Bioorganic & Medicinal Chemistry 18 (2010) 4224–4230

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and in vitro evaluation of novel 1,2,4-triazine derivatives as neuroprotective agents

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ARTICLE INFO

Article history:
Received 3 March 2010
Revised 29 April 2010
Accepted 30 April 2010
Available online 8 May 2010

Keywords:
Alzheimer's disease
Oxidative stress
PC12 cells
1,2,4-Triazine

ABSTRACT

The role of novel triazine derivatives against oxidative stress exerted by hydrogen peroxide on differentiated rat pheochromocytoma (PC12) cell line was examined and a consistent protection from H_2O_2-induced cell death, associated with a marked reduction in caspase-3 activation, was observed. Moreover, activation of NF-κB, a known regulator of a host of genes that involves in specific stress and inflammatory responses by H_2O_2, was greatly impaired by triazine pretreatment in differentiated PC12 cells. Neuroprotective effect of such compounds may represent a promising approach for treatment of neurodegenerative diseases.

1. Introduction

Oxidative stress-induced cell damage has long been implicated both in the physiological process of aging and in a variety of neurodegenerative diseases such as Alzheimer's disease (AD).[1,2] Oxidative damage mediated by reactive oxygen species (ROS) which can be generated by cell lysis, oxidative burst or in the presence of an excess of free transition metals, can attack proteins, deoxynucleic acid and lipid membranes; hence, disrupt cellular function and integrity. Hydrogen peroxide (H_2O_2), one of the main reactive oxygen species, is produced during the redox process and is considered as a messenger in intracellular signaling cascades.[3] In addition, it is well known that H_2O_2 can cause lipid peroxidation and DNA damage and induce apoptosis in many different cell types.[4,5] In this pathway, NF-κB is the first transcription factor reported to respond directly.[6] NF-κB, which is a redox-sensitive transcription factor, regulates expression of genes that are involved in cellular differentiation, proliferation, apoptosis, oxidative response, inflammation, and immune response.[7] Its activation is evident by many distinct stimuli including bacterial lipopolysaccharide, oxidative stress, and amyloid beta (Aβ).[8] Several evidences have shown that NF-κB activation may act as a pro-apoptotic factor,[9] and reduction in NF-κB activation protect PC12 cells and hippocampal neurons against Aβ-induced toxicity.[10] Kutuk and Basaga have also reported that aspirin prevents H_2O_2-induced apoptosis in HeLa cells through NF-κB inactivation.[11]

1,2,4-Triazine nucleus is a prominent structural core system present in numerous biologically active compounds, possessing a wide range of biological activities. Many investigations revealed the anticancer potential of triazine derivatives.[12] Triazine derivatives have been also introduced as anti-inflammatory,[13,14] radical scavenger,[15] β-sheet breaker,[16] and antifungal[17] agents. 5,6-Diphenyl-1,2,4-triazine-3-thiol is one of the triazine derivatives that its anti-inflammatory action has been reported by Saxena et al.[18]

In the present investigation, some of the novel triazine derivatives were synthesized and evaluated for their neuroprotective effect.

2. Results and discussion

2.1. Synthesis of 1,2,4-triazine derivatives

The synthetic pathway for the preparation of triazine derivatives (**5**) is outlined in Scheme 1. 5,6-Diaryl-1,2,4-triazine-3-thiol (**4**) was prepared by cyclization of compound **3** with thiosemicarbazide as reported previously.[18] The method for the preparation of benzil[20] was used for the preparation of 1,2-bis(4-chlrophenyl)ethanedione (**3**). Compound **2** was prepared as reported.[19] Alkylation of compound **4** was carried out by using alkyl iodides in the

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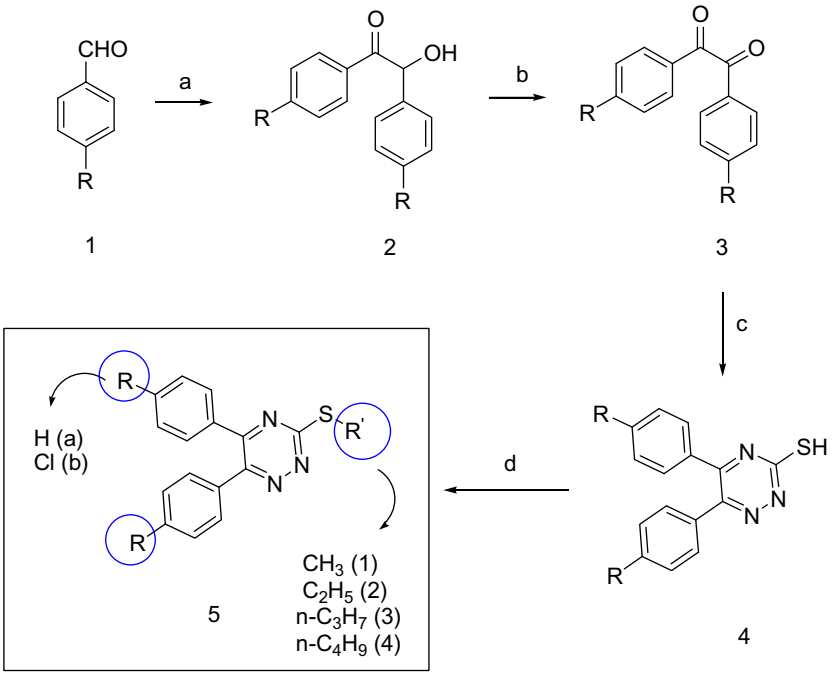
doi:10.1016/j.bmc.2010.04.097



Scheme 1. Synthesis pathway for the preparation of triazine derivatives. Reagents and conditions: (a) NaCN, EtOH, reflux, 2 h; (b) CuSO4, pyridine, 80 °C; (c) thiosemicarbazide, H_2O/dioxane (2:1), reflux, 2–5 h; (d) alkyl iodide, TEA, CH_3OH, 2 h.

presence of triethylamine in methanol to give compounds **5a**(**1**–**4**) and **5b**(**1**–**4**).

2.2. Protection against oxidative stress-induced cell death in PC12 neurons

The present investigation was aimed to determine the protective role of triazine derivatives **5** in oxidative stress-induced cell death. The PC12 cell line is a useful and widespread model for studying neuronal differentiation in sympathetic neurons and other neurobiochemical and neurobiological events.[21] Therefore, differentiated PC12 cells were chosen as in vitro models and H_2O_2 as an oxidative agent.[22] Differentiated PC12 cells seeded in 96-well plates were treated with different concentrations (0.5–20 μM) of compounds and were exposed to H_2O_2 3 h later. After 24 h, the cells were assayed for cell viability. Figure 1B showed that H_2O_2 significantly reduced cell viability to 25%, compared to control, whereas pretreatment with **5a-1** and **5a-2** (10 μM) protected neurons against H_2O_2-induced cell death (47% and 54%, compared to control, respectively). However, synergistic effects with H_2O_2 were observed for **5a-3** and **5a-4** in reducing cell viability. Preincubation with **5b(1–4)** increased cell viability to 83, 40, 45 and 50%, compared to control, respectively. Similar results were obtained from undifferentiated PC12 cells. Undifferentiated PC12 cells seeded in 96-well plates were treated with different concentrations (0.5–20 μM) of compounds and were exposed to H_2O_2 3 h later. After 24 h, the cells were assayed for cell viability. Figure 1D showed that H_2O_2 significantly reduced cell viability to 29%, compared to control, whereas pretreatment with **5a(1–2)** (10 μM) protected neurons against H_2O_2-induced cell death up to 53% and 59%, respectively, compared to control. Preincubation with **5b**(**1**–**4**) (10 μM) increased cell viability to 88%, 58%, 60% and 63%, respectively, compared to control.

Compounds **5a(1–2)** and **5b(1–4)** alone did not show any toxicity effect at 0.5–10 μM in both undifferentiated and differentiated PC12 cells after 24 h (Fig. 1A and C).

According to the chemical structure of compounds, it seems that there is an inverse correlation between the number of carbon atoms substituted on thiol group and the neuroprotective effect of compounds. In other words, increase in the length of thioalkyl moiety, from methyl to *n*-butyl, decreases protective effect of compounds against H_2O_2-induced cell death in both series (**a** and **b**). However, **5b** derivatives possess significant advantages over its **5a** counterparts. A possible explanation for this difference could be due to optimum lipophilicity of **5b** in comparison with **5a** series. Comparison of the activity of two triazine series showed that b series was more active than their homologous a series where H group is substituted by chlorine in 4-position of phenyl ring. All of the compounds showed parallel activity except **5a-2**. The most active compound was **5b-1**, in which thioalkyl group in 3-position of triazine ring was the smallest alkyl and the lipophilicity of the compound was increased by chlorine substitution.

The neuroprotective effect of these compounds was further confirmed by morphological observations (Fig. 1E). After incubation with 50 ng/ml NGF for 6 days, the PC12 cells displayed a neuronal phenotype that resembled to sympathetic neurons. While the H_2O_2-treated cells showed heterogeneity in their shape and were mostly detached from the cultured plate surface, the **5b-1** pretreated cells showed remarkable resistance to damaging effects of H_2O_2 and this was evident through their almost uniform neuronal shape, being mostly attached to the plate surface, and restoration of networks that were formed between neurons after differentiating by NGF (Fig. 1E). On the contrary, **5a-3** and **5a-4** treated cells were completely damaged.

For further investigation, apoptosis assays and western blot analysis were performed to determine their mechanism of action, at their best protective concentration (10 μM), against oxidative stress-induced cell death.

2.3. Morphological evaluation of apoptosis

Acridine orange/ethidium bromide (AO/EB) staining discriminates live cells from dead ones on the basis of membrane integrity. AO is a cell-permeable nucleic acid selective cationic dye which is taken up by both viable and nonviable cells and emits green fluorescence if intercalates into double stranded nucleic acid (DNA). EB

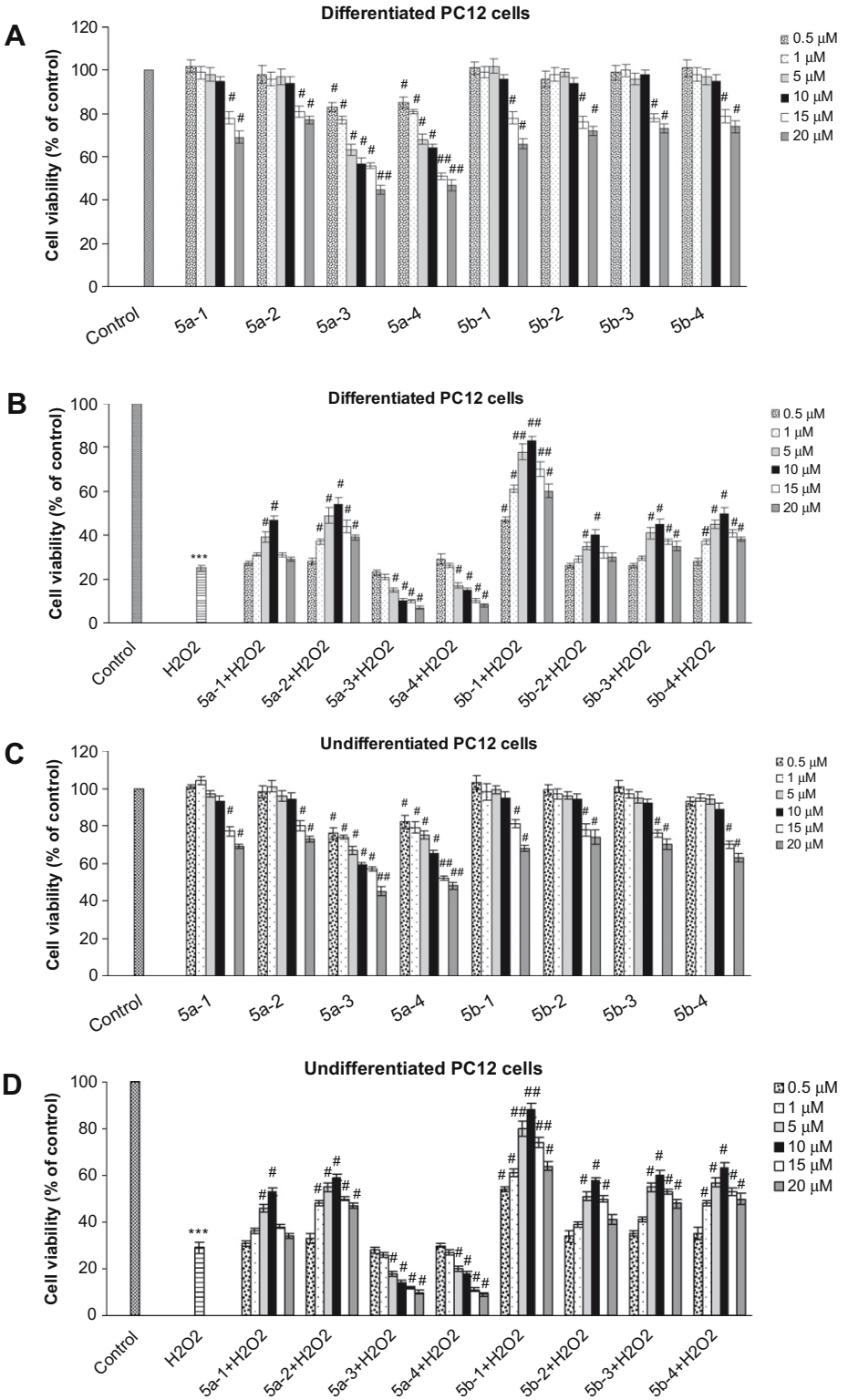


Figure 1. Effect of triazine derivatives on cell viability and morphology. Differentiated and/or undifferentiated PC12 cells were treated with different concentrations (0.5–20 μM) of compounds for 3 h. After 24 h, cell viability was determined by MTT assay in the absence (A and C) and/or presence (B and D) of H_2O_2. (E) Morphological evaluation of differentiated PC12 cells after 24 h exposure to H_2O_2 in the presence of triazine derivatives (10 μM). Viability was calculated as the percentage of living cells in treated cultures compared to those in control cultures. Each value represents the mean ± S.D (n = 3). *Significantly different from untreated cells. #Significantly different from H_2O_2-treated cells. (B) Morphological evaluation of PC12 cells.

intercalates and stains DNA, providing a red–orange fluorescent. Although it does not stain healthy cells, it can be used to identify cells that are in the early or final stages of apoptosis which have much more permeable membranes. The result obtained from AO/EB double staining is represented in Figure 2. Four types of cells could be distinguished according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei: (1) Viable cells which have uniform bright green

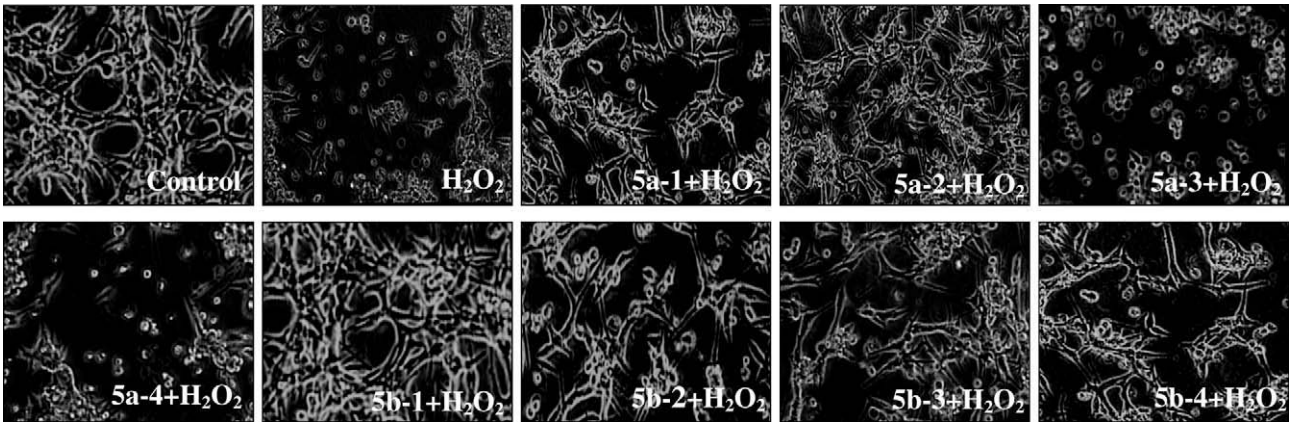


Fig. 1 (*continued*)

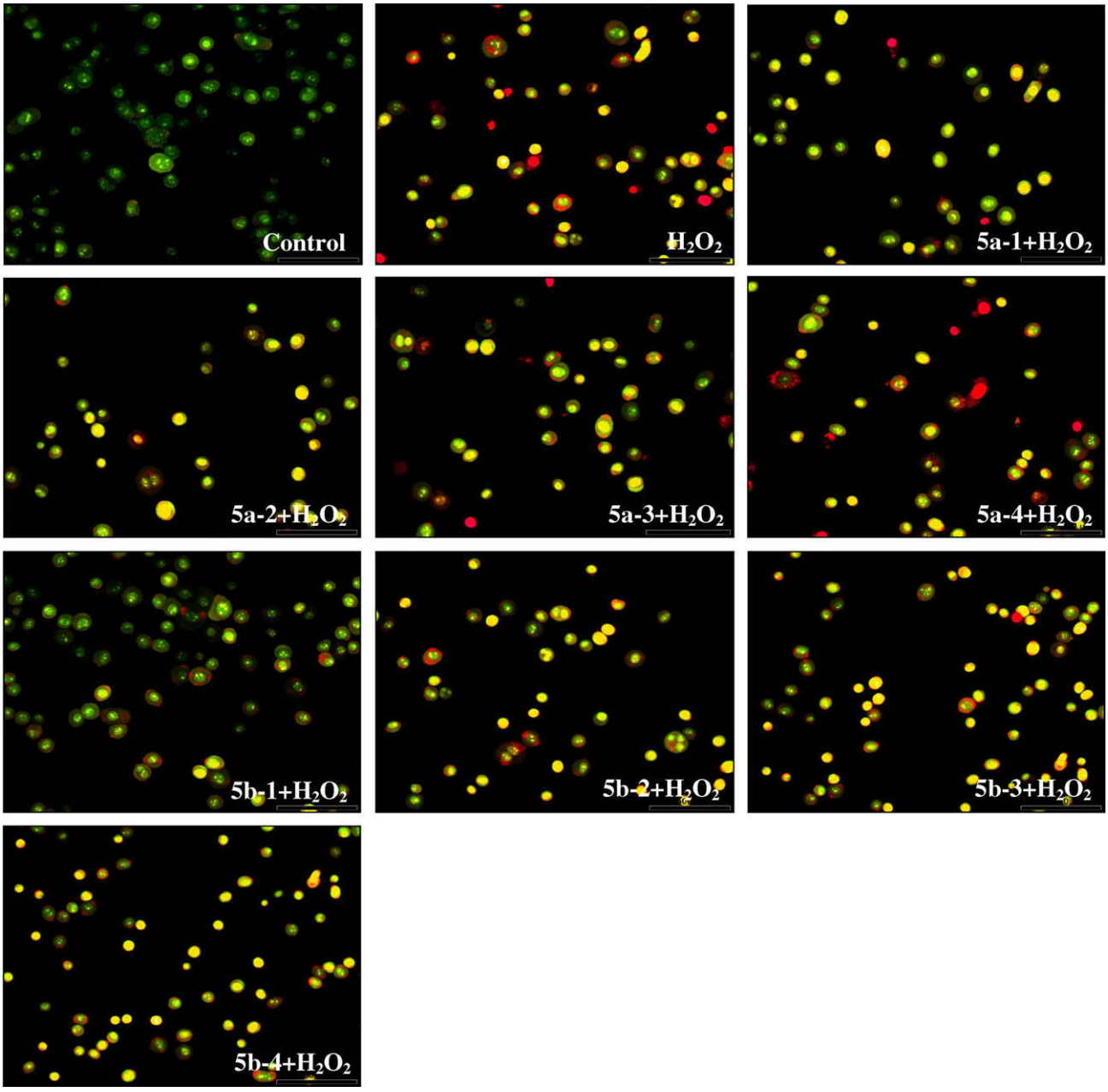


Figure 2. Morphological evaluation of cells, using AO/EB double staining. Differentiated PC12 cells were exposed to triazine derivatives (10 μM) for 3 h followed by exposure to 150 μM H_2O_2 for 24 h. The morphological patterns of apoptotic cells are described in the text.

nuclei with organized structure. (2) Early apoptotic cells (which still have intact membranes but have started to undergo DNA cleavage) have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments. (3) Late apoptotic cells which have orange to red nuclei with condensed or fragmented chromatin. (4) Necrotic cells which have uniformly red nuclei. Quantitative analysis of the stained cells indicated that pretreatment of the cells with **5a-1** and **5a-2** has significantly decreased the extent of cell apoptosis to about 35–45% from 70% observed among the cells exposed solely to H_2O_2. Preincubation of the cells with **5b-1** prevented the extent of apoptosis to about 20%, while **5b(2–4)** pretreatment decreased apoptotic proportion to about 40–50%. The difference observed between MTT results and the proportion of apoptosis was obtained from AO/EB staining test shows the involvement of necrosis in this process.

2.4. Inhibition of caspase-3 activation in neuron-like PC12 cells

Another important finding, which confirmed the protective effect of these derivatives, was the data obtained from western blot analysis of procaspase-3 and caspase-3. The progress of apoptosis is regulated by a series of signal cascades. Caspases are cysteine-dependent enzymes that play central role in the induction, transduction and amplification of intracellular apoptotic signals and previous studies have demonstrated efficacy of caspase inhibitors in preventing H_2O_2-induced apoptosis in PC12 cells.[23,24] To verify whether these triazine derivatives interfere apoptosis via suppressing caspase-3, the level of cleaved caspase-3 was measured by western blot analysis in the presence of compounds. As shown in Figure 3, H_2O_2-induced the appearance of cleaved active caspase-3, showing the involvement of caspase-3 in H_2O_2-induced cell death in PC12 neurons. In the cells pretreated with **5a**(**1**–**2**), band of cleaved (active) caspase-3 was weaker compared to H_2O_2-treated cells by about 1.91- and 1.64-fold, respectively. Caspase-3 level was decreased to 4.59-, 2.00-, 1.92-, and 1.83-fold, compared to H_2O_2-treated cells by **5b-1**, **5b-2**, **5b-3**, and **5b-4**, respectively, demonstrating the ability of these compounds to suppress oxidative stress-induced apoptosis in differentiated PC12 cells.

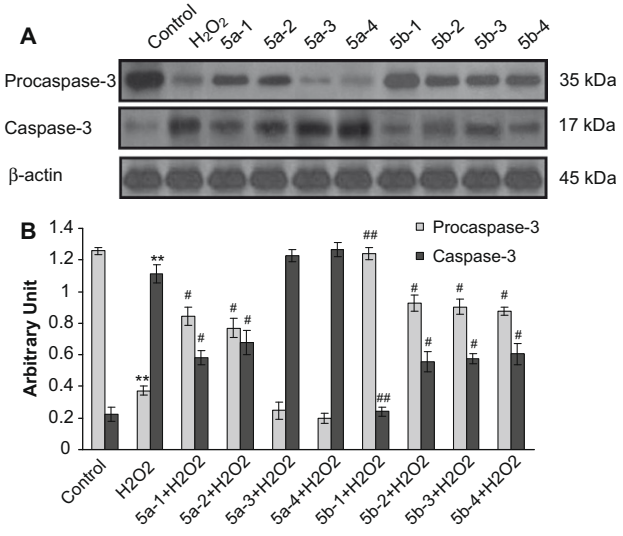


Figure 3. Caspase-3 activity in PC12 cells pretreated with triazine derivatives. Differentiated PC12 cells were pretreated with triazine derivatives for 3 h and then exposed to H_2O_2 for 24 h. Twenty micrograms of proteins were separated on SDS–PAGE, western blotted, probed with anti-caspase antibody and reprobed with anti-β-actin antibody. (One representative western blot was shown; $n = 3$). (B) The densities of corresponding bands were measured and the ratio was calculated. The median of three independent experiments is shown. *Significantly different from untreated cells. #Significantly different from H_2O_2-treated cells.

2.5. Inhibition of NF-κB nuclear translocation in PC12 cells

Although many signals and metabolic events are important in the regulation of cell death, the intracellular redox level, in particular, has been shown to play a critical role. Redox regulation is an interesting and important issue which tightly associates with oxidative stress. Redox-sensitive NF-κB is increased in states of oxidative stress and regulates the expression of many cellular signaling molecules.[25] The link between oxidative stress and NF-κB was established mainly from the inhibition of NF-κB activation by cellular oxidants.[26,27] NF-κB is normally located in the cytosol, bound and inhibited by I-κB. Following oxidative stress, NF-κB is released, translocates to the nucleus and activates transcription of downstream inflammatory mediators. Since H_2O_2 was first suggested as an endogenous NF-κB activator, investigation of the effect of triazine derivatives on NF-κB inactivation was of our interest. We found that in those cells exposed to H_2O_2, the level of NF-κB in nucleus in comparison to control was increased 6.02-fold, as determined by western blot (Fig. 4). This increase was prevented by **5a-1** and **5a-2** by about 1.79-and 1.56-fold, compared to H_2O_2-treated cells, respectively. Interestingly, **5b-1**, **5b-2**, **5b-3**, and **5b-4** treatments resulted in 4.04-, 2.00-, 1.89-, and 1.78-fold reduction of NF-κB, respectively, compared to H_2O_2. This inhibition is associated with the prevention of cell death induced by H_2O_2.

2.6. Inhibition of H_2O_2-induced ROS generation

Many studies have shown that oxidative stress is a major cause of cellular injuries in a variety of human diseases including neurodegenerative disorders. ROS such as hydrogen peroxide, superoxide anion, and hydroxyl radical readily damage biological molecules, which can ultimately lead to apoptotic or necrotic cell death.[5,4] Thus, removal of excess ROS or suppression of their generation may be effective in preventing oxidative cell death.

ROS levels produced by H_2O_2 were determined based on DCF fluorescence. The fluorescence intensity of DCF in cells treated with H_2O_2 (150 μM) was increased by 5.07-fold compared to untreated control cells. Pretreatment of the cells with **5a**(**1**–**2**) (10 μM) prevented the accumulation of ROS by 1.50- and 1.62-fold, respec-

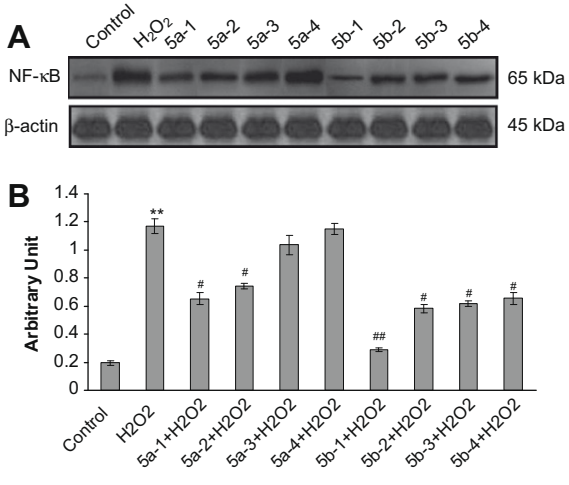


Figure 4. Western blot analysis to measure the effects of triazine derivatives on the nuclear levels of NF-κB in PC12 cells. (A) Differentiated PC12 cells were pretreated with compounds for 3 h and then exposed to H_2O_2 for 24 h. Twenty micrograms of nuclear proteins were separated on SDS–PAGE, western blotted, probed with anti-NF-κB antibody and reprobed with anti-β-actin antibody. (One representative western blot was shown; $n = 3$). (B) The densities of NF-κB bands were measured and the ratio was calculated. The median of three independent experiments is shown. *Significantly different from untreated cells. #Significantly different from H_2O_2-treated cells.

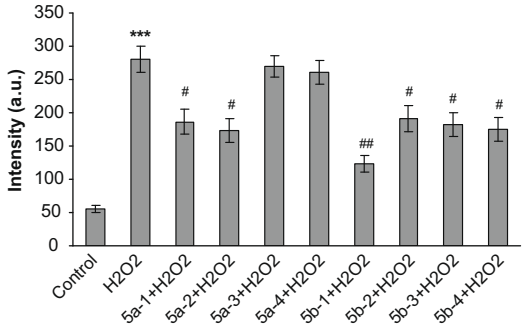


Figure 5. Inhibitory effect of compounds on accumulation of intracellular ROS. Differentiated PC12 cells were incubated 3 h with triazine derivatives (10 μM) and then exposed to H_2O_2 for 24 h. Intracellular levels of reactive oxygen species were measured with DCFH-DA. The median of three independent experiments is shown. *Significantly different from control cells. #Significantly different from H_2O_2-treated cells.

tively, compared to H_2O_2-treated cells (Fig. 5). Preincubation of the cells with **5b**(**1**–**4**) (10 μM) reduced intracellular ROS by 2.26-, 1.46-, 1.53-, and 1.60-fold, respectively, compared to H_2O_2-treated cells. It seems that in this signaling pathway ROS act as second messengers. Because, ROS have been associated with neurodegenerative disease and recent studies have demonstrated that ROS, over a narrow concentration range, might function as second messengers in cell-signaling pathways.[3] Several data have shown that ROS activates NF-κB.[28] In addition, caspase-3 a family of cysteine protease, can be activated by ROS. So the suppressive effect of our compounds on translocation of NF-κB and also the expression of active forms of caspase-3 shows that these triazine derivatives suppress NF-κB activating via suppression of ROS production.

3. Conclusion

In summary, our results clearly indicate that triazine derivatives prevent the H_2O_2-induced cell death in differentiated PC12 cells. It seems that in this cytoprotection, inhibitions of caspase-3 and NF-κB via suppression of ROS production are the main factors which protect neurons against oxidative stress-induced apoptosis. However, the complete molecular milieu that links all these events need to be elucidated. A further study of the detailed mechanisms is now in progress in our laboratory that will pave the way to exploiting preventive or therapeutic strategies for neurodegenerative diseases.

4. Experimental

4.1. Instrumentation and chemicals

Melting points were determined with a Reichert-Jung hot-stage microscope and are uncorrected. ^{1}H NMR (500 MHz) spectra were recorded on a Brucker spectrometer using $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts (δ) are reported in ppm relative to TMS as internal standard. Infrared spectra were acquired on a Nicolet Magna 550-FT spectrometer. Mass spectra were obtained with a Finnigan Mat TSQ-70 spectrometer. Elemental microanalyses were within ±0.4% of the theoretical values for C, H, and N.

Antibodies directed against caspase-3, NF-κB, and β-actin were obtained from Cell Signaling Company. All the other reagents, unless otherwise stated, were from Sigma–Aldrich (St. Louis, MO).

4.2. Synthesis

4.2.1. General procedures for preparation of triazine derivatives (5)

To a stirring solution of compound **4** (1 mmol) in methanol (50 ml), alkyl iodide (1.2 mmol), and triethylamine (1 ml) were added and the mixture was stirred for 2 h at room temperature. The solvent was removed under reduced pressure. Water (20 ml) and dichloromethane (50 ml) were added to the mixture. The organic phase was separated, dried (sodium sulfate), filtered and evaporated under reduced pressure and the residue was crystallized from methanol–water to yield 3-thioalkyl-5,6-diaryl-1,2,4-triazines. The spectroscopic and analytical data of compounds are as follows:

4.2.1.1. 3-Thiomethyl-5,6-diphenyl-1,2,4-triazine (5a-1). Mp: 89–91 °C, ^{1}H NMR ($CDCl_3$) δ: 2.70 (s, 3H, SCH_3), 7.30–7.45 (m, 6H), 7.50–7.58 (m, 4H). Mass: *m*/*z* (%), 279 (M^+, 7), 178 (25), 176 (100), 100 (5), 85 (15), Anal. Calcd for $C_{16}H_{13}N_3S$: C, 68.79; H, 4.69; N, 15.04. Found: C, 68.57; H, 4.73; N, 14.96.

4.2.1.2. 3-Thioethyl-5,6-diphenyl-1,2,4-triazine (5a-2). Mp: 70–72 °C, ^{1}H NMR ($CDCl_3$) δ: 1.52 (t, J = 7.5 Hz, 3H, SCH_2–CH_3), 3.37 (q, J = 7.5 Hz, 2H, SCH_2–CH_3), 7.28–7.48 (m, 6H), 7.55–7.62 (m, 4H). Mass: *m*/*z* (%), 293 (M^+, 5), 178 (100), 176 (30), Anal. Calcd for $C_{17}H_{15}N_3S$: C, 69.59; H, 5.15; N, 14.32. Found: C, 69.48; H, 4.99; N, 14.58.

4.2.1.3. 3-Thio-*n*-propyl-5,6-diphenyl-1,2,4-triazine (5a-3). Mp: 69–71 °C, ^{1}H NMR ($CDCl_3$) δ: 1.10 (t, J = 7.4 Hz, 3H, SCH_2–CH_2–CH_3), 1.88–1.98 (m, 2H, SCH_2–CH_2–CH_3), 3.31 (q, J = 7.5 Hz, 2H, SCH_2–CH_2–CH_3), 7.25–7.47 (m, 6H), 7.48–7.60 (m, 4H). Mass: *m*/*z* (%), 307 (M^+, 8), 264 (12), 178 (20), 176 (100), 152 (5), 105 (37), 77 (19), Anal. Calcd for $C_{18}H_{17}N_3S$: C, 70.33; H, 5.57; N, 13.67. Found: C, 70.51; H, 5.65; N, 13.75.

4.2.1.4. 3-Thio-*n*-butyl-5,6-diphenyl-1,2,4-triazine (5a-4). Mp: 45–47 °C, ^{1}H NMR ($CDCl_3$) δ: 0.81 (t, J = 7.3 Hz, 3H, SCH_2–CH_2–CH_2–CH_3), 1.28–1.38 (m, 2H, SCH_2–CH_2–CH_2–CH_3), 1.78–1.96 (m, 2H, SCH_2–CH_2–CH_2–CH_3), 3.40 (q, J = 7.5 Hz, 2H, SCH_2–CH_2–CH_2–CH_3), 7.34–7.48 (m, 6H), 7.51–7.59 (m, 4H). Mass: *m*/*z* (%), 321 (M^+, 10), 264 (7), 178 (35), 176 (100), 149 (5), 85 (15), Anal. Calcd for $C_{19}H_{19}N_3S$: C, 70.99; H, 5.96; N, 13.07. Found: C, 71.09; H, 5.77; N, 12.85.

4.2.1.5. 3-Thiomethyl-5,6-(4-dicholrophenyl)-1,2,4-triazine (5b-1). Mp: 137–139, °C ^{1}H NMR ($CDCl_3$) δ: 2.70 (s, 3H, SCH_3), 7.34 (d, J = 9.0 Hz, 2H), 7.37 (d, J = 8.5 Hz, 2H), 7.45 (d, J = 9.0 Hz, 2H), 7.50 (d, J = 8.5 Hz, 2H). Mass: *m*/*z* (%), 347 (M^+, 17), 245 (100), 176 (45), 123 (15), 63 (42), Anal. Calcd for $C_{16}H_{11}Cl_2N_3S$: C, 55.09; H, 3.18; N, 12.05. Found: C, 55.39; H, 3.24; N, 11.89.

4.2.1.6. 3-Thioethyl-5,6-(4-dichlorophenyl)-1,2,4-triazine (5b-2). Mp: 115–117 °C, ^{1}H NMR ($CDCl_3$) δ: 1.48 (t, J = 7.4 Hz, 3H, SCH_2–CH_3), 3.39 (q, J = 7.4 Hz, 2H, SCH_2–CH_3), 7.38 (d, J = 9.0 Hz, 2H), 7.40 (d, J = 8.5 Hz, 2H), 7.45 (d, J = 9.0 Hz, 2H), 7.50 (d, J = 8.5 Hz, 2H). Mass: *m*/*z* (%), 361 (M^+, 9), 247 (65), 245 (100), 209 (5), 175 (20), Anal. Calcd for $C_{17}H_{13}Cl_2N_3S$: C, 56.36; H, 3.62; N, 11.60. Found: C, 56.45; H, 3.78; N, 11.75.

4.2.1.7. 3-Thio-*n*-propyl-5,6-(4-dichlorophenyl)-1,2,4-triazine (5b-3). Mp: 88–90 °C, ^{1}H NMR ($CDCl_3$) δ: 1.15 (t, J = 7.5 Hz, 3H, SCH_2–CH_2–CH_3), 1.88–1.95 (m, 2H, SCH_2–CH_2–CH_3), 3.30 (q, J = 7.5 Hz, 2H, SCH_2–CH_2–CH_3), 7.30 (d, J = 9.1 Hz, 2H), 7.37 (d, J = 8.3 Hz, 2H), 7.45 (d, J = 9.1 Hz, 2H), 7.48 (d, J = 8.3 Hz, 2H). Mass: *m*/*z* (%), 375 (M^+, 5), 333 (9), 248 (64) 246 (100), 176 (35), Anal. Calcd for $C_{18}H_{15}Cl_2N_3S$: C, 57.45; H, 4.02; N, 11.17. Found: C, 57.29; H, 4.19; N, 10.87.

4.2.1.8. 3-Thio-*n*-butyl-5,6-(4-dicholorophenyl)-1,2,4-triazine (5b-4). Mp: 90–91 °C, ^{1}H NMR ($CDCl_3$) δ: 0.80 (t, J = 7.5 Hz, 3H, SCH_2–CH_2–CH_2–CH_3), 1.31–1.40 (m, 2H, SCH_2–CH_2–CH_2–CH_3),

1.78–1.98 (m, 2H, SCH_2–CH_2–CH_2–CH_3), 3.40 (q, J = 7.4 Hz, 2H, SCH_2–CH_2–CH_2–CH_3), 7.31 (d, J = 9.0 Hz, 2H), 7.38 (d, J = 8.5 Hz, 2H), 7.45 (d, J = 9.0 Hz, 2H), 7.50 (d, J = 8.3 Hz, 2H). Mass: m/z (%), 389 (M^+, 42), 332 (30), 247 (98), 208 (18), 176 (100), 100 (5), 85 (15), Anal. Calcd for $C_{19}H_{17}Cl_2N_3S$: C, 58.46; H, 4.39; N, 10.76. Found: C, 58.47; H, 4.23; N, 10.96.

4.3. Cell culture, differentiation, and treatment conditions

Rat pheochromocytoma (PC12) cells obtained from Pasteur Institute (Tehran, Iran) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Aldrich), supplemented with 10% fetal bovine serum and 1% antibiotic mixture comprising penicillin–streptomycin, in a humidified atmosphere at 37 °C with 5% CO_2. Growth medium was changed three times a week. The cells were differentiated by treating with nerve growth factor (NGF) (50 ng/ml) every other day for 6 days.

Differentiated PC12 cells were pretreated with different concentrations (0.5–20 μM) of triazine derivatives for 3 h and incubated with H_2O_2 (150 μM), as an oxidative agent, in the presence of compounds, for an additional 24 h.

4.4. Measurement of cell viability

Cell viability was determined by the conventional MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay. The dark blue formazan crystals formed in intact cells were solubilized in dimethyl sulfoxide (DMSO) and the absorbance was measured at 550 nm. Results were expressed as the percentages of reduced MTT, assuming the absorbance of control cells as 100%.

4.5. AO/EB double staining

Apoptosis was determined morphologically after staining the cells with AO/EB followed by fluorescence microscopy inspection. Briefly, differentiated PC12 cells were seeded in a 96-well plate and were treated with triazine derivatives (10 μM) followed by adding H_2O_2 (150 μM). After 24 h, the cells were harvested and washed three times with PBS and were adjusted to a density of 10^6 cells/ml of PBS. AO/EB solution (1:1 v/v) was added to the cell suspension in a final concentration of 100 μg/ml. The cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany). All experiments were repeated three times and the number of stained cells was counted in 10 randomly selected fields.

4.6. Western blot analysis

The total proteins were electrophoresed in 12% SDS–PAGE gel, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced ElectroChemiLuminescence (ECL) reagents (Amersham Bioscience, USA) and subsequent autoradiography. Quantification of the results was performed by densitometric scan of films. Data analysis was done by Image.J. Protein concentrations were determined according to the Bradford's method.[29] A standard plot was generated by using bovine serum albumin. Nuclear and cytoplasmic proteins were isolated as described by Kutuk and Basaga.[11]

4.7. Measurement of intracellular ROS

The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) was used to monitor intracellular accumulation of ROS. For this purpose, the DCFH-DA solution (10 μM) was added to the suspension of differentiated PC12 cells (1×10^6/ml) and the mixture was incubated at 37 °C for 1 h. The cells were then washed twice with PBS and finally, the fluorescence intensity was measured by Varian-spectrofluorometer, model Cary Eclipse with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

4.8. Data analysis

All data are represented as the mean ± S.D. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by a specific post-hoc test to analyze the difference. The statistical significances were achieved when P <0.05 ($^*P$ <0.05, $^{**}P$ <0.01, and $^{***}P$ <0.001).

Acknowledgments

M.A. and F.K. thank National Elite Fund, Iran, for the award of Young Scientist Research Fellowship. This work was supported partially by Shahid Beheshti University of Medical Sciences Research Funds, Tehran University of Medical Sciences Research Council and INSF (Iran National Sciences Foundation). The authors thank Fatemeh Shaerzadeh for her excellent technical assistance.

References and notes

1. Götz, M. E.; Künig, G.; Riederer, P.; Youdim, M. B. *Pharmacol. Ther.* **1994**, *63*, 37.
2. Butterfield, D. A.; Drake, J.; Pocernich, C. B.; Castegna, A. *Trends Mol. Med.* **2001**, *7*, 548.
3. Behl, C.; Davis, J. B.; Lesley, R.; Schubert, D. *Cell* **1994**, *77*, 817.
4. Halliwell, B.; Aruoma, O. I. *FEBS Lett.* **1991**, *281*, 9.
5. Yoshikawa, A.; Saito, Y.; Maruyama, K. *Biochem. Biophys. Res. Commun.* **2006**, *344*, 394.
6. Zingarelli, B.; Sheehan, M.; Wong, H. R. *Crit. Care Med.* **2003**, *31*, 105.
7. Janssen-Heininger, Y. M.; Poynter, M. E.; Baeuerle, P. A. *Free Radical Biol. Med.* **2000**, *28*, 1317.
8. Akama, K. T.; Van Eldik, L. J. *J. Biol. Chem.* **2000**, *275*, 7918.
9. Karin, M.; Lin, A. *Nat. Immunol.* **2002**, *3*, 221.
10. Jang, J. H.; Surh, Y. J. *Free Radical Biol. Med.* **2005**, *38*, 1604.
11. Kutuk, O.; Basaga, H. *Free Radical Res.* **2003**, *37*, 1267.
12. Bekircan, O.; Küxük, M.; Kahveci, B.; Kolayli, S. *Arch. Pharm. (Weinheim)* **2005**, *338*, 365.
13. Misra, U.; Hitkari, A.; Saxena, A. K.; Gurtu, S.; Shanker, K. *Eur. J. Med. Chem.* **1996**, *31*, 629.
14. Brzozowski, Z.; Saczewski, F. *Eur. J. Med. Chem.* **2002**, *37*, 709.
15. Iwashita, A.; Maemoto, T.; Nakada, H.; Shima, I.; Matsuoka, N.; Hisajima, H. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 961.
16. Kim, W.; Kim, Y.; Min, J.; Kim, D. J.; Chang, Y. T.; Hecht, M. H. *ACS Chem. Biol.* **2006**, *1*, 461.
17. Hunt, J. C. A.; Briggs, E.; Clarke, E. D.; Whittingham, W. G. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5222.
18. Saxena, S.; Verma, M.; Saxena, A. K.; Shanker, K. *Arzneim.-Forsch./Drug Res.* **1994**, *44*, 766.
19. Adams, R.; Marvel, C. S. *Org. Synth. Coll.* **1941**, *1*, 94.
20. Clark, H. T.; Dreger, E. E. *Org. Synth. Coll.* **1941**, *1*, 87.
21. Guroff, G. In *Cell Culture in Neurosciences*; Bottenstein, J. E., Ed.; Plenum Press: New York, 1985; pp 245–272.
22. Tamango, E.; Bardini, P.; Obbili, A.; Vitali, A.; Borghi, R.; Zaccheo, D. *J. Neurobiol. Dis.* **2002**, *10*, 279.
23. Miller, D. K. *Semin. Immunol.* **1997**, *9*, 35.
24. Chen, L.; Liu, L.; Yin, J.; Luo, Y.; Huang, S. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 1284.
25. Zingarelli, B. *Crit. Care Med.* **2005**, *33*, 414.
26. Eftekharzadeh, B.; Khodagholi, F.; Abdi, A.; Maghsoudi, N. *Carbohydr. Polym.* **2010**, *79*, 1063.
27. Guzhova, I. V.; Darieva, Z. A.; Melo, A. R.; Margulis, B. A. *Cell Stress Chaperones* **1997**, *2*, 132.
28. Ruiz-Ramos, R.; Lopez-Carrillo, L.; Rios-Perez, A. D.; De Vizcaya-Ruíz, A.; Cebrian, M. E. *Mutat. Res.* **2009**, *674*, 109.
29. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.