In vitro and *In vivo* Assessment of the Antioxidant Activity of Melatonin and Related Indole Derivatives

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Abstract. Effects of melatonin and some structurally related indole compounds were studied by *in vitro* methods such as (i) an inhibition of the hyaluronic acid degradation and (ii) a standard lipid peroxidation assay. *In vivo* approach was based on the alloxan model of hyperglycaemia.

Reduction of the viscosity of a hyaluronic acid solution in the reaction mixture was inhibited by tryptamine (91 % inhibition), as well as by indole-3-carboxylic acid and indomethacin (80 % and 77 % inhibition, respectively). Lipid peroxidation with *tert*-butyl hydroperoxide as a source of radicals was followed by the formation of thiobarbituric acid reactive substances. Tested drugs inhibited lipid peroxidation in the order: tryptamine (59 %) > indole-2-carboxylic acid (38 %) > indomethacin (26 %) > melatonin and indole-3-carboxylic acid (13 %). *In vivo*, alloxan-induced hyperglycaemia was reduced in mice pretreated with drugs tested. The highest protective effect was observed with indomethacin (52 % inhibition), followed by tryptamine and melatonin (18 % and 16 % inhibition, respectively).

Key words: Antioxidant activity — Indole derivatives — Lipid peroxidation — Alloxan-induced hyperglycaemia

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), the main pineal gland hormone acts as an endogenous synchronizer co-ordinating biological rhythms (Humlová and Illnerová 1990) and is known as potent free radical scavenger and antioxidant (Abuja et al. 1997; Reiter et al. 1998a; Qi et al. 1999).

Melatonin has been shown to scavenge the most reactive and cytotoxic oxygen species (Reiter et al. 2000), namely the hydroxyl radical (Tan et al. 1993; Reiter et al. 1997, 1998a; Brömme et al. 2000; Ebelt et al. 2000) with which the melatonin yields cyclic 3-hydroxymelatonin. The formation of this compound was confirmed in two different cell-free *in vitro* systems; 3-hydroxymelatonin was identified also in the urine of both rats and humans as a valuable biomarker of hydroxyl radical generation (Tan et al. 1998). Melatonin has been found to be able to scavenge also the peroxyl radical (Pieri et al. 1994), the peroxynitrite anion (Gilad et al. 1997), the nitric oxide (Noda et al. 1999) and the singlet oxygen (Cagnoli et al. 1995). Furthermore, melatonin may stimulate antioxidative enzymes as superoxide dismutase, glutathione peroxidase and glutathione reductase as well as inhibit the pro-oxidative enzyme, nitric-oxide synthase (Reiter et al. 1998a).

There are reports in the literature that melatonin is effective in inhibiting oxidative damage *in vitro* (Abuja et al. 1997; Reiter 1998b; Ebelt et al. 2000; Cabrera et al. 2000) and in various animal disease models *in vivo* (Lagneux et al. 2000; Qi et al. 1999; Willis and Armstrong 1999; Cabrera et al. 2000) protecting nuclear and mitochondrial DNA, membrane lipids and possibly cytosolic proteins (Reiter et al. 1997, 2000). Recently, Acuña-Castroviejo et al. (2001) have reviewed a novel mechanism of action of melatonin at the mitochondrial level and have noticed that melatonin improves the bioenergetics of the cell by improving of mitochondrial respiration and ATP synthesis by increasing the rate of electron transport across the electron transport chain.

The aim of this study is to evaluate the antioxidant effectiveness of melatonin in comparison with other structurally related indole compounds (Fig. 1) both *in vitro* and *in vivo*. Firstly, the scavenging activity of selected compounds in cell free *in vitro* system was tested using a system generating hydroxyl radicals. Secondly, the level of malondialdehyde formation was used as an index of oxidative destruction of mitochondrial membrane lipids *in vitro*. Alloxan-induced hyperglycaemia in mice was used as an *in vivo* model of free radical pathology to test the antioxidant activity of tested compounds.

Materials and Methods

Animal. Male Wistar strain rats weighting 200–250 g and female NMRI strain mice weighting 27 ± 3 g (BioTest, Konárovice breed, Czech Rep.) were placed in plastic cages in a room at a constant temperature of $22 \pm 2\,°C$ with 12-h light and dark cycles and fed standard pellet diet (Velaz, Czech Rep.) and water *ad libitum.*

Chemicals. Hyaluronic acid was purchased from Contipro (Czech Rep.); acetonitrile was product of Merck (Germany); alloxan tetrahydrate and other reagents were purchased from Sigma (MO, USA).

Tested substances. Melatonin, tryptamine, indole-2-carboxylic acid, indole-3-carboxylic acid, indomethacin (all Sigma, MO, USA) (see also Fig. 1).
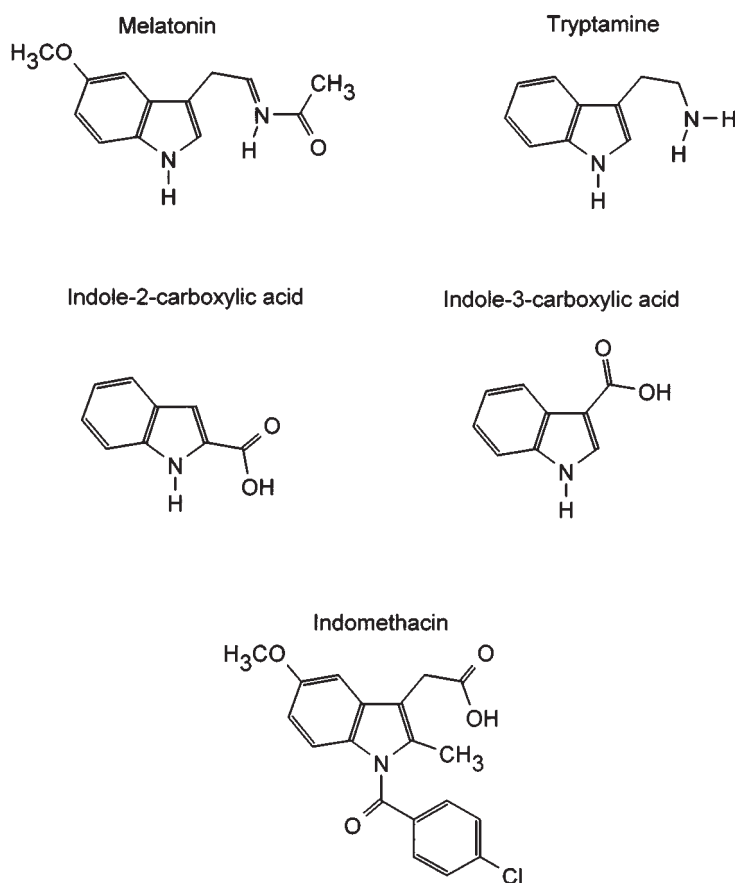
Figure 1. Chemical structures of melatonin and related derivatives used in this study.

Degradation of hyaluronic acid (cell-free in vitro study)

The antioxidant activity of the indole derivatives was evaluated by the hyaluronic acid (HA) degradation method (Kataoka et al. 1997). Tested drugs were dissolved in 100 mmol·l^{-1} Na$_2$HPO$_4$ (pH 9.4) except melatonin which was at first dissolved in acetonitrile (final concentration of acetonitrile in the reaction mixture was 0.4%). HA was dissolved in 100 mmol·l^{-1} NaH$_2$PO$_4$ (pH 4.4). The Fenton reaction system containing Fe^{2+}-EDTA and H$_2$O$_2$ was used for generation of hydroxyl radicals. The reaction mixture (25 ml total volume) contained 0.25% HA, 5.0 μmol·l^{-1} FeSO$_4$-EDTA, 80 mmol·l^{-1} Na$_2$HPO$_4$ (pH 9.4), 20 mmol·l^{-1} NaH$_2$PO$_4$ (pH 4.4), 20 mmol·l^{-1} H$_2$O$_2$, and the substances tested (1 mmol·l^{-1} or 1.5 mmol·l^{-1}). After incubation at 37℃ for 45 min, the reaction was stopped by the addition of DMSO. Degradation of HA was determined by the decrease in viscosity (expressed

in mPa·s) of the reaction mixture. Viscosity was measured using an Ubbelohde vis-
cometer U II (Technosklo, Czech Rep.). Each measurement was carried out twice in
duplicate. Inhibitory effects of drugs tested were expressed as changes in viscosity
of reaction mixtures relatively to viscosity of unaffected HA (in %).

Inhibition of mitochondrial lipid peroxidation

Isolation of mitochondria. Rat liver was quickly removed and placed into an ice-cold
3 mmol·l^{-1} Tris-HCl buffer (pH 7.4) containing 0.25 mol sucrose and 0.1 mmol·l^{-1}
EDTA and homogenised in a mixer (ETA Mira, Czech Rep.) (Haraguchi et al.
1995, 1997). The mitochondria were prepared by differential centrifugation of liver
homogenate (Hermle Z360K centrifuge, Germany) twice at $1000 \times g$ for 10 minutes
and, subsequently, of the postnuclear supernatant (four times at $10,000 \times g$ for 15
minutes). Crude mitochondria were washed 3 times with buffer (50 mmol·l^{-1} Tris-
HCl buffer, 100 mmol·l^{-1} KCl, 1 mmol·l^{-1} EDTA (pH 7.4)) (Shayiq et al. 1991)
and suspended in total volume of 50 mmol·l^{-1} Tris-HCl buffer corresponding to
fourfold volume of the original liver weight giving final protein concentration of
approximately 4 mg protein *per* ml as measured by the biuret method (Gornall
1949). Mitochondrial suspension was stored at $-60\,$℃ for further processing (Shen
et al. 1994).

Mitochondrial peroxidation. Lipid peroxidation was induced by a lipid soluble per-
oxyl radical generating system with *tert*-butyl hydroperoxide (*t*BH) (1 ml of mito-
chondrial suspension, 20 μl of *t*BH and 20 μl of tested drug both dissolved in DMSO
giving the final concentration of 1.5 mmol·l^{-1} and 2 mmol·l^{-1}, respectively). All
samples were made in triplicate in three separate experiments ($n = 9$) and incu-
bated at 37 ℃ under gentle shaking for 60 minutes in a water bath (Elphan 357,
Poland) (Mathiesen et al. 1995).

Thiobarbituric acid assay. The degree of peroxidation was determined spectro-
photometrically at 535 nm by the yield of thiobarbituric acid reactive substances
(TBARS) as described by Haraguchi et al. (1995, 1997). Separate experiments were
performed to detect whether the test compounds did not interfere with the reaction.
Inhibition of TBARS formation was calculated as $100 \times (A_1 - A_t)/(A_1 - A_2)$, where
A_1, A_2, and A_t are absorbance values for unprotected samples, blanks, and test
samples, respectively (Mathiesen et al. 1995).

Model of alloxan diabetes

Alloxan-induced hyperglycaemia (Nukatsuka et al. 1989) in female mice (6 animals
in each group) was used as the criterion for diabetes. On day 0, alloxan dissolved in
isotonic saline was administered i.v. in a dose of 120 mg·kg^{-1} to mice deprived of
food for 17 h. The drug tested (dissolved in 0.5 % methylcelulose) was administered
in a dose of 50 mg·kg^{-1} i.p. 30 min before alloxan administration. One of the control
groups received alloxan alone (alloxan control), the other control group received
the vehicle and alloxan (vehicle control). Glycaemia was estimated in a drop of
blood from the *v. caudalis lateralis* using a Glucochir apparatus (Meta Brno, Czech

Rep.) on day 0 (before pre-treatment) and on day 2 when the induced glycaemia increaseing was the highest (Štětinová and Grossmann 2000).

Lowering of glycaemia on day 2 in animals pre-treated with tested drugs was taken as an indication of the effect of the substances tested. This effect was expressed as the percentage of inhibition of hyperglycaemia relatively to glycaemia of the vehicle control.

Statistical analysis

Data obtained were analysed using Jarque-Berra normality test for combined sample skewness and kurtosis, Bartlett's test and Fisher-Snedecor test of the homogeneity of variance followed by an appropriate Student's *t*-tests at 5 % level of significance.

Results

Degradation of hyaluronic acid

Initial viscosity of HA reaction mixture with vehicle only (without the substances tested) was decreased by 56 % as a consequence of HA degradation caused by radicals formed by Fenton reaction system. In the case of the vehicle used for
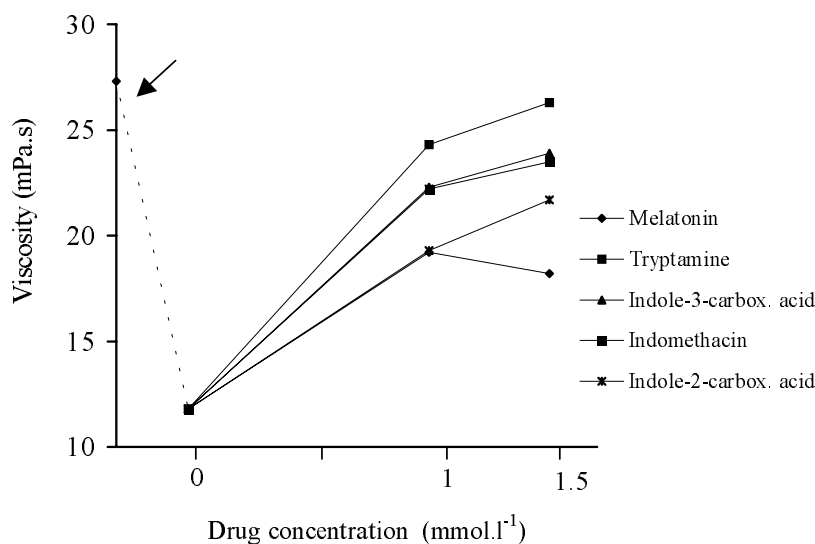

Figure 2. Effect of melatonin and other indole derivatives on HA degradation induced by the Fenton system. Arrow indicates the initial viscosity of the HA reaction mixture without drugs tested. Dashed line shows decrease in the viscosity induced by the Fenton system. Effect of tested drugs at final concentrations of 1 and 1.5 mmol·l^{-1} in reaction mixture are plotted, each point is the mean of four determinations (values did not differ by more than 1.5 %).

Table 1. Antioxidant effects of indole derivatives *in vitro* and *in vivo* expressed as % of inhibition changes induced by free radicals

Drugs	Lipid peroxidation[a]	Alloxan hyperglycaemia[b]
Melatonin	13	16 [*+]
Tryptamine	59 [*]	18 [*]
Indole-2-carboxylic acid	38 [*]	7 [*]
Indole-3-carboxylic acid	13	3
Indomethacin	26	52 [*+]

[a] Inhibitory effect of melatonin and other derivatives at concentration of 2 mmol·l^{-1} on t-butyl hydroperoxide-induced lipid peroxidation in rat mitochondria. Values represent the result of three separate estimations (with each point being done in triplicate), $n = 9$. Control (unprotected sample): 100 % refers to 0.876 ± 205 nmol TBARS/mg protein of absorbance 0.180 ± 0.047 nm. Significant differences (calculated from original values of absorbance) versus the unprotected samples are labeled with [*], $p < 0.05$.

[b] Protective action of tested drugs 50 mg·kg^{-1} i.p. against alloxan-induced hyperglycaemia in mice females, ($n = 6$). Vehicle control: 100 % refers to 20.9 ± 2.1 mmol·l^{-1} blood glucose. Significant differences (calculated from original values of blood glucose level) versus alloxan control group are labeled with [*], versus the vehicle control group are labeled with [+], $p < 0.05$.

melatonin (i.e. Na$_2$HPO$_4$ and acetonitrile), the vehicle exhibited its own effect on the viscosity of the reaction mixture. Hence, for the estimation of antioxidant effect of melatonin, the values measured were corrected for this effect.

All compounds tested inhibited the HA degradation initiated by Fenton reaction system (Fig. 2); percentage of the inhibition (in concentration of 1.5 mmol·l^{-1}) showed that tryptamine had the highest antioxidant potential in this system (91 % inhibition), followed by indole-3-carboxylic acid and indomethacin (80 % and 77 % inhibition, respectively). The least effective were indole-2-carboxylic acid and melatonin (64 % and 41 % of inhibition, respectively).

Inhibition of mitochondrial lipid peroxidation

Protective effect of compounds tested at concentration of 2 mmol·l^{-1} was determined spectrophotometrically by formation of TBARS as described by Haraguchi et al. (1995, 1997). The lipid peroxidation in rat mitochondria induced by a free radical generating system with tBH was about 10 times higher in the unprotected samples than that in the blank. As shown in Table 1, under these conditions, tryptamine and indole-2-carboxylic acid appear to be the most effective antioxidants inhibiting significantly formation of TBARS by 59 % and 38 %, respectively. The antioxidant activity of the other compounds decreased in the following order: indomethacin (26 %) > melatonin = indole-3-carboxylic acid (13 %).

Model of alloxan diabetes

The mean glycaemia estimated on day 0 (before any treatment of animals) was 5.9 ± 0.1 mmol·l^{-1}. Alloxan was effective in producing the hyperglycaemia in following way: in the group of animals which obtained nothing else than alloxan (alloxan control animals) the glycaemia on day 2 was increased by approximately 420%; in the group of animals which obtained both the alloxan and the vehicle (vehicle control animals), an increase by 220% was observed (in comparison with the respective values of the glycaemia on day 0). Pre-treatment of animals with the drugs tested showed that indomethacin and melatonin significantly reduced the elevated values of the blood glucose – by 52% and 16% of this for the vehicle control, respectively. Tryptamine and indole-2-carboxylic acid induced reduction of glucose levels by 18% and 7%, respectively, indole-3-carboxylic acid increased alloxan induced hyperglycaemia by 3% only (Table 1).

Discussion

The results obtained show that the most pronounced antioxidant effect of tested drugs is demonstrated using the cell-free and enzyme-free simple chemical reaction system generating hydroxyl radicals *in vitro* (HA degradation method). Tryptamine, which represents a core of the melatonin molecule (see Fig. 1), exhibited the highest inhibiting effect in this system – 91% HA degradation, followed by other compounds in order: indole-3-carboxylic acid > nonsteroidal antinflammatory drug indomethacin > indole-2-carboxylic acid > melatonin.

In the case of lipid mitochondrial peroxidation induced by *t*BH, the effect of tested compounds (of 1.6 or 2 times higher concentrations than those used in HA degradation) was lower in comparison with the effect in the cell-free system. However, the order of the tested drugs, expressing their antioxidant effect, was practically the same as tryptamine, being the most effective. Lower effect of tested compounds in this system is probably due to their interaction with other components of the reaction mixture, namely with the proteins. Similar effect has been observed in an *in vitro* experiments with melatonin and system consisting of glutathione and alloxan in presence of ferrous ions – the IC$_{50}$ concentration (i.e. the concentration causing 50% inhibition of lipid peroxidation) was higher when a more complex system with liposomes was used (the IC$_{50}$ increased from 23 to 750 μmol·l^{-1}) (Brömme et al. 2000).

The way by which the peroxyl or alkyl radicals are produced during the process of the lipid peroxidation in biomembranes has been well characterized by Darley-Usmar and coworkers (1995). The evaluation of the radical-scavenging effect of melatonin seems to be difficult as the *in vitro* tests often yield contradictory results. In our *in vitro* systems, a lower protecting effect of melatonin has been found (13% inhibition, see Table 1). This result seems to be in line with recent finding of Antunes et al. (1999) who have found that melatonin is not able to trap peroxyl radicals. On the other hand, Pieri et al. (1994) have reported that melatonin

scavenges (in a cell-free *in vitro* system) the peroxyl radicals twice more effectively than vitamin E. Hence, it may seems that melatonin scavenges the hydroxyl radicals active in the initiation phase of the lipoperoxidation rather than the peroxyl or alkyl radicals formed in the next phase of lipoperoxidation mechanism.

As an *in vivo* approach, a model of alloxan-induced hyperglycaemia in mice was measured. It is known that the alloxan selectively destroys the insulin-producing pancreatic β cells due to the formation of reactive free radicals. It has been suggested that alloxan induces the formation of reactive oxygen species, above all the hydroxyl radicals (Heikkila 1977; Ebelt et al. 2000) or, possibly the peroxyl radicals (Stefek and Trnkova 1996) as well as the alloxan anion radicals (Nukatsuka et al. 1989), while the superoxide and hydrogen peroxide radicals are formed initially in processes leading to pancreatic β cell damage (Brömme et al. 1999). The hydroxyl radicals formed in this way are believed to mediate the alloxan diabetogenic action (Brömme et al. 2000).

In the organism, the difference in the absorption, distribution, biotransformation and excretion of drugs tested may significantly influence the resultant effect. Here, it has been found that the magnitude order of the antioxidant effect observed in the *in vitro* experiments has changed. In addition to indomethacin, the effect of compounds tested *in vivo* was lower. The effect of indomethacin may be explained by its relatively slower metabolism (plasma half-life in rats is about 4 h (Hucker et al. 1966)) allowing indometacin to act for longer time. For example, the half-life of melatonin (which exhibit lower effect) is short with its distribution half-life α of 2 min, and with the elimination half-life β of 20 min (Claustrat et al. 1998). Another fact which may influence the extent of the antioxidant effect *in vivo* may be the bioavailability of the respective compounds or, the actual concentration in the cell.

Melatonin and tryptamine possess both hydrophilic and lipophilic characteristics (Cabrera et al. 2000) and their ability to penetrate readily the biological membrane could be probably the reason of their effect *in vivo*, although, *in vitro* melatonin inhibits the radical-induced HA degradation and the level of lipid peroxidation relatively less effectively than other compounds. In contrast, both indole carboxylic acids relatively effective *in vitro*, hardly penetrate biological tissues (most likely due to their acidic character which is reflected in their low *in vivo* activity).

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