

MINIREVIEW

Melatonin mitigates mitochondrial malfunction

Abstract: Melatonin, or *N*-acetyl-5-methoxytryptamine, is a compound derived from tryptophan that is found in all organisms from unicells to vertebrates. This indoleamine may act as a protective agent in disease conditions such as Parkinson's, Alzheimer's, aging, sepsis and other disorders including ischemia/reperfusion. In addition, melatonin has been proposed as a drug for the treatment of cancer. These disorders have in common a dysfunction of the apoptotic program. Thus, while defects which reduce apoptotic processes can exaggerate cancer, neurodegenerative disorders and ischemic conditions are made worse by enhanced apoptosis. The mechanism by which melatonin controls cell death is not entirely known. Recently, mitochondria, which are implicated in the intrinsic pathway of apoptosis, have been identified as a target for melatonin actions. It is known that melatonin scavenges oxygen and nitrogen-based reactants generated in mitochondria. This limits the loss of the intramitochondrial glutathione and lowers mitochondrial protein damage, improving electron transport chain (ETC) activity and reducing mtDNA damage. Melatonin also increases the activity of the complex I and complex IV of the ETC, thereby improving mitochondrial respiration and increasing ATP synthesis under normal and stressful conditions. These effects reflect the ability of melatonin to reduce the harmful reduction in the mitochondrial membrane potential that may trigger mitochondrial transition pore (MTP) opening and the apoptotic cascade. In addition, a reported direct action of melatonin in the control of currents through the MTP opens a new perspective in the understanding of the regulation of apoptotic cell death by the indoleamine.

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Introduction

Apoptosis is a form of programmed cell death that physiologically plays a role in embryogenesis, metamorphosis, differentiation, proliferation/homeostasis, and as a defensive mechanism to remove infected, mutated, or damaged cells [1]. Under normal conditions, a balance between apoptosis and cell survival is important in the development of multicellular organisms and in the regulation and maintenance of cell populations in tissues. In fact, dysfunction of the apoptotic program is implicated in a variety of pathological conditions. Thus, defects in apoptosis can result in cancer, autoimmune diseases and the spread of viral infections, while neurodegenerative disorders, AIDS and ischemic diseases are caused or enhanced by excessive apoptosis [2]. As a consequence, modulation of the different molecular pathways of the apoptotic process has emerged as an attractive therapeutic strategy for these diseases [3]. In particular, recent studies have focused on the extrinsic or mitochondrial pathway of apoptosis which leads to mitochondrial membrane permeabilization (MMP) and translocation of a number of soluble proteins localized in the matrix and in the intermembrane space to the cytosol [4]. The cause of MMP is the opening of a nonspecific pore

in the inner mitochondrial membrane, known as mitochondrial transition pore (MTP), as a consequence of a rise in matrix calcium levels [5]. Several factors are known to greatly enhance the sensitivity of the MTP to calcium, of which the most potent and relevant are oxidative stress, ATP depletion, mitochondrial depolarization, among others [6].

Melatonin is a highly conservative molecule found in organism from unicells to vertebrates [7]. First discovered as the main secretory product of the pineal gland, it is known to be present in the blood, where its concentrations exhibit a circadian rhythm; it is also found in high concentrations in others body fluids and tissues and is differentially distributed in subcellular organelles as well [8–10]. Its wide extracellular and intracellular distribution may explain the complexity of melatonin's role in modulating a diverse number of physiological processes through different mechanisms of action. Classically, the effects of melatonin were considered to be receptor mediated; more recently, nonreceptor mediated actions, including its free radical scavenging activities, have been uncovered [11–13]. Although two distinct receptors/binding sites have been identified, i.e. membrane [14] and nuclear [15, 16], they may not act separately [17]. New recent studies suggest that

calreticulin may represent a new class of high-affinity melatonin-binding sites involved in some functions of the indoleamine including genomic regulation [18]. For example, some of the antioxidant properties of melatonin are because of a genomic effect in regulating protein expression and activities of antioxidant enzymes [19] as well as the inducible (iNOS) and mitochondrial (mtNOS) isoforms of nitric oxide synthase [20, 21]. Melatonin inhibits nNOS activity due its binding to the calcium-calmodulin complex [22]. Some compounds structurally related to melatonin including its neural metabolite, *N*-acetyl-5-methoxykynurenamine (AMK), also inhibit nNOS activity in rat striatum in a dose-dependent manner. This suggests that the effect of melatonin on cerebral nNOS may be mediated, at least in part, through its metabolites [22, 23]. AMK and *N*¹-acetyl-*N*²-formyl-5-methoxykinuramine (AFMK) are formed during the enzymatic metabolism of melatonin in the brain [24], but also as secondary products when melatonin acts as free radical scavenger of reactive oxygen (ROS) and reactive nitrogen species (RNS). Interestingly, these metabolites are also efficient antioxidants [25, 26].

The recent discovery that mitochondria are a target for melatonin opened a new perspective to understand the mechanism of action of this indoleamine [27]. Melatonin has a direct role in mitochondrial homeostasis [9, 28, 29], which may explain the protective effect of this molecule in diseases such as Parkinson's disease, Alzheimer's disease, epilepsy, aging, ischemia-reperfusion and sepsis, all of which have mitochondrial dysfunction as a primary or secondary cause of the condition [30]. As apoptosis is a mechanism involved in the cell death described in these diseases, it was expected that melatonin may exhibit antiapoptotic effects [27]. In fact, several findings document a role for melatonin in modulating experimentally induced apoptosis by a variety of agents. The indoleamine inhibits apoptosis in immune cells [31, 32], peripheral tissues [33, 34] and prevents neuronal cell death in models of Parkinsonism [35–37], Alzheimer's disease [38–41] and ischemia-reperfusion injury [42–44]. The mechanism by which melatonin reduces apoptosis seems to be related to its antioxidant and free radical scavenging properties. However, recently, a new mechanism has revealed that the antiapoptotic effects of melatonin may be explained by a direct interaction with the MTP [45]. Interestingly, melatonin acts as a proapoptotic agent in cancer models [46], and, therefore, it appears to have differential actions in regulating the apoptotic process in normal and cancer cells [47].

Mitochondria and cell death

Apoptosis and necrosis are two forms of cell death, with clearly distinguishable morphological and biochemical features [48]. Apoptosis is morphologically characterized by cytoplasmic contraction, chromatin condensation, nuclear fragmentation, internucleosomal DNA fragmentation, plasma membrane bleb formation, apoptotic body formation and retention of organelle integrity [49]. Many of these changes are activated specifically by a set of cysteine proteases called caspases. They possess an active site, cysteine, and cleave substrates after aspartic acid residues [1]. Apoptotic cells are rapidly sequestered by phagocytes or

by neighboring cells before they can lyse, spill their contents and cause an inflammatory reaction [50].

In contrast to apoptosis, necrosis does not involve any regular DNA or protein degradation pattern and is accompanied by swelling of the entire cytoplasm (oncosis) and of the mitochondrial matrix, both of which occur shortly before the cell membrane ruptures [51].

These two types of cellular demise can occur concurrently in tissues or cell cultures exposed to the same stimulus [52] and, often, the intensity of the same initial insult dictates the prevalence of either apoptosis or necrosis and it can also vary among experiments [53]. This suggests that while some early events may be common to both types of cell death, a downstream controller may be required to direct cells toward the organized execution of apoptosis [54]. Thus, the early phase of both modes of cell death may involve a similar change in MMP [51].

The cause of the MMP is the opening of a nonspecific pore in the inner mitochondrial membrane, known as the MTP. Opening of the MTP allows the passage of any molecule of >1500 Da across the inner mitochondrial membrane; it can be rapidly closed by chelation of calcium. Because the MTP also allows rapid passage of protons, its opening is accompanied by depolarization of the mitochondria and uncoupling of oxidative phosphorylation. In addition, the equilibration of small solutes across the inner mitochondrial membrane leaves behind high concentrations of proteins in the matrix and these exert a colloidal osmotic pressure that is responsible for the extensive swelling of mitochondria associated with MTP opening [5].

If the MTP remains open, ATP levels can be totally depleted leading to cell necrosis. On the contrary, transient opening of the MTP may be involved in the intrinsic pathway or mitochondrial-mediated apoptosis through the release of proteins usually confined to the mitochondrial compartment. Known as apoptogenic proteins, these released molecules include cytochrome *c* [55], AIF [56], HtrA2/Omi [57], SMAC/Diablo [58] and EndoG [59] of which cytochrome *c* has been the most intensively studied. Upon intrinsic apoptotic stimulation, cytochrome *c* is released into the cytosol where it triggers the formation of the apoptosome, a multimeric molecule composed of apoptotic protease activating factor-1 (Apaf-1), dATP and cytochrome *c* [60]. At present, the only known function of the apoptosome is the recruitment and activation of caspase 9 [61]. The caspase 9/apoptosome complex targets and activates caspase 3. This is considered the point of no return in the apoptotic signaling cascade [4]. However, mitochondria play an important role in apoptosis even in the absence of the MTP opening as release of proapoptotic factors from the intermembrane space of mitochondria may occur through changes in the outer membrane permeability. These are induced by proapoptotic proteins such as Bax and Bid, two members of the Bcl-2 protein family [62].

The exact composition of the MTP is not known; it is currently believed to involve cytosolic proteins (hexokinase), outer membrane proteins (peripheral benzodiazepine receptor, voltage-dependent anion channel or VDAC), intermembrane proteins (creatine kinase), inner membrane proteins (adenine nucleotide translocator or ANT); and also matrix proteins (cyclophilin D) [6].

It appears that any major change in energy balance (absence of oxygen, depletion of ATP, depletion of NADH/NADPH, disruption of the $\Delta\Psi_m$) or changes in the redox balance (oxidation/depletion of reduced glutathione, excessive production of ROS/RNS) may induce MTP opening. In addition, determined signal transduction pathways triggered via intracellular or cell surface receptors can result in MTP opening. Thus, second messengers such as increases in cytosolic calcium concentration, ceramide and caspase 1-like enzymes facilitate MTP [51].

The Bcl-2 family of proteins are potent regulators of apoptosis. This family is divided into three groups, based on structural similarities and functional criteria. Members of group I (Bcl-2 and Bcl-x_L) possess antiapoptotic activity, whereas members of groups II (Bax and Bak) and group III (Bid) promote cell death [62]. One hypothesis proposes that permeabilization of the mitochondrial outer membrane to small proteins occurs through interaction of a Bcl-2 family member with the MTP. Bax has been shown to induce the MTP in cells upon induction of apoptosis via an interaction with VDAC. However, other experiments suggested the involvement of ANT in Bax-mediated apoptosis [63].

Apoptotic cell death can also be triggered when death signals, i.e. tumor necrosis factor (TNF) or Fas ligand, interact with the death receptors at the plasma membrane, resulting in the recruitment of adaptor molecules such as the Fas-associated protein with the death domain, which is responsible for activating caspase 8. Activated caspase 8 can directly activate caspase 3 and caspase 7, but it can also cleave Bid. The cleaved C-terminal Bid (truncated Bid or tBid) translocates to the mitochondria and induces the release of cytochrome c, linking the death receptor pathway with the mitochondrial pathway [64]. Interaction of tBid with the mitochondria does not seem to require the activation of the MTP or Bax, although tBid and Bax can function synergistically [65]. In addition, Bid-induced cytochrome c release can be antagonized by Bcl-2 death repressor protein [64].

Mitochondria, free radicals and cell death

Cells possess multiple sites for ROS/RNS production and a number of mechanisms for their detoxification [66]. Small fluctuations in the steady-state concentrations of ROS/RNS may play a role in intracellular signaling [67]; however, uncontrolled increases in these metabolites lead to free radical-mediated chain reactions which indiscriminately target proteins, lipids and DNA resulting in cell death [66].

Mitochondria are considered the main source of free radicals in the cell and oxidants produced by the electron transport chain (ETC) have been implicated in cell death [68]. Most available data indicate that the origin of excessive ROS generation is a consequence of an impairment of the ETC [68].

The major consequence of an increased ROS production is the subsequent decreased availability of intracellular antioxidants such as NAD(P)H or GSH, leading to an imbalance in the redox status. This, in turn, results in damage to the mitochondrial respiratory chain and a further elevation of free radical generation [69]. Other major consequence of a reduction in the mitochondrial

GSH content is the opening of the MTP because of the oxidation of critical sulfhydryl groups present in the channel [6].

The ROS produced by mitochondria can be discharged into the cytoplasm where they induce calcium release from the endoplasmic reticulum, which leads to mitochondrial calcium loading. The increase in the concentration of mitochondrial calcium can induce opening of the MTP [70]. Other consequence due to the accumulation of calcium in the mitochondria include the induction of mtNOS causing a rise in nitric oxide (NO[•]) and peroxynitrite (ONOO⁻) production which induce (cyclosporine-insensitive) cytochrome c release associated with peroxidation of mitochondrial lipids [71].

Melatonin and mitochondria

In vitro and in vivo experiments have shown that melatonin can influence mitochondrial homeostasis. Thus, melatonin increases the activities of the brain and liver mitochondrial respiratory complexes I and IV in a time-dependent manner after its administration to rats [28]. Melatonin also counteracts ruthenium red-induced inhibition of complexes I and IV in brain and liver mitochondria [28].

Further experiments indicate that the indoleamine, but not other endogenous antioxidants such as vitamins C and E, regulates the glutathione redox status in isolated brain and hepatic mitochondria, correcting it when it is disrupted by oxidative stress [9]. Under normal conditions, melatonin reduces mitochondrial hydroperoxide levels and stimulates the activity of the two enzymes involved in the GSH-GSSG balance, i.e. glutathione peroxidase (GPx) and glutathione reductase (GRd) [9]. Melatonin is also able to counteract the oxidative damage induced by high doses of t-butyl hydroperoxide (t-BHP), restoring GSH levels and GPx and GRd activities and scavenging hydroperoxides. However, vitamins C and E have no such effect under these conditions [9]. These results are in agreement with other data showing the effects of melatonin on GSH homeostasis in brain tissue [72] and in gastric mucosa and testis [73]. As a result of the interaction of melatonin with complexes I and IV and the subsequent promotion of electron flux through the ETC, melatonin increases ATP production under basal conditions and counteracts cyanide-induced depletion of ATP associated with complex IV inhibition [29]. Although the indoleamine also reportedly stimulates metabolism of isolated mitochondria from frog oocytes [74], other experiments have shown that melatonin reduces the oxygen consumption of liver mitochondria [75], an effect that may protect this organelle from excessive oxidative damage [76–79].

The antioxidant and free radical scavenging capacity of melatonin protects proteins of the ETC and mtDNA from the ROS/RNS-induced oxidative damage [80]. Melatonin also interacts with lipid bilayers, reducing lipid peroxidation and stabilizing mitochondrial inner membranes [81], an effect that may improve ETC activity [30]. In a model of sepsis induced by the administration of lipopolysaccharide in rats, melatonin prevented functional deterioration which occurs as a result of mtNOS-induced mitochondrial failure. In this situation, melatonin administration also reduced

both mtNOS activity and NO[•] production and also counteracted the inhibition of complexes I and IV [21].

Other studies have described one possible mechanism by which melatonin increases the activity of the complex IV; this protective action may be due, at least in part, to an effect on the expression of mtDNA. Melatonin increases the expression of mtDNA encoded polypeptide subunits I, II and III of complex IV in mitochondria from rat liver in a time-dependent manner which correlates with the increase in complex IV activity [27]. In experiments with fresh mitochondria prepared from rats treated for 10 days with melatonin, the indoleamine reduced the levels of mRNA in these animals, compared with non-melatonin-treated controls [27]. These effects were also produced by AMK and this compound was more potent than melatonin itself [82]. Interestingly, AMK is a metabolite of melatonin and its in vivo production could be responsible for some of the apparent actions of melatonin [83].

Collectively, these results may help to explain the protective effects of melatonin in neurodegenerative diseases and other disorders which involve mitochondrial dysfunction. Thus, melatonin prevents the inhibition of mitochondrial complex I activity induced by MPTP [84] and limits dopamine autooxidation [85]. Melatonin is also neuroprotective in in vitro models of Alzheimer's disease through its stimulatory effects on complex V activity [9, 28, 38]. Furthermore, the antiepileptic properties of melatonin may be due to the regulation of the central GABA-benzodiazepine receptor complex and inhibition of the glutamate-mediated response [86]. However, other studies reveal that melatonin acts by inhibiting ROS-induced mitochondrial dysfunction in vivo [78, 87] as well as in cultured cells [88]. In the senescence accelerated mouse, either chronic or acute melatonin administration restores the activity of the mitochondrial complexes [89–91]. Treatment with melatonin before injury protects against mitochondrial dysfunction induced by ischemia–reperfusion of rat liver [92] and restores hepatic energetic status by inhibiting both activation of iNOS and the production of TNF α [93].

As mitochondrial dysfunction can lead to ATP depletion, depolarization and initiation of apoptotic processes, it is possible that the antiapoptotic effects of melatonin in the situations described above may be a result of its protective actions [27, 30, 82]. However, recent findings have shown that the interaction of melatonin with mitochondria in terms of antiapoptotic agent is more complex than described here.

Melatonin, mitochondria and apoptosis

Mitochondrial dysfunction associated with the loss of calcium homeostasis and enhanced cellular oxidative stress have long been recognized to play a major role in cell death associated with excitotoxicity [94], a well-known process that has been implicated in neurodegeneration in Huntington's disease, Alzheimer's disease, Parkinsonism, epilepsy and disorders such as ischemia–reperfusion [95]. Excitotoxicity results from the over-stimulation of ionotropic glutamate receptors, in particular, the *N*-methyl-D-aspartate (NMDA) and the α -amino-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors which lacks the GluR2

subunit [96, 97]. Over-stimulation can occur as a result of an increase in the liberation of excitatory aminoacids from the presynaptic neuron. However, energy depletion caused by mitochondrial dysfunction can result in neuronal depolarization, opening of NMDA receptors and the influx of calcium then activates several intracellular enzymes, including phospholipase A2, NOS, xanthine dehydrogenase, calcineurin and endonucleases, many of which elicit the generation of endogenous ROS. Additionally, when taken up by mitochondria, calcium can induce MTP opening and cell death [6].

Melatonin is a potent antiexcitotoxic agent which has been documented in both in vivo and in vitro experiments [86]. Electrophysiological experiments demonstrate the antagonism of melatonin on the NMDA receptor [98–101]. This effect is dose-dependent and, as a consequence of the treatment, the NMDA receptor channel pore remains closed, thereby preventing the opening of L-type calcium channels and calcium influx [102]. Other experiments have shown that melatonin also inhibits activation of nNOS through its binding to the calcium–calmodulin complex, reducing the production of both NO[•] and ONOO[−] as well as the presynaptic release of additional glutamate [22]. Some synthetic melatonin-related kynurenes also reduce striatal NMDA excitability in a dose-dependent manner; some of these kynurenes were 100 times more potent than melatonin in this action. The effects of these drugs were linked to their inhibition of nNOS activity and a reduction in NO[•] production and were not because of an interaction with melatonin membrane receptors [22, 23]. Further experiments demonstrate that melatonin is able to diminish the rises in cytosolic calcium induced by NMDA in cultured mouse striatal neurons [45]. Taken together, these results show that melatonin limits cytosolic calcium rises and, as a consequence, the concomitant production of free radicals; additionally, melatonin reduces the associated mitochondrial membrane depolarization [103]. Other experiments carried out using rat brain astrocytes [104] and cultured PC12 cells [105] show that melatonin prevents ROS-induced calcium overload and mitochondrial membrane depolarization. In these two reports, melatonin indirectly inhibited the opening of the MTP and blocked MTP-dependent cytochrome c release, the downstream activation of caspase 3 and the cell death by apoptosis [104]. In a recent in vivo experiment as well, melatonin was reported to inhibit caspase 3 activation in the mouse brain damaged by ischemia–reperfusion [106]. However, recordings have been obtained from the inner mitochondrial membrane of rat liver mitoplasts using the patch-clamp approach and have demonstrated a direct effect of melatonin on the MTP activity at the single channel level. These results showed that melatonin strongly inhibits MTP currents in a dose-dependent manner with an IC₅₀ of 0.8 μ M [45].

Studies in peripheral tissues have suggested that melatonin inhibits apoptotic processes via its antioxidant properties. For example, melatonin protects against cyclosporin A-induced hemolysis in human erythrocytes because of depuration resulting from O₂^{•−} produced by mitochondria [107]. Melatonin is also highly protective against mitochondrial ROS-induced cardiotoxicity resulting from doxorubicin treatment. In this study, pretreatment with

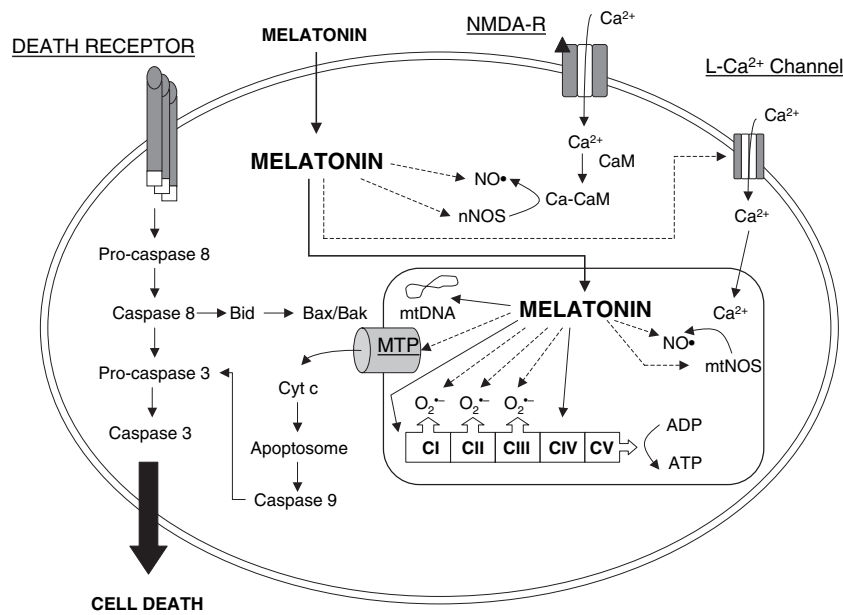


Fig. 1 Melatonin is a highly lipophilic molecule that readily crosses cellular membranes and enters a variety of sub-cellular compartments including the mitochondria. Once there, melatonin exerts its effects by a number of mechanisms. This property may explain how the indoleamine exerts its differential antiapoptotic effects. The intracellular and mitochondrial actions of melatonin, as currently understood, are summarized in this figure. NMDA-R, NMDA receptor; CaM, calmodulin; CaCaM, calcium-calmodulin complex; CI-CV, complexes I-V

melatonin prevented the release of lactate dehydrogenase and restored membrane potential [108].

Many lines of evidence indicate an antiapoptotic effect of melatonin on thymic cells. The methoxyindole reduces DNA fragmentation induced by glucocorticoids in cultured thymocytes [31]. A reduction in glucocorticoid-receptor mRNA levels in the intact thymus as well as in cultured thymocytes that were treated with melatonin seem to be the most likely mechanism whereby melatonin inhibits glucocorticoid-induced cell death [32]. Other studies reported that melatonin inhibits DNA fragmentation and the release of cytochrome *c* from mitochondria of mouse thymocytes treated with dexamethasone. Melatonin may act by inhibiting the mitochondrial pathway, presumably through the regulation of Bax protein levels [109], although melatonin was ineffective per se on this parameter.

Interestingly, proapoptotic effects of melatonin have been noted in a number of tumor cell lines [47]. In MCF-7 breast tumor cell studies conducted in the absence of exogenous steroid hormones, treatment with melatonin produced a 64% reduction in the cellular ATP levels through a membrane receptor-modulated pathway [110]. These findings in tumor cells are in contrast to the described actions of melatonin in normal cells and suggest melatonin's potential use in killing cancer cells while preserving the function of normal cells.

Concluding remarks

The actions of melatonin on mitochondria may be mediated via at least three mechanisms (Fig. 1). First, antioxidant and free radical scavenging properties of the indoleamine protect the organelle from oxidative damage. Secondly, its actions at the mtDNA level increase the expression of complex IV. Thirdly, a direct interaction of melatonin with the MTP was found recently. These effects suggest that melatonin, because of these direct and indirect mitochondrial actions, may have utility as an antiapoptotic agent for normal cells.

In addition, some of the products that are produced when melatonin detoxifies reactive species [25, 111–114], especially AMK and AFMK, are also both efficient antioxidants [25, 83, 115] that may be found in mitochondria; these metabolites can also act at the mitochondrial genomic level, resulting in a cascade of protective reactions. Given that these compounds exert the same actions as melatonin, they also could act as antiapoptotic drugs in normal cells and as proapoptotic agents in cancer models. In all the studies where comparisons were made, melatonin's metabolites AMK and AFMK were more potent than melatonin itself [82]. Therefore, these compounds may exert the same regulatory effects on apoptotic processes in a more efficient manner than melatonin.

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