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Sulforaphane interaction with amyloid beta 1-40 peptide studied by electrospray ionization mass spectrometry

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RATIONALE: Aggregation of amyloid beta 1-40 (Aβ) in the brain causes Alzheimer's disease (AD) and several small molecules are known to inhibit the aggregation process. Sulforaphane (SFN) is a natural isothiocyanate which is known to prevent various neurodegenerative processes. However, its interaction with Aβ is yet to be explored. Such studies could provide new mechanistic insights for its neuroprotective properties.
METHODS: Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) and in-source fragmentation experiments were performed on an Orbitrap mass spectrometer. The solution of Aβ and SFN was incubated and analyzed by mass spectrometry. Isotopic distribution patterns, accurate mass values and theoretical product ions were used to analyze the mass spectrometry data. The nature of binding of SFN and its binding sites with Aβ were evaluated by LC/MS and trypsin digestion experiments.
RESULTS: ESI-MS analysis of the incubated solution of Aβ and SFN showed a 1:1 complex of [Aβ+SFN]. LC/MS analysis revealed that the solution contains three different [Aβ+SFN] complexes due to covalent binding of SFN to Aβ at three different sites. The in-source fragmentation experiments revealed that SFN is binding to free NH_2 groups (N-terminal amino acid and lysines) in Aβ. Trypsin digestion experiments further confirmed the SFN binding sites in Aβ.
CONCLUSIONS: The interaction of SFN, an anticancer agent, with Aβ was studied using ESI-MS. SFN is found to bind covalently and specifically with the free NH_2 group of N-terminal aspartic acid and the ε-amino group of lysine at positions 16 and 28. Aggregation assay studies showed a lesser inclination of Aβ to aggregate when SFN is present. Hence the present study helps in understanding the mechanism of the action of SFN on the Aβ peptide. Copyright © 2014 John Wiley & Sons, Ltd.

Sulforaphane (SFN) is a naturally occurring isothiocyanate found in cruciferous vegetables such as broccoli and cabbages, that exhibits anticancer, antidiabetic, antioxidant and antimicrobial properties.[1–4] It belongs to a family of isothiocyanates (ITCs) such as benzyl-ITC (BITC) and phenethyl-ITC (PEITC), which are potential anticancer agents. Tubulin protein was found to be a potential target for ITCs[5–8] and covalent binding by cysteines in this case inhibited cell growth. Among the ITCs, the level of modification follows the order BITC > PEITC > SFN.[6] ITCs are also known to react with the hydroxyl groups of amino acids, especially with tyrosines in proteins.[7] Such molecules that can specifically recognize and then covalently react with target proteins in a biological system are useful in disease-related research.

Amyloid plaques and neurofibrillary tangles are the two pathological hallmarks of Alzheimer's disease (AD). A key event in AD pathogenesis is the conversion of amyloid beta 1-40 peptide (Aβ) from its soluble form into aggregated forms in the brain.[9,10] However, the mechanism by which Aβ aggregates induce toxicity is still not clear. Several small naturally occurring molecules have been found to interact with Aβ and prevent its toxicity.[11] On the other hand, the use of SFN for inhibiting neurodegenerative diseases is still a current topic of interest to scientists. Recently, *in vitro* and *in vivo* studies have demonstrated the ability of SFN to prevent various neurodegenerative processes that underlie stroke, traumatic brain injury, AD and Parkinson's disease (PD). These studies demonstrated the neuroprotective effects of SFN against oxidative stress and confirmed the ability of SFN to enhance the proteosome activities and to protect the neuronal cells from Aβ 1-42 mediated cytotoxicity.[12,13] Recently, Tarozzi *et al.* reviewed the potential protective effects of SFN against neurodegenerative diseases, and its neuroprotective effects against Aβ toxicity.[14] However, in order to exert protective effects towards neurodegenerative disorders, SFN must travel through the blood brain barrier (BBB) and accumulate in the central nervous system (CNS). Jazwa *et al.*[15] demonstrated that SFN is able to cross the BBB and accumulate in cerebral tissues of mice such as the ventral midbrain and striatum with a maximum increase and disappearance after 15 min and 2 h, respectively. Clarke *et al.*[16] detected metabolites, but not SFN alone, in the CNS in an *in vivo* experimental model after 2 h and 6 h. However, these metabolites are unstable and readily dissociate back to SFN under physiological conditions. This shows that, although SFN has poor ability to cross the BBB, the metabolites of SFN prolong the presence of unmodified SFN

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in the CNS. Hence, studying the interaction of SFN with Aβ is of biological importance, because unmodified SFN can reach the brain to some extent.

Small molecules have been widely used as covalent inhibitors of enzymes.[17] However, there are not many reports on the covalent targeting of neuropeptides, except for transerythrin amyloidosis inhibitors.[18] Recently, Ma *et al*.[19] showed that P9-NCS peptide can covalently react with Lys 16 of Aβ and inhibit neurotoxic fibrillization. Hence, small molecules that covalently react with proteins like Aβ are likely to be important in diseases related to protein aggregation.

In recent years, mass spectrometry (MS) has been used to investigate Aβ interaction with small molecules and aggregation mechanism studies. It has advantages over other new strategies used to study the development of drugs aimed at Aβ inhibition,[20] and it is a useful technique for the identification of potential protein targets of ITCs in proteomics.[5] Recently, we have studied the interactions of antiepileptic drugs with Aβ and showed the binding region of Aβ with the antiepileptic drug lacosamide.[21] However, there are no reports on the use of MS to study the interaction of SFN with Aβ. Hence, we undertook this study on the interaction of SFN with Aβ. The present study was also aimed at investigating the binding sites of SFN on Aβ peptide. This type of study helps to understand the mechanism of action of SFN on Aβ.

EXPERIMENTAL

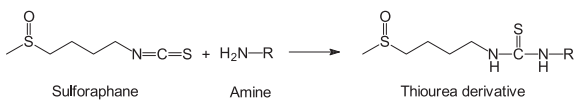
Materials

Aβ 1-40 peptide ($C_{194}H_{295}N_{53}O_{58}S$, 4327.1484 Da), L-SFN ($C_6H_{11}NOS_2$, 177.0282 Da; Scheme 1) and trypsin were purchased from Sigma Aldrich (Steinhem, Germany). All solvents used were of HPLC grade (Merck, Mumbai, India). The stock solution of Aβ (100 μM) was prepared in 20 mM (pH 7.4) ammonium acetate buffer and that of SFN (500 μM) in dimethyl sulfoxide. The working solutions of Aβ (10 μM) and SFN (10, 20, 30 40 and 50 μM) were prepared by diluting the stock solutions in milli Q water (Merck Millipore, Billerica, MA, USA).

ESI-MS analysis

Electrospray ionization (ESI) mass spectra were recorded using an Exactive Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) in positive ion mode. Data was acquired using Xcalibur software (Thermo Scientific). The spray voltage was 4000 V; capillary voltage 30 V; capillary temperature 250 °C; sheath gas (nitrogen) flow rate 40 (arbitrary units); tube lens offset voltage 150 V; skimmer voltage 30 V; and vaporizer temperature 50 °C. The mass spectrometer was scanned in the mass range *m/z* 170–2500 and the resolution was 50 000 (FWHM).

The working solutions of Aβ and SFN were mixed (1:1, v/v) by vortexing for 1 min and then incubated at room temperature for 1 h. The resulting sample solution was directly infused into the ESI source of the mass spectrometer at a flow rate of 5 μL/min using a syringe pump (Harvard Apparatus, Holliston, MA, USA).



Scheme 1. Reaction of the NCS group of sulforaphane with the free amine group of the Aβ peptide.

Trypsin digestion

Trypsin was added to the Aβ peptide or mixture of Aβ peptide and SFN (1:5 ratio), at 5% wt. The sample was incubated at 37 °C for 12 h and after that quenched by adding 2.5% acetic acid. These samples were stored in a freezer (–20 °C) until subjecting them to LC/ESI-MS analysis.

LC/MS analysis

The Aβ sample was incubated with SFN (37 °C for 24 h), and the trypsin-digested Aβ samples (with or without SFN) were analyzed by LC/ESI-MS. The samples (10 pmol of Aβ and 50 pmol of SFN) were injected using an autosampler (injection volume 2 μL) onto a C18 chromatographic column (Kromasil; 250 × 4.6 mm, 5 μm; VDS optilab, Wiesenweg, Berlin, Germany). The mobile phase consisted of water with 1% formic acid as component A and acetonitrile as component B. A linear gradient was run as follows: 0 min 10% B; 5 min 10% B; 25 min 40% B; 35 min 10% B; at a flow rate of 0.8 mL/min. The total run time was 35 min per sample. Typical MS conditions were: sheath gas (N_2) flow rate 50 arbitrary units; auxiliary gas (N_2) flow rate 10 arbitrary units; spray voltage 4.5 kV; capillary temperature 270 °C; capillary voltage 32 V; and tube lens voltage 135 V. The collision energy used for in-source collision-induced dissociation (CID) experiments was 100 eV.

RESULTS AND DISCUSSION

The positive ion ESI mass spectrum of a 10 μM solution of Aβ peptide showed a multiply charged envelope including signals at *m/z* 1444.06 $[Aβ+3H]^{3+}$, 1083.29 $[Aβ+4H]^{4+}$, 866.84 $[Aβ+5H]^{5+}$, 722.53 $[Aβ+6H]^{6+}$ and 619.46 $[Aβ+7H]^{7+}$ (Fig. 1(a)). The ESI mass spectrum of a solution containing Aβ and SFN (1:1 molar ratio), recorded after incubating for 1 h at room temperature, showed peaks at *m/z* 1503.07 (3+), 1127.55 (4+), 902.24 (5+) and 752.04 (6+), corresponding to the Aβ+SFN complex (Fig. 1(b)). However, ions relating to the Aβ+2SFN complex were not found in the spectrum. The accurate masses (from high-resolution mass spectrometry (HRMS) data) obtained for the above ions confirmed that they belonged to the Aβ+SFN complex. The mass of Aβ was measured as 4328.1557 Da with an error of 0.2 ppm (exact mass 4328.1564 Da), and the mass of the Aβ+SFN complex was measured as 4505.1839 with an error of 0.2 ppm (exact mass 4505.1846). It is important to note that the experimentally obtained molecular weight of the Aβ+SFN complex from the ESI mass spectrum is essentially the same as the sum of molecular weights of the individual Aβ and SFN molecules. This raises the question of whether the formed Aβ+SFN complex is a simple

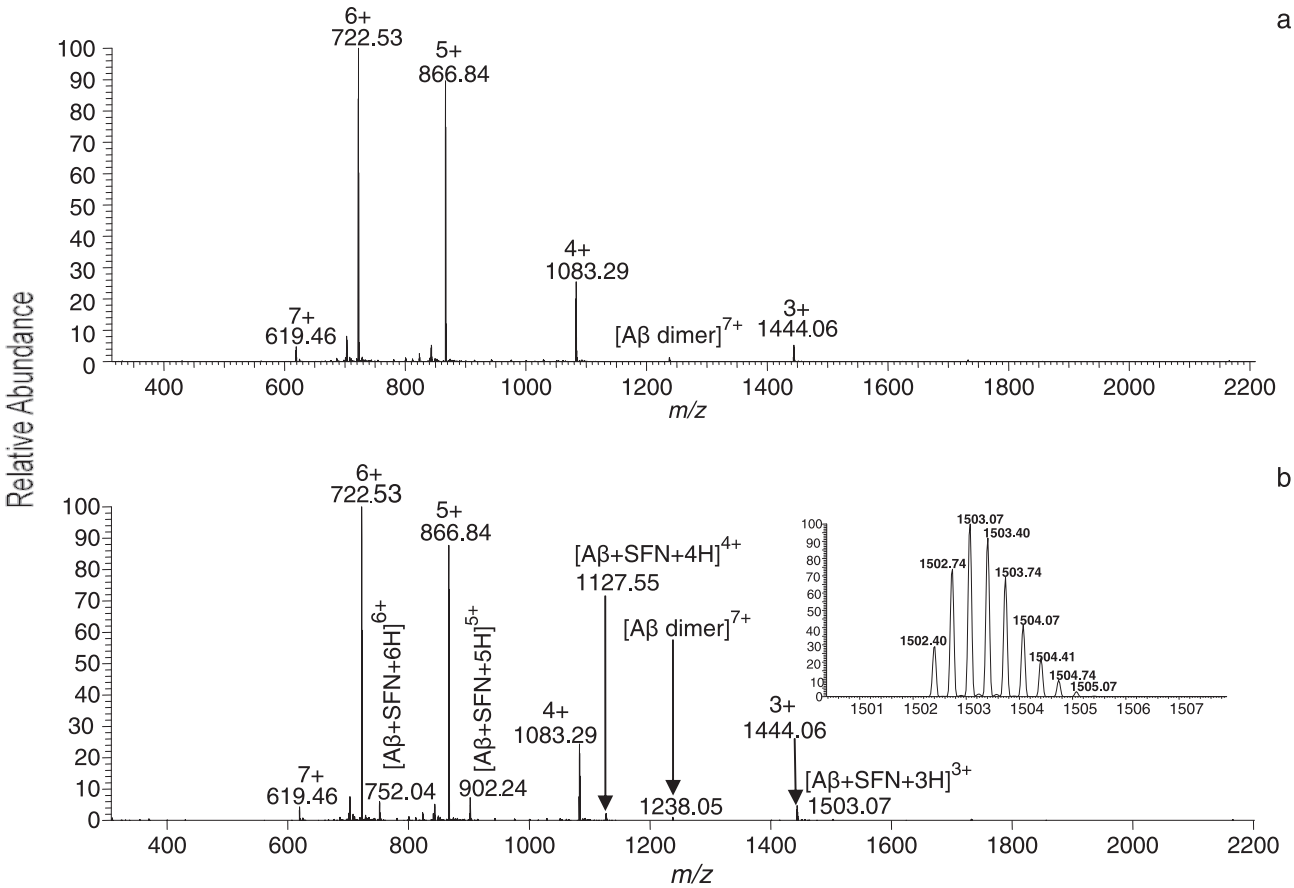


Figure 1. ESI mass spectra of (a) Aβ and (b) Aβ+SFN in 1:1 molar ratio. Inset shows expanded spectrum of $[Aβ+SFN+3H]^{3+}$.

proton-bound complex or a covalently modified peptide (Scheme 1). We have carried out LC/MS analysis of the solution to confirm the actual mode of binding of SFN to Aβ. If the complex is a proton-bound dimer (weakly bound), it would separate in LC and Aβ and SFN would appear at different retention times. If, however, the complex is covalently bound, it would be expected to appear as a single peak.

LC/MS analysis of Aβ+SFN samples

The Aβ+SFN solution (1:5) was incubated at 37 °C for 24 h and then subjected to LC/MS analysis. The LC/MS chromatogram showed four peaks, a peak at a retention time (RT) of 16.5 min (Fig. 2(a)), with minor peaks at RTs 17.5, 19.0 and 20.3 min (Fig. 2(b)). The ESI mass spectrum of the peak eluting at 16.5 min corresponded to Aβ. The other three minor peaks showed similar spectra and the accurate masses of the ions showed that they all corresponded to the Aβ-SFN complex (Fig. 3). The exact mass of Aβ-SFN is 4505.1839 and the measured accurate masses for the peaks eluting at RTs 17.5, 19.0 and 20.3 min were 4505.2069 (5.1 ppm), 4505.2061 (4.9 ppm) and 4505.2054 (4.8 ppm), respectively. This suggests that SFN can bind to three different sites in Aβ, resulting in three positional isomers of the Aβ-SFN complex. The experiments were repeated at various concentrations of Aβ/SFN (1:1, 1:2, 1:3, 1:4 and 1:5) with similar data being found in all cases. The LC/MS analysis of all these samples showed the same peaks due to Aβ and three Aβ-SFN complexes at the same retention times, and there were no additional peaks. There was no significant change in the peak heights of the three modified Aβ peptides in the chromatogram (even at the high concentration of 1:5); however, the MS signals were better when the experiment was performed at 1:5 concentrations. These experiments cannot, however, provide information on the exact site of binding of SFN to Aβ. Thus, we next studied the fragmentation of each SFN-modified Aβ (using in-source CID experiments) to locate the SFN binding sites in Aβ.

In-source CID experiments

The Orbitrap instrument used in the present experiments does not have tandem mass spectrometry capability; however, it is possible to study fragmentation using an HCD cell or in-source fragmentation, which allows fragmentation without mass selection. This mode of experiment is called "all ion fragmentation" (AIF), and it has been used in proteomics applications.[22,23] In such experiments, especially when aimed at structural elucidation of unknown molecules, the target compound must be either pure or well separated in chromatography before subjecting it to AIF to avoid interfering product ions from impurities or co-eluting compounds in the spectra. In the present study, all three isomeric Aβ-SFN complexes were well separated by LC. Thus, we performed AIF using an HCD cell and in-source fragmentation with appropriate collision energy. Initial

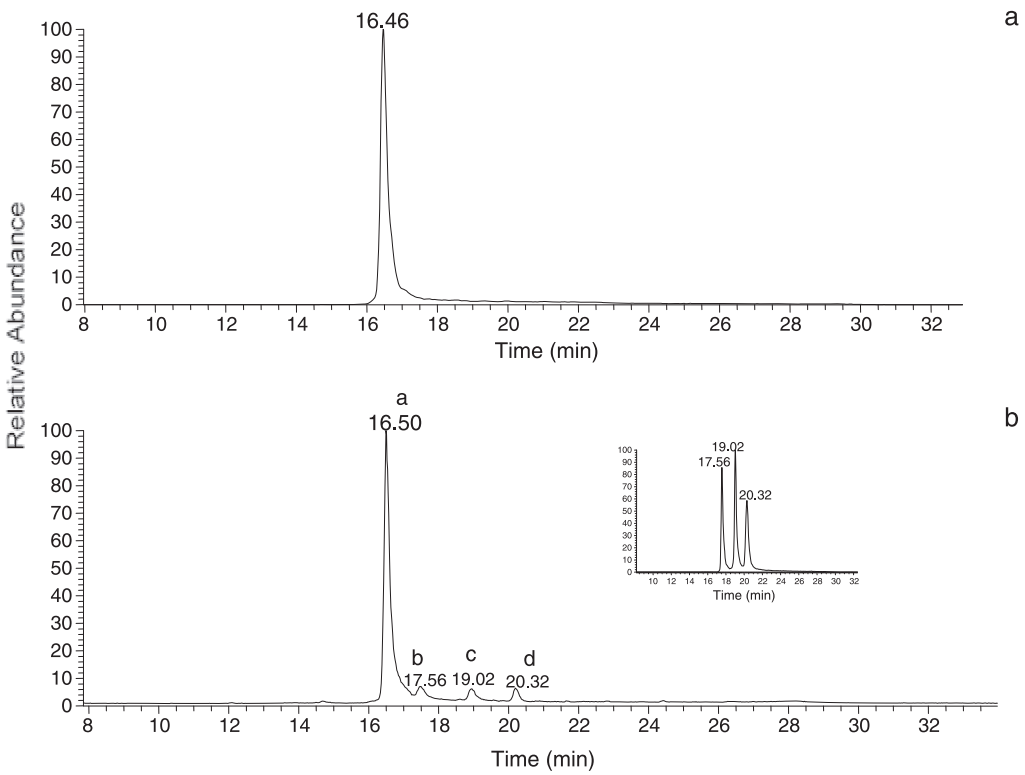


Figure 2. LC/MS chromatograms of (a) Aβ alone and (b) Aβ with SFN in the molar ratio of 1:5 incubated at 37 °C for 24 h, where peak a = Aβ peptide, peak b = SFN-modified Aβ peptide 1, peak c = SFN-modified Aβ peptide 2, and peak d = SFN-modified Aβ peptide 3. The inset shows the ion chromatogram of the *m/z* 1503 ion (3+) corresponding to SFN-modified Aβ peptide.

experiments with the HCD cell showed a few, low-abundance product ions (singly charged), whereas in-source CID experiments showed a higher number of product ions with better signals (multiply charged). Thus, we restricted our investigation to in-source CID experiments.

The experiments were performed at different collision energies (CEs) and it was found that a CE of 100 eV was suitable to obtain reasonable fragmentation with reproducible signals. The AIF spectra thus obtained, hereafter called CID spectra, for the three peaks at RTs of 17.5, 19.0 and 20.3 min are given in Fig. 4. The charge-state information (from the isotopic pattern) and the accurate masses of all the product ions were used in interpretation of the spectra. The product ions in the spectra were characterized by comparing them with an *in silico* fragmentation list obtained from bioinformatics tools ("Fragment ion calculator"[24]). For the *in silico* analysis, the Aβ peptides with SFN modification at the N-terminal amino acid, Lys-16 or Lys-28, were used because isothiocyanates (ITCs) were expected to bind with free amino groups. The spectra showed b series of ions, and the exact masses (theoretical) and accurate experimental masses for all the observed b ions along with mass errors are shown in Table 1.

Peak at RT 17.5 min

The CID spectrum (Fig. 4(a)) showed a dominant and diagnostic product ion at *m/z* 293, which corresponds to the b_1 ion with SFN modification, denoted as a $*b_1$ ion (hereafter, the product ions with SFN modification are shown with an asterisk). The presence of this ion confirms that the peak eluting at RT 17.5 min corresponds to the Aβ peptide with SFN modification on the N-terminal amino acid (Asp). The other product ions in the spectrum were exclusively b ions. The exact theoretical masses (theoretical) and the accurate masses (experimental) for all the observed b ions are shown in Table 1. The $*b_{29}$–$*b_{38}$ (triply charged) ions were observed in the mass spectrum, together with other unknown ions in the mass range *m/z* 550–1100, which correspond to the loss of 293 Da from doubly charged $^{\#}b_{10}$ to $^{\#}b_{19}$ ions and triply charged $^{\#}b_{29}$. We presume that these $[*b\text{-}293]^{2+}$ ions might have been formed from an intermediate doubly charged product ion (y_{39}^{2+}) that formed after the loss of 293 Da from the $[A\beta+SFN+3H]^{3+}$ ion (shown in Fig. 4(a); the (*b-293) ions are marked with the hash symbol).

Peak at RT 19.0 min

The CID spectrum of the peak eluting at RT 19.0 min (Fig. 4(b)) showed a series of b ions with or without SFN modifications. The ions b_{10}, b_{11}, b_{13}, b_{14}, b_{15} (doubly charged) were found to be without SFN modification. This clearly proves that the N-terminal amino acid is free (unmodified). The next b ions in the series, i.e., $*b_{16}$, $*b_{17}$, $*b_{18}$, $*b_{19}$, $*b_{23}$, $*b_{29}$, $*b_{30}$, $*b_{31}$, $*b_{32}$, $*b_{33}$, $*b_{34}$, $*b_{35}$, $*b_{36}$, $*b_{38}$ (triply charged), were shifted by 177 *m/z* units (SFN modified). This proves that this peak corresponds to the Aβ peptide modified with SFN at the Lys-16 amino acid. The ε-amino group of Lys must be involved in the covalent binding with SFN.

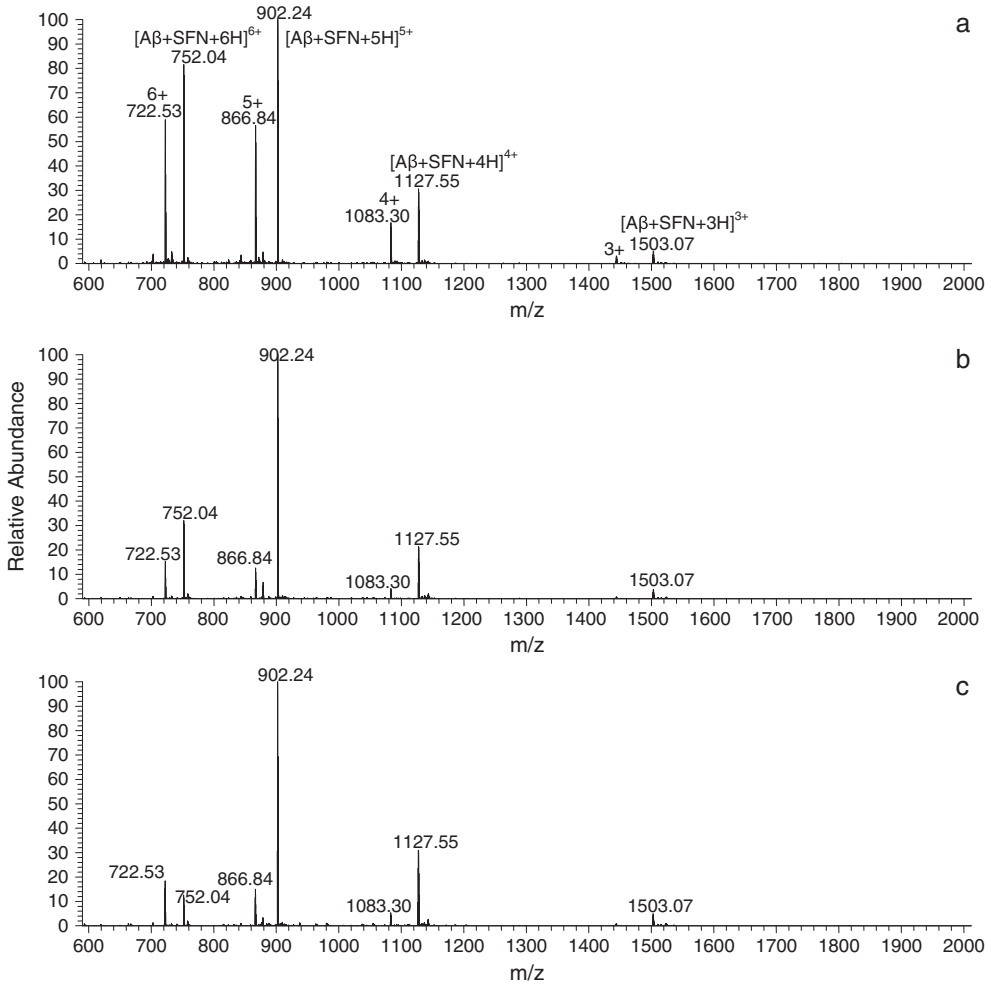


Figure 3. ESI mass spectra of SFN-modified Aβ peptides eluted at RTs (a) 17.5 min, (b) 19.0 min, and (c) 20.3 min.

Peak at RT 20.3 min

The CID spectrum of this peak (Fig. 4(c)) showed neither the product ion at *m/z* 293 nor the b series of ions with SFN modification between b_1 to b_{16}, which were characteristic of the SFN-modified Aβ peaks eluting at RTs 17.5 and 19.0 min. The spectrum included b_{16}–b_{18} (both doubly and triply charged species) and b_{19}, b_{20} and b_{23} ions (triply charged species) without SFN modification, and SFN-modified b series of ions from the 28th amino acid (i.e. triply charged species of $*b_{28}$ to $*b_{39}$). The presence of these b series of ions indicated that the SFN modification could be on the ε-amino group of Lys-28.

Although the data reported above demonstrate that Lys-28 might be modified with SFN, SFN modification on Ser-26 (hydroxyl group) cannot be ruled out because the detected b ions can also be obtained when Ser-26 is modified with SFN. We thus performed trypsin digestion experiments to obtain unequivocal confirmation of the SFN modification in Aβ. Since there were three different SFN-modified Aβ peptides existing together in the SFN-treated Aβ solution, trypsin digestion of the solution would result in a mixture of tryptic peptides with and without SFN modification. It should then be possible to verify whether Ser-26 is modified with SFN by the LC/ESI-MS analysis of the tryptic peptides.

LC/MS analysis of the trypsin-digested (Aβ+SFN) sample

Trypsin digestions of Aβ alone and Aβ incubated with SFN were performed and the resulting samples were analyzed by LC/MS. The chromatographic conditions used for the analysis of SFN-modified Aβ peptides were also found to be suitable for the analysis of tryptic peptides. Trypsin cleavage of the Aβ peptide alone should result in four tryptic peptides, i.e., [1–5], [6–16], [17-28] and [29-40], and all four peptide peaks could be seen in the LC/MS analysis of the trypsin-digested Aβ. The LC chromatogram also showed two additional peaks due to missed cleavages corresponding to the [1–16] (a very low abundance peak) and [17-40] peptides (Fig. 5(A)). As the trypsin-digested Aβ-SFN solution consists of three different isomeric Aβ-SFN complexes existing together, it should result in a mixture of tryptic peptides with and without SFN modification (theoretically 22 peptides expected; shown in Table 2). The LC chromatogram is given in Fig. 5(B), and the LC peaks were identified from their ESI mass spectra. Of the 22 expected peptide fragments, 15 were identified in the LC/MS analysis. The observed tryptic peptides are shown in Table 2 (in bold). In-source CID data was then used to further confirm the binding site. In-source CID of the SFN-modified 17-40 peptide showed unmodified b series of ions (singly charged b_2, b_3, b_4, b_5, b_7, and b_8) and SFN-modified

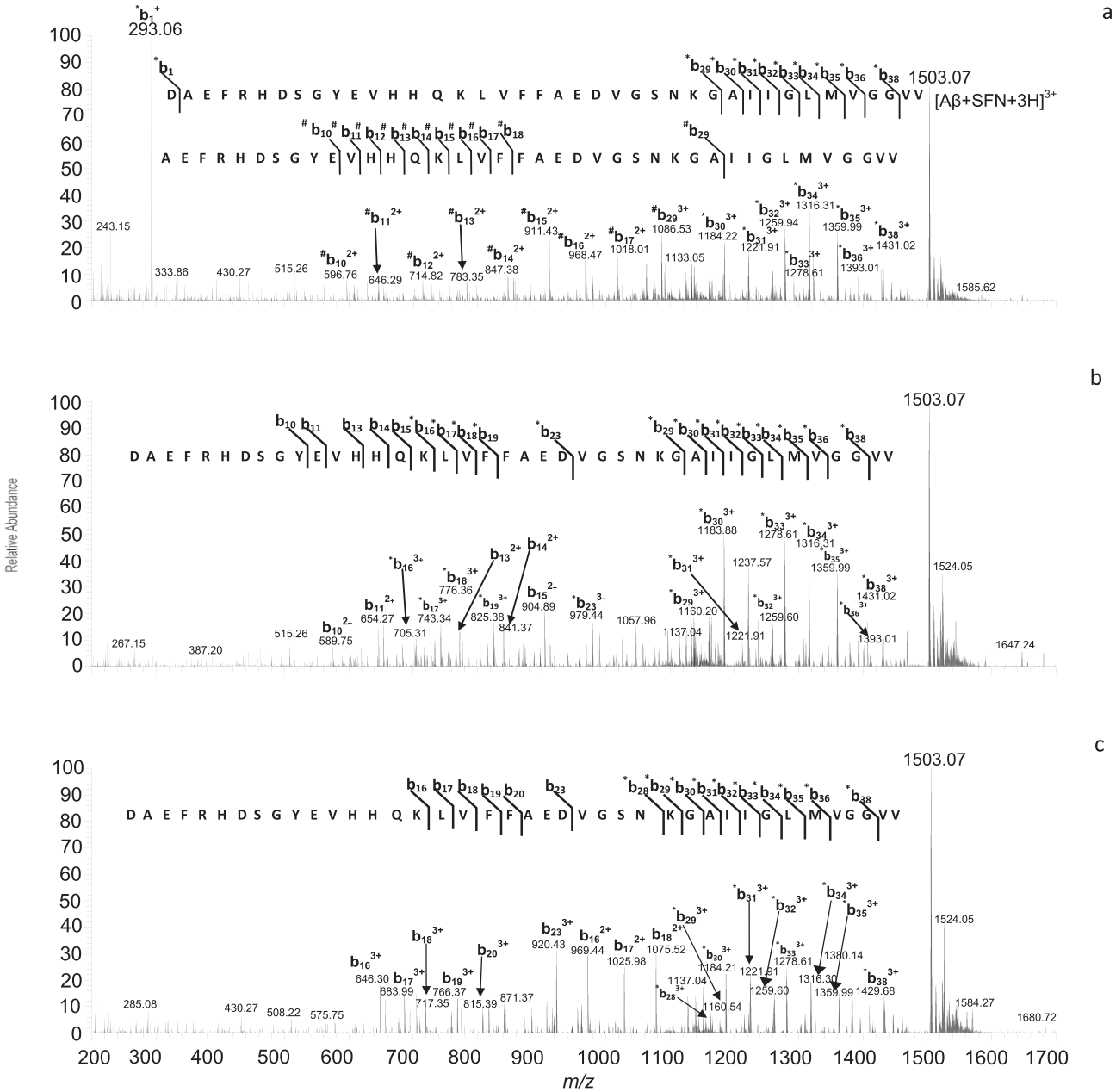


Figure 4. LC/MS/MS spectra (all ion fragmentation/in-source CID) of SFN-modified Aβ, eluted at RTs (a) [17.56 min] N-terminal binding with Asp-1, (b) [19.02 min] binding with Lys-16, and (c) [20.32 min] binding with Lys-28.* indicates increment of 177 *m/z* units, i.e., addition of SFN molecule. # indicates ions formed by the loss of N-terminal amino acid covalently bound with SFN, aspartic acid.

b ions (doubly charged, *b_{14}, *b_{15}, *b_{16}, *b_{17}, *b_{18}, *b_{19}, *b_{20}, *b_{21}, *b_{22} and *b_{23}). Modified b ions (singly charged) were also observed: *b_{12}, *b_{14}, *b_{15}, *b_{16}, *b_{17},*b_{18}, *b_{19} and *b_{21} (shown in Fig. 6). From the HRMS data of these ions (Table 3), it can be confirmed that the 12th position of the 17-40 peptide was modified with SFN (Lys-28 in the Aβ peptide). Hence trypsin digestion followed by LC/MS/MS analysis confirmed Lys-28 as the SFN-binding site in the peak eluting at 20.3 min.

Although ITCs are known to react with cysteines, primary/secondary amines (Lys and Pro) and hydroxyl groups (Tyr) to form the corresponding thiocarbamates, the present experimental data confirms that the SFN binds covalently to Aβ at the NH_2 group of the N-terminal amino acid (Asp), and the ε-NH_2 groups of lysine at the 16 and 28 positions. Ma *et al.*[19] showed that P9-NCS peptide can covalently react with Lys-16 of Aβ and inhibit neurotoxic fibrillization. With this background, we are of the opinion that SFN may also play a significant role in the inhibition of Aβ peptide aggregation. With a view to understanding the role of SFN in the aggregation of Aβ, preliminary Aβ aggregation experiments were performed in the presence of SFN.

Influence of SFN on Aβ aggregation

The mass spectrometry-based screening assay developed by Van Breemen *et al.*[25] was used to study the effect of SFN on the aggregation of Aβ. Samples of Aβ were incubated with and

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Table 1. The accurate masses of product ions obtained in the CID spectra of Aβ-SFN compounds. (* indicates SFN-modified ion and # indicates ions formed by the loss of 293 Da)

Ions	Ion formula with charge state	Theoretical mass (*m/z*)	Observed mass (*m/z*)	Error (ppm)
*b1	$C_{10}H_{17}N_2O_4S_2^{1+}$	293.0624	293.0634	3.3
b9	$C_{42}H_{60}N_{14}O_{16}^{2+}$	508.2150	508.2172	4.3
b10	$C_{51}H_{69}N_{15}O_{18}^{2+}$	589.7467	589.7507	6.9
b11	$C_{56}H_{76}N_{16}O_{21}^{2+}$	654.2680	654.2713	5.1
b12	$C_{61}H_{85}N_{17}O_{22}^{2+}$	703.8022	703.8063	5.8
b13	$C_{67}H_{92}N_{20}O_{23}^{2+}$	772.3316	772.3365	6.3
b14	$C_{73}H_{99}N_{23}O_{24}^{2+}$	840.8612	840.8669	6.9
b15	$C_{78}H_{107}N_{25}O_{26}^{2+}$	904.8904	904.8961	6.3
b16	$C_{84}H_{119}N_{27}O_{27}^{2+}$	968.9378	968.9410	3.2
b17	$C_{90}H_{130}N_{28}O_{28}^{2+}$	1025.4799	1025.4837	3.7
b18	$C_{95}H_{139}N_{29}O_{29}^{2+}$	1075.0141	1075.0175	3.2
b19	$C_{104}H_{148}N_{30}O_{30}^{2+}$	1148.5483	1148.5552	6.0
b20	$C_{113}H_{158}N_{31}O_{31}^{3+}$	815.0574	815.0602	3.4
b21	$C_{116}H_{163}N_{32}O_{32}^{3+}$	838.7364	838.7398	4.0
b22	$C_{121}H_{170}N_{33}O_{35}^{3+}$	881.7506	881.7540	3.7
b23	$C_{125}H_{174}N_{34}O_{38}^{2+}$	1379.6358	1379.6438	5.7
b24	$C_{130}H_{183}N_{35}O_{39}^{2+}$	1429.1700	1429.1745	3.1
*b28	$C_{151}H_{221}N_{42}O_{46}S_2^{3+}$	1140.8557	1140.8584	2.4
*b29	$C_{153}H_{224}N_{43}O_{47}S_2^{3+}$	1159.8628	1159.8675	4.0
*b30	$C_{156}H_{229}N_{44}O_{48}S_2^{3+}$	1183.5419	1183.5451	2.7
*b31	$C_{162}H_{240}N_{45}O_{49}S_2^{3+}$	1221.2365	1221.2399	2.7
*b32	$C_{168}H_{251}N_{46}O_{50}S_2^{3+}$	1258.9312	1258.9337	2.0
*b33	$C_{170}H_{254}N_{47}O_{51}S_2^{3+}$	1277.9384	1277.9425	3.2
*b34	$C_{176}H_{265}N_{48}O_{52}S_2^{3+}$	1315.6331	1315.6358	2.1
*b35	$C_{181}H_{274}N_{49}O_{53}S_3^{3+}$	1359.3132	1359.3182	3.7
*b36	$C_{186}H_{283}N_{50}O_{54}S_3^{3+}$	1392.3360	1392.3406	3.3
*b37	$C_{188}H_{286}N_{51}O_{55}S_3^{3+}$	1411.3432	1411.3524	6.5
*b38	$C_{190}H_{289}N_{52}O_{56}S_3^{+3}$	1430.3504	1430.3523	1.4
*b16	$C_{90}H_{131}N_{28}O_{28}S_2^{3+}$	705.3038	705.3084	6.5
*b17	$C_{96}H_{142}N_{29}O_{29}S_2^{3+}$	742.9984	743.0015	4.2
*b18	$C_{101}H_{151}N_{30}O_{30}S_2^{3+}$	776.0212	776.0245	4.2
*b19	$C_{110}H_{160}N_{31}O_{31}S_2^{3+}$	825.0440	825.0483	5.2
*b20	$C_{119}H_{170}N_{32}O_{32}S_2^{3+}$	874.4028	874.4048	2.3
*b23	$C_{131}H_{186}N_{35}O_{39}S_2^{3+}$	979.1024	979.1062	3.9
#b10	$C_{52}H_{71}N_{15}O_{18}^{2+}$	596.7545	596.7571	4.3
#b11	$C_{57}H_{80}N_{16}O_{19}^{2+}$	646.2887	646.2910	3.5
#b12	$C_{63}H_{87}N_{19}O_{20}^{2+}$	714.8181	714.8217	5.0
#b13	$C_{69}H_{94}N_{22}O_{21}^{2+}$	783.3476	783.3513	4.7
#b14	$C_{74}H_{102}N_{24}O_{23}^{2+}$	847.3769	847.3820	6.0
#b15	$C_{80}H_{114}N_{26}O_{24}^{2+}$	911.4244	911.4295	5.6
#b16	$C_{86}H_{125}N_{27}O_{25}^{2+}$	967.9664	967.9716	5.3
#b17	$C_{91}H_{134}N_{28}O_{26}^{2+}$	1017.5006	1017.5063	5.6
#b18	$C_{100}H_{143}N_{29}O_{27}^{2+}$	1091.0348	1091.0405	5.2
#b19	$C_{109}H_{152}N_{30}O_{28}^{2+}$	1164.5690	1164.5730	3.4
#b29	$C_{146}H_{213}N_{42}O_{44}^{3+}$	1086.1901	1086.1960	5.4
b16	$C_{84}H_{120}N_{27}O_{27}^{3+}$	646.2943	646.2983	6.0
b17	$C_{90}H_{131}N_{28}O_{28}^{3+}$	683.9890	683.9936	6.7
b18	$C_{95}H_{140}N_{29}O_{29}^{3+}$	717.0118	717.0162	6.0
b19	$C_{104}H_{149}N_{30}O_{30}^{3+}$	766.0346	766.0399	6.8
b23	$C_{125}H_{175}N_{34}O_{38}^{3+}$	920.0930	920.0993	6.9

without SFN (1:5), where Aβ without SFN serves as the control sample. After incubation for 24 h at 37 °C, the supernatant was filtered through molecular weight cut off filters (vivaspin 10 000 Da membrane filters, Sartorius Stedim Biotech, Goettingen Germany) to remove aggregates. The supernatant was subjected to LC/ESI-MS analysis using the same chromatographic conditions as described for the LC/MS analysis, and the peak areas of the monomeric Aβ were recorded from the LC/MS chromatograms. The calibration curve was linear for the range 0.25 and 100 μM ($R^2 = 0.99$) and described by the equation $y = 179056.3x - 167202.6$, from which the concentration of free monomer was determined (see Supplementary Fig. S1, Supporting Information). When Aβ was incubated in the presence of SFN, the monomer concentration was higher than

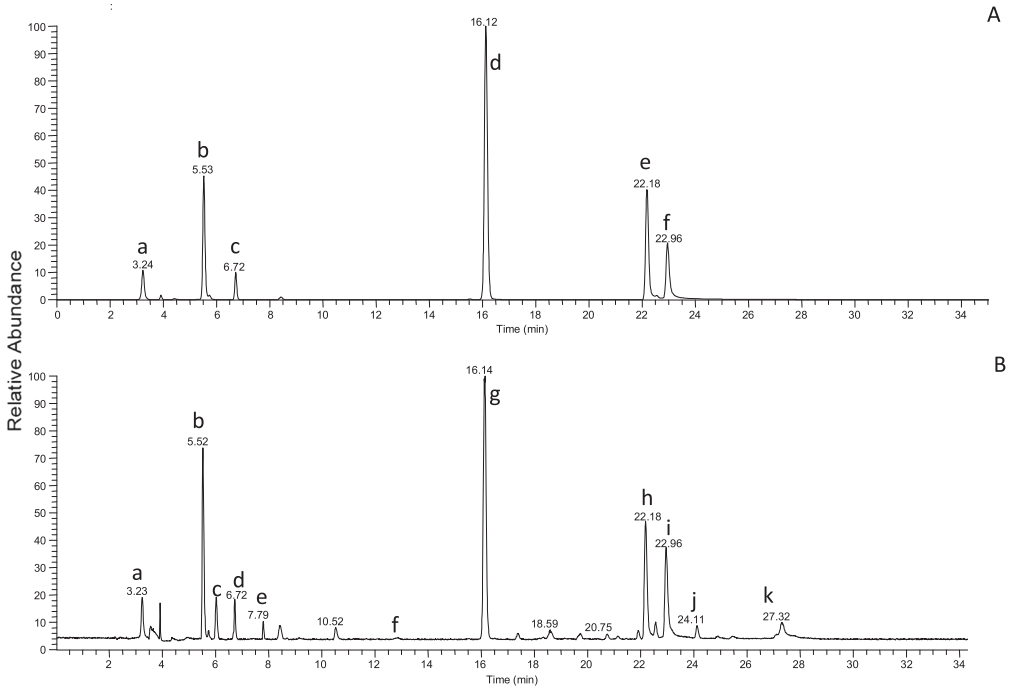

Figure 5. LC/MS chromatograms of (A) trypsin-digested Aβ peptide without SFN, where a = Aβ [6-16], b = [1-16], c = [1-5], d = [17-28], e = [29-40], and f = [17-40] and (B) trypsin-digested Aβ peptide with SFN (in 1:5 molar ratio), where peak a = [6-16], b = [1-16], c = SFN, d = [1-5], e = [1-16+SFN], f = [1-28+SFN], g = [17-28], h = [29-40], i = [17-40], j = [17-28+SFN], and k = [17-40+SFN].

Table 2. Theoretical (A) and observed (B) tryptic digested peptides from three SFN-modified Aβ peptides (superscript number on tryptic peptide indicates the position of the amino acid modified)

A. Theoretical (*in silico*) tryptic peptides

Modified peptide	Without missed cleavage	With missed cleavages (one/two)
Aβ 1-40 (N-terminal amino acid modified)	(1-5)+SFN, 6-16, 17-28, 29-40	(1-16)+SFN, (1-28)+SFN, (6-28)+SFN, (6-40)+SFN,17-40
Aβ 1-40 (16-Lys-modified)	1-5, (6-16)+SFN, 17-28, 29-40	(1-28)+SFN, (6-28)+SFN, (17-40)+SFN, (6-40)+SFN 6-16, 1-16, 29-40.
Aβ 1-40 (28-Lys-modified)	1-5, 6-16,(17-28)+SFN, 29-40	(1-28)+SFN,(6-28)+SFN, (17-40)+SFN, (6-40)+SFN 6-16, 1-16, 29-40.

B. Tryptic peptides observed in LC/MS analysis

RT (min)	Tryptic peptide	Ion formula with charge state	Theoretical mass (*m/z*)	Observed mass (*m/z*)	Error (ppm)
5.99	**(6-16)-SFN at Lys^{16}**	$C_{63}H_{94}N_{20}O_{20}S_2^{2+}$	757.3192	757.3190	0.2
6.67	**(1-16)-SFN at Lys^{16}**	$C_{90}H_{133}N_{28}O_{29}S_2^{3+}$	711.3073	711.3064	1.2
7.79	**(1-16)-SFN at Asp^{1}**	$C_{90}H_{133}N_{28}O_{29}S_2^{3+}$	711.3073	711.3059	1.9
11.68	**(1-5)-SFN at Asp^{1}**	$C_{33}H_{52}N_9O_{11}S_2^{1+}$	814.3222	814.3224	0.2
12.85	**(6-28)-SFN at Lys^{16}**	$C_{124}H_{185}N_{34}O_{38}S_2^{3+}$	940.7671	940.7665	0.7
12.88	**(1-28)-SFN at Asp^{1}**	$C_{151}H_{224}N_{42}O_{47}S_2^{4+}$	860.3962	860.3967	0.6
19.98	**(6-40)-SFN at Lys^{28}**	$C_{173}H_{272}N_{46}O_{50}S_3^{4+}$	972.4824	972.4823	0.1
24.11	**(17-28)-SFN at Lys^{28}**	$C_{67}H_{105}N_{15}O_{20}S_2^{2+}$	751.8545	751.8560	1.9
27.32	**(17-40)-SFN at Lys^{28}**	$C_{116}H_{191}N_{27}O_{32}S_3^{2+}$	1285.1650	1285.1648	0.2
3.23	**6-16**	$C_{57}H_{83}N_{19}O_{19}^{2+}$	668.8051	668.8054	0.5
5.52	**1-16**	$C_{84}H_{122}N_{27}O_{28}^{3+}$	652.2979	652.2983	0.7
6.72	**1-5**	$C_{27}H_{41}N_8O_{10}^{1+}$	637.2940	637.2944	0.6
16.14	**17-28**	$C_{61}H_{94}N_{14}O_{19}^{2+}$	663.3404	663.3398	1.0
22.18	**29-40**	$C_{49}H_{89}N_{12}O_{13}S^{1+}$	1085.6387	1085.6361	2.4
22.96	**17-40**	$C_{110}H_{180}N_{26}O_{31}S^{2+}$	1196.6509	1196.6498	0.9

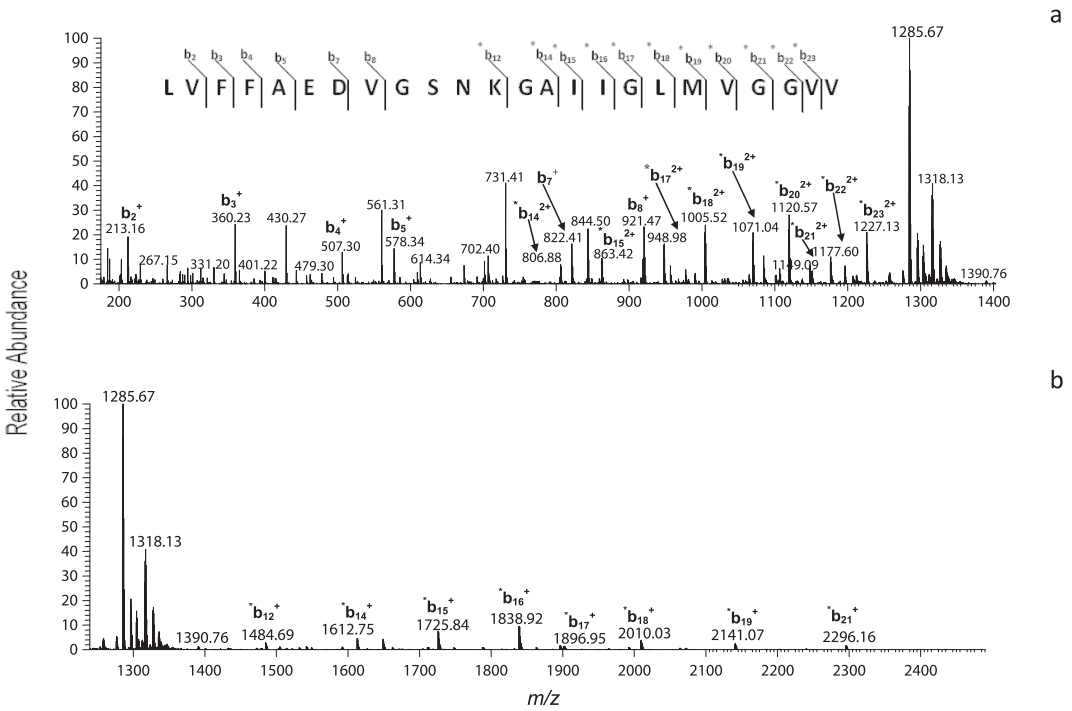

Figure 6. Expanded MS/MS (in-source CID) spectra of SFN-modified tryptic peptide [17-40]: (a) *m/z* 200–1400 and (b) *m/z* 1200–2400. Ions with * indicate SFN-modified Aβ ions.

Table 3. The accurate masses of product ions obtained in the CID spectrum of Aβ 17-40 tryptic peptide fragment modified with SFN

Ions	Formula of ions	Theoretical mass (*m/z*)	Observed mass (*m/z*)	Error (ppm)
b2	$C_{11}H_{21}N_2O_2^{1+}$	213.1598	213.1603	2.6
b3	$C_{20}H_{30}N_3O_3^{1+}$	360.2282	360.2291	2.6
b4	$C_{29}H_{39}N_4O_4^{1+}$	507.2966	507.2982	3.2
b5	$C_{32}H_{44}N_5O_5^{1+}$	578.3337	578.3357	3.5
b7	$C_{41}H_{56}N_7O_{11}^{1+}$	822.4032	822.4065	4.0
b8	$C_{46}H_{65}N_8O_{12}^{1+}$	921.4716	921.4744	3.0
*b12	$C_{67}H_{102}N_{15}O_{19}S_2^{1+}$	1484.6912	1484.6951	2.6
*b14	$C_{72}H_{110}N_{17}O_{21}S_2^{1+}$	1612.7498	1612.7539	2.5
*b15	$C_{78}H_{121}N_{18}O_{22}S_2^{1+}$	1725.8339	1725.8382	2.5
*b16	$C_{84}H_{132}N_{19}O_{23}S_2^{1+}$	1838.9179	1838.9221	2.3
*b17	$C_{86}H_{135}N_{20}O_{24}S_2^{1+}$	1895.9394	1895.9435	2.7
*b18	$C_{92}H_{146}N_{21}O_{25}S_2^{1+}$	2009.0235	2009.0267	1.6
*b19	$C_{97}H_{155}N_{22}O_{26}S_3^{1+}$	2140.0640	2140.0677	1.8
*b21	$C_{104}H_{167}N_{24}O_{28}S_3^{1+}$	2296.1538	2296.1645	4.6
*b14	$C_{72}H_{111}N_{17}O_{21}S_2^{2+}$	806.8785	806.8819	4.2
*b15	$C_{78}H_{122}N_{18}O_{22}S_2^{2+}$	863.4206	863.4237	3.6
*b17	$C_{86}H_{136}N_{20}O_{24}S_2^{2+}$	948.4733	948.4765	3.3
*b18	$C_{92}H_{147}N_{21}O_{25}S_2^{2+}$	1005.0154	1005.0184	3.0
*b19	$C_{97}H_{156}N_{22}O_{26}S_3^{2+}$	1070.5356	1070.5388	3.0
*b20	$C_{102}H_{165}N_{23}O_{27}S_3^{2+}$	1120.0698	1120.0730	2.8
*b21	$C_{104}H_{168}N_{24}O_{28}S_3^{2+}$	1148.5806	1148.5833	2.4
*b22	$C_{106}H_{171}N_{25}O_{29}S_3^{2+}$	1177.0913	1177.0948	3.0
*b23	$C_{111}H_{180}N_{26}O_{30}S_3^{2+}$	1226.6255	1226.6292	3.0

*indicates SFN modified

in the control sample. The monomer concentration was reduced to 63.6% in the control sample, whereas it was 85.5% in the presence of SFN (see Supplementary Fig. S2, Supporting Information). This reveals that SFN inhibits fibril formation of Aβ to some extent. The inhibition capacity may be mainly attributed to the covalent binding of SFN to Aβ.

CONCLUSIONS

The accumulation of aggregated Aβ peptide in the brain is a hallmark of AD and it is thought to play a role in the neurotoxicity associated with the disease. Several small molecules have been found to interact with Aβ fibrils and prevent its toxicity. In this work, we studied the interaction of SFN, a known anticancer agent, with Aβ to explore the type of interactions and also the binding sites on Aβ peptide. LC/ESI-MS, in-source CID experiments were employed in the study, and the experimental results revealed that SFN binds to Aβ to form three different 1:1 complexes. The SFN bound covalently and specifically with the free amino group of the N-terminal amino acid and with the ε-amino group on the Lys at the 16 and 28 positions, and formed the corresponding thiourea derivative. The aggregation assay studies showed lower inclination of Aβ to aggregate when SFN was present. Further investigations towards gaining insight into the action of SFN and other isothiocyanates on Aβ aggregation in biological matrices are in progress.

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