Reactions of Indole Derivatives with Cardioprotective Activity with Reactive Oxygen Species. Comparison with Melatonin

Ioanna Andreadou,* Anna Tsantili-Kakoulidou, Eleni Spyropoulou, and Theodora Siatra

Department of Pharmaceutical Chemistry, School of Pharmacy, University of Athens; Panepistimiopolis, Zografou, Athens 157 71, Greece. Received March 17, 2003; accepted June 10, 2003

We have previously reported on the synthesis of novel indole derivatives containing an amine-triazole moiety (1a—d, 2a—c), and their antioxidant activity on *in vitro* non-enzymatic rat hepatic microsomal lipid peroxidation. Some of the compounds showed protective activity against oxidative injury of ischemic myocardium. In the present paper we investigated the interactions of these derivatives with reactive oxygen species, in order to find a mechanism of their antioxidant capacity and to identify structural characteristics responsible for these properties. These interactions were compared with melatonin, which is also an indole derivative. The antioxidant profiles of the compounds were established by different *in vitro* protocols as follows: 1) by the interaction of the compounds with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical, 2) their scavenging effects on superoxide anions using an enzymic system of xanthine–xanthine oxidase, 3) their inhibitory effects on xanthine oxidase and 4) their ability to scavenge hydroxyl radicals by comparison with dimethyl sulfoxide (DMSO) for ˙OH. All compounds were found to interact with DPPH, most of them to be superoxide anion scavengers and to be strong hydroxyl radical scavengers. Derivatives 1a and 1d substituted on the nitrogen of the indolic nucleus were found to have better antioxidant properties than the reference compounds used and melatonin.

Key words indole derivative; melatonin; reactive oxygen species; free radical; antioxidant activity

The heart is the most susceptible of all the organs to premature aging and free radical mediated oxidative stress.[1)] Basic and clinical research has clearly documented the role of free radical damage and the progression of cardiovascular disease. This may be the result of myocardial ischemia, acute ischemia-reperfusion injury, endothelial damage of hyperhomocysteinemia, as well as chronic oxidative damage secondary to lipid peroxidation.[1—4)]

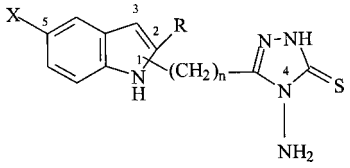
In this aspect, the antioxidant potential is very important in the development of cardiovascular agents. A number of experimental protocols have been developed in order to assess the antioxidant activities *in vitro*.[5—7)]

We have previously reported on the synthesis of a series of indole derivatives that contain a triazole moiety and their antioxidant activity on *in vitro* non-enzymatic rat hepatic microsomal lipid peroxidation [Table 1, **1a**—**d**, **2a**—**c**].[8)] Furthermore, we have reported on the cardioprotective efficacy of one of the above derivatives (**1a**), the compound, 3-[(1*H*-1-indolyl)methyl]-4-amino-4,5-dihydro-1*H*,1,2,4-triazole-5-thione. This molecule showed to reduce the myocardial infarct size in rabbits, *in vivo*. Its beneficial effect on ischemic myocardium might be attributed to its antioxidant and free radical scavenging activity as measured by its interaction with DPPH and its ability to scavenge hydroxyl radicals *in vitro*.[9)]

In the present paper, we considered it of interest to extend our investigation on the interactions of all the indole derivatives **1a**—**d**, **2a**—**c**, with reactive oxygen species (ROS), in order to have a better insight on the mechanism of their antioxidant capacity and to identify structural characteristics responsible for these properties. The redox potential of the compounds was assessed by their interaction with the stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH).[10)] Their ability to scavenge hydroxyl radicals by comparison with dimethyl sulfoxide (DMSO) for ˙OH and their quenching effects on superoxide anions using the enzymic system of xanthine–xanthine oxidase as well as their inhibition on xanthine oxidase were determined and evaluated in order to elucidate the mechanism of the antioxidant action.

Furthermore, melatonin, which is also an indole derivative (Fig. 1), was included in our study. Melatonin influences the cardiovascular system and may contribute in cardioprotection in isolated rat heart following myocardial ischemia.[11)] However, it did not affect myocardial infarct size *in vivo* in rabbits.[12)] Concerning the antioxidant activity, available data suggest that melatonin acts directly as free radical scavenger

Table 1. Structures of the Synthesized Compounds[a)]



Compound	Position of the triazole moiety	X	R	*n*
1a	1	H	H	1
1b	1	Br	H	1
1c	1	Cl	H	1
1d	1	NO_2	H	1
2a	3	H	H	1
2b	3	H	H	2
2c	3	H	CH_3	1

a) From ref. 8.

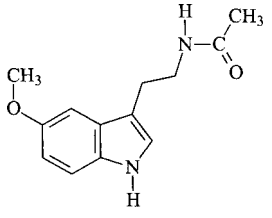


Fig. 1. Structure of Melatonin

* To whom correspondence should be addressed. e-mail: ioannandread@yahoo.com

and/or by inhibiting their formation while, it may interfere in the endogenous defense system.[13] However, relevant studies *in vitro* are rather contradictory and it seems that it exerts only limited antioxidant activity in systems *in vitro*.[14] Therefore, we found it of interest to compare its antioxidant activity to that produced by compounds **1a**—**d** and **2a**—**c** under the same experimental conditions.

Results and Discussion

Oxygen-derived free radicals and their metabolites may contribute to the extension of irreversible cellular injury, which occurs in reperfusion of previously ischemic myocardium. Therefore, therapy directed against the effects of reactive oxygen species may provide protection to the ischemic myocardium, which undergoes subsequent reperfusion.[15]

The investigated compounds are hybrid molecules containing an indole and an amine–triazole moiety (Subclasses 1, 2), (Table 1). The triazole nucleus is connected *via* a methylene bridge either on the indole nitrogen (Subclass 1), or at position 3 of the indole (Subclass 2). Compounds differentiate also in substitution at position 5 of the indole nucleus. Melatonin is substituted at position 3 and has an $-OCH_3$ substituent at position 5 of the indole nucleus (Fig. 1).

The ability of the compounds to interact with DPPH was examined. DPPH is a stable free radical, converting into a stable diamagnetic molecule by acceptance of an electron or hydrogen radical. Due to its odd electron, DPPH shows a strong absorption band at 517 nm. Reduction of DPPH leads to pairing off the electron and the absorption decreases stoichiometrically with respect to the number of electrons taken up. The change in absorbance produced during this reaction has been widely used to assess the ability of compounds to act as free radical scavengers.[16]

The effect of different concentrations (5—200 μM) of the tested compounds and of melatonin on their interaction with DPPH (200 μM) is shown in Table 2. Experiments were performed also with ascorbic acid as reference substance. Compound **1a** with no substitution at position C5 of the indolic nucleus showed strong inhibitory activity with IC_{50} value lower than Vitamine E, as previously reported,[8] and close to that of ascorbic acid. Lower but comparable IC_{50} values were obtained for compounds **1b**—**d**, while a tenfold reduced activity was observed for compounds **2a**—**c** in which the triazole moiety is connected *via* a methylene bridge at position C3 on the indole nucleus. In contrast, melatonin, tested for the first time in this protocol, showed a very weak scavenging activity on DPPH. In the highest concentration used (200 μM) the inhibition did not exceed 22%, so an IC_{50} value could not be obtained. Figures 2 and 3 show the DPPH radical scavenging activity of the compounds as a function of time in comparison with DPPH and melatonin. Inhibition started at the 1st minute, after the addition of DPPH, and seems to level off after 15 min for compounds **1a**—**d** and **2a**—**c**, while time did not have any influence in the inhibition produced by melatonin.

During postischemic reperfusion, oxygen enters the cell at high tension and combined with hypoxanthine in the presense of xanthine oxidase. This combination has as result the generation of superoxide anions and hydroxyl radicals that cause lipid peroxidation and damage to cellular membranes.[17] It has been shown that pretreatment of the hearts with antioxidants can ameliorate ischemic reperfusion injury presumably by reducing the formation of detrimental free radicals, such as superoxide anions and hydroxyl radicals.[18] In our previous work we have demonstrated that the tested compounds exhibit significant inhibitory activity against lipid peroxidation in rat microsomes.[8]

In order to further elucidate the possible mechanism of this inhibition, in the present study we investigated their abil-

Table 2. Effect of Different Concentrations (5—200 μM) of the Synthesized Compounds on Their Interaction with DPPH (200 μM)

Compound	Percentage of Interaction with DPPH					
	[DPPH] μM[a]					IC_{50}
	5	10	50	100	200	
1a	25	43	89	91	93	12.1 ± 1.6[b]
1b	7	16	36	49	66	94.8 ± 8.6
1c	9	14	36	60	84	66.7 ± 9.1
1d	12	13	20	48	87	91.4 ± 9.8
2a	10	14	25	31	49	190.8 ± 32.9
2b	4	9	28	32	52	176.5 ± 18.9
2c	8	14	29	37	51	159.2 ± 16.8
Melatonin	1	6	19	21	22	$\gg 200$
Ascorbic acid	19	47	94	96	97	11.2 ± 2.7

a) Based on absorbance values of samples with the tested compounds against controls containing equal volume of the solvent. Standard deviation of absorbance values was less than $\pm 10\%$, $n=3$—5. *b*) From ref. 9.

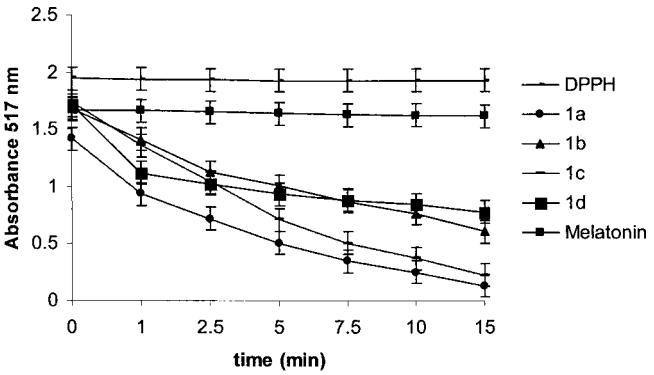


Fig. 2. DPPH Reduction, as Evaluated by the Decrease in Absorbance, at 517 nm, as a Function of Time at 200 μM Concentration of Compounds **1a**—**d** and Melatonin

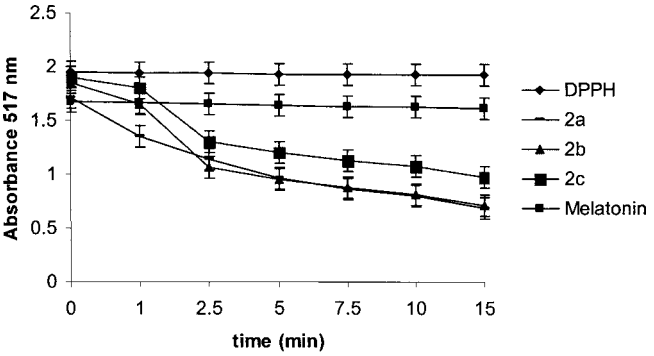


Fig. 3. DPPH Reduction, as Evaluated by the Decrease in Absorbance, at 517 nm, as a Function of Time at 200 μM Concentration of Compounds **2a**—**c** and Melatonin

ity to scavenge superoxide anions, generated by the enzymic xanthine–xanthine oxidase system in presence of nitro blue tetrazolium (NBT). The ability of the indole derivatives as well as of melatonin to scavenge superoxide anions as measured by the reduction of NBT is illustrated on Fig. 4. The compounds were tested in two concentrations (1, 0.5 mM) and the order of activity on superoxide anion inhibition was: **1a**>melatonin>**1d**>**2b**>**2a**>**1c**>**1b**>**2c**. **1a** showed slightly higher activity than melatonin, **1d**, which contains a nitro group at position 5 of the indolic nucleus, followed, while the halogen substitution at the same position (**1b**, **c**) is detrimental for the activity. Practically inactive was also compound **2c**, with a CH_3 group at position 2 of the indolic nucleus. Allopurinol, used as a reference compound, showed strong scavenging activity equal to 84 and 80% at the concentrations tested.

To clarify if the scavenging activity of the compounds **1a**, **d**, **2a**, **b** and melatonin, was due directly to their superoxide anion radical scavenging activity or/and to their inhibition of the enzyme xanthine oxidase, a further test was performed to explore their inhibitory activity against xanthine oxidase to produce uric acid.[19] The results are shown in Table 3. Melatonin completely inhibited uric acid production by xanthine oxidase at the concentration of 1 mM while at 0.5 mM it lost its inhibitory effect almost completely. Compound **1a** produced 50% inhibition at the concentration of 1 mM, while at 0.5 mM it lost its inhibitory activity. No inhibitory activity was found for compound **1d**. Allopurinol used as a reference compound showed strong inhibitory activity at the concentrations of 1 and 0.5 mM, equal to 87 and 81.5%, respectively. These findings suggest that the inhibition of superoxide anion radical should be attributed to both radical scavenging and inhibitory activity of the xanthine oxidase. For the nitro-derivative, compound **1d**, inhibition of the superoxide anion generation is totally governed by its radical scavenging activity.

In addition to superoxide anions, other ROS, such as hydroxyl radicals, may contribute to myocardial injury.[20] It has been shown that H_2O_2 causes cell injury and death in cardiac myoblasts in rats and this procedure is mediated by the generation of hydroxyl radicals.[21] The competition of the compounds with DMSO for hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity.[5] The effect of a standard concentration of the examined compounds (3 mM) on the $HO^{\cdot}$ mediated oxidation of different concentrations of DMSO and their corresponding k_s values are shown in Table 4. All compounds exhibit significant $^{\cdot}OH$ scavenging activity with reaction rate constants (k_s) much higher than that corresponding to DMSO and mannitol,[22] both known hydroxyl radical scavengers. Especially compounds **1a** and **d** proved to be very strong hydroxyl radical scavengers, with reaction rate constants 132.6±1.7, and 100.6±0.9, respectively (Table 4), much higher than that of the other substances tested, including melatonin, DMSO and mannitol. In the same class of compounds a halogen substitutent at position 5 of the indolic nucleus (compounds **1b**, **c**) proved to be an unfavourable characteristic for this type of activity. Melatonin, which is considered a strong hydroxyl radical scavenger,[23] showed a high rate constant equal to 43±3.5.

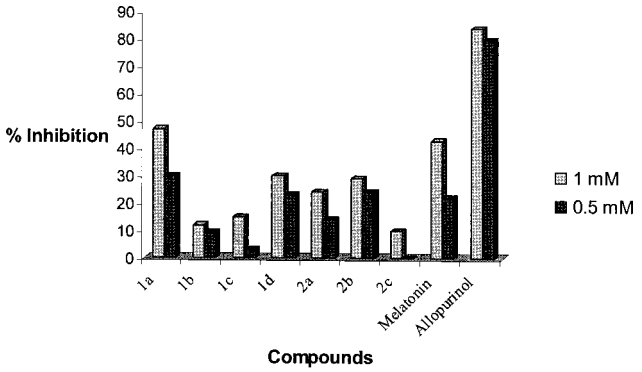


Fig. 4. Percent Inhibition of the Synthesized Compounds on Xanthine–Xanthine Oxidase Generated Superoxide Anion Radical

Table 3. The Inhibitory Effects of the Examined Compounds on Xanthine Oxidase

Compound	Percentage inhibition	
	1 mM	0.5 mM
1a	47	0
1d	0	0
2a	49	22
2b	72	44
Melatonin	100	8
Allopurinol	87	81

Table 4. Effect of the Examined Compounds (3 mM) on the $HO^{\cdot}$ Mediated Oxidation of DMSO (12.5, 6.25, 3.125 mM) and Their k_s Values

Compound	Percentage of inhibition			k_s ($\times 10^9\,M^{-1}\,s^{-1}$)
	[DMSO] (mM)[a]			
	12.5	6.25	3.125	
1a	95	100	100	132.6 (±1.7)[b]
1b	30	33	45	6.5 (±0.5)
1c	60	65	85	25.8 (±0.1)
1d	87	91	99.5	100.6 (±0.9)
2a	40	55	75	11.1 (±0.2)
2b	73	80	88	36.7 (±3.8)
2c	54	62	86	21.5 (±1.9)
Melatonin	56	66	75	43.0 (±3.5)
Mannitol				1.7[b]
DMSO				7[b]

a) Based on absorbance values of samples with the tested compounds, against controls containing equal volume of the solvent. Standard deviation of absorbance values was less than ±10%, n=3—5. *b*) From ref. 9.

In conclusion, derivatives **1a** and **d**, substituted at the indolic nitrogen N1, exhibit better antioxidant properties in comparison with melatonin and compounds **2a**—**c** which have a free hydrogen at N1. Among them, compound **1a** with no further substitution of the indolic nucleus showed the best antioxidant profile. This finding indicates that the presence of hydrogen on the indolic nitrogen was not essential for reactive oxygen species scavenging activity.

Concerning the pathways for reaction of melatonin with hydroxyl radicals and superoxide anions two principal pathways have been proposed. The pathway proposal by Reiter *et al.*,[24,25] does not involve the hydrogen of the indolic nitrogen, while according to Turjanski *et al.*,[26] abstraction of that hy-

drogen is the first step in the pathway for radical scavenging activity. The strong radical scavenging activity observed of compounds **1a** and **d**, which does not possess hydrogen at the indolic nitrogen, may provide further evidence to support the first mechanism.

Experimental

Compounds **1a**—**d** and **2a**—**c** were synthesized and identified as previously reported.[8] Melatonin was purchased from Sigma. Xanthine oxidase and all reagents for biochemical assays were purchased from Sigma Aldrich Chemie, Steinheim, Germany. The solvents were supplied by Labscan Ltd. (Unit T26, Dublin Ireland) and were of analytical grade.

Measurement of Activity in Reduction of DPPH The method has been previously described in detail.[6] Briefly, to a solution of DPPH (final concentration 200 μM) in absolute ethanol, an equal volume of the compound dissolved in ethanol was added at various concentrations (5—200 μM). Ethanol was added to the control solution. Absorbance was recorded at 517 nm after 20 min. The % inhibition and IC_{50} values are reported in Table 2. In another set of experiments, a 1 ml aliquot of the compounds (200 μM) dissolved in ethanol was mixed with 1 ml 200 μM DPPH in ethanol in a cuvette and the time course of the optical density change at 517 nm was followed for 15 min.[27] Plots of absorbance versus time are illustrated in Figs. 2 and 3. Each experiment was performed at least in triplicate and the standard deviation in absorbance values was less than $\pm$10%.

Quenching of the Superoxide Anion Radical The $O_2^{\cdot-}$ quenching capacity of the synthesized compounds was tested by estimation of the reduction product of nitro blue tetrazolium (NBT), as described previously.[7] The incubation system contained 200 μM xanthine, 600 μM NBT, in 0.1 M phosphate buffer (pH 7.4). The tested substances were dissolved in 0.1% dimethyl formamide (DMF) in buffer, and added to the reaction mixture (300 μl, final concentrations 1 and 0.5 mM). An equal volume of the solvent system was added to the control mixture. The reaction was initiated with the addition of 0.07 units/ml of xanthine oxidase. After incubation (25 °C, 10 min), absorbance was recorded at 560 nm, against blank samples, which did not contain the enzyme. DMF was tested and found not to interfere with the assay at the concentration used (0.1% v/v). Each experiment was performed at least in triplicate and the deviation in absorbance values was less than $\pm$10%.

Inhibitory Effects on the Activity of Xanthine-Oxidase Inhibitory effects on the activity of xanthine oxidase were estimated by the method of Chang *et al.*[28] In briefly, xanthine 6.084 mg was dissolved in 200 ml of 0.1 M phosphate buffer pH 7.8 to make a 200 μM xanthine buffer solution. 990 μl of xanthine buffer, 2 μl of xanthine oxidase and 10 μl of DMSO were incubated for 4 min at room temperature and the formation of uric acid was estimated at 295 nm against a blank sample which did not contain the enzyme but 2 μl of water instead. Aliquots (10 μl) of 2 concentrations (1, 0.5 mM) of test samples dissolved in DMSO were added to xanthine buffer solution (990 μl) pH 7.8, and incubated with 2 μl of xanthine oxidase (0.04 units) for 4 min at room temperature. Blank samples were prepared using 2 μl water instead of the enzyme. Optical density was recorded every 0.2 min during 4 min and the tests were performed in triplicate. % Inhibition was expressed by comparing the initial velocity (V) in presence (V_i) and absence (V_o) of the tested compounds:

$$\% = \left(1 - \frac{V_i}{V_o}\right) \times 100$$

Competition with DMSO for Hydroxyl Radicals The hydroxyl radicals generated by the Fe^{3+}/ ascorbic acid system were detected by the determination of formaldehyde produced from the oxidation of dimethyl sulfoxide (DMSO). The reaction mixture contained EDTA (0.1 mmol/l), Fe^{3+} (167 μmol/l, as a 1 : 2 mixture with EDTA), ascorbic acid 20 μmol/l and DMSO (12.50, 6.25, 3.125 mmol/l), in phosphate buffer (50 mmol/l, pH 7.4). The tested compound was dissolved in phosphate buffer and added to the reaction mixture (final volume 750 μl) at the concentration of 3 mmol/l. The mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 250 μl trichloroacetic acid (17.5% w/v) and the formaldehyde formed was detected spectrophotometrically.[9] The same experiments were repeated in absence of DMSO. Each experiment was performed at least in triplicate and the standard deviation in absorbance values was less than $\pm$10%. The determination of the second order reaction rate constants k_s is succeeded using the following equation:

$$A_o/A = 1 + \frac{k_s[s]}{k_{DMSO}[DMSO]}$$

A_o: absorbance at 412 nm before the addition of the tested compound
A: absorbance after the addition of the tested compound
[DMSO]: concentration of the DMSO (12.5, 6.25, 3.125)
[S]: concentration of the tested compound
k_{DMSO}: $7 \times 10^9\ M^{-1}\ s^{-1}$

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