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Research paper

Thiosemicarbazone modification of 3-acetyl coumarin inhibits Aβ peptide aggregation and protect against Aβ-induced cytotoxicity

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ABSTRACT

Aggregation of amyloid β peptide (Aβ) is an important event in the progression of Alzheimer's disease. Therefore, among the available therapeutic approaches to fight with disease, inhibition of Aβ aggregation is widely studied and one of the promising approach for the development of treatments for Alzheimer's disease. Thiosemicarbazone compounds are known for their variety of biological activities. However, the potential of thiosemicarbazone compounds towards inhibition of Aβ peptide aggregation and the subsequent toxicity is little explored. Herein, we report synthesis and x-ray crystal structure of novel compound 3-acetyl coumarin thiosemicarbazone and its efficacy toward inhibition of Aβ(1-42) peptide aggregation. Our results indicate that 3-acetyl coumarin thiosemicarbazone inhibits Aβ(1-42) peptide aggregation up to 80% compared to the parent 3-acetyl coumarin which inhibits 52%. Further, 3-acetyl coumarin thiosemicarbazone provides neuroprotection against Aβ-induced cytotoxicity in SH-SY5Y cell line. These findings indicate that thiosemicarbazone modification renders 3-acetyl coumarin neuroprotective properties.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss, decline in language skills, and thinking abilities [1]. It is characterized by the presence of extracellular beta amyloid plaques and intracellular neurofibrillary lesions in the brain [2]. Aβ peptide formed due to the abnormal processing of amyloid precursor protein (APP) by β and γ-secretases [3]. Usually, two main forms of Aβ peptide namely Aβ(1-40) and Aβ(1-42) are present in the brain [4]. Among the two common alloforms of Aβ, Aβ(1-42) is more prone to aggregation than Aβ(1-40) [5]. The amyloid cascade mechanism is the most widely established hypothesis of overall disease pathology [6]. This mechanism proceeds through the formation of dimers and small oligomers followed by protofibrils and fibrils via primary nucleation process [7]. Amyloid aggregates are stable, highly ordered structures mainly composed of crossed β sheet core and are responsible for neurodegeneration [8]. Aβ peptide aggregates are considered to be more toxic to neuronal cells compared to monomeric Aβ peptide, while some reports have suggested that the soluble Aβ peptide are the main cytotoxic agents rather than monomeric Aβ peptide [9–11]. Recently, the information of toxic oligomeric species via fibril-catalyzed secondary nucleation process of monomeric Aβ peptide has been demonstrated [12,13].

The formation of Aβ peptide and its aggregation was explored to develop the treatment of AD. This includes designing of inhibitors to block the expression of amyloid precursor protein (APP), prevent the proteolytic cleavage of APP into Aβ peptide or inhibit Aβ peptide aggregates [14]. The inhibition of Aβ peptide aggregation is an attractive target as this process is directly implicated in AD pathology. A number of inhibitors of Aβ peptide aggregation have been reported which includes polyamines [15], chaperones [16], metal chelators [17], and nutraceuticals [18]. Metal chelators such as clioquinol and its analog PTB2 are found to be effective in preventing metal-dependant Aβ peptide aggregation, deposition and toxicity [19]. Similarly, inhibition of Aβ peptide aggregation by tanshinone, bathocuproine, diamine, trientine and

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bathophenanthroline is well known [20]. Polyphenolic compounds such as apomorphine, curcumin, rosmarinic acid, resveratrol and catechin showed inhibition of Aβ peptide aggregation [21].

The presence of planar aromatic moieties with long chain functional groups in the inhibitors are crucial for its hydrophobic, hydrogen bonding, and π-stacking interactions with Aβ peptide. Recently Kanai et al. designed non peptidic inhibitors of Aβ peptide aggregation which possess side chains of Leu^2, Val^3, Phe^4 and Phe^5 residues of Aβ(16-20) peptide (cyclo-[KLVFF]) without backbone amide bonds [22]. Based on this study, a pharmacophore with one or two aromatic rings along with side chain functionality is required for inhibition of Aβ peptide aggregation [22].

Coumarins are important group of naturally occurring compounds known for anti-coagulant, anti-tumor, antiviral, and anti-inflammatory activities [23]. Coumarin derivatives also exhibit good cell permeability [24]. Besides this Moss et al. have reported the inhibition of Aβ peptide aggregation by several coumarin analogs through functionalization of the aromatic center [25]. On the other hand thiosemicarbazone compounds and their metal conjugates are known for various biological activities such as anticancer, antiviral, and antitumor, DNA replication and protease inhibitor [26]. Thiosemicarbazone compounds are well known metal chelator and their biological derivatives are correlated to their ability to control redox properties of metal ions [27]. Thiosemicarbazone chelates metal ion as bidentate N,S-donor ligands while can function as tridentate species if third donor site is present in the remaining part of the ligating system [28]. Interestingly copper-bis(thiosemicarbazonato) complexes have shown to reduce levels of Aβ peptide by up-regulation of Aβ peptide degrading metalloproteases [29]. In addition to this recent report revealed that the copper-bis(thiosemicarbazonato) complexes have the potential to enhance neurogenerative processes by increased metal bioavailability [30].

Hence, we report the synthesis of novel 3-acetyl coumarin thiosemicarbazone and investigate the effect thiosemicarbazone modification on Aβ(1-42) peptide aggregation by several techniques such as ThT fluorescence assay, turbidity assay, ANS fluorescence assay and native gel electrophoresis (see Scheme 1). We also evaluated effect of 3-acetyl coumarin thiosemicarbazone on Aβ induced cytoxicity in SH-SY5Y cells. Our results indicate that thiosemicarbazone derivative of 3-acetyl coumarin was able to reduce toxicity of Aβ peptide in SH-SY5Y cells by inhibiting formation of higher aggregate.

2. Chemistry

In our study, we first carried out synthesis of ACT and the compound was characterized using various techniques such as UV–Vis spectroscopy, IR, ^{1}H NMR, ESI Mass and x-ray single crystal diffraction. ACT exhibits a band in the far UV region at 351 nm with high molar absorptivity due to n–π transition. IR spectra of the parent compound 3-acetyl coumarin shows a strong band at 1718 cm^{-1} due to lactone carbonyl stretching frequency while a band at 1683 cm^{-1} is assigned to the C=O stretching of acetyl group [39]. On condensing with thiosemicarbazide, a new stretching frequency at 1604 cm^{-1} was observed indicating formation of imine bond C=N.

X-ray crystal structure of compound ACT (CCDC Number 972476) was solved (Fig. 1). Crystal data for ACT and structure refinement parameters are given in Table 1, and bond lengths (Å) and bond angles (°) are given in Table 2. The chromene ring is almost planar, with a maximum deviation of 0.065 (2) Å from the mean plane for one of the C atoms. The unit cell of the crystal system has P1 space group. The symmetric unit contains two ACT molecules where the packing is dominated by van der Waals interactions of the planar ligands. The crystal structure of ACT shows that the thiocarbonyl sulphur and the imine nitrogen N (1) atom in the thiosemicarbazone side chain are trans to each other across the C(12)–N(2) bond. The structural arrangement corresponds to the E-isomer.

3. Results and discussion

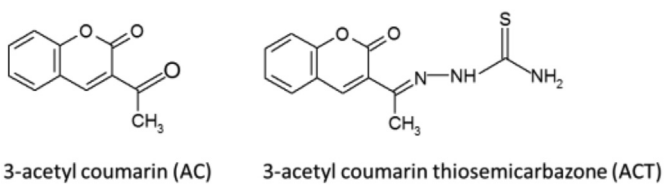
In the all experiments, we have used Aβ(1-42) peptide. We added 1% HFIP to Aβ(1-42) peptide monomer to induce aggregation. This method of aggregation in the presence of dilute HFIP exhibits following advantages; i) It is highly reproducible and hence more amenable to quantitative evaluation. ii) Reduction in the time course of aggregation from several days to few minutes [36]. Thus, we investigated the effect of AC and ACT on HFIP induced aggregation of Aβ(1-42) peptide by using ThT, turbidity, ANS assay and native gel electrophoresis.

3.1. Study of inhibition of Aβ(1-42) peptide aggregation by ThT fluorescence assay

ThT assay is routinely used to quantitate the formation of higher oligomers and aggregates [40,41]. Aβ aggregate formed in the presence of dilute HFIP exhibits an abolished lag phase, during which nucleation events occur, and yields a more uniform aggregate population consisting of predominantly soluble aggregate structures (Fig. 2).

In the presence of 20 μM Aβ(1-42) peptide alone significant increase in the ThT fluorescence intensity was observed and it reaches plateau phase at 40 min (Fig. 2). When Aβ(1-42) peptide was incubated in the presence of AC (1:1 M ratio), almost 50% decrease in the ThT fluorescence intensity was observed at 40 min. This result showed the potential of AC to inhibit the Aβ(1-42) peptide aggregation. When Aβ(1-42) was incubated in the presence of ACT at 1:1 M ratio, almost 80% reduction in the ThT fluorescence was observed at 40 min. Result obtained from ThT fluorescence assay reveled that ACT profoundly prevents the formation higher aggregates of Aβ(1-42) peptide compared to parent AC.

Further, we studied dose dependent effect of AC and ACT on the aggregation of Aβ(1-42) peptide (Fig. 3). Upon incubation of 20 μM



Scheme 1. Structures of 3-acetyl coumarin and 3-acetyl coumarin thiosemicarbazone.

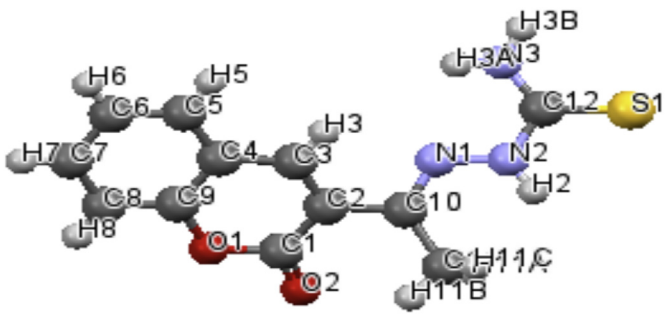


Fig. 1. ORTEP diagram of compound (ACT) showing the atom numbering scheme.

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Table 1
Crystal data and structure refinement for ACT.

Formula	$C_{12}H_{11}N_3O_2S$
Fw	261.30
T(K)	293(2)
Wavelenght (Å)	0.71073
Crystal system	Triclinic
Space group	P-1
Unit cell dimension	
a (Å)	6.6725 (7)
b (Å)	7.2645 (7)
c (Å)	13.7150 (14)
α (°)	96.184 (3)
β (°)	93.121 (4)
γ (°)	114.361 (4)
V (Å^3)	598.48 (10)
Z	2
d (calc) (Mg/m^3)	1.450
Abs coeff (mm^{-1})	0.268
Crystal size (mm^3)	0.48 × 0.41 × 0.30
Crystal color	Yellow
Φ range [deg]	1.50–27.52
Index ranges	−8 < h < 8
	−9 < k < 9
	−17 < l < 17
No. of reflns collected	11,872
No. of indep reflns	2681
R(int)	0.0237
GOF on F^2	1.028
R1/wR2[a,b], [I > 2σI)]	0.0391/0.0971
R1/wR2[a,b], all data	0.0517/0.1049
Largest diff peak and hole [e Å^{-3}]	0.215 and −0.298

[a] R1 = SII*Fo*I − I*Fc*II/SI*Fo*I.
[b] wR2 = {S[w($F_O^2 - F_c^2$)^2]/ S [w(F_O^2)2]} where w = $q/s^2(F_O^2) + (qp)^2 + bp$. GOF = S = {S[w($F_O^2 - F_c^2$)^2]/ (n − p)$^{1/2}$}.

Aβ(1-42) peptide alone resulted in the significant increase in the ThT fluorescence intensity after 40 min (Fig. 3). This increase in the ThT fluorescence intensity indicate formation of Aβ(1-42) peptide aggregates. Further to study dose dependent effect, Aβ(1-42) peptide was incubated at different molar ratio of AC and ACT (1:0.25, 1:0.5, 1:0.75, 1:1). 40% and 53% reduction in the ThT fluorescence intensity was observed at (1:0.25) molar ratio for AC and ACT respectively.

Table 2
Relevant bond lengths (Å) and bond angles (°) for ACT.

Bond length (Å)	Bond angles (°)
S1 C12 1.6786 (16)	C10 N1 N2 117.36 (14)
N1 C10 1.282 (2)	C12 N2 N1 119.12 (14)
N1 N2 1.3688 (17)	C1 O1 C9 122.57 (12)
N2 C12 1.352 (2)	O2 C1 O1 116.11 (14)
N3 C12 1.310 (2)	O2 C1 C2 126.59 (15)
O1 C1 1.3731 (18)	O1 C1 C2 117.28 (14)
O1 C9 1.3755 (19)	C3 C2 C1 119.67 (14)
O2 C1 1.201 (2)	C3 C2 C10 121.68 (14)
C1 C2 1.459 (2)	C1 C2 C10 118.54 (14)
C2 C3 1.346 (2)	C2 C3 C4 121.57 (14)
C2 C10 1.483 (2)	C9 C4 C5 117.93 (14)
C3 C4 1.431 (2)	C9 C4 C3 118.00 (14)
C4 C9 1.388 (2)	C5 C4 C3 124.06 (14)
C4 C5 1.400 (2)	C6 C5 C4 120.28 (16)
C5 C6 1.373 (2)	C5 C6 C7 120.42 (17)
C6 C7 1.384 (3)	C8 C7 C6 120.59 (15)
C7 C8 1.375 (3)	C7 C8 C9 118.69 (16)
C8 C9 1.382 (2)	O1 C9 C8 117.44 (14)
C10C11 1.495 (2)	O1 C9 C4 120.47 (13)
	C8 C9 C4 122.08 (15)
	N1 C10C2 114.01 (14)
	N1 C10C11 124.54 (15)
	C2 C10C11 121.37 (14)
	N3 C12 N2 116.75 (14)
	N3 C12 S1 124.73 (13)
	N2 C12 S1 118.52 (13)

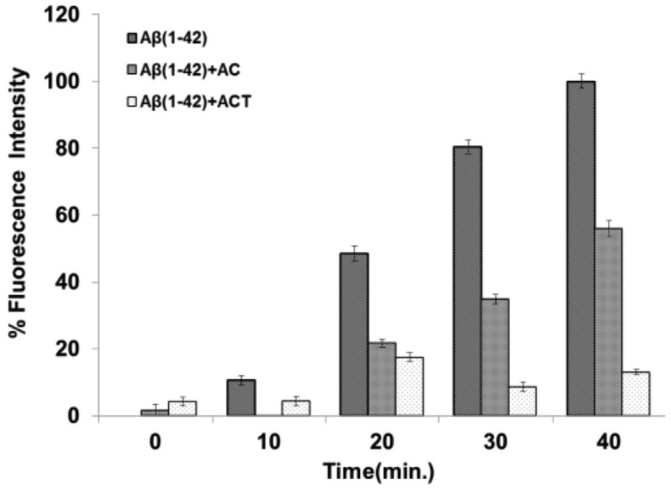

Fig. 2. ThT fluorescence assay showing inhibition of Aβ(1-42) aggregation by AC and ACT. Readings were recorded at 10 min time intervals. Concentration used in the experiment is 20 μM Aβ(1-42) using PBS, pH 7.4 at 37 °C with equimolar amount of AC and ACT in (10% DMSO in PBS pH 7.4). Experiment was performed in the triplicate (n = 3).

Subsequently, the reduction in the ThT fluorescence intensities for both AC and ACT were observed with increase in the molar ratio from 0.25 to 1. Interestingly, the reduction in the ThT fluorescence intensity in case of ACT was higher at 1:1 M ratio compared to that of AC. For ACT, 80% reduction in the ThT fluorescence intensity was observed at molar ratio 1:1 while in case of AC, it is only 52%. These result demonstrate that ACT profoundly prevents the formation higher aggregates of Aβ(1-42) peptide compared to AC.

3.2. Study of inhibition of Aβ(1-42) peptide aggregation by turbidity assay

The aggregation of Aβ peptide can also be followed by simple spectroscopic technique such as turbidity assay (Fig. 4). Incubation of 20 μM Aβ(1-42) peptide alone shows profound increase in the turbidity at 405 nm after 40 min (Fig. 4). This increase in the turbidity or absorbance can be correlated with increase in the

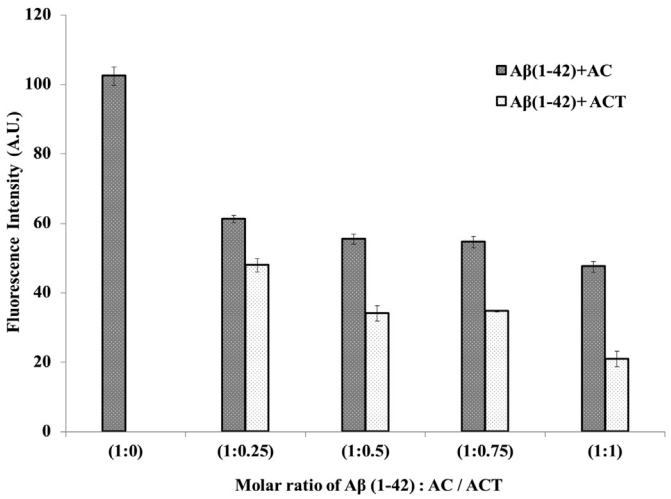

Fig. 3. ThT fluorescence assay showing dose dependent studies of AC and ACT on aggregation of Aβ(1-42) peptide. Concentration used in the experiment is 20 μM Aβ(1-42) using PBS, pH 7.4 at 37 °C with different ratios of AC and ACT (Aβ: AC/ACT = 1:0, 1:0.25, 1:0.5, 1:0.75 and 1:1) in (10% DMSO in PBS, pH 7.4). Experiment was performed in triplicates (n = 3).

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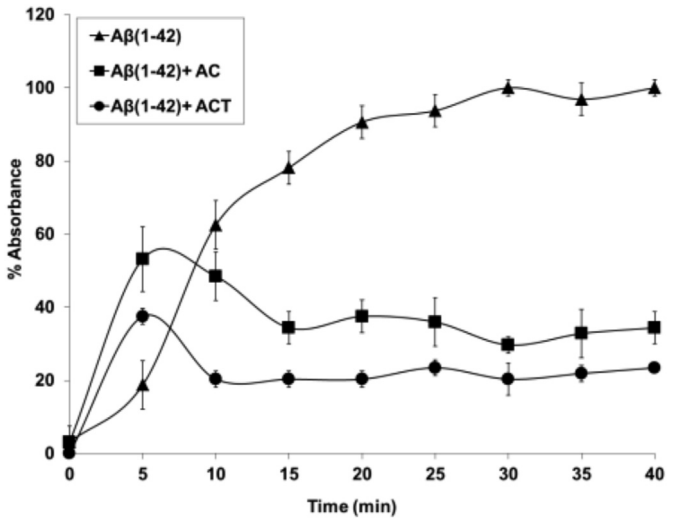

Fig. 4. Turbidity assay showing inhibition of Aβ(1-42) aggregation by AC and ACT in (1:1 ratio) in PBS, pH 7.4. Experiment was performed in triplicate (n = 3) and standard deviation was calculated.

formation of Aβ(1-42) peptide aggregates. When Aβ(1-42) peptide was incubated with AC in (1:1 ratio), 59% reduction in the turbidity was observed at 40 min while in the presence of ACT (in 1:1 ratio), turbidity reduced to 78% at 40 min. This significant reduction in the turbidity supports inhibition of Aβ(1-42) peptide aggregation in the presence of ACT.

3.3. Study of inhibition of Aβ(1-42) peptide aggregation by ANS fluorescence assay

Further, aggregation of Aβ(1-42) peptide was studied using a dye 1-Anilinonaphthalene-8-Sulfonic acid (ANS) (Fig. 5) [42]. ANS assay is used specifically to study the hydrophobic interaction between Aβ peptide and the test compound. Incubation of ANS dye with 20 μM Aβ(1-42) peptide alone (in 1:1 M ratio) resulted in the increase in the intensity of ANS fluorescence due to exposure of hydrophobic region of Aβ peptide aggregates to ANS dye (Fig. 5). When Aβ(1-42) peptide (20 μM) was incubated with AC at (1:1 ratio), almost 40% reduction in the ANS fluorescence intensity was observed at 40 min. This reduction in the ANS intensity suggests decrease exposure of hydrophobic regions of the Aβ(1-42) peptide aggregates to ANS dye. Interestingly, when Aβ(1-42) peptide (20 μM) was incubated with ACT at (1:1 M ratio), the intensity of ANS fluorescence was found to reduce by ~86% which suggest that ACT inhibits the formation of Aβ(1-42) peptide aggregates through hydrophobic interactions. Results obtained from ANS fluorescence assay indicate that both the AC and ACT binds to hydrophobic surfaces of intermediates formed during Aβ(1-42) peptide aggregation and prevents formation of higher aggregates. Interestingly thiosemicarbazone modification improves the inhibition of the formation of higher aggregates than that of parent 3-acetyl coumarin.

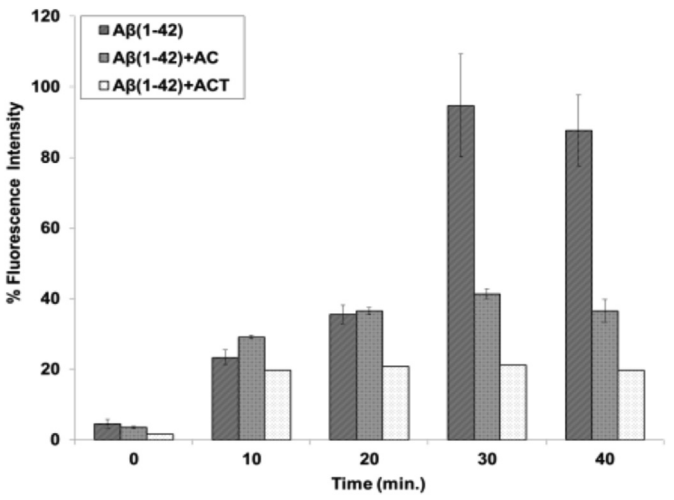

Fig. 5. ANS fluorescence assay showing inhibition of Aβ(1-42) aggregation by AC and ACT. Readings were recorded at every 10 min time intervals. Concentration used in the experiment is 20 μM Aβ(1-42) using PBS, pH 7.4 at 37 °C with equimolar amounts of AC and ACT in (10% DMSO in PBS, pH 7.4). Experiment was performed in triplicate (n = 3) and standard deviation was calculated.

3.4. Study of inhibition of Aβ(1-42) peptide aggregation by native gel electrophoresis

Native gel electrophoresis is found to be particularly suitable for studying formation of different Aβ species. Previously, Sharma et al. reported the formation of different oligomeric species in the range of 10–30 kD and the effect of bifunctional compounds on the aggregation of Aβ(1-42) peptide using native gel electrophoresis [43]. Similarly, in the present case, we have studied inhibitory potential of AC and ACT on Aβ(1-42) peptide aggregation using native gel electrophoresis (Fig. 6). Aβ(1-42) peptide alone shows formation of different oligomeric species in the range of 10–90 kD. Among the oligomeric species observed, two prominent bands around 40 kD show changes upon incubation with test compounds (Band C and Band D). While other oligomeric species have weak intensities to judge impact of test compounds (Band B and Band E). We also observed faint band for monomeric Aβ peptide around 4.5 kD. In the presence of AC the Band D disappeared while the intensity of B

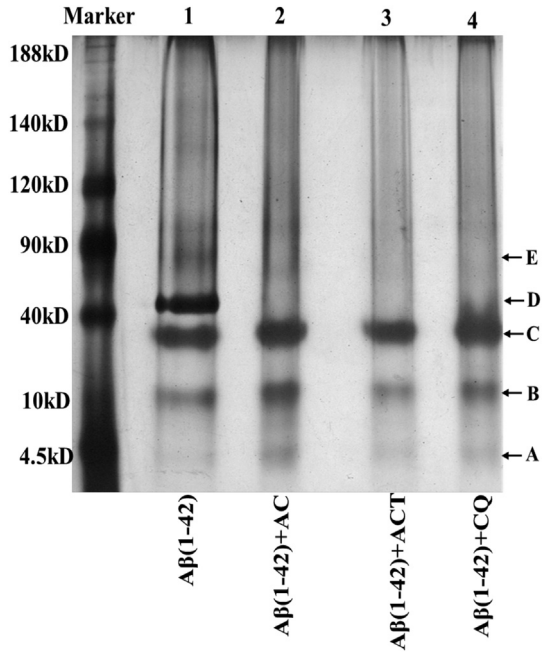

Fig. 6. (A) Native gel electrophoresis of 20 μM Aβ(1-42) alone and in the presence of AC, ACT and clioquinol in 1:1 M ratio incubated for 40 min before electrophoresis. Lanes [1]: 20 μM Aβ(1-42) [2]; Aβ(1-42) + AC(1:1) [3]; Aβ(1-42) + ACT(1:1) [4]; Aβ(1-42) + clioquinol(CQ)(1:1).

and C is slightly increased. On the other hand the intensity of Band B increased in the presence of AC. A large diffuse smear corresponding to higher aggregate (Band E) remains unchanged in the presence of AC. In case of ACT, intensities of Band E and Band B are reduced considerably. The results obtained for both AC and ACT are comparable to that obtained for clioquinol (CQ), which is used as positive control in this experiment. These results indicate that ACT is better inhibitor of Aβ peptide aggregation compared to AC.

3.5. Cytotoxicity of 3-acetyl coumarin and 3-acetyl coumarin thiosemicarbazone in SH-SY5Y cells

Aβ(1-42) peptide aggregates show toxicity to neuronal cells [9–11]. The development of compounds that control the neurotoxicity induced by Aβ(1-42) peptide aggregates are therefore desired. First, we examined toxicity of both AC and ACT on SH-SY5Y cells at various concentrations ranging from 10 to 200 μM. The IC_{50} for AC is 75 μM and that of ACT was found to be in the range of 185 μM (Fig. 7).

3.6. Effects of compounds AC and ACT on Aβ(1-42) peptide induced toxicity in neuronal cell culture

Further, we conducted a neurotoxicity assay to examine whether AC and ACT can protect SH-SY5Y neuronal cells from Aβ(1-42) peptide induced toxicity. Based on the cytotoxicity data, we used fairly non-toxic concentrations of AC and ACT for further experiments (Fig. 8). First, we evaluated the cytotoxicity of the cells treated with Aβ(1-42) peptide (20 μM). Cells treated with Aβ(1-42) peptide alone shows around 28% cell viability (Fig. 8). Interestingly, the cells treated with Aβ(1-42) peptide in the presence of AC showed increase in the cell viability ranging from 77% to 96% at a concentration range of 5–20 μM. Moreover, the cells treated with Aβ(1-42) peptide in the presence of ACT showed increase in the cell viability ranging from 95% to 104% for the same concentration range as AC. The ability of ACT to rescue neurotoxicity induced by Aβ(1-42) peptide at (1:0.5) molar ratio is higher than AC ($^{*}p < 0.05$). Slightly higher neuroprotection observed for ACT is expected based on the results obtained from the ThT assay. Nevertheless, our results are encouraging because both compounds demonstrated significant neuroprotection against Aβ(1-42) peptide induced toxicity at all tested concentrations ($^{**}p < 0.01$). However, it may be noted that ACT is less cytotoxic at higher concentrations compared to AC which underlines the significance of thiosemicarbazone modification (Fig. 8). This result indicates that ACT could be a potential inhibitor for Aβ(1-42) peptide induced toxicity as well as aggregation while AC could also provide neuroprotection to some extent.

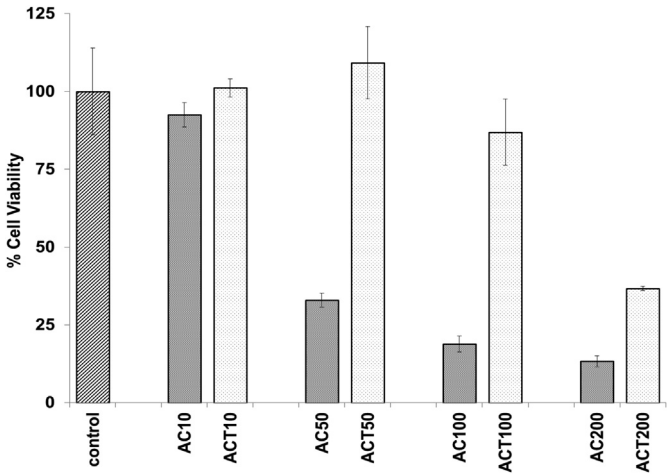

Fig. 7. Cytotoxicity studies of AC and ACT in human neuroblastoma SH-SY5Y cells at different concentrations. Experiment was performed in triplicate (n = 3) and results were expressed as mean% cell viability ± standard deviation.

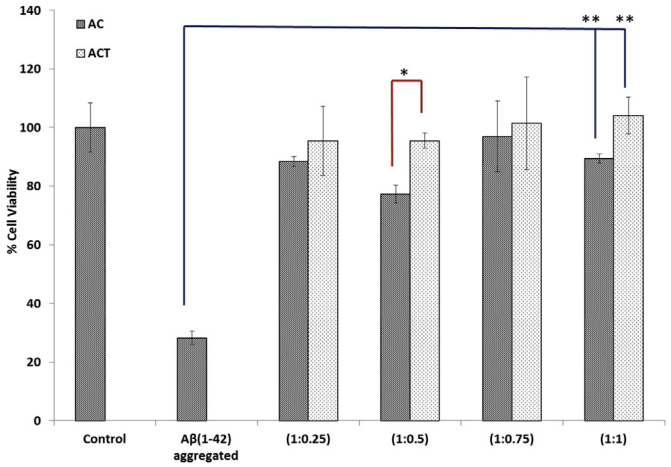

Fig. 8. Effects of compounds AC and ACT on Aβ(1-42) induced toxicity in neuronal cell culture. The concentration of Aβ(1-42) peptide was 20 μM and the ratio of peptide to compound was 1:0.25, 1:0.5, 1: 0.75, 1:1. Experiment was performed in triplicate (n = 3). Results were expressed as mean% cell viability ± standard deviation (n.s. = $^{*}p < 0.05$, $^{**}p < 0.01$).

4. Conclusions

The inhibition of Aβ peptide aggregation is a promising strategy for the prevention and treatment of AD. In our study, thiosemicarbazone derivative of 3-acetyl coumarin (ACT) was synthesized to investigate the effect of thiosemicarbazone modification on the inhibition of aggregation of Aβ(1-42) peptide and subsequent toxicity. The result obtained from dose dependent ThT fluorescence assay showed that ACT profoundly inhibits the formation of higher aggregates of Aβ peptide compared to AC. Turbidity assay shows increase in the aggregation of Aβ(1-42) peptide during initial stages for both AC and ACT but later on aggregation was reduced significantly. Result obtained from both ThT and turbidity support inhibition of Aβ peptide aggregates by ACT compared to AC. Studies using ANS showed that ACT interacts with hydrophobic regions of Aβ peptide and reduces the formation of higher aggregates than that of parent AC. Furthermore ability of ACT to abrogate the formation of high molecular weight aggregates was confirmed by native gel electrophoresis. Overall, our results indicate that thiosemicarbazone derivatization provides promising strategy for the inhibition of Aβ aggregation while it may provide some benefit in rescuing Aβ(1-42) induced toxicity in neuronal SH-SY5Y cells. Due to the ease of synthesis, established drug properties, inhibition of peptide aggregation and its ability to rescue Aβ induced neurotoxicity, thiosemicarbazone modification can be used as excellent tool to improve therapeutic promise of drug molecules in the treatment of AD.

5. Experimental protocol

All reagents and solvents were procured and used as received. 3-acetyl coumarin and thiosemicarbazide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Bicinchoninic Acid Solution (BCA), 1,8-ANS (*1-anilinonaphthalene-8-sulfonic acid*) and

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silver staining kit were purchased from Sigma, USA. Electrophoresis kit purchased from BioRad, USA. Aβ(1-42) peptide purchased from Invitrogen, USA. All reagents for electrophoresis were purchased from Invitrogen, USA. 4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylanilinechloride (ThT) was purchased from Himedia, India. IR spectra were recorded in KBr pellets on IRAffinity-1, Shimadzu. ^{1}H NMR spectra of ACT was measured on a Varian-Mercury 300 MHz spectrometer. Elemental analysis (CHNS) was carried on Vario ELIII. An ESI mass spectrum was recorded on Varian Inc., Model 410.

5.1. Synthesis of 3-acetyl coumarin thiosemicarbazone (ACT)

ACT was prepared by mixing of 3-acetyl coumarin with thiosemicarbazide in 1:1 ratio in 30 mL methanol. The solution was reflux for 1 h. The yellow color product was filtered off and recrystallized from DMF-Methanol (1:1 v/v). The compound was dried in vacuum desiccator. Yellow colored fine single crystals suitable for x-ray analysis were collected (Yield: 85%). Elemental analysis, Found (calcd) for C12H11N3O2S: C, 53.26 (55.16); H, 4.56 (4.24); N, 15.56 (16.08); S, 11.51 (12.26). UV–Vis (DMSO, nm) 351 (ε, 1.13×103 M^{-1} cm^{-1}). IR (ν, cm^{-1}): ν (C═O lactone) 1718, ν (C═N) 1604, ν (NH_2) 3388, ν (NH) 3155, ν (C═S) 1230. ^{1}H NMR δ3.20(S, 3H, CH_3), 7.21–7.89 (m, 4H, Ar–H), 8.32(S, 1H, pyran-H), 10.44 (S, 1H, H–C═S) ppm. ESI Mass (m/z) Found (calcd.) 261.05 (261.3).

5.2. Single crystal x-ray crystallography

Data were collected with a Bruker SMARTAPEX CCD diffractometer (Mo-Kα radiation graphite-monochromated radiation, λ = 0.71073 A) controlled by APEX2 software package [31]. Data integration and global cell refinement were performed with the program SAINT [32]. Data were corrected for absorption by the multi scan empirical method implemented in SADABS [33]. The structure was solved by direct methods using SHELXS 97 [34]. Refinement based on F2, was carried out by full matrix least squares with SHELXL-97 software. Non hydrogen atoms were refined anisotropic thermal parameters. The hydrogen atoms were placed in their geometrically generated positions and allowed to ride on their parent atoms with an isotropic thermal parameter 20% higher to that of the atom of attachment.

5.3. Preparation of Aβ(1-42) monomer stock solution

We have used method adapted by Nichols et al. for the preparation of monomeric Aβ(1-42) peptide solution [35]. We used HFIP/NaOH treatment for the preparation monomeric Aβ(1-42) peptide. Briefly, lyophilized Aβ(1-42) peptide was dissolved in HFIP and stored at −80 °C. Further HFIP was removed by lyophilization and peptide was then stored at −80 °C. Prior to use Aβ(1-42) peptide reconstituted in 60 mM NaOH and diluted to a final concentration of 20 μM in phosphate buffered saline pH 7.4 and the concentration of Aβ(1-42) peptide was determined by BCA method. This is robust method to generate monomeric solution of Aβ(1-42) peptide, which we have used for all the experiments reported in the manuscript. We have confirmed monomeric state of Aβ(1-42) peptide before proceeding experiments using HPLC and ThT assay.

5.4. ThT aggregation assay

Monomeric 20 μM Aβ(1-42) peptide was combined with equimolar concentration of compounds, 20 μM ThT and incubated at 4 °C for 15 min. After incubation, 1% HFIP (v/v) was added to Aβ peptide solution in order to initiate rapid aggregation [36]. This reaction was carried out at 37 °C, and ThT fluorescence was continuously monitored with excitation and emission of 440 nm and 495 nm respectively using (Hitachi F-2500, spectrofluorometer). Plateau fluorescence values were taken as representative of aggregate formation.

Different ratios of Aβ:AC/ACT (1:0, 1:0.25, 1:0.5, 1:0.75 and 1:1) were used to evaluate dose dependent inhibition Aβ(1-42) peptide aggregation. All the samples were incubated at 37 °C for 40 min.

5.5. Turbidity assay

All solutions were prepared using phosphate buffered saline pH 7.4. 20 μM Aβ(1-42) peptide combined with compound AC and ACT in (1:1) ratio and incubated at 4 °C for 15 min. After incubation 1% HFIP (v/v) was added to each solution to induce rapid aggregation. The solutions were added to individual wells in a 96-well plate and the turbidity was measured by light scattering at 405 nm for 40 min time intervals on a plate reader (Biotek SYNERGY HT, microplate reader) [37].

5.6. ANS extrinsic fluorescence assay

The assay was conducted by adding ANS (1-anilinonaphthalene-8-sulfonic acid), Aβ(1-42) peptide and AC and ACT to a quartz cuvette at final concentration of 20 μM (200 μL) using PBS pH 7.4 at 37 °C. 1% HFIP (v/v) was added to the each Aβ(1-42) peptide solution. Upon mixing, the fluorescence emission was recorded at 500 nm with excitation at 390 nm using a (Hitachi F-2500, spectrofluorometer) at 10 min time interval. The fluorescence intensity was monitored for 40 min [38].

5.7. Native gel electrophoresis

20 μM Aβ(1-42) peptide was incubated in PBS pH 7.4 in the presence and in the absence of AC and ACT in 1:1 ratio. 1% HFIP was added to each solution to initiate rapid aggregation and incubated for 40 min at 37 °C. 10 μL from each reaction mixture was diluted with 10 μL of PBS pH 7.4 and 10 μL sample was loaded onto a 12% Native PAGE. Subsequently gel was stained by silver staining method.

5.8. Cytotoxicity studies

Toxicity studies were carried out using human SH-SY5Y neuroblastoma cell line. Cells were obtained from National Centre for Cell Science, Pune, India. Cell viability was determined using the MTT assay. The cells were seeded in a 96-well plate at a density of 10^5 cells/mL in Dulbecco's Modified Eagle's Medium + Ham's F12 containing 10% fetal bovine serum and a 0.1% antibiotic solution for 24 h at 37 °C and 5% CO_2 for adherence. ACT at concentration of 10, 50, 100, 200 μM dissolved in DMSO were added to the cells in media without FBS. DMSO was used as a control. MTT assay was carried out after 24 h incubation. A MTT solution (20 μL, 5 mg/mL) was prepared in PBS pH 7.4 then added to each well and incubated for 3 h. The purple formazan crystals formed were dissolved by addition of 150 μL of DMSO for 5 min absorbance was measured using Biotek SYNERGY HT, microplate reader. IC_{50} was determined by using ED50V10 excel add-on software.

Effect of compounds AC and ACT on Aβ(1-42) mediated toxicity in neuronal cell culture was determined by MTT assay as described previously. Cells were incubated with Aβ(1-42) (20 μM) in the presence and in the absence of different molar ratios of AC and ACT (1:0, 1:0.25, 1:0.5, 1:0.75 and 1:1) for 3 days. After this, 20 μl MTT (5 mg/ml) was added to the cells and incubated for 3 h. Cell viability was measured after dissolving the crystals in DMSO and reading the absorbance at 570 nm as mentioned above.

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