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Chemical and Physical Properties and Potential Mechanisms: Melatonin as a Broad Spectrum Antioxidant and Free Radical Scavenger

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Abstract: Melatonin was found to be a potent free radical scavenger in 1993. Since then over 800 publications have directly or indirectly confirmed this observation. Melatonin scavenges a variety of reactive oxygen and nitrogen species including hydroxyl radical, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrite anion. Based on the analyses of structure-activity relationships, the indole moiety of the melatonin molecule is the reactive center of interaction with oxidants due to its high resonance stability and very low activation energy barrier towards the free radical reactions. However, the methoxy and amide side chains also contribute significantly to melatonin's antioxidant capacity. The N-C=O structure in the C3 amide side chain is the functional group. The carbonyl group in the structure of N-C=O is key for melatonin to scavenge the second reactive species and the nitrogen in the N-C=O structure is necessary for melatonin to form the new five membered ring after melatonin's interaction with a reactive species. The methoxy group in C5 appears to keep melatonin from exhibiting prooxidative activity. If the methoxy group is replaced by a hydroxyl group, under some in vitro conditions, the antioxidant capacity of this molecule may be enhanced. However, the cost of this change are decreased lipophility and increased prooxidative potential. Therefore, in *in vivo* studies the antioxidant efficacy of melatonin appears to be superior to its hydroxylated counterpart. The mechanisms of melatonin's interaction with reactive species probably involves donation of an electron to form the melatoninyl cation radical or through an 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. Melatonin also has the ability to repair damaged biomolecules as shown by the fact that it converts the guanosine radical to guanosine by electron transfer. Unlike the classical antioxidants, melatonin is devoid of prooxidative activity and all known intermediates generated by the interaction of melatonin with reactive species are also free radical scavengers. This phenomenon is defined as the free radical scavenging cascade reaction of the melatonin family. Due to this cascade, one melatonin molecule has the potential to scavenge up to 4 or more reactive species. This makes melatonin very effective as an antioxidant. Under *in vivo* conditions, melatonin is often several times more potent than vitamin C and E in protecting tissues from oxidative injury when compared at an equivalent dosage (μmol/kg). Future research in the field of melatonin as a free radical scavenger might be focused on: 1), signal transduction and antioxidant enzyme gene expression induced by melatonin and its metabolites, 2)**,** melatonin levels in tissues and in cells, 3)**,** melatonin structure modifications, 4)**,** melatonin and its metabolites in plants and, 5)**,** clinical trials using melatonin to treat free radical related diseases such as Alzheimer's, Parkinson's, stroke and heart disease.

Key words: Melatonin, Metabolites, Antioxidant, Free radical scavenger, Plants, Reactive oxygen species, Reactive nitrogen species, Structure-activity relationship

INTRODUCTION

Melatonin (N-acetyl-5-methoxyindolamine) is a derivative of tryptophan, an essential amino acid for mammals. The structure of melatonin was described in 1958 by Lerner *et al.* [1]. Early studies indicated that melatonin was uniquely synthesized and secreted by the pineal gland in vertebrates [2]. In recent years, numerous publications have shown its extrapineal origin including retina, Harderian glands, gut, ovary, testes, bone marrow and lens [3-10]. However, it is not clear whether extrapineal melatonin is released into circulation or limited to the organs or tissues which synthesize it. Melatonin was also identified in bacteria [11], alga [12], fungi [13] and other plants [14, 15]. From an evolutionary point of view, melatonin's wide distribution implies that it plays a primary role in the

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function and survival of organisms. It has been reported that the high melatonin-containing tobacco is more resistant to UV irradiation than that is the low melatonin tabacco strain [16]. In a unicellular organism (*Gonyaulax polyedra*) when the ambient temperature of the culture is decreased, the organism increases melatonin production to adapt to cold stress to ensure survival [17].

To clarify the mechanism by which melatonin functions in defense of environmental stresses, we found in 1993 that melatonin is a potent free radical scavenger [18]. Thus, melatonin can reduce the toxicity of a wide variety of environmental and chemical insults which initiate oxidative stress. Although melatonin has several important physiological actions including the control of circadian rhythms, sleep induction, regulation of seasonal reproduction and immune enhancement [19], the most basic function of melatonin is speculated to be its antioxidant actions which protect organisms from oxidative stress [20]. In excess of 800 publications have directly or indirectly documented the potent antioxidant and free radical scavenging ability of melatonin. Melatonin detoxifies numerous reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), peroxyl radicals (ROO·) and singlet oxygen ($^1O_2$), and also reactive nitrogen species (RNS) such as nitric oxide radical (NO·) and peroxinitrite ($ONOO^-$); it also neutralizes hypochlorous acid (21, 22). Melatonin prevents injuries induced by oxidative stress at the molecular, cellular, tissue, organ and organ system levels (23). However, the multiple mechanisms by which melatonin interacts with different reactive species are far from clarified. Also, the question as to how an antioxidant in such presumed low concentrations contributes so significantly to the antioxidant defense system is still to be determined. In this article we review recent developments regarding melatonin's chemical and physical properties and its structure-activity relationships which relate to its free radical scavenging capacity. We also consider hypotheses regarding the antioxidant actions of melatonin and suggest potentially important future research in the field of melatonin as a free radical scavenger.

THE STRUCTURAL PROPERTIES OF MELATONIN AS A FREE RADICAL SCAVENGER

Melatonin is an indoleamine. It contains a indole heterocycle and two side chains, namely, a 5-methoxy group and 3-amide group. The chemical structure of melatonin is illustrated in Fig. **1**.

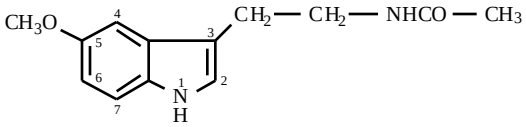


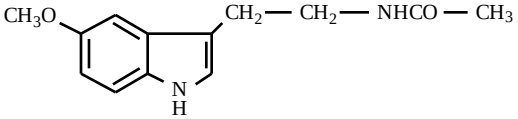
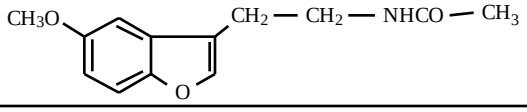
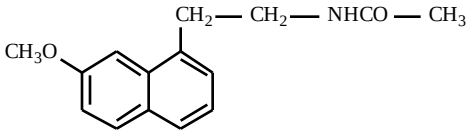
Fig. (1). The structure of melatonin. The numbers identify the atom's positions in the indole heterocycle.

Indole Heterocycle

It is obvious that the core structure for melatonin required to scavenge free radicals is the indole heterocycle. The electron-rich indole moiety with high resonance stability and electroreactivity determines melatonin's potent free radical scavenging capacity [24]. If the indole moiety is replaced by structurally similar moieties such as benzofurane and naphthalene, the antioxidant activity of these agents decreases substantially when compared with melatonin [25]. Even when the closest atom in the periodic table is replaced, for example, if the oxygen replaces the nitrogen in the indole ring, the antioxidant activity of benzofurane is decreased by 40% (Table 1). Indole compounds appear to be endowed with antioxidant properties greater than that of their benzofurane or naphthalene analogs in specific experimental conditions.

In chemical reactions, the levels of the activation energy barrier reflect whether these reactions will readily occur or not. Using computational approaches and calculating the relative free energy of radical reactions indicate that the carbon atoms at positions 2, 3, 4, 6 and 7 of the indole heterocycle are suitable sites for hydroxyl radical or nitrogen

Table 1. A Comparison of the Antioxidant Activities of Melatonin and Its Analogs. The Antioxidant Activity of the Melatonin Defined as 1 and the Antioxidant Activities of Melatonin Analogs are Expressed as a Ratio to that of Melatonin.

Compound	Structures	Activity
		10^{-4} M
1		1.0
2		0.6
3		0.7

Modified from [25]

Table 2. Relative Free Energy Values (ΔG^o, kcal mol-1) for the Interactions Between Reactive Nitrogen Species or the Hydroxyl Radical with the Indole Heterocyclic Moiety of Melatonin in Aqueous Phase

	C2	C3	C4	C6	C7
Mel-HO$^{\bullet}$	1.1	4.4	2.4	2.2	2.2
Mel-NO	2.4	-	5.9	1.4	0.0

Cn = the carbon position in the indole ring, Mel-HO$^{\bullet}$ = interaction of melatonin with HO$^{\bullet}$, Mel-NO = interaction of melatonin with nitric oxide, - = not determined, The data from references [26] and [27].

radical reactions [26, 27]. The relative free energy values (or activation energy barrier) ($^{\Delta}G^o$, kcal/mol) for these carbon atoms to react with hydroxyl and nitrogen radicals to form adducts are quite low (Table 2). These low activation energy barriers suggest that the addition of $^{\bullet}$OH or NO to any of the mentioned carbon atoms should occur quite rapidly in line with general expectations. From the kinetic point of view, carbon C2 with a low energy barrier of about 1 kcal/mol seems the most favorable site for $^{\bullet}$OH addition [26]. On other hand, carbon C7 appears to have no activation energy barrier to react with nitrogen radicals [27]. However, conventional chemistry theory suggests that the most reactive site in the indole ring is carbon C3, and despite the fact that there is a side chain, the electrophilic addition should primarily occur in carbon atom C3 [28]. This theory is supported by experimental observations which show that the original reaction of $^{\bullet}$OH indeed occurred at carbon atom C3 rather than at C2 [29].

Since radical reactions are very complex and the target sites or the resulting adducts vary according to environmental conditions such as pH, temperature and the reactants' concentrations, it is not surprising that the primary reaction site of a radical attack will also vary from one carbon to another in the indole heterocycle during different experimental situations. Thus, the resulting products of melatonin's interaction with free radicals have been reported to be 2-hydroxy, 3-hydroxy, 6-hydroxy, and 7-hydroxy melatonin, cyclic 2 or 3-hydroxymelatonin, N-nitrosomelatonin and N^1-acetyl-N^2 formyl-5-methoxyknuramine (AFMK), respectively [29-36].

Side Chains

Methoxy and aminoactyl side groups are connected at the C5 and C3 positions, respectively, of the indole moiety in the melatonin molecule. These side chains appear to

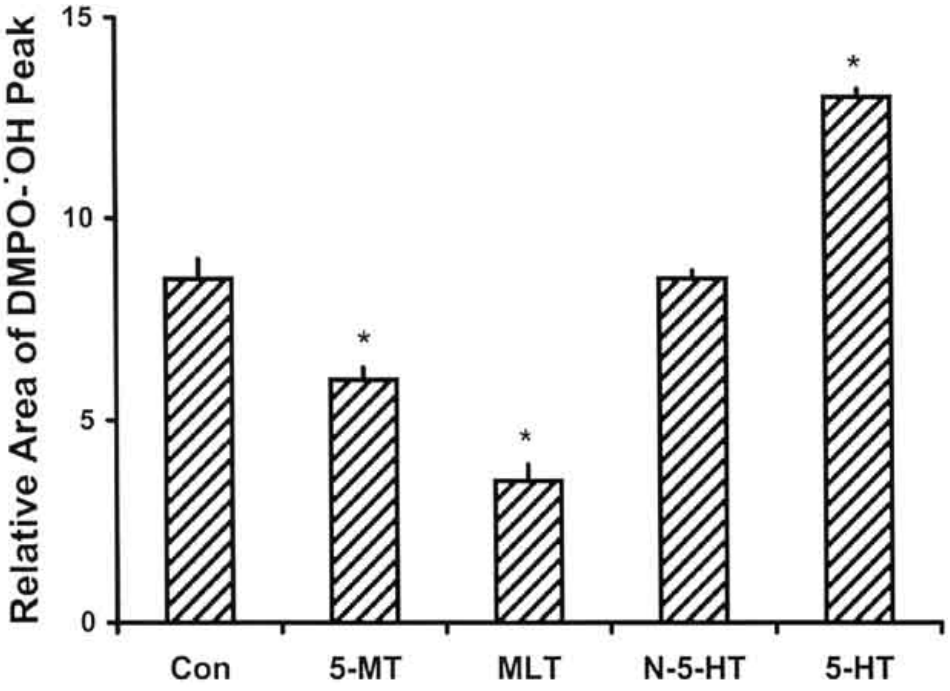


Fig. (2). Effects of melatonin and its analogs on the formation of DMPO-$^{\bullet}$OH adducts. The amount of DMPO-$^{\bullet}$OH is positively related to the number of $^{\bullet}$OH. The DMPO-$^{\bullet}$OH was measured using HPLC with an electro-chemical detector. $^{\bullet}$OH was generated by irradiation of hydrogen peroxide with UV light (254nm). The data are expressed as mean ± SE of six separate experiments. Con = control, 5-MT = 5-methoxytryptamine, MLT = melatonin, N-5-HT = N-acetyl-5-hydroxytryptamine, 5-HT = 5-hydroxytryptamine, * $p < 0.01$ versus control (18).

contribute significantly to the free radical scavenging capacity and they limit prooxidative actions of melatonin. Initially, Tan *et al*. [18] investigated the influence of the side chains on the •OH scavenging ability by comparing melatonin with several analogs. It was found that the methoxy group as well the acetyl group of the amide were essential for melatonin to display potent •OH scavenging activity. The •OH scavenging capacity of 5-methoxytryptamine, which is devoid of a acetyl group, was about 50 % that of melatonin (Fig. **2**). Furthermore, a compound lacking both the methoxy and acetyl groups, namely 5-hydroxytryptamine, was a prooxidant rather than an antioxidant. It was speculated that the acetyl group has a synergistic effect on the antioxidative ability and the methoxy group is essential for this reaction [18].

Initially it was difficult to understand how the side chains would be involved in such a reaction. When Tan *et al*. [29] elucidated the pathway of melatonin's interaction with •OH and with the formation of cyclic 3-hydroxymelatonin, the function of the N-acetyl group became apparent. The formation of cyclic 3-hydroxymelatonin requires melatonin to scavenge two •OH and this reaction also requires the acetyl group to be intact on the side chain. When melatonin interacts with the first •OH it forms the cyclic 3-hydroxy melatoninyl radical. The unpaired electron captured from the •OH shifts from the newly formed heterocycle moiety and localizes at the carbonyl structure of the acetyl group. The highly localized unpaired electron will easily interact with the second •OH to yield the stable final product. If a melatonin analog lacks this nitrogen connected carbonyl structure or related structures such as 5-methoxytryptamine (one acetyl group less than melatonin), it may also lack the ability to capture the second •OH. This would explain stoichiometrically why the •OH scavenging capacity of 5-methoxytryptamine is about half that of melatonin (Fig. **2**), i.e., melatonin scavengers two •OH and 5-methoxytryptamine scavenges one •OH.

In a carefully designed experiment to explore the structure-activity relationships of melatonin and its analogs, Gozzo *et al*. [25] selected a variety of different structures to replace melatonin's side chains (Table 3). Upon examination of Table 3, it is apparent that once the side chain at C5 is fixed, the replacement of the C3 side chain will substantially modify the antioxidant activity of the resulting molecules. A significant aspect of this is, if a molecule lacks a N-carbonyl

Table 3. Comparison of the Antioxidant Activities of Melatonin Analogs
The Antioxidant Activity of Melatonin is Defined as 1 and the Antioxidant Activities of Melatonin Analogs are Expressed as a Ratio to that of Melatonin. The NHCO structure is Bolded.

Compound	R1	R2	Activity	
			10^{-4} M	10^{-5} M
1 (MLT)	CH_2-CH_2-**NHCO**-CH_3	5-OMe	1	-
2	CH_2-CH_2-**NHCO**-$C(CH_3)_3$	5-OMe	0.9	-
3	CH_2-CH_2-**NHCO**-Ph	5-OMe	1.3	-
4	CH_2-CH_2-**NHCO**-C_8H_{17}	5-OMe	>3.5	1
5	CH_2-CH_2-NH_2	5-OMe	0.4	-
6	CH_2-CH_2-CO-CH_3	5-OME	1.2	-
7	CH_2-CH_2-**NHCO**-CH_3	5-H	0.6	-
8	CH_2-CH_2-**NHCO**-$C(CH_3)_3$	5-H	1.2	-
9	CH_2-CH_2-**NHCO**-Ph	5-H	1.1	-
10	CH_2-CH_2-**NHCO**-C_8H_{17}	5-H	2	-
11	CH_2-CH_2-NH_2	5-H	0.4	-
12	CH_2-CH_2-CO-CH_3	5-H	0.6	-
13	CH_2-CH_2-**NHCO**-CH_3	6-OMe	>3.5	1
14	CH_2-CH_2-**NHCO**-$C(CH_3)_3$	6-OMe	1.8	-
15	CH_2-CH_2-NH_2	6-OMe	0.9	-
16	CH_2-CH_2-**NHCO**-CH_3	5-OH	>3.5	0.8
17	CH_2-CH_2-**NHCO**-Ph	5-OH	>3.5	>3.5
18	CH_2-CH_2-NH_2	5-OH	>3.5	1.1

MLT = melatonin, - = not determined. Modified from [25].

structure in this side chain as compound 5, 11 and 15, the antioxidant activity decreases several-fold compared to a compound with this structure. The only exception is compound 18. Its antioxidant activity, compared to that of compound 16 is not changed significantly. However, compared to compound 17 its antioxidant activity is indeed reduced approximately 3-fold.

Recently, Poeggeler *et al.* [37] compared melatonin's redox chemistry with that of several structural analogs including tryptamine, N-acetyltryptamine, serotonin, N-acetylserotonin, 5-methoxytryptamine, 6-chloromelatonin and 2-iodomelatonin. They concluded that the absence of either the O-methyl or the N-acetyl residue (note, it contains a N-carbonyl structure) causes a marked diminution in the capacities for scavenging $^{\bullet}OH$ and ABTS cation radical as well as in chemiluminescence emitted during oxidation. The hypothesis that the acetyl residue produces synergistic effects in terms of the antioxidant capacity of melatonin are supported by the evidence obtained from these structure-activity relationship studies. It appears that the carbonyl connected to the nitrogen atom may be an important functional structure for this activity.

Another important side chain of the melatonin molecule is the methoxy group at C5. Analysis of the data from the original publication of Tan *et al.* [18] (Fig. **2**) leaves an impression that the methoxy group is necessary for the antioxidative activity of indole analog. This was correct under the specific experimental condition of this study, i.e., when 254 nm UV light was used to homolyze H_2O_2 to form $^{\bullet}OH$. Under other experimental conditions, the indole analogs lacking a methoxy group have been shown to possess antioxidative activity [25]. Several in vitro experiments indicate that replacement of the hydroxyl group at position 5 or 6 increases the indoleamine's antioxidant capacity [38-41] (this is not the case *in vivo*, the hydroxylation changes the physical property and decreases lipophilicity of this indole). This is not unexpected since phenolic structures are good hydrogen donors. However, the cost for this is the decreased lipophilicity and potential prooxidative action (see below). The methoxy group does modify the antioxdative efficacy of these indole compounds, namely to improve their performance as antioxidants [18, 25, 37]. The mechanism is not currently known, but it suggests that the 5-methoxy group may share electrons with the indole to make the molecule more likely to react with radical species. An important contribution of the methoxy group is to prohibit prooxidative reactions of the indoles. The protective methoxy group at 5 position completely prevents quinone-imine formation and radical and redox reaction-promoting activities of the hydroquinone-semiquinone-quinone-imine type. This type of reactivity is common for the pro- and antioxidative activities of antioxidants containing hydroxyl groups [42-44].

The lack of prooxidative actions for melatonin has been confirmed by several investigators [18, 37, 41, 45-48]. If the methoxy group is replaced by a hydroxyl group (as in serotonin and other hydroxyindoles) the dual behavior (prooxidation and antioxidation) is observed [37, 41, 45, 46, 48]. This unshield hydroxyl group may form O-centered radical intermediates [49] and induce peroxidative reactions. Recently, Ng *et al.* [41] and Poeggeller *et al.* [37, 48] confirmed again that all hydroxyindoles tested in their studies exhibited excellent antioxidant abilities and inevitably also possessed prooxidant actions. Conversely, all methoxyindoles with free radical scavenging capacity tested were devoid of prooxidant activity.

Recently, Turjanski *et al.* [50] concluded that the 5-methoxy and the N-acetyl groups do not significantly affect the antioxidant properties of melatonin. Their conclusion is based solely on a computational model and is devoid of any experimental prove. Furthermore, in this computational model the authors have not taken into consideration the fact that one melatonin molecule may scavenge more than one $^{\bullet}OH$, depending on the presence of a N-carbonyl structure containing side chain. After scavenging the first $^{\bullet}OH$, the molecular structure of melatonin is modified [29] and the relative free energy which is required for it to react with a second $^{\bullet}OH$ should be altered accordingly. If the computational model had considered this option, the conclusion may be different than currently presented.

The methoxy and acetyl side chains are not only important chemically but also physically. The physical property of being both lipophilic and hydrophilic [51, 52] enables the molecule to cross membranes with ease but also to distribute in sufficiently high portions in the lipid and the aqueous phases of the cell. Thus, melatonin effectively protects molecules in various compartments of the cell including the membrane, cytosol, mitochondrion and nucleus against oxidative insults. Modifications of the side chains, such as hydroxylation in C5, influences both the chemical and the physical properties of melatonin, thus, altering its antioxidant efficacy in *in vivo* situations.

THE POTENTIAL REACTIONS OF MELATONIN AS A FREE RADICAL SCAVENGER

Radical reactions are very complex and not fully understood currently. Thus, the mechanisms of melatonin interactions with free radicals also are not totally clarified. However, based on the experimental observations and theoretical hypotheses, several possibilities has been proposed [53].

Electron Donation

Melatonin, like other antioxidants, possesses a specific electron reduction potential. The reduction potential of melatonin was examined using cyclic voltammetry (CV) in our laboratory as well as by others [54]. Our finding indicate that melatonin exhibits an anodic wave at a maximum potential value [Ep(a)] of 0.73 V during the forward scan (Fig. **3**). No wave was detected during the reverse scan which indicates that the oxidation products were unstable or a consequence of fast second order decay of the radicals. The presence of an anodic wave on CV indicates the ability of melatonin to donate an electron and thus provides a physical parameter for the electron donation hypothesis. The one electron donation mechanism to explain melatonin's scavenging action was first proposed by Hardeland *et al.*

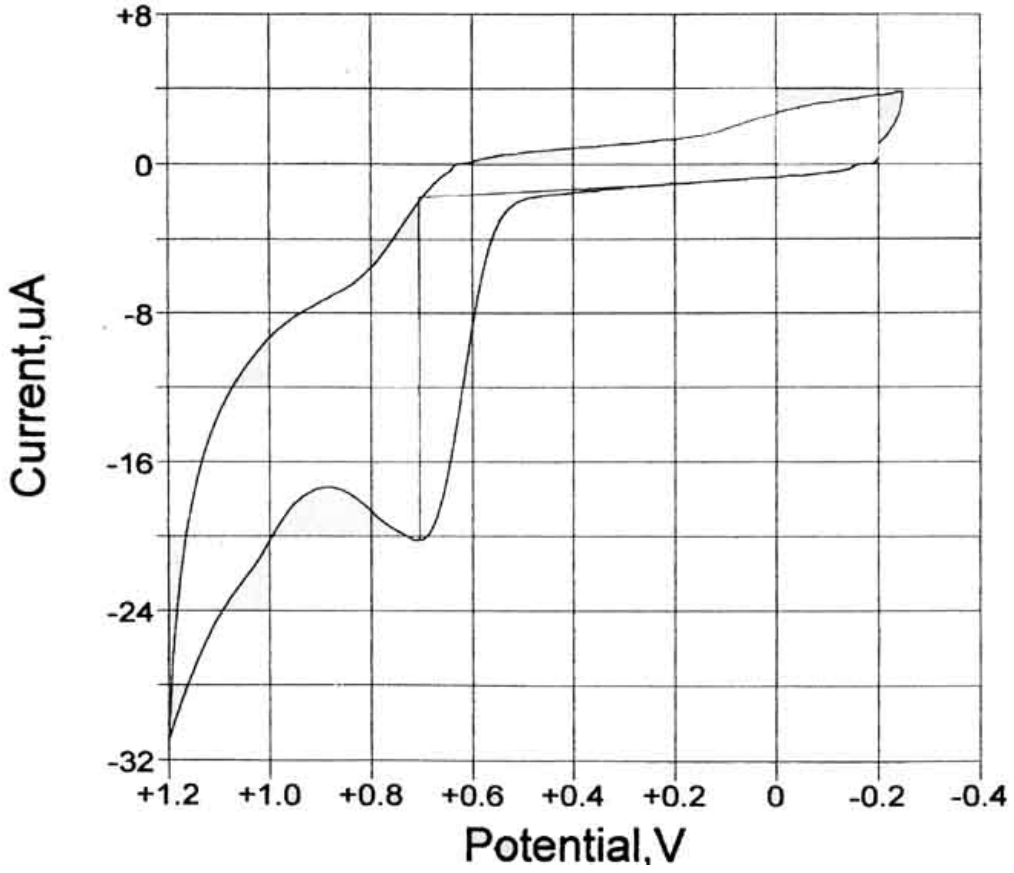


Fig. (3). Cyclic voltammetry of melatonin. The potential range is from the -0.3 to 1.3 V at a rate of 100 mV/s vs the Ag/AgCl reference electrode. An anodic wave at Ep(a) of 730 mV is apparent.

[55]. The key feature of this hypothesis is the formation of melatonin cation (melatoninyl) radical. The resonance stabilized and relatively long-lived melatoninyl cation radical then reacts with $O_2^{\bullet -}$ to form AFMK (Fig. **4**).

The melatoninyl radical theory or one electron abstraction reaction proposed by Hardenland *et al.* [55] is supported by several experimental observations. Scaiano *et al.* [56], Stasica *et al.* [57] and Mahal *et al.* [54] using a pulse radiolysis system, claimed to have found evidence for the existence of the melatoninyl radical. These authors defined a spectrum using spectrophotomeric monitoring systems when melatonin interacted with free radicals. The resulting spectrum strongly resembled that for a indolyl-type radical of various indole derivatives (a pronounced maximum absorbence at λ = 345 nm and a weaker maximum absorbence at λ = 530 nm). The other evidence which favors the melatoninyl cation radical theory is melatonin's capability of easily undergoing a single electron donation reaction with $ABTS^{\bullet +}$ [37, 58]. The melatoninyl radical theory has been employed by several research groups to explain their observations [59-63], without which it would have been difficult to explain the experimental results. All the evidence which supports the existence of the melatoninyl cation radical are indirect. Thus, the formation of melatoninyl cation radical from the interaction of melatonin and free radicals still awaits definitive proof; this could come in the form of a ESR melatoninyl radical signal [53].

Tesoriere *et al.* [64] recently observed that melatonin undergoes two one-electron donation steps to reduce the reactive oxoferryl hemoglobin (oxoferryl-Hb) molecule.

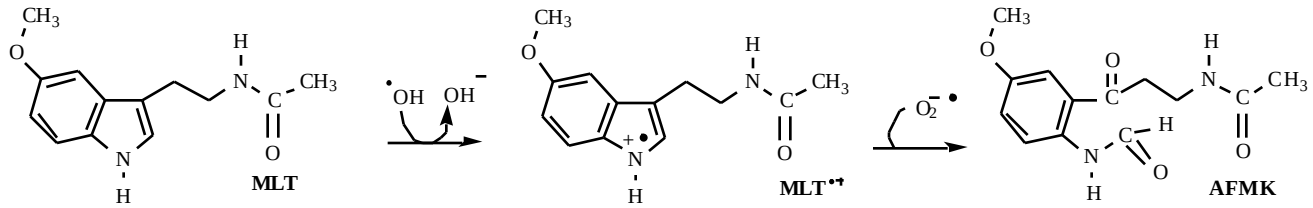


Fig. (4). The proposed pathway of melatoninyl cation radical formation [55]. MLT = melatonin, $MLT^{\bullet +}$ = melatoninyl cation radical.

Oxoferryl-Hb was induced by the interaction of H_2O_2 and hemoglobin. Once melatonin was introduced to oxoferrel-Hb, melatonin underwent one electron donation to reduce one oxoferryl-Hb to met-Hb and generate a melatoninyl cation radical. The melatoninyl cation radical then interacts with the second oxoferryl-Hb via radical addition. The heme oxygen of oxoferryl-Hb is added to C3 position of the melatoninyl cation radical and the melatoninyl cation radical donates a second electron. The resulting products from the second electron donation were again the met-Hb and cyclic 3-hydroxymelatonin (Fig. **5**). Thus, the authors suggest that melatonin is "a two-electron reducing molecule." The experimental stoichiometric factor of oxoferryl-Hb (reduced): melatonin (consumed) being about 2, in the range of the melatonin concentrations 5-50uM, fully support the speculation of melatonin being a two-electrons reducing molecule.

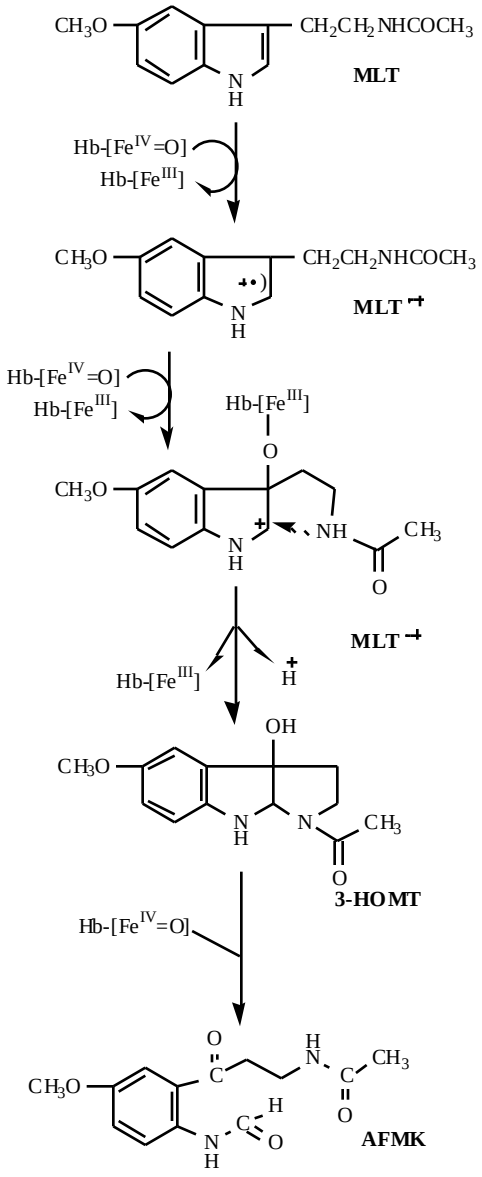


Fig. (5). The proposed two one electron transfer steps during melatonin's interaction with reactive oxoferrylhemoglobin [64]. MTL = melatonin, $MLT^{\bullet+}$ = melatoninyl cation radical, 3-HOMT = cyclic 3-hydroxymelatonin, AFMK= N^1-acetyl-N^2-formyl-5-methoxykynuramine.

Hydrogen Donation

Besides lacking ESR data to definitively prove the existence of the melatoninyl cation radical, Turjanski *et al.* [50] also suggested that the one electron abstraction from melatonin to form melatoninyl cation radical was thermodynamically unfavorable on the basis of their computational model. They suggest that instead of donating an electron to form a melatoninyl cation radical, melatonin might donate a hydrogen atom from the NH structure of the pyrrole ring to generate a neutral melatonin radical. The neutral melatonin radical could scavenge a $O_2^{\bullet-}$ to form the final product AFMK, just as does the melatoninyl cation radical. There is yet no experimental evidence to prove or disprove this scenario. However, indirect evidence does not favor the hydrogen donation hypothesis.

It is commonly accepted that a chain breaking antioxidant should exhibit the ability to donate its labile hydrogen atom to the relatively unreactive alkylperoxyl radical ($ROO^{\bullet}$) such as is the case with vitamin E. Melatonin is not considered as a classical chain break antioxidant as indicated in a study performed by Antunes *et al*. [65] and its ability to scavenge $ROO^{\bullet}$ appears somewhat limited [66, 67]. There is at least one report which claimed that melatonin was more effective than vitamin E in scavenging $ROO^{\bullet}$ [68], but this observation might relate to the unique experimental conditions which did not apply in other studies. The possible site of hydrogen donation in melatonin is the NH group [50]. Practically, the N-H bond in the pyrrole ring is much stronger than those in diarylamines in which the N-H group is free to rotate with respect to the aromatic ring. The N-H bond in a pyrrole ring is orthogonal with the p electron system which can therefore provide no resonance stabilization when the N-H bond is stretched. Therefore, melatonin's indole hydrogen more readily undergoes electron donation rather than hydrogen transfer.

Addition

Another possible mechanism of melatonin's interaction with free radicals is via an addition reaction, especially for $^{\bullet}OH$. These radicals are among the most reactive chemical species known. They are powerful electron acceptors; they abstract H-atoms from most molecules and they add readily to double bonds. The barrier to their addition to double bonds is usually even less than that of H-abstraction, so that competitive addition is often favored [69]. The addition reaction of melatonin in scavenging $^{\bullet}OH$ has been proven by Tan *et al*. [29]. The favorite position for the $^{\bullet}OH$ addition is at C3. The pathway is illustrated in Fig. **6**. In this pathway, one melatonin molecule scavenges two $^{\bullet}OH$ to form the thermodynamically stable final product cyclic 3-hydroxymelatonin. The initial $^{\bullet}OH$ is added to C3 of the indole moiety of the melatonin molecule to form the 3-hydroxymelatonin neutral radical. After addition, the double bond between C3 and C2 is converted to a single bond and the unpaired electron appears to concentrate at C2 to balance the valence required. The electrophilic C2 attracts the nitrogen atom in the side chain to form a 5 membered ring and the resulting intermediate is cyclic 3-hydroxymelatonin

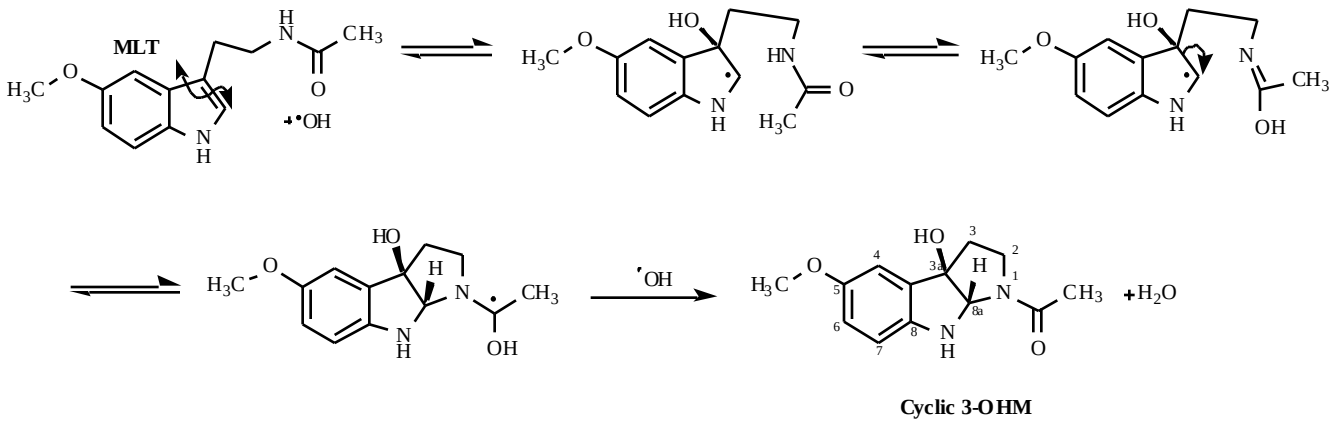


Fig. (6). The proposed reaction pathway during melatonin's interaction with two •OH and the formation of cyclic 3-hydroxymelatonin [29]. MTL = melatonin, Cyclic 3-OHM = cyclic 3-hydroxymelatonin.

radical. After intramolecular rearrangement, the unpaired electron appears to shift to the carbonyl structure adjacent to the nitrogen which is the member of the newly formed 5 membered ring. Then, this structure scavenges the second •OH to form cyclic 3-hydroxymelatonin. Cyclic 3-hydroxymelatonin was identified by both mass spectrometry (MS) and proton nuclear magnetic resonance (NMR) both in vitro and *in vivo* [29].

The addition reaction was also speculated in the initial step of melatonin's interaction with hypochlorous acid on the C3 carbon [32]. Interestingly, the novel melatonin metabolite, i.e.; cyclic 3-hydroxymelatonin, has also been found when melatonin scavenges peroxynitrite and reduces oxoferryl-Hb [31, 64]. It seems that cyclic 3-hydroxymelatonin is a common metabolite of melatonin's interaction with variety of reactive species rather than the specific product of melatonin scavenging the •OH [29]. Based on what is known, it is clear that the nitrogen atom in the side chain is necessary for the formation of new 5-member-ring and the N-C=O structure promotes scavenging of the second oxidant. This observation correlates well with the results of structure-activity studies regarding melatonin's interaction with free radicals [18, 25, 37, 56].

Substitution

Another alternative for melatonin's interaction with free radicals is substitution, especially for the highly reactive •OH. Roberts *et al.* [30] reported that melatonin scavenges •OH to form 2, 3 or 7-hydroxymelatonin, respectively. This suggests that the •OH substitutes for the hydrogen associated with the carbon atoms at positions 2, 3 or 7, respectively.

Nitrosation

Melatonin was frequently reported to be a nitrogen centered free radical scavenger [31, 59, 70]. However, the nitrosation was not considered a likely reaction of melatonin with these radicals since no nitrosated melatonin metabolite had been detected previously [59]. However, recent studies have shown the presence of nitrosated products when melatonin interacts with nitrogen-centered radicals [27]. Turjanski *et al.* [34, 35] have also proposed both theoretically and experimentally, that melatonin reacts with NO and that the N-nitrosomelatonin is the main product of this reaction in both aprotic or aqueous media. However, the mechanism of interaction between melatonin and nitrogen radicals requires further investigation.

Repair

As with any antioxidant, it will be more effective in reducing oxidative damage if it exhibits not only the ability to neutralize oxidants but also it has the ability to repair a biomolecule that has been oxidized. Mahal *et al.* [54] claimed that melatonin may function in the repair of some molecules. They found that melatonin (at pH 7.0) repaired the guanosine radical (G•) induced by oxidation. The rate constant for this reaction is quite high, roughly 3×10^9 dm^3 mol^{-1} s^{-1}, and the mechanism presumably is via electron transfer (G• + MelH → G-H + Mel•, G• = guanosine radical, Mel• = melatonin radical). The major advantage of repair function becomes obvious when various types of attacking radicals are involved in the destructive process. The antioxidant can effectively protect a specific biomolecule via its repair function rather than reacting with a variety of possible attacking radicals [71].

MELATONIN FAMILY AS FREE RADICAL SCAVENGERS

The melatonin family includes only melatonin and the metabolites which are generated by the interaction of melatonin with the reactive oxygen species and reactive nitrogen species (some of these metabolites can be generated enzymatically). The members of this family include melatonin, 6-hydroxymelatonin, cyclic 3-hydroxymelatonin, AFMK and N-acetyl-5-methoxykynuramine (AMK). Each of these family members has been reported to be a free radical scavenger. 6-Hydroxymelatonin was thought to be an exclusively enzymatic metabolite of melatonin formed by hepatocytes [2]. However, Zhang *et al.* [31] showed that 6-

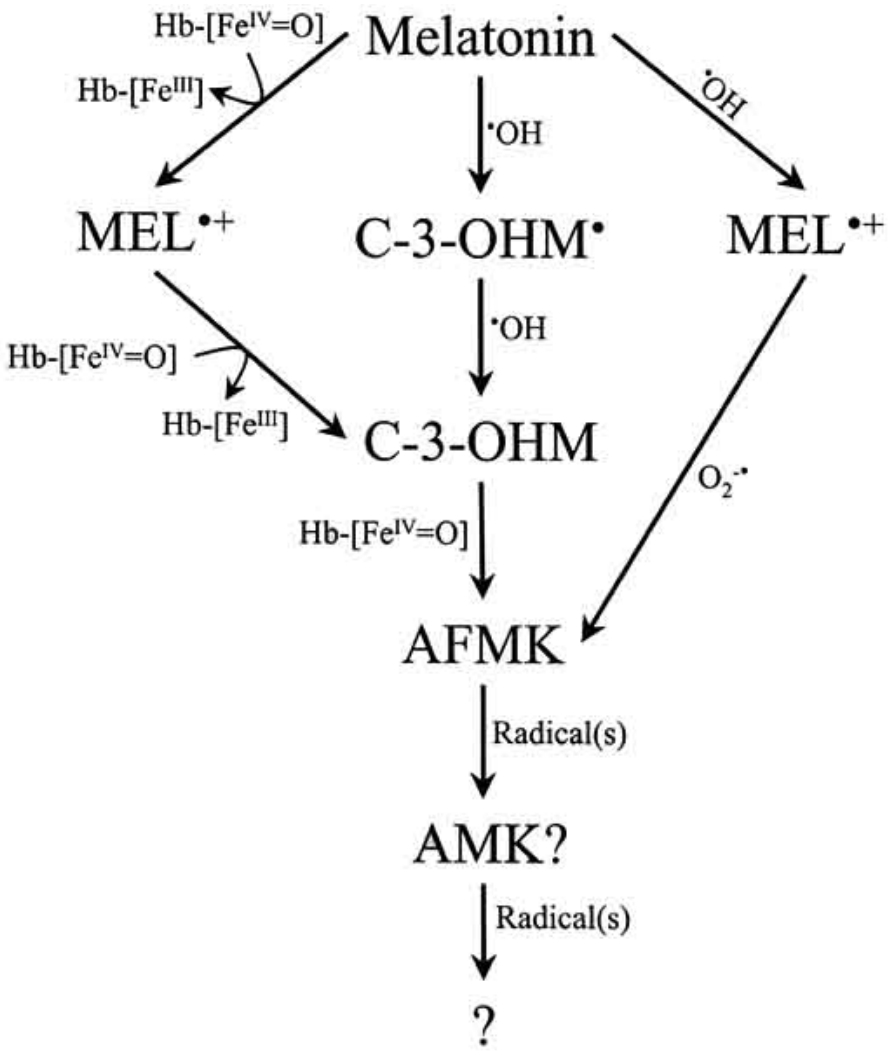


Fig. (7). The proposed free radical scavenging cascade reaction of melatonin. C-3-OHM$^{\bullet}$ = cyclic 3-hydroxymelatonin radical, C-3-OHM = cyclic 3-hydroxymelatonin, MEL$^{\bullet+}$, = melatoninyl cation radical.

hydroxymelatonin is also the major product of melatonin's interaction with nitrogen centered reactants. Several in vitro and *in vivo* studies have shown that 6-hydroxymelatonin effectively scavenges free radicals and protects against oxidative tissue damage [72-75]. The scavenging mechanism of 6-hydroxymelatonin may be similar to melatonin since the molecules have similar structures. In some in vitro studies, it was reported that the antioxidant capacity of 6-hydroxymelatonin is even more potent than that of its parent molecule melatonin [38, 39]. However, under *in vivo* conditions, the tissue protective effect of melatonin is always better than that of 6-hydroxymelatonin [72, 74, 75] probably due to the unique physical properties of melatonin and the fact that it does not act as a prooxidant.

AFMK can be formed from melatonin via several enzymatic and oxidative pathways [64, 76, 77]. It may be one of the primary metabolites of melatonin. Using cyclic voltammetry we were able to show that AFMK has the ability to donate two electrons at potentials [EP(a)] of 456 and 668 mV, respectively [76]. AMFK protects against $^{\bullet}$OH induced DNA damage and lipid peroxidation in in vitro studies [76, 78]. The ability of AFMK to protect against free radical mutilation of DNA is equivalent to that of melatonin [78] while the lipid peroxidative protection of AFMK is less than that of melatonin [76] probably due to its distribution which is not favored in lipid. AFMK also significantly reduces neuronal cell death induced by H_2O_2, glutamate or β-amyloid peptide [76]. The mechanism of how AFMK functions as a free radical scavenger and what is the functional structure(s) of electron donation is still unknown and deserves further investigation.

AMK is a deformyl product of AFMK which is generated both by enzymatic and by nonenzymatic means [53]. AMK was observed to protect against DNA damage induced by $^{\bullet}$OH even more effectively than did melatonin under in vitro conditions (unpublished observations).

Table 4. Comparisons of the Antioxidant Effects of Melatonin with Classical Antioxidants (Vitamin C and E) in *in vivo* Animal Studies

Species	Toxin	μmol/kg			Ratio (μmol) Vit/Mel	Damaged Organs or Tissues	Protective Effects			Ref.
		Mel	Vit E	Vit C			Mel	Vit E	Vit C	
Rat	Phosphine	43		170	4.0	Brain (MDA+4HDA) Brain (8-OHdG) Liver (MDA+4HDA) Liver (8-OHdG)	- 27% - 44% - 21% - 33%		- 6% - 17% - 21% - 22%	[79]
Rat	Extra hepatic Bile Duct Ligation	2		35	17.5	Plasma (MDA) Liver (MDA)	- 63% - 49%	- 19% - 12%		[80]
Rat	Chlorpyrifos-ethyl	43	349 +	1136	34.5	Erythrocytes (MDA) APO	Reduced equal % Increased equal %			[81]
Rat	$KBrO_3$	172*	3488*		20.0	Kidney (8-OHdG)	- 25%	- 25%		[82]
Mouse	Ethanol	172*	523*		3.0	Liver (mtDNA) Heart (mtDNA) Brain (mtDNA)	Preserved as equal % Preserved as equal % Preserved as equal %			[83]
Mouse	Doxorubicin	21	580		28.0	Heart (MDA) Long Term Survivor	- 35% (14)/20	- 26% (12)/20		[84]

Mel = Melatonin; Vit = Vitamin; APO = Antioxidant defense potential; mtDNA = Mitochondrial DNA; 8-OHdG = 8-hydroxy-2-deoxyguanosine; (12) and (14) indicate the numbers of surviving animals (20 total); Ref. = References.

*Total dosage.

Cyclic 3-hydroxymelatonin was believed to be an exclusively non-enzymatic metabolite generated by the interaction of melatonin with reactive species. Surprisingly, if the concentration of reactive species, e.g., oxoferryl-Hb, is very high, cyclic 3-hydroxymelatonin interacts with it to form AFMK. At low concentrations of reactive species it remains inactive [64] (Fig. **5**). The reaction of cyclic 3-hydroxymelatonin with oxidants seems to depend on the level of reactive species and may be conserved to interact with excessive reactive species as a last resort.

The family effort of melatonin in scavenging free radicals is different from classical antioxidants such as vitamin C, E and glutathione. These antioxidants scavenge free radicals at the ratio of one molecule to one molecule. Conversely, the interaction of melatonin and its metabolites with damaging reactants is defined as a scavenging cascade reaction [53]. One melatonin molecule, via this cascade, is estimated to scavenge possibly up to 4 reactive species (Fig. **7**). The cascade reaction and the unique physical properties of melatonin make it a potent antioxidant when compared with classical antioxidants, particularly in *in vivo* situations. In *in vivo* comparative studies, the efficacy of melatonin as a antioxidant is often several-fold greater than that of vitamin C and E in terms of protection from tissue damage, regardless of whether the comparison is with molar concentrations or mg/kg administered to animals [79-84] (Table 4). This is consistent with the cascade reaction of the melatonin family with free radicals.

There is no doubt that the melatonin concentrations used in these studies were well above the physiological levels. Blood levels of melatonin, which are often used to define physiological concentrations, are lower than in tissues and other body fluids [85-87]. Clearly, serum melatonin levels themselves likely do not reflect the total antioxidative capacity of melatonin. The antioxidant capacity of melatonin is in fact a combination of the levels of melatonin and its related metabolites. In the studies cited above, the free radical generating agents were also administered in pharmacological concentrations. It is not practical to expect that physiological levels of any antioxidant can protect the damage induced by pharmacological levels of oxidants.

FURTHER RESEARCH REGARDING MELATONIN AS AN ANTIOXIDANT

Signal Transduction and Antioxidant Enzyme Gene Expression Induced by Melatonin and its Metabolite

Melatonin has been shown to increase the activity of an antioxidative enzymes including glutathione peroxidase (GPx) [88]. Even though there is one report to the contrary [89], under conditions of oxidative stress the stimulatory effect of melatonin on GPx is obvious [90, 91]. Other antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) also have been reported to be stimulated by melatonin under basal conditions or under conditions of oxidant stress [92, 93]. A remaining question is whether this stimulatory action is the result of the direct action of melatonin on the existing enzymes or through signal transduction mechanisms which regulate gene expression and increase the production of these enzymes. It is known that melatonin has the ability to increase mRNA levels for several enzymes probably by regulating gene expression [94-96]. Again, it remains to be determined whether signal transduction is mediated by melatonin membrane receptors or through the nuclear binding sites. Recently, melatonin was shown to act via membrane receptors which participate in the signal transduction pathway for MAPK [97, 98]; this pathway is activated in response to a diverse array of extracellular stimuli leading to the regulation of gene expression. Additionally, however, melatonin nuclear binding sites have also be identified and been speculated to participate in gene regulation [99, 100].

A final question is whether enzyme stimulation or/and the activation of signal transduction pathways are a consequence of melatonin itself or whether the effect is due to the oxidative metabolites of melatonin since it is quickly metabolized to AFMK, 6-hydroxymelatonin or cyclic 3-hydroxymelatonin under conditions of oxidative stress [53]. The levels of melatonin and its metabolites determine the redox status in human serum [101]. Changes in the cellular redox state mediate the binding activities of some critical transcription factors such as AP-1 and NF-kB and regulate gene expression of antioxidative enzymes [102, 103]. Thus, melatonin and its oxidative metabolites may play an essential role in modifying the signal transduction pathways and gene regulation to protect organisms from oxidative stress.

The mRNAs of the key enzymes required for melatonin synthesis, including N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT), have been identified in numerous tissues including pineal gland, brain, lung, heart, lever, spleen, gut and testes [104]. This implies that these tissues and organs have the potential to synthesize melatonin. We hypothesize that melatonin synthesis may be inducible during conditions of elevated oxidative stress. Oxidative stress could change the ratio of melatonin to its oxidative metabolites, e.g., decreased melatonin levels or increased oxidative metabolite levels. The signal generated by low melatonin or high oxidative metabolites may result in the positive induction of NAT and/or HIOMT through gene expression thereby increasing the production of melatonin, a potent antioxidant. If this assumption can be verified it may provide an explanation for the phenomenon of ischemia/reoxygenation preconditioning. Melatonin has been shown to protect against the ischemia/reperfusion injury in brain [105], retina [106], heart [107-111] liver [112], gut [113, 114] and vasculature [115].

Melatonin Levels in Tissues and in Cells

Even through melatonin was discovered 4 decades ago, its levels in tissues and cells are only poorly defined. Most data on melatonin levels in tissues are speculations which are based on information regarding serum melatonin levels. It widely accepted that melatonin concentrations in serum are in the picomolar range; these are most frequently measured using radioimmunoassay. The available reports regarding melatonin levels in tissues, cells or other body fluids are variable and range from picomolar to micromolar levels; these are usually estimated using radioimmunoassay, HPLC and mass spectroscopy [85-87]. It is more difficult to obtain reliable data on intracellular melatonin levels. Melatonin is a amphiphilic molecule and many endogenous substances share the chemical, physical and structural properties of melatonin. These factors make it difficult to isolate and purify melatonin from tissues. To obtain reliable tissue levels will require new methods of extraction.

Based on available data, however, it seems that the tissue melatonin concentrations are considerably higher than they are in serum (85-87). Since melatonin can freely cross the membrane, the question is what mechanism(s) is involved to maintain the melatonin gradient between serum and tissues. A possibility is that melatonin binds to a specific protein. We speculate that there are melatonin binding proteins in some tissues. These melatonin binding proteins function as carriers of melatonin and serve to concentrate melatonin. When intracellular free melatonin is consumed, bound melatonin may be released to establish equilibrium with the consumed free melatonin. This hypothesis is based on the discovery of melatonin binding proteins in different cell lines and in various regions of the human brain [116]. Melatonin has also been reported to bind to the intracellular protein, calmodulin [117]. This observation also supports the possibility of high melatonin levels related to protein binding.

Melatonin Structure Modifications

The unique structure of melatonin makes it a potent free radical scavenger both physically and chemically. The direct free radical scavenging ability of melatonin seems independent of its receptor-mediated actions. The structure-activity relationships have revealed that the structural modification, especially of the side chains, modifies the antioxidative capacity of this indole (25, 37). Based on what is known of melatonin's structure-activity relationship regarding its antioxidant activity, it is feasible to synthesize new compounds endowed with better antioxidant properties and possibly devoid of any affinity for the known melatonin receptors. Such new compounds could have a higher therapeutic efficacy in free radical-related diseases with reduced side effects resulting from the receptor-mediated actions.

Melatonin and its Metabolites in Plants

A novel area for melatonin research relates to melatonin in plants [53]. Melatonin was identified in all parts of plants including the flowers, seeds, leaves, stems and roots [118, 119]. Melatonin levels in plants seem to be much higher than the concentrations measured in the serum of animals using the methods currently available [118]. The high melatonin levels in plants are speculated to participate the antioxidant defense system to protect plants against hostile surroundings including the extremely cold, heat, drought as well as from soil, water or air contamination [53]. The question is whether all plant melatonin is synthesized by the plant itself or whether it is absorbed from the surroundings materials such as soil. It has been reported that at least one plant has the ability to synthesize melatonin and this is regulated by light intensity, i.e., light intensity increases melatonin biosynthesis [120]. This is not unreasonable, since photosynthesis and light irradiation promote the generation of reactive species. However, it is difficult to rule out the possibility that plants may also absorb melatonin. For example, the soil is the mixture of the decomposed plants and microbiological organisms such as bacteria and fungi. All of these contain melatonin. Especially fungi have been reported to contain high levels of melatonin [121]. The melatonin from the decomposed material may be absorbed by the plants and become an alternate source of plant melatonin. We speculate that this reabsorption of melatonin

is part of a natural melatonin recycling. This hypothesis clearly requires prove at this point.

To date, there is no evidence for the existence of oxidized melatonin metabolites such as AFMK and cyclic 3-hydroxymelatonin in plants. It is predicted that the plants will contain high levels of these melatonin metabolites since melatonin presumably is continuously consumed during the detoxification of the oxidants generated from photosynthesis and the cellular respiratory chain reaction. On the other hand, plants have no means to excrete these metabolites as do animals and the metabolites would be accumulated unless plants can reconvert these metabolites back to melatonin. Further research in this area could provide valuable information on the significances of plant derived dietary supplements, nutrition, agriculture and phytoremediation against environmental contamination. The speculation is that high melatonin containing plants or melatonin treated plants may more readily adapt to or resist soil contaminants such as lead, aluminum and chromium. Thus, these pollutant-resistant plants could extract and concentrate these contaminates from the soil. Once the pollutant-rich plants are removed, the soil would be cleaned.

Clinical Trials

Research regarding the mechanisms of melatonin as an antioxidant supports the use of this indole in clinical trials in diseases where free radicals have been implicated such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, stroke, sepsis and heart attack. The etiologies of these diseases at least partially involve free radicals [122]. Several small scale clinical trials have been carried out to examine the therapeutic effects of melatonin on Alzheimer's disease [123-125], erythopoietin plus iron induced renal injury [126], sepsis [127] and asphyxia [128]. These trials suggest positive therapeutic effects of melatonin. The number of patients in these trials preclude definitive conclusions regarding the utility of melatonin in these conditions. Melatonin is also a food supplement and in the US is once surpassed vitamin C in total annual sales [129]. There are anecdoted reports of the therapeutic effects of melatonin, but many unanswered questions remain. To clarify these issues, double blind trials using melatonin with well defined clinical endpoints would provide important data regarding the future of melatonin in the field of medicine.

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