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Resveratrol-mediated cleavage of amyloid β_{1-42} peptide; potential relevance to Alzheimer's disease

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Abstract

Aggregation of amyloid β_{1-42} ($A\beta_{1-42}$) peptide within the brain is considered one of the main causes of the neuropathological changes associated with Alzheimer's disease (AD). Resveratrol is a well-known antioxidant but has also been reported to bind to $A\beta_{1-42}$ peptide, thereby reducing aggregation. However, little is known of the precise mechanism by which resveratrol reduces $A\beta_{1-42}$ peptide aggregation. Using the thioflavin-T (ThT) assay, the ability of resveratrol to reduce the extent of $A\beta_{1-42}$ peptide aggregation was confirmed. The findings of the present study demonstrate that interaction of resveratrol with $A\beta_{1-42}$ peptide resulted in the cleavage of $A\beta_{1-42}$ peptide into smaller fragments, as detected by matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry. Atomic force microscopy (AFM) analyses revealed $A\beta_{1-42}$ peptide, under control conditions, aggregated into oligomers, protofibrils and fibrils, whereas there was a distinct lack of these structures when $A\beta_{1-42}$ peptide was incubated with resveratrol. Following 10 days' incubation of $A\beta_{1-42}$ peptide with resveratrol particles with a mean z-height of 1.940 nm (range 0.675 - 3.275 nm) were observed, which are characteristic of shorter peptide species. In cell-based studies, resveratrol significantly reduced the cytotoxicity of $A\beta_{1-42}$ peptide towards SH-SY5Y human neuroblastoma cells, suggesting a protective effect of the polyphenol. We therefore propose a novel mechanism by which resveratrol disrupts $A\beta_{1-42}$ aggregation by mediating fragmentation of $A\beta_{1-42}$ into smaller peptides, which have no propensity to aggregate further.

Keywords: Resveratrol; Aβ₁₋₄₂ peptide; cleavage; MALDI-TOF, Alzheimer's disease

1. Introduction

Alzheimer's disease (AD), the most common cause of dementia, is a terminal progressive neurodegenerative disease, which destroys brain neurones causing loss of memory and a severe decline in cognitive function (Regitz *et al.*, 2015). To date, there is no effective treatment for AD, and strategies are aimed at managing, rather than curing or preventing, the condition (Bhushan *et al.*, 2018). The aetiology of AD is not completely understood: aspects of the pathology of the disease have been identified and include aggregation of Aβ₁₋₄₂ peptide into fibrils, and subsequent deposition of Aβ₁₋₄₂ peptide-containing plaques, which is

recognised as the major cause of AD (Li *et al.*, 2011), together with the formation of tau neurofibrillary tangles (Fiala and Veerhuis, 2010).

Resveratrol is a stilbene compound first identified in the roots of white hellebore, *Veratrum grandiflorum* (Aggarwal *et al.*, 2004). Resveratrol has received much interest due to its benefits to human health (Baur and Sinclair, 2006, Sales and Resurreccion, 2014), which include anti-cancer, anti-inflammatory, anti-aging and cardio-protective properties. Resveratrol has also been reported to possess anti-oxidant properties, chelating properties and scavenging capability towards reactive oxygen species (Belguendouz *et al.*, 1997).

A number of studies have reported resveratrol inhibits aggregation of A β ₁₋₄₂ monomers *in vitro*. However, the precise and definitive mechanism(s) by which the polyphenol inhibits aggregation is unknown. Feng *et al.*, (2009) suggested that inhibition of A β ₁₋₄₂ monomers into fibrils was due to direct binding of the polyphenol to A β ₁₋₄₂ (Feng *et al.*, 2009), consistent with atomic force microscopy studies reporting resveratrol was able to bind to the N-terminus of A β ₁₋₄₂ monomers (Fu *et al.*, 2014). In discrete molecular dynamics simulations, Nedumpully-Govindan *et al.*, (2016) reported resveratrol interacted with the islet amyloid polypeptide amylin *via* a combination of hydrogen bonding, π - π stacking and hydrophobic interactions (Nedumpully-Govindan *et al.*, 2016) whilst, in studies with the A β ₁₇₋₃₆ peptide, Wang *et al.*, (2017) reported resveratrol bound only to the hydrophobic residues near the peptide termini (Wang *et al.*, 2017).

In other studies employing surface plasmon resonance and NMR analyses, Ge *et al.*, (2012) confirmed direct binding between resveratrol and A β monomers and fibrils (Ge *et al.*, 2012) and also reported that resveratrol stained senile plaques in brain slices from Alzheimer's disease patients.

Studies also documented the ability of resveratrol derivatives to bind amyloid beta, and subsequently disrupt aggregation. Xu *et al.*, (2017) reported deferiprone-resveratrol hybrids inhibited aggregation of A β ₁₋₄₂ (Xu *et al.*, 2017), Puksasook *et al.*, (2017) reported prenylated resveratrol derivatives inhibited aggregation of A β ₁₋₄₂ (Puksasook *et al.*, 2017), and Lan *et al.*, (2018) reported a resveratrol-indazole hybrid inhibited aggregation of A β ₁₋₄₂ (Lan *et al.*, 2018).

Whilst multiple studies report the ability of resveratrol, and resveratrol derivatives, to bind to amyloid beta monomers and fibrils, and to inhibit amyloid beta aggregation, the precise mechanism by which resveratrol actually disrupts aggregation is not well understood. In this study we investigated the ability of resveratrol to inhibit aggregation of the peptide by disruption of A β ₁₋₄₂ structure and report a novel mechanism of resveratrol-mediated fragmentation of A β ₁₋₄₂ peptide.

2. Materials and Methods

2.1. Materials

Ultra pure A β ₁₋₄₀ peptide recombinant type was purchased from rPeptide, UK. A β ₁₋₄₂ was purchased from the American Peptide Company, CA, USA. 2,2'-Azobis (2-methyl propionamidine) dihydrochloride (AAPH), Thioflavin T (ThT), resveratrol, trifluoroacetic acid (TFA), Dulbecco's Phosphate Buffered Saline (PBS), polycarbonate filters (Whatman[®] Nuclepore[™] Track-Etched membranes, 25 mm diameter with pore sizes of 0.05 μ m, 0.1 μ m, 0.2 μ m) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, UK. Acetonitrile (ACN) (HPLC grade) and Milli-Q water (HPLC grade) were purchased from Fischer Scientific, UK. Hexafluoroisopropanol (HFIP) was from SynQuest Laboratories, Inc., UK. α -Cyano-4-hydroxycinnamic acid (CHCA) was purchased from Fluka, UK. Syringe filters (diameter 13 mm PTFE and pore size 0.22 μ m NSTR) and centrifuge tube filters (Ultracel-30 with 30 kDa pore size) were purchased from Fisher. The SH-SY5Y cell line was obtained from

ATCC and cell culture grade DMSO, PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, 0.25 % trypsin, 0.53 mM EDTA in HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ and thiazolyl blue tetrazolium bromide (MTT) (suitable for cell culture ≥ 97.5 % TLC) were obtained from Sigma-Aldrich. Eagle's Minimum Essential Medium (1X), Ham's F-12 medium, foetal bovine serum (FBS), 1 % L-glutamine, 1 % penicillin/streptomycin, 1 % non-essential amino acids and phenol red free OPTI-MEM medium (1X) were obtained from Invitrogen (UK). 96-well polystyrene plates for cell culture were from Thermo Scientific (UK). All other reagents were of analytical grade and commercially available.

2.2. Preparation of monomeric $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ peptides

Monomeric $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ peptides were produced as described previously (Attanasio *et al.*, 2013, Orre *et al.*, 2013). $\text{A}\beta$ peptide was dissolved in TFA and filtered using a 13 mm PTFE syringe filter (0.22 μm pore size). Subsequently, TFA was evaporated using dry nitrogen gas. $\text{A}\beta$ peptide was then treated with 2 ml of 1 % (v/v) HFIP, the sample mixed at room temperature for 15 min and then evaporated to dryness. $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ peptide were freeze-dried under vacuum for 2 h at 20 mbar and -50 $^{\circ}\text{C}$ (Christ[®], Osterode, Germany) and stored under desiccated conditions at -20 $^{\circ}\text{C}$ until use.

2.3. Aggregation of $\text{A}\beta_{1-42}$ peptide

Aggregation of $\text{A}\beta_{1-42}$ peptide was monitored using the ThT assay as described previously (Reyes Barcelo *et al.*, 2009, Bohrmann *et al.*, 2012). Lyophilised $\text{A}\beta_{1-42}$ peptide was dissolved to a concentration of 20 mM in DMSO and then diluted to 200 μM in Milli-Q water. ThT, 20 μM , was prepared in 50 mM glycine in Milli-Q water, pH 8.5. Resveratrol, 20 mM, was prepared in DMSO:PBS (4.5:5.5) for studies with $\text{A}\beta_{1-42}$ peptide:resveratrol at 1:100. In studies with $\text{A}\beta_{1-42}$ peptide:resveratrol at 1:0.01, 1:0.1, 1:1 and 1:10, the 20 mM stock solution was diluted in DMSO:PBS (4.5:5.5) such that 5 μl provided the required resveratrol

concentration. A β ₁₋₄₂ peptide solution, 5 μ l, was added to 5 μ l of resveratrol solution and then immediately transferred into individual wells of black 96-well plates containing 90 μ l ThT solution. In control studies, resveratrol was incubated with ThT in the absence A β ₁₋₄₂ peptide, and with A β ₁₋₄₂ peptide in the absence of ThT. Plates were sealed using adhesive tape and incubated at 37 °C for 10 days. Aggregation was monitored by measuring ThT fluorescence using a Tecan Safire microplate reader (MTX lab systems, USA), with a 3 s shake prior to each fluorescence reading, at excitation and emission wavelengths of 440 nm and 495 nm respectively.

2.4. High performance liquid chromatography

High performance liquid chromatography (HPLC) was carried out to investigate the effect of resveratrol on A β ₁₋₄₂ peptide aggregation over time. A β ₁₋₄₂ peptide (100 μ M) was incubated at 37 °C with resveratrol at an A β ₁₋₄₂ peptide:resveratrol molar ratio of 1:100, and samples were removed at 0, 16, 20 and 22 h for detection of A β ₁₋₄₂ peptide. Separation was performed as described by the American Peptide Company using a PLRP-S column and a HPLC LC 200 system (PerkinElmer, UK) fitted with a UV-detector. Mobile phase A was 0.05 M pH 6.8 Tris in Milli-Q water, while mobile phase B was 0.05 M Tris pH 6.8 in 80 % (v/v) ACN. The HPLC column was equilibrated in 80 % mobile phase A and 20 % mobile phase B. Samples were loaded and gradient elution was performed with a change in mobile phase from 20 % mobile phase B to 100% mobile phase B over 20 min. A β ₁₋₄₂ peptide was detected at 215 nm.

2.5. Mass laser desorption ionization time-of-flight (MALDI-TOF) spectrometry

The effect of resveratrol on the aggregation of A β ₁₋₄₂ peptide over time was analysed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry, as

peptide aggregation decreases the amount of detectable free monomer (McGowan *et al.*, 2005, Zovo *et al.*, 2010).

A β ₁₋₄₂ peptide (100 μ M) and resveratrol (10 mM) were incubated in PBS, pH 7.4, at 37 °C for 10 days (Stine *et al.*, 1996). Matrix was prepared as a saturated solution of α -cyano-4-hydroxycinnamic acid (CHCA). A saturated solution of CHCA was prepared by dissolving 10 mg in 1 ml of 70% ACN:30% of 0.1% TFA in Milli-Q water as described by Zoho and colleagues (Zovo *et al.*, 2010).

A β ₁₋₄₂ peptide samples were mixed with matrix at a ratio of 1:10, spotted onto metal plates, air-dried for 20 min and immediately analysed using a MALDI-TOF spectrometer (Shimadzu Biotech Axima Confidence, Japan) equipped with a 337 nm nitrogen laser operating at 3.0 Hz in linear mode geometry. The laser intensity was in the range of 5000 - 6000 W.cm⁻² and the consecutive laser shots were between 1000 and 4000. The delay in the pulsed ion extraction time was 700 ns with ions accelerated at 20 kV. The mass/charge (m/z) data were collected as three repetitions of 100 consecutive laser irradiation clusters, producing three spectra per sample per well. The systematic artefact bias was avoided by randomization of samples for data acquisition.

2.6. Atomic force microscopy

Atomic force microscopy (AFM) was used to study A β ₁₋₄₂ peptide aggregation as described previously (Kowalewski and Holtzman, 1999, Nichols *et al.*, 2005, Cohen *et al.*, 2006). A β ₁₋₄₂ peptide (100 μ M) and resveratrol (10 mM) were incubated in PBS, pH 7.4, at 37 °C for 10 days (Stine *et al.*, 1996). Freshly cleaved mica was mounted on a metal disc and 20 μ l samples were deposited on the mica for 20 min (Ostapchenko *et al.*, 2012). Samples were washed five times with filter sterilised Milli-Q water, air dried at room temperature and analysed using a Picoforce Nanoscope V Multimode atomic force microscope (Bruker) using

tapping mode with scan rates between 0.5 and 1.5 Hz and a resonant frequency range of 270 - 460 kHz. Image capturing was conducted after specifying representative areas. The resolution of the images was 512 x 512 points and the height and amplitude error mode images (5 μm x 5 μm) were used after flattening using Nanoscope software v7.2.

2.7. SH-SY5Y cell culture

SH-SY5Y neuroblastoma cells were maintained as described by (Ross *et al.*, 1983). SH-SY5Y cells were cultured in growth medium composed of a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F12 Medium, containing 10 % foetal bovine serum (FBS) at 37 °C and in an atmosphere of 5 % carbon dioxide (CO₂).

2.8. Measurement of cell viability using the MTT assay

The effects of A β ₁₋₄₂ peptide and resveratrol on the viability of SH-SY5Y cells were determined as previously described (Abe and Matsuki, 2000). Cells were plated at a density of 1.5 x 10⁴ cells/well in 96-well plates and left to adhere overnight. Cells were incubated with either 10 μM A β ₁₋₄₂ peptide, 1 μM resveratrol or 10 μM A β ₁₋₄₂ peptide and 1 μM resveratrol combined in 100 μl growth medium for 48 h at 37 °C, 5 % CO₂. Twenty five microliters of MTT (5 mg.ml⁻¹) were then added to each well and the cells incubated for a further 4.5 h at 37 °C in 5 % CO₂. Growth medium was then removed, monolayers washed three times with PBS and 100 μl of 50 % *N,N*-dimethyl-formamide and 20 % SDS added to each well. Plates were incubated overnight at 37 °C and the absorbance measured at a test wavelength of 570 nm and a reference wavelength of 655 nm using a Polar Star Omega plate reader.

2.9. Statistical analysis

Data are expressed as mean $\pm$ standard deviation of at least three independent experiments, each containing at five replicates. Multiple comparisons were made using

repeated measures ANOVA and Student's t-test was carried out using IBM SPSS software v20 and Design-Expert[®] software v9. Differences were considered to be statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Resveratrol reduces aggregation of A β ₁₋₄₂ peptide

Studies were carried out to investigate the ability of resveratrol to inhibit aggregation of monomeric A β ₁₋₄₂ peptide at A β ₁₋₄₂ peptide:resveratrol molar ratios of 1:0.01, 1:0.1, 1:1, 1:10 and 1:100. These studies revealed significant reductions in ThT fluorescence following resveratrol treatment, with reductions reflecting a concentration-dependent anti-aggregation effect of the polyphenol (Fig 1). It is notable that, at an A β ₁₋₄₂ peptide:resveratrol molar ratio of 1:100, fluorescence emission was almost completely abolished with resveratrol treatment, suggesting that the new non-aggregated structures formed were no longer able to bind ThT, unlike the starting monomeric A β ₁₋₄₂ peptide. It should be noted however, that there is a slight possibility of interaction between resveratrol and thioflavin, which is a potential limitation in this study.

Figure 1. Effect of resveratrol on the aggregation of Aβ₁₋₄₂ peptide. Aβ₁₋₄₂ peptide was prepared by solubilising in TFA, evaporating to dryness using dry nitrogen gas and solubilising in 1 % (v/v) HFIP. The solvent was then evaporated and the Aβ₁₋₄₂ peptide stored at -20 °C. The thioflavin T assay was carried out in black 96-well plates and fluorescence measured using excitation and emission wavelengths of 440 nm and 495 nm respectively. Data are represented as mean ± standard deviation of three independent experiments each containing at five replicates and were analysed by repeated measures ANOVA to compare fluorescence at each individual time point with fluorescence at day 0. * p < 0.05, ** p < 0.01 and *** p < 0.001 and NS not significant.

In control studies, where resveratrol was incubated with ThT over 10 days in the absence of Aβ₁₋₄₂ peptide, fluorescence levels were low (due to the absence of aggregated Aβ₁₋₄₂ peptide) and there was no significant change in the level of fluorescence over time (data not shown). Furthermore, when monomeric Aβ₁₋₄₂ peptide was incubated with resveratrol over 10 days, fluorescence levels were low (due to the absence of ThT) and there was no significant change in the level of fluorescence over time (data not shown).

The results of the current study are consistent with those of Guo *et al.*, (2010) who demonstrated that resveratrol inhibited A β ₁₋₄₂ aggregation (Guo *et al.*, 2010), with an effective dose of 40.8 nM reducing aggregation by 50% and those of Richard *et al.*, (2011) who reported resveratrol inhibited both A β ₁₋₄₂ and A β ₁₋₄₀ fibril formation (Richard *et al.*, 2011). The findings are also consistent with the study of Lu *et al.*, (2012), which reported that resveratrol inhibited A β ₁₋₄₂ aggregation by 69.73 % (Lu *et al.*, 2012) and the study of Kantham *et al.*, (2017) which demonstrated resveratrol significantly inhibited formation of A β ₁₋₄₂ oligomers and fibrils and that the polyphenol reduced A β ₁₋₄₂-induced paralysis in a *C. elegans* model (Kantham *et al.*, 2017). Similar findings have also been observed *in vivo*, where it was reported that resveratrol markedly reduced amyloid plaque formation in 45 day old Tg19959 mice (Walle, 2011).

3.2. Resveratrol causes fragmentation of A β ₁₋₄₂ peptide

The temporal effect of resveratrol treatment on monomeric A β ₁₋₄₂ peptide was analysed using high-performance liquid chromatography (HPLC). In control studies without resveratrol, the retention time of intact monomeric A β ₁₋₄₂ peptide was 8.47 min and no peaks corresponding to fragments of A β ₁₋₄₂ peptide were observed (Fig. 2). Over time, there was a decrease in the peak height corresponding to A β ₁₋₄₂ peptide monomer (Fig. 2 and Supplementary Fig 1), reflecting a decrease in the amount of monomeric A β ₁₋₄₂ peptide. This is explained by the fact that the peptide may undergo auto-hydrolysis or aggregation (Hosia *et al.*, 2005, Grasso *et al.*, 2009). Auto-hydrolysis can produce fragments which are smaller and more soluble, hence eluting with lower retention times, as apparent in the HPLC profile in Fig 2. A β ₁₋₄₂ peptide aggregates were not detected by HPLC, as the higher order aggregates are not amenable to

separation by HPLC due to low solubility, consistent with the finding previously reported (Zhang *et al.*, 2013).

When monomeric A β ₁₋₄₂ peptide was incubated with resveratrol, the retention time of A β ₁₋₄₂ peptide at the start of the incubation was 8.47 min (Fig. 2), consistent with the control condition. In this condition there was also a time-dependent decrease in the peak height corresponding to A β ₁₋₄₂ peptide monomer, but this decrease was accompanied by a time-dependent left shift in the retention time (Fig. 2) suggesting that additional degradation is occurring, mediated by resveratrol.

It is noticeable that the rate of A β ₁₋₄₂ monomer disappearance in the presence of resveratrol is faster than in the control, lacking resveratrol. In control studies, intact monomeric A β ₁₋₄₂ peptide was still detectable at 22 h (Fig. 2), however when A β ₁₋₄₂ peptide was incubated with resveratrol, no intact peptide was detected at 22 h (Fig. 2). To exclude the possibility that the major peak with a retention time of 8.47 min was due to resveratrol, analyses of the absorption characteristics of both A β ₁₋₄₂ peptide and resveratrol were carried out. Resveratrol possessed significant absorbance between 250 nm and 400 nm, but minimal absorbance at 215 nm, indicating that resveratrol was unlikely to interfere with the HPLC analysis of A β ₁₋₄₂ peptide (data not shown).

Figure 2. Superimposed HPLC analyses of (A) Aβ₁₋₄₂ peptide, 100 μM, and (B) Aβ₁₋₄₂ peptide, 100 μM, incubated with resveratrol at a 1:100 molar ratio, at 0 h and 22 h.

The findings of the present study are consistent with those of Naslund *et al.*, (1996), who confirmed loss of residues from the N-terminal of Aβ₁₋₄₂ peptide resulting in a more lipophilic peptide, with an increased retention time, while loss of residues from the C-terminal of Aβ₁₋₄₂ peptide gave peaks with a decreased retention time (Naslund *et al.*, 1996). In our

studies, when A β ₁₋₄₂ peptide was incubated with resveratrol for 20 h, peaks were observed at retention times between 1 – 4 min, indicating more hydrophilic (i.e. polar) peptide fragments.

The A β peptide exists in several forms, and many studies have been carried out using the A β ₁₋₄₀ peptide rather than the A β ₁₋₄₂ form. Therefore, we initially investigated the ability of resveratrol to cleave monomeric A β ₁₋₄₀ peptide. The MALDI-TOF analysis of A β ₁₋₄₀ in control conditions (without resveratrol) at 24 h showed a peak corresponding to a m/z ratio of 4331, indicating intact monomeric A β ₁₋₄₀ peptide (Fig. 3 A). However, following 24 h incubation with resveratrol, peptide fragments with m/z ratio between 1032 - 1826 were observed, with no intact monomeric A β ₁₋₄₀ peptide detected (Fig. 3 B). The differences between the consecutive peaks from left to right (Fig. 3) are 128, 138, 137, 99, 129 and 163, corresponding to the residual mass of glutamine (Q), histidine (H), histidine (H), valine (V), glutamic acid (E) and tyrosine (Y), respectively, with the peak at m/z 1826 consistent with the DAEFRHDSGYEVHHQ fragment. The absence of peaks corresponding to the fragments right of the cleavage sites suggests that the peptides have been hydrolysed to small fragments in the matrix region of the MALDI spectrum, below m/z 1000. The absence of hydrolysis products on the first day of incubation of monomeric A β ₁₋₄₀ peptide with resveratrol suggests that there is a lag time before the start of hydrolysis, which might be driven by oxidation products of resveratrol. The possible sites of oxidation of resveratrol are the m-hydroquinone moiety, to produce m-semiquinone radicals and the phenol moiety which produces phenoxy radicals (Sako *et al.*, 2004). Resveratrol is proposed to modulate A β aggregation by the same mechanism as the oxidation product of dopamine, dopamine quinone (by forming a covalent adduct with a tyrosine residue of A β) (Kapurniotu, 2004) or as baicalein (Zhu *et al.*, 2004) by forming a Schiff base with a lysine residue of A β (Petkova *et al.*, 2002).

Figure 3. MALDI-TOF spectra of A β ₁₋₄₀ peptide in the absence and presence of resveratrol. A β ₁₋₄₀ peptide solution (100 μ M) was freshly prepared in 0.1 % DMSO in Milli-Q water. (A) A β ₁₋₄₀ peptide solution was mixed with matrix (70 % ACN: 0.1 % TFA) at an A β ₁₋₄₀ peptide:matrix ratio of 1:10 and samples spotted onto metal plates, air-dried for 20 min and immediately analysed using a MALDI-TOF spectrometer (Shimadzu Biotech Axima Confidence, Japan). (B) MALDI-TOF analysis of A β ₁₋₄₀ peptide after 10 days incubation with 10 mM resveratrol at 37 °C.

MALDI-TOF analysis of freshly prepared A β ₁₋₄₂ peptide solution (100 μ M), revealed a peak at m/z 4517.49 attributed to the monomeric form of A β ₁₋₄₂ peptide (Fig. 4 A). This is consistent with the literature, reporting detection of A β ₁₋₄₂ peptide monomers using MALDI-TOF (Hosia *et al.*, 2005, Grasso, 2011). The intact monomeric A β ₁₋₄₂ peptide was still detected after 20 h, however, it was not the major peak at this time point (Fig. 4 B). Following incubation of monomeric A β ₁₋₄₂ peptide with resveratrol for 20 h, there was a complete absence of intact A β ₁₋₄₂ peptide (no peak observed at m/z 4517.49) but a peak at m/z 2258.2 at half the original value, which is attributed to the diprotonated molecular ion (M + 2H)²⁺, presumably arising due to a decrease in the pH on the addition of resveratrol. New multiple protein fragments were also detected (Fig. 4 C) which were consistent with the formation of smaller fragments peptides.

Figure 4. MALDI-TOF spectra of $A\beta_{1-42}$ peptide in the absence and presence of resveratrol. $A\beta_{1-42}$ peptide solution (100 μ M) was freshly prepared in 0.1 % DMSO in Milli-Q water. (A) $A\beta_{1-42}$ peptide solution was mixed with matrix (70 % ACN: 0.1 % TFA) at an $A\beta_{1-42}$ peptide:matrix ratio of 1:10 and samples spotted onto metal plates, air-dried for 20 min and immediately analysed using a MALDI-TOF spectrometer (Shimadzu Biotech Axima Confidence, Japan). (B) MALDI-TOF analysis of $A\beta_{1-42}$ peptide after 20 h incubation at 37 $^{\circ}$ C. (C) MALDI-TOF analysis of $A\beta_{1-42}$ peptide after 20 h incubation with 10 mM resveratrol at 37 $^{\circ}$ C.

The intensity of the A β ₁₋₄₂ peptide (M+H)⁺ peak was dramatically decreased after 20 h of incubation at 37 °C, indicating a decrease in the monomeric form of A β ₁₋₄₂ peptide, due to aggregation and the likely formation of larger species (Fig. 4). Bartolini *et al.*, (2007) reported that fibrils, protofibrils and oligomers are not detectable by MALDI-TOF due to their inability to ionise (Bartolini *et al.*, 2007), in agreement with our findings.

The possible protein sequences for the fragment with m/z of 2317.11 and the fragment with m/z of 2414.41 derived from A β ₁₋₄₂ peptide were identified using ProteinCalculator (v3.4) (<http://protcalc.sourceforge.net/cgi-bin/protcalc>). The theoretical isotope patterns for the sequences were matched with the experimental isotope patterns from MALDI-TOF (Table 1). The fragment at m/z of 2317.10 does not seem to correspond to any specific peptide sequence and it may have been chemically modified by resveratrol. The fragment with m/z of 2414.40 revealed one matched sequence corresponding to fragment A β ₅₋₂₄, Table 1. This corresponds to cleavage between Phe4 - Arg5 of the A β ₁₋₄₂ peptide and between Val24 - Gly25. The latter is adjacent to the site in A β ₁₋₄₀ where auto-hydrolysis has been reported to cleave between Asp23 - Val24 residues (Hosia *et al.*, 2005, Lyons *et al.*, 2016). Of note, this is the first study to report cleavage at Phe4 - Arg5, which may be the primary site for resveratrol catalysed cleavage of the A β ₁₋₄₂ peptide, and represents a novel mechanism by which the polyphenol disrupts A β ₁₋₄₂ peptide aggregation.

Table 1. Comparison of the experimental (MALDI-TOF) and theoretical isotope patterns (online ChemCalc protein calculator) for the observed peptide fragments.

Fragment	Experimental isotope pattern	Theoretical isotope pattern	Matched sequences
2414.4			RHDSGYEVHHQKLVFFAEDV Molecular formula: C ₁₀₉ H ₁₅₇ N ₃₁ O ₃₂ Molecular weight: 2413.60567 Monoisotopic mass: 2412.16109 (M) 2413.16891 (M+H)

Coskuner and Wise-Scira, 2013 reported that the Arg5 residue in A β ₁₋₄₂ peptide forms stable salt bridges with various residues (Coskuner and Wise-Scira, 2013). The cleavage of A β is known to be dependent on its secondary structure (Naldi *et al.*, 2012, Rudinskiy *et al.*, 2016). An interaction between the guanidinium group and resveratrol may weaken the salt bridge to destabilise the A β ₁₋₄₂ secondary structure and prevent aggregation. This may be followed by hydrolysis. This is in line with our finding that resveratrol exhibited anti-aggregation activity well before the appearance of peptide fragments. Therefore, interference with this amino acid (by forming bonds between resveratrol and the Arg residue) could be a possible explanation for the mechanism of resveratrol action (Coskuner and Wise-Scira, 2013). Rudinskiy *et al.*, (2016) highlights the importance of cleavage occurring at Gly25 as the A β ₁₋₂₅ peptide has been implicated as a fragment that becomes internalised by neuronal cells and which may be associated with early events of A β aggregation (Rudinskiy *et al.*, 2016). In the case of resveratrol treatment, cleavage at Phe4 - Arg5 will truncate this fragment further to produce A β ₅₋₂₄ (RHDSGYEVHHQKLVFFAEDV), which could prevent aggregation of the peptide.

3.3. Morphological examination of A β ₁₋₄₂ peptide using atomic force microscopy

The effect of resveratrol on aggregation of the A β ₁₋₄₂ peptide was analysed using atomic force microscopy (AFM). In control AFM studies, without resveratrol, the fresh (Day 0) A β ₁₋₄₂ peptide possessed a mean z-height of 1.932 nm (range 0.846 - 7.520 nm) and samples were free of A β ₁₋₄₂ fibrils and large aggregates (Fig. 5 A), consistent with literature findings (Parbhu *et al.*, 2002). By day 1, the A β ₁₋₄₂ peptide oligomers could be observed, the mean z-height of which was 4.238 nm (range 1.334 - 23.367 nm) (data not shown). At day 3, larger A β ₁₋₄₂ peptide oligomers were observed, which possessed a mean z-height of 5.825 nm (range 1.311 - 23.521 nm) and very few A β ₁₋₄₂ peptide monomers were detected (data not shown), again similar to the literature (Parbhu *et al.*, 2002). Subsequently, on day 4, larger A β ₁₋₄₂ peptide oligomers, with a mean z-height of 8.161 nm (range 4.278 - 16.252 nm) and protofibrils up to 1 μ m in length were detected (data not shown). Analysis on day 7 revealed the presence of fibrils with mean z-height of 12.145 nm (range 4.250 - 43.847 nm), and lengths between 1 - 4 μ m (data not shown). Analysis on day 10 revealed the presence of larger fibrils with mean z-height of 18.706 nm (range 2.644 - 35.924 nm), and lengths between 1.24 - 4.5 μ m observed at day 10 (Fig. 5 B). Analysis on day 10 of A β ₁₋₄₂ in the presence of resveratrol revealed only cleaved peptides (Fig. 5 C).

Using AFM, Ge *et al.*, (2012), confirmed that co-incubation of resveratrol with A β ₁₋₄₂ peptide monomer significantly reduced the aggregation of A β ₁₋₄₂ peptide, which is consistent with our findings (Ge *et al.*, 2012). The results are also consistent with the findings of Fu *et al.*, (2014) who assessed the influence of resveratrol on the aggregation of A β ₁₋₄₂ peptide using AFM, and proposed that resveratrol binds to the N-terminus of the A β ₁₋₄₂ peptide monomers and caps the height of the oligomers (Fu *et al.*, 2014). However, whilst the findings of the present study, and others', reveal resveratrol disrupts A β ₁₋₄₂ peptide aggregation, Phan *et al.*, (2019) report resveratrol accelerated the formation of amyloid beta fibrils, before decreasing

fibrillation (Phan *et al.*, 2019). It is possible this difference may be due to differences in the way A β_{1-42} peptides were prepared, with peptides prepared in ammonia solution having a greater propensity to form fibrils than peptides prepared in HFIP. Furthermore, there are slight differences in the experimental conditions, i.e. solvents, under which A β_{1-42} peptide aggregation was studied.

Figure 5. Visualisation of the effect of resveratrol on A β ₁₋₄₂ peptide aggregation by atomic force microscopic analysis. A β ₁₋₄₂ peptide (0.2 mM) was incubated with resveratrol (20 mM) at an A β ₁₋₄₂ peptide:resveratrol molar ratio of 1:100 at 37 °C for 10 days. Samples, 20 μ l, of A β ₁₋₄₂ peptide (A, B) or A β ₁₋₄₂ peptide incubated with resveratrol (C) were then deposited on freshly cleaved mica for 20 min, washed five times with filter sterilised Milli-Q water and air dried at room temperature. Atomic force microscopic analysis was carried out using a Picoforce Nanoscope V Multimode atomic force microscope at day 0 (A) and day 10 (B, C) using tapping mode with scan rates between 0.5 and 1.5 Hz and a resonant frequency range of 270 - 460 kHz. Scanned areas shown are 5 $\times$ 5 μ m.

3.4 Resveratrol reduces A β ₁₋₄₂ peptide toxicity towards SH-SY5Y cells

The SH-SY5Y human neuroblastoma cell line was chosen to assess the effect of A β ₁₋₄₂ peptide on cell viability since SH-SY5Y cells have been reported to be sensitive to A β ₁₋₄₂ peptide (El-Agnaf *et al.*, 1998). Exposure of SH-SY5Y cells to A β ₁₋₄₂ peptide resulted in a significant ($P < 0.01$) reduction in cell viability, from 100 % to 78 %, whilst exposure to resveratrol did not affect viability (Fig. 6). Interestingly, when SH-SY5Y cells were co-incubated with A β ₁₋₄₂ peptide and resveratrol (1:10 molar ratio), no A β ₁₋₄₂ peptide-mediated toxicity was observed (Fig. 6). This finding is consistent with the finding that resveratrol cleaves A β ₁₋₄₂ peptide into smaller fragments, thereby reducing toxicity towards cells. There is evidence that A β ₁₋₄₂ peptide-mediated neurotoxicity is induced by A β aggregation (Barrow *et al.*, 1992), therefore disruption of the aggregation process is likely to reduce A β ₁₋₄₂ peptide-mediated neurotoxicity, as observed in the current study.

Figure 6. Effect of Aβ₁₋₄₂ peptide and resveratrol on SH-SY5Y cell viability. SH-SY5Y cells in 100 μl growth medium were plated into 96-well plates at a density of 1.5×10^4 cells/well in 96-well plates and left to adhere overnight at 37 °C in 5 % CO₂. Cells were then incubated with 100 μl growth medium containing either 10 μM Aβ₁₋₄₂ peptide, 1 μM resveratrol or 10 μM Aβ₁₋₄₂ peptide and 1 μM resveratrol combined for 48 h at 37 °C and 5 % CO₂. MTT solution (25 μl, 5 mg.ml⁻¹) was then added to each well and cells incubated for a further 4.5 h. The growth medium was then removed, cell monolayers washed three times with PBS and 100 μl of 50 % *N,N*-dimethyl-formamide and 20 % SDS (pH 4.8) added to each well. Plates were incubated overnight at 37 °C and the absorbance measured at a test wavelength of 570 nm and a reference wavelength of 655 nm using a Polar Star Omega plate reader. Data were analysed using Student's t-test and are expressed as mean ± standard deviation of four independent experiments, each containing five replicates. * *P* < 0.01 and NS = not significant.

4. Conclusions

We report a novel mechanism by which treatment of Aβ₁₋₄₂ peptide with the polyphenol resveratrol caused fragmentation and prevented Aβ₁₋₄₂ peptide-mediated toxicity towards SH-SY5Y cells. The sites of cleavage in the Aβ₁₋₄₂ peptide are identified between Arg5 and His6 and between Val24 and Gly25. The finding that resveratrol cleaves Aβ₁₋₄₂ peptide into fragments, preventing peptide aggregation, is of interest and of potential application to the treatment of Alzheimer's disease.

Whilst the current study provides evidence for resveratrol-mediated fragmentation of A β ₁₋₄₂ peptide, the polyphenol and its derivatives also demonstrate additional anti-amyloidogenic properties. Cleavage of amyloid precursor protein (APP) by β -secretase (BACE1) results in production of A β ₁₋₄₂ peptide. Choi *et al.*, (2011) report oligomeric resveratrol significantly inhibited β -secretase activity (Choi *et al.*, 2011) and Koukoulitsa *et al.* (2016) demonstrated the polyphenol also inhibited β -secretase (Koukoulitsa *et al.*, 2016). Furthermore, a recent study by Chen *et al.*, (2019) demonstrated resveratrol reduced expression of APP and its cleavage products in a transgenic mouse model (Chen *et al.*, 2019), however, to date, no studies report resveratrol-mediated fragmentation of holo-APP. Taken together, such findings suggest a multimodal mechanism by which resveratrol potentially influences A β ₁₋₄₂ peptide levels in the brain.

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Supplementary Figure 1. Superimposed HPLC analyses of (A) Aβ₁₋₄₂ peptide, 100 μM, and (B) Aβ₁₋₄₂ peptide, 100 μM, incubated with resveratrol at a 1:100 molar ratio over time.