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ORIGINAL ARTICLE

3-(1H-pyrazole-1-yl/1H-1,2,4-triazole-1-yl)-N-propananilide Derivatives: Design, Synthesis and Neuroprotectivity Potential Against 6-OHDA Induced Neurotoxicity Model

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ABSTRACT

Objectives: Excessive amounts of neuroapoptosis are the underlying cause of neurodegenerative diseases. Bax is a pro-apoptotic member of the B-cell lymphoma-2 family that activates caspases which are the members of the cysteine protease family that play a significant role in the initiation and execution phases of apoptosis. The aim of this study was to design and synthesize a group of N-propananilide derivatives bearing pyrazole or 1,2,4-triazole ring were designed and synthesized to analyze the neuroprotectivity potential against 6-hydroxy-dopamine (6-OHDA). Four compounds possessed protectivity at lower doses were subjected to further studies on caspase-3 and Bax pathway.

Materials and Methods: Designed compounds were synthesized by reacting 1H-pyrazole or 1H-1,2,4-triazole with propananilide intermediates in Dimethylformamide. The neuroprotective activity of the title compounds was analyzed against 6-OHDA-6-OHDA-induced neurotoxicity model. Then, caspase-3 and Bax levels were determined for the selected compounds by Western blot study.

Results: All twelve 3-(1H-pyrazole-1-yl/1H-1,2,4-triazole-1-yl)-N-propananilide derivatives possessed neuroprotectivity against the 6-OHDA-induced neurotoxicity model ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$). Compounds 7, 10, 11, and 12 were found to be more active at lower doses. They were subjected to further studies and the results revealed that their protecting activity arose from the decreasing levels of Bax, one of the pro-apoptotic proteins, and c expression levels and caspase-3 proteins.

Conclusion: All designed and synthesized derivatives possessed neuroprotectivity against 6-OHDA-induced neurotoxicity in the SH-SY5Y cell line and compounds 7, 10, 11, and 12 revealed that their neuroprotectivity originated from the decreasing Bax expression levels and caspase-3 activation.

Keywords: Neuroprotectivity, caspase-3, Bax protein, 1, 2, 4-triazole, 1H-pyrazole

INTRODUCTION

Physiologically, cell proliferation and death should occur in multicellular organisms.[1] Apoptosis is a programmed cell death process characterized by biochemical and morphological changes that eventually lead to cell death.[2] Central to the execution of apoptosis is a group of proteolytic enzymes known as caspases, which are cysteine proteases. Recently, 14 members of the caspase family have been identified, and 11 of them are found in humans.[3,4] Caspases, directly and indirectly, operate the apoptosis process both directly and

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indirectly.[4] In particular, caspase-3 is a principal effector in the initiation and execution phases of apoptosis. Once caspase-3 is activated in cells, it cleaves a wide range of substrates, leading to biochemical and morphological changes associated with apoptosis.[5]

Proteins of the B-cell lymphoma-2 (Bcl-2) and the mitochondrial pathway are critical intermediates in this pathway, one of the four major pathways that lead to caspase activation.[5] When cells receive apoptotic signals; Bax which is a pro-apoptotic protein that belongs to the Bcl-2 family, undergoes a multistep process to trigger the activation of caspases to execute the apoptotic program leading to cell death.[2,4,5]

Neuronal cells have a different cycle than other cells as they live longer to maintain their routine pathways.[6] Sometimes, redundant amounts and rates of neuronal apoptosis (neuroapoptosis) can occur and trigger neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis (MS), and amyotrophic lateral sclerosis.[3,7]

Based on these findings, it is suggested that apoptosis triggered by increased caspase-3 and Bax plays a significant role in the pathogenesis of neurological disorders. Therefore, inhibition of 6-OHDA-induced neurodegeneration and activation of caspases may be important targets in the treatment of neurodegenerative diseases.

We reported the cholinesterase activity of a group of *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole rings and investigated the neuroprotective potential of the most active derivatives in our previous studies.[8] Based on the promising results on neuroprotectivity and cholinesterase inhibition, we designed *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole rings and tested their cholinesterase activity, but the results were negligible. Therefore, we aimed to focus on their neuroprotective activities. We tested the neuroprotective activity of the synthesized compounds in SH-SY5Y neurotoxicity model induced by 6-OHDA, which is a neurotoxic agent used for neurotoxicity assays based on its ability to be autoxidized to yield potentially toxic products and reactive oxygen species.[9] After conducting neuroprotection studies with selecting poteint compounds with more potential, we further analyzed the underlying mechanism *via* immunoblotting studies of the caspase-3 and Bax proteins for the most active ones.

MATERIALS AND METHODS

Thin-layer chromatography was performed on silica gel plates (Merck, Kieselgel 60F254) and detected using 254 nm ultraviolet (UV) light. The structures of the compounds were verified using infrared spectra (Perkin Elmer Fourier transform infrared spectrometer 100 with attenuated total reflectance (ATR) attachment, Perkin Elmer Inc., Massachusetts, USA), mass spectra [atmospheric pressure chemical ionization-electrospray ionization (APCI-ESI), (Thermo MSQ Plus LC/MS, Thermoscientific Inc., San Jose, CA, USA), and NMR spectra (Varian As 400 Mercury Plus nuclear magnetic resonance (NMR), Varian Inc., Palo Alto, CA, USA). Melting points were determined using a melting point apparatus on a Stuart SMP30 and were uncorrected. Elemental analysis was performed using a TruSpec Micro Instrument LECO CHNS 932. All the starting materials and reagents used for the synthesis were commercial products with high-quality properties.

General procedure for the synthesis of the compounds 1a-6a

Substituted aniline (1 eq.) and K_2CO_3 (1 eq.) were dissolved in the mixture of acetone: water (1:2). 3-chloropropionyl chloride (1 eq.) was added dropwise in an ice bath, and the mixture was stirred at room temperature for 2 h. The reaction mixture was then poured into cold water, and the precipitate was filtered and washed with water.[10]

General procedure for the synthesis of the compounds 1-12

ω-Chloro-*N*-propananilides (1 eq.), pyrazole or triazole (1 eq.), and K_2CO_3 (1 eq.) were dissolved in dimethylformamide (DMF) and allowed to react under reflux. After monitoring the end of the reaction, DMF was evaporated under reduced pressure, and the residue was extracted using chloroform and water. The organic phases were evaporated after drying over anhydrous Na_2SO_4.[11] Crude products with various mobile phases were purified by column chromatography.

N-(2-chlorophenyl)-3-(1H-pyrazol-1-yl propanamide (1)

Yield, 30%; *m.p.*, 102 °C; IR (ATR) ν_{max}. (cm^{-1}): 3279 (NH), 1650 (amide I), 1533 (amide II); ^1H NMR ($CDCl_3$, 400 MHz) δ 3.04 (t, 2H, *J*=6.3 Hz, CH_2), 4.53 (t, 2H, *J*=6.3 Hz, CH_2), 6.21 (t, 1H, *J*=2.1 Hz, Py-H), 7.03 (td, 1H, *J*=7.8; 1.6 Hz, Ph-H), 7.22-7.26 (m, 1H, Ph-H), 7.33 (dd, 1H, *J*=8.1; 1.5 Hz, Ph-H), 7.45 (d, 1H, *J*=2.3 Hz, Py-H), 7.54 (d, 1H, *J*=1.5 Hz, Py-H), 7.91 (brs, 1H, NH), 8.27 (d, 1H, *J*=8.3 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 123 (100), 250 (73) $[M+H]^+$, 252 (23) $[M+2+H]^+$; Anal. Calcd for $C_{12}H_{12}ClN_3O$ (249): C: 57.72; H: 4.84; N: 16.83. Found C: 57.60; H: 4.99; N: 16.42.

N-(3-chlorophenyl)-3-(1H-pyrazol-1-yl)propanamide (2)

Yield, 72%; *m.p.*,76 °C; IR (ATR) ν_{max}. (cm^{-1}): 3236 (NH), 1685 (amide I), 1590 (amide II); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.95 (t, 2H, *J*=6.1 Hz, CH_2), 4.51 (t, 2H, *J*=6.1 Hz, CH_2), 6.25 (t, 1H, *J*=2.1 Hz, Py-H), 7.04-7.07 (m, 1H, Ph-H), 7.19 (t, 1H, *J*=8.0 Hz, Ph-H), 7.26-7.29 (m, 1H, Ph-H), 7.46 (d, 1H, *J*=2.3 Hz, Py-H), 7.57-7.64 (m, 2H, Ph-H and Py-H), 8.53 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 250 (100) $[M+H]^+$, 252 (23) $[M+2+H]^+$; Anal. Calcd for $C_{12}H_{12}ClN_3O$ (249): C: 57.72; H: 4.84; N: 16.83. Found C: 57.38; H: 5.07; N: 16.46.

N-(4-chlorophenyl)-3-(1H-pyrazol-1-yl)propanamide (3)

Yield, 59%; *m.p.*, 151 °C; IR (ATR) ν_{max}. (cm^{-1}): 3246 (NH), 1677 (amide I), 1538 (amide II); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.83 (t, 2H, *J*=6.4 Hz, CH_2), 4.40 (t, 2H, *J*=6.4 Hz, CH_2), 6.09-6.10 (m, 1H, Py-H), 7.09-7.13 (m, 2H, Ph-2H), 7.36-7.42 (m, 4H, Ph-2H and Py-2H), 9.25 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 123 (97), 250 (100) $[M+H]^+$, 252 (32) $[M+2+H]^+$; Anal. Calcd for $C_{12}H_{12}ClN_3O$ (249): C: 57.72; H: 4.84; N: 16.83. Found C: 57.52; H: 4.88; N: 16.50.

N-(2-methoxyphenyl)-3-(1H-pyrazol-1-yl)propanamide (4)

Yield, 62%; *m.p.*, 88 °C; IR (ATR) ν_{max}. (cm^{-1}): 3374 (NH), 1681 (amide I), 1534 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.98 (t, 2H, *J*=6.5 Hz, CH_2), 3.83 (s, 3H, OCH_3), 4.53 (t, 2H, *J*=6.5 Hz, CH_2), 6.19 (t, 1H, *J*=2.1 Hz, Py-H), 6.84 (dd, 1H, *J*=8.1; 1.4 Hz, Ph-H), 6.91-6.95 (m, 1H, Ph-H), 7.03 (td, 1H, *J*=7.8; 1.7 Hz, Ph-H), 7.45-7.46 (m, 1H, Py-H), 7.51-7.52 (m, 1H, Py-H), 7.84 (brs, 1H, NH), 8.29-8.31 (m, 1H, Ph-H) ppm; MS (APCI) *m/z* (% intensity): 123 (100), 246 (86) $[M+H]^+$; Anal. Calcd for $C_{13}H_{15}N_3O_2$ (245): C: 63.66; H: 6.16; N: 17.13. Found C: 63.30; H: 6.48; N: 16.84.

N-(3-methoxyphenyl)-3-(1H-pyrazol-1-yl)propanamide (5)

Yield, 66%; *m.p.*, 96 °C; IR (ATR) ν_{max}. (cm^{-1}): 3262 (NH), 1692 (amide I), 1491 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.94 (t, 2H, *J*=6.1 Hz, CH_2), 3.78 (s, 3H, OCH_3), 4.51 (t, 2H, *J*=6.2 Hz, CH_2), 6.23 (t, 1H, *J*=2.1 Hz, Py-H), 6.62-6.65 (m, 1H, Ph-H), 6.91 (d, 1H, *J*=7.8 Hz, Ph-H), 7.15-7.19 (m, 2H, Ph-2H), 7.45 (dd, 1H, *J*=2.3; 0.7 Hz, Py-H), 7.55-7.56 (m, 1H, Py-H), 8.14 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 246 (100) $[M+H]^+$; Anal. Calcd for $C_{13}H_{15}N_3O_2$ (245): C: 63.66; H: 6.16; N: 17.13. Found C: 63.70; H: 6.24; N: 16.70.

N-(4-methoxyphenyl)-3-(1H-pyrazol-1-yl)propanamide (6)

Yield, 14%; *m.p.*, 127 °C; IR (ATR) ν_{max}. (cm^{-1}): 3297 (NH), 1654 (amide I), 1529 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.91 (t, 2H, *J*=6.2 Hz, CH_2), 3.77 (s, 3H, OCH_3), 4.51 (t, 2H, *J*=6.2 Hz, CH_2), 6.23 (t, 1H, *J*=2.1 Hz, Py-H), 6.82 (d, 2H, *J*= 9.0 Hz, Ph-2H), 7.31 (d, 2H, *J*= 9.0 Hz, Ph-2H), 7.45 (d, 1H, *J*=2.3 Hz, Py-H), 7.55 (d, 1H, *J*=1.9 Hz, Py-H), 7.93 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 178 (100), 246 (91) $[M+H]^+$; Anal. Calcd for $C_{13}H_{15}N_3O_2$ (245): C: 63.66; H: 6.16; N: 17.13. Found C: 63.25; H: 5.98; N: 16.83.

N-(2-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (7)

Yield, 7%; *m.p.*, 98 °C; IR (ATR) ν_{max}. (cm^{-1}): 3129 (NH), 1691 (amide I), 1507 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.04 (t, 2H, *J*=6.2 Hz, CH_2), 4.59 (t, 2H, *J*=6.2 Hz, CH_2), 7.03-7.07 (m, 1H, Ph-H), 7.23-7.27 (m, 1H, Ph-H), 7.34 (dd, 1H, *J*=8.1; 1.5 Hz, Ph-H), 7.67 (brs, 1H, NH), 7.95 (s, 1H, Tr-H), 8.17 (s, 1H, Tr-H), 8.24 (d, 1H, *J*=8.4 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 251 (100) $[M+H]^+$, 253 (30) $[M+2+H]^+$; Anal. Calcd for $C_{11}H_{11}ClN_4O$ (250): C: 52.70; H: 4.42; N: 22.35. Found C: 52.79; H: 4.79; N: 22.18.

N-(3-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (8)

Yield, 38%; *m.p.*, 167 °C; IR (ATR) ν_{max}. (cm^{-1}): 3122 (NH), 1690 (amide I), 1540 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.82 (t, 2H, *J*=6.3 Hz, CH_2), 4.42 (t, 2H, *J*=6.3 Hz, CH_2), 6.86-6.89 (m, 1H, Ph-H), 7.05 (t, 1H, *J*=8.1 Hz, Ph-H), 7.24-7.27 (m, 1H, Ph-H), 7.53 (t, 1H, *J*=2.0 Hz, Ph-H), 7.76 (s, 1H, Tr-H), 8.06 (s, 1H, Tr-H), 9.47 (brs, 1H, NH) ppm; MS (APCI) *m/z* (% intensity): 251 (100) $[M+H]^+$, 253 (29) $[M+2+H]^+$; Anal. Calcd for $C_{11}H_{11}ClN_4O$ (250): C: 52.70; H: 4.42; N: 22.35. Found C: 52.77; H: 4.49; N: 21.98.

N-(4-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (9)

Yield, 59%; *m.p.*, 136 °C; IR (ATR) ν_{max}. (cm^{-1}): 3124 (NH), 1697 (amide I), 1547 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.95 (t, 2H, *J*=6.1 Hz, CH_2), 4.57 (t, 2H, *J*=6.1 Hz, CH_2), 7.26 (d, 2H, *J*=8.8 Hz, Ph-2H), 7.38 (d, 2H, *J*=8.8 Hz, Ph-2H), 7.62 (brs, 1H, NH), 7.95 (s, 1H, Tr-H), 8.16 (s, 1H, Tr-H) ppm; MS (ESI) *m/z* (% intensity): 249 (100) $[M-H]^-$, 253 (28) $[M+2-H]^-$; Anal. Calcd for $C_{11}H_{11}ClN_4O$ (250): C: 52.70; H: 4.42; N: 22.35. Found C: 52.79; H: 4.37; N: 22.05.

N-(2-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (10)

Yield, 84%; *m.p.*, 81 °C; IR (ATR) ν_{max}. (cm^{-1}): 3112 (NH), 1688 (amide I), 1537 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.99 (t, 2H, *J*=6.2 Hz, CH_2), 3.84 (s, 3H, OCH_3), 4.58 (t, 2H, *J*=6.3 Hz, CH_2), 6.84 (dd, 1H, *J*=8.1; 1.4 Hz, Ph-H), 6.93 (td, 1H, *J*=7.8; 1.4 Hz, Ph-H), 7.04 (td, 1H, *J*=7.8; 1.7 Hz, Ph-H), 7.75 (brs, 1H, NH), 7.93 (s, 1H, Tr-H), 8.16 (s, 1H, Tr-H), 8.25 (dd, 1H, *J*= 8.1; 1.6 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 247 (100) $[M+H]^+$; Anal. Calcd for $C_{12}H_{14}N_4O_2$ (246): C: 58.53; H: 5.73; N: 22.75. Found C: 58.11; H: 5.88; N: 22.51.

N-(3-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (11)

Yield, 33%; *m.p.*, 152 °C; IR (ATR) $\nu_{max.}$ (cm^{-1}): 3114 (NH), 1675 (amide I), 1555 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.79 (t, 2H, *J*=6.4 Hz, CH_2), 3.62 (s, 3H, OCH_3), 4.40 (t, 2H, *J*=6.4 Hz, CH_2), 6.44-6.46 (m, 1H, Ph-H), 6.86-6.89 (m, 1H, Ph-H), 7.00 (t, 1H, *J*=8.1 Hz, Ph-H), 7.12 (t, 1H, *J*=2.2 Hz, Ph-H), 7.74 (s, 1H, Tr-H), 8.05 (s, 1H, Tr-H), 9.23 (brs, 1H, NH), ppm; MS (ESI) *m/z* (% intensity): 247 (100) $[M+H]^+$; Anal. Calcd for $C_{12}H_{14}N_4O_2$ (246): C: 58.53; H: 5.73; N: 22.75. Found C: 58.35; H: 5.71; N: 22.41.

N-(4-methoxyphenyl)-3-(1H-1,2,4-triazol-1-yl)propanamide (12)

Yield, 49%; *m.p.*, 129 °C; IR (ATR) ν_{max}. (cm^{-1}): 3113 (NH), 1682 (amide I), 1557 (amide II); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.90 (t, 2H, *J*=6.2 Hz, CH_2), 3.76 (s, 3H, OCH_3), 4.55 (t, 2H, *J*=6.2 Hz, CH_2), 6.81 (d, 2H, *J*=9.0 Hz, Ph-2H), 7.29 (d, 2H, *J*=9.0 Hz, Ph-2H), 7.67 (brs, 1H, NH), 7.93 (s, 1H, Tr-H), 8.15 (s, 1H, Tr-H), 8.25 (dd, 1H, *J*= 8.1; 1.6 Hz, Ph-H) ppm; MS (ESI) *m/z* (% intensity): 178 (29), 247 (100) $[M+H]^+$; Anal. Calcd for $C_{12}H_{14}N_4O_2$ (246): C: 58.53; H: 5.73; N: 22.75. Found C: 58.40; H: 5.72; N: 22.34.

Cell lines and treatments

The SH-SY5Y cell line (human dopaminergic neurons) was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium; (Biological Ind., Israel) containing 10% fetal bovine serum (Panbiotech, Germany), and 2 mM L-glutamine (Biological Ind., Israel) according to the manufacturer's instructions. Cells were seeded at a density of 2 x 10^4 cells per well for the cell viability assay; for the Western blot (WB) study cells were seeded at 5 x 10^5 per well. The following day, cells were pre-treated with desired concentrations of the synthesized compounds for 12 h and then treated with 50 µm 6-OHDA (Sigma Aldrich, UK) for 12 h. Dimethyl sulfoxide (DMSO)-treated cells were used as a negative experimental control.

While 6-OHDA was prepared in sterile water as a 1000X stock solution, the synthesized compounds were prepared in DMSO. The final DMSO concentration was below 0.1%.

Cell viability assay

The potential neuroprotective properties of the synthesized compounds against 6-OHDA-induced neurotoxicity were assessed using a water-soluble tetrazolium salt (WST) reagent (Roche, Switzerland) according to the manufacturer's instructions. The cell viability assay was performed by replacing the old media with a WST/medium mixture (1:9). The absorbance was measured using a microplate reader at 440 and 690 nm (Varioscan, Thermo Fisher Scientific, US). The cell viability is presented as a percentage of cell viability compared to DMSO-treated cells. This experiment was conducted in triplicate.

WB analysis

Cells were harvested using a Radioimmunoprecipitation Assay buffer containing a protease inhibitor cocktail (Roche, Switzerland). The bicinchoninic acid (BCA, Thermo Fisher Scientific, US) protein assay was used to determine total protein levels according to the manufacturer's instructions. Equal amounts of protein were used in WB studies. After denaturation of protein samples in 4X Laemmli buffer (Bio-Rad, US) at 95 °C for 5 min, the samples were first separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis then transferred to polyvinylidene fluoride membranes (EMD Millipore, Thermo Fisher Scientific, US). Next, the membranes were blocked with blocking buffer (phosphate-buffered saline-0.1% tween-20 with 5% non-fat dry milk). In this study, anti-β-actin (1:10000, Sigma-Aldrich-A5316, UK) was used as a mouse monoclonal antibody, and anti-caspase-3 (1:3000, CST-9665, US) and anti-Bax (1:3000, CST-2774, US) antibodies were used as rabbit monoclonal antibodies. Besides, We used goat anti-rabbit (1.5000, Thermo Fisher Scientific-31460, US) and Goat anti-mouse (1:5000, Thermo Fisher Scientific-31430, US) antibodies as secondary antibodies. Fusion-FX7 (Vilber Lourmat, Thermo Fisher Scientific, USA) and Clarity ECL substrate solution (Bio-Rad-1705061, USA) were used to determine the chemiluminescence signal.

Statistical analysis

Data were shown as means ± standard deviation. The experiment was conducted using three independent biological replicates and two technical replicates. Student's *t*-test was used to determine significant differences between groups ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$).

Acetylthiocholine esterase (AChE) activity

AChE enzyme activity was investigated with slight modifications based on the study of Ellman et al.[12,13] Thiocholine, the product of enzymatic hydrolysis, does not have a distinct chromophore for UV detection, therefore 5,5'-Dithiobis(2-nitrobenzoic acid) [Ellman's reagent, DTNB, Sigma-Aldrich, (St. Louis, MO, USA)] was used for the evaluation of enzyme activity. All compounds were tested at 30 μM and 300 μM concentrations. Galantamine was used as a positive control. Each concentration was studied three times. All solutions were adjusted to 20 °C before use. First, 3.0 mL of phosphate buffer (0.1 M; pH 8.0), then 100 *μL* of substrate solution (dissolved in 2% DMSO and then diluted to lower than 0.2% DMSO in the aqueous assay medium), 100 *μL* of enzyme solution [(2.5 units/*μL*) (from electric eel, E.C.3.1.1.7., Type VI-S, Sigma-Aldrich)] were added into the cuvette and incubated for 5 min. After the required fractional amounts of the DTNB solution (0.01 M, 100 ml) and 20 *μL* of the acetylthiocholine (ATC) (0.075 M) were added, the cuvette was mixed immediately and rapidly. The enzymatic hydrolysis of ATC was detected at 412 nm by UV-visible absorption (Shimadzu UV/160-A spectrophotometer). The *in vitro* results for AChE are presented in Table 1.

RESULTS

Chemistry

Synthesis of target compounds was carried out in two steps (1a-6a and 1-12). In the first step, ω-chloro-*N*-propananilide derivatives were obtained.[10] Subsequently, these derivatives were combined with pyrazole or triazole to obtain the final compounds.[11] Column chromatography was used for purification studies. Structure characterization studies of the synthesized compounds were carried out successfully.

The neuroprotective effects of the final compounds against neurotoxicity and cell death

To investigate the potential neuroprotective effects of compounds against neurotoxicity induced by 6-OHDA, we first pre-treated cells with different concentrations of compounds (1, 5, 10, 25, and 50 μM) for 12 h; then treated them with 6-OHDA for additonal12 h. After conducting cell viability experiments, we showed that while 6-OHDA decreased cells' viability 46.30±0.66% compared to the DMSO-treated cells (control cells), the compounds have a potential protective effecs against 6-OHDA induced toxicity. Especially, compounds 1, 5, 7, 10, and 12

Table 1. AChE inhibition results of the title compounds

Compound	Inhibition %	
	30 μM	300 μM
1	22.08±2.63	65.56±1.35
2	12.91±0.68	67.86±0.02
3	14.35±0.02	69.16±0.11
4	16.31±0.9	70.30±1.84
5	15.18±0.65	66.61±1.01
6	25.81±0.16	67.09±0.08
7	29.08±0.68	83.03±0.99
8	17.37±0.67	64.20±1.71
9	13.56±1.65	68.70±1.08
10	23.07±0.47	82.20±0.53
11	18.18±0.46	66.50±0.9
12	30.22±0.56	86.24±0.52
Galantamine	80.7	95.9

AChE: Acetylthiocholine esterase

presented their activities at lower concentration. While 6-OHDA decreased cell viability to 46.30±0.66%, the viability was restored to 40±0.76, and 70.09±1.44% at 1 and 5 concentrations of compound 1, respectively (Figure 1, Supplementary Table 1). While compound 5 enhanced cell viability to 98.78±2.23 (5 μM) and 99.68±4.04% (10 μM), the cell viability with treatment with compound 7 reached up to to 104.33±3.77 and 111.12±3.02% at 1 and 5 μM concentrations, respectively (Figure 1, Supplementary Table 1). The viability of cells with the treatment of complund 10 was found to be increased to to 101.97±3.11 (1 μM) and 107.54±2.25 (5 μM) (Figure 1, Supplementary Table 1). Compound 12, another molecule that shows activity at lower concentrations, increased cells viability up to 10 μM (103.07±2.24; 1 μM , 106.49±2.59; 5 μM, 113.69±2.14%; 10 μM) (Figure 1, Supplementary Table 1). The compounds 2, 6 and 11 presented their activity in a dose dependent manner. For compound 3 and 4, the activity was found to be increased up to 25 μM in a dose dependent manner (Figure 1, Supplementary Table 1). While compound 9 enhanced cell viability to 10 μM in a dose dependent manner, compound 8 presented its activity at higher concentrations as at 10-, 25 and 50 μM (Figure 1, Supplementary Table 1). For this analysis, Student's *t*-test was used for determination of significancy between groups ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$). To investigate the potential mechanism for protective effects of selected compounds against 6-OHDA induced toxicity model, we checked some proteins' levels as Bax and cleaved caspase 3 *via* Western blot. The results obtained, showed that while 6-OHDA increased cleaved caspase-3 and Bax proteins' levels, compouns 7, 10, 11, and 12 decreased these proteins levels leadindg to potential neuroprotection.

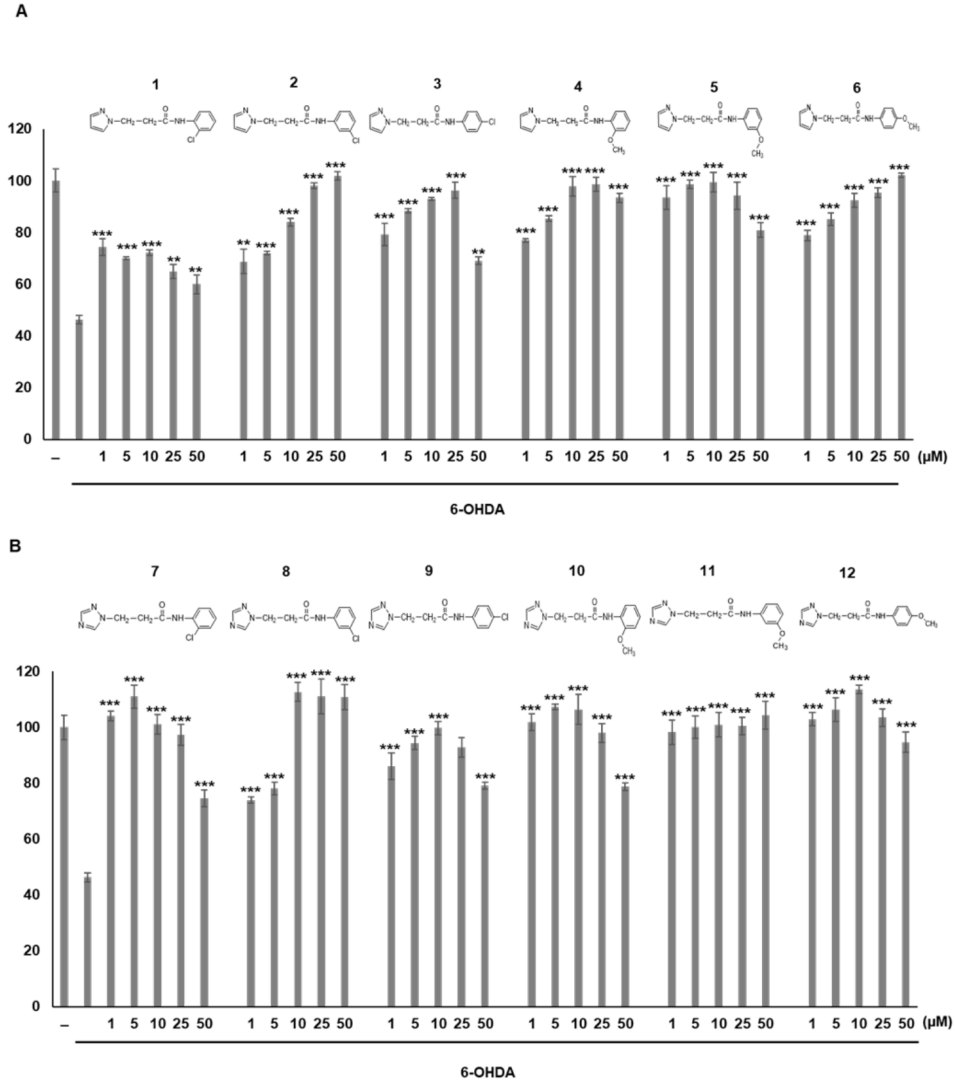
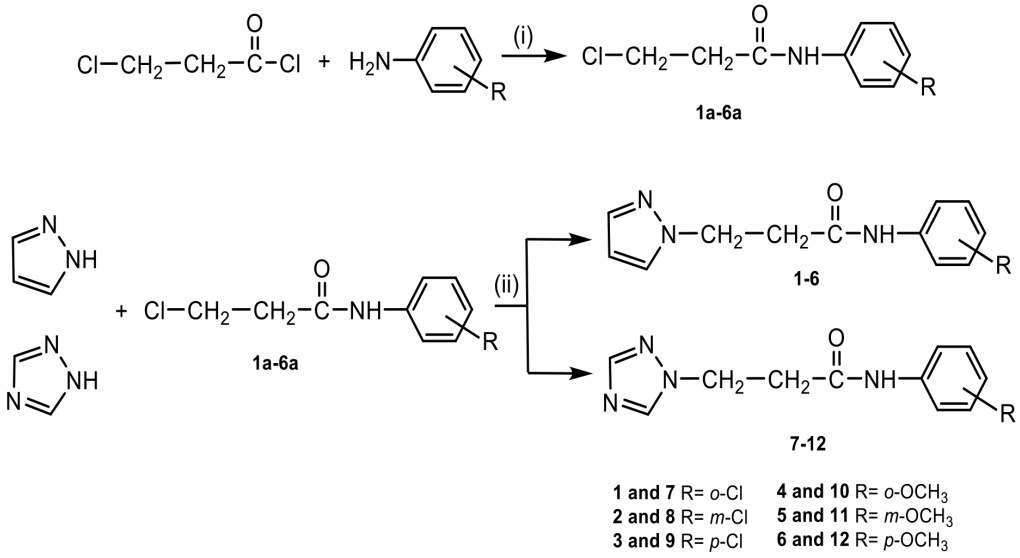


Figure 1. Synthesized compounds protect cells against 6-OHDA-induced toxicity. SH-SY5Y cells were pre-treated with increasing doses of compounds for 12 h and then treated with 6-OHDA for 12 h. The viability of cells was performed by WST-1 reagent. The absorbance of cells treated with DMSO as solvent control was considered 100% and cell viability of the applied concentrations was calculated. The WST-1 assay was performed by triplicate samples. ANOVA analysis with Dunnett's post hoc test was used to determine the significance of the differences compared to the control cells ($^{*}p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$)

6-OHDA: 6-Hidroksi-dopamine, WST: Water-soluble tetrazolium salt, DMSO: Dimethyl sulfoxide, ANOVA: Analysis of variance



Scheme 1. Synthesis pathway of compounds 1-12
DMF: Dimethylformamide

DISCUSSION

Chemistry

In this study, 12 substituted *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole were synthesized.[10,11] After the acylation of substituted anilines with 3-chloropropionyl chloride (compounds 1a-6a), they were reacted with pyrazole or 1,2,4-triazole in DMF to form 1-6 and 7-12, respectively (Scheme 1). The structures of the final compounds were verified using spectral methods, and purity percentages were determined by elemental analysis. Strong stretching bands were noticed regarding the carbonyl group at 1697-1650 cm^{-1} and 1590-1491 cm^{-1} as amide I and amide II bands, respectively (Supplementary Figures 1-12, Supplementary Table 1). In addition, stretching signals in the H bond region (3374-3112 cm^{-1}) verifying the existence of the amide functional group were observed.[14,15]

The chemical shifts and coupling constants of ^{1}H NMR spectra were consistent with the molecular structures. While the signals of methylene protons neighboring the carbonyl group were observed at δ 2.79 - 3.04, signals of methylene protons neighboring heterocyclic ring systems were observed at δ 4.40 - δ 4.59 region because the deshielding effect of pyrazole/1,2,4-triazole rings is greater than that of the carbonyl functional group.[16] The NH proton of the amide function was noticed as a broad singlet from δ 7.50 to 9.50 depending on the position and type of substituent (Supplementary Figures 1-12).

The mass spectra of the title compounds were recorded using ESI or APCI, and $[M+1]^+$ and $[M-1]^-$ signals were determined according to their molecular weights. Additionally, the expected molecular formulas and purity of the compounds were supported within ±0.4% range according to the elemental analysis results (Supplementary Figures 1-12, Supplementary Table 1).

All molecules except 10 had registry numbers, but no corresponding scientific data was available.

The neuroprotective effects of the final compounds against neurotoxicity

The potential neuroprotective activity of the titles compounds was analyzed in a 6-OHDA-induced neurotoxicity model. First, cells were pre-treated with desired concentrations of compounds (1, 5, 10, 25, and 50 μM) for 12 h; then they were subjected to 6-OHDA treatment for another 12 h. The cell viability assays, performed using WST-1 reagent, showed that all final compounds possessed remarkable neuroprotective effects against 6-OHDA-induced cells, and compounds 1, 5, 7, 10, and 12 reduced the toxic effect of 6-OHDA more effectively at lower concentrations. 6-OHDA treatment decreased cell viability by 46.30±0.66% (Figure 1, Supplementary Table 1). The viability was revitalized to an extent of 74.40±0.76, and 70.09±1.44% at 1 and 5 concentrations of compound 1, respectively. The highest concentration of compound 1 did not reverse cell viability as much as the lowest concentration. For this analysis, Student's *t*-test was used for determination of significancy between groups ($p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq 0.005$).

Compound 5 treatment increased cell viability to 98.78±2.23 and 99.68±4.04% at 5 and 10 μM concentrations, respectively. The treatment with compound 7 ameliorated cell viability to 104.33±3.77 and 111.12±3.02% at 1 and 5 μM concentrations, respectively; compound 10 increased cell viability to 101.97±3.11 and 107.54±2.25% at 1 and 5 μM concentrations, respectively. Compound 12, another synthesized molecule that increased cell viability at lower doses, enhanced cell viability up to a concentration of 10 μM (103.07±2.24, 106.49±2.59, 113.69±2.14 % at the 1, 5, and 10 μM concentration, respectively). Treatment with compounds 2, 6, and 11 increased cell viability in a

dose-dependent manner which means that the highest doses (50 μM) did not exert any cytotoxic effects on SH-SY5Y cells (101.98±1.97% for 50 μM concentration of compound 2, 102.21±2.26% for 50 μM concentration of compound 6, 104.35±2.26% for 50 μM concentration of compound 11).

Compound 3 increased cell viability up to a concentration of 25 μM in a dose-dependent manner. The viability decreased dramatically to 69.09±0.98% at the highest concentration, which may be due to the cytotoxic effect of the highest dose of this compound. While compound 4 increased cell viability up to a concentration of 25 μM (98.60±3.51%), it was slightly decreased at 50 μM concentration (93.36±3.38%). Furthermore, treatment with compound 9 triggered cell viability in a dose-dependent manner up to a concentration of 10 μM, and it was shown that a minor and moderate decrease at higher concentrations (92.95±0.90% at 25 μM and 79.20±1.95% at 50 μM). Finally, compound 8 did not increase cell viability at lower concentrations, whereas treatments at higher concentrations attenuated 6-OHDA toxicity at higher doses (113.02±4.73%, 111.03±1.48% and 111.20±5.20% at 10-, 25 and 50 μM, respectively) (Supplementary Table 1).

The protective effects of the final compounds against cell death

6-OHDA has been used in neurotoxicity models for Parkinson's both *in vitro* and *in vivo*[17] based on its ability to activate caspase-3 and increase the level of Bax protein in several studies.[18–20]

Based on the significant neuroprotective activity of the final derivatives, we selected derivatives 7, 10, 11, and 12 for further investigation because they also displayed impressive neuroprotectivity at lower concentrations. For this purpose, we used the same experimental conditions used in the cell viability assays. Consistent with the literature, treatment with 6-OHDA alone increased the Bax and cleavage caspase-3 protein levels. This increase in Bax and cleaved caspase-3 was significantly attenuated in cells pre-treated with 7, 10, 11, and 12 (Figure 2). 6-OHDA, a 6-hydroxylated analog of dopamine, causes degeneration of dopaminergic neurons. Treatment with 6-OHDA induces cellular damage through a reaction with nucleophiles, such as protein and DNA, leading to apoptotic cell death.[21] Our results revealed that compounds 7, 10, 11, and 12 protect SH-SY5Y cells against 6-OHDA toxicity by decreasing pro-apoptotic Bax expression and caspase-3 activation.

AChE activity

All compounds were screened for AChE inhibitory potential using Ellman's slightly modified colorimetric method, and galantamine was the standard drug.[12,22] The results demonstrated that all synthesized compounds exhibited weak AChE inhibitory activity (Table 1). As shown in Table 1, the title compounds exhibited 64.4-86.2% inhibition at 300 μM and 13.62-30.22% inhibition at 30 μM on AChE. Among the series, compounds 7, 10, and 12 showed better activity than the other tested compounds at both concentrations. In particular, compound 12, named *N*-(4-methoxyphenyl)-2-(1*H*-1,2,4-triazol-1-yl)propanamide, was found to be the most potent derivative of all the synthesized derivatives, with an 86.2% inhibition at 300 μM and a 30.2% inhibition at 30 μM on AChE (Table 1).

CONCLUSION

In this study, we designed and synthesized twelve substituted *N*-propananilide derivatives bearing pyrazole or 1,2,4-triazole rings based on the neuroprotective results of the compounds from our previous study.[8] In terms of neuroprotective potential, triazole derivatives generally exhibit better activity than pyrazole derivatives. Furthermore, compounds that have potential against AChE have neuroprotective effects. Additionally, compounds 7, 10, 11, and 12 provide neuroprotection against 6-OHDA-induced neurotoxicity by decreasing the pro-apoptotic protein Bax and cleaved caspase-3, which play central roles in cellular apoptosis.

In conclusion, we can state that adding one methylene group to the linker increases the neuroprotective effect of the designed

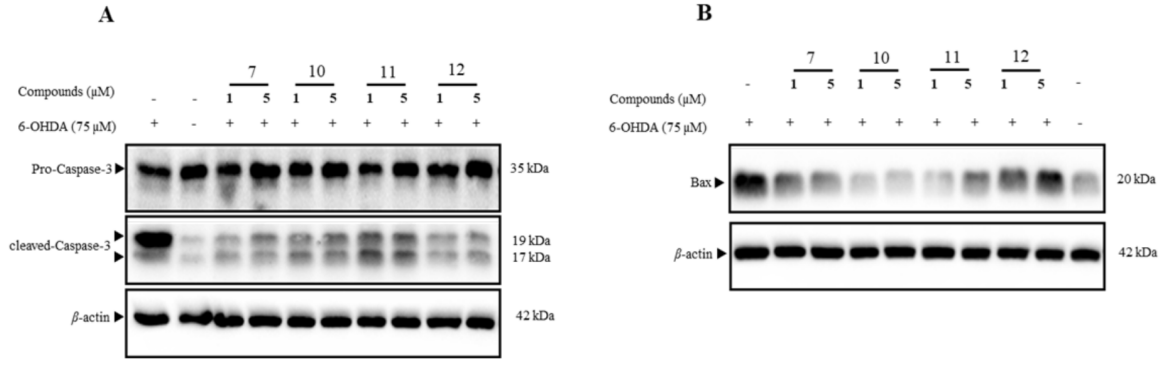


Figure 2. Selected compounds prevent 6-OHDA-induced neurotoxicity. SH-SY5Y cells were first treated with the desired concentration of selected compounds, then treated with 6-OHDA. While DMSO was used as a solvent control; 6-OHDA was used as a positive control which led to activating the cleavage form of caspase-3 and increased the level of Bax. The levels of pro-caspase 3, cleavage-caspase 3, and Bax were determined by WB using antibodies against them. β-actin was used as a loading control. The experiments were repeated three times independently; with one representative result shown

6-OHDA: 6-Hidroksi-dopamine, DMSO: Dimethyl sulfoxide, WB: Western blot

compounds but decreases the AChE inhibitory activity; therefore, adding another methylene group to the intermediate chain to test the neuroprotective activity of the pyrazole/1,2,4-triazole butyramide derivatives will be our future focus.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Surgical and Medical Practices: A.H.T., M.S., S.Y., S.P., P.B., V.A., Concept: A.H.T., M.S., Design: A.H.T., V.A., Data Collection or Processing: A.H.T., M.S., S.Y., S.P., P.B., V.A., Analysis or Interpretation: A.H.T., M.S., S.Y., S.P., P.B., V.A., Literature Search: A.H.T., M.S., S.Y., Writing: A.H.T., M.S., S.Y., S.P., P.B., V.A.

Conflict of Interest: The authors have no conflicts of interest to declare.

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