J Pineal Res 1998; 24:96–101
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Journal of Pineal Research
ISSN 0742-3098

Comparison of the antioxidant activity of melatonin and pinoline in vitro

Pähkla R, Zilmer M, Kullisaar T, Rägo L. Comparison of the antioxidant activity of melatonin and pinoline in vitro. J. Pineal Res. 1998; 24:96–101.

Abstract: Several recent experiments have shown that melatonin is an efficient antioxidant and free radical scavenger. In the present study the antioxidative effect of melatonin was compared with that of pinoline. Pinoline (6-methoxy-tetrahydro-β-carboline) can be formed in the mammalian body under physiological conditions from 5-hydroxytryptamine or as a tricyclic metabolite of melatonin. Both melatonin and pinoline inhibited lipid peroxidation and showed comparable activity in a total antioxidant status test. Melatonin and pinoline concentration-dependently scavenged hydroxyl radicals with IC_{50} 11.4±1.0 μM for melatonin and 62.3±3.8 μM for pinoline. These results support the importance of the indolic part of the molecule and the 5-methoxy group common to both compounds in terms of the ability of these molecules to quench the hydroxyl radicals. As pinoline has been shown to exert an antidepressant-like effect in behavioral experiments and has been reported to have a low toxicity, this compound should be further studied as a potential antidepressant with pronounced antioxidative effects. These results further support the importance of pineal gland in antioxidative protection.

Rein Pähkla,[1] Mihkel Zilmer,[2] Tiiu Kullisaar,[2] and Lembit Rägo[1]

Departments of [1]Pharmacology and [2]Biochemistry, University of Tartu, EE2400 Tartu, Estonia

Key words: pinoline – 6-methoxy-tetrahydro-beta-carboline – melatonin – antioxidant – hydroxyl radical – lipid peroxidation

Address reprint requests to Rein Pähkla, Department of Pharmacology, University of Tartu, Ülikooli 18, EE2400 Tartu, Estonia. E-mail: rein@sam.ee

Received July 22, 1997; accepted September 25, 1997

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is the main neuroendocrine product of the pineal gland. Besides the well-known circadian and reproductive effects of melatonin, several recent experiments have shown that melatonin also is an efficient antioxidant and free radical scavenger. For example, melatonin has been shown to quench the highly toxic hydroxyl radical (•OH) in vitro [Tan et al., 1993]. Melatonin also has been shown to scavenge the peroxyl radical that is generated during the peroxidation of lipids [Pieri et al., 1994, 1995]. In addition, melatonin provides antioxidative protection against lipid peroxidation in different in vitro assay conditions [Chen et al., 1995; Melchiorri et al., 1995]. There is an accumulating evidence supporting melatonin's role in antioxidative protection in vivo [Reiter et al., 1996; Reiter, 1997].

Structure-activity studies comparing the free radical scavenging ability of melatonin with several chemically related molecules showed the importance of the methoxy group at position 5 of the indole nucleus and the N-acetyl group on the side chain [Tan et al., 1993; Scaiano, 1995]. A methoxy group of the same location is present on an another compound, 6-methoxy-tetrahydro-β-carboline; however, rather than the aliphatic side chain containing the acetyl group, the β-carboline has a third closed ring (Fig. 1).

6-Methoxy-1,2,3,4-tetrahydro-β-carboline (6-MeO-THBC, 5-methoxytryptoline), named "pinoline" from the words "pineal β-carboline," can be produced from 5-hydroxytryptamine (5-HT) through the nonenzymatic Pictet-Spengler cyclization [Callaway et al., 1994]. Formation of pinoline requires O-methylation of 5-HT catalyzed by the enzyme 5-hydroxyindole-O-methyltransferase (5-HIOMT), an essential enzyme in the biosynthetic pathway of melatonin [Morton and Potgeiter, 1982; Hardeland et al., 1993]. Therefore, the endogenous synthesis of pinoline is presumably largely confined to the pineal gland. Alternatively, pinoline can be formed from melatonin following its metabolism to 5-methoxytryptamine by the enzyme, aryl acylamidase [Hardeland et al., 1993; Airaksinen et al., 1993].

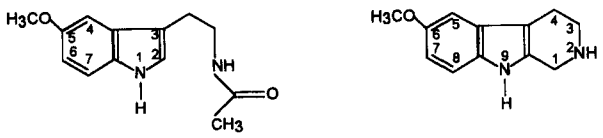

Fig. 1. Structures of melatonin. (N-acetyl-5-methoxytryptamine) and pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline). Carbon atoms of both compounds are numbered according to the numbering systems for tryptamines and β-carbolines, respectively.

There are only few published data in the literature concerning the anti- or pro-oxidative properties of β-carboline compounds. Tse et al. [1991] found that harman and related β-carbolines and dihydro-β-carbolines inhibited lipid peroxidation in a hepatic microsomal preparation incubated with oxygen radical producing systems. Kawashima et al. [1995] found that 1-aryl substituted 1,2,3,4-tetrahydro-β-carboline compounds has potent effects against lipid peroxidation. To our knowledge, no studies on the ability of pinoline as a free radical scavenger have been published as yet.

The aim of the present experiment was to compare the antioxidative effects of pinoline and melatonin by using different in vitro assay systems. Special attention was paid to the determination of the hydroxyl radical scavenging capacity of the molecule since previous studies have shown melatonin to scavenge the •OH with great efficiency. An abstract of some of these findings has previously been published [Pähkla et al., 1997a].

Materials and methods

Materials

Pinoline and melatonin were purchased from Sigma Chemical Co. (St. Louis, MO). "Total Antioxidant Status" kit (cat. No. NX2332) was purchased from Randox Laboratories, Ltd. (Crumlin, UK). All other reagents were standard laboratory reagents of analytical grade. Pinoline and melatonin were dissolved in protonated NaCl solution, made immediately before experiment from HCl and NaOH solutions (pH of the mixture was about 3–4), and then diluted with water to the final volume; the pH of the solution was adjusted to 7.5 with NaOH.

Lipid peroxidation inhibition

Antioxidative capacity of melatonin and pinoline was determined using the inhibition of lipid peroxidation of a linolenic acid standard by a sample containing the investigated compounds [Starkopf et al., 1995]. The concentration of investigated compounds selected, i.e., 42 μM, is from earlier studies 2× IC_{50} value of melatonin to reduce 5,5-dimethyl-pyrroline N-oxide adduct formation with hydroxy radicals [Tan et al., 1993]. Peroxidation of the linolenic acid standard solution was initiated by incubation in the presence of 100 μM $FeSO_4$ for 60 min at 37°C. Lipid peroxidation was assessed photometrically by absorbance at 534 nm via the content of thiobarbituric acid reactive substances which were measured by the method of Ohkawa et al. [1979] with slight modifications [Starkopf et al., 1995]. The antioxidative capacity was calculated as percent inhibition of the linolenic acid standard solution peroxidation. Significant differences between mean values of pinoline and melatonin were determined using Student's t-test.

Total antioxidant status determination

The total antioxidative capacity of pinoline and melatonin was determined by using the standard "Total Antioxidant Status" kit. In this test, ABTS (2,2′azino-di-[3 ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$. This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree that is proportional to their concentration and antioxidant capacity. Both compounds were used at concentrations of 42 μM. The values of this test are expressed as Trolox Units (0–2.5 mmol/l). Significant differences between mean values were determined using Student's t-test.

Hydroxyl radical scavenging

The assay was performed as described by Barreto et al. [1994], using terephthalic acid (THA) as a chemical dosimeter of •OH. THA dosimeter solution contained 10 mM THA in a 10 mM sodium phosphate buffer at pH 7.5; the investigated compounds were dissolved as described above and equal amounts of 0.1 N HCl and NaOH solutions were used as controls. Hydroxyl radicals were generated via the Fenton reaction by adding $CuSO_4$ and H_2O_2 to the THA dosimeter solution in order to achieve the final concentrations of 10 μM. The reaction product of THA with hydroxyl radicals was measured fluorometrically at 312 nm excitation and 426 nm emission. The data are expressed as per cent inhibition of THA–•OH peaks. IC_{50} values were determined by sigmoidal dose-response (variable slope) analysis with the multipurpose data analysing program, Prism 2.0 (GraphPad Software Inc., San Diego, CA).

Results

Lipid peroxidation inhibition

The results of this experiment revealed that both compounds potently inhibited peroxidation of the linolenic acid standard solution. The antioxidative capacities of 42 μM of melatonin and pinoline were 21.4±0.6% and 13.6±0.7%, respectively (Fig. 2). The data are expressed as the mean value ± SEM of five separate determinations done in triplicate. Student's t-test revealed that under these assay conditions melatonin was significantly more potent than pinoline ($P<0.001$).

Total antioxidant status

Both compounds showed comparable activity in this test. The antioxidative potencies of 42 μM of melatonin and pinoline were 1.55±0.24 mmol/l (n=7) and 1.43±0.23 mmol/l (n=6), respectively (Fig. 3). The data are expressed as the mean value ± SEM. While this test indicated melatonin was also slightly more potent than pinoline, the values did not differ significantly.

Hydroxyl radical scavenging

Melatonin and pinoline concentration-dependently scavenged •OH induced by the Fenton reaction and thereby reduced the formation of THA–•OH. Results of the experiment are presented in Figure 4. The concentrations required to reduce THA–•OH formation by 50% were 11.4±1.0 μM for melatonin and 62.3±3.8 μM for pinoline, respectively.

Discussion

The results of the present experiments demonstrate that both melatonin and pinoline exerted antioxidant effects in all three assay systems. In reference to the inhibition of lipid peroxidation, melatonin was about twice as potent as pinoline. The data concerning the potency of melatonin are in good agreement with the other published studies showing the inhibition of lipid peroxidation by melatonin [Marshall et al., 1996; Daniels et al., 1996]. When determining total antioxidant status, melatonin was only slightly more potent than pinoline. Poeggeler et al. [1995] showed that in this test, melatonin works synergistically with the other antioxidants and implied that similar cooperativity exists in vivo. Considering the structural similarity between pinoline and melatonin and similar potency in different assay systems, we can expect that pinoline also may act synergistically with other antioxidants.

The most important action in antioxidative defence mechanisms exerted by melatonin is considered to be its ability to scavenge the highly toxic hydroxyl radical [Reiter et al., 1996; Reiter, 1997]. The ability of melatonin to reduce hydroxyl radical formation was first shown by Tan et al. [1993]. The

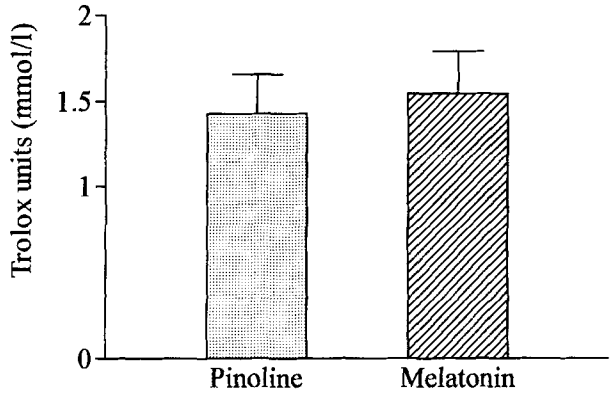

Fig. 3. Activity of pinoline and melatonin in total antioxidant status test. The data are expressed as the mean value ± SEM of six (melatonin) and seven (pinoline) separate determinations.

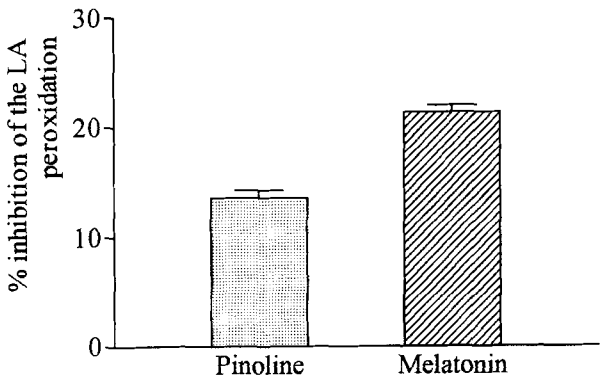

Fig. 2. Inhibition of the linolenic acid standard solution peroxidation by 42 μM of melatonin and pinoline (mean ± SEM, n=5).

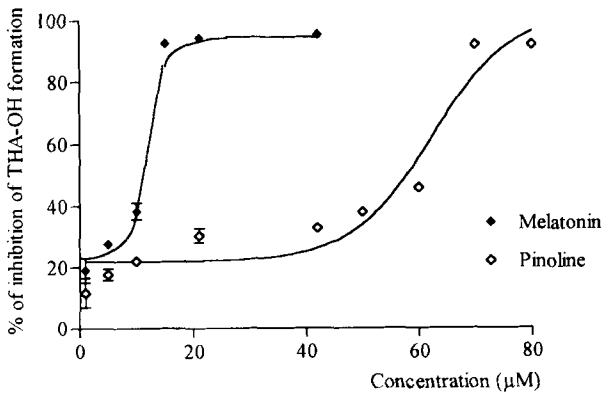

Fig. 4. Effect of melatonin and pinoline in reducing the formation of THA–•OH adducts. Each point represents the mean value ± SEM of 2–5 different determinations.

IC_{50} value of melatonin to reduce the spin trapping agent DMPO–•OH adduct formation in this experiment was 21 μM. This value was more than five times higher than the IC_{50} value for glutathione. Matuszak et al. [1997] recently confirmed that melatonin efficiently reduced the DMPO–•OH adduct formation. In this work, however, the rate constant for scavenging •OH by melatonin was determined but not the IC_{50} value. Marshall et al. [1996] tried to determine the •OH scavenging potency of melatonin but due to technical difficulties they could not test melatonin in their assay system. Results of our experiment (IC_{50} 11.4 μM for melatonin) are in a good agreement with the results of Tan et al. The small difference in the IC_{50} value is most probably due to differences in the assay systems (e.g., a different dosimeter solution).

The present study is the first to show that pinoline also has a pronounced antioxidant action. The potency of pinoline to reduce terephthalic acid–•OH adduct formation was about five times lower than that of melatonin. However, considering the fact that the •OH scavenging ability of melatonin is possibly many times better than that of the well-known antioxidants glutathione and mannitol, pinoline could still be considered a very good •OH scavenger. As melatonin and pinoline share the indole nucleus and the methoxy group at the same position, we confirmed that these portions of the molecule are requirements for their ability to quench the •OH. The present results also show that alterations in the side chain of melatonin (e.g., cyclization) lead to a reduction in the •OH scavenging potency; this is also consistent with the findings of Tan et al. [1993].

A major advantage of melatonin is its ability to penetrate into all tissues and accumulate intracellularly, particularly in the nuclei of cells [Menendez-Palaez et al., 1993; Reiter et al., 1994]. Thus, melatonin is considered to protect nuclear DNA against radical-related damage. This protective action of melatonin is demonstrated also in in vivo experiments [Tang et al., 1997]. Recently, we showed that, like melatonin, pinoline penetrates into all tissues and accumulates in cell nuclei [Pähkla et al., 1996a]. Therefore, it can be expected that pinoline at higher doses may have similar protective action against DNA oxidative damage like that of melatonin.

The concentration of pinoline in mammalian tissues is relatively low. Pinoline has been found in rat brain [Shoemaker et al., 1978; Barker et al., 1981], adrenal glands [Barker et al., 1981], human and bird pineal gland [Kari, 1981; Kari et al., 1983; Langer et al., 1984, 1986], in the rabbit, pig and human retina [Leino et al., 1983; Leino, 1984], and in human serum and cerebrospinal fluid [Rimón et al., 1984] in concentrations ranging from 1 ng/g up to 20 μg/g tissue. A major problem in the determination of pinoline and other β-carbolines in biological samples is their formation during the extraction procedure. It has been suggested that many reported findings of β-carbolines as constituents of various tissues derive from artifactual formation of these compounds [Faull et al., 1982; Bosin et al., 1983]. In recent years there have been no studies published which have attempted to confirm the presence of pinoline in tissues.

Considering the low physiological concentrations of pinoline in the blood and in tissues, the antioxidative action is probably not the main action of pinoline. However, in pharmacological doses these concentrations are reached easily. Recently, we showed the clear and dose-dependent antidepressant-like effect of pinoline in rat behavioral experiments [Pähkla et al., 1996b]. The main neurochemical mechanism of this action is most probably the inhibition of neuronal serotonin (5-HT) reuptake [Pähkla et al., 1997b] and an increase in brain 5-HT levels [McIsaac et al., 1972]. These effects are common to most clinically effective antidepressants. In addition, pinoline appears rather non-toxic in acute toxicity studies. The LD_{50} in mice for pinoline following intraperitoneal administration was 235 mg/kg [Airaksinen et al., 1978]. In comparison, the LD_{50} for intraperitoneally administered tricyclic antidepressants amitriptyline and imipramine are 65 mg/kg and 90 mg/kg, respectively [Tobe et al., 1981].

Thus, considering the antidepressant-like effect of pinoline in behavioral experiments combined with its low acute toxicity and pronounced antioxidative action, this compound should be further studied as a potential antidepressant. A second major finding of the current report relates to the additional evidence that supports the role of the pineal gland in protection against free radical generating agents. In addition to melatonin, pinoline is another compound possibly synthesized in the pineal gland or occurring as a metabolite of melatonin, which has substantial antioxidant action.

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