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Research Paper

Curcumin and a hemi-analogue with improved blood–brain barrier permeability protect against amyloid-beta toxicity in *Caenorhabditis elegans* via SKN-1/Nrf activation

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Keywords
amyloid-beta; blood–brain barrier permeability; *Caenorhabditis elegans*; curcumin analogues; SKN-1/Nrf

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Abstract

Objectives This study aims to investigate the blood–brain barrier (BBB) permeability of curcumin analogues with shortened linkers and their ability to protect against amyloid-beta toxicity in a whole organism model.

Method Four curcumin analogues were synthesized. These analogues and curcumin were evaluated for their BBB permeability in the parallel artificial membrane permeability assay. The transgenic *Caenorhabditis elegans* GMC101 that expresses human $A\beta_{1-42}$ was treated with the compounds to evaluate their ability to delay Aβ-induced paralysis. Expression of *skn-1* mRNA was examined on nematodes treated with selected efficacious compounds. *In vitro* Aβ aggregation in the presence of the compounds was performed.

Key findings The four analogues showed improved BBB permeability vs curcumin in the PAMPA with the hemi-analogue **C4** having the highest permeability coefficient. At 100 μM, analogues **C1** and **C4** as well as curcumin significantly prolonged the survival of the nematodes protecting against Aβ toxicity. However, only curcumin and **C4** showed protection at lower concentrations. *skn-1* mRNA was significantly elevated in nematodes treated with curcumin and **C4** indicating SKN-1/Nrf activation as a possible mode of action.

Conclusions Analogue **C4** provides a new lead for the development of a curcumin-based compound for protection against Aβ toxicity with an improved BBB permeability.

Introduction

Alzheimer's disease is a common cause of dementia in the ageing population. It is a neurodegenerative condition marked by progressive cognitive impairment leading to overt disability, morbidity and death. At the cellular level, the hallmarks of the disorder are the deposition of toxic amyloid beta (Aβ) oligomers, increased oxidative stress, the development of neurofibrillary tangles and glial activation, multiple events that interact to cause neuronal cell dysfunction and death.[1] Due to the complex interplay between these multiple causative processes, the pathogenesis of Alzheimer's disease is not precisely understood. However, the negative or toxic effects of Aβ in the intra- and intercellular environments of neurons and glial cells are evident in numerous studies.[2–4]

Disease-modifying agents that address these multiple factors of Alzheimer's disease and extend beyond symptomatic control are being intensively studied and sought after. A notable example is curcumin, a naturally occurring phenolic diarylheptanoid constituent of the spice turmeric (*Curcuma longa*). Curcumin has demonstrated remarkable properties *in vitro* such as the ability to inhibit Aβ aggregation or cause its disaggregation[5,6] and to reduce Aβ-induced toxicity in various cell culture preparations.[7–9] Despite these promising attributes, little has been done to address its ability to penetrate the blood–brain barrier (BBB) and be available in efficacious concentrations in the brain. Sensitive multiphoton microscopy has shown evidence of curcumin BBB penetration but its presence in the mouse brain was weak and required the accumulation of curcumin via repeated intravenous administrations.[10] Cheng *et al.*[11] using the Mardin-Darby canine kidney cell monolayer expressing human multidrug resistance 1 (MDR1) as their BBB model reported low curcumin permeability rate (1.8×10^{-6} cm/s) even when the compound

was formulated as nanoparticles. A recent study[12] reported that curcumin conjugated to a nanoliposome and a transactivator of transcription (TAT) peptide showed a higher permeability coefficient (2.2×10^{-5} cm/s) across the human cerebral microvascular (hCMEC/D3) cell monolayer but it remains to be tested whether conjugation with such large moieties would retain curcumin bioactivity.

We hypothesized that replacing the heptadienedione linker of curcumin with a single carbonyl cross-conjugated dienone thus shortening the molecule, reducing its molecular weight and increasing its lipophilicity would result in better BBB permeability. Shortening the linker may also improve its stability as this portion of the molecule has been known to contribute to its degradation and low bioavailability.[13] We were also interested to determine whether such modified curcumin analogues would still retain or improve the protective properties of curcumin against Aβ. To this end, four analogues were prepared and evaluated in a transgenic *Caenorhabditis elegans* that expresses $A\beta_{1-42}$ and manifests paralysis as a result of Aβ toxicity to its bodywall muscle cells.[14] BBB permeability of the analogues was determined using the parallel artificial membrane permeability assay for blood–brain barrier (PAMPA-BBB).

The protective effect of curcumin has been attributed to its Aβ binding property.[5,6] In another perspective, Michael acceptor motifs (alpha, beta-unsaturated carbonyl) are embedded within the structures of curcumin and the analogues in this study. Michael acceptor moiety-bearing compounds are known to activate the Kelch-like ECH-associated protein 1-Nuclear Factor (erythroid-derived 2)-like 2 (Keap1-Nrf2) pathway, a master regulator of cytoprotective responses to endogenous and exogenous electrophilic and oxidative stressors.[15,16] Accordingly, to elucidate the possible mechanism(s) underlying the protection against Aβ by the compounds, two mechanisms were explored in this study: their Aβ aggregation inhibitory potential, and their ability to induce the SKN-1 pathway, the nematode orthologous version of the mammalian Nrf2 pathway.

Materials and Methods

General experimental details for synthesis

Reagents (synthetic grade or better) were obtained from Sigma-Aldrich Chemical Company Inc. (Singapore, Singapore), Acros Organics (Geel, Belgium) and Biobasic Canada Inc. (Toronto, Canada) and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Bruker Biospin AG, Fallanden, Switzerland). Chemical shifts (δ) were reported in parts per millions (ppm) and the coupling constants in Hz. Electrospray Ionisation Mass Spectra (ESI-MS) were obtained using a Waters 2695 Separations Module with Micromass Quattro Micro mass spectrometer (Waters Corporation, Milford, MA, USA) and accurate *m*/*z* for molecular ions were reported. Melting points of the compounds were obtained using a digital melting point apparatus Stuart SMP10 (Staffordshire, UK) and were uncorrected. All synthesized compounds were purified by flash column chromatography on silica gel 60 (230–400 mesh). All reactions were monitored by thin layer chromatography on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). The purities of compounds **C1**, **C2** and **C3** (higher than 95%) were confirmed through HPLC on a Agilent Tech 1120 Compact HPLC system with the use of a Purospher STAR RP-18e column (4.6 × 100 mm, 5 μm) at 1 ml/min on two different solvent systems.

Synthesis of curcumin analogues C1, C2 and C3

To a solution of 4-hydroxybenzaldehyde (2 mol) in glacial acetic acid was added a ketone (1 mol) such as acetone (for **C1**), cyclopentanone (for **C2**) and cyclohexanone (for **C3**). The resultant mixture was saturated with anhydrous HCl and stirred at room temperature for 2 h. After standing for 48 h, the mixture was poured into cold water and the suspension was extracted with ethyl acetate (50 ml × 3). The collected organic extract was dried with anhydrous Na_2SO_4, evaporated *in vacuo* to give a solid residue and purified by column chromatography on silica gel using hexane: ethyl acetate as eluting solvents. The compounds were recrystallized from ethyl acetate or ethanol.

1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one (C1)

Greenish yellow powder. $C_{19}H_{18}O_5$. Yield: 54.6%. Melting point: 134–138 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.85 (s, 6H, –OC$\underline{H}_3$ × 2), 6.84 (d, J = 8 Hz, 2H, arom), 7.16 (d, J = 15 Hz, 2H, CH=C$\underline{H}$–CO × 2), 7.37 (d, J = 1.5 Hz, 2H, arom), 7.65 (d, J = 15 Hz, 2H, arom-C$\underline{H}$ = CH × 2), 9.71 (s, 2H, –O$\underline{H}$ × 2). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 188.5, 149.9, 148.4, 143.2, 126.8, 123.8, 123.4, 116.1, 111.9, 111.8, 56.17; ESI-MS *m*/*z*: 327.24 $[M + H]^{+\cdot}$ HPLC (MeOH : H_2O = 55 : 45) t_R (min): 5.593. Purity: 99.84%. HPLC (ACN : H_2O = 40 : 60) t_R (min): 4.697. Purity: 99.36%.

2,5-bis (4 hydroxy-3-methoxybenzylidene) cyclopentanone (C2)

Yellow powder, Yield: 87.0%, m.p. 196–198 °C; ^{1}H NMR (DMSO-d_6): δ 3.07 (4H, s, –CH_2CH_2–), 3.84 (s,

6H, OCH_3 × 2), 6.89 (d, J = 9 Hz, 2H, CH=CH–CO × 2), 7.17 (d, J = 9 Hz, 2H, arom-CH=CH × 2), 7.25 (s, 2H, arom), 7.36 (s, 2H, arom), 9.72 (br s, 2H, OH × 2). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 195.3, 149.0, 148.2, 135.2, 133.3, 127.6, 125.2, 116.3, 115.0, 56.0, 26.3; ESI-MS m/z: 353.29 $[M + H]^+$. HPLC (MeOH : H_2O = 65 : 35) t_R (min): 3.973. Purity: 99.73%. HPLC (ACN : H_2O = 50 : 50) t_R (min): 3.097. Purity: 99.80%.

2,6-bis (4-hydroxy-3-methoxy benzylidene) cyclohexanone (C3)

Yellow powder, Yield: 73.3%, m.p. 174–176 °C;^{1}H NMR (DMSO-d_6): δ 1.71–1.75 (m, 2H), 2.9 (t, 4H), 3.81 (s, 6H, OCH_3 × 2), 6.85 (d, J = 9 Hz, 2H, CH=CH–CO × 2), 7.04 (d, J = 9 Hz, 2H, arom-CH=CH × 2), 7.12 (s, 2H, arom), 7.57 (s, 2H, arom), 9.57 (s, 2H, OH × 2). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 189.0, 148.3, 147.9, 136.6, 134.0, 127.4, 124.7, 116.0, 115.2, 56.1, 28.4, 23.03. ESI-MS m/z: 367.27 $[M + H]^+$. HPLC (MeOH : H_2O = 65 : 35) t_R (min): 4.870. Purity: 98.84%. HPLC (ACN : H_2O = 50 : 50) t_R (min): 4.177. Purity: 98.84%.

Caenorhabditis elegans strain and culture conditions

The transgenic strain GMC101 (dvIs100 (unc-54p::A-beta-1-42::unc-54 3′-UTR + mtl-2p::GFP). mtl-2p::GFP) and the food source *Escherichia coli* (*E. coli*) strain OP50 were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minnesota, MN, USA). The GMC101 strain constitutively produces a full-length human $A\beta_{1–42}$ in bodywall muscle cells that aggregates *in vivo* and upshifting L4 or young adults worms from 16 to 25 °C induces paralysis.[14] The nematodes were routinely propagated at 16 °C on solid nematode growth medium (NGM) seeded with spots of OP50 as the food source.

Media preparation

Curcumin and **C4** were purchased from Sigma-Aldrich Inc. (Singapore, Singapore). The curcumin analogues (**C1**, **C2** and **C3**) were synthesized as described in the previous section. Stock solutions of all compounds were made with 100% DMSO. The final concentration of DMSO did not exceed 1% in the media. All compounds were added directly into the molten NGM to a final concentration of 100, 10 and 1 μM; NGM with 1% of DMSO served as vehicle control. All media were spotted with 600 μl of OP50 food source.

Aβ-induced paralysis assay

Paralysis assays were performed as described previously.[14] In brief, egg-synchronized population of transgenic GMC101 strain was prepared by allowing gravid adult nematodes to lay eggs on the NGM plate containing either a vehicle or test compound at the desired concentrations for 2 h at 16 °C. Adult nematodes were then removed and plates with eggs were allowed to develop into L3 larvae stage at 16 °C. At 68 h post-egg lay, the test plates were temperature upshifted to 25 °C to induce transgene expression. Scoring for paralysis was performed at 2 h intervals beginning from 24 h post-temperature upshift. Nematodes were scored as paralysed if they failed to respond to a touch-provoked movement with platinum wire. At least three independent experiments were performed for each test compounds. Approximately 55–100 worms were grown on each assay plate. Survival curves were generated using Prism 5 (GraphPad Software Inc., La Jolla, CA, US). The delays in paralysis were assessed by comparing the median survival time at which 50% nematodes were paralysed, represented as median ±95% confident interval, between nematodes fed with vehicle or compounds. Significant differences between groups were assessed using the log-rank (Mantel-Cox) analysis. A P value <0.05 was considered statistically significant.

Parallel artificial membrane permeability assay for blood–brain barrier

Porcine brain lipid (PBL) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Dodecane, caffeine and donepezil were sourced from Sigma-Aldrich (St. Louis, MO, USA) while DMSO was from BioBasic Canada Inc. The 96-well donor plate microplate (PVDF membrane, pore size 0.45 μM) and the acceptor microplate were both from Merck Millipore Bioscience (Bedford, MA, USA). The method by Di *et al.*[17] was followed. Stock solutions of each reference and test compound were prepared in DMSO at 1 mM and then diluted with phosphate buffered saline (PBS) to obtain the donor solution with the final concentration of 10 μM. 300 μl of the compounds were added to the donor wells. The lipid membrane of the PAMPA-BBB model was prepared by dissolving 20 mg of PBL in 1 ml dodecane. The filter membrane of the acceptor microplate was coated with 4 μl of the PBL solution and then filled with 300 μl of PBS. Thereafter, acceptor plate was gently placed into the donor plate and incubated at room temperature for 18 h. Upon completion of incubation, the plates were separated and the concentration of the compounds in both donor and acceptor wells were quantified by the UV

absorbances of the compounds (curcumin and **C2**, λ_{max} 420 nm; **C1** and **C3**, λ_{max} 395 nm; **C4**, λ_{max} 340 nm). Permeability rates (P_e) and membrane retention (R) were calculated using the following equation:

$$P_e = -\frac{2.303 V_A}{A.t}\left[\frac{V_A}{V_A + V_D}\right] \cdot \log\left\{1 - \frac{V_A + V_D}{V_D(1-R)} \cdot \frac{C_A(t)}{C_D(0)}\right\}$$

$$R = 1 - \frac{C_D(t)}{C_D(0)} - \frac{V_A}{V_D} \cdot \frac{C_A(t)}{C_D(0)}$$

where A is the active surface area of the membrane (0.24 cm^2), t is the incubation time (in s), V_A and V_D are the volumes (cm^3) of the acceptor and donor compartments, C_A and C_D are the compound concentrations (mol/cm^3) in the acceptor and donor compartments. Experiments were carried out in replicates ($n = 6$) in three independent experiments and P_e values were reported as means ± standard deviations.

Inhibition of $A\beta_{1-40}$ peptide aggregation

Synthetic full-length $A\beta_{1-40}$ peptides (Sigma-Aldrich) were pretreated with HFIP (1,1,1,3,3,3-Hexafluoro-2-propanol) to ensure it is in a monomer state, subsequently evaporated under stream of nitrogen gas and redissolved in dimethylsulfoxide (DMSO) to keep as stock. The peptide stock solutions were later diluted in PBS (pH 7.4) to the final concentration of 10 μM used in experiments. Thioflavin T (ThT) assay was performed as follows: 10 μM of $A\beta_{1-40}$ peptide incubated with 10 μM test compounds or plain PBS was mixed with 20 μM ThT at 37 °C for 24 h. The ThT fluorescence intensity was measured on LS 45 Luminescence Spectrometer (Perkin-Elmer, Waltham, MA, USA) with excitation and emission wavelengths at 440 and 484 nm, respectively. Each assay was run in triplicates in three independent experiments.

Isolation of RNA and cDNA synthesis

Egg synchronized transgenic GMC101 worms were exposed to media containing vehicle, curcumin or **C4** (10 and 100 μM) at 16 °C for 68 h and were upshifted to 25 °C for transgene induction. At 15 h post-temperature upshift, about 8000 worms were harvested for total RNA extraction. Total RNA was extracted with Nucleospin^R RNA Plus kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. Purity and concentration of total RNA were determined by using Bioanalyser (UV spectrophotometer Q3000, Quawell). RNA samples with the purity above 1.8 and 2.0 were qualified for further tests. First strand cDNA was synthesized from 2 μg RNA using HiSenscript™ RH(−) cDNA Synthesis kit (Intron Biotechnology Inc., Gyeonggi-do, Korea).

Real-time quantitative PCR

For quantitative gene expression analysis, total RNA samples, derived from three biological replicates of each treatment were pooled. qRT-PCR experiments were performed using KAPA SYBR® FAST quantitative PCR (qPCR) kit (Kapa Biosystems, Wilmington, MA, USA) on Eco™ Real-Time PCR system (Illumina Inc, San Diego, CA, USA). Briefly, the reaction consists of 0.2 μM each gene-specific primer and 100 ng of cDNA as the template. Each pooled sample was run in reaction mixture with a final volume of 20 μl in triplicate, following the manufacturer's instructions. Relative gene expression of *skn-1* was determined using the Pfaffl method[18] and normalized to reference gene *cdc-42*. The specific primer sequences were as follows: *skn-1*: forward (5′ → 3′): AGTGTCGGCGTTCCAGATTT, reverse (5′ → 3′): GTCGACGAATCTTGCGAATC; *cdc-42*: forward (5′ → 3′): CTGCTGGACAGGAAGATTACG, reverse (5′ → 3′): CTCGGACATTCTCGAATGAAG. Data were presented as means ± SD, and differences between groups were assessed by using one-way ANOVA in GraphPad Prism 5.

Results

Preparation of curcumin analogues C1, C2, C3 and C4

Three analogues were prepared by acid-catalysed aldol condensation of vanillin with various ketones (acetone, cyclopentanone and cyclohexanone; Figure 1) in moderate yields and high purity. The NMR, mass spectra and HPLC chromatograms of the compounds are given in the Appendix S1. They were considered analogues with a shortened linker connecting the two aromatic rings. The substituents on the aromatic rings (the 4-hydroxyl and 3-methoxyl) were maintained in these compounds with the focus being variation to the linker. The linker was acyclic as in **C1** or cyclic (**C2** and **C3**) imparting the compounds with variation in size and lipophilicity. The commercially obtained **C4** was considered a 'hemi-analogue' of curcumin as it constituted a symmetric half of the curcumin molecule.

In vitro passive BBB permeability of curcumin and analogues

We first evaluated the passive permeability of the compounds in the PAMPA-BBB model. PAMPA evaluates the ability of a compound to permeate the BBB by measuring the rate (P_e) at which a compound transverses a brain lipid-coated filter membrane that mimics the BBB from a donor compartment to an acceptor compartment filled

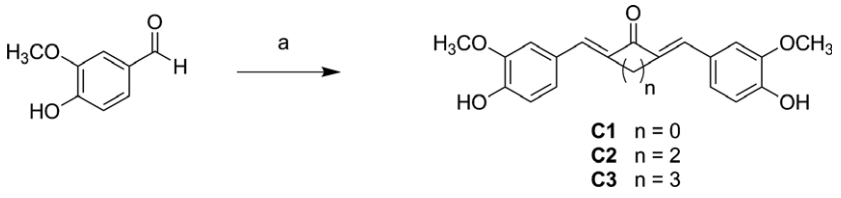


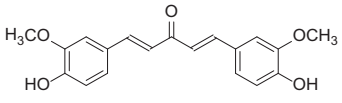
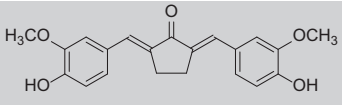
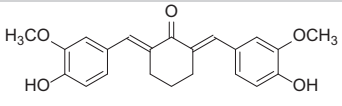
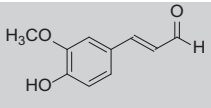
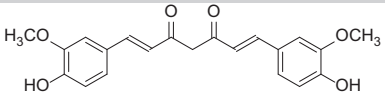
Figure 1 Synthesis of curcumin analogues. Reagent and condition: (a) Acetone, cyclopentanone, or cyclohexanone (2 eq), HCl, acetic acid, rt, 48 h.

with phosphate buffer (pH 7.4). Donepezil (a high permeability compound, $P_e > 4$ cm/s) and caffeine (a low permeability compound, $P_e < 2$ cm/s) were used to validate the assay by comparing permeability rates obtained in the assay with literature values (Table 1). Curcumin showed a low P_e while the four analogues interestingly demonstrated significantly higher P_e values. **C4** with the smallest molecular size (and the lowest lipophilicity) showed the highest P_e.

Protective effect of curcumin analogues in Aβ-induced paralysis in GMC101 *Caenorhabditis elegans*

We next evaluated the ability of curcumin and the analogues to delay Aβ-induced paralysis in the transgenic *C. elegans* GMC101 that expresses the $A\beta_{1-42}$ peptide in its bodywall cells. Initially, the nematodes were treated with each compound at the highest possible concentration (with solubility and toxicity being the limiting factor) of 100 μM. The nematodes were exposed to the compounds from the egg stage and throughout the assay (Figure 2a). The compounds-treated nematodes showed normal development to adulthood; thus, the compounds were deemed non-toxic. Upon temperature upshift from 16 to 25 °C, Aβ was expressed in the worms and scoring of nematode survival was performed post 24 h when worm paralysis became pronounced. Curcumin, **C1** and **C4** at 100 μM significantly delayed paralysis of the worms (Figure 2b). The median survival times of nematodes treated with these compounds were also increased significantly ($P < 0.001$, Table 2). In

Table 1 Blood–brain barrier permeability of curcumin analogues

Compound	Structure	P_e ($\times 10^{-6}$ cm/s)	Lipophilicity[c]
C1		8.2 ± 1.3	2.64
C2		6.1 ± 2.1	3.13
C3		4.9 ± 1.6	3.69
C4		18.0 ± 4.7	1.23
Curcumin		0.60 ± 0.67	2.25

Controls	Experimental P_e ($\times 10^{-6}$ cm/s)	Literature P_e ($\times 10^{-6}$ cm/s)
Caffeine	1.90 ± 0.62	1.3[a]
Donepezil	21.8 ± 4.1	12.0[b]

[a]Taken from Ref. [17]. [b]Taken from Ref. [19]. [c]Calculated lipophilicity (cLogP) using ChemBioDraw 14.0 (CambridgeSoft Corp., Cambridge, MA, USA).

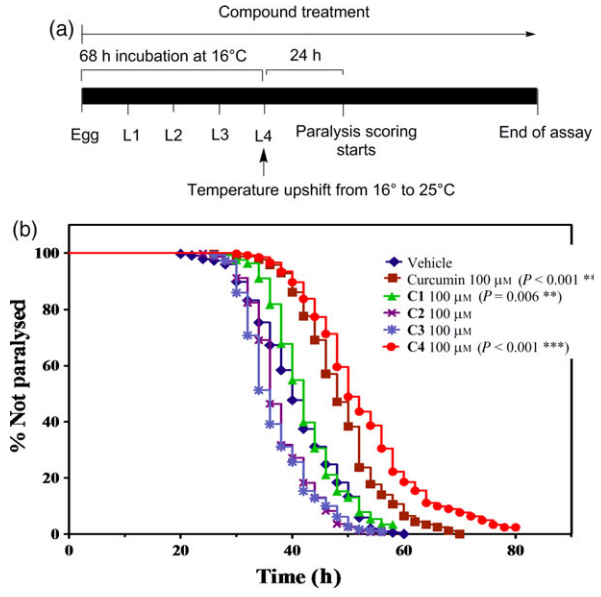


Figure 2 (a) Experimental protocol for the GMC101 paralysis assay. (b) Effects of curcumin and analogues at 100 µM in the Aβ-induced paralysis assay. Time refers to hours after temperature up-shift. Aβ expression was induced by upshifting temperature from 16 to 25 °C. Worms began to show paralysis 24 h post-temperature upshift and were scored at 2 h intervals. An asterisk (*) indicates significant differences between vehicle and treatment (**$P < 0.01$; ***$P < 0.001$, paired log rank survival test). Data are expressed as percentage of non-paralysed worms derived from at least three independent experiments of >100 worms in each experiment. The plots shown are representatives of three experiments.

Table 2 Median survival times of GMC101 nematodes treated with compounds at 100 µM in the Aβ-induced paralysis assay

	Survival time in hours			
Treatment	Estimate[a]	95% CI	χ^2 (df)	*P* value
Vehicle	40	39.4; 40.6	Overall[d]: 2481.26 (7)	<0.001
Curcumin	48	47.2; 48.8[b]		
C1	42	41.5; 42.5[c]		
C2	36	35.6; 36.4		
C3	36	35.5; 36.5		
C4	50	49.1; 50.9[b]		

Median survival time is the period at which 50% worms survive. Comparisons were done between vehicle and treatment. [a]Median survival time (50% non-paralysed worms). [b]$P < 0.001$ compared to vehicle. [c]$P < 0.01$ compared to vehicle. [d]Overall log-rank test.

contrast, cyclic linker-bearing analogues **C2** and **C3** failed to show protective effect at 100 µM. At lower concentrations (1 and 10 µM), curcumin and **C4** retained their protective effect in a concentration-dependent manner while **C1** did not show protection (Figure 3). Thus, curcumin and **C4** were deemed as the more efficacious compounds.

Aβ aggregation inhibition by curcumin and C4

Efficacious compounds curcumin and **C4** were evaluated for their ability to inhibit Aβ aggregation when Aβ monomers were incubated in the presence of the compounds *in vitro*. Aβ aggregation was monitored for 24 h using the fluorescent Thioflavin T as indicator of aggregation levels of the peptide. At the 24th h post-incubation, curcumin at 10 µM showed a slight inhibition (14.8%) but this reduction in Aβ aggregation was not significant (Figure 4a). **C4** also did not inhibit Aβ aggregation significantly.

Upregulation of skn-1 by curcumin and C4

Experiments using GMC101 were repeated using the same protocol (Figure 1b) but without scoring for paralysed worms. The RNA of the nematodes under curcumin and **C4** treatment (10 and 100 µM) was extracted and probed for *skn-1* mRNA levels at the 15th h post-temperature upshift when Aβ was expressed and the number of nematodes experiencing paralysis was pronounced. RT-PCR analysis showed *skn-1* levels were significantly increased in worms treated with curcumin at 10 µM ($P < 0.01$) and **C4** at 10 µM ($P < 0.05$) and 100 µM ($P < 0.001$) compared to the vehicle control (Figure 4b).

Discussion

In vitro, curcumin has consistently shown protection against Aβ-induced toxicity in various cell lines as well as the ability to rescue long-term potentiation in rat hippocampus impaired by Aβ.[6,8,20,21] Its *in vivo* performance however has been hampered by inherently suboptimal physicochemical properties such as low chemical and metabolic stability, and inconsistent BBB permeability.[22] In this study, we explored the possibility of improving curcumin BBB permeability via curcumin analogues with shortened linkers while retaining curcumin protective activity. Such dibenzylidene ketone analogues as **C1** and **C2** have been shown to retain the Aβ binding property of curcumin[23,24] but their ability to protect against Aβ toxicity in a physiological context as in the whole organism *C. elegans* has hitherto not been tested.

Using the PAMPA model, we demonstrated for the first time that the curcumin analogues **C1**, **C2**, **C3** and **C4** showed improved passive BBB permeability compared to curcumin. It would seem that the higher lipophilicities (vs curcumin) of **C1**, **C2** and **C3** may have contributed to their improved BBB permeability with the exception of the smaller hemi-analogue **C4** which possessed the lowest lipophilicity (cLogP 1.23) showing the highest permeability coefficient of the four compounds. This indicates that

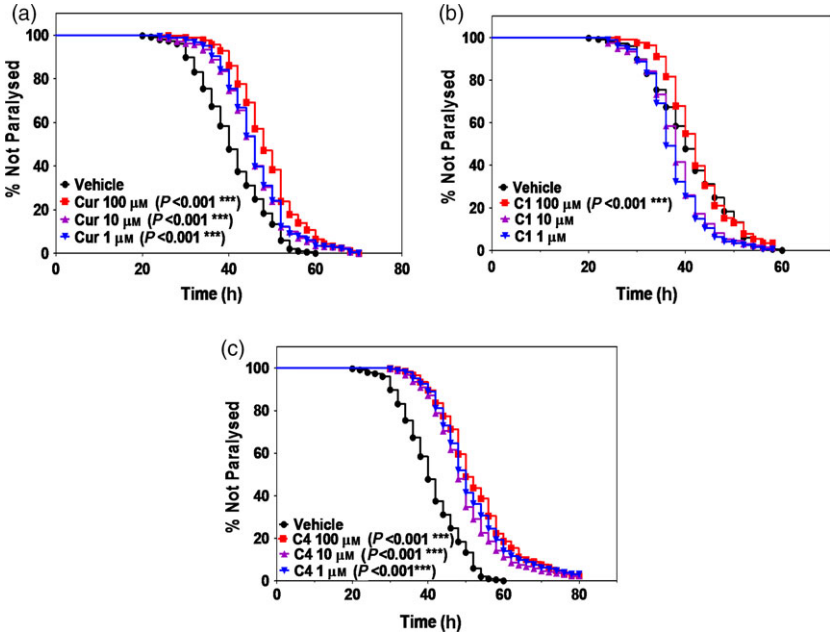


Figure 3 Effects of curcumin (a), **C1** (b) and **C4** (c) at 1–100 μM on the Aβ-induced paralysis in GMC101. An asterisk (*) indicates significant differences between vehicle and treatment (***$P < 0.001$, paired log rank survival test). Data are expressed as percentage of non-paralysed worms from at least three independent assays of >100 worms in each experiment. The plots shown are representative of three experiments.

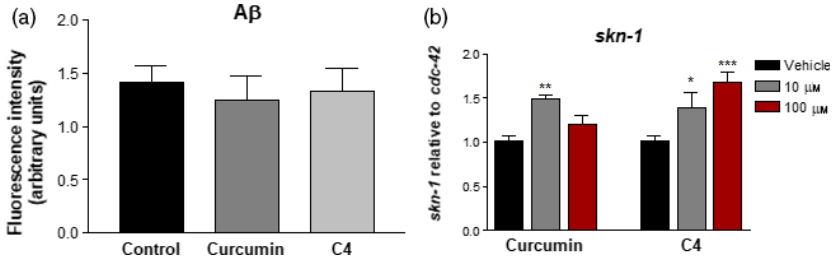


Figure 4 Mechanistic evaluation of curcumin and **C4** protection against Aβ toxicity. (a) $A\beta_{1-40}$ aggregation inhibitory effect was evaluated by ThT fluorescence assay. $A\beta_{1-40}$ (10 μM) was incubated at 37 °C for 24 h in the presence and absence of curcumin or **C4** (10 μM). The fluorescence intensities were corrected by subtracting the background reading. Data are presented as means ± SD from three independent experiments with three replicates for each experiment ($n = 3$). (b) RT-qPCR probing of *skn-1* mRNA was conducted. GMC101 nematodes were cultured on NGM containing vehicle or test compounds (10 and 100 μM) at 16 °C for 68 h. Aβ expression was then induced by upshifting temperature from 16 to 25 °C. At 15 h post-temperature up-shift, total RNA was extracted from the worms (~8000) and the gene expression was analysed using qPCR. *skn-1* gene expression was normalized with reference gene *cdc-42*. Data are presented as means ± SD ($n = 3$) from three independent experiments. Statistical test was performed by one-way ANOVA with post hoc Tukey's. An asterisk (*) indicates significant differences between vehicle and treatment (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

molecular size in the present instance played a larger role towards BBB permeability compared to lipophilicity. A caveat, however, in the present study is that these curcumin analogues merely showed better passive permeability which does not take into account active transport protein-mediated permeation by which a cell-based BBB model would offer.[25,26] In light of recent findings of interactions of curcumin derivatives with efflux proteins,[27,28] the possibility that the BBB permeation of these compounds may also involve active transporters requires further confirmation with a cell-based BBB assay.

Having found these analogues to have improved BBB permeability, we evaluated their ability to confer protection against Aβ in the GMC101 nematode that manifests paralysis as a result of Aβ toxicity. At the highest screening concentration (100 μM), curcumin showed protective effect in delaying the onset of Aβ-induced paralysis and its effect was dose-dependent thus confirming on previous tissue/cell-based studies of curcumin protective activity. The ability of curcumin to interact with Aβ and inhibit its aggregation has been linked to its ability to attenuate Aβ toxicity. However, analogues **C2** and **C3** which were reported as

strong Aβ binders comparable to curcumin[23,24] failed to show any protection in delaying paralysis onset of the worms. Similarly, **C1** which showed protection at 100 μM, was inefficacious at lower concentrations. This draws parallel to a study by Park *et al.*[8] which showed that the 7-carbon linker of curcumin was necessary for its protection against Aβ-induced death in PC-12 cells and that shorter linkers (as that of **C1**, **C2** and **C3**) would result in inactivity. Reinke and Gestwicki[29] proposed that an Aβ-binding compound requires (i) a linker of optimal length and flexibility and (ii) two aromatic rings attached to both ends of the linker. In clear contrast, **C4** (being half the structure of curcumin) lacked such structural requirements but showed significant and dose-responsive paralysis delay and a better median survival time than curcumin. Taken together, this suggests that the protective effect shown by the curcumin and **C4** may not be attributed to Aβ binding mechanism or necessitating the full structural motif of curcumin.

To confirm on this conjecture, curcumin and **C4** were evaluated for their ability to inhibit Aβ aggregation *in vitro*. Curcumin did not lower Aβ aggregation significantly despite its reputed ability to bind Aβ possibly because the concentration (10 μM) used was insufficient for this property to be apparent. The hemi-analogue **C4** failed to inhibit Aβ aggregation as expected for its lack of the structural requirements of an Aβ-binding compound. However, working on the premise of curcumin and **C4** having a Michael acceptor motif within their structures and that Michael acceptor compounds are potent Nrf2 activators, the *skn-1* (the nematode ortholog of Nrf2) gene was measured on the compound-treated worms. We showed that indeed *skn-1* mRNA levels of worms treated with curcumin and **C4** were elevated significantly. Thus, this indicates that the ability of the compounds to modulate oxidative stress response mechanism via SKN-1 may likely be their mode of protection against Aβ toxicity. A means of Aβ toxicity is the ability of the peptide to induce oxidative stress and modify cellular redox state.[4] For example, the Met-35 residue of $A\beta_{1-42}$ peptides located in the lipid bilayer has been shown to undergo one-electron oxidation to generate sulfuranyl free radical leading to oxidative modification of cellular proteins.[30] Induction of SKN-1 by **C4** or curcumin would thus lead to upregulation of downstream effectors such as glutathione-S-transferases to remove the oxidative stressor caused by the presence of Aβ. Similar studies but without the presence of Aβ showed the ability of plumbagin[31] and allyl isothiocyanate[32] (both electrophilic Nrf2 inducers) to prolong the lifespan and increase oxidative stress resistance of wild type *C. elegans* via SKN-1 activation. The notion that curcumin and **C4** alleviate Aβ toxicity through oxidative stress modulation in the present finding requires further investigation into the possible oxidative modification(s) within the nematode when exposed to Aβ and how these compounds may be able to modify these changes.

In conclusion, a simplified curcumin analogue **C4** has been identified in the present study with improved passive BBB permeability and the ability to protect against Aβ toxicity. **C4** represents a potential lead for the further optimization of similar curcumin-like compounds with therapeutic potentials and promising BBB targeting property in central neurodegenerative diseases such as Alzheimer's disease.

Declarations

Conflict of interest

The Authors declare that they have no conflict of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. ^{1}H NMR, ^{13}C NMR, and mass spectra and HPLC purities of **C1**, **C2**, and **C3**.