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MINI REVIEW

Malaria: therapeutic implications of melatonin

Abstract: Malaria, which infects more than 300 million people annually, is a serious disease. Epidemiological surveys indicate that of those who are affected, malaria will claim the lives of more than one million individuals, mostly children. There is evidence that the synchronous maturation of *Plasmodium falciparum*, the parasite that causes a severe form of malaria in humans and *Plasmodium chabaudi*, responsible for rodent malaria, could be linked to circadian changes in melatonin concentration. In vitro melatonin stimulates the growth and development of *P. falciparum* through the activation of specific melatonin receptors coupled to phospholipase-C activation and the concomitant increase of intracellular Ca^{2+}. The Ca^{2+} signaling pathway is important to stimulate parasite transition from the trophozoite to the schizont stage, the final stage of intraerythrocytic cycle, thus promoting the rise of parasitemia. Either pinealectomy or the administration of the melatonin receptor blocking agent luzindole desynchronizes the parasitic cell cycle. Therefore, the use of melatonin antagonists could be a novel therapeutic approach for controlling the disease. On the other hand, the complexity of melatonin's action in malaria is underscored by the demonstration that treatment with high doses of melatonin is actually beneficial for inhibiting apoptosis and liver damage resulting from the oxidative stress in malaria. The possibility that the coordinated administration of melatonin antagonists (to impair the melatonin signal that synchronizes *P. falciparum*) and of melatonin in doses high enough to decrease oxidative damage could be a novel approach in malaria treatment is discussed.

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Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a ubiquitous substance secreted by the pineal gland of all mammals, including man. In addition, its presence has been confirmed in many plants [1, 2], Chinese herbs [3], and unicellular organisms [4, 5].

Melatonin participates in diverse functions of the body including sleep and circadian rhythm regulation, immunoregulation and free radical scavenging, and may have anticancer actions [6–9]. Melatonin also protects organisms against both bacterial and viral infections by a variety of mechanisms [10–12] and has been shown to be beneficial for reversing symptoms of septic shock [13].

Malaria, a disease caused by the protozoan species *Plasmodium*, infects more than 300 million people and results in the death of more than one million people annually [14]. It has been estimated that nearly half of the earth's population is at the risk for contracting this deadly illness. Human malaria is caused by four different species of the protozoan parasite *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* [15]. The most severe form is caused by *P. falciparum*in which causes the variable clinical symptoms including fever, chills, headache, muscular aching, and weakness, vomiting, cough, diarrhea, and abdominal pain [16, 17]. Other symptoms related to organ failure may supervene, such as acute renal failure, generalized convulsions, and circulatory collapse, followed by coma and death. In endemic areas it is estimated that about 1% of patients, mostly children and pregnant women, with *P. falciparum* infection die of the disease [18].

It is known that malaria affects several organs including kidney, heart, spleen, heart, and cerebral tissue [16, 17]. Malaria has been shown to increase the generation of reactive oxygen species (ROS) in the tissues [19] and additionally to decrease the level of critical antioxidant enzymes including catalase, glutathione peroxidase,

glutathione reductase, and superoxide dismutase [20]. Malarial infection activates immune effector and regulatory cells, thereby causing intravascular lesions in target organs, lungs, kidney, and brain thus accounting for the broad systemic complications which inevitably accompany progression of the disease [21, 22].

Melatonin has been shown to modulate the cell cycle of *P. falciparum* and *P. chabaudi* (a parasite that causes rodent malaria) [23, 24]. In addition, treatment with melatonin is beneficial for inhibiting apoptosis and liver damage resulting from the oxidative stress caused by malarial infections [25].

The aim of this mini review is to discuss the possible role of melatonin and melatonin receptors in the pathogenesis of malaria as well as the protective function against malarial hepatotoxicity displayed by melatonin. The possibility of developing two-tiered or multimodal approaches for melatonin's use as a therapeutic strategy in combating malarial infection is considered.

Melatonin synthesis and metabolism

In all mammals, circulating melatonin is synthesized primarily in the pineal gland [26]. In addition, melatonin is also locally found in various cells, tissues, and organs including lymphocytes [27], human and murine bone marrow [28, 29], the thymus [30], the gastrointestinal tract [31], skin [32], and the eyes [33], where it plays either an autocrine or paracrine role [34].

Tryptophan serves as the precursor for the biosynthesis of melatonin [35]. It is taken up from the blood and converted into 5-hydroxytryptophan which is subsequently decarboxylated to yield serotonin. Serotonin is acetylated to form *N*-acetylserotonin through the action of arylakylamine *N*-acetyltransferase (AANAT), one of the key enzymes in melatonin synthesis. *N*-acetylserotonin is then converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT). There is evidence that HIOMT is responsible for the amplitude of the nocturnal peak of melatonin, whereas AANAT is responsible for the timing of the peak. In the Siberian hamster, the amplitude of the nocturnal peak was related to HIOMT activity rather than to AANAT [36, 37]. In the rat, *N*-acetylserotonin is present in vast excess during the night [38]. Thus, although AA-NAT is the rhythm-generating enzyme [39] it is not rate limiting for nocturnal production.

Pineal melatonin biosynthesis is regulated by the light–dark (LD) cycle via the retinohypothalamic tract [40]. Special melanopsin-containing retinal ganglion cells [41] project to the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN then projects through other neuronal circuits including the hypothalamic paraventricular nucleus, the medial forebrain bundle and the reticular formation to influence intermediolateral horn cells of the spinal cord, which are the preganglionic neurons that innervate the superior cervical ganglion (SCG) [42]. The postganglionic fibers that arise from SCG regulate pineal melatonin biosynthesis by releasing norepinephrine (NE) at its pinealocyte receptor sites. NE, not only by interacting mainly with β_1 but also with α_{1B}-adrenergic receptors in the pineal gland, activates the adenylyl cyclase-cyclic AMP pathway which in turn regulates expression of enzymes in the melatonin biosynthetic pathway [35]. α_{1B}-Adrenergic receptors potentiate β_1-adrenergic activity by producing a sharp increase in intracellular Ca^{2+} and activation of protein kinase C (PKC) and of prostaglandin synthesis [43–45]. The subcellular mechanisms involved in initiation and termination of AANAT activity have been elucidated in great detail [46]. Cyclic AMP stimulates AANAT expression and phosphorylation via protein kinase A, which also allows AANAT to be stabilized by binding of 14-3-3 proteins [47, 48]. The nocturnal exposure to bright light suppresses melatonin production immediately by degradation of pineal AANAT [49].

Once formed melatonin is not stored within the pineal gland, but diffuses out either into the cerebrospinal fluid (CSF) or directly into the blood [50]. Melatonin released into the CSF via the pineal recess reaches high concentrations in the third ventricle, 20–30 times higher than that found in the blood [51].

Melatonin in blood is metabolized mainly in the liver where it is hydroxylated in the C6 position by cytochrome P_{450} monooxygenases (CYPA2 and CYP1A) [26]. It is then conjugated with sulfate to form 6-sulphatoxymelatonin, the main melatonin metabolite found in urine. Melatonin is also deacetylated in neural tissues [52] and is also metabolized to form the kynuramine derivative N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) [53]. Interestingly, this metabolite shares melatonin's antioxidant and anti-inflammatory properties [54]. Melatonin is also converted into cyclic 3-hydroxymelatonin in a process that directly scavenges two hydroxyl radicals [55].

Melatonin receptors

Inasmuch as melatonin freely diffuses through all biological membranes, it exerts its actions in almost all cells. Some of melatonin's actions are receptor mediated while many others, e.g., free radical scavenging, are receptor-independent [55, 56].

Molecular cloning of the first high affinity melatonin receptor (MT_1) by Reppert and coworkers [57] was accomplished using a cDNA library constructed from a dermal cell line of melanophores, the first tissue in which melatonin's action had been demonstrated. This initial finding led to the discovery that there is another G_i-protein coupled melatonin receptor in humans. The second receptor (MT_2) [58] is 60% identical in amino acid sequence to the MT_1 receptor. Yet, a third melatonin-related receptor, now called GPR50, shares 45% of the amino acid sequence with MT_1 and MT_2, but does not bind melatonin and has an unidentified natural ligand in the pituitary and hypothalamus [59]. It is unusual in that it lacks *N*-linked glycosylation sites and additionally has a C-terminal that is over 300 amino acids in length.

A fourth melatonin binding site was identified in mammals [60] (MT_3, initially called ML-2). Unlike the picomolar membrane receptors it binds melatonin in the nanomolar range and has a specific pharmacologic profile and fast kinetics of association/dissociation [61]. It has now been purified from hamster kidney and characterized as the

analog of quinone reductase type 2 [62]. Melatonin can also exert some of its physiological actions by binding with nuclear receptors, such as ROR α 1, ROR α 2, and RZR β that belong to the retinoic acid receptor family [63]. In addition, melatonin interacts with intracellular proteins such as calmodulin [64, 65] or tubulin [66, 67]. These interactions are most likely related to some of the physiological effects of melatonin, but critical data regarding this point have yet to be obtained.

A combination of reagents derived from the molecular clones and pharmacologic tools have revealed a considerable amount of information about the MT_1 and MT_2 receptors [68]. Many G protein-coupled receptors (GPCR), including the MT_1 and MT_2 receptors, exist in living cells as dimers. The relative propensity of the MT_1 homodimer and MT_1/MT_2 heterodimer formation are similar, whereas that of the MT_2 homodimer is three- to four-fold lower [69, 70]. It is of considerable interest that the GPR 50 receptor, although lacking the ability to bind melatonin, abolishes high affinity binding of the MT_1 receptor through heterodimerization [71, 72]. Thus, the GPR50 receptor may have a role in melatonin function by altering binding to the MT_1 receptor.

Luzindole was the first ligand to be identified as a competitive melatonin receptor antagonist [73] and has since been used extensively in the field to validate melatonin receptor action. It is relatively receptor type non-selective (MT_1/MT_2 affinity ratio = 16/26) and was the first antagonist used for demonstration that melatonin receptors are involved in the inhibition of dopamine release in rabbit retina and the phase shift of circadian rhythms in rodents [74]. It must be noted that recent data indicate that luzindole is an effective antioxidant in vitro [75] inhibiting the formation of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation by 80% at a concentration of 10 μM. Therefore, some of the beneficial findings with luzindole may be unrelated to melatonin's actions on receptors.

Melatonin and molecular mechanisms of *Plasmodium* survival

The development of malarial parasites is a complex process that involves both intracellular and extracellular phases. The life cycle of *Plasmodium* is initiated by the bite of the female *Anopheles* mosquito, which bites mainly between sunset and sunrise. At this time moderate to severe skin response to mosquito bite occurs thus contributing to protection against malaria. Since itch in an individual follows a 24-hr cycle and it is about 100-fold higher at midnight than midday it has been speculated that jet lag would contribute to the enhanced susceptibility to malarial infection seen after transmeridian flights [76].

The spread of malarial parasites follows the *Anopheles* mosquito's aspiration of blood and simultaneous injection of saliva that contains the infecting sporozoites. The blood borne sporozoites then invade hepatocytes, where they develop into asexual forms known as merozoites [77]. After entering into the circulation the merozoites invade erythrocytes where they proceed through three distinct stages of maturation, referred to as ring, trophozoite, and schizont [78]. Some merozoites undergo differentiation into gametocytes, which trigger a sexual stage once ingested by mosquito.

In humans, *Plasmodium* spends most of its life span within red blood cells and hepatocytes [79, 80]. Within erythrocytes *Plasmodium* multiplies and maturates into forms that are ready to invade other cells. The periodic fever peaks, which occur generally every 48 (*P. vivax* and *P. ovale*) or 72 (*P. malariae*) hours are the most striking trait of the malarial infection. The *P. falciparum* infection is usually associated with 36–48 hr fever peaks intervals or with a continuous subfebrile fever. Fever results from a burst in the number of merozoites in the host's bloodstream, the parasites displaying a synchronous development to produce such a population increase. The timing of appearance of the invertebrate-infective forms in circulation matching the time of vector's feeding behavior which ensures infection propagation has been termed ''the Hawking effect'' [79–82].

The malarial erythrocyte rupture and reinvasion process is an extremely synchronized event. Early studies demonstrated that the parasite's life cycle changes concomitantly with changes in the LD cycles to which the host was exposed. *Plasmodium falciparum* synchrony loss in culture indicates the involvement of host's physiology in the maintenance of the infection rhythm [83].

Malaria parasites appear to sense environmental chemical cues and regulate their life cycle in response to them. Several molecules derived from the host/vector can be recognized by the *Plasmodium* including tryptophan-derived metabolites such as melatonin [23, 24, 84, 85]. It has been shown that melatonin is capable of synchronizing the life cycle of *P. falciparum* and *P. chabaudi in vitro* and that this effect is abolished by co-incubation with the MT_1/MT_2 melatonin receptor antagonist luzindole. The synchrony is also lost *in vivo* in pinealectomized mice and after the injection of luzindole. Moreover, synchrony in pinealectomized mice is restored by melatonin administration [84]. Thus, circulating melatonin has been proposed as the signal that modulates the *Plasmodium* cell cycle.

In erythrocytes calcium homeostasis is controlled by plasma membrane Ca^{2+} ATPase [86]. During its development in erythrocytes, *Plasmodium* generates high levels of Ca^{2+} and uses it for inducing signaling events. In the *Plasmodium*, genome 30 proteins containing the Ca^{2+} binding EF-hand motif have been identified [79]. In studies using fluorescent dyes and genetic encoded Ca^{2+} probes, the role of Ca^{2+} in the control of gene expression and cell cycle regulation of *Plasmodium* has been thoroughly investigated and has been found to involve generation of inositol triphosphate ($InsP_3$) [24, 87].

Upon activation of specific receptors, melatonin is coupled to phospholipase C which causes the release of Ca^{2+} from intracellular stores of *Plasmodium* grown in vitro which in turn synchronizes parasite activity [84, 85]. The increase in Ca^{2+} caused by melatonin was blocked by luzindole and by U 73122 (an inhibitor of phospholipase C). Both drugs completely inhibited the increase in parasitemia [84, 85]. It remains to be established whether

the melatonin receptors of *Plasmodium* are coupled to G-proteins, as in mammals, or to other signal-transduction pathways, and which of the many signaling events that are controlled by Ca^{2+} are involved in the effects of melatonin on *Plasmodium* maturation. In mammalian cells, the effects of Ca^{2+} on the cell cycle are often dependent on calcineurin-regulated pathways, and that calcineurin is thought to be expressed in *Plasmodium* [88].

Evidence relating to luzindole's actions deserves careful consideration. As mentioned above, besides MT_1/MT_2 receptor antagonist properties, luzindole is a strong antioxidant [75]. Thus, it can potentially have two different and opposing actions: (a) it could inhibit melatonin action on membrane receptors, while (b) actually potentiating melatonin's antioxidant activity. The use of MT_1/MT_2 melatonin agonists such as ramelteon could be useful for unraveling this question as ramelteon displays no relevant antioxidant capacity in the ABTS radical cation assay, as compared with luzindole or melatonin [89].

In another study 2-aminoethyl diphenylborinate (2-APB), an $InsP_3$ inhibitor, was used for investigating the molecular mechanism of melatonin action cycle [90]. 2-APB inhibits store operated Ca^{2+} channels or capacitative calcium entry. *Plasmodium falciparum* were maintained in continuous in vitro cell culture in adult erythrocytes and 2-APB (75 μg) was added in the presence of melatonin (80 μg). Melatonin was found to induce an increase in cytosolic calcium concentration in *Plasmodium*, as measured by the fluorescent Ca^{2+} indicator Fluo-3AM, an effect inhibited by 2-APB [90]. It was thus proposed that melatonin activates phospholipase C and production of $InsP_3$ via a receptor-mediated mechanism. This in turn releases Ca^{2+} from the endoplasmic reticulum, thus enhancing Ca^{2+} signaling and influencing the *P. falciparum* cell cycle [90]. In a study of erythrocytes obtained from Balb/C mice infected with *P. chabaudi* in vitro, increases in doses of melatonin from 1 to 100 nM were found (a) to enhance the ability of *Plasmodium* to invade the erythrocytes; (b) to stimulate the maturation of the parasites in their transition from the trophozoite to schizont forms. Addition of 10–100 nM melatonin markedly reduced the percentage of ring and trophozoite stages, whereas the percentage of the mature form schizont was doubled [85]. Collectively, these studies suggest that melatonin receptor blockade might represent a means for desynchronizing the cell cycle of *Plasmodium*, thus making available a new therapeutic strategy for combating malaria.

In addition to melatonin, the melatonin metabolite AFMK has been found to modulate the cell cycle of *P. falciparum* and *P. chabaudi*. AFMK is formed endogenously by oxygenases, such as indoleamine-2,3-dioxygenase and myeloperoxidase, and when melatonin functions as a free radical scavenger [55]. It was suggested that some of melatonin that reached malaria infected cells can be converted into AFMK. With uninfected RBC, the level of melatonin degradation into AFMK is quite low, approximately 0.1% but with infected cells this percentage reaches 5–7% [23]. The presence of AFMK (500 nM or 1 μM) caused an increase in schizont forms. It also increased calcium concentration in the cytosol of *P. falciparum* and *P. chabaudi*. As in the case of melatonin, the synchronizing effect of 1 μmol/L AFMK was abrogated by the melatonin receptor antagonist luzindole, although it cannot be affirmed that luzindole acts directly on AFMK-binding site in infected cells because melatonin and AFMK are distinctively different molecules [23]. It is tempting to speculate that interaction between orphan GPCRs and GPCR takes place and influence each other response [71].

In contrast to the findings with *P. falciparum* and *P. chabaudi*, melatonin was not able to modulate the cell cycle, nor to elicit an elevation in intracellular calcium concentration of the intraerythrocytic forms of *P. berghei* or *P. yoelii*, two rodent parasites that show an asynchrononous development in vivo [83]. Further, melatonin did not affect hepatic infection by *P. berghei* sporozoites. These data may provide an explanation as to why infections by these parasites are asynchronous.

Melatonin, oxidative damage, and hepatic apoptosis in malarial infection

Malarial infection affects several organs including the liver, kidney, heart, spleen, lungs, and cerebral tissue [21, 91, 92]. ROS and oxidative stress have been hypothesized to play major roles in the development of systemic complications associated with malaria.

Further insights into how melatonin may be involved in the pathogenesis of malaria are now being provided by work showing that melatonin has protective effects against hepatic dysfunction occurring in malaria [25]. Jaundice, hepatocyte dysfunction, and hepatic encephalopathy are common in malarial patients [93]. The histopathological changes reported in the malaria patients include hepatocyte necrosis, bile stasis, granulomatous lesions, and malarial nodules.

Malarial infection develops mitochondrial pathology and mitochondrial oxidative stress to promote hepatocyte apoptosis [94] an effect prevented by melatonin [25]. Melatonin's potent antioxidant actions have been demonstrated in a number of oxidative stress conditions, including the ability to neutralize-free radicals at the mitochondrial level [54, 95]. Melatonin also may be beneficial because of its ability to preserve mitochondrial oxidative phosphorylation [96].

Malarial infected liver tissues show characteristic apoptotic features, such as chromatin condensation, disappearance of nucleoli, cytoplasmic vacuoles, etc. Melatonin treatment (20 mg/kg) mitigated apoptosis by attenuating all these apoptotic features [25]. It also inhibited caspase-3 activation, a common critical event for apoptosis. These findings support the conclusion that melatonin is effective against oxidative stress induced apoptosis and liver damage during malaria [25].

Conclusions

Studies discussed above underline the complexity of melatonin's effects on malaria. Melatonin administration may have both enhancing and inhibitory effects on malaria development (Fig. 1). Via its receptors melatonin increases the ability of *Plasmodium* to invade erythro-

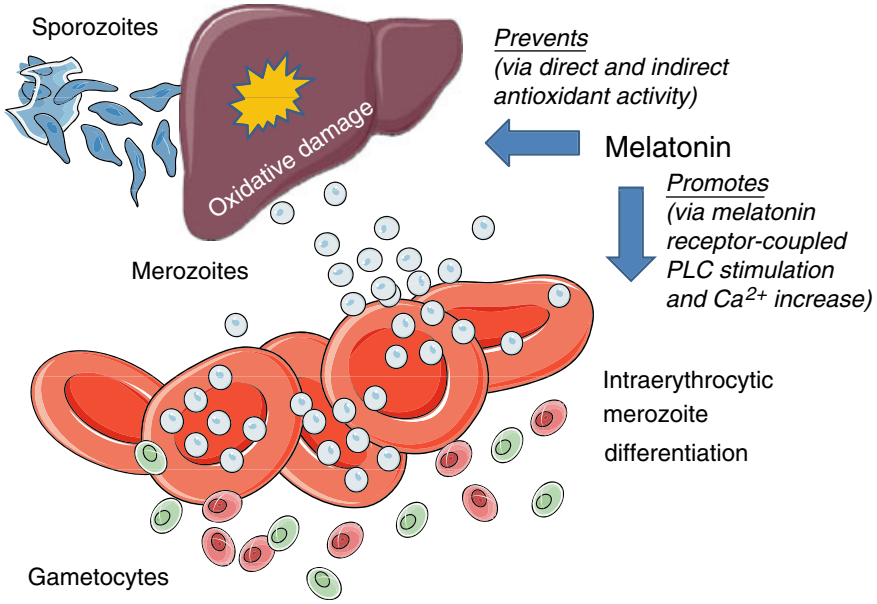

Fig. 1. Scheme summarizing the possible effect of melatonin on malaria infection. Via its receptors endogenous melatonin is a signal to increase the ability of *Plasmodium* to invade erythrocytes, a process that involves phospholipase C activation and release of Ca^{2+} from the intracellular storage sites. The Ca^{2+} signaling pathway is important to stimulate parasite transition from the trophozoite to the schizont stage, the final stage of intraerythrocytic cycle, thus promoting the rise of parasitemia. On the other hand, exogenous melatonin administration inhibits free radical-mediated mitochondrial-dependent hepatocyte apoptosis and liver damage induced by malarial infection, indicating that appropriate antioxidant doses of melatonin could be particularly useful to limit ROS production and ROS-induced hepatocyte apoptosis.

cytes, a process that involves phospholipase C activation and release of calcium from the intracellular storage sites. On the other hand, melatonin inhibits free radical-mediated mitochondrial-dependent hepatocyte apoptosis and liver damage induced by malarial infection, indicating that appropriate antioxidant doses of melatonin could be particularly useful to limit ROS production and ROS-induced hepatocyte apoptosis and hence to protect liver from apoptosis and dysfunction. A major argument in favor of the promoting effect of *Plasmodium* infection via melatonin receptors was provided by the inhibition of this melatonin action in vivo and in vitro by the MT_1/MT_2 receptor antagonist luzindole. However, luzindole is a strong antioxidant per se making it necessary further studies necessary that employ melatonin antagonists devoid of antioxidant activity or MT_1/MT_2 agonists like ramelteon which lacks such free radical scavenger properties [89].

As melatonin effects on ROS production under these circumstances may not be receptor-mediated the association of melatonin antagonists (to impair the synchronizing effect of melatonin on *Plasmodia*) with pharmacological doses of melatonin (to impair ROS production and scavenge ROS that are generated) might have therapeutical significance in the treatment of malaria, a deadly disease that annually affects millions of people on a worldwide basis. Future investigations should consider two-tiered or multimodal approaches to the use of melatonin, melatonin agonists, and melatonin antagonists as new therapeutic strategies in combating malarial infection.

Competing interest statement and disclosure statement

S.R. Pandi-Perumal is a stockholder and the President and Chief Executive Office of Somnogen Inc., a New York Corporation. He declared no competing interests that might be perceived to influence the content of this article. All remaining authors declare that they have no proprietary, financial, professional, nor any other personal interest of any nature or kind in any product or services and/or company that could be construed or considered a potential conflict of interest that might have influenced the views expressed in this manuscript.

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