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Synthesis, Anticholinesterase and Antioxidant Activity of Thiosemicarbazone Derivatives

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ABSTRACT

In this research, we report the synthesis and evaluation of novel thiosemicarbazones as anti-Alzheimer's agents. The structural clarification of the newly synthesized compounds was carried out by ^{1}H NMR, ^{13}C NMR, and MS analyses. According to the *in vitro* cholinesterase inhibition assay, compounds showed more inhibitory potential against AChE than BuChE. The *in vitro* antioxidant activity of the synthesized compounds was measured via two different methods. According to ferrous ion-chelating assay compound 2b demonstrated 5.26% activity when compared to BHT (2.57%). DPPH radical scavenging activity assay revealed that compound 2b showed the most potent antioxidant activity with an IC_{50} value of 43.91 ± 0.021µM. Among the synthesized compounds, compound 2b was found as the most potent antioxidant agent.

Keywords: Thiosemicarbazone, AChE, BuChE, Antioxidant.

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Introduction

Free radicals are highly very and unstable molecules with one or more unpaired electron in valency shell. They attack to other compounds to abstract electrons due to their electron deficiency [1,2]. Targets of free radicals are important macromolecules including lipids, nucleic acids and proteins. The attacked molecule loses its electron and becomes a free radical itself, initiating a chain reaction cascade therefore damaging cells and tissues in the body [3-5].

There are other reactive species that can oxidize compounds including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Antioxidant defense system is capable of reduce the amount of these free radicals in the body, otherwise, excessive generation of these reactive species leads to a condition known as oxidative stress [6,7]. Oxidative stress is a common denominator in several chronic and degenerative diseases such as atherosclerosis, cancer, rheumatoid arthritis, chronic fatigue syndrome, Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis, and multiple sclerosis [8-16].

There are several hypotheses for the neurodegeneration process of Alzheimer's Disease (AD). According to the amyloid hypothesis, the accumulation of Aβ in the brain initiates the onset and progression of AD. In the brain of patients with AD, there is often a significant extent of oxidative damage related to the deposition of extracellular amyloid-β (Aβ) plaques and intracellular tau neurofibrillary tangles. Thus, Aβ is a major target in the therapy of AD. However, the etiology mechanisms underlying factors initiate Aβ aggregation are not clear yet [17,18]. Cholinergic hypothesis, the oldest known hypothesis for the neurodegeneration process in AD, suggests that there is a reduced rate of production and transportation of the neurotransmitter acetylcholine in the brains of individuals suffering from AD. Cholinesterase inhibitors such as donepezil, galantamine and rivastigmine increase the quantity of acetylcholine at cholinergic synapses by counteracting the effects of these acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [19, 20].

Thiosemicarbazones are sulfur and nitrogen-containing compounds with potential pharmacological activities, resulting in significant attention in the field of medicinal chemistry. Thiosemicarbazone derivatives have been reported to be potent metal chelators both *in vitro* and *in vivo*. Metal ions such as Cu, Zn and Fe are associated with metal-induced Aβ fibrils aggregation in the pathogenesis of AD, thus metal ion chelators may serve as therapeutic agents [21-23]. Thiosemicarbazones have been also reported as antioxidant and cholinesterase inhibitors [24-28]. Due to the multifactorial nature of AD, here, we report the synthesis of potential multi-targeted compounds with cholinesterase inhibitory and antioxidant effects.

Materials and Methods

Chemistry

Synthesis of 2-[(3-methylthiophen-2-yl) methylidene] hydrazine-1-carbothioamide (1)

2-Methylthiophene-2-carbaldehyde and thiosemicarbazide were dissolved in ethanol. The mixture was refluxed for three hours. After this time, the mixture was set in an ice bath and the precipitated product was filtered.

General synthesis of target 2a-2f compounds

Compound 1 and appropriate aldehyde derivative were dissolved in ethanol. Following that, the mixture was refluxed for five hours. After this time, the mixture was set in an ice bath and the precipitated product was filtered.

2-((3-Methylthiophen-2-yl)methylene)-N-(5-methylfuran-2-yl)methylene)hydrazine-1-carbothioamide (2a): Yield: 74 %, M.p.= 194.1 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ: 2.26 (6H, s, CH_3), 6.93-6.95 (2H, m, Aromatic CH), 7.42-7.44 (1H, m, Aromatic CH), 7.53-7.55 (1H, m, Aromatik CH), 8.17 (1H, br.s., Aromatik CH), 8.35 (1H, s, Aromatik CH), 11.29 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 13.27, 14.94, 127.10, 127.20, 129.59, 129.68, 130.26, 132.48, 132.54, 136.56, 138.72, 140.54, 177.68.

2-((3-Methylthiophen-2-yl)methylene)-N-(5-nitrofuran-2-yl)methylene)hydrazine-1-carbothioamide (2b): Yield: 78 %, M.P.= 150.1 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ: 2.28 (3H, s, CH_3), 6.95 (2H, s, Aromatic CH), 7.44-7.55 (2H, m, Aromatic CH), 8.17-8.36 (2H, m, Aromatik CH), 11.30 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 14.11, 116.65, 120.53, 123.65, 126.03, 128.40, 131.38, 134.28, 137.65, 140.91, 166.41, 188.04. HRMS (m/z): $[M+H]^+$ calcd for $C_{12}H_{10}N_4O_3S_2$: 323.0267; found: 323.0270.

2-((3-Methylthiophen-2-yl)methylene)-N-(5-hydroxymethylfuran-2-yl)methylene) hydrazine-1-carbothioamide (2c): Yield: 79 %, M.P.= 98.6 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ: 2.27 (3H, s, CH_3), 4.50 (2H, s, CH_2), 6.60-6.61 (1H, m, Aromatic CH), 6.93-6.95 (1H, m, Aromatic CH), 7.42-7.44 (1H, m, Aromatik CH), 7.54-7.55 (2H, m, Aromatik CH), 8.16-8.19 (1H, m, Aromatik CH), 8.35 (1H, s, Aromatik CH), 11.30 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 14.88, 56.39, 125.40, 127.10, 127.20, 129.58, 129.68, 130.26, 132.48, 136.55, 138.77, 140.53, 177.68. HRMS (m/z): $[M+H]^+$ calcd for $C_{13}H_{13}N_3O_2S_2$: 308.0522; found: 308.0517.

2-((3-Methylthiophen-2-yl)methylene)-N-((thiophen-2-yl)methylene)hydrazine-1-carbothioamide (2d): Yield: 69 %, M.P.= 201.1 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ: 2.26 (2H, s, CH_3), 6.93-6.95 (1H, m, Aromatic CH), 7.41-7.44 (1H, m, Aromatic CH), 7.53-7.55 (1H, m, Aromatik CH), 7.81 (2H, s, Aromatik CH), 8.15-8.17 (1H, m, Aromatik CH), 8.35 (1H, s, Aromatic CH), 11.30 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 15.13, 127.15, 129.58, 130.14, 130.34, 132.40, 132.67, 136.59, 138.79, 140.32, 140.65, 177.66.

2-((3-Methylthiophen-2-yl)methylene)-N-(5-methylthiophen-2-yl)methylene)hydrazine-1-carbothioamide (2e): Yield: 70 %, M.P.= 190.0 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ: 2.26 (6H, s, CH_3), 6.93-6.95 (1H, m, Aromatic CH), 7.43-7.46 (1H, m, Aromatic CH), 7.54-7.55 (1H, m, Aromatik CH), 8.17-8.19 (1H, m, Aromatik CH), 8.35 (2H, s, Aromatik CH), 11.30 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 13.26, 14.97, 126.42, 127.10, 127.20, 129.58, 129.68, 130.26, 132.48, 132.54, 136.52, 138.75, 177.68. HRMS (m/z): $[M+H]^+$ calcd for $C_{13}H_{13}N_3S_3$: 308.0344; found: 308.0359.

2-((3-Methylthiophen-2-yl)methylene)-N-(3-methylthiophen-2-yl)methylene)hydrazine-1-carbothioamide (2f): Yield: 71 %, M.P.= 185.6 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ: 2.27 (6H, s, CH_3), 6.94-7.02 (2H, m, Aromatic CH), 7.43-7.67 (2H, m, Aromatic CH), 8.17-8.35 (2H, m, Aromatik CH), 11.30 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 14.94, 16.51, 127.11, 128.40, 129.59, 130.26, 131.37, 132.48, 136.55, 137.65, 138.76, 140.52, 177.72. HRMS (m/z): $[M+H]^+$ calcd for $C_{13}H_{13}N_3S_3$: 308.0344; found: 308.0349.

Cholinesterase Inhibition Assay

Inhibition potential of acetylcholinesterase and butyrylcholinesterase was determined by Ellman's modified spectrophotometric technique [29]. "Equine serum BuChE" (EC 3.1.1.8, Sigma) and electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) enzymes were employed. The reaction's substrates were butyrylthiocholine chloride and acetylthiocholine iodide obtained from Sigma Aldrich at Saint Louis, USA. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Aldrich at Saint Louis, United States America) was also used as a coloring reagent. The reaction mixture contained 50 mM Tris HCl buffer (pH 8.0), 6.8 mM DTNB, 10 µl of BuChE/AChE solution, and 2 µl of sample solutions in a 96-well microplate. Following that, acetythiocholine chloride or butyrylthiocholine chloride was added in 10 µl amount to the respective AChE or BuChE enzyme solution to start the enzymatic reactions. The hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion from the reaction of DTNB with thiocholine. This reaction catalyzed by enzymes and recorded at 412 nm, utilizing a 96-well microplate reader (Varioskan Flash, Thermo Scientific, USA). The reaction mixture was incubated for 15 min at 27 °C. Periodic test lasting 75 seconds was obtained. The Varioskan Flash software's SkanIt Software 2.4.5 RE was used to assess the measurements and computations. By comparing the sample reaction rates to those of the blank sample (DMSO and methanol) and applying the formula (E-S)/E x 100 (E: the activity of the enzyme without the test sample, S: the activity of the enzyme with the test sample) the percentage of AChE and BuChE inhibition was calculated. Three replicates of each experiment were conducted. Galantamine hydrochloride obtained from the Sigma-Al, USA has been utilized as a reference material.

Antioxidant Activity Assay

Ferrous ion-chelating effect

The ferrous ion-chelating effect of all the synthesized compounds 2a-2f and reference galantamine was measured through the method of Chua [30]. In brief, various dilutions of compounds were dissolved in ethanol (80%). After that 2 mM $FeCl_2$ solution (200 µL) was added and the mixture was incubated. 800 µL of 5 mM ferrozine (Sigma, St. Louis, MO, USA) was added into the mixture to start the reaction. The mixture was left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was evaluated at 562 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA) against ethanol (80%) as blank. The inhibition ratio of ferrozine-Fe^{2+} complex formation was calculated according to following equation:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

A_{blank}: the absorbance of the control reaction (containing only $FeCl_2$ and ferrozine)

A_{sample}: the absorbance of the compounds/reference

Rutin and butylated hydroxytoluene (BHT) were used as references and they were obtained from Sigma Aldrich (USA) in this assay. Analyses were run in triplicates and the results were expressed as average values with S.E.M. [31,32].

DPPH Radical Scavenging Activity Assay

Blois's UV method [33] was utilized to measure the 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity of the compounds. 40 µM and 100 µM concentrations of the tested compounds and gallic acid (reference) were prepared in 20 µL methanol. 180 µL of 0.15 mM DPPH solution in methanol was added to each solution. The rest amount of DPPH was measured at 520 nm using a Unico 4802 UV–visible double beam spectrophotometer against ethanol (80%) as blank. The percent DPPH radical scavenging activity was calculated as given below:

$I\% = [(A_{control} - A_{sample})/A_{control}] \times 100$

$A_{control}$: the absorbance of the control reaction

A_{sample}: the absorbance of the compounds/reference

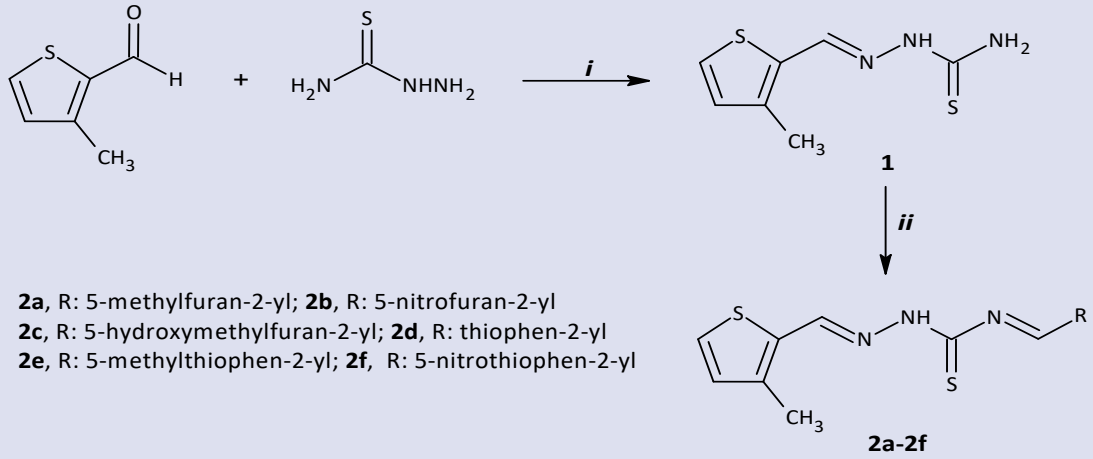
Experiments were run in triplicate and the results were expressed as average values with S.E.M. (standard error mean).

Results and Discussion

Chemistry

Herein, a novel series of thiosemicarbazones (2a-2f) were synthesized according to the two-steps synthetic procedure as illustrated in Scheme 1. Firstly, intermediate thiosemicarbazone compound 1 was afforded via the reaction of starting material 3-methylthiophene-2-carbaldehyde with thiosemicarbazide. In the second step, target thiosemicarbazone compounds 2a-2f were synthesized in good yields through the reaction between compound 1 and various commercially available heterocyclic aldehydes.

In the ^{1}H NMR spectra of the compounds (2a-2f), the protons belonging to CH_3 group attached to thiophen ring, were observed at between 2.26-2.28 ppm. NH protons of thioamide structure were detected at between 11.29-11.30 ppm. The other aromatic and aliphatic protons were observed at the expected regions. In ^{13}C NMR spectra of the compounds, peaks due to all aliphatic and aromatic carbons were in accordance with the chemical structures of the compounds. In our previous study [34], In the ^{1}H NMR spectra of the compounds, the protons belonging to N=CH, and CO-NH were detected as paired peaks with respect to the presence of the E and Z forms of the compounds. Also, In the ^{13}C NMR spectra of the compounds, the carbons belonging to -N=CH- were observed as paired peaks due to E and Z isomer forms. Accordingly, it can be claimed that title compounds (2a-2f) were obtained as a single isomer.



Scheme 1. Synthesis of thiosemicarbazide 1 and thiosemicarbazones 2a-2f. (*i*) Ethanol, reflux; (*ii*) aldehyde derivatives, ethanol, reflux

Anticholinesterase Activity

The newly prepared thiosemicarbazones 2a-2f were screened *in vitro* for their ability to inhibit cholinesterase enzymes (AChE and BuChE) using Ellman's method with minor modifications [31]. The inhibition potencies of the compounds were compared to the well-known AChE inhibitor galantamine. The results were presented in Table 1. At the concentration of 50 µM, Galantamine showed anti-AChE and anti-BuChE activity with 97.89% and 62.48%, respectively. In the series, compound **2c** demonstrated the most inhibitory activity against AChE with 18.29%. Tested compounds showed weak inhibitory activity against cholinesterase enzymes.

Table 1. % Cholinesterase inhibitory activities of the synthesized compounds 2a-2f at 50 µM reaction concentrations.

Compound	AChE	BuChE
2a	3.68 ± 0.009	NA*
2b	11.86 ± 0.002	NA
2c	18.29 ± 0.013	4.86 ± 0.006
2d	16.96 ± 0.009	7.18 ± 0.002
2e	14.80 ± 0.005	NA
2f	7.27 ± 0.006	NA
Gal HBr	97.89 ± 0.01	62.48 ± 0.01

*NA: non-active

Antioxidant Activity

Ferrous ion-chelating effects

The ferrous ion chelating activities of the compounds 2a-2f, rutin and BHT are shown in Table 2. The results showed that the compounds 2a, 2b and 2e demonstrate a marked capacity for iron binding. The ferrous ion-chelating activity of the most active compounds and references decreased in the order of rutin > 2b > 2a > 2e > BHT, which were 13.21, 5.26, 4.13, 2.59 and 2.57 (%), at 50 µM concentration, respectively.

DPPH radical scavenging activity

The DPPH radical-scavenging activity of the newly synthesized compounds 2a-2f was determined using gallic acid as a reference and IC_{50} of the most active compounds were calculated (Table 2). According to the results, compounds 2b, 2c and 2f indicated free radical-scavenging effects of 59.25%, 41.28% and 43.95%, respectively, compared with that of gallic acid of 70.29% at a concentration of 50 µM IC_{50} values of the most potent compounds 2b, 2c and 2f were found 43.91, 59.26, 58.01 µM, respectively, while that of gallic acid was found 29.48 µM It can be claimed that 5-nitrofuran (2b), 5-hydroxymethylfuran (2c) and 5-nitrothiophene (2f) groups have a positive contribution on the DPPH radical-scavenging activity.

The -NH group of thiosemicarbazone scaffold in the structure of 2a-2f compounds, is thought to react with DPPH free radicals by giving hydrogen atom according to the (DPPH• + R-NH → DPPH-H + R-N•) reaction. It can be stated that weaker hydrogen bonds are necessary for higher antioxidant activity. In the light of this, nitro (-NO_2) group bearing compounds 2b and 2f and hydroxymethyl (-CH_2OH) possessing compound 2c showed the strongest DPPH radical-scavenging activity of all the tested compounds. Because the bond strength between nitrogen and hydrogen atoms weakens in the presence of electron-withdrawing groups near the donor atom, which causes easier loss of the hydrogen atom. [35]

Table 2. DPPH free radical-scavenging activity and ferric ion chelating effect (inhibition % ± S.E.M) of synthesized compounds at 50 µM and IC_{50} values (µM)

Compound	DPPH	ION CHELATING	IC_{50} (DPPH) µM
2a	26.65 ± 0.014	4.13 ± 0.003	> 60
2b	59.25 ± 0.021	5.26 ± 0.001	43.91 ± 0.021
2c	41.28 ± 0.005	NA*	59.26 ± 0.005
2d	26.43 ± 0.004	0.62 ± 0.001	> 200
2e	39.87 ± 0.023	2.59 ± 0.002	> 200
2f	43.95 ± 0.032	0.54 ± 0.001	58.01 ± 0.032
Gallic Acid	70.29 ± 0.005	4.13 ± 0.003	29.48 ± 0.014
RUTIN	-	13.21 ± 0.007	-
BHT	-	2.57 ± 0.004	-

*NA: non-active

Conclusions

In this study, six thiosemicarbazone derivative were synthesized and examined in terms of anti-cholinesterase and antioxidant activities. All tested compounds showed weak inhibitory activity against cholinesterase enzymes. The ferrous ion-chelating activity of the compounds 2a, 2b and 2e were higher than reference BHT at 50 µM concentration. In addition, compounds 2b, 2c and 2f containing electron-withdrawing groups showed more effective antioxidant activity than the reference drug according to the DPPH method.

Conflicts of interest

There are no conflicts of interest in this work.

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