Journal of Enzyme Inhibition and Medicinal Chemistry, August 2006; 21(4): 405–411

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healthcare

Investigation of the *in vitro* antioxidant behaviour of some 2-phenylindole derivatives: discussion on possible antioxidant mechanisms and comparison with melatonin

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(Received 26 July 2005; in final form 1 September 2005)

Abstract

Oxidative stress has been implicated in the development of many neurodegenerative diseases and also responsible from aging and some cancer types. Indolic compounds are a broad family of substances present in microorganisms, plants and animals. They are mainly related to tryptophan metabolism, and present particular properties that depend on their respective chemical structures. Due to free radical scavenger and antioxidant properties of indolic derivatives such as indolinic nitroxides and melatonin, a series of 2-phenyl indole derivatives were prepared and their *in vitro* effects on rat liver lipid peroxidation levels, superoxide formation and DPPH stable radical scavenging activities were determined against melatonin, BHT and α-tocopherol. The compounds significantly inhibited (72–98%) lipid peroxidation at 10^{-3} M. These values were similar to that observed with BHT (88%). Possible structure–activity relationships of the compounds were discussed.

Keywords: *Indoles, antioxidant mechanism, lipid peroxidation, free radical, synthesis, 2-phenylindoles*

Introduction

Reactive oxygen species (ROS) play an important role in physiological processes, but when in excess, ROS cause oxidative damage to molecules. Under physiological conditions, the production and detoxification of ROS are more-or-less balanced [1]. However, any internal or external pathological factor may disrupt this balance, leading to conditions referred to as oxidative stress, playing a significant role in the pathogenesis of several diseases [2]. Free radicals are chemically reactive species that can attack and damage several biomolecules such as DNA and enzymes. Oxidative damage to DNA by ROS is a continuous problem that cells must guard against to survive. ROS include singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical. Oxidative stress has been implicated in the development of neurodegenerative diseases like Parkinson's, Alzheimer's and Huntington's diseases, epileptic seizures, stroke and is also responsible from aging and some cancer types. In the last decade, melatonin and related indole derivatives have been widely studied as a scavenger of ROS, a secondary product of the aerobic metabolism within cells [3–7].

Indolic compounds are a broad family of substances present in microorganisms, plants and animals [8]. N-Substituted indole-2-carboxamide and 3-acetamide derivatives show promising antioxidant activity against $O_2^{\cdot-}$ [9,10]. Also N-H and N-substituted indole ester derivatives [11] have anti-superoxide formation activity at a concentration of 10^{-4} M.

Comparison of indolinic nitroxides with vitamin E and Trolox (a hydrophilic analogue of vitamin E), showed that where the only difference between the nitroxides was the length of the hydrocarbon chain in the 2-position of indole (Figure 1) then all the

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ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/14756360500381210

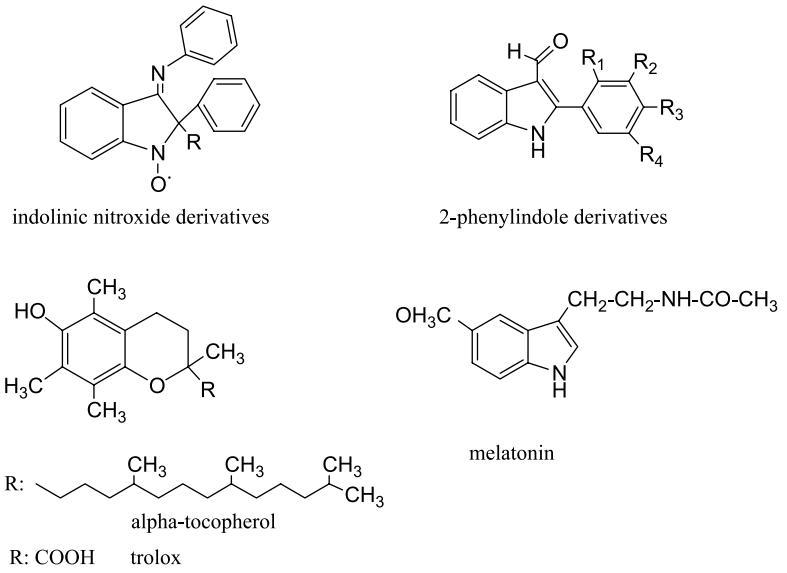

Figure 1. Structure of some antioxidants and 2PI derivatives.

nitroxides were effective in preventing oxidation of bovine serum albumin. Also the results clearly demonstrate that indolinic compounds are efficient antioxidants, protecting both lipids and proteins from peroxidation. The indole structure influences the antioxidant efficacy in biological systems [12].

A series of 2-phenylindole (2PI) **1a**–**g** and 2-phenylindole-3-aldehyde (2PIA) **2a**–**f** derivatives were prepared in order to evaluate their *in vitro* antioxidant properties by determining superoxide anion formation, DPPH free radical scavenging activity and lipid peroxidation (LP) on rat liver homogenate. Also possible structure–activity relationships of the compounds were discussed by comparing with melatonin, alpha-tocopherol, BHT and previously published antioxidant indole derivatives.

Materials and methods

Uncorrected melting points were determined with a Büchi SMP-20 apparatus. The ^{1}H NMR spectra were measured with a Varian 400 MHz using TMS internal standart and DMSO-d_6. ESI Mass spectra were determined on a Waters micromass ZQ. Chromatography was carried out using Merck silica gel 60 (230–400 mesh ASTM). Xanthine, xanthine oxidase, cytochrome c, 2,2,diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene, α-tocopherol and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The chemical reagents used in synthesis were purchased from Sigma (Germany) and Aldrich (USA).

For the preparation of the 2PI derivatives, the Fischer indole synthesis [13] was used. Phenyl hydrazine and acetophenone derivatives were reacted to give hydrazones. Intramolecular cyclization of the hydrazones was performed by $ZnCl_2$. The 2PI **1a**–**g** then were treated with $POCl_3$ and DMF to give 2-PIA **2a**–**f** [14] (Figure 2).

General procedure for the preparation of the 2-phenyl indole-3-aldehydes (2a–f)

$POCl_3$ (6 mmol) was added dropwise to DMF (21 mmol) at −15°C under nitrogen. After addition of 2PI derivative (6 mmol), the mixture was stirred at r.t for 0.5 h. The solution was then poured into ice and NaOH (40%) was added until the mixture became basic. The crystals were filtered and recrystallised from ethanol. The synthesis and physical data for compounds **2d**, **2e** and **2f** that were not stated in the literature are given below.

Compound 2d. yield 88%, m.p: 256–258°C. ^{1}H NMR (d_6-DMSO) δ: (7.21, 2H, m; 7.50, 1H, s; 7.80, 2H, dd; 8.08, 1H, s; 8.18, 1H, d: aromatic-H), 9.95 (1H, s, C*H*O); MS: m/z (%) 292 (63.38) (M + 2), 290 (98.16) (M($^+$, 289 (16.14), 264 (28.38), 262 (42.65), 227 (45.19), 165 (28.14).

Compound 2e. yield 61%, m.p: 149–250°C. ^{1}H NMR (d_6-DMSO) δ: (7.12, 1H, d; 7.23, 2H, m; 7.47, 1H, d; 7.63, 2H, d; 7.90, 1H, s; 8.18, 1H, d: aromatic-H), 9.97 (1H, s, C*H*O). MS: m/z (%) 237 (6.29) (M + 1), 205 (12.16), 169 (18.10), 147 (100.00), 137 (12.15), 119 (39.08).

Compound 2f. yield 79%, m.p: 255–256°C. ^{1}H NMR (d_6-DMSO) δ: (7.44, 2H, m; 7.65, 1H, m; 8.03, 1H, d; 8.14, 1H, d; 8.20, 1H, d; 8.54, 1H, s: aromatic-H), 10.01 (1H, s, C*H*O); MS: m/z (%) 302 (22.27) (M + 2), 301 (72.26) (M + 1(, 231 (36.35), 182 (28.13), 158 (100.00), 137 (29.19), 129 (68.59).

Antioxidant activity studies

Superoxide radical scavenging activity. The ability of 2PI and 2PIA derivatives to scavenge superoxide anion formation was determined spectrophotometrically on the basis of inhibition of cytochrome c reduction according to the modified method of McCord et al. [15]. Superoxide anion $O_2^{\cdot-}$ was generated in the xanthine/xanthine oxidase system. The activity of the enzyme was detected by its ability to reduce cytochrome c which causes an increase in absorbance at 550 nm. The incubation mixture (1 ml, total volume) consisted of phosphate buffer (pH 7.8, 0.05M), xanthine oxidase (0.32 Units/ml), xanthine (50 μM), cytochrome c (60 mM) and different concentrations of 2PI and 2PIA derivatives in 100 μl. The reaction was started by the addition of xanthine oxidase to this mixture. The absorbance was measured spectrophotometrically at 550 nm for 3 min for cytochrome c reduction. Each experiment was in triplicate, and the results are expressed as a percentage of the control.

DPPH free radical scavenging activity. The free radical scavenging activities of 2PI and 2PIA derivatives were tested by their ability to bleach the stable radical 2,2,diphenyl-1-picrylhydrazyl (DPPH) [16]. This assay has often been used to estimate the anti-radical activity of antioxidants. When DPPH$^{\cdot}$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from violet to yellow) were measured at 517 nm on a visible spectrophotometer. The reaction mixture contained 100 μM DPPH in methanol and different concentrations of synthesized compounds. Absorbance at 517 nm was determined after 30 min at room temperature and the scavenging activity were calculated as a percentage of the radical reduction. Each experiment was performed in triplicate and BHT was used as a reference compound. The radical scavenging activity was obtained from the equation:

$$\text{Radicalscavengingactivity}\% = \{(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{control}}\} \times 100$$

where: $\text{OD}_{\text{control}}$:absorption of the blank sample; $\text{OD}_{\text{sample}}$:absorption of tested extract solution.

Assay of lipid peroxidation. The effect of the 2PI and 2PIA derivatives on rat liver homogenate induced with $FeCl_2$-ascorbic acid was determined. LP was examined by the method of Mihara et al. [17]. Procedures involving the animals and their care conformed to institutional guidelines, in compliance with National and International laws and guidelines for the use of animals in biomedical research. Animals were starved for 24 h. prior to sacrifice and then sacrificed by decapitation under anaesthesia. The livers were immediately removed, washed in ice-cold distilled water then immediately homogenized with a Teflon homogenizer. LP was measured spectrophotometrically by estimation of thiobarbituric acid reactants (TBARS). Amounts of TBARS were expressed in terms of nmol malondialdehyde (MDA/g tissue). A typical optimized assay mixture contained 0.5 ml of liver homogenate, 0.1 ml of Tris–HCl buffer (pH 7.2), 0.05 ml of 0.1 mM ascorbic acid, 0.05 ml of 4 mM $FeCl_2$ and 0.05 ml of various concentrations of indole derivatives, melatonin or α-tocopherol, and was incubated for 1 h at 37°C. After incubation, 3.0 ml of H_3PO_4 and 1 ml of 0.6% TBA were added and the mixture shaken vigorously. The mixture was boiled for 30 min. After cooling the mixture to room temperature, n-butanol was added and mixed vigorously. The n-butanol phase was separated at 3000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm against a blank, which contained all reagents except the liver homogenate.

Results

In the present study, we have investigated the antioxidant capacity of the synthesized 2PI and 2PIA derivatives in three different *in vitro* assays; superoxide anion $\left(O_2^{\cdot-}\right)$, DPPH stable radical scavenging activity and LP (Table I).

The inhibitory effects of the compounds on LP levels were determined by measuring the formation of 2-thiobarbituric acid reactive substances. All the compounds were evaluated against melatonin which was chosen as a reference compound because of its high anti-oxidant capacity. As seen in Table I, compounds **1a**–**f**, **2e**, **2f** significantly inhibited (**72**–**98%**) LP at 10^{-3} M and 10^{-4} M concentrations. These values were similar to those observed with BHT and melatonin at 10^{-3} M concentration. Additionally, compound **2b** showed slight inhibition by about 12% at the 10^{-3} M concentration. However the remainder of the compounds, especially 2PIA derivatives, had no effects on LP.

The superoxide anion radical scavenging activities of 2PI and 2PIA derivatives at different concentration were investigated as presented in Table I. The results showed that compounds **1f** and **1 g** have

Table I. Effects of the 2-phenylindole derivatives on LP levels and scavenging activity of superoxide and DPPH radical[a].

Compounds	R_1	R_2	R_3	R_4	R_5	Concentration in incubation medium (M)	Superoxide anion ($O_2^{\cdot-}$) scavenging activity (percent of control)	LP (Percent of control)	DPPH free radical scavenging activity (percent of control)
Control[b]							100	100	100
1a	H	H	H	H	H	10^{-3}	98 ± 5	4.0 ± 1	73 ± 6
						10^{-4}	116 ± 7	5.0 ± 2	88 ± 2
1b	H	H	F	H	H	10^{-3}	84 ± 2	7.0 ± 2	100 ± 7
						10^{-4}	93 ± 4	22 ± 2	109 ± 4
1c	H	H	Cl	H	H	10^{-3}	75 ± 2	7.0 ± 2	96 ± 2
						10^{-4}	108 ± 4	6.0 ± 1	99 ± 4
1d	H	Cl	Cl	H	H	10^{-3}	49 ± 5	5.0 ± 1	95 ± 3
						10^{-4}	101 ± 4	13 ± 2	106 ± 4
1e	H	H	NH_2	H	H	10^{-3}	49 ± 6	2.0 ± 1	11 ± 2
						10^{-4}	69 ± 1	4.0 ± 2	12 ± 4
1f	H	NO_2	Cl	H	H	10^{-3}	14 ± 6	3.0 ± 2	4 ± 2
						10^{-4}	116 ± 6	5.0 ± 2	5 ± 1
1g	OH	H	H	CH_3	H	10^{-3}	6 ± 4	3.0 ± 1	62 ± 2
						10^{-4}	95 ± 5	5.0 ± 2	109 ± 3
2a	H	H	H	H	CHO	10^{-3}	156 ± 14	110 ± 2	103 ± 3
						10^{-4}	131 ± 11	103 ± 2	112 ± 2
2b	H	H	F	H	CHO	10^{-3}	158 ± 12	88 ± 2	103 ± 1
						10^{-4}	130 ± 6	103 ± 2	110 ± 1
2c	H	H	Cl	H	CHO	10^{-3}	148 ± 10	111 ± 2	103 ± 4
						10^{-4}	127 ± 13	103 ± 2	111 ± 3
2d	H	Cl	Cl	H	CHO	10^{-3}	162 ± 15	110 ± 3	104 ± 1
						10^{-4}	140 ± 16	108 ± 4	112 ± 2
2e	H	H	NH_2	H	CHO	10^{-3}	281 ± 22	12 ± 1	99 ± 3
						10^{-4}	128 ± 8	28 ± 2	111 ± 2
2f	H	NO_2	Cl	H	CHO	10^{-3}	131 ± 12	12 ± 2	103 ± 3
						10^{-4}	139 ± 14	15 ± 4	109 ± 4
BHT						10^{-3}		12 ± 2	7 ± 3
						10^{-4}			25 ± 4
melatonin						10^{-3}		13 ± 3	
						10^{-4}		14 ± 3	
α-tocopherol						10^{-3}		3 ± 1	
						10^{-4}		11 ± 3	
SOD						30 IU	24 ± 2		
						45 IU	11 ± 1		

[a] Each value represents the mean ± S.D. of 2–4 independent experiments.
[b] DMSO, control for compounds and BHT.

a strong scavenger effect on superoxide anion at 10^{-3} M concentration. Additionally, these compounds had comparable scavenger effects on superoxide radical as that of SOD (89% inhibitor at 45 IU). Compounds **1b**, **1c**, **1d** and **1e** also decreased the level of superoxide anion by about 16%, 25%, 51%, 51% at 10^{-3} M concentration, respectively. Compounds **1a**, **2a**–**f** had no scavenging effect on superoxide anion at 10^{-3} M and 10^{-4} M concentrations. Compound **1e** had a scavenging effect on superoxide anion at 10^{-4} M concentration by about 31%. Compounds **1f** and **1g** have a promising antioxidant activity by scavenging superoxide radical. However, compounds **2a**, **2b**, **2c**, **2d** which are 2PIA derivatives increased the superoxide anion level at 10^{-4} and 10^{-3} M concentrations.

The scavenging effect of 2PI and 2PIA derivatives on the DPPH radical was also examined. As seen in Table I, compounds **1e** and **1f** had the highest DPPH scavenger activity at 10^{-3} M and 10^{-4} M concentrations (88–96%). These compounds were found to be as effective as BHT, a well known antioxidant used as positive control. Compound **1g** showed a slight scavenger effect on DPPH by about 38%. Compounds **1f** and **1g** scavenged superoxide and DPPH radical and inhibited lipid peroxidation at 10^{-3} M concentrations. The remainder of the compounds showed different patterns of effect on these parameters. The inhibitory effects of compounds were noted at the level of superoxide radical but not DPPH radical. Such contradictory results have also been found in previous studies [18,19]. Therefore, the observation of different effects of synthetic compounds on superoxide anion and DPPH radical scavenger activity was not surprising since the mechanism of production of oxidative stress by these methods were different [20–22].

Discussion

Due to its free radical scavenging and antioxidant properties, all the melatonin-related compounds are under investigation to discover the ideal antioxidant compound showing the highest antioxidant activity with the lowest side effects. Indole derivatives have a heterocyclic aromatic ring structure with high resonance stability and several different substituents on the ring, and this led the researchers to suspect antioxidant activity in these compounds on theoretical founds (Figure 1).

The mechanism of melatonin's interaction with reactive species probably involves donation of an electron to form the melatoninyl cation radical or through a radical addition at the site C3. Other possibilities include hydrogen donation from the nitrogen atom or substitution at position C2, C4 and C7 and nitrosation [23]. The mechanisms by which melatonin protects against lipid peroxidation most likely involve direct or indirect antioxidant and free radical scavenging activities of this indoleamine The most important among them seems to be the ability of melatonin to scavenge such toxic species, like $ONOO^-$, which is sufficiently reactive to initiate the breakdown of lipids, and $NO^{\cdot}$ [24,25]. 2PI derivatives have redox properties because of the presence of an electron-rich aromatic ring system which allows the indoleamine to easily function as an electron donor. However, the mechanisms by which the indole ring interacts with free radicals is still only partially understood. For 2PI derivatives the possible antioxidant mechanism may be toward carbon-centered

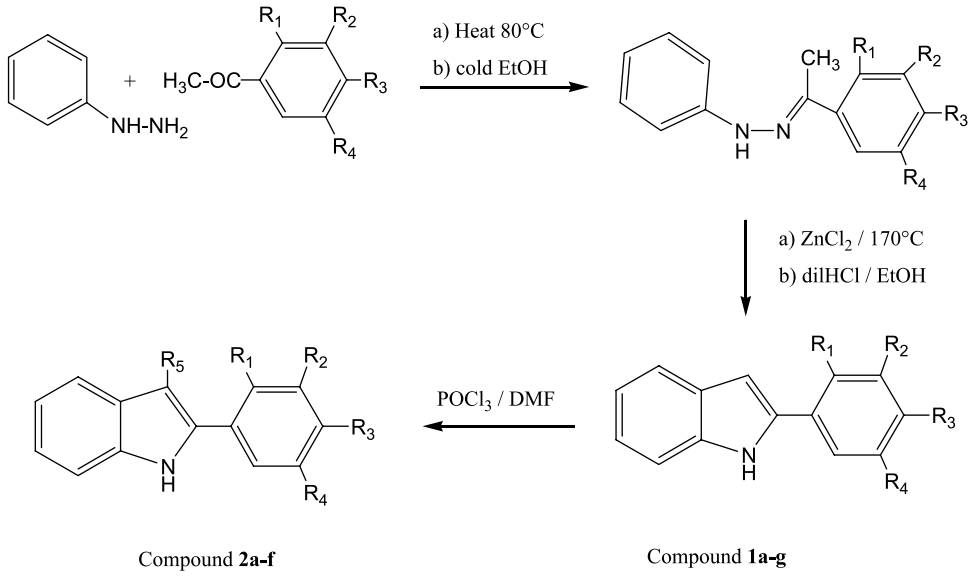

Figure 2. Synthetic route for the preparation of 2PI and 2PIA derivatives.

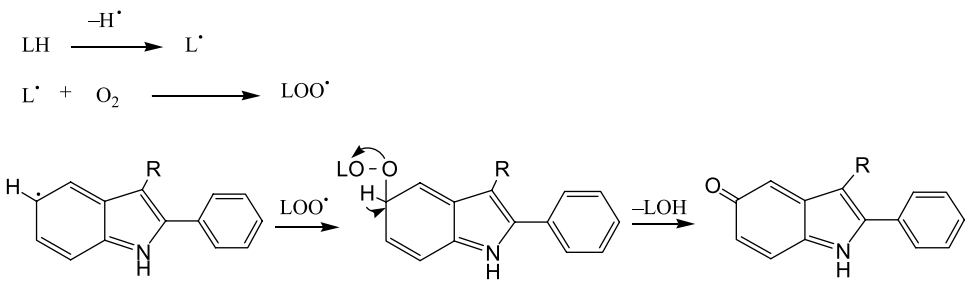

Figure 3. Proposed antioxidant mechanism of 2PI derivatives (adapted from Antosiewicz et al., Free Rad Biol Med 1997;22:249–55).

radicals as shown in Figure 3 that is adopted from Antosiewicz et al. [12]. Compounds bearing electron-withdrawing groups such as F, Cl, NO_2 (**1b**, **1c**, **1d**, **1f**) had the highest reduction in LP values since these groups are inductive electron withdrawing due to their electronegativity and they help indole ring to easily interacts with free radicals.

All of the examined compounds, and melatonin, had no significant inhibitory effect on superoxide anion formation except compounds **1f** and **1g**. This shows that melatonin and 2PI derivatives act as potent hydroxyl radical scavengers *in vitro*. The different effects of the compounds on LP and superoxide anion radical formation is not surprising, since the mechanism of production of oxidative stress (or reactive oxygen species) in these methods are different [20–22]. The results suggest that the antioxidant properties of 2PI derivatives in part, may involve a direct effect on the scavenging of hydroxyl radicals.

We suggest that hydroxyl radical can react with 2PI derivatives by abstraction of an electron. The resulting indolyl cation radical may combines with an $O_2^{\bullet-}$, a reaction by which the electrons are paired and electrical charges are neutralized, so that the radical reaction chain may be terminated. The occurrence of carbon centered radicals may explain the possible antioxidant mechanism of indole derivatives [26] that was proved by electroanalytical studies in our laboratory [27]. There was no significant activity pattern obtained from the LP experiments. The scavenging activity of the compounds against hydroxyl radicals was greater than against superoxide anion radicals.

During the oxidation of indolic compounds, it was observed that an electron was removed from the nitrogen atom (–NH) of the pyrrole ring, generating a radical cation [28–30]. Due to this capacity of the pyrrole ring, the antioxidant activity of the 3-substituted indolic compounds was affected by both functional groups present in the molecule [8]. Thus, the carbonyl group bound to C3 of the indolic ring might be responsible for the absence of antioxidant activity being found for 2-PIA (except for **2e**–**f**) derivatives, the only compounds that do not show significant antioxidant activity.

The *in vitro* experimental findings for the indole derivatives show that there are many derivatives that had good or in some cases better antioxidant properties than melatonin. It should be considered to synthesise more derivatives to discover their free radical scavenging activities and biologically evaluate their synthesised compounds. Further investigations should be done to reveal the exact mechanism of action and cytotoxic effects of new indole derivatives.

Acknowledgements

This work was supported by Ankara University Research and Development Grant 99-03-00-03.

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