Behaviour of 9-Ethyl-9H-carbazole Hydrazone Derivatives Against Oxidant Systems: Protective Effect on Amyloid β-Induced Damage

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Abstract: Antioxidants are helpful in prevention of several diseases related with oxidative stress including neurodegenerative disorders. In recent studies, carbazoles were given proof of promising antioxidant activities. In this article, 9-ethyl-9H-carbazole hydrazone derivatives were synthesized, characterized and their in vitro antioxidant activity and possible cytotoxic effects were investigated. Furthermore, protective effect of the synthesized derivatives against amyloid β-induced damage in PC12 neuronal cells was examined by using MTT assay. The newly synthesized carbazoles were found to have radical scavenging activity with a varying potency both in cell-free and cell-based in vitro assays. Several compounds, especially such as 3d and 3e, 3m and 3n bearing two halogen groups on the phenyl ring, were found to have cytotoxic activity. However, their cytotoxic activities were not higher than that of melatonin. Several compounds also significantly protected neuronal PC12 cells against amyloid β-induced damage, which can be defined as neuroprotective agents. (4-(2-((9-ethyl-9H-carbazol-3-yl)methylene)hydrazinyl)benzonitrile) 3r was found as the most active compound with both radical scavenging activity and neuroprotective effects against amyloid β-induced damage. These findings might provide an alternative strategy for developing novel carbazole derivatives for management of neurodegenerative diseases, such as Alzheimer’s disease.

Keywords: carbazole, antioxidant, cytotoxicity, alzheimer’s disease, β-amyloid.

INTRODUCTION

ARBazoLES represent a family of tricyclic compounds which are widely found in nature. Several studies have been conducted to reveal biological effects of these compounds since last decade. Therefore, synthesis of substituted carbazoles have attracted considerable attention.1,2 Carbazole derivatives have been reported to demonstrate diverse biological activities such as antimicrobial, anti-tumor, antiepileptic, antihistaminic, antioxidant, anti-inflammatory, analgesic, neuroprotective and pancreatic lipase inhibition properties.2,3 Carbazole structure is already present in many drugs such as carvedilol,4 an antihypertensive; carprofen,4 a non-steroidal anti-inflammatory agent, carazostatin,5 an antioxidant (Figure 1).

N-substituted carbazoles have been also well known for their variety of pharmacological activities, such as antioxidant6 and antitumor7 properties.

![Figure 1: Biologically active compounds in carbazole scaffold.](image-url)

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Antioxidant effects of carbazole derivatives are specially focused in many studies. Thus, 1,3-dihydroxy-2-carboxycarbazole analogues exhibited antioxidative properties, promising neuroprotective activities in a cellular model of Alzheimer’s disease (AD) and inhibitory activity against Aβ aggregation. In another study 1- and 3-substituted carbazole derivatives were observed with significant neuroprotective effects on neuronal cells HT22 against cell injury. Substituents introduced to the carbazole ring system play crucial roles in their biological activities. It was found that bulky groups favour the neuroprotective activity,\(^{[9]}\) 3-Oxygenated and 3,4-dioxygenated carbazole alkaloids and their related carbazoles such as 3,8-dihydroxycarbazoles carbazomadurin A and B showed higher antioxidant activities.\(^{[10]}\) Bandgar et al.\(^{[11]}\) examined antioxidative properties, promising neuroprotective activities in a cellular model of Alzheimer’s disease (AD) and inhibitory activity against Aβ aggregation.

Due to the plaque formation caused by cytosolic accumulation of amyloid β-peptide (Aβ), many studies have focused on designing new molecules that are able to inhibit Aβ aggregation. Saturnino et al.\(^{[18]}\) found out two promising N-alkyl carbazole derivatives by docking simulations which also exhibited the higher activity in vitro. N-substituted carbazoles have been reported as neuroprotective agents with potent anti-oxidative activity performing good neuroprotective effects on neuronal cells HT22 against cell injury induced by glutamate or homocysteic acid.\(^{[9]}\)

In the light of these observations, it was taken an interest in synthesizing a new class of carbazole-hydrazone derivatives in order to study the possible antioxidant activity and protection of neuronal PC12 cells against amyloid β-induced damage. Herein, we report the synthesis of 9-ethyl-9H-carbazole hydrazine derivatives starting from 9-ethyl-9H-carbazol-3-carboxaldehyde and phenyl hydrazine derivatives. Their potential antioxidant activity was investigated by evaluating their reducing effect against oxidation of a redox sensitive fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA), in a cell-based in vitro model and their radical scavenging activity was assessed via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay in a cell-free in vitro model. MT1 (4-5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate cytotoxic activity of the compounds and neuroprotective effect was examined in PC12. All the analogue compounds were characterized on the basis of \(^1\)H and \(^13\)C NMR and mass spectra and elemental analysis.

**EXPERIMENTAL**

The chemical reagents used in synthesis were purchased from Sigma (Germany) and Aldrich (USA). Uncorrected melting points were determined with a Buchi SMP-20 apparatus. The \(^1\)H and \(^13\)C NMR spectra were measured with a Varian 400 MHz using TMS internal standard and DMSO-d_6 as solvent. ESI Mass spectra were determined on a Waters micromass ZQ. FT-IR spectra were recorded on a Waters micromass ZQ. FT-IR spectra were recorded on Jasco 420 Fourier. Elemental analyses were performed using CHNS-932 (LECO). All spectral analyses were performed at Ankara University Faculty of Pharmacy Central Laboratory.

RPMI 1640 was obtained from Atocel (Australia). Amyloid-β peptide (residues 25 to 35, Aβ25-35) was purchased from Tocris Bioscience (USA). Penicillin / streptomycin, sterile phosphate-buffered saline (PBS), and tryptophan EDTA 0.25 % were purchased from Shell Max. Fetal bovine serum and horse serum were acquired from Thermo Scientific (USA), while dimethyl sulfoxide was obtained from Merck (Germany).

**Figure 2.** General formula of (a) Antioxidant 3-(substituted)-aryl-5-(9-methyl-3-carbazole)-1H-2-pyrazolines and (b) synthesized 9-ethyl-9H-carbazole hydrazone derivatives.
Chemistry
For the synthesis of new carbazole imine compounds (3a–3r), phenyl hydrazine derivatives and 9-ethyl-9H-carbazole-3-carboxaldehyde were heated in the presence of ethanol. All the compounds were characterized on the basis of spectral data. The characterization results of the novel compounds 3e and 3m–r are given as follows. Compounds 3a–d and 3f–l were previously described by Aboul-Enein.[24]

General Procedure for the Synthesis of Compounds
9-Ethyl-9H-carbazole-3-carboxaldehyde (0.1 mmol) was reacted with phenyl hydrazine or its derivatives (0.13 mmol) in 20 mL of EtOH for 3 h on the hot water bath. On cooling, the precipitate was collected washed with cold EtOH to give the final compounds with 45–87 % yield.

3-((2-(3,5-CHLOROPHENYL)HYDRAZONOMETHYL)-9-ETHYL-9H-CARBAZOLE (3e)
Yield 58.6 %; m.p. 154–157 °C; 1H NMR (DMSO): δ = 1.30 (3H, t, CH3), 4.41 (2H, q, CH2), 6.79 (1H, d, J = 1.6 Hz, H-4''), 7.06 (1H, d, J = 1.2 Hz, H-2', 6''), 7.20 (1H, t, J = 7.6 Hz, H-6), 7.46 (1H, t, J = 8 Hz, H-7), 7.58-7.61 (2H, m, H-5, 8'), 7.85 (1H, dd, J = 8.8 Hz, J = 1.6 Hz, H-2), 8.09 (1H, s, -NH2), 8.21 (1H, d, J = 7.2 Hz, H-1), 8.40 (1H, d, J = 1.2 Hz, H-4), 10.69 (1H, s, -CHOH). 13C NMR: δ = 13.7, 37.0, 109.3, 109.4, 109.8, 116.8, 119.0, 120.6, 122.1, 122.3, 128.3, 125.9, 126.0, 134.6, 139.9, 140.9, 147.8. ESI-MS: m/z = 383 [M+1] (100 %), 385 [M+1+2] (65 %), 387 [M+1+4] (37 %). Found, %: C 64.47; H 4.58; N 10.73. C21H17N3Cl2O 0.5 H2O Calcd, %: C 71.08; H 6.45; N 11.74.

3-((2-(3,5-FLUOROPHENYL)HYDRAZONOMETHYL)-9-ETHYL-9H-CARBAZOLE (3m)
Yield 81.3 %; m.p. 112–116 °C; 1H NMR (DMSO): δ = 1.31 (3H, t, CH3), 4.44 (2H, q, CH2), 6.46-6.51 (1H, m, H-6'), 7.11–7.22 (2H, m, H-6', 3'), 7.29-7.34 (1H, m, H-4'), 7.45 (1H, td, J = 7.2 Hz, H-7), 7.59-7.63 (2H, m, H-5, 8'), 7.84 (1H, dd, J = 8.4 Hz, J = 1.6 Hz, H-2), 8.25 (1H, d, J = 7.2 Hz, H-1), 8.32 (1H, s, -NH2), 8.41 (1H, d, J = 10.4 Hz, H-10), 3.21 (1H, s, -CHOH). ESI-MS: m/z = 350 [M+1] (100 %). Found, %: C 71.85; H 5.32; N 10.54. C21H17N3F2O 0.2 H2O Calcd, %: C 71.06; H 4.97; N 11.90.

3-((2-(3,5-FLUOROPHENYL)HYDRAZONOMETHYL)-9-ETHYL-9H-CARBAZOLE (3n)
Yield 66.5 %; m.p. 148–151 °C; 1H NMR (DMSO): δ = 1.30 (3H, t, CH3), 4.43 (2H, q, CH2), 6.40-6.46 (1H, m, H-4'), 6.71 (2H, dd, J = 10.4 Hz, J = 2.4 Hz, H-2', 6''), 7.20 (1H, t, J = 7.6 Hz, H-6), 7.45 (1H, td, J = 7.2 Hz, J = 1.2 Hz, H-7), 7.58-7.62 (2H, m, H-5, 8'), 7.85 (1H, dd, J = 8.4 Hz, J = 1.6 Hz, H-2'), 8.07 (1H, s, -NH2), 8.22 (1H, d, J = 7.6 Hz, H-1), 8.42 (1H, d, J = 1.2 Hz, H-4), 10.63 (1H, s, -CHOH). 13C NMR: δ = 13.7, 37.0, 92.2, 92.5, 92.8, (94.3, 94.6), (109.2, 109.3), (118.9, 119.0), 120.6, (122.1, 122.3), 123.9, (125.9, 126.1), (139.8, 139.9), 140.4, 148.1, 148.3, 148.4, (162.2, 162.3), (164.5, 164.7). ESI-MS: m/z = 350 [M+1] (100 %). Found, %: C 71.39; H 5.23; N 11.98. C21H17N3F2O 0.2 H2O Calcd, %: C 71.46; H 4.97; N 11.90.
**4-[2-((9-ETHYL-9H-CARBAZOL-3-YL)-METHYL)HYDRAZINYL]BENZONITRILE (3r)**

Yield 86.9 %; m.p. 195–199 °C; 1H NMR (DMSO): δ = 1.30 (3H, t, CH₃), 2.12 (3H, s, CH₃(phenyl)), 4.42 (2H, q, CH₂), 7.17–7.23 (3H, m, H-6,2',6'), 7.45 (1H, td, J = 8.4 Hz, J = 1.2 Hz, H-7), 7.59-7.63 (4H, m, H-5,8,3',5'), 8.71 (1H, dd, J = 8.8 Hz, J = 1.6 Hz, H-2), 8.15 (1H, s, -NH), 8.21 (1H, d, J = 7.6 Hz, H-1), 8.42 (1H, d, J =1.2 Hz, H-4), 10.89 (1H, s, -CH=N). 13C NMR: δ = 13.7, 37.0, 98.4, 109.3, 109.4, 111.8, 119.1, 120.2, 120.5, 122.1, 122.3, 123.9, 125.9, 126.0, 133.5, 139.8, 139.9, 141.5, 148.8. ESI-MS: m/z = 339 [M+1] (100 %). Found, %: C 77.02; H 5.65; N 16.29.

**Biological Activity**

**CELL CULTURE AND REAGENTS**

CHO-K1 cells were purchased from ATCC and maintained at 37 °C in 5 % CO₂ atmosphere. Dulbecco’s modified Eagle’s medium (DMEM) / F12 (Sigma, St. Louis, MO) (1 : 1) nutrient mixture medium supplemented with 10 % foetal bovine serum, 1 % of a 100 U / mL penicillin-streptomycin solution, 2 mM L-glutamine and 1 mM sodium pyruvate was used in all cell incubations. Hyclone characterized fetal bovine serum (FBS) was purchased from Thermo Scientific. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 7-ethoxyresorufin, sodium pyruvate and L-glutamine were obtained from Sigma (St. Louis, MO).

**CYTOTOXIC EFFECTS VIA THE MTT ASSAY**

CHO-K1 cells were seeded in 96-well plates (5 x 10³ cells / well) and incubated at 37 °C in a humid atmosphere containing 5 % CO₂ for 24 h. Cells were treated with the newly synthesized compounds (10 µM) for 24 h. Control (medium only), vehicle control and positive control (15 µM Triton X-100) were included in every experiment. Following the exposure period, the medium was removed, cells were washed with phosphate buffered saline (PBS) and then incubated with MTT (1 mg mL⁻¹) for 4 h at 37 °C. MTT solution was removed and formazan crystals were dissolved in DMSO. The absorbance was recorded at 550 nm on a microplate reader. The ratio of the absorbance of treated samples to the absorbance of control (taken as 100 %) was expressed as % cell viability.

**ANTIOXIDANT ACTIVITY ON ROS-INDUCED DCFH-DA OXIDATION**

For estimation of intracellular ROS, DCFH-DA was used as a probe. In cellular systems the non-fluorescent probe DCFH-DA readily crosses the cell membrane and undergoes hydrolysis by intracellular esterases to non-fluorescent 2′,7′-dichlorofluorescin (DCFH). In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescin (DCF) which can be detected by a fluorescent microplate reader.

Cells were seeded in black 96-well plates at a density of 5 x 10³ cells / well and incubated at 37 °C in a humid atmosphere containing 5 % CO₂ for 24 h for cell attachment. The medium was removed and then cells were incubated with DCFH-DA (20 µM) containing medium for 30 minutes. Cells were washed with PBS to remove excess DCFH-DA. 10 µM synthesized compounds and 10 µM cumene hydroperoxide (CMHP) were added into medium. The production of fluorescent DCF was evaluated by monitoring fluorescence intensity at 488 nm excitation, 530 nm emission wavelengths for 60 minutes.

**DPHH FREE RADICAL SCAVENGING ACTIVITY**

Free radical scavenging activity of newly synthesized compounds was tested by their ability to bleach the stable radical DPPH. DPPH presents a maximum of absorbance at 515 nm; when DPPH reacts with an antioxidant compound, which can donate hydrogen, this absorbance diminishes and can be measured on a visible spectrophotometer. 20 µL of the samples (100 µM) were added to 180 µL of DPPH solution (150 µM in methanol-water (80 : 20, v / v) in 96-well plates, incubated for 30 minutes at room temperature then DPPH reduction was estimated at 517 nm. Percentage inhibition by the sample treatment was determined by comparison with a DMSO-treated control group. All experiments were carried out in triplicate. MLT and butylated hydroxytoluene (BHT) were used as a reference compounds. The radical scavenging activities were expressed as the percent of inhibition and were calculated by the below formula:

\[
\text{Radical scavenging activity (%) = } \left[1 - \frac{A_0 - A_t}{A_0}\right] \times 100
\]
where \( A_0 \) is the absorbance of the control (blank) and \( A_1 \) is the absorbance of the test compound.

INHIBITION OF NEUROTOXICITY INDUCED BY A\(\beta\) IN PC12 CELLS

Cell viability after exposure to A\(\beta_{25-35}\) was determined by MTT assay, which was performed as previously described with some modifications. \[27\] Briefly, the cells were seeded in collagen-coated 96-well plates at a density of 5 \times 10^4 cells / well and were allowed to adhere at 37 °C for 48 h. Three different concentrations of carbazole derivatives (5, 25 and 100 \(\mu\)M) were added in duplicate and incubated for 3 h. Maximum concentration of DMSO in the wells was kept below 0.5 %. A\(\beta_{25-35}\) was then added at a final concentration of 5 \(\mu\)M for an additional 24 h in the presence or absence of test compounds. After 24 h, the medium was replaced with 20 \(\mu\)L of MTT 0.5 mg mL \(^{-1}\) dissolved in RPMI and incubated for an additional 2 h at 37 °C. Afterwards, formazan crystals were solubilized in 200 \(\mu\)L DMSO. The optical density was measured at 570 nm with background correction at 655 nm using a Bio-Rad microplate reader (Model 680, Bio-Rad). Each experiment was repeated 3–5 times. The percent inhibition of cell damage was calculated with reference to the absorbance of control wells not treated with A\(\beta\) (assumed as 100 % protection) and the absorbance of wells treated with A\(\beta\) in the absence of any test compound (assumed as 0 % protection).

Statistical Analysis

Unpaired Student t-test was performed to evaluate the significance of the differences between groups. Differences with \( p < 0.05 \) were considered as statistically significant.

RESULTS AND DISCUSSION

Chemistry

Synthesis of all described carbazole hydrazone derivatives were carried out according to the method described in literature\[24\] (Scheme 1). 9-Ethyl-9H-carbazole-3-carboxaldehyde was heated with several phenyl hydrazine derivatives in the presence of ethanol to obtain carbazole hydrazone derivatives.

Cytotoxic Effects of the Synthesized Compounds

The failure of early identification of toxic effects of compounds resulted in attrition of new drug candidates,\[19\] which cause important losses for pharmaceutical industry. Therefore, screening for cytotoxicity has become one of the most important steps in early phases of drug discovery. In the present study cytotoxicity of the synthesized carbazole-hydrazone derivatives were evaluated in CHO-K1 cells by using a tetrozolium salt, MTT. The assay based on the reduction of MTT only by metabolically active cells to a colored formazan product that can be quantified spectrophotometrically.\[20\] Almost all compounds (10 \(\mu\)M) were found to have no cytotoxic effect more than melatonin (MLT), an endogenous indole compound with known antioxidant and anticarcinogenic activity (Figure 2) on CHO-K1 cells when incubated for 24 hours. Only 3e and 3k decreased cell viability more than MLT. Among halogenated carbazole derivatives chloro- and fluoro-substituted ones were found to be more cytotoxic than bromo-substituted compounds. The substances without any halogen group and with methoxy substitution on aromatic ring 3p, 3q, 3r, did not have any effects on cell viability (Figure 3).

Antioxidant Effects of the Synthesized Compounds

The protective effect of synthesized carbazole-hydrazone derivatives (10 \(\mu\)M) against cumene hydroperoxide induced DCFH-DA oxidation was determined in CHO-K1 cells that were preloaded with the fluorescent probe. Oxidation of the probe located in the cytosol was detected after 60 min of incubation (Figure 4). All of the carbazole derivatives, except 3b, 3c, 3g and 3j had potent reducing activity against DCFH oxidation, even higher than MLT. Compounds 3o, 3p, 3q and 3r bearing no halogen substituent, were found to have reducing activity where 3r was found to be the most potent antioxidant among all tested compounds. Among the halogenated derivatives fluoro-substituted ones were found to have better antioxidant effects compared to chloro- and bromo-halogenated derivatives.

Free radical scavenging activity of the newly synthesized compounds was further investigated in a cell free in vitro model where an active radical, DPPH was used.
The radical scavenging activity of the compounds was detected via decrease in the absorbance of DPPH. All of the synthesized compounds had radical scavenging activity in tested concentration (100 µM) where MLT was found to have the lowest effect (Figure 5). Among mono-halogenated derivatives m-substituted derivatives were found to have higher activity and p-halogenation was found to decrease antioxidant activity which was similar to our previous findings.[21,22] 3r was found to be the most potent radical scavenger among all tested compounds similar to its reducing effect found in DCFH assay (Figure 4).

Antioxidant molecules have proved to be effective in AD mouse models and small clinical studies. Clinical trials for AD prevention and treatment by antioxidants are still under discussion. Hence, advance studies are necessary to determine if antioxidants may decrease the risk or slow the progression of the disease for AD patients.[23]

Protection of Neuronal PC12 Cells Against Aβ-Induced Damage

Neuroprotective capacity of synthesized derivatives was assessed in PC12 neuronal cells challenged with Aβ, which is a well-established cell model of neurodegeneration. None of the compounds showed any cytotoxicity against the cells when incubated with the cells alone (data not shown). Several carbazole derivatives showed significant protection against Aβ at 25 and 100 µM including 3d, 3e, 3h and 3n. It was remarkable to note that compound 3r showed a high protection against Aβ damage at the lower dose of 25 µM (Figure 6).
Comounds with electron withdrawing moieties on the phenyl ring including those bearing Cl (3–3f), Br (3–3l) and F (3–3n) showed much better protective effects in PC12 cells compared to derivatives bearing electron donating groups including the ones containing methoxy (3o) and methyl moieties (3p–3q) on the phenyl ring. Among halogen containing synthesized compounds, the ones bearing two halogen groups on the phenyl ring, including 3d and 3e with 3,4-dichlorophenyl and 3,5-dichlorophenyl rings, respectively, as well as 3m and 3n, with 2,5-dibromophenyl and 3,5-dibromophenyl rings, respectively, were more effective than those bearing only one halogen group.

CONCLUSION

A series of carbazole-hydrazone derivatives was synthesized and evaluated for their cytotoxicity, antioxidant potential and protective effect against amyloid β-induced damage in neuronal PC12 cells. The results pointed out that almost all compounds were found to have no cytotoxic effect more than the known antioxidant, melatonin (MLT).

Most of the carbazole derivatives were found to have reducing potential against DCFH oxidation even higher than MLT. This higher reducing potential may be related with halogen- substituted aromatic moieties. Compounds bearing halogen substituents were found to have higher activities, especially fluoro-substituted compounds that had the highest reducing potential.

The synthesized novel compounds also exhibited good DPPH radical scavenging activity. Particularly m-substituted derivatives were found to have higher activity than rest of the compounds. The most significant compound was 3r regarding the antioxidant activity tests. Furthermore, compound 3r showed a high protection against Aβ damage at the lower dose of 25 µM. Correlation between the activities of 3r as antioxidant and Aβ damage protection is promising for further development of carbazole-hydrazone derivatives as future AD drug.

Antioxidant molecules have proved to be effective in AD mouse models and small clinical studies. Clinical trials for AD prevention and treatment by antioxidants are still under discussion. Hence, advance studies are necessary to determine if antioxidants may decrease the risk or slow the progression of the disease for AD patients.12

These preliminary results are promising and we are confident that the N-alkyl carbazole derivatives may represent new leads in the search for novel AD therapy.

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