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Original article

Synthesis and in vivo anti-mutagenic activity of novel melatonin derivatives

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Abstract

Extensive literature suggests that melatonin play a role against the degenerative effect of central neurotoxins by its acting as free radical scavenger. This study aimed at evaluation of the anti-mutagenic activity of novel synthesized indole derivatives **2**, **4a**, and **8** in albino male mice in comparison with the parent melatonin. Efficacy of melatonin and its derivatives to influence cyclophosphamide (CP)-induced genotoxicity was tested using micronuclei (MN) formation in the bone marrow cells and determination of DNA, RNA and protein levels as well as cholinesterase and peroxidase activities in several organs of male mice. Following intragastrical injection of melatonin or one of its derivatives daily for 1 week, CP was given intraperitoneally, i.p., as a single dose of 25 mg/kg BW. Pyridazin-4-yl thiadiazoloindole derivative **8**, diaminothiophen-5-yl thiadiazoloindole derivative **4a** and melatonin were significantly able to reduce the number of micronucleated polychromatic erythrocytes (MnPCEs) in the bone marrow cells induced by CP ($P < 0.0001$, $P < 0.001$, $P < 0.01$, respectively). However, reduction of MN formation in the bone marrow cells was not significant when thiadiazoloindole derivative **2** was administered ($P = 0.14$). Examination of the protective effect of melatonin and its derivatives on the levels of DNA, RNA and protein as well as enzyme activities showed that compound **8** had the ability to inhibit the clastogenic effect of CP in several organs of male mice. These findings suggest that compounds **4a**, **8** and melatonin were able to reduce the mutagenicity effect of CP in male mice. The ability of compounds **4a**, **8** and melatonin to reduce CP-related genotoxicity is possibly attributed to their antioxidant activity.

Keywords: Melatonin; Genotoxicity; Cyclophosphamide; Thiadiazole; Thiophene; Pyridazine

1. Introduction

Melatonin, the pineal gland indole, is a potent endogenous antioxidant. It is claimed to detoxify a variety of free radicals and reactive oxygen intermediates [1]. Melatonin prevents lipid membrane peroxidation and apoptosis [2], and protects the DNA from damage induced by free radicals [3]. Moreover, melatonin stimulates gene expression of antioxidative enzymes [4,5]. Cyclophosphamide (CP) is a chemotherapeutic cancer medication. Serious side effects have been reported with the use of CP. It is a clastogenic agent which has been shown to produce gene mutations, chromosome aberrations, micronuclei (MN) formation and sister chromatid exchanges in rats, mice, Chinese hamsters and fish species [6,7]. Melatonin leads to strong inhibition of the clastogenic activity of CP [8] and ameliorates bladder damage induced by CP by diminishing the oxidative stress and blocking iNOS and peroxynitrite production [9]. Also, the use of melatonin as antimutagenic agent for human protection against carbamazepine-induced chromosome damage was investigated [10]. The development of synthetic compounds capable of mimicking the effects of melatonin has progressed considerably during the past decade. These compounds are structurally diverse, and range from simple indole derivatives and its bioisosteres to phenylalkyl amides and constrained melatoninergic agents [11,12].

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In continuation with previous studies on the biologically active indole derivatives of melatonin [13,14], this work aimed to study the possible protective effect of novel synthesized indole derivatives against the CP-induced genotoxicity in comparison with the parent melatonin. The frequency of MN in bone marrow cells and the rate of damage in DNA, RNA, protein, peroxidase and cholinesterase in several organs of male mice were investigated to verify the goal of the present study.

2. Results and discussion

2.1. Chemistry

To improve the essential pharmacophoric features of the melatonin molecule, we have outlined the synthesis of several indole derivatives containing an important heterocyclic moiety starting with melatonin. In fact, therapeutic agents containing thiadiazole and thiophene have attracted the attention of researchers in pharmaceutical chemistry; these heterocycles have been found to show various biological activities such as antimicrobial, anti-inflammatory, antiviral, antituberculosis, anti-tumor and antioxidant activities [15–19].

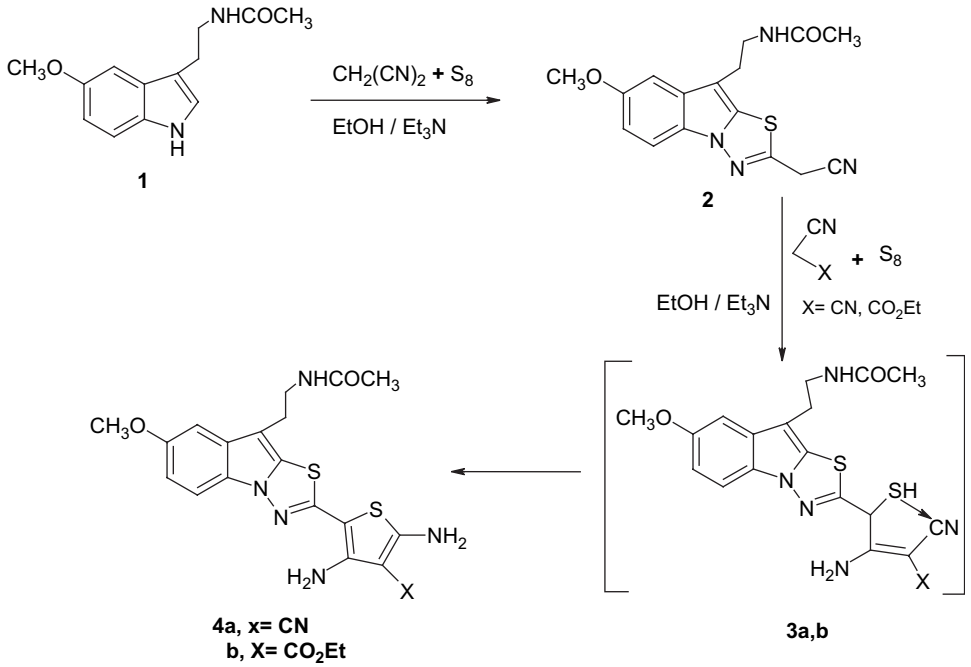
In a recent communication from this laboratory we have reported about the synthesis of thiadiazoloindole derivative (**2**) by the reaction of melatonin (**1**) with equimolar amounts of malononitrile and elemental sulfur in refluxing ethanolic triethylamine solution (Scheme 1) [14]. Compound **2** seemed to be a key precursor for many interesting chemistry. The reactivity of acetonitrile moiety of compound **2** towards the formation of thiophenes was studied. Thus, when compound **2** was allowed to react with either malononitrile or ethyl cyanoacetate and elemental sulfur, upon boiling under reflux in ethanolic triethylamine solution, it yielded the corresponding diaminothiophen-5-yl thiadiazoloindole derivatives **4a**,**b**, respectively, via the intermediacy of **3a**,**b** (Scheme 1). Compound **2** was fused with acetophenone in the presence of ammonium acetate to form the condensed product, butyronitrilylthiadiazoloindole derivative **5**. The reaction of compound **5** with equimolar amount of elemental sulfur in refluxing ethanolic triethylamine solution afforded the corresponding thiophen-3-yl thiadiazoloindole derivative **6** (Scheme 2). Elucidation of the proposed structures **4a**,**b**, **5** and **6** was based on its correct elemental analyses and compatible spectroscopic data (*cf.* Section 3).

Pyridazines and their derivatives possess various biological activities in addition to their powerful antioxidant and free radical scavenging properties [20,21]. Compound **5** with its methyl group in β-position to the electronegative cyano group showed marked reactivity towards electrophilic reagents. Thus, it is easily coupled with benzendiazonium chloride at 0–5 °C in ethanolic sodium acetate solution to afford the isolated coupling product **7** in 78% yield (Scheme 2). Compound **7** readily undergoes intramolecular cyclization when heated under reflux in sodium ethoxide solution to give the pyridazin-4-yl thiadiazoloindole derivative **8**. Confirmation of the structures of all products was obtained through studying their spectral and analytical data (*cf.* Section 3).

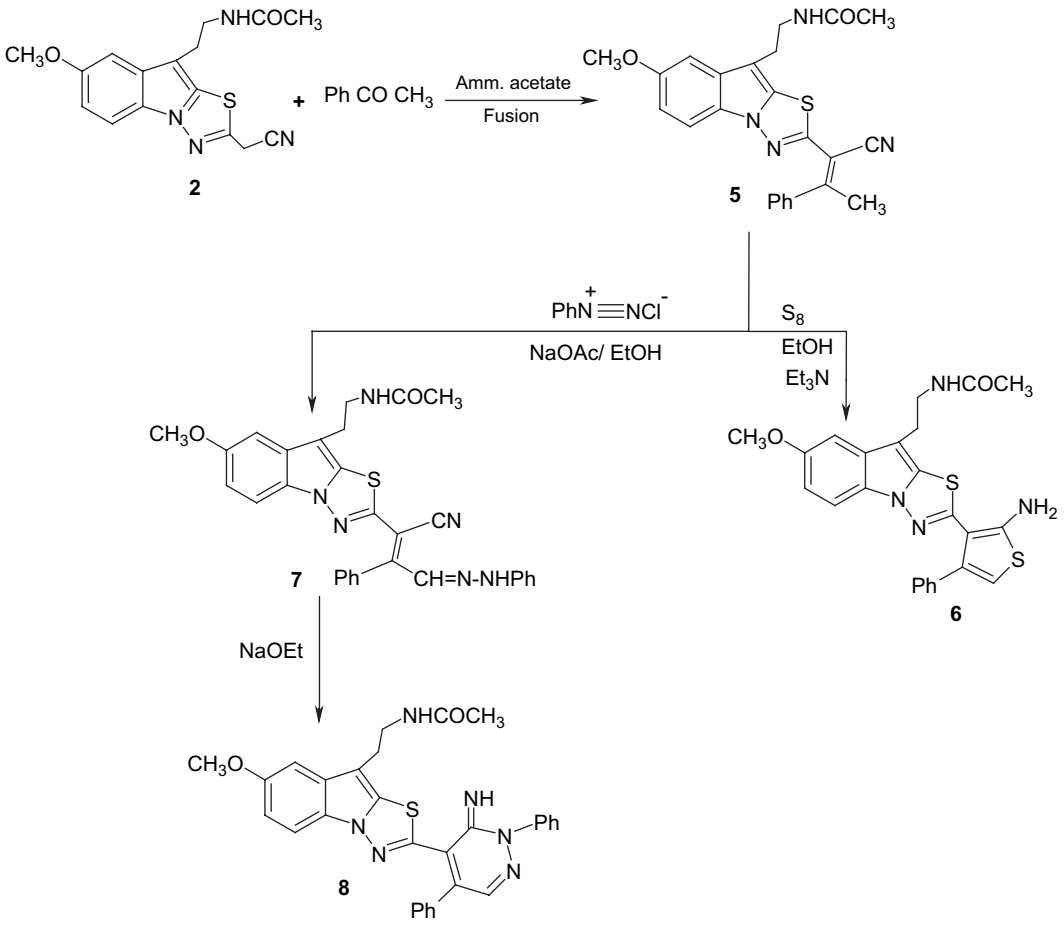
2.2. Genotoxicity assays

2.2.1. Micronuclei assay

Cytogenetic methods are probably the most sensitive and efficient means of detecting the effects of genotoxins [7]. The MN test has been used successfully as a mutagenic assay since it is simple, reliable, sensitive, and is not strongly dependent on any karyotypic characteristics [22]. In the present study, we have used the MN assay to determine whether



Scheme 1.



Scheme 2.

melatonin and its novel synthesized indole derivatives **2**, **4a** and **8** could protect male mice from the toxicity of CP.

After CP supplementation, the number of MnPCEs in the bone marrow cells of male mice was highly significantly increased compared with the values of control, melatonin and its indole derivatives **2**, **4a**, and **8** (Fig. 1). Pyridazin-4-yl thiadiazoloindole derivative **8**, diaminothiophen-5-yl thiadiazoloindole derivative **4a** and melatonin were able to significantly reduce the number of MnPCEs in the bone marrow cells induced by CP ($P < 0.0001$, $P < 0.001$, $P < 0.01$, respectively) (Fig. 1). Compound **8** inhibited the rise of MnPCEs by about 65%, while compound **4a** inhibited the rise of MnPCEs by about 44% and melatonin by about 41%. On the other hand, thiadiazoloindole derivative **2** slightly

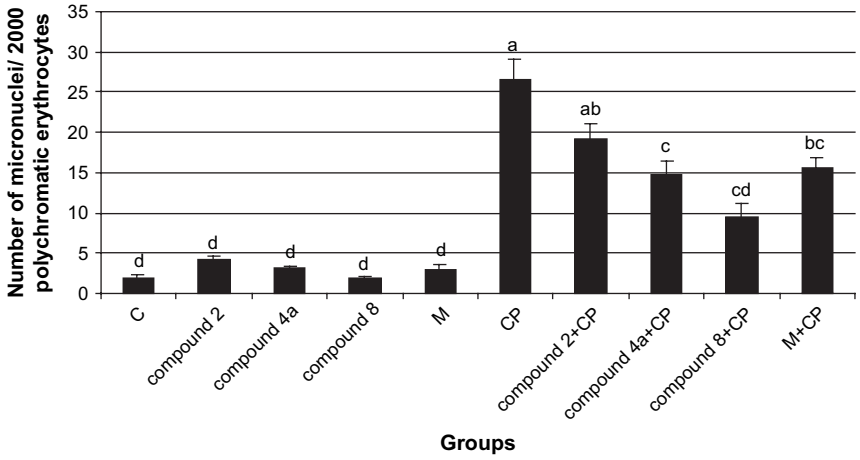


Fig. 1. Effect of melatonin and its derivatives **2**, **4a** and **8** on cyclophosphamide-induced micronuclei in albino male mice. a–d within columns: values with different superscripts differ ($P < 0.05$, Scheffé-test). C = control, M = melatonin, CP = cyclophosphamide.

Table 1
The amount of DNA content in mice tissues 24 h post-cyclophosphamide administration with and without melatonin and its indole derivatives **2**, **4a** and **8**

Groups	DNA level (mg/g tissues)							
	Brain		Kidney		Liver		Testes	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C	0.24^{b}	0.01	0.25^{b}	0.01	0.23^{c}	0.01	0.24^{bc}	0.01
Compound **2**	0.33^{a}	0.02	0.28^{bc}	0.01	0.27^{bc}	0.01	0.32^{ab}	0.02
Compound **4a**	0.35^{a}	0.02	0.34^{ab}	0.02	0.33^{ab}	0.02	0.35^{a}	0.01
Compound **8**	0.37^{a}	0.01	0.38^{a}	0.03	0.35^{a}	0.02	0.39^{a}	0.01
M	0.32^{a}	0.01	0.25^{bc}	0.01	0.26^{bc}	0.01	0.26^{bc}	0.01
CP	0.18^{c}	0.01	0.19^{c}	0.01	0.20^{c}	0.01	0.20^{c}	0.01
Compound **2** + CP	0.21^{b}	0.01	0.35^{ab}	0.01	0.25^{ab}	0.01	0.28^{bc}	0.01
Compound **4a** + CP	0.22^{b}	0.01	0.35^{ab}	0.01	0.29^{bc}	0.01	0.30^{b}	0.02
Compound **8** + CP	0.25^{b}	0.01	0.36^{ab}	0.02	0.32^{ab}	0.01	0.31^{ab}	0.02
M + CP	0.21^{b}	0.01	0.24^{b}	0.01	0.23^{c}	0.01	0.23^{c}	0.01

Superscripts a–c within columns: values with different superscripts differ ($P < 0.05$, Scheffé-test). C = control, M = melatonin, CP = cyclophosphamide.

decreased the MN formation induced by CP, while reduced the MN formation in the bone marrow cells by about 28%.

2.2.2. Changes in DNA content

Administration of CP significantly decreased the content of DNA in several organs of male mice (Table 1). However, the damage in DNA content was not shown in the brain of albino male mice treated either with melatonin or its indole derivatives **2**, **4a**, and **8** (Table 1). Furthermore, the ability of compound **8** to inhibit the damage in DNA content induced by CP was significantly observed in kidney, liver and testes of albino male mice. Similarly, compounds **4a**, **2** and melatonin decreased the damage in the DNA induced by CP in kidney tissues (Table 1).

2.2.3. Changes in RNA content

Table 2 shows that RNA was damaged in male mice tissues treated with CP. However, RNA content was increased in the tissues of all tested organ when albino male mice were supplemented with compound **8**. Similarly, compound **4** and melatonin had the same effect on the RNA content in liver and testes. Furthermore, the ability of compound **8** to inhibit the damage in RNA content induced by CP was markedly shown in the brain and liver of albino male mice (Table 2).

2.2.4. Changes in total protein and enzyme activities

The present study showed low values of protein and peroxidase as well as cholinesterase in CP group (Tables 3 and 4). This reduction caused by CP was significantly inhibited in the brain, liver and testes when male mice were administered compound **8** (Tables 3 and 4). The same trend was found for total protein in the brain and liver with compounds **4a** and **2** (Table 3).

Our present results indicated that CP significantly increased the number of MN in bone marrow cells and made a high reduction in DNA, RNA, protein and peroxidase as well as cholinesterase levels of male mice when compared with the values of control, melatonin and its derivatives. It is known that exposure of DNA to free radical-generating systems causes extensive strand breakage and degradation of deoxyribose [23], probably due to the formation of very toxic hydroxyl radicals. Reactive oxygen radicals have been suggested to be involved in the action of a number of DNA-damaging drugs and other xenobiotics [24–26]. We suggest also that CP may induce genotoxicity due to stimulation of the somatic cells of male mice to form reactive oxygen radicals which are involved in the pathway of DNA-, RNA- and protein-damaging.

Pyridazin-4-yl thiadiazoloindole derivative **8**, diaminothiophen-5-yl thiadiazoloindole derivative **4a** and melatonin were able to significantly reduce the number of the MnPCEs in the bone marrow cells induced by CP. Our present study established that compounds **8**, **4a** and melatonin could also inhibit the damage in DNA, RNA, protein and peroxidase as well as cholinesterase levels induced by CP in several organs of male mice. There are several studies which tried to explain the protective effect of melatonin against CP-induced genotoxicity.

Table 2
The amount of RNA content in mice tissues 24 h post-cyclophosphamide administration with and without melatonin and indole derivatives **2**, **4a**, and **8**

Groups	RNA level (mg/g tissues)							
	Brain		Kidney		Liver		Testes	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C	0.16^{bc}	0.01	0.17^{b}	0.01	0.19^{c}	0.01	0.19^{c}	0.01
Compound **2**	0.19^{ab}	0.01	0.23^{ab}	0.01	0.30^{bc}	0.01	0.21^{bc}	0.01
Compound **4a**	0.19^{ab}	0.01	0.28^{ab}	0.01	0.46^{a}	0.01	0.27^{ab}	0.01
Compound **8**	0.21^{a}	0.01	0.47^{a}	0.01	0.48^{a}	0.05	0.36^{a}	0.02
M	0.18^{ab}	0.01	0.38^{ab}	0.06	0.44^{a}	0.01	0.34^{a}	0.03
CP	0.13^{c}	0.01	0.13^{b}	0.01	0.16^{c}	0.01	0.13^{bc}	0.01
Compound **2** + CP	0.16^{bc}	0.01	0.22^{ab}	0.01	0.34^{ab}	0.02	0.19^{bc}	0.01
Compound **4a** + CP	0.16^{bc}	0.01	0.24^{ab}	0.01	0.49^{a}	0.03	0.20^{bc}	0.01
Compound **8** + CP	0.17^{ab}	0.01	0.40^{a}	0.11	0.51^{a}	0.03	0.20^{bc}	0.01
M + CP	0.15^{c}	0.01	0.19^{b}	0.01	0.28^{bc}	0.01	0.17^{c}	0.01

Superscripts a–c within columns: values with different superscripts differ ($P < 0.05$, Scheffé-test). C = control, M = melatonin, CP = cyclophosphamide.

Table 3
The amount of total protein in mice tissues 24 h post-cyclophosphamide administration with and without melatonin and indole derivatives **2**, **4a**, and **8**

Groups	Protein level (g/g tissues)							
	Brain		Kidney		Liver		Testes	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C	2.50^{ab}	0.14	5.27^{c}	0.16	7.32^{bc}	0.13	4.39^{b}	0.09
Compound **2**	2.98^{ab}	0.06	7.87^{ab}	0.47	9.95^{ab}	0.63	5.25^{ab}	0.18
Compound **4a**	2.76^{ab}	0.19	7.17^{ab}	0.22	9.81^{ab}	0.61	5.87^{a}	0.07
Compound **8**	3.20^{a}	0.12	8.68^{a}	0.16	10.23^{a}	0.38	6.02^{a}	0.12
M	2.48^{ab}	0.09	6.39^{bc}	0.35	8.42^{bc}	0.23	4.98^{ab}	0.27
CP	2.00^{c}	0.02	3.54^{d}	0.06	6.24^{c}	0.16	2.27^{c}	0.16
Compound **2** + CP	2.71^{ab}	0.09	4.94^{cd}	0.22	9.40^{ab}	0.55	3.49^{bc}	0.19
Compound **4a** + CP	2.53^{ab}	0.09	4.14^{cd}	0.24	9.26^{b}	0.44	3.11^{c}	0.16
Compound **8** + CP	3.15^{a}	0.09	4.96^{cd}	0.39	10.03^{ab}	0.60	4.31^{b}	0.29
M + CP	2.37^{bc}	0.09	4.42^{cd}	0.19	8.42^{bc}	0.33	2.96^{c}	0.12

Superscripts a–d within columns: values with different superscripts differ ($P < 0.05$, Scheffé-test). C = control, M = melatonin, CP = cyclophosphamide.

Table 4
The amount of peroxidase and cholinesterase activities in mice brain 24 h post-cyclophosphamide administration with and without melatonin and indole derivatives **2**, **4a**, and **8**

Groups	Peroxidase activity (U/mg tissues/min)		Cholinesterase activity (U/mg tissues)	
	Mean	SEM	Mean	SEM
C	1.68^{e}	0.07	2.51^{bc}	0.08
Compound **2**	1.06^{e}	0.15	3.30^{ab}	0.15
Compound **4a**	6.30^{b}	0.14	3.76^{a}	0.10
Compound **8**	9.74^{a}	0.27	4.21^{a}	0.24
M	3.38^{cd}	0.28	3.13^{ab}	0.06
CP	0.96^{e}	0.14	2.11^{c}	0.07
Compound **2** + CP	1.24^{e}	0.23	2.73^{bc}	0.12
Compound **4a** + CP	4.10^{c}	0.19	2.85^{bc}	0.09
Compound **8** + CP	5.50^{abc}	0.35	3.03^{ab}	0.12
M + CP	2.54^{d}	0.11	2.63^{bc}	0.09

Superscripts a–e within columns: values with different superscripts differ ($P < 0.05$, Scheffé-test). C = control, M = melatonin, CP = cyclophosphamide.

Melatonin is a potent scavenger of both reactive hydroxyl "OH" [27] and peroxyl "ROO" radicals [28] and it increases the concentrations of endogenous antioxidants such as glutathione [29,30]. Melatonin also stimulates the antioxidative enzyme glutathione peroxidase [5,26,31]. In addition, *in vitro* studies showed that melatonin may act synergistically with other chain-breaking antioxidants such as glutathione, Trolox (water-soluble vitamin E analogue), and ascorbate to suppress the formation of free hydroxyl radicals [32].

In the present study, we have found that compounds **4a**, **8** and melatonin were able to increase levels of DNA, RNA and protein as well as stimulate the synthesis of the antioxidant enzyme peroxidase. In agreement with these results, Zavodnik et al. [33] suggested that melatonin may be able to stimulate the nucleus (in the transcription–translation pathway) to produce antioxidative enzymes. Through actions that lead to stimulation of antioxidative enzymes, melatonin protects cells from a wide variety of toxins and drugs that generate free radicals and associated toxic agents. Moreover, our present results indicate that compounds **4a** and **8** have the strongest activity against genotoxicity of CP and antioxidant activity which exceeded that of the parent melatonin. Theses results indicate that the presence of the powerful antioxidant and free radical scavengers, pyridazine and thiophene moieties with their phenyl and amino side chains [18,20,21,34] in addition to the pharmacophoric features of the melatonin moiety, the suitably spaced methoxy and amide side chains and the indole moiety [1], increased the antioxidant properties of melatonin. On the other hand, the presence of thiadiazole ring alone with acetonitrile side chain fused to melatonin moiety in compound **2** could not affect melatonin activity.

The mechanism that how can the cell be protected by the antioxidants such as compounds **4a**, **8** and melatonin against the genotoxicity of CP is not clear. Menendez-Pelaez and Reiter [35] suggested that melatonin easily enters each subcellular compartment. Both endogenously synthesized and exogenously administered melatonin have been shown to be in higher concentrations in the nucleus than in the cytosol of mammalian tissues [35,36]. The present data provide additional evidence for the rapid diffusion of compounds **4a**, **8** and melatonin into the nucleus where they protect DNA and consequently RNA as well as protein against CP-induced genotoxicity.

2.3. Conclusion

Our present results have clearly demonstrated that CP increased MN formation and inflicted damage to DNA, RNA, protein and peroxidase as well as cholinesterase *in vivo* in albino male mice. This toxic effect was completely abolished by treatment with pyridazin-4-yl thiadiazoloindole derivative **8**, diaminothiophen-5-yl thiadiazoloindole derivative **4a** and melatonin, as scavengers of the reactive oxygen radicals or through acting synergistically with other chain-breaking antioxidants such as peroxidase enzyme.

3. Materials and methods

3.1. Synthetic methods and spectral data

The starting pure powder of melatonin was obtained as a gift from *Amoun Pharmaceutical Industries, Egypt*. The appropriate precautions in handling moisture-sensitive compounds were undertaken. All melting points of the new compounds were measured using an electrothermal capillary melting point apparatus and are uncorrected. The IR spectra are expressed in cm^{-1} and recorded in KBr pellets on a Pa-9721 IR spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a Varian EM-390 90 MHz spectrometer in DMSO-d_6 as solvent, using TMS as internal reference and chemical shifts (δ) are expressed in ppm. Mass spectra were recorded on a GCMS-QP 1000 Ex spectra mass spectrometer operating at 70 eV. Elemental analyses were performed with all final compounds by Microanalytical Data Unit at The National Research Centre, Cairo, Egypt. All the results of the elemental analyses were within ±0.4% of the theoretical values. The reactions were followed using TLC analyses which were performed using Merck 60 F254 aluminum sheets and visualized by UV light (254 nm). The starting compound **2** was prepared according to the published procedure [14].

3.1.1. *N-[2-(2-(3-cyano-2,4-diaminothiophen-5-yl)-6-methoxy-1,3,4-thiadiazolo[3,2-a]indol-4-yl)ethyl]acetamide (**4a**) and N-[2-(2-(2,4-diamino-3-ethoxycarbonylthiophen-5-yl)-6-methoxy-1,3,4-thiadiazolo[3,2-a]indol-4-yl)-ethyl]acetamide (**4b**): general procedure*

To a mixture of thiadiazoloindole **2** (1.64 g, 5 mmol) and malononitrile (0.33 g, 5 mmol) or ethyl cyanoacetate (0.56 g, 5 mmol) in ethanol (30 ml) containing a catalytic amount of triethylamine (0.5 ml), elemental sulfur (0.16 g, 5 mmol) was added. The reaction mixture, in each case, was heated under reflux for 4 h until all starting materials had disappeared as indicated by TLC, then left to cool at room temperature, poured onto ice and neutralized with dilute hydrochloric

acid, whereby the resulting solid product in each case was collected by filtration and crystallized from the proper solvent.

*3.1.1.1. Compound **4a***. Reddish brown crystals from EtOH, yield: 1.55 g (73%), mp: 145–147 °C, $C_{19}H_{18}N_6O_2S_2$ (426.522). IR (ν/cm^{-1}): 3428–3335 (NH, $2NH_2$), 3030 (CH aromatic), 2985, 2870 (CH_3, CH_2), 2228 (CN), 1695 (C=O), 1643 (C=C). ^{1}H NMR (δ ppm): 1.72 (s, 3H, $COCH_3$), 2.72 (t, 2H, CH_2), 3.49 (t, 2H, CH_2), 3.78 (s, 3H, OCH_3), 4.95, 5.12 (2s, $2NH_2$, D_2O-exchangeable), 7.15–7.63 (m, 3H, C_6H_3), 8.42 (s, 1H, NH, D_2O-exchangeable). ^{13}C NMR (δ): 23.6 ($CO\mathit{C}H_3$), 25.5, 38.2 ($2CH_2$), 55.7 (OCH_3), 131.6 (C-2), 111.8 (C-3), 100.3 (C-4), 152.9 (C-5), 110.7 (C-6), 109.6 (C-7), 131.5, 128.7 (fused aromatic-C), 133.2, 126.3, 133.7, 127.5 (thiophene-C), 135.6 (thiadiazole-C), 170.5 (C=O). MS (EI), *m/z* (%): 426 ($M^{+\bullet}$, 50%).

*3.1.1.2. Compound **4b***. Pale brown crystals from acetone, yield: 1.65 g (70%), mp: 163–164 °C, $C_{21}H_{23}N_5O_4S_2$ (473.582). IR (ν/cm^{-1}): 3432–3334 (NH, $2NH_2$), 3035 (CH aromatic), 2985, 2876 (CH_3, CH_2), 1730,1695 (2C=O, ester C=O, $COCH_3$), 1640 (C=C). ^{1}H NMR (δ ppm): 1.13 (t, 3H, ester CH_3), 1.70 (s, 3H, $COCH_3$), 2.72 (t, 2H, CH_2), 3.52 (t, 2H, CH_2), 3.73 (s, 3H, OCH_3), 4.25 (q, 2H, ester CH_2), 5.15, 5.42 (2s, $2NH_2$, D_2O-exchangeable), 7.25–7.68 (m, 3H, C_6H_3), 8.47 (s, 1H, NH, D_2O-exchangeable). MS (EI), *m/z* (%): 472 ($M^{+\bullet} - 1$, 35%).

*3.1.2. N-[2-(2-(β-phenylbutyronitrilo-α-yl)-6-methoxy-1,3,4-thiadiazolo[3,2-a]indol-4-yl)ethyl]acetamide (**5**)*

To a dry mixture of thiadiazoloindole **2** (1.64 g, 5 mmol) and acetophenone (0.60 g, 5 mmol), ammonium acetate (0.77 g, 10 mmol) was added. The reaction mixture was heated in an oil bath (130 °C) for 2 h and then left to cool, triturated with ethanol and poured onto ice water. The formed solid product was collected by filtration, dried and crystallized from methanol to yield 1.46 g (68%) of compound **5**, mp: 114–116 °C, $C_{24}H_{22}N_4O_2S$ (430.532). IR (ν/cm^{-1}): 3435 (NH), 3030 (CH aromatic), 2978, 2870 (CH_3, CH_2), 1698 (C=O), 1647 (C=C). ^{1}H NMR (δ ppm): 1.74 (s, 3H, $COCH_3$), 2.70 (t, 2H, CH_2), 3.09 (s, 3H, CH_3), 3.47 (t, 2H, CH_2), 3.82 (s, 3H, OCH_3), 7.20–7.98 (m, 8H, C_6H_3, C_6H_5), 8.23 (s, 1H, NH, D_2O-exchangeable). MS (EI), *m/z* (%): 430 ($M^{+\bullet}$, 47%).

*3.1.3. N-[2-(2-(2-amino-4-phenylthiophen-3-yl)-6-methoxy-1,3,4-thiadiazolo[3,2-a]indol-4-yl)ethyl]acetamide (**6**)*

To a solution of compound **5** (0.86 g, 2 mmol) in ethanol (30 ml) containing a catalytic amount of triethylamine (0.5 ml), elemental sulfur (0.064 g, 2 mmol) was added. The reaction mixture was heated under reflux for 3 h until all starting materials had disappeared as indicated by TLC. The reaction mixture was concentrated under *vacuum* and then left to cool at room temperature, poured over ice/water mixture and neutralized with dilute hydrochloric acid. The formed solid product was filtered off, dried and crystallized from ethanol to yield 0.66 g (72%) of compound **6**, mp: 172–174 °C, $C_{24}H_{22}N_4O_2S_2$ (462.603). IR (ν/cm^{-1}): 3448, 3345 (NH, NH_2), 3027 (CH aromatic), 2985, 2872 (CH_3, CH_2), 1693 (C=O), 1648 (C=C). ^{1}H NMR (δ ppm): 1.81 (s, 3H, $COCH_3$), 2.70 (t, 2H, CH_2), 3.38 (t, 2H, CH_2), 3.80 (s, 3H, OCH_3), 5.22 (s, NH_2, D_2O-exchangeable), 7.23–7.92 (m, 8H, C_6H_3, C_6H_5), 8.45 (s, 1H, NH, D_2O-exchangeable). MS (EI), *m/z* (%): 462 ($M^{+\bullet}$, 32%).

*3.1.4. N-[2-(2-(β-phenyl-γ-phenylhydrazonbutyronitrilo-α-yl)-6-methoxy-1,3,4-thiadiazolo[3,2-a]indol-4-yl)ethyl]-acetamide (**7**)*

A solution of compound **5** (0.86 g, 2 mmol) in ethanol (30 ml) containing sodium acetate (0.5 g) was cooled to 0–5 °C, then treated gradually with continuous stirring with a cold solution of benzenediazonium chloride salt (2 mmol) [prepared by the addition of sodium nitrite solution (0.14 g, 2 mmol) to a cold solution of aniline (0.18 g, 2 mmol) in concentrated hydrochloric acid (3 ml)]. After the addition of the diazonium salt, the reaction mixture was stirred at room temperature for 1 h. The precipitated product, separated upon dilution with cold water, was filtered off, washed several times with water, dried and crystallized from ethanol to yield 0.83 g (78%) of compound **7**, mp: 160–162 °C, $C_{30}H_{26}N_6O_2S$ (534.643). IR (ν/cm^{-1}): 3455, 3436 (2NH), 3032 (CH aromatic), 2984, 2882 (CH_3, CH_2), 2220 (CN), 1690 (C=O), 1658 (C=N), 1638 (C=C). ^{1}H NMR (δ ppm): 1.78 (s, 3H, $COCH_3$), 2.74 (t, 2H, CH_2), 3.36 (t, 2H, CH_2), 3.88 (s, 3H, OCH_3), 7.28–7.98 (m, 13H, C_6H_3, $2C_6H_5$), 8.87, 8.48 (s, 2H, 2NH, D_2O-exchangeable). MS (EI), *m/z* (%): 533 ($M^{+\bullet} - 1$, 52%).

*3.1.5. N-[2-(2-(2,5-diphenyl-3-iminopyridazin-4-yl)-6-methoxy-1,3,4-thiadiazolo[3,2-a]indol-4-yl)ethyl]-acetamide (**8**)*

To ethanolic sodium ethoxide solution [prepared by dissolving sodium metal (0.5 g) in 30 ml absolute ethanol] compound **7** (1.06 g, 2 mmol) was added and the solution was heated under reflux for 5 h. The reaction mixture was then cooled at room temperature, poured onto ice and neutralized with dilute hydrochloric acid (to pH 6). The formed solid product was filtered off, washed several times with water, dried and crystallized from 1,4-dioxane to yield 0.74 g (70%) of compound **8**, mp: 242–243 °C, $C_{30}H_{26}N_6O_2S$ (534.643). IR (ν/cm^{-1}): 3455, 3436 (2NH), 3025 (CH aromatic), 2980, 2878 (CH_3, CH_2), 1693 (C=O), 1660 (C=N), 1640 (C=C). ^{1}H NMR (δ ppm): 1.76 (s, 3H, $COCH_3$), 2.75 (t, 2H, CH_2), 3.42 (t, 2H, CH_2), 3.85 (s, 3H, OCH_3), 7.30–7.96 (m, 13H, C_6H_3, $2C_6H_5$), 8.74, 8.46 (s, 2H, 2NH, D_2O-exchangeable). ^{13}C NMR (δ): 22.8 ($CO\mathit{C}H_3$), 24.8, 40.6 (2 CH_2), 55.4 (OCH_3), 132.3 (C-2), 110.5 (C-3), 111.3 (C-4), 152.3 (C-5), 110.9 (C-6), 110.2 (C-7), 133.0, 129.1 (fused aromatic-C), 169.5 (C=O), 150.6, 148.7, 112.2, 110.2, 112.8, 111.4, 129.0, 129.5, 130.8, 130.2 (2 phenyl-C). 169.2, 168.5, 165.3, 134.7 (pyridazine-C), 137.2 (thiadiazole-C), MS (EI), *m/z* (%): 534 ($M^{+\bullet}$, 63%).

3.2. Bioassay

3.2.1. Animals

Fifty adult male albino mice with body weight BW ranging from 25 to 30 g, purchased from the Animal House at National Research Centre, Cairo, Egypt, were used in this study. The animals were housed in plastic cages, 10 mice per cage, with free access to standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum* at the Animal House Laboratory, National Research Center, Dokki, Cairo, Egypt. After an acclimation period of 1 week, the animals were housed individually in filter-top polycarbonate cages housed in a temperature-controlled and artificially illuminated room free from any source of chemical contamination.

3.2.2. Chemicals

A pure powder of melatonin was obtained as a gift from *Amoun Pharmaceutical Industries, Egypt*. CP was purchased from Sigma, USA. Solutions of melatonin and its novel synthesized indole derivatives **2**, **4a**, and **8** were made by dissolving in 3% Tween solution. The dose of melatonin (10 mg/kg BW) was chosen based on previous studies [9] and the equimolar doses 14.16, 18.38 and 23.04 mg/kg BW of compounds **2**, **4a** and **8**, respectively, were used.

3.2.3. Experimental design

The mice were randomly divided into 10 groups ($n = 5$ per group) and treated intragastrically for 1 week as follows: *Group* I, control group: animals were treated with solvent vehicle control (3% Tween). *Group II*, animals were treated with melatonin (10 mg/kg BW). *Group III*, animals were treated with compound **2** (14.16 mg/kg BW). *Group IV*, animals were treated with compound **4a** (18.38 mg/kg BW). *Group V*, animals were treated with compound **8** (23.04 mg/kg BW). *Group VI*, animals were injected intraperitoneally, i.p., with CP alone (25 mg/kg BW, as a single injection) 24 h prior to their sacrifice. *Groups VII, VIII, IX and X* were treated in the same way as groups *II, III, IV* and *V* except that CP was injected 30 min after last administration of melatonin, compounds **2**, **4a** and **8**, respectively. All animals were sacrificed by cervical dislocation after 24 h of CP injection. Bone marrow was extracted immediately and processed for the MN assay. Estimation of various biochemical parameters like DNA, RNA, protein, cholinesterase and peroxidase levels was carried out in the brain, kidney, liver and testes of the male mice.

3.2.4. Genotoxicity assays

3.2.4.1. Micronuclei test. The bone marrow cells, resuspended in a small volume of fetal calf serum on a glass slide, were used for smear preparation. The smears of bone marrow cells were prepared from each mouse. After air-drying, the slide was fixed in methyl alcohol for 10 min and stained with 5% Giemsa stain for 10 min. All glass slides were coded before observation. From each animal, 2000 PCEs (1000 per slide) were examined for MnPCEs under 1000× magnification using a Nikon microscope [37].

3.2.4.2. Determination of nucleic acid levels. Determination of DNA content: DNA content was determined in mice tissues (brain, kidney, liver and testes) according to Dische [38] and Peares [39]. The method relies on using diphenylamine reagent (1% acetic acid containing 2.75% conc. H_2SO_4). The optical density of the resulting blue color solution (DNA gives blue color when heated with diphenylamine reagent) was read at wavelength of 600 nm using a spectrophotometer.

Determination of RNA content: according to Schneider [40], RNA content was determined in mice tissues using orcinol reagent (1% conc. HCl containing 0.5 g $FeCl_3$). Optical density of the resulting green color solution with orcinol reagent was read at wavelength of 660 nm.

3.2.4.3. Determination of protein level and enzyme activities. Determination of total protein: protein was determined according to the method of Peters [41]. The optical density of the resulting blue color solution was read at wavelength of 545 nm.

Determination of cholinesterase activity: cholinesterase enzyme activity was determined according to Jakobs et al. [42]. A volume of the working reagent solution was mixed with the sample. The absorbance was read at wavelength 405 nm.

Determination of peroxidase activity: peroxidase activity measurements were carried out according to Miranda et al. [43]. The reaction mixture consisted of 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and a suitable amount of the enzyme preparation. The change in absorbance at 470 nm due to guaiacol oxidation was followed at 30 s intervals. One unit of peroxidase activity was defined as the amount of enzyme which increases the O.D. 1.0/min under standard assay conditions.

3.2.5. Statistical analysis

Data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS users guide) followed by Scheffé-test to assess significant differences between groups. The values are expressed as mean ± S.E.M.

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