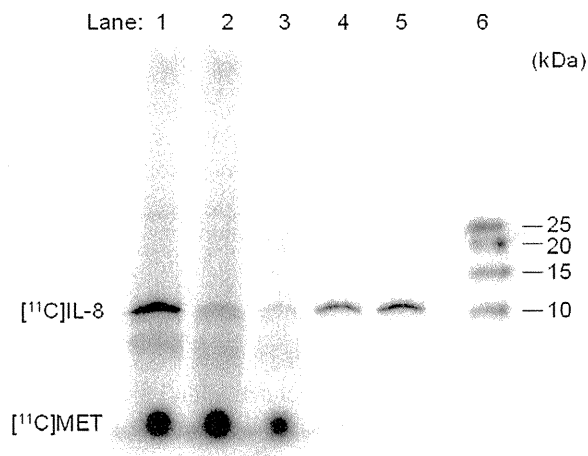


Fig. 5. Purification of [¹¹C]IL-8 with cation exchange and ultrafiltration spin column. Sample at each purification step was analyzed with SDS-PAGE ARG. Lane 1: crude reaction solution. Lane 2: reaction solution passed through cation exchange resin with HEPES (pH=7.6). Lane 3: washing solution with 0.1 N NaCl-HEPES (pH=7.6). Lane 4: eluate from the resin with 0.5 N NaCl-HEPES (pH=7.6). Lane 5: filtrate after ultrafiltration. Lane 6: marker.

In this study, it was also investigated whether synthesis methods of [¹¹C]MET had an influence on the yield of [¹¹C]IL-8 because ingredients such as ions and solvents in the prepared [¹¹C]MET solution vary according to the synthetic method. In this respect, we compared two methods for [¹¹C]MET synthesis, which use L-homocysteine thiolactone or L-homocysteine as a precursor (Fig. 4). As a result, radiosynthesis using [¹¹C]MET prepared from L-homocysteine thiolactone as a precursor afforded [¹¹C]IL-8 in a good radiochemical yield of 63%, while [¹¹C]MET from L-homocysteine gave [¹¹C]IL-8 in a low yield of 20%.

The ARG of SDS-PAGE gel revealed that not only [¹¹C]IL-8 but also [¹¹C]MET and incomplete products of [¹¹C]IL-8 existed as radioactive components in the protein synthesis solution (Fig. 5). In the reaction solution (pH 7.6), [¹¹C]IL-8 has a net positive charge because the isoelectric point of IL-8 is 8.6, while that of other proteins in the solution ranges lower than 7.6. The reaction mixture was therefore subjected to a strong cation exchange chromatography to isolate [¹¹C]IL-8 from other components. After passing the mixture through a cation exchange spin column, [¹¹C]IL-8 was effectively entrapped in the column (74%). The [¹¹C]IL-8 was eluted with 0.5 N NaCl-HEPES (pH=7.6) to make it useful for an injectable solution. This simple purification gave rise to the efficient isolation of [¹¹C]IL-8 from the reaction with a yield of approximately 40%. Although some amount of radioactivity was lost during the purification, [¹¹C]IL-8 was conveniently isolated in excellent radiochemical purity of 97%. This high radiochemical purity was sufficient for use of [¹¹C]IL-8 as a radioactive probe in animals. A preliminary imaging study with [¹¹C]IL-8 was performed using high-sensitivity PPIS, demonstrating higher retention of radioactivity in the liver and kidney in mice (Fig. 6).